1995-06-01

The Role of T Lymphocytes in the hu-PBMC-SCID Mouse Model of Epstein-Barr Virus-Associated Lymphoproliferative Disease

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A Dissertation Presented

By

Mary Amanda Cromwell

Submitted to the Faculty of the
University of Massachusetts Graduate School of
Biomedical Sciences, Worcester
in partial fulfillment of requirements for the degree of:

DOCTOR OF PHILOSOPHY

JUNE 1995

IMMUNOLOGY

THE ROLE OF T LYMPHOCYTES IN THE HU-PBMC-SCID MOUSE MODEL OF
EPSTEIN-BARR VIRUS-ASSOCIATED LYMPHOPROLIFERATIVE DISEASE

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DEDICATION AND ACKNOWLEDGEMENTS

This dissertation is dedicated to my children, Elana and Timothy, who entered the world during its creation, and to my husband Duane. Their unconditional love, support and patience allowed me to keep my perspective over the past eight years.

I would like to acknowledge the contribution of Dr. Ruth Hesselton and thank her for her support and encouragement during the pursuit of this thesis. I would also like to acknowledge everyone working in the laboratory and office, who make the Sullivan lab a fun, interesting and unique place to work. I am grateful to the other graduate students for their support, conversations, and friendship. I would also like to acknowledge Ana Diaz for her assistance in many areas involving mice.

Finally, I am indebted to Dr. John Sullivan for his generosity, guidance, patience and encouragement.

ABSTRACT

Epstein-Barr virus (EBV) is associated with a spectrum of benign and malignant lymphoproliferative disorders, including acute infectious mononucleosis (IM), Burkitt's lymphoma (BL) and immunosuppression-associated B cell lymphoproliferative disease (LPD). Immunosurveillance mediated by virus-specific cytotoxic T lymphocytes is believed to protect immunocompetent hosts from EBV-associated lymphoma and LPD. Due to the lack of an adequate animal model, however, the precise immunologic mechanisms which provide this protection have not been directly demonstrated *in vivo*.

Human peripheral blood mononuclear cell-reconstituted C.B.-17-*scid/scid* mice (hu-PBMC-SCID mice) develop EBV-positive LPD following intraperitoneal injection of PBMC from EBV-seropositive donors. The SCID mouse disease mirrors human EBV-associated LPD in morphology, presence of the EBV genome, clonality, and patterns of expression of latent viral cellular differentiation antigens. The hu-PBMC-SCID mouse provides a unique small animal model of EBV$^+$ LPD, and it was used in this study to examine the role of CD8$^+$ CTL in controlling LPD. Survival time increase significantly when EBV-specific cytotoxic T-cell lines (CTL) are adoptive transferred into hu-PBMC-SCID mice, demonstrating suppression of LPD *in vivo* by a CTL-mediated virus-specific mechanism. Survival time also increases significantly with administration of alloreactive CTL lines, suggesting that a non-virus-specific mechanism also contributes to control of EBV-associated LPD by CTL.

NOD-SCID mice reconstituted with PBMC from donors with latent EBV infection develop EBV$^+$ LPD with significantly less frequency than do C.B.17-SCID mice reconstituted with PBMC from the same donors. Administration of anti-CD8 mAb to these mice depletes human CD8$^+$ cells and increases the incidence of LPD to 100%, demonstrating that CD8$^+$ T cells are neccessary for protection from EBV-associated LPD. Adoptive transfer of human CD8$^+$ T cells, but not CD4$^+$ T cells, prevents LPD in CD8-depleted NOD-SCID mice. *In vivo* depletion of CD4$^+$ T cells prevents engraftment of human T cells, and LPD does not develop in most mice after CD4$^+$ cell depletion. These studies are the first to

directly demonstrate both the protective role of CD8$^+$ T cells and a requirement for CD4$^+$ T cells in EBV-associated LPD in an *in vivo* model.

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ABBREVIATIONS

APC	Antigen presenting cell
BL	Burkitt's lymphoma
B-LCL	B-lymphoblastoid cell line
CALLA	Common acute lymphoblastic leukemia antigen
CD	Cluster designation
CTL	Cytotoxic T lymphocytes
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
E:T	Effector cell to target cell ratio
FITC	Fluorescein isothiocyanate
HuIg	Human immunoglobulin
hu-PBMC-SCID	human peripheral blood mononuclear cell-reconstituted SCID mouse
IDDM	Insulin-dependent diabetes mellitus
Ig	Immunoglobulin
IL-2	Interleukin-2
IM	infectious mononucleosis
i.p.	intraperitoneal(ly)
LPD	Lymphoproliferative disease
L.U.	Lytic unit
mAb	Monoclonal antibody
MHC	Major histocombatibility complex
MuIg	Murine immunoglobulin
PBMC	Peripheral blood mononuclear cell

NBF	Neutral-buffered formalin
NK	Natural killer
NOD	Non-obese diabetic
PE	Phycoerythrin
PBS	Phosphate-buffered saline
SCID	Severe combined immunodeficiency
SPF	Specific pathogen free

CHAPTER I. INTRODUCTION

A. The Epstein-Barr virus (EBV)

1. Biology of EBV.

Epstein-Barr virus (EBV) was first detected in 1964 in cultured cells from African Burkitt's lymphoma (16). It was subsequently detected in lymphoid cell lines from patients with various malignancies, infectious mononucleosis, and apparently healthy individuals (43). EBV is a human lymphotropic herpesvirus which infects over 90% of the world population. It is the human prototype virus for the genus *Lymphocryptovirus*, which are a member of the gamma herpesvirus family. A member of the *Lymphocryptovirus* genus is endemic in each species of old world primates, but none are found in other species (38).

The EBV genome has been completely sequenced (3). It consists of a 172 Kb double-stranded DNA, which is linear in virions but circularizes soon after infection of cells. The genome contains certain features shared by all lymphocryptoviruses, including 3Kb internal direct repeat (IR) sequences and 0.5 Kb terminal direct repeat sequences (TR) (38). The number of iterations of each of these repeat sequences varies between virus isolates, but is usually maintained through sequential passage. This is an important characteristic that can be exploited to assist in determining the clonality of EBV-carrying lesions (see below).

The host cell range for EBV is largely limited to B lymphocytes and the squamous epithelial cells of the oropharynx. Primary infection with EBV begins in the oropharyngeal epitheliun, which is permissive for viral replication (51, 80). B lymphocytes are subsequently infected, probably by trafficking through the oropharynx (2). EBV infection of B lymphocytes is usually latent, although replication can be induced *in vitro* and may occur *in vivo* under certain circumstances. In less developed cultures primary infection typically occurs in the first year of life and is asymptomatic. When infection is delayed until late childhood or early adulthood, as it often is in the developed world, it can result in acute infectious mononucleosis (IM), a benign, self-limiting disease which is marked by sore throat,

lymphadenopathy and fatigue. In either case primary infection is followed by lifelong, persistent infection.

EBV is maintained in the persistently-infected human host as a productive infection of the oropharyngeal epithelium and a latent infection of B cells (43). It is unclear what relationship exists between these two sites of infection. Persistence is thought to be maintained by a reservoir of latent virus being fed to the site of replication, because variations in the virus that would be generated during replication are not found in sequential isolates from saliva of infected individuals over a period of years (41). Furthermore, maintainence of latent infection in B cells does not appear to require continual reinfection via the oropharynx; treatment with acyclovir, which blocks viral replication in the oropharynx, does not affect the number of EBV-carrying cells in the blood, and virus can be detected in throat washings soon after treatment is stopped (98). It is difficult to reconcile the evidence for B cells as the reservoir of latent virus with data that suggests that B cells turn over rapidly, with as much as 1% of the total pool being renewed daily (41). One possibility is that EBV-infected B cells are spared from clearance by virus-induced mechanisms, such as the upregulation of *bcl-2* gene expression by the EBV LMP-1 latent protein (23). Induction of *bcl-2* expression has been shown to protect B cells from programmed cell death (apoptosis) (23).

Two remarkable biologic properties of the virus are its abilities to induce activation and growth transformation of infected B lymphocytes. Activation occurs following attachment of the EBV gp350/220 glycoprotein to its receptor, CD21, which is also the C3d complement receptor (CR2) (17). Synthesis of B cell activation molecules, such as CD23 and CD30, accompanies activation, and does not require expression of EBV latent gene products (85). EBV-induced growth transformation, or immortalization, occurs in mature B cells which have undergone Ig gene rearrangements (11). It occurs after B cell activation and requires synthesis of EBV latent gene products.

Growth transformation causes cells to proliferate indefinitely *in vitro*, and permits the propagation of permanent B-lymphoblastoid cell lines (B-LCL) which carry the virus as a latent infection (38). These cell lines provide an *in vitro* system in which to study EBV biology and virus-specific cellular immunity, whereas cultivation of infected epithelial cells has proven to be

more difficult. EBV can also infect and immortalize lymphocytes from some non-human primates. Cell lines established from the cotton-top tamarin produce high titers of virus; one of these, the B95.8 cell line, is used routinely as a source of cultured virus (44). The immortalizing property of EBV also underlies the function of EBV in its associated lymphoproliferative diseases.

Much of the existing knowledge of the biology of EBV has been gathered from the study of *in vitro* EBV-transformed B-LCL. Viral gene expression in these latently infected cell lines is limited to the latent proteins: six nuclear proteins, EBNAs 1-6, and three latent membrane proteins, LMP-1, LMP-2A and LMP-2B (38). (The EBNA protein are sometimes referred to as EBNA-1, -2, -3A-C, and -LP.) This pattern of latent gene expression has been termed the latency III program; latency programs I and II have been demonstrated in other EBV-carrying cell lines and tumor biopsies (69). The latency I program is expressed in primary BL cells and some BL cell lines, and permits expression of only one of the latent proteins, EBNA-1. The latency II program, found *in vivo* in Hodgkin's disease and nasopharyngeal carcinoma (NPC), and inducible in BL lines, permits expression of EBNA-1 plus the LMPs. It is often assumed that the phenotype of infected cells *in vivo* is identical to that of B-LCL, but recent evidence suggests that the latency II program may more accurately represent the phenotype of latently-infected B cells *in vivo*, which have a non-activated (ie: CD23$^-$) phenotype (45). EBNA-2 induces the expression of CD23 in B-LCL (96); since EBNA-2 is not expressed in the latency II program, it would be expected to lead to the CD23$^-$ phenotype.

2. Diseases associated with EBV.

EBV has been associated with malignancy since its initial detection in Burkitt's lymphoma (BL) cells (16). It has since been associated with a variety of benign and malignant lymphoproliferative disorders of B lymphocyte and epithelial cell origin, including IM, oral hairy leukoplakia, nasopharyngeal carcinoma (NPC), and B cell lymphoproliferative diseases in immunodeficient hosts (2). More recently EBV has also been detected in T cell lymphomas (13), Hodgkin's lymphoma (19) and several tumors of non-lymphoid and non-epithelial cell origin (37, 42).

EBV is consistently found in association with two types of B lymphocyte tumors: endemic BL and immunoblastic lymphoproliferative disease (LPD)

occurring in immunocompromised patients. In BL, host immune control of EBV infection is believed to be suppressed by pre-existing malaria; the sporadic form of BL, which occurs in areas where malaria is not endemic, is usually negative for EBV. Patients susceptible to EBV-associated LPD include those with congenital immunodeficiency diseases, recipients of allogeneic organ transplants receiving immunosuppressive therapy, and patients with AIDS (5, 21, 55, 56, 71, 81).

Several characteristics distinguish BL from EBV-associated LPD: BL is monoclonal; BL cells express CD10 (CALLA), an antigen found on immature B cells and certain leukemias and do not express CD23 or the EBV latent proteins, with the exception of EBNA-1; expression of MHC antigens and many adhesion molecules is very low to absent. Finally, BL cells always carry a chromosomal translocation involving *c-myc*. Many of these features appear to allow BL to elude host immunosurveillance mechanisms. In contrast, EBV-associated LPD is usually, but not always, oligo- or polyclonal, and it expresses cell surface and latent viral antigens in a pattern that is very similar to B-LCL. *C-myc* translocations are not found in LPD. BL is also easily distinguished from LPD by its distinctive morphology.

3. Immune responses to EBV.

a. The immune reponse in acute infectious mononucleosis. The cellular immune response to primary EBV infection during acute IM is dominated by a highly activated and expanded population of "atypical" lymphocytes (76). These cells are primarily of the $CD3^+/CD8^+$ cytotoxic/suppressor T cell phenotype and are unusual in their apparent lack of specificity for virus and restriction by host MHC antigens (75, 90). Rigorous analysis of T cell subpopulations in acute IM PBMC has revealed the presence of distinct alloreactive and EBV-specific components (87). Cytotoxic T cell (CTL) responses to other human viruses such as cytomegalovirus (61), measles (36), mumps (35), and influenza (91), are typically MHC-restricted and virus specific. Generation of virus-specific CTL is only one of several potential cellular immune responses to these infections; the importance of CTL in protecting the host from disease is not clear in every case. For example, in acute measles virus infection, NK cells represent a major component of the cytotoxic response (33).

b. The immune response in latent infection. *In vitro* stimulation of peripheral blood mononuclear cells (PBMC) from latently-infected (ie: EBV-seropositive) individuals with autologous EBV-transformed B-lymphoblastoid cell lines (B-LCL) usually results in the induction of EBV-specific, MHC class I-restricted CTL. This *in vitro* stimulation procedure demonstrates the presence of memory CTL in latently infected individuals.

By using vaccinia recombinants which express individual latent genes in target cells, several EBV latent gene products have been identified as target antigens for *in vitro*-stimulated EBV-specific CTL (66). The most frequent responses have been mapped to the EBNA 3 family of proteins, with others identified against EBNA-2, LMP-1 and LMP-2; no CTL responses to EBNA-1 have been identified so far. The dominance of particular antigens in the response made by individuals is often correlated with expression of certain HLA alleles; for example, the MHC class I allele HLA B8 frequently presents an epitope of EBNA-3A.

Although the EBV-specific CTL responses just described are generally believed to protect the host against EBV-associated LPD, their role has been difficult to assess directly due to the lack of an adequate animal model. The cotton-top tamarin, or marmoset, is the only primate species known to reliably produce LPD on exposure to EBV (77); however, the fact that it is a protected species severely limits its use as an experimental model.

B. The severe combined immunodeficient (SCID) mouse.
1. The *scid/scid* mutation.

The *scid* mutation in mice was described by Bosma in 1983 (6). The mutation arose spontaneously in the C.B.-17lcr inbred strain, which is an immunoglobulin congenic partner of BALB/c. The mutation gave rise to a severe combined immunodeficiency (SCID) syndrome with many of the features of human SCID, though for different reasons, as was later discovered. A C.B.-17 strain homozygous for *scid* (C.B.-17 *scid/scid*) was established by selective breeding; the mutation segregated as a single autosomal recessive gene which has since been mapped to the centromeric end of chromosome 16 (7).

2. The SCID mouse phenotype.

C.B.-17 *scid/scid* mice, or SCID mice, fail to generate T and B lymphocyte antigen receptors, apparently because of a defect in double-stranded DNA repair (24). As a result, they are severely deficient in humoral and cell-mediated immunity. SCID mice are unresponsive to T cell-dependent and T cell-independent antigens, and their spleen cells fail to proliferate in mixed lymphocyte culture or in response to mitogens. They accept allogeneic skin grafts, and as Mosier first described in 1988, they also accept grafts of human peripheral blood mononuclear cells (PBMC) (47).

C. The hu-PBMC-SCID mouse.

1. Engraftment of human PBMC in SCID mice.

Reconstitution of SCID mice with human PBMC was accomplished by Mosier, et al. by injecting the cells into the peritoneal cavity (47). The majority of "reconstituted" mice, or hu-PBMC-SCID mice, spontaneously produced human immunoglobulin (HuIg) after receiving $2x10^7$ or more cells. Transfer of a critical number of cells was found to be required for detectable levels of HuIg to be made, for reasons that are unclear. One possibility is that a large number of cells is needed to overcome resistance to engraftment by intact components of the murine immune system, ie: many of the transferred cells do not survive for long in the murine host. Varying degrees of low level graft versus host disease (GVHD) have been reported (30, 34), but generally it does not appear to pose a significant problem.

Studies of the distribution and phenotype of human cells persisting in hu-PBMC-SCID mice have yielded wildly variable results (29, 40, 84). The predominant cell type has consistently been identified as single positive T cells; most groups report detecting fairly low percentages of these cells (<10%) outside of the peritoneal cavity(30, 84). The majority of T cells recovered from lymphoid organs two weeks or more after engraftment express the CD45RO+, or memory cell, phenotype (88). Most of these cells also express MHC class II (DR) antigens, which is indicative of an activated phenotype (29). Primary and secondary humoral and cellular immune responses to several antigens have been demonstrated in the hu-PBMC-SCID mouse, but are mostly very weak (39, 74, 79, 88).

The hu-PBMC-SCID mouse has been used extensively to study the pathogenesis of human infectious and autoimmune diseases (34, 44, 48, 72, 88, 92). It also provides a small animal model in which to study mechanisms of T cell control of EBV-associated LPD.

2. EBV-associated LPD in hu-PBMC-SCID mice.

Reconstitution of SCID mice with 50×10^6 or more PBMC from EBV-seropositive donors has been found to lead to the development of B cell lymphomas of human origin (46, 47). The initial report of this phenomenon suggested that these tumors resembled BL (47), but this was not borne out in subsequent studies. Several groups have since reported that these lymphomas are always positive for the EBV genome, are usually oligoclonal, and express a pattern of latent viral and cell surface antigens that mirrors those found on B-LCL (12).(49, 52, 57, 58). Tumor morphology has been repeatedly reported to be immunoblastic rather than BL-like. These findings have made it clear that the tumors which arise in hu-PBMC-SCID are remarkably similar to EBV-associated LPD arising in immunocompromised human hosts, and are not in most cases related to BL.

3. The NOD/LtSz-*scid/scid* mouse.

The apparently low level of human cell engraftment in hu-PBMC-SCID mice has prompted efforts to improve the model. Treatment of mice with irradiation or anti-asialo GM1 antibody before reconstitution to deplete NK cell activity has been partially successful in improving engraftment (11). This finding suggests that host inate immunity influences human cell engraftment, and led to an examination of the effect of host background strain on engraftment in mice with the *scid* mutation (20, 28).

The NOD/Lt inbred mouse strain is deficient in NK cell, macrophage, and complement activities (78); this strain was used as genetic stock onto which the *scid* mutation was backcrossed to create the NOD/LtSz-*scid/scid* mouse (hereafter referred to as NOD-SCID) (78). NOD-SCID mice lack T and B lymphocyte function and have the defects in inate immunity which accompany the NOD/Lt background strain (Table 1). In a comparison of four inbred mouse strains homozygous for the *scid* mutation, the highest level of PBMC engraftment were seen in the NOD-SCID strain (28). The majority of engrafted cells were found to have a CD3$^+$ (T cell) phenotype. The NOD-SCID

mouse therefore provides an improved model for studies of T cell function in human diseases.

D. Objectives of the study.

The primary objectives of the study which constitutes this thesis were 1) to determine the degree to which the tumors arising in hu-PBMC-SCID mice resembled either BL or immunosupression-induced LPD and 2) to demonstrate whether or not EBV-specific CTL could prevent EBV-associated LPD in the hu-PBMC-SCID mouse. The first objective was achieved by others and published during the course of the project. My results and conclusions are consistent with those published and are included here.

The results of experiments addressing the second objective were not definitive, and led to additional studies using the PBMC-reconstituted NOD-SCID mouse. The objectives of these experiments were: 3) to determine whether reconstituted NOD-SCID mice were susceptible to EBV-expressing LPD; and 4) to determine the role of CD8$^+$ human T cells in preventing LPD in this model. In the process of addressing these objectives, an important role for CD4$^+$ T cells in the NOD-SCID model was discovered.

Table 1. Immunologic defects in NOD/LtSz-SCID mice.

Immune function:	Mouse strain:		
	C.B.-17-SCID	NOD/LtSz-SCID	NOD/Lt
Mature B and T lymphocytes	no	no	yes
NK cell function	normal/high	low (endogenous and induced) decreased	low (endogenous and induced) decreased
APC[1]	normal		
Serum C' activity	normal/high	absent	absent
Reconstitution of spleen with human T cells	low (<10% of spleen cells)	high (>25% of spleen cells)	N A
Spontaneous IDDM	no	no	yes[2]

[1]Antigen presenting cell (APC) function was assessed LPS-stimulated IL-1 secretion by BM-derived macrophages.

[2] Insulin-dependent diabetes mellitus (IDDM) in NOD/Lt mice is T cell dependent and T cell mediated.

CHAPTER II. MATERIALS AND METHODS

A. SCID mice

C.B.17-*scid/scid* (C.B.17-SCID) and NOD/LtSz-*scid/scid* (NOD-SCID) mice were bred in the University of Massachusetts Medical Center breeding colony, which is maintained under specific-pathogen free (SPF) conditions. C.B.17-SCID breeding pairs used to establish the colony were provided by Dr. Donald Mosier and the Medical Biology Institute, La Jolla, CA. NOD-SCID breeding pairs were provided by Dr. Leonard Shultz, the Jackson Laboratory, Bar Harbor, ME. Mice were 6-12 weeks old when used in experiments. Age matched mice of either sex were used. Experimental animals were maintained under SPF conditions.

B. PBMC donors.

PBMC were obtained from healthy, EBV-seropositive volunteer donors as by-products of plateletpheresis (Model 30, Haemonetics, Inc. Braintree, MA) in the University of Massachusetts Hospital blood donor room or as heparinized blood by standard venipuncture. Heparinized blood from acute IM patients was obtained from the Student Health Services at the University of Massachusetts, Amherst. All patients had clinical and laboratory evidence of acute IM, with fever, sore throat, lymphocytosis, splenomegaly and at least 20% atypical lymphocytes on a differential white blood cell count. All patients were positive for heterophile antibody by the Monospot test. Acute IM blood was obtained within five days of the first positive Monospot test and within ten days of the onset of symptoms. Written informed consent was obtained from each patient before entry into the study.

C. Isolation of donor lymphocytes.

PBMC from pheresis of peripheral blood were isolated under sterile conditions by density gradient centrifugation using Ficoll-Hypaque (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Cells were recovered from the interface and washed twice with phosphate-buffered saline (PBS). The cells were then examined for viability by trypan blue dye exclusion and the lymphocytes enumerated. PBMC that were to be injected into SCID mice were

suspended in PBS at a concentration of 1×10^8/ml. Blood from acute IM patients was diluted in RPMI1640 medium supplemented with 20U/ml human recombinant IL-2 and incubated at 37°C overnight. PBMC were isolated from the acute IM blood the next morning according to the described protocol.

D. Reconstitution of SCID mice.

C.B.17-SCID or NOD-SCID mice were injected intraperitoneally (i.p.) with 5-6×10^7 PBMC within 2 hr of isolation. From 2 to 13 mice (mean = 5 mice) were reconstituted with each acute IM donor, and from 2 to 32 mice (mean=15 mice) were reconstituted with each latently-infected PBMC donor. PBMC from some donors were used to reconstitute mice in multiple experiments whereas other donors were used only once. Blood was collected from the tail vein two to six weeks after the PBMC injection and by cardiac puncture at the time of terminal sacrifice, and assayed for the presence of human immunoglobulin (HuIg) by one of the methods described below. Mice with serum HuIg levels of at least 1 ug/µl were considered to be successfully reconstituted. Individual mice were excluded from the analyses if: (1) serum HuIg was < 1.0 µg/µl (37/240 C.B.17-SCID mice), or (2) the mouse was not examined or could not be evaluated immediately after its death. In addition, 10% of mice that were not tested for HuIg and did not develop LPD were excluded (one mouse); this represents the proportion of mice predicted to be negative for HuIg. Only 1/116 C.B.17-SCID mice which developed LPD and were tested for HuIg was negative for HuIg (0.86%).

E. Immunoglobulin assays.

1. Murine immunoglobulin.

Mice were bled from the tail vein before being used in reconstitution experiments, and the serum was tested for levels of murine immunoglobulin (MuIg) using a commercial semi-quantitative, filter-based enzyme-linked immunoassay kit (Mouse Ab-Stat, Sang-Stat Medical Corp, Menlo Park, CA). The assay was scored as: negative = 0-1ug/ml, +/- = 1-5 ug/ml, 1+ = 5-10 ug/ml, 2+ = 10-50 ug/ml, 3+ or 4+ = >50 ug/ml. Mice with greater than 1 ug/ml of MuIg were considered to be "leaky" (8) and were not used in reconstitution experiments.

2. Human immunoglobulin.

Two methods were used to measure human immunoglobulin levels in post-reconstitution SCID mouse sera. For testing of the C.B.17-SCID mice, a semi-quantitative immunoassay similar to the mouse Ig test was used (Human Ab-Stat, Sang-Stat Medical Corp, Menlo Park, CA). When this kit later became unavailable, an in-house quantitative competetive ELISA was used to test most of the NOD/LtSz-SCID sera (32) Briefly, assay plates (Nunc microwell) were coated with 50ml of human gamma globulin (Cappel) at 0.25mg/ml in PBS containing 0.05% sodium azide for 2 hr at 37°C, washed with ddH_2O and blocked for 30 min. at room temperature with borate-buffered saline containing 0.05% Tween 20 and 0.25% bovine serum albumin. HuIg standard (Cappel) was serially diluted 1:3 to create a range of 0.4 mg/ml to 100 mg/ml. Test samples, controls and standards (30-50 ml) were mixed 1:1 with 2x alkaline phosphatase-conjugated goat anti-human Ig antibody (1:5000 dil.) in a 96-well PVC assay plate and incubated for 30 min. at RT. Fifty ml of the sample plus conjugate mixture was added to the Ag-coated plates and incubated 2 hr at room T. Plates were washed 3 times with ddH_2O and 75ul of 3 mM NPP substrate was added (Bio-Rad). Plates were read at 405 nm on a microtiter plate reader after 1 hr. HuIg concentrations were calculated by construction of a standard curve.

F. Termination of experiments.

1. Tissue harvest.

At scheduled timepoints or when moribund, reconstituted mice were anesthetized with ketamine-rompun, bled by cardiac puncture and euthanized by cervical dislocation. Autopsies were performed to confirm the presence of LPD, and tumor tissue was excised and preserved in 10% neutral buffered formalin (NBF) and/or by quick freezing in liquid nitrogen. To assess human PBMC engraftment and *in vivo* depletion, peritoneal cells were collected by open lavage with 5-6 ml PBS, and spleens were harvested. Cells recovered by lavage and spleen cells were analyzed for human lymphocyte surface markers by flow cytometry.

2. Histopathology.

Tissues were fixed in 10% NBF and processed for histopathology by standard automated processing and paraffin embedding procedures. Sections

were cut at 5-6m thickness and stained with hematoxylin-eosin for microscopic examination. Additional slides were cut from some tumor tissue for standard immunohistochemistry and stained with immunoperoxidase-conjugated anti-CD45 monoclonal antibody (this procedure was performed in the laboratory of Dr. Leonard Shultz, the Jackson Laboratory, Bar Harbor, ME).

G. DNA Analysis.

    1. Isolation of tumor DNA.

Genomic DNA was isolated from quick-frozen tumor tissue using a modification of a previously described method (67). The frozen tissue was homogenized to a cell suspension in buffer containing 10 mM Tris, 10 mM EDTA and 100 mM NaCl using a tissue homogenizer, followed by the addition of an equal volume of buffer containing 0.4mg/ml proteinase K and 1% SDS, and was incubated for 1 hour at 37°c. Cellular DNA was purified by three 25:24:1 phenol/chloroform/isoamyl alcohol extractions and ethanol precipitation followed by micro-centifugation.

    2. Southern blotting.

DNA samples were digested with restriction endonucleases and buffers obtained from Stratagene (LaJolla,CA). DNA was digested with BamHI for hybridization with the EBV-BamW and BamNJ probes, or with HindIII for detection of Ig J_H gene rearrangements. Restriction digests were separated on 0.75% agarose gels at 1.0 V/cm in Tris-acetate buffer (40 mM Tris-acetate, 1 mM EDTA) for 16-18 hours. For the J_H analysis, the gels was denatured, neutralized and blotted onto nylon membranes according to the manufacturer's protocols (Oncor, Inc., Gaithersburg,MD). The immobilized DNA was preincubated in a 50% formamide prehybrization solution (Hybrisol I, Oncor, Inc.). Analysis of the EBV genome was according to standard Southern blot methods (73). All probes were labeled with ^{32}P-dCTP by the random primer method (random primer labeling kit, Bio-Rad Laboratories, Richmond, CA), and washed according to standard or product (Oncor) protocols.

    3. Probes.

The Ig J_H probe used to detect rearrangments of Ig heavy chain J region genes is a commercially-available 5.6 kB BamHI-HindIII fragment of the human immunoglobulin J_H gene (Oncor,Inc.) (63). The EBV-BamW probe is a

3.1 kb BamHI fragment from the EBV internal repeat (Bam W) in pBR322 (a gift from E. Kieff) (25). The BamNJ probe, used to distinguish circular and linear episomal forms of the EBV genome and to corroborate tumor clonality, is an 8.4kb BamHI fragment containing the terminal repeats inserted into pUC18 (62, 95).

H. Flow cytometry.

Single cell suspensions of tumor cells were prepared by teasing apart fresh tumor tissue with two scalpel blades in PBS or RPMI 1640 medium. Spleen cells were prepared by dissociation between the frosted ends of 2 glass microscope slides and passage through a tissue strainer (Falcon Cell Strainer, Becton Dickinson Labware, Lincoln park, NJ). Red blood cells were lysed with a hypotonic ammonium chloride solution. The remaining nucleated cells were washed in PBS, examined for viability by trypan blue dye exclusion and lymphocytes counted. The cells were then stained with fluorescein (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies (mAbs) according to the manufacturer's instructions, either immediately or after several weeks in culture. MAbs which recognize CD45 (anti-HLe-1), CD3 (anti-Leu 4), CD4 (anti-Leu-3a), CD8 (anti-Leu-2a), CD19 (anti-Leu-12), CD20 (anti-Leu-16), CD23 (anti-Leu-20), and CD10 (anti-CALLA, common acute lymphoblastic leukemia antigen) (Becton-Dickinson, San Jose, CA) were used for direct immunofluorescent staining, and unlabelled anti-CD30 (anti-Ki-1, DAKO Corp., Carpinteria, CA) and FITC-labelled goat anti-mouse Ig (Coulter Immunology, Hialeah, FL) were used for indirect staining. Cell surface phenotypes were determined by flow cytometric analysis using a FACScan instrument (Becton-Dickinson).

I. Cell lines.

1. B-lymphoblastoid cell lines.

B-lymphoblastoid cell lines were initiated from donor PBMC by induction with supernatant from the EBV-producing cell line B95.8 (50). PBMC were cultured at a concentration of $1-2 \times 10^6$/ml in 48-well tissue culture plates. Culture medium was RPMI 1640 medium supplemented with 15% fetal bovine serum (FBS) (RPMI/15%), 10% B95.8 supernatant and 2 ug/ml cyclosporin A; the cultures were fed twice weekly with RPMI/15%, and observed for clusters of proliferating cells. After approximately 3-4 weeks in culture, the B-LCL were usually well established and were transferred to 25 cm^2 tissue culture flasks and maintained at a concentration of $4-8 \times 10^5$ cells/ml. B95.8 supernatant was produced by seeding B95.8 cells at a concentration of 4×10^5/ml and culturing for ten days in RPMI/15% without feeding, and harvesting the supernatant.

The supernatant was centrifuged in 50 ml polypropylene centrifuge tubes at 1000xg for 20 minutes three times to remove cellular contamination, and frozen in aliquots at -80°C.

2. Cytotoxic T lymphocyte lines.

All CTL lines were initiated with PBMC which had been depleted of NK cells by incubating with anti-CD16 mAb (Leu 11b, Becton Dickinson) for 30 min. on ice, washing once with PBS, and incubating for 45-60 min. at 37°C with rabbit complement (Cedarlane Laboratories, Hornsby, Ontario) diluted 1:3-1:5 with RPMI.

a. Generation of EBV-specific CTL. EBV-specific cytotoxic T cell lines were generated by culturing NK cell depleted PBMC with mitomycin c-treated autologous B-LCL at a ratio of 40:1 in RPMI/15%, in 24-well tissue culture plates. Cultures were restimulated weekly with autologous B-LCL, at a responder to stimulator ratio of 5:1, and were fed with 1/2 volume fresh medium twice weekly. Beginning at day 10 and thereafter, the medium was supplemented with 20U/ml recombinant human interleukin-2 (rhIL-2, Boehringer Ingelheim). Cell lines were tested for surface phenotype and cytotoxic activity after 3-4 weeks in culture.

b. Generation of alloreactive CTL. MHC class I alloreactive cytotoxic T cell lines were generated by culturing NK cell-depleted PBMC with mitomycin c-treated allogeneic B-LCL at a ratio of 40:1 in RPMI/15%, in 24-well tissue culture plates. Cultures were restimulated weekly at a responder to stimulator ratio of 5:1 with allogeneic B-LCL and fed twice weekly. Beginning at day 10 and thereafter, the medium was supplemented with 20U/ml rhIL-2. Cell lines were tested for surface phenotype and cytotoxic activity after 3-4 weeks in culture. Allogeneic stimulator cells were always mismatched for all identified class I alleles, to avoid the possibility of stimulating an EBV-specific cytotoxic response which was restricted to an allele shared by the responder and the stimulator. Tissue typing of cell lines was performed by the University of Massachusetts Hospital HLA laboratory.

J. Cytotoxicity assay.

The cytotoxic activity of the CTL lines against various target cells was determined in standard ^{51}Chromium-release assays performed immediately

prior to injection into mice. Target cells were autologous B-LCL, HLA class I-mismatched B-LCL or single cell suspension of tumor cells from a hu-PBMC-SCID mouse. Tumor cells originated from the PBMC of the same individual from whom the CTL were generated. Target cells were incubated with 100 mCi/10^6 cells of ^{51}Cr (as sodium chromate; DuPont-NEN) for 1 hr at 37°C, washed three times and suspended at a concentration of 1×10^5 cells/ml in RPMI/15%.

    1. Target cells.

Target cells (100ml) were added to wells of 96-well culture plates. The concentration of CTL was adjusted to 2×10^6/ml, and appropriate dilutions made to achieve the effector to target ratios used in the assay. The diluted CTL were added to wells in 100ml volume. The plates were centrifuged for 3 min. at 500xg and incubated for 4 hr at 37°C, after which 100ml of supernatant was harvested and counted in a gamma counter. Maximum ^{51}Cr release was determined by counting 50ml of labeled target cell suspension. Spontaneous ^{51}Cr release was determined by incubating target cells with culture medium alone. Percent specific lysis was calculated as: 100 x (experimental cpm - spontaneous release cpm)/ (maximum release cpm - spontaneous release cpm). CA-Cricket Graph III software (version 1.5.2; Computer Associates International, Inc., Islandia, NY) was used to plot % specific lysis for Figures 8-10.

    2. Calculation of lytic units.

Relative cytolytic activity of the CTL lines was determined by calculating lytic units (L.U.) as previously described (97). Percent specific lysis values were plotted versus the log of the effector cell number. A lysis value of 50% was used to determine the number of lytic units per 10^6 effector cells. CA-Cricket Graph III software was used to generate L.U. plots.

K. Adoptive transfer of CTL lines.

The number of CTL injected for each twice-weekly treatment was determined by the calculated number of L.U. per 10^6 cells. Each CTL injection contained approximately 100-200 L.U. of activity against either autologous B-LCL (for EBV-specific CTL) or allogeneic B-LCL (for alloreactive CTL). CTL were

washed and suspended in an appropriate volume of PBS for each mouse to be injected with 0.25-0.5 ml volume.

L. Production and administration of monoclonal antibodies.
    1. Hybridomas.

For *in vivo* depletion of human lymphocyte subsets, monoclonal antibodies were produced as ascites in the peritoneal cavity of C.B.-17 scid mice. The OKT4 (CRL 8002) hybridoma secretes an IgG2b anti-CD4 mAb, and the OKT8 hybridoma (CRL 8014) secretes an IgG2a anti-CD8 mAb; both were purchased from the American Type Culture Collection (ATCC). The B73.1 hybridoma secretes an IgG1 anti-CD16 antibody, and was a gift from Dr. Giorgio Trinchieri of The Wistar Institute (Philadelphia, PA) The S-S.1 hybridoma secretes an IgG2a anti-SRBC mAb (used as a control), and was also purchased from ATCC (#TIB111). Hybridoma cells were expanded in tissue culture flasks in the medium specified by the supplier. In general, medium was supplemented with b-mercaptoethanol and sodium pyruvate in addition to the routine growth medium supplements. Cells were maintained at a density of 1-$2x10^5$/ml, fed with fresh medium, and expanded until numbers were sufficient to inject into mice for ascites production.
    2. Production of ascites.

Hybridoma cells were harvested while in log phase growth, washed with PBS, and injected i.p. into C.B.17-SCID mice which had been primed with 0.5ml of pristane 10 days earlier. Between 0.5 and $5x10^6$ cells were injected into each animal. After 10-14 days, when the mice exhibited signs of ascites accumulation, the ascites was tapped with a 20-22 gauge Vacutainer needle into sterile test tubes. Ascites was tapped over several days and those containing the same Ab were pooled. Mice were sacrificed by cervical dislocation.
    3. Antibody purification and administration.

MAb was purified from pooled ascites by caprylic acid precipitation of non-Ig proteins followed by ammonium sulfate precipitation of Ig. Precipitated Ig was dissolved in PBS and dialyzed against PBS to remove residual salts. A sample of the purified Ab was analyzed by SDS-PAGE to determine the degree of purity. All Ab preparations were at least 90% pure Ig. Concentration was

determined spectrophotometrically. Abs were filter sterilized before injecting 0.2-0.5 mg i.p. into reconstituted SCID mice.

4. *In vivo* depletion.

To deplete subsets of human lymphocytes *in vivo*, SCID mice of either strain were injected with OKT4, OKT8 or B73.1 mAbs 0 to 3 days after after PBMC reconstitution. In one experiment, Ab was injected six weeks after PBMC reconstitution (Fig. 12). In some depletion experiments, control mice were injected with the IgG2a control mAb S-S.1.

M. Isolation and adoptive transfer of T cell subsets.

Purified populations of T cells were obtained by immunomagnetic isolation using mAb-coated beads (Dynabeads M-450, Dynal A.S., Oslo, Norway). PBMC were positively selected for CD4$^+$ or CD8$^+$ cells with the Ab-coated beads according to the manufacturer's instructions, and the cells detached from beads using the Detach-a-bead reagent (Dynal). The resulting populations were greater than 95% pure CD4$^+$ and CD8$^+$ cells when assessed by flow cytometry.

N. Statistical analyses.

Kaplan-Meier estimates of cumulative survival and logrank (Mantel-Cox) comparison were calculated using Statview and Survival Tools for StatView software (Abacus Concepts, Inc., Berkley, CA). P-values for significance of tumor frequency was calculated by Chi-square analysis, also using StatView.

CHAPTER III. RESULTS

A. Experiments in C.B.17-SCID mice.

    1. Immune reconstitution of C.B.17-SCID mice with peripheral blood mononuclear cells from individuals with acute or latent Epstein-Barr virus infection.

    Immune reconstitution of C.B.17-SCID mice consisted of intraperitoneal injection of 5×10^7 human PBMC. The PBMC donors belonged to one of the following groups: (1) individuals with serological evidence of past EBV infection (17 donors; these will be referred to as latently infected), or (2) patients in the acute phase of infectious mononucleosis (32 donors; referred to as acutely infected or IM). PBMC from the acutely infected donors contain higher percentages of activated CD8 T lymphocytes and a 10- to 1000-fold higher percentage of EBV-infected B lymphocytes than PBMC from latently infected individuals (45, 89, 99). Several potential indicators of human PBMC engraftment were investigated, including measurements of human cell numbers in peripheral blood, peritoneal wash and spleen, and human immunoglobulin in serum. Flow cytometric analysis four to eight weeks after reconstitution revealed significant numbers (>5%) of human cells consistantly present only in the peritoneal cavity (data not shown). Occasionally, larger numbers of human cells were recovered from spleens.

    a. Production of human immunoglobulin. Injection of C.B.17-SCID mice with human PBMC resulted in the spontaneous secretion of human immunoglobulin (HuIg), which could be detected in the serum by two weeks (Fig. 1). Mice with HuIg levels of 1 μg/μl or higher were considered to be successfully reconstituted. Successful reconstitution occurred in 90% of mice injected with PBMC from latently-infected donors and 75% of mice injected with PBMC from acutely-infected donors.

    b. Incidence of lymphoproliferative disease. LPD of human B cell origin developed in 216 of 314 PBMC-reconstituted SCID mice (Table 2). Lesions consisted of single or multiple-site tumors of lymphoid organs, liver, lung, heart and/or muscle. The incidence of LPD was similar (p=0.29) in mice reconstituted with PBMC from acutely infected or latently infected donors. The

average time from reconstitution to the development of LPD was 11.5 weeks for both groups. Mice which developed LPD made higher levels of HuIg than mice which did not develop LPD (Fig. 1); this was presumably due to Ig secretion by the tumor cells.

PBMC donors varied considerably in their ability to generate LPD in SCID mice, with incidence of LPD ranging from 0% to 100% for individual donors (Table 3). When a single donor was used in more than one experiment, the incidence of LPD often varied between experiments. PBMC from donor B, for example, generated LPD in 83% of mice in experiment 2 and in 0% of mice in experiment 3; similar results were seen with PBMC from donor G. These results are likely to reflect changes in the number of EBV-infected B lymphocytes present in the circulation at the time PBMC were donated. All but one of the 17 PBMC donors produced tumors in one or more experiments; the donor which did not generate tumors was used in only one experiment. In experiments conducted by Dr. R. M. Hesselton, mice reconstituted with 50 x 10^6 PBMC (23 mice with two donors) or spleen cells (10 mice with one donor) from EBV-seronegative donors did not develop LPD. Mice injected with 20 x 10^6 or fewer cells from EBV-seropositive donors were also free of LPD (data not shown).

c. Characterization of LPD in hu-PBMC-SCID mice. EBV appears to be involved in the pathogenesis of two human B cell tumors. These are Burkitt's lymphoma (BL) and immunoblastic B cell lymphomas which arise as a complication of immunosuppression. To determine whether the SCID mouse tumors resembled one of these types of B cell tumors, cells from hu-PBMC-SCID mouse tumor masses were analyzed for surface antigen phenotype, presence and clonality of the EBV genome, tumor cell clonality, and histopathology.

i. Expression of cell surface antigens. The pattern of surface antigen expression on hu-PBMC-SCID mouse tumors were similar to those expressed on B-lymphoblastoid cells (Table 4A and B). However, the levels of expression of antigens found on resting B cells (CD19 and CD20) and of B cell activation antigens (CD23 and CD30) were less intense on the tumor cells than would be expected on B-LCL (Table 4B). The absence of CD10 expression is significant, because CD10 (or CALLA) is strongly expressed on BL cells but not B-LCL (Table

4A)(69). The SCID mouse tumors are further distinguished from BL by their strong expression of MHC class II antigens, which are weakly expressed in BL.

ii. Presence of the EBV genome and replicating viral forms. The EBV genome could be demonstrated in all of the SCID mouse tumors examined using Southern blot analysis with the BamW probe. Because it detects internal repeat sequences (Fig. 2), this probe is highly sensitive for detection of the EBV genome. A representative group of tumors is shown in Figure 3, including four originating from acute IM PBMC (lanes 2, 3, 8 and 9) and five from latently-infected PBMC (lanes 1, 4, 5, 6 and 7).

The EBV genome exists in two distinct forms: during latency it is circular, and when replicating and in complete virions it is linear. Circularization occurs by the joining of tandem repeat sequences, or terminal repeats (TRs) at each end of the linear genome. The number of TRs varies among EBV episomes, resulting in different numbers and sizes of terminal repeat fragments (Fig. 2). Analysis of the terminal fragments by Southern blot can be used to distinguish circular and linear genomes and to establish the clonality of tumors carrying the EBV genome. The BamNJ probe hybridizes to sequences on both terminal fragments, and therefore detects linearized and circular forms of the genome. The fused termini of circularized genomes yield high molecular weight bands (8-21 kb) on Southern blot analysis; the number of these bands correlates to the clonality of the tumor with respect to the number of terminal repeats in the viral genome. The linearized termini of relicating genomes result in a ladder of smaller bands (4-6 kb) which differ in size by 0.5 kb increments.

Analysis of tumor DNA using the BamNJ probe revealed the presence of linear (replicating) forms in 5 of 9 tumors originating from 9 different PBMC donors (Fig.4,A and B). Two of 5 tumors from mice reconstituted with PBMC from donors with latent infection, and 3 of 4 tumors from mice reconstituted with PBMC from donors with acute IM exhibited linear (or replicating) forms of virus. The presence of replicating virus does not appear to be correlated with either latent of acute infection in the PBMC donor. As expected, all of the tumors analyzed also contained circularized EBV genome.

Most of the tumors studied (8/9) had evidence of multiple EBV episomes (ie: contained more than one fused terminal band). Of these eight, two had a

single dominant band (tumors 6 and 9) with multiple fainter bands, while the others had two or more (tumors 1-5 and 8) . One tumor from a mouse reconstituted with an acute IM donor, appeared to have a single species of EBV present (tumor 7). These results are consistent with the interpretation that the majority of tumors arising in hu-PBMC-SCID mice are oligoclonal, with occasional monoclonal lesions. It has yet to be proven, however, whether determinations of tumor clonality based on EBV TR analysis are perfectly correlated with those based on pathologic criteria such as morphology and Ig gene rearrangements.

iii. Clonality and histopathology. To confirm their clonality, the tumors shown in Figure 4 were also analyzed by Southern blot using a probe which detects rearrangements of the Ig J_H locus. Tumors which were oligoclonal for the EBV-TR also had multiple bands on the JH blot, and these were less distinct than the others (tumors 1, 4, 5, 6, 8 and 9). Tumor 7, which was monoclonal for the EBV-TR, had two distinct rearranged bands plus one germline-sized band on the JH blot, which is consistent with monoclonality. The other two tumors which had distinct rearranged bands on the JH blot (tumors 2 and 5) had single dominant bands with the BamNJ probe; these results are consistent with each other and with an interpretation of a single dominant clone of B cells in the tumor, with perhaps several minor populations of EBV-infected cells present as well.

The germline-sized bands which are seen in two of the tumors (tumors 3 and 5) are probably due to human T cells infiltrating the tumors. Most circulating B lymphocytes have rearranged J_H genes on both chromosomes (14) (1). This would explain the faintness of the bands for tumor 3 on the BamNJ blot, since T cells are not likely to be infected with EBV.

Histologically, the tumors typically had the appearance of immunoblastic large cell lymphomas, with some plasmacytic areas and small lymphocytes (Fig. 6, A and B). The morphology of the hu-PBMC-SCID tumors was very similar to what is reported for LPD in immunosuppressed patients. Surprisingly, only one tumor was found to have a unique Burkitt's lymphoma-like morphology (Fig. 6C). Tumors from mice reconstituted with PBMC from donors with acute IM or from latently infected donors were morphologically indistinguishable from each other.

Table 2. Incidence of LPD in hu-PBMC-SCID mice reconstituted with PBMC from EBV-infected donors (C.B.17-SCID strain).

Type of PBMC EBV infection:	mice with LPD/total	% with LPD	x^2 p value[1]	mean days to LPD (S.E.)
acute IM	77/118	65.3		83 (2)
			0.294	
latent	139/196	70.9		83 (3)
total	216/314	69%		

[1]x^2 p value compares percentages of mice developing LPD after reconstitution with PBMC from donors with acute IM or latent EBV infection.

Table 3. Donor-related variation in the incidence of LPD in hu-PBMC-SCID mice (C.B.17-SCID strain).

Donor	Experiment	Mice developing LPD/total	% LPD
A		3/4	75
B	1	4/8	50
	2	5/6	83
	3	0/3	0
C		3/4	75
D		3/4	75
E		6/7	86
F		3/4	75
G	1	3/5	60
	2	0/7	0
	3	0/6	0
H		2/2	100
I	1	6/6	100
	2	4/5	80
	3	7/9	78
J		1/4	25
K		5/5	100
L		10/11	91
M		3/5	60
N		9/13	69
O		0/9	0
P	1	21/22	95
	2	6/10	60
Q	1	12/12	100
	2	17/18	94
	3	6/8	75

Table 4. Surface phenotype of B-LCL, BL, and hu-PBMC-SCID tumor cells.
A. Surface phenotype of EBV-transformed B-lymphoblastoid cell lines (B-LCL) and Burkitt's lymphoma cells (BL).

Antigen	B-LCL	BL
CD10 (CALLA)	-	+++
CD19 / CD20	+++	+++
CD23	+++	-
CD30	+++	-
HLA class I and II	+++	+

B. Surface phenotype of tumor masses from hu-PBMC-SCID mice (C.B.-17-SCID strain).

Surface antigen (# tumors tested)	B lymphocyte distribution	% cells expressing Ag (SE)[1]
CD45 (10)	all B lymphocytes	93.4 (1.7)
CD10 (8)	immature B cells, BL	2.9 (1.3)
CD19 (7)	mature B cells, B-LCL, BL	66.1 (10.8)
CD20 (5)	mature B cells, B-LCL, BL	33.8 (18.9)
CD23 (8)	activated B cells, B-LCL	23.9 (3.5)
CD30 (3)	activated B cells, B-LCL	37.9 (7.1)
HLA-DR (8)	B cells, B-LCL	85.8 (4.0)

[1] Suspensions of tumor cells were incubated with fluoroscein- or phycoerythrin-labeled monoclonal antibodies and analyzed by flow cytometry. Percentages of cells expressing Ag are those cells which displayed higher levels of fluorescence when stained with Ag-specific Ab than with control Ab.

Human Ig levels in hu-PBMC-SCID mice (C.B.17-SCID strain)
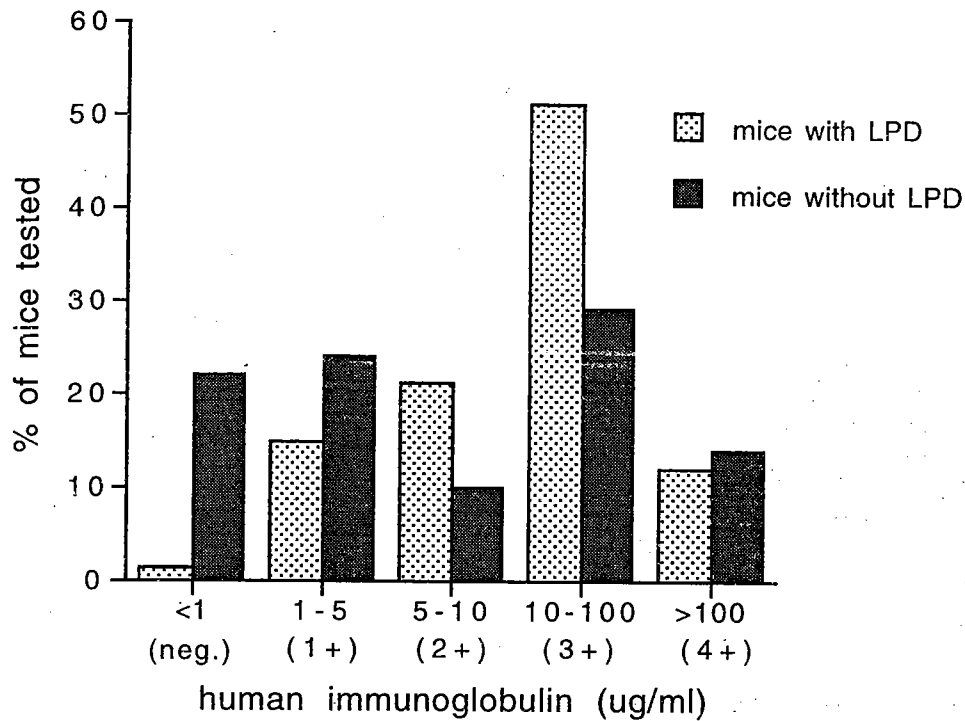
Figure 1. Human Ig levels were measured in serum of C.B.-17-scid mice two to four weeks after PBMC reconstitution, using a semi-quantitative assay described in Materials and Methods.

Figure 2. EBV genome map and BamNJ probe hybridization patterns A. Map of the EBV genome and location of BamNJ and BamW probes. B. Hybridization of linear and circular EBV episomes with the BamNJ probe demonstrates the clonality of the infection and the presence of replicating forms of virus.

LATENT EBV DNA   REPLICATING EBV DNA

Figure 3. LPD in hu-PBMC-SCID mice carries the EBV genome. DNA from hu-PBMC-SCID tumor masses was digested with BamHI and hybridized with EBV-BamW. The BamW internal repeat band is visible in the EBV-infected Raji and B95.8 cell lines. All tumor masses were from the C.B.17-SCID strain.

Murine   Raji B95.8   1   2   3   4   5   6   7   8   9

W →

Figure 4. LPD in C.B.17-hu-PBMC-SCID mice contains linear and circular forms of the EBV genome. (A) and (B) are two exposures of a Southern blot of DNA from C.B.17-SCID human LPD. DNA was digested with BamHI and hybridized with BamNJ Bands larger than 8 kb are the fused termini of circular, non-replicating EBV; 4-6 kb bands are the termini of linear, replicating EBV. The B95.8 cell line contains latent and replicating episomes. Tumors 1 through 9 represent the same tumors shown in Fig. 3, but are rearranged according to the type of PBMC donor (latent= tumors from mice reconstituted with PBMC from latently-infected donors; acute= tumors from mice reconstituted with PBMC from donors with acute IM).

Figure 5. Immunoglobulin J_H gene rearrangements in LPD from hu-PBMC-SCID mice. DNA from hu-PBMC-SCID LPD was digested with HindIII and hybridized with the J_H probe. Germline J_H is visable as an 11kb fragment in the lane labelled "T cell" (DNA from the T cell line C8166). Raji is a monoclonal Burkitt's lymphoma cell line. (Tumors 1 through 9 are in the same order as in Fig. 4.)
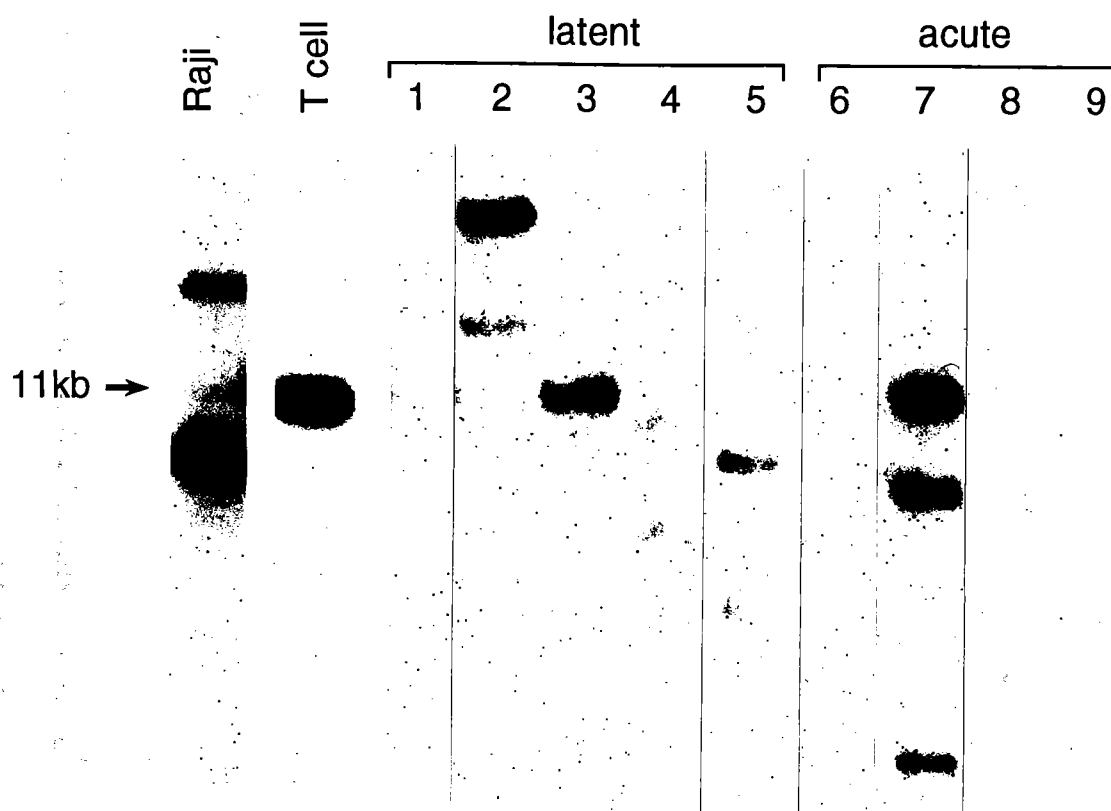
Figure 6. Morphology of representative tumors arising in hu-PBMC-SCID mice (C.B.17-SCID strain). A. A typical tumor consisting predominantly of immunoblasts (arrow); B. A minority of tumors contained areas of plasmacytic cells (arrow); C. A single mouse produced several tumors with morphology typical of BL. Magnification=400x with water immersion; bar = 5 microns.
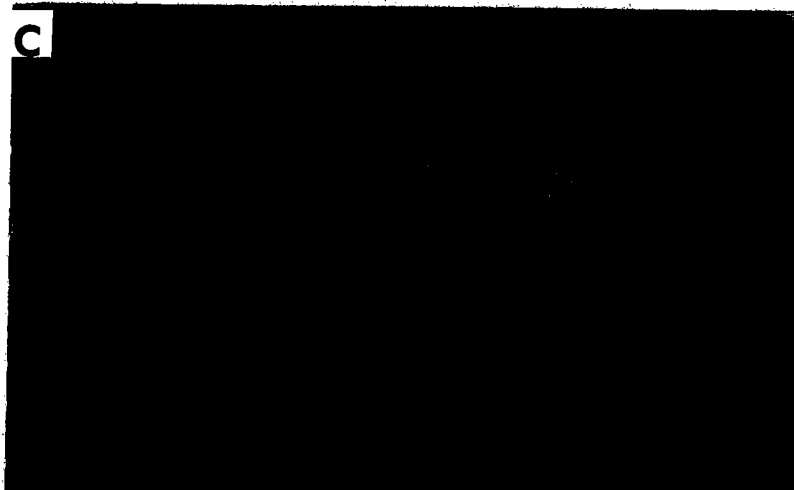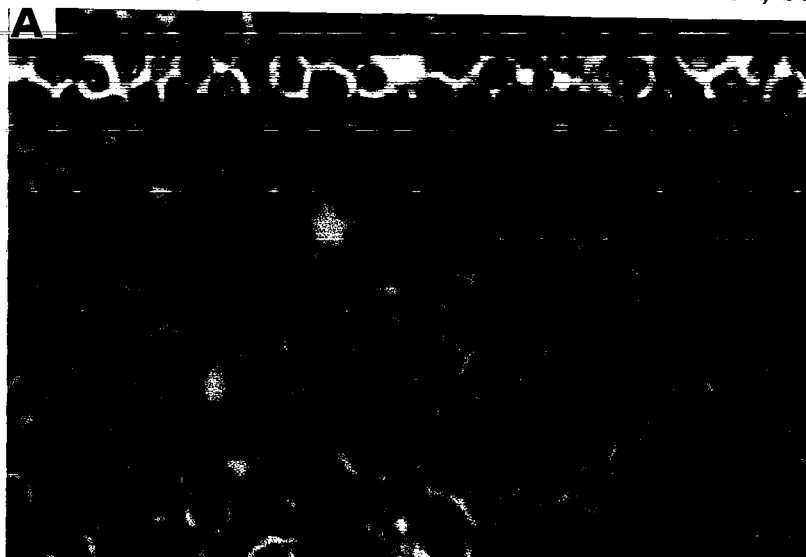
2. Adoptive transfer of cytotoxic T lymphocyte lines into hu-PBMC-SCID mice.

The preceding results indicate that reconstitution of the SCID mouse with PBMC from EBV-seropositive donors could be used as a small animal model of EBV-associated LPD. The experiments which follow were conceived and designed to answer the question: can EBV-specific CTL prevent the outgrowth (from PBMC) of EBV-positive LPD *in vivo*? The approach taken was to provide reconstituted SCID mice with adoptive immunotherapy in the form of EBV-specific CTL lines or alloreactive CTL lines.

a. Phenotype, activity and specificity of CTL lines. T cell lines with cytotoxic activity directed against the EBV latent proteins are easily established from the PBMC of an EBV seropositive individual by multiple rounds of stimulation *in vitro* with autologous B-LCL. Cytotoxic T cell lines which recognize HLA class I alloantigens can similarly be established by stimulation of PBMC with class I-mismatched B-LCL. CTL lines generated in this manner are nearly always HLA class I-restricted and CD8 positive. Flow cytometric analysis of the CTL lines used in the adoptive transfer experiments which follow demonstrate that, typically, 87% or more of the cells were CD3+ and 80% or more were CD8+ (Fig. 7). Occasionally cell lines were generated in which 50% or more of the cells were CD4+; these cells invariably exhibited very low levels of lysis in cytotoxicity assays and were not used for adoptive transfer.

The specificity of CTL lines for EBV antigens and their HLA restriction is demonstrated by their ability to recognize and lyse autologous ^{51}Cr-labelled B-LCL in cytotoxicity assays (Fig. 8). Autologous B-LCL and hu-PBMC-SCID tumor cells generated from the same individual's PBMC were lysed equally well by EBV-specific CTL. Substantially lower levels of lysis of HLA-mismatched B-LCL by these CTL indicated that most of the lytic activity is HLA restricted. Alloreactive CTL lines recognized and lysed allogeneic B-LCLs (which were also the stimulator cells) to a much greater extent than autologous B-LCLs (Fig. 10).

The lytic activity of the CTL lines against their specific target cells was standardized by determining the lytic units (L.U.) of activity for each cell line just prior to their injection into hu-PBMC-SCID mice (Figs. 9 and 10).

b. Delay of LPD with CTL immunotherapy. SCID mice were reconstituted with PBMC from EBV-seropositive donors, and treated with CTL lines established from the same donor's PBMC. Immunotherapy consisted of 100-200 L.U. of EBV-specific CTL injected i.p. twice per week, with the initial injection occurring on day 0 to day 3 after PBMC reconstitution (Table 5). To control for virus specificity and HLA restriction of CTL-induced effects, a group of mice was injected with alloreactive CTL on the same schedule. The alloreactive CTL were generated from the same PBMC donor and exhibited very low levels of lysis against autologous B-LCL (Fig. 10). Van Kuyk et al have shown persistence of CTL clones for only 3 to 4 days after injection into hu-PBMC-SCID mice (92); therefore, in these experiments, mice were given CTL twice weekly.

The cumulative survival of hu-PBMC-SCID mice receiving EBV-specific CTL in two experiments was significantly increased compared to untreated mice (p=0.006; Table 5 and Fig.11). Survival increased even more significantly when mice were treated with alloreactive CTL (p=0.0008;). Either type of CTL immunotherapy resulted in a delay in tumor outgrowth, but did not completely prevent tumors from developing. Surviving mice were sacrificed on day 90 (untreated and allo-CTL treated) or day 95 (EBV-CTL treated) and examined for LPD. One of 23 untreated mice (4.3%), 3/13 mice treated with EBV-specific CTL (23.1%), and 2/9 mice treated with alloreactive CTL (22.2%) were free of tumors at the end of the experiment. The differences in tumor frequency were not statistically significant by X^2 analysis or Fischer's exact test.

Table 5. Survival of hu-PBMC-SCID mice (C.B.17-SCID strain) after adoptive transfer of CTL.

CTL specificity	expt (n).	CTL therapy inititated[1]	duration of therapy (weeks)[2]	mean survival (S.E.) (days)	P-value for survival vs. untreated[3]
EBV	1 (6)	day 0	10.6	77.2 (3.48)	0.006
	2 (7)	day 3	6.4		
Allo	(9)	day 0	6.6	78.0 (2.27)	0.0008
untreated	(23)			64.3 (2.10)	

[1] day after reconstitution

[2] treated mice received 100-200 LU of CTL twice weekly.

[3] by logrank (Mantel-Cox) test; EBV-CTL value was calculated for expt.1 + expt. 2 vs. untreated controls.

Fig. 7. Flow cytometric analysis of CTL lines. Percentages of cells expressing CD3, CD4 or CD8 are indicated in each quadrant.

EBV-specific CTL line:
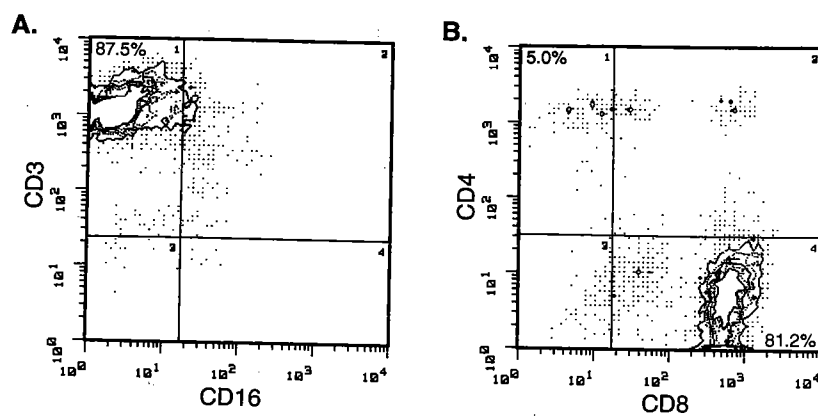
Alloreactive CTL line:
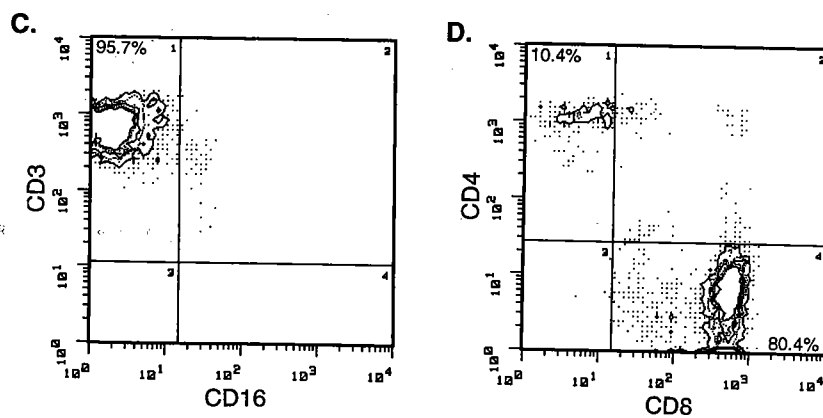
Figure 8. Lysis of autologous B-LCL, HLA-class I-mismatched B-LCL and hu-PBMC-SCID mouse tumor cells by EBV-specific CTL lines.

Lysis of hu-PBL-SCID tumor cells by EBV-specific CTL lines

% specific lysis
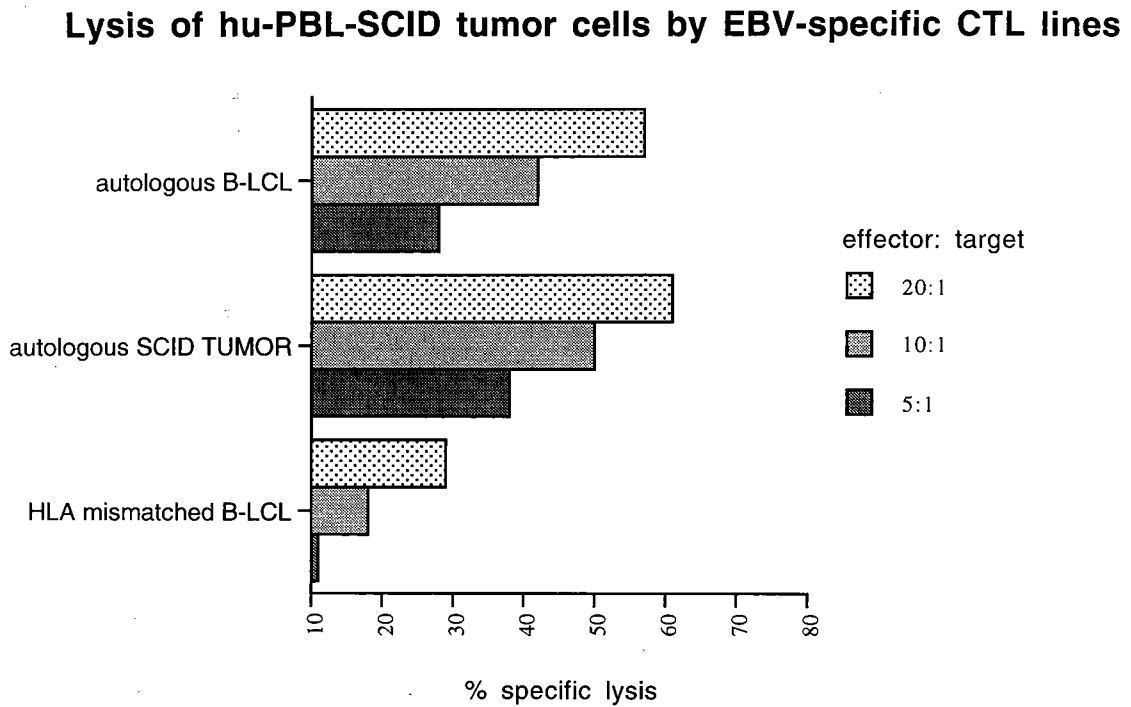
Figure 9. Determination of lytic units (L.U.) of EBV-specific activity/10^6 CTL. EBV-specific CTL lines averaged 36 L.U./10^6 CTL. Mice were injected 2x/week with between 3x10^6 and 6x10^6 CTL (100-200 L.U.).

Lytic activity of EBV-specific CTL
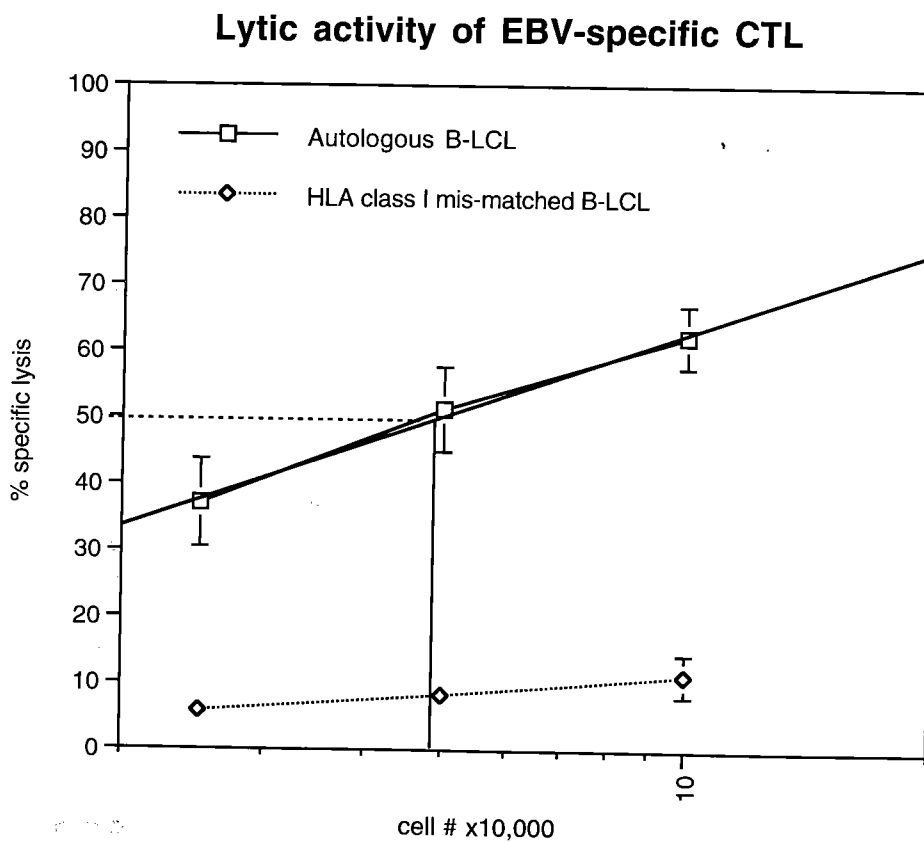
Figure 10. Determination of lytic units (L.U.) of alloreactivity/10^6 CTL. Alloreactive CTL averaged 20 L.U./10^6 CTL . Mice were injected 2x/week with approximately 100 L.U.

Lytic Activity of Alloreactive CTL
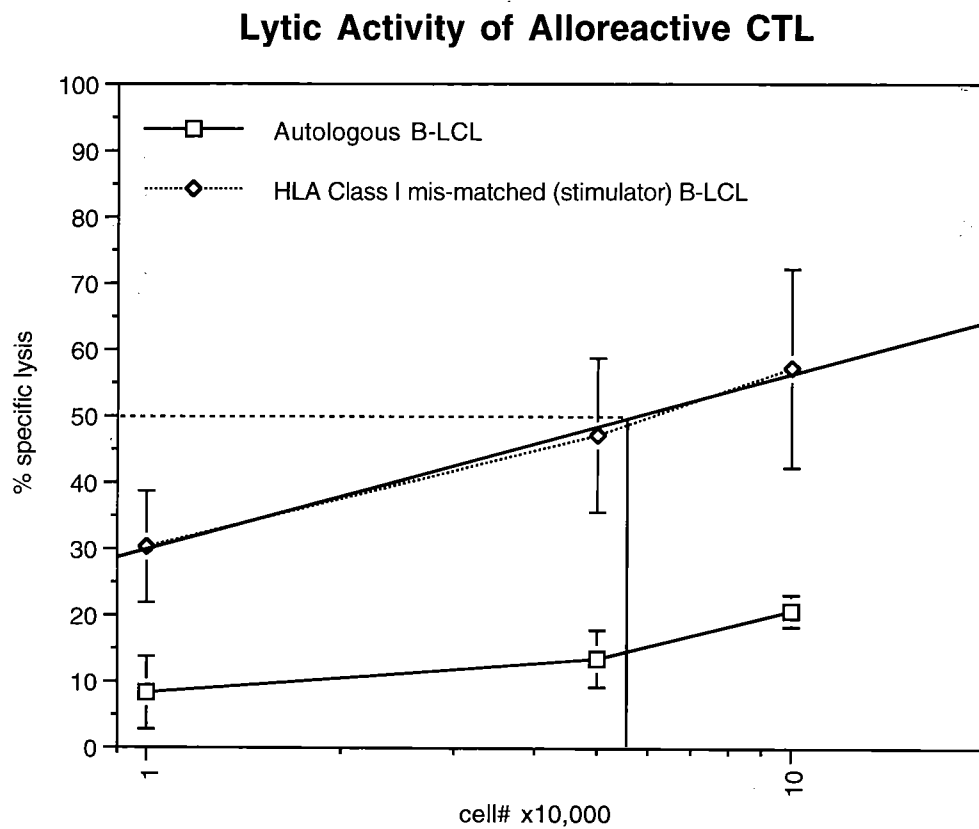
Figure 11. Increased survival of hu-PBMC-SCID mice treated with CTL.
Mortality was due to LPD in all cases. Reconstituted mice injected twice weekly
with EBV-specific CTL lines or alloreactive CTL lines survived significantly
longer than did PBS-injected mice (p=0.006 and 0.0008 respectively).
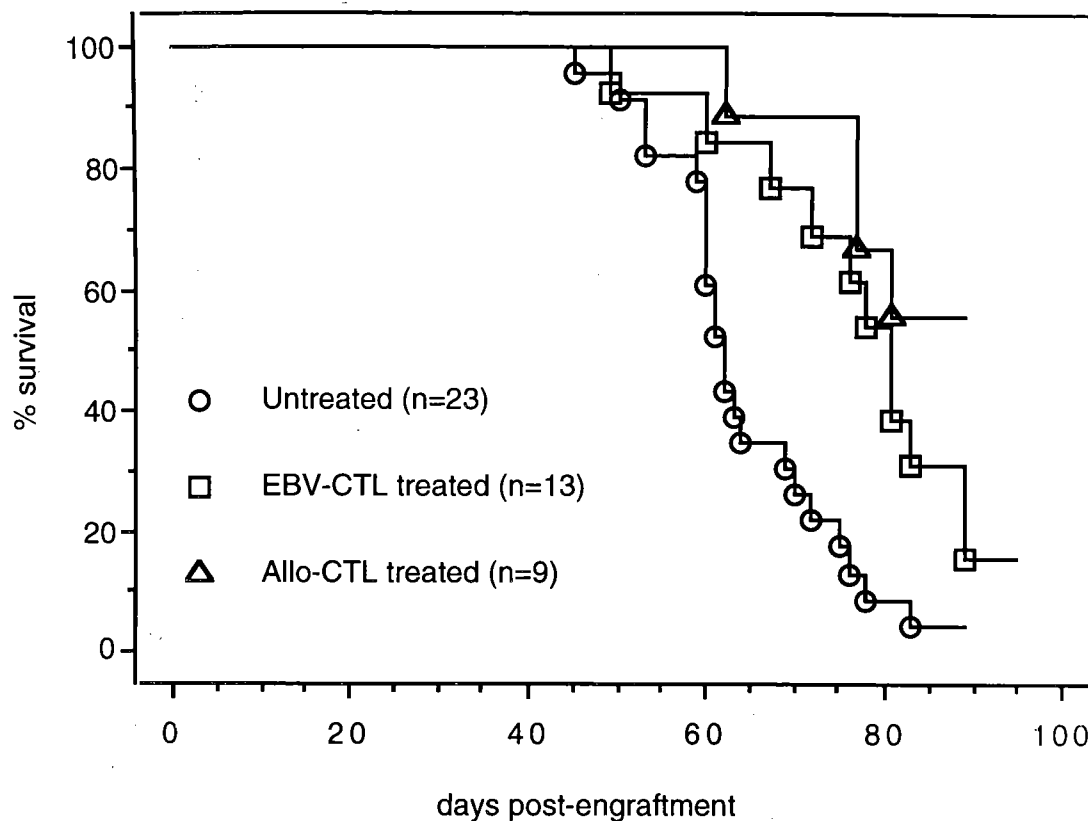
B. Experiments in NOD-SCID mice.

1. Immune reconstitution of NOD-SCID mice with PBMC from individuals with acute or latent EBV infection.

a. High levels of human T lymphocyte engraftment. Initial experiments characterizing immune reconstitution of the NOD-SCID strain of mice were performed by Dr. R. M. Hesselton; a breeding colony of NOD-SCID mice was subsequently established at the U. Mass. Medical Center Animal Medicine facility. In these experiments, human PBMC engraftment in spleens of NOD-SCID mice was significantly increased (5-10 fold) after injection of human PBMC, compared to reconstituted C.B.17-SCID mice (Fig. 12) (22). The human cells in the spleen were almost entirely CD4 and CD8 single-positive T cells, and expressed the CD45RO (activated/memory cell) phenotype (22). Further reconstitutions of NOD-SCID mice with human PBMC were performed to determine whether EBV-associated LPD would also develop in this strain of SCID mouse, and with what frequency. Human cells in the spleens of reconstituted NOD-SCID mice were again found be almost exclusively CD3$^+$ (Table 6).

b. Comparison of the frequency of lymphoproliferative disease. NOD-SCID mice were reconstituted according to the same protocol used for C.B.17-SCID mice, using PBMC from six donors with acute IM and three donors with latent EBV infection. The overall frequency of LPD was significantly lower in reconstituted NOD-SCID mice than in C.B.17-SCID mice (58% vs. 69%, X^2 p=0.029) (Tables 2 and 7). In contrast to the findings in reconstituted C.B.17-SCID mice, the frequency of LPD was significantly higher in NOD-SCID mice engrafted with cells from donors with acute IM than in NOD-SCID mice engrafted with cells from latently-infected donors (Table 7). The two latently-infected PBMC donors used to reconstitute NOD-SCID mice were known to generate a high frequency of LPD in C.B.17-SCID mice. When the results of reconstitution of C.B.17-SCID and NOD-SCID mice with just these two donors are compared, the difference in the frequency of LPD is striking: 89% of C.B.17-SCID mice developed LPD compared to 50% of NOD-SCID mice (Table 8).

c. Characterization of LPD in reconstituted NOD-SCID mice.

i. Cell surface phenotype. Tumor masses from reconstituted NOD-SCID mice were analyzed by flow cytometry to determine if their surface

antigen phenotype differed in any way from masses found in C.B.17-SCID mice. Tumors arising in the two strains of mice did not differ markedly in expression of any of the surface antigens tested (Table 4 and 9). A substantial percentage of human T cell were identified in masses from NOD-SCID mice; however, comparisons cannot be made with C.B.17-SCID masses as these were not stained with the CD3 antibody.

ii. Presence of EBV genome and replicating viral forms. Evidence of EBV replication was found in 12/23 tumors from NOD-SCID mice (Fig. 13); this frequency is similar to that seen in C.B.17-SCID mice (5/9 tumors, Fig. 4). Several of the tumors in Figure 13 appear not to carry the EBV genome; these tumors were determined by surface marker analysis to be murine thymic lymphomas, which frequently occur in older NOD-SCID mice and are associated with the expression of ecotropic murine leukemia provirus Emv30 (60). The murine tumors were also found to metastasize from the thymus to other sites, usually lymph nodes in the abdomen. Surface marker staining of NOD-SCID tumor masses occasionally revealed a predominance of CD3+ human T cells.

Not surprisingly, the tumor masses arising in NOD-SCID mice appear to be identical to those arising in C.B.17-SCID mice with regard to cell surface phenotype, presence and clonality of the EBV genome, and frequency with which replicating episomes are found. LPD from the two SCID strains were also found to be very similar histologically (data not shown).

d. Wasting disease in PBMC-reconstituted NOD-SCID mice. A phenomenon which was not seen in C.B.17-SCID mice was observed in some reconstituted NOD-SCID mice in necropsies performed at least eight weeks after reconstitution. Affected mice were small in size and appeared anemic and cachectic, with pale internal organs and no body fat. Spleens were usually very small, pale to white, and fibrous. In general, very few viable cells could be recovered from these spleens. These mice often had a scaley dermatitis and hair loss. Mice affected by this syndrome were free of human LPD, and always produced greater than 1 mg/ml of human Ig. The syndrome has not been observed in unreconstituted NOD-SCID mice.

Table 6. Surface phenotype of spleen cells from hu-PBMC-SCID mice (NOD-SCID strain).

Surface antigen (# spleens tested)	lymphocyte distribution	% cells expressing Ag (SE)[1]
untreated mice:		
CD45 (6)	all lymphoid cells	48.2 (11.9)
CD3 (7)	immature B cells, BL	45.9 (11.0)
CD4 (4)	helper T cells	36.0 (12.0)
CD8 (4)	cytotoxic T cells	26.0 (4.1)
anti-CD8-treated mice:		
CD45 (7)		19.1 (4.2)
CD3 (10)		11.5 (2.7)
CD4 (4)		16.2 (3.4)
CD8 (7)		≤0.1 (0)
Goat anti-mouse Ig (6)	control for Ab-coated cells	1.9 (0.9)

[1]Suspensions of tumor cells were incubated with fluoroscein- or phycoerythrin-labeled monoclonal antibodies and analyzed by flow cytometry. Percentages of cells expressing Ag are those cells which displayed higher levels of fluorescence when stained with Ag-specific Ab than with control Ab.

Table 7. Incidence of LPD in NOD-SCID mice injected with PBMC from EBV-infected donors.

PBMC EBV infection type:	mice with LPD/total	% with LPD	X^2 p value[1]	mean days to LPD (S.E.)
acute IM	47/73	67.1		88.2 (4.1)
latent	29/58	50.0	0.049	69.3 (3.5)
total	76/131	58.0		

[1]X^2 p value compares percentages of mice developing LPD after reconstitution with PBMC from donors with acute IM or latent EBV infection.

Table 8. Comparison of incidence of LPD in C.B.17-SCID and NOD-SCID mice.[1]

PBMC donor	SCID strain	Mice with LPD/total	% with LPD	X^2 p value[2]
CH	C.B.-17	35/38	92	
PM	C.B.-17	27/32	84	
	total C.B.-17-SCID	62/70	89	
CH	NOD	4/12	33	
PM	NOD	25/46	57	
	total NOD-SCID	29/58	50	<0.0001

[1] Reconstituted with PBMC from donors with latent EBV infection.
[2] X^2 p value compares incidence of LPD in C.B.-17-SCID and NOD-SCID mice.

Table 9. Surface phenotype of tumor masses from hu-PBMC-SCID mice (NOD-SCID strain).

Surface antigen (# tumors tested)	B lymphocyte distribution	% cells expressing Ag (SE)[1]
CD45 (16)	all B lymphocytes	70 (3)
CD3 (26)	T cells	20 (3)
CD4 (16)	helper/inducer T cells	14 (3)
CD8 (4)	cytotoxic T cells	19 (3)
CD20 (23)	B cells, B-lymphoblastoid cells	21 (3)
CD23 (4)	activated B cells, B-lymphoblastoid cells	24 (12)
CD10 (2)	immature B cells, BL (CALLA)	0.10 (0.1)

[1]Suspensions of tumor cells were incubated with fluoroscein- or phycoerythrin-labeled monoclonal antibodies and analyzed by flow cytometry. Percentages of cells expressing Ag are those cells which displayed higher levels of fluorescence when stained with Ag-specific Ab than with control Ab.

12. Recovery of human cells from spleens of hu-PBMC-SCID mice.
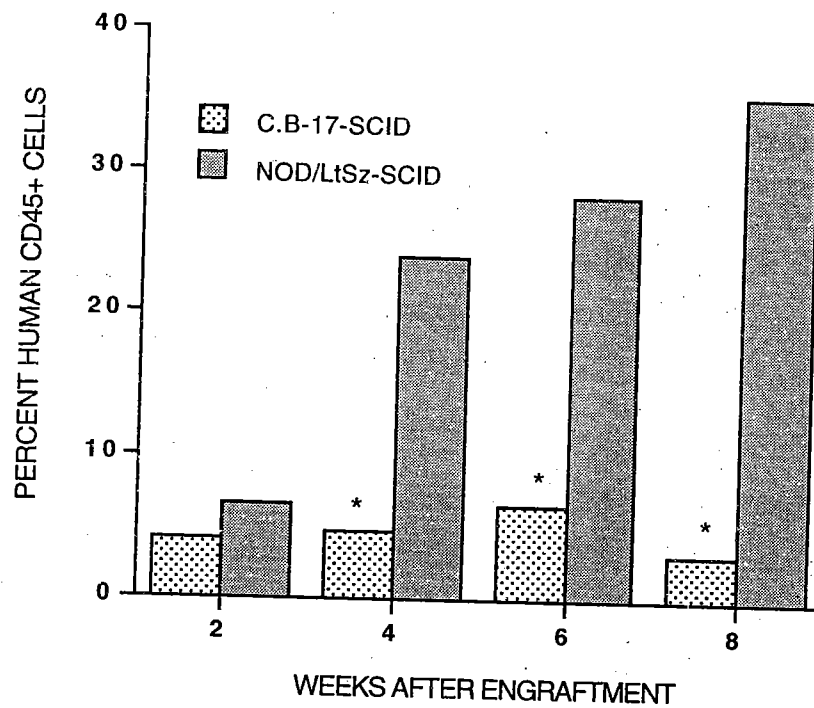
Figure 12. Percentage of human cells present in spleens of C.B.17-SCID and NOD-SCID mice after i.p. injection of with 20 x 106 human PBMC. Between 6 and 10 animals were tested at each time point. Statistically significant comparisons are indicated by *: p< 0.05 for NOD-SCID vs. C.B.17-SCID. From Hesselton, et al., 1995, J. Infect. Dis. (61).

Figure 13. LPD in hu-PBMC-SCID mice (NOD-SCID strain) contains linear and circular forms of the EBV genome.Tumor DNA was digested with BamHI and hybridized with the BamNJ probe. Lat.: tumors from mice reconstituted with PBMC from latently-infected donors. IM: tumors from mice reconstituted with PBMC from donors with acute IM

EBV-BamNJ Southern blot:
Hu-PBMC-SCID Tumor DNA
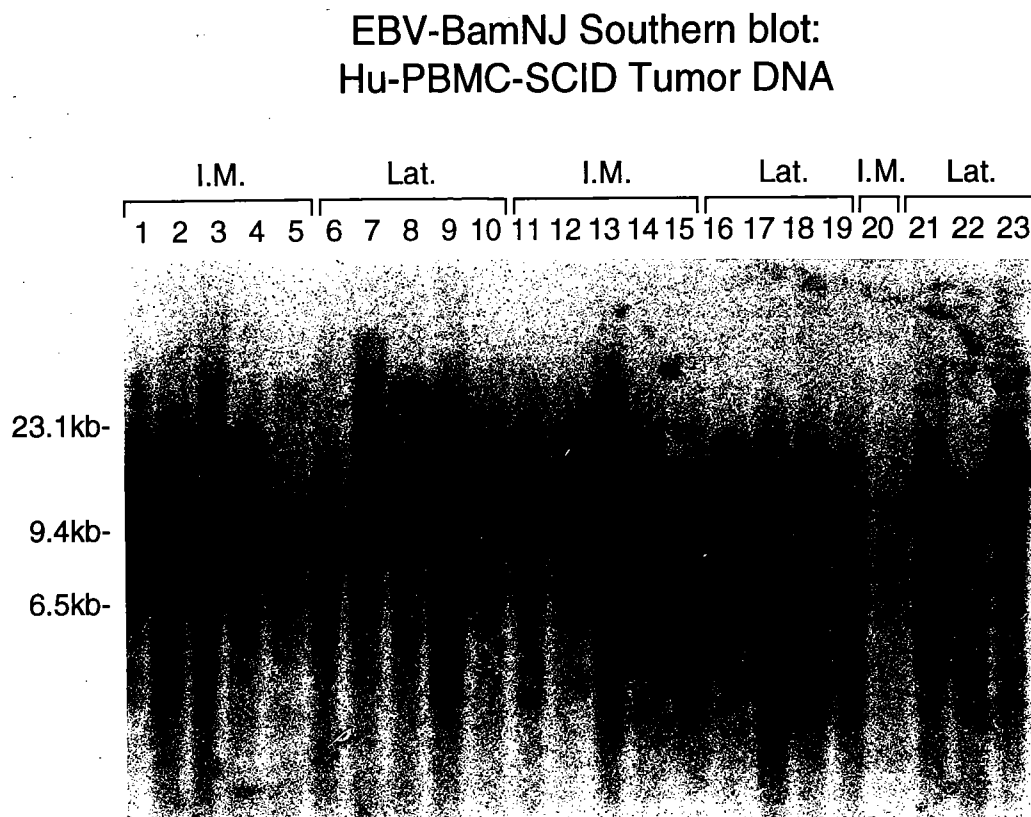
2. CD8$^+$ T cells protect NOD-SCID mice from LPD.

a. Depletion of engrafted CD8$^+$ cells increases the frequency of LPD to 100%. EBV-associated LPD is believed to be prevented in healthy human hosts by EBV-specific CTL. In this context, the coincidence of high levels of T cell engraftment and relatively low LPD in reconstituted NOD-SCID mice suggests a causal relationship between the two observations. To investigate this possibility, monoclonal antibodies were employed to deplete CD4$^+$ and CD8$^+$ human T cell subsets from reconstituted mice. Ab-mediated depletion was successful when purified OKT4 (anti-CD4) and OKT8 (anti-CD8) antibodies were injected i.p. into mice with established PBMC grafts (Fig. 14.). One week after the Ab injections, spleens of mice receiving a single injection of OKT4 or OKT8 contained less than 0.5% of cells bearing the respective surface marker.

Treatment with OKT4 at early timepoints after reconstitution (0-3 days) had another significant effect: one injection of OKT4 completely prevented the engraftment of all human T cells in the spleen (Fig. 15, G-I). Injection of OKT8 at early timepoints, as at the later timepoint, prevented only CD8 T cells from engrafting in the spleen (Fig. 15, D-F). The possibility that the presence of CD4 or CD8 cells was simply being masked by Ab coating was ruled out in two ways: first, the percentage of CD3$^+$ T cells was in all cases very similar to the percentage of either CD4$^+$ or CD8$^+$ when the reciprocol population had been depleted; i.e. all of the CD3$^+$ cells were also CD4$^+$ when CD8 T cells were depleted, and vice versa. Second, cells were stained with with fluorescein-labelled goat-anti-mouse Ig, which would bind to cells coated with mouse mAb. No significant staining was ever seen with the goat anti-mouse reagents in Ab-treated animals (Table 6). These results indicate that CD4$^+$ T cells or their secreted products are required for efficient human lymphocyte engraftment. It is important to note that all of the OKT4-treated mice produced HuIg, although at lower levels than untreated mice without LPD, indicating that B cells are indeed engrafting in these mice.

When Ab-treated mice were observed for a period of up to 16 weeks, the incidence of LPD was significantly increased in anti-CD8-treated mice compared to control mice (100% vs. 47%, p<0.0001;Table 10, groups A and B). The incidence of LPD decreased significantly in anti-CD4-treated mice (11% vs. 47%,

p=0.007; groups A and C). Although very few to no CD16$^+$ cells are detectable in hu-PBMC-SCID mice of either strain, the possible influence of NK cells on LPD was investigated by injecting mice with a mAb which recognizes the NK cell marker CD16 (B73.1). As expected, anti-CD16 treatment did not have a significant effect on the incidence of LPD (47% vs. 35%; Table 10, groups A and D). The effects of OKT4 and OKT8 treatment on cumulative survival were as expected: OKT4 significantly increased survival time and OKT8 significantly decreased survival time, while B73.1 had no effect (Table 11). These results suggest that CD8$^+$ T cells provide protection from LPD in about half of reconstituted NOD-SCID mice, and that CD4$^+$ T cells or their secreted products are required for the generation of LPD.

Table 10. CD8^{+} T cells protect PBMC-reconstituted NOD-SCID mice from EBV-associated lymphoproliferative disease.

Treatment group[1] (PBMC-reconstituted mice)	Mice with tumor/total	% mice with tumor	P-value vs control[2]	P-value vs anti-CD8[3]
A. control[4] (4 experiments)	16/34	47		
B. anti-CD8 mAb (3 experiments)	20/20	100	<0.0001	
C. anti-CD4 mAb (2 experiments)	2/19	11	0.007	
D. anti-CD16 mAb (2 experiments)	6/17	35		
E. anti-CD8 + CD8^{+} T cells (1 experiment)	1/10	10	0.034	<0.0001
F. anti-CD8 + CD4^{+} T cells (1 experiment)	7/9	78	0.10	0.03

[1] NOD-SCID mice in were injected ip with PBS or mAbs once, on day 0-3 after PBMC-reconstitution; mice in groups C and D were also injected i.p. with purified CD4^{+} or CD8^{+} T cells on days 15 and 34.

[2] X^2 P-value for LPD incidence vs. control.

[3] X^2 P-value for LPD incidence vs. anti-CD8-treated mice (group B).

[4] Control mice were injected with PBS in 3 experiments and control MAb in one experiment.

Table 11. Effect of CD4 and CD8 T cell depletion and reconstitution on survival of PBMC-reconstituted NOD-SCID mice.

Treatment group (n)[1]	mean days survived (SE)	P-value vs control[2]	P-value vs anti-CD8[3]
A. control (34)[4] (4 experiments)	73.9 (4.0)		
B. anti-CD8 mAb (20) (3 experiments)	48.7 (0.8)	<0.0001	
E. anti-CD 4 mAb (19) (2 experiments)	111 (5.9)	<0.0001	
F. anti-CD16 mAb (17) (2 experiments)	79 (4.9)	NS	
C. anti-CD8 + CD8+ T cells (10) (1 experiment)	63.5 (6.4)	NS	0.003
D. anti-CD8 + CD4+ T cells (9) (1 experiment)	41.3 (1.7)	0.0002	0.002

[1] NOD-SCID mice were injected ip with PBS or mAbs once, on day 0-3 after PBMC-reconstitution; mice in groups C and D were also injected ip with purified CD4+ or CD8+ T cells on days 15 and 34.

[2] T-test p-values for cumulative survival of treated groups vs. control group. NS=not significant.

[3] T- test p-values for cumulative survival of treated groups vs mice receiving anti-CD8 only (group B).

[4] Control mice were injected with PBS in 3 experiments and control MAb in one experiment.

Figure 14. Antibody-mediated depletion of human T cell subsets in mice with established PBMC grafts. NOD-SCID mice were injected i.p. with OKT4 (anti-CD4), OKT8 (anti-CD8), or PBS six weeks after PBMC reconstitution. Spleen cells were analyzed for the presence of human lymphocytes one week later.

PBMC Only:

PBMC + Anti-CD8 mAb:
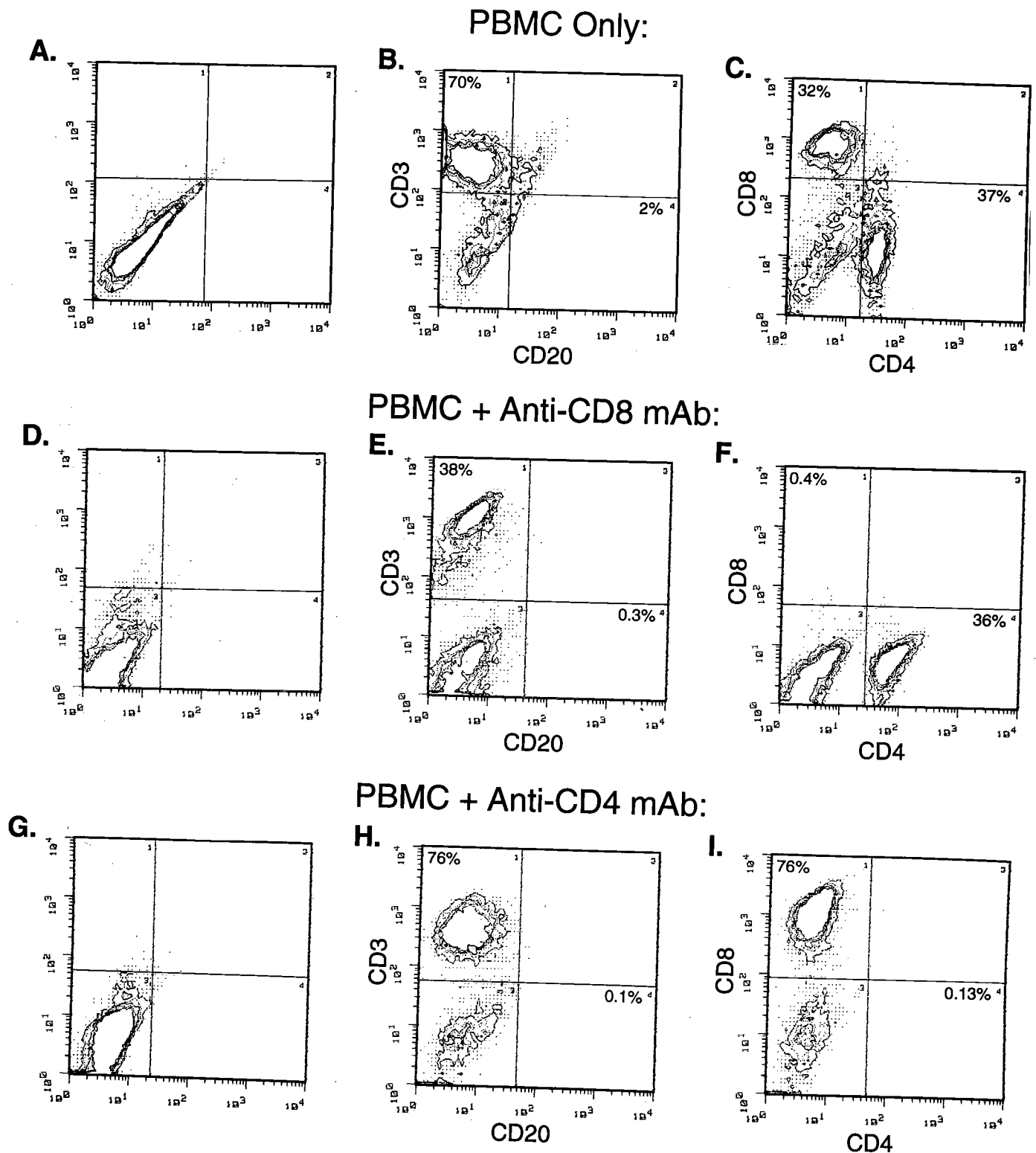
PBMC + Anti-CD4 mAb:

Figure 15. Anti-CD8 treatment at the time of PBMC reconstitution prevents engraftment of CD8 T cells in the spleen, and anti-CD4 mAb prevents engraftment of all human cells. NOD-SCID mice were injected i.p. with MAbs 0-3 days after PBMC reconstitution; spleens were analyzed for the presence of human cells 4.5 weeks later.
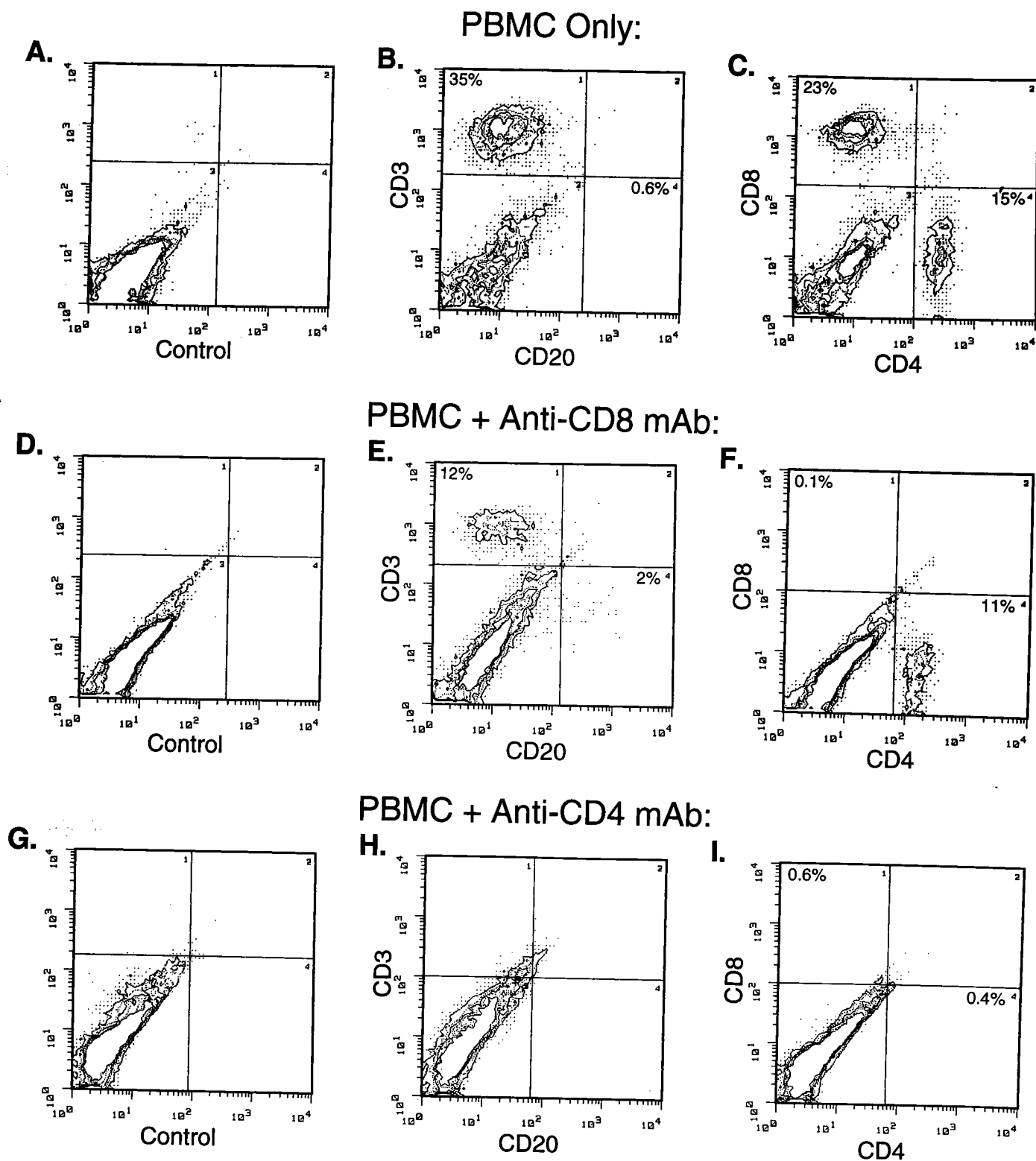
Figure 16. Adoptively transferred CD4+ or CD8+ T cells traffic to the spleen of CD8-depleted mice. NOD-SCID mice were injected i.p. with OKT8 mAb immediately after reconstitution and with purified CD4+ or CD8+ cell populations (5-10 x 10^6 per injection) on days 16 and 34 after reconstitution. Spleens were analyzed for the presence of human cells at sacrifice (6 weeks for mouse receiving CD4+ cells and 10 weeks for mouse receiving CD8+ cells).

ADOPTIVE TRANSFER OF HUMAN T CELLS AFTER DEPLETION OF CD8 T CELLS

Anti-CD8 + CD4 T Cells:

Anti-CD8 + CD8 T Cells:
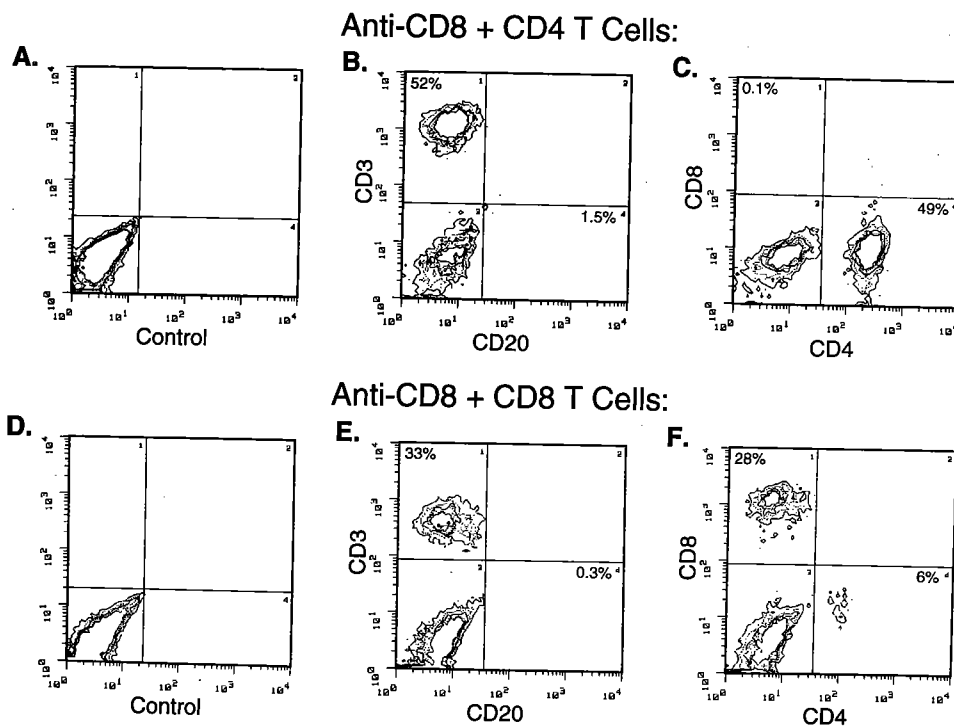
b. Adoptive transfer of CD8+, but not CD4+, T cells restores protection from LPD The increase in LPD and decrease in survival which accompanied depletion of CD8+ cells may be attributable to the absence of cells which have the effector functions of CD8+ T cells (ie: antigen-specific cytolytic activity) or which secrete specific cytokines; it may also simply be the result of a reduction in the total number of engrafted T cells. In the latter case, replenishment with T cells of either phenotype should restore the protection from LPD which is seen in about half of control mice; if the former is the case, replenishment of CD8+ cells would be required to provide such protection. The requirement for CD8+ T cells for protection was established by adoptive transfer of T cell subsets into CD8-depleted mice. The 50×10^6 PBMC injected to reconstitute each mouse would be expected to contain approximately 22×10^6 (44%) CD4+ T cells and 16.5×10^6 (33%) CD8+ T cells. To replace the depleted CD8+ cells with an approximately equal number of purified CD4+ or CD8+ cells, mice were injected with 10×10^6 and 5×10^6 CD4+ cells on days 16 and 34 after reconstitution, or with 7.5×10^6 and 8.5×10^6 CD8+ cells on days 16 and 34 after reconstitution.

Adoptive transfer of CD8+ T cells provided a highly significant level of protection from LPD: only one of 10 mice developed LPD after receiving anti-CD8 followed by CD8+ T cells, compared to 20/20 receiving anti-CD8 alone (p=0.0001, Table 10). In contrast, 7 of 9 mice receiving CD4+ T cells after CD8 depletion developed LPD (p=0.03 vs. anti-CD8 alone, Table 10). The two mice in this group which did not develop LPD became moribund and were sacrificed in the fourth week of the experiment. Both animals had gross indications of graft vs. host disease (GVHD), which was probably caused by the injection of additional CD4+ T cells. The level of protection provided by CD8+ T cells was significantly higher than that provided by CD4+ T cells (X^2 p=0.003).

FACS analysis at the time of sacrifice revealed a large population of CD4+ cells in spleens from mice receiving CD4+ T cells (Fig. 16A-C). Likewise, adoptive transfer of CD8+ T cells resulted in a large CD8+ population, as well as a smaller CD4+ population, in the spleen when analyzed at the time of sacrifice (Fig 16B), confirming that the purified T cell subpopulations also traffic to and populate the spleen.

The effects of antibody treatments on the incidence of LPD were expected to also be reflected in survival times. As expected, cumulative survival time of anti-CD8-treated mice was significantly shorter than both control and anti-CD8 plus CD8 T cell-treated groups, and survival of anti-CD4-treated mice was significantly longer (Table 11). The shortest mean survival times were seen in the group that received CD4$^+$ T cells after CD8 depletion; this was due to the short survival of two mice which developed GVHD. Surprisingly, though, survival time was not significantly longer for mice receiving CD8$^+$ T cells after CD8-depletion than for controls. The reason for this appears to be that, although most of the mice in this group did not develop lethal LPD, they did develop a wasting syndrome (described earlier) at about the same time control mice were developing tumors.

Mice treated with anti-CD4 Ab had the longest survival times. While several of these mice eventually developed LPD, none of the others had signs of the wasting disease, which appears to be a T cell mediated phenomenon. These results of these experiments do not distinguish whether CD4$^+$ or CD8$^+$ T cells are responsible, since depletion of CD4$^+$ cells prevents engraftment of CD8$^+$ cell, and depletion of CD8$^+$ cells usually leads to death due to LPD before the onset of the wasting syndrome.

CHAPTER IV. DISCUSSION

A. Experiments in C.B.17-SCID mice.

    1. Comparison of immune reconstitution with PBMC from two types of EBV-infected donors.

        a. Frequency and characterization of LPD. Reconstitution of C.B.17-SCID mice with PBMC from EBV-infected donors in most cases led to the development of LPD that shared many of the characteristics of immunosuppression-associated immunoblastic B cell lymphoma in humans. The frequency of LPD and survival time of affected mice were similar when mice were reconstituted with PBMC from donors with acute IM or with latent EBV infection. In all cases LPD carried the EBV genome (Fig. 3), expressed surface antigens similar to those found on B-LCL (Table 4), were predominantly oligoclonal with respect to immunoglobulin gene rearrangements (Fig. 5) and EBV genome (Fig. 4), and typically displayed immunoblastic morphology (Fig. 6). Each of these characteristics of LPD arising in hu-PBMC-SCID mice is similar to immunosuppression-induced LPD, and distinguished the SCID mouse tumors from Burkitt's lymphoma. Similar findings have also been reported by several other groups(10, 46, 52, 58, 70).

    The observation that reconstitution with PBMC from donors with either acute or latent EBV infection results in similar high frequencies of LPD suggests that the transplanted CD8 T cells do not suppress EBV-induced B cell proliferation to a great extent in this model, as they are thought to do in the human host. If it is assumed that CD8+ T cells have an effect on LPD, one would predict that reconstitution with PBMC from donors with acute and latent infection would have different outcomes due to the differences in composition of their T cells and the proportion of EBV-infected B cells. In acute IM, a high percentage of T cells in peripheral blood are activated (86), making them highly susceptible to activation-induced apoptosis (33). In addition, the percentage of B cells that are infected with EBV is 10- to 10,000-fold higher during acute IM than in latent infection (45, 89, 99). If the T cells from acute IM patients undergo apoptosis at a high rate compared to those

from latently infected donors after transplantation into SCID mice, then a higher rate of LPD (up to 100%) would be predicted in mice receiving acute IM PBMC due to the large number of EBV-infected B cells present. However, if the cytotoxicity directed against EBV-infected cells present in acute IM PBMC is preserved after transplantation, then in mice receiving these cells, the rate of LPD would be predicted to be lower than 100%. The rate of LPD after reconstitution with acute IM PBMC was lower that 100%, but not dramatically so. This could be a result of the effects of acute IM activated T cells, or simply due to decreased survival of B cell tumor precursors, which are also activated by EBV infection. The results of these experiments do not distinguish between these two possible effects on the development LPD.

Several lines of evidence support the conclusion that the injected human T cells do not significantly affect the development of LPD in reconstituted SCID mice. First, the most common sites for LPD in hu-PBMC-SCID mice are the liver and hepato-pancreatic and mesenteric lymph nodes (our unpublished observations). In contrast, the majority of transplanted T cells colonize the peritoneal cavity, forming foci on the serosal peritoneum (31). A study which used fluorescence *in situ* hybridization techniques to detect human cells in hu-PBMC-SCID mice revealed that most of the human T cells remained in the peritoneal cavity, with very low percentages of T cells being detected in lymphoid organs (40). Others, however, have reported finding that from 0% to 57% of hu-PBMC-SCID spleen cells stained positively for human T cell antigens (34, 84). Second, T cells recovered from reconstituted SCID mice have been reported to be anergic to activation via the T cell receptor (84). The presence of T cells in the PBMC inoculum has been shown to be required for HuIg production and the generation of LPD in hu-PBMC-SCID mice (93). These findings suggest that human T cells transplanted into SCID mice provide help for Ig production and B cell proliferation in the form of cytokine secretion, but they may not be capable of providing other effector functions or of normal trafficking outside of the peritoneal cavity, both of which are neccessary for suppression of B cell proliferation. The results of reconstitution using PBMC from donors with acute IM and latent EBV infection described above are consistent with this interpretation, which in turn implies decreased survival of B cells from acute IM PBMC donors.

b. Comparison of rates of reconstitution. The rate of successful reconstitution of C.B.17-SCID mice was higher when PBMC from donors with latent EBV infection were used than when PBMC were from donors with acute IM. That is, a higher percentage of mice injected with PBMC from latently-infected donors produced significant levels of human Ig (90% with latent vs. 75% with acute). A possible reason for this difference again lies in the T cell composition of the injected PBMC. As previously mentioned , T cells are neccessary for human Ig production and tumor induction in hu-PBMC-SCID mice; mice injected with purified B cells do not make human Ig, nor do they develop LPD (93). If the acute IM T cells have a shorter half-life in SCID mice as a result of increased apoptosis, then it is possible that in some mice the number of surviving T cells is insufficient to support Ig production. The frequency of such mice would be higher among those injected with acute IM PBMC than those injected with latently-infected PBMC.

Another possible reason for the difference in reconstitution success relates to the fact that successful reconstitution is dependent upon the number of B cells injected (46): While the percentage of B cells in PBMC from acute IM donors was one-forth to one-half that of latently-infected donors (1-4% vs. 4-8%), a relatively large proportion of these cells are EBV-infected and hence activated. The rate of LPD in mice reconstituted with acute IM donors indirectly implies that these activated B cells may have a shorter survival time after injection. Either of these factors (increased apoptosis of T cells or B cells due to activation) could be responsible for the lower rate of reconstitution with acute IM PBMC.

c. Variability in the ability of PBMC donors to generate LPD. Substantial variability in the ability to generate LPD in hu-PBMC-SCID mice was seen among individual latently-infected PBMC donors (Table 2). This was also the case with acute IM donors. Similar variability was also reported by Picchio et al., along with the finding that replicating forms of EBV were always present in tumors from mice reconstituted with so-called "high incidence"donors (57). More than half of tumors from mice reconstituted with "intermediate-low incidence" donors also had evidence of EBV replication. My analysis of EBV replication did not include a large enough sample to corroborate or refute these findings. Picchio suggests that lower

levels of EBV-specific immunity in high incidence donors might allow activation of EBV replication and new infection of B cells. The previously-mentioned evidence that T cell immunity is not active after reconstitution argues against this theory. An alternative explanation is that the variability in frequency of LPD reflects differences in the number of EBV-infected B cells in the PBMC inoculum; this would also explain the variability seen between different experiments using the same donor, since the frequency of EBV-infected B cells is known to fluctuate over time in infected individuals (45).

2. Cytotoxic T lymphocytes delay LPD in hu-PBMC-SCID mice.

EBV-specific cytotoxic T cells are often cited as the main factor in protecting immunocompetent, infected individuals from EBV-associated LPD. This protection has not been demonstrated *in vivo* due to the lack of an adequate animal model of the disease. Such a model is now provided by the hu-PBMC-SCID mouse.

The results of adoptive transfer of CTL lines into hu-PBMC-SCID mice described here demonstrate that cytotoxic T cells can delay, and in some cases, prevent EBV-associated LPD. This delay was seen when the injected CTL specifically recognized EBV latent proteins, and also when CTL specifically recognized HLA class I alloantigens (Fig. 11 and Table 5). The delay in LPD observed following injection of EBV-specific CTL was presumably due to HLA class I-restricted CTL lysis of EBV-infected tumor cell precursors *in vivo*. However, since lysis of autologous EBV-infected target cells by the alloreactive CTL lines *in vitro* was very low (compared to lysis of allogeneic target cells), it is unlikely that delay of LPD following *in vivo* administration of alloreactive cells is mediated by direct lysis of tumor cell precursors by HLA-restricted recognition of viral antigens. While the allogeneic targets are infected with EBV, they do not share any HLA class I alleles with the CTL; the mechanism for the delay of LPD following the administration of alloreactive CTL is therefore neither virus specific nor HLA-restricted. These results demonstrate contributions by two distinct mechanisms of tumor suppression *in vivo*.

Two mechanisms could be responsible for the non-specific suppression of LPD by alloreactive CTL. The first possibility is that the CTL secrete cytokines which suppress tumor outgrowth . HIV-1-specific CD8+ CTL have been shown to produce tumor necrosis factor-α, interferon-γ,

granulocyte/macrophage colony-stimulating factor, and low amounts of interleukins 2, 3, and 4 (59). One or more of these cytokines could either directly suppress EBV-transformed B cells or have an indirect effect by influencing intact murine inate immune functions. An example of the latter is the prevention of tumors by low concentrations of IL-2 in hu-PBMC-SCID mice, due to activation of murine NK cells (4). Expression of a transfected interferon-g gene in tumor cells leads to growth inhibition of the tumor when T cells are also present (18). Growth inhibition is also seen in the absence of T cells when tumor necrosis factor is expressed in tumor cells. (53).

Despite a relatively large L.U. dosage of CTL, adoptive immunotherapy did not completely protect mice from LPD. Previously published studies provide clues to why protection by CTL was not complete in hu-PBMC-SCID mice. HIV-1-specific CTL injected into hu-PBMC-SCID mice were cleared from the peritoneal cavity by day five (92). Cells recovered from the peritoneal cavity through day four retained cytotoxic activity, indicating that CTL function was not inhibited after adoptive transfer (ie: the CTL did not appear to be anergic).

There are several reasons why CTL might have such a short survival time after injection into SCID mice. First, endogenous NK cell activity is retained in the SCID mouse, and may even be higher than in non-SCID mice. Gamma-irradiation of SCID mice prior to reconstitution to inactivate endogenous NK cell activity has been shown to increase human immunoglobulin levels. While the authors suggest that mouse NK cells may suppress Ig production by B cells or B cell engraftment, they may also reduce longevity of injected T cells and CTL lines. Depletion of endogenous NK cells prior to reconstitution might therefore increase the lifespan of T cells in SCID mice and the effectiveness of CTL immunotherapy. The results of experiments in NOD-SCID mice, described below, support this view. Second, the absence of growth factors, especially IL-2, could be causing the CTL to be short-lived. Hu-PBMC-SCID mice could not be given IL-2 in these experiments, because its proliferative effect on murine NK cells has been shown to prevent EBV-induced LPD (4).

Other groups have reported using EBV-specific CTL to treat LPD in SCID mice which had been injected with EBV-transformed lymphoblastoid cell

lines (9, 65). Boyle et al reported results which were similar to those described here: CD8$^+$ CTL conveyed partial protection from tumors, and significantly delayed tumor outgrowth in unprotected mice. Rencher et al reported complete protection from LPD with injection of CD8$^+$ or mixed T cell lines (65). In both of these studies, however, LPD was induced by injecting B-LCL, as opposed to PBMC in the study reported here. Recently, the phenotype of the cells which are latently infected with EBV *in vivo* has been described (45). The cells bear the phenotype of resting B cells, and lack expression of the lymphoblastoid antigens found on B-LCL. The EBV-infected cells in the PBMC injected into SCID mice must undergo a phenotypic change before they develop into LPD, and are therefore distinct from B-LCL. This distinction could explain why CTL are less effective in protecting PBMC-injected mice from LPD than they are B-LCL-injected mice.

Evidence for protection from EBV-associated LPD has also been reported in human patients treated with EBV-specific T cells (26, 68) or purified donor T cells (54) following T-depleted bone marrow transplantation. Infused EBV-specific CTL persisted in recipients for extended periods, EBV DNA levels decreased, and LPD was resolved. While it is more difficult to assess the effects of CTL treatment in these human studies, there are several reasons it is unlikely that the outcome of these CTL infusions was due to a recovery of host immunity. First, the timing of the therapeutic effects was very closely correlated with CTL infusions; second, virus-specific T cell immunity is generally impaired for up to a year after BMT, and third, spontaneous resolution of EBV-induced LPD occurs very rarely (68).

Infusion of unmanipulated donor T cells was also effective in inducing remission of LPD, but was associated with the clinical complications of graft-versus-host disease and lethal inflammatory responses (54). This toxicity is thought to be due to the presence of alloreactive T cells in the infusion, and may be avoided by the use of EBV-specific CTL (27).

B. Experiments in NOD-SCID mice.

1. Immune reconstitution of NOD-SCID mice with PBMC from EBV-infected individuals.

a. High levels of human T lympocyte engraftment. Recent studies in NOD-SCID mice have demonstrated high levels of splenic engraftment when human T cell lines (78), spleen cells (20) or 20×10^6 PBMC (28) are injected intraperitoneally. These findings are confirmed here in experiments in which mice were injected with 50×10^6 PBMC from donors with latent EBV infection. Close to 50% of spleen cells from reconstituted NOD-SCID mice expressed the human CD45 surface marker, and most of these also expressed the T cell marker CD3. In results not presented, spleen cells from more than 20 reconstituted C.B.17-SCID mice were found to contain very few (0% to 5%) human CD45+ cells. Large numbers of human cells are able to survive and populate the spleens of PBMC-reconstituted NOD-SCID because this SCID mouse strain has few intact components of the inate immune response available to resist the xenogeneic PBMC graft. The comparison made here of PBMC-reconstitution of C.B.17- and NOD-SCID mice demonstrates the ability of the C.B.17-SCID's inate immunity to prevent the xenograft from becoming well-established.

b. Frequency and characterization of LPD. NOD-SCID mice were reconstituted with latently- infected PBMC to determine what effect the large number of engrafted T cells would have on development of LPD. The results of initial reconstitution experiments suggested that these T cells provided mice with a substantial degree of protection against LPD, since the frequency of LPD was significantly lower in NOD-SCID mice than in C.B.17-SCID mice. The role of CD4 and CD8 T cells in the control of LPD was clarified in later experiments.

The high frequency of LPD in NOD-SCID mice reconstituted with PBMC from acute IM donors is consistent with earlier speculation that the acute IM T cells undergo apoptosis following transplantation into C.B.17-SCID mice, and hence do not influence the course of LPD. Apoptosis of T cells during acute viral infection has been shown to be correlated with virus-induced immune suppression, such as that which can occur with EBV infection (64). It is possible that apoptosis-induced suppression of T cell immunity in acute IM-PBMC-reconstituted NOD-SCID mice was responsible for the observed increase in incidence of LPD compared the mice receiving latently-infected PBMC.

LPD arising in reconstituted NOD-SCID mice was very similar to LPD in C.B.17-SCID mice in every characteristic analyzed. The predominant cell surface phenotype was nearly identical, as was the proportion of tumors with evidence of EBV replication; the morphology of tumors arising in the two mouse strains was also identical. While this was not an unexpected finding, it suggests that similar events lead to the development of tumors in each of these models.

2. CD4$^+$ and CD8$^+$ T lymphocytes have distinct roles in the outcome of PBMC reconstitution of NOD-SCID mice.

a. CD8$^+$ T lymphocytes protect PBMC-reconstituted NOD-SCID mice from LPD. The outcome of *in vivo* depletion of CD8$^+$ T cells demonstrate that CD8$^+$ T cells protect about half of reconstituted NOD-SCID mice from developing LPD; specific depletion of CD8$^+$ T cells increased the frequency of LPD from 47% to 100% and decreased survival time. Anti-CD16 antibody had little effect on LPD or survival, suggesting that NK cells do not provide signficant protection from LPD in this model. However, since CD16$^+$ cells are rarely detected in either spleen or peritoneal lavage cells of reconstituted mice, it was not possible to confirm that treatment with anti-CD16 Ab depleted NK cells from reconstituted mice.

The increase in LPD after treatment with anti-CD8 Ab was due specifically to the depletion of CD8$^+$ T cells, rather than to a reduction in the total number of engrafted T cells. This was shown by replenishing the depleted (CD8) cell population with either purified CD4$^+$ or CD8$^+$ T cells. Only CD8$^+$ cell replenishment prevented LPD and increased survival time; replenishment with CD4$^+$ T cells had no effect on the frequency of LPD and decreased survival time. The decrease in survival time was apparently due to graft versus host disease (GVHD) caused by the increased number of CD4$^+$ T cells. The condition of CD8-depleted mice which were replenished with CD4$^+$ cells at autopsies was clinically and histologically consistant with GVHD.

The adoptively transferred purified CD8$^+$ T cells provided a higher level of protection from LPD than did the CD8$^+$ T cells in the initial PBMC injection, even though the number of CD8$^+$ cells was the same in each case. This is apparent when the frequency of LPD in control mice is compared to that in mice depleted of CD8$^+$ T cells and then replenished with purified CD8$^+$

T cells (Table 10, groups E and A; 90% protected vs. 53%protected)). The purified CD8$^+$ cells were injected into mice with established T cell grafts, the CD4$^+$ T cell component of which would produce cytokines capable of enhanceing the survival and function of the CD8$^+$ cells injected at later timepoints. In contrast, the CD8$^+$ T cells resident in the PBMC injected into untreated control mice would be subject to some degree of resistance by the host immune system, and it is likely that fewer of these cells would survive long-term. Although the point was stressed earlier that the NOD-SCID mouse presents fewer obstacles to human PBMC engraftment due to defects in inate immunity, some proportion of injected cells is presumeably eliminated by host resistance; this is evident in the small percentage of mice which do not become successfully reconstituted, ie: do not produce HuIg.

b. CD4$^+$ T cells are required for T cell engraftment and development of LPD in NOD-SCID mice. The results of *in vivo* depletion of CD4$^+$ T cells demonstrate that CD4$^+$ T cells are required for engraftment of CD8$^+$ T cells in the spleen and for the progression of EBV-infected B cells to LPD. However, it is apparent that some B cells do engraft in the absence of CD4$^+$ T cells, since these mice produced levels of HuIg which, although lower than those of control mice without LPD, were significant.

The CD4 and CD8 depletion results disagree with those reported by Veronese, et al. for the C.B.17-SCID mouse (93). They found that LPD was prevented when all T cells were removed from PBMC prior to injection, or when mice were treated with agents which prevent T cell activation such as cyclosporin A. However, they also found that removal of either CD4$^+$ or CD8$^+$ T cells separately did not prevent tumors from forming (84). They concluded from this that either CD4$^+$ or CD8$^+$ T cells could provide factor(s) neccessary for EBV$^+$ B cell proliferation and progression to LPD. My results have led me to a very different conclusion, namely, that the only T cells that are required for development of LPD are CD4$^+$ T cells.

A possible explanation for the difference in our findings lies in the methods used to remove T cell subsets. In the Veronese et al. study, CD4$^+$ or CD8$^+$ T cells were removed from PBMC by immunomagnetic separation before they were injected into mice; the authors state that residual contamination with cells bearing the depleted phenotype was 1% or less. It

seems unlikely that 1% residual $CD4^+$ cells could support B cell proliferation; however, mice receiving CD4-depleted PBMC in those experiments did take more than twice as long to develop LPD, suggesting that the residual $CD4^+$ cells might have expanded *in vivo* over that period, eventually providing the level of "help" needed for LPD to progress. The other important difference between these two studies is the use of different SCID mouse strains. The results reported here do not, however, provide an obvious solution to why the two strains would yield different outcomes in these types of experiments.

A requirement for $CD4^+$ T cells for development of EBV-associated LPD has not been previously reported. However, similar roles for both $CD4^+$ and $CD8^+$ T cells have been described in a murine model of EBV infection, murine herpesvirus-68 (MHV-68). MHV-68 shares many molecular and biological properties with EBV. $CD8^+$ T cells have been shown to be important for recovery from primary infection of mice with this virus, (15). Acute lymphoproliferation in the spleen is also seen in acute infection, and is prevented by depletion of $CD4^+$ T cells. Like EBV, MHV-68 establishes a latent infection in B lymphocytes, and chronically infected mice develop LPD similar to that seen in immunocompromised EBV-infected patients (82, 83). It is not yet known what influence $CD4^+$ and $CD8^+$ T cells have on development of LPD in this model, but the results in the NOD-SCID mouse would predict that depletion of CD4 cells will prevent LPD. Depletion of CD8 cells would be predicted to increase the incidence of LPD, which is relatively low (9%) in MHV-68-infected mice.

3. Comparison of immune reconstitution of C.B.17-SCID and NOD-SCID mice.

Human PBMC engraftment is enhanced in the NOD-SCID mouse in comparison to the C.B.17-SCID mouse. It is likely that much of this enhancement can be attributed to reduced NK cell activity in the NOD-SCID strain. Evidence for this includes the finding that depletion of murine NK cells results in higher levels of human Ig production in reconstituted C.B.17-SCID mice (94). However, depletion of NK cell activity from another strain of SCID mouse, the C57BL/6JSz-*scid*, resulted in only a small increase in PBMC engraftment compared to untreated mice (20), suggesting that additional factor also play a significant role. Complement components and macrophage

function, both of which are defective in the NOD-SCID, are potential contributors to the low level of PBMC engraftment achieved in the C.B.-17 SCID mouse.

The experiments in NOD-SCID mice which are described here demonstrate an important role for CD8+ T cells in controlling EBV-induced LPD. The results do not, however, distinguish between the several possible mechanism of control, ie: direct lysis of EBV-infected cells by CD8+ CTL, or alternative mechanism. Healthy individuals who are latenly infected with EBV have a relatively large EBV-specific component within the CTL precursor pool of their PBMC, and it is reasonable to assume that the precursors maintain this function when transferred into the SCID mouse. It is known that human B lymphocytes are stimulated to spontaneously produce Ig when injected into SCID mice; also, expansion of EBV-infected B cell tumor precursors occurs without much difficulty in the C.B.17-SCID environment. Control of B cell outgrowth by EBV-specific CTL would required these cells to expand in reponse to EBV antigens. One could therefore determine whether an EBV-specific CTL-mediated mechanism is responsible for tumor control by measuring an increase in the frequency of EBV-specific CTL in PBMC after residence in the NOD-SCID mouse.

The observation that alloreactive CTL lines protect against LPD in the C.B.17-SCID model suggests a second potential mechanism; that is, secretion of of one or more cytokines by CD8+ T cells that act directly or indirectly on tumor cells or precursors to control LPD. It might be possible to identify such a mechanism by neutralizing specific cytokines *in vivo* by administration of an antibody. Alternatively, one or a combination of candidate cytokines might be found which could, when administered to hu-PBMC-SCID mice, mimic the effect of CD8+ T cells in preventing LPD.

PBMC-reconstituted C.B.17-SCID and NOD-SCID mice provide distinct and complementary models for the study of EBV-associated LPD and the role of T cells on its development. In the healthy EBV-infected human host, an equilibrium is established between virus-induced lymphoproliferation and the anti-viral immune response. Of the two models, the reconstituted NOD-SCID mouse more closely resembles this state of equilibrium. As a result, about half of the mice are protected from disease; the fact that a large

percentage are still susceptible to tumors points to a relative tilting of the equilibrium in favor of the EBV-infected cells. The equilibrium is shifted in favor of protective T cell immunity when additional numbers of CD8$^+$ T cells are injected into the system, and as a result, tumor incidence decreases dramatically. This situation thus models that of the healthy human system, in which the host is protected from EBV-induced LPD.

In the C.B.17-SCID model, the equilibrium appears to be tilted in favor of the virus; following transplantation, the infected human B cells seem to have a survival advantage over the cells which provide protective immunity against virus-induced proliferation. The result is the outgrowth of tumors in a majority of C.B.17-SCID mice; this situation is similar to but more dramatic than what is thought to be present in the immunosuppressed human host. The incidence of tumors in human organ transplant recipients, for example, is approximately 1%, which is far lower than what is found the C.B.17-SCID mouse model.

In addition to providing a model for EBV-related LPD and immunotherapy testing, the hu-PBMC-SCID mouse provides a new model in which to test pharmacotherapeutics and candidate vaccines for viruses which only infect human or for which there is no alternative animal model. These types of studies are already being pursued in the context of HIV-1 infection (28, 59).

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