

## METHODS

## Fine-tuning the EBV+ hu-PBL-SCID Xenogeneic Chimera Model Using *In Vivo* Superinfection

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Our purpose was to establish a reproducible xenogeneic chimera model to observe tumors similar to the well-known human posttransplant lymphoproliferative disease (LPD). First we followed the original protocol injecting Epstein-Barr virus positive (EBV+) human peripheral blood lymphocytes (PBL) intraperitoneally into immunodeficient (SCID) mice. Human cells showed T cell phenotype in majority one week after the transfer, whereas one month later a shift towards B cell phenotype was evident according to immunohistochemical and flow cytometric analysis. At this stage the intraperitoneal mass of cells suggested a biologically malignant behaviour infiltrating the liver and the spleen of the host animal. Immunohistochemistry indicated proliferating human lymphatic cells expressing EBV associated proteins and characteristic patterns of invasion within the affected organs. Eventually LPD was lethal to the host animals in 46-67 days. However, the microscopic appearance of experimental LPD was different from the human haemopoietic malignancies: the basic structures of lymphatic organs were preserved and the human T and B cells repopulated the normally T and B dependent areas in mice. The phenotypes of the proliferating cells were human and characteristic for the mature T- and B-lymphocytes. No dominant clone developed during

*in vitro* culturing of the biologically invasive mass of cells removed from the tumor-bearing mice. The results of microscopical, immunological, and flow cytometrical analysis suggested a mature but uncontrolled proliferation of human lymphocytes in SCID mice. The original method for the induction of posttransplant LPD in SCID mice was modified in our further experiments to standardise the experimental technique increasing the efficiency of B cell proliferation and the reducing the number of unspecific factors. Subsequent *in vivo* EBV superinfection was carried out after the intraperitoneal transfer of a reduced quantity of human PBL from different donors. The same disease developed in our modified chimera model as by the use of original protocol except for some valuable differences. All hosts developed LPD regardless the significantly reduced amount of transplanted PBL and it was lethal in a shorter period of time (41-43 days) compared to the original model. The decreased quantity of transplanted human lymphatic cells was formerly insufficient using the original protocol. Therefore this modified and standardised protocol can lead to a more predictable and reproducible model allowing us to examine fine details of posttransplant lymphoproliferative disease. (Pathology Oncology Research Vol 6, No 4, 280-286, 2000)

**Keywords:** SCID mice, lymphoproliferative disease, EBV superinfection, B cell proliferation

### Introduction

Epstein, Achong and Barr described first the "Epstein-Barr virus" (EBV) in 1964.<sup>9</sup> EBV is the 4<sup>th</sup> member of the herpes virus family (gamma herpesviridae) contains double stranded DNA and it is a polyclonal stimulator of

B-lymphocytes. EBV is a rather ubiquitous infective agent, 70-90% of the human population is seropositive.<sup>5</sup> The causal aetiology of EBV is evident and proved in some disease, in some others the correlation is revealed by means of statistics only. Relations to many known human diseases such as infectious mononucleosis, Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, gastric carcinoma and posttransplant lymphoproliferative disease (LPD) were drawn.<sup>32</sup>

The LPD may develop in EBV seropositive and immunodeficient humans. These immunodeficiencies by origin can be "natural" (e.g. in AIDS, Duncan's disease), or

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“artificial” (as a result of chronic immunosuppressive treatment e.g. in posttransplant medication). Among transplanted patients the occurrence of LPD is approximately 1%.<sup>3</sup> As chronic immunosuppression becomes part of everyday medicine the problem of LPD grows alike. “Type A” LPD in younger patients has symptoms similar to infectious mononucleosis and infiltrates organs like the graft, lymph nodes, bone marrow, liver, gastrointestinal tract, lungs, heart, nasal pharynx, thyroid glands and the central nervous system. “Type B” LPD develops in senior patients in the form of solid tumors within the central nervous system and in the liver or elsewhere in the abdomen.<sup>7</sup> Both types are progressive and may be lethal. Biologic behaviour generally beats histological grading since the patients are immunocompromised. Ceasing immunosuppression usually heals LPD, but simultaneously triggers graft rejection or the progression of the primary disease. Different animal models are available for experimental LPD, but the induction of lymphoid chimeras in genetically immunodeficient mice is the most similar to the human disease. The C.B.17 scid/scid mice were first described in 1983 by Bosma and were kept inbred ever since. They share a spontaneous point mutation concerning the centromeric region of the 16<sup>th</sup> chromosome. Among the results is a special defect of V(D)J recombinase system, namely of the ligase within the complex.<sup>5</sup> Immunoglobulin and T cell receptor gene rearrangements suffer mistakes, even deletions that remain uncorrected.<sup>11</sup> As neither functional B nor T cells develop, these animals lack specific immune reactions, are susceptible to infections and can easily host xenogeneic tissues. There are basically two different methods to create chimeras by repopulating SCID mice with human lymphoid cells.<sup>15</sup> One involves simultaneous transfer of human embryonic bone marrow or liver tissue and human embryonic thymus tissue into infant SCID mice and the model is often referred to as “SCID-hu”. It ensures a permanent co-operation of xenogeneic cells.<sup>8,14</sup>

**Table 1. Chimeras created following the original protocol, mice indicated as “\*” were subjects of early measurements**

No. of mouse	No. of donor	LPD emergence	Survival (days)
1.	1.	*	*
2.	1.	+	55
3.	2.	*	*
4.	2.	+	46
5.	2.	+	67
6.	3.	*	*
7.	3.	*	*
8.	3.	-	>122
6.	3.	-	>122
10.	3.	-	>122

The other way is to transfer human PBL into mature SCID mice generally via an intraperitoneal injection and the model is called “hu-(PBL)-SCID”. Although this method provides only a transient chimera condition for about six months, it is convenient and usually stable for a period enough to examine some human diseases *in vivo*.<sup>8,12,13,14</sup> In our experiments we used the latter method to create chimeras and characterised them by immunohistochemistry and flow cytometry. Using *in vivo* superinfection it was possible to fine-tune and standardise the EBV+ hu-(PBL)-SCID xenogeneic chimera model reducing unspecific effects of the transplanted cells (e.g. graft versus host reactions).

### Materials and Methods

#### Mice

C.B.17 scid/scid homozygotic mice were kept inbred in the animal facility of the University of Pécs, Faculty of Medicine under specific pathogen free (SPF) conditions. During the time of experiments the animals were kept in a laminar cabinet in the Department of Immunology and Biotechnology. Ciprofloxacin (Ciprobay, Bayer) containing water was administered *per os* as antibiotic profilaxis.

#### Transplantation of human PBL

Buffy-coats from six different blood donors with their informed consent were the sources of human PBL. According to attached qualifications both EBV+ and EBV- buffy-coats were used. A suspension enriched in lymphocytes was obtained by density gradient centrifuging (20°C, 20 minutes, 2000/min.) over Ficoll solution ( $\rho=1.077 \text{ g/cm}^3$ ). Cell count was completed in Burker chamber after tripan-blue staining. Intraperitoneal cell transfers were achieved with  $3-6 \times 10^7$  cells according to the original protocol. Following the modified protocol PBL was transferred at a smaller quantity that is  $1-2 \times 10^7$  cells per inoculation without changing any other circumstances. The viability of PBL cells was higher than 98% in all cases.

#### In vivo superinfection

The B95-8 marmoset cell line (ATCC CRL 1612), secreting complete infective EB virions was cultured in DMEM containing 20% FCS for fourteen days in the Virus Laboratory of the State Office of Public Health, Pécs, Hungary. Supernatant of the culture was centrifuged (20°C, 5 minutes, 1000/min) and physically filtered through a Whatman GF/C pad before administering 1.5 ml intraperitoneally into the hosts, eleven days after intraperitoneal PBL transfer. In each of the three pairs of chimeras created following the modified protocol only one was subjected to subsequent *in vivo* superinfection.

An extra SCID mouse was given intraperitoneally 1.5 ml of EBV+ B95-8 supernatant without former PBL transfer as a negative control.

### Antibodies

The following anti-human monoclonal antibodies were used: anti- CD3, CD4, CD5, CD8, CD10, CD19, CD20, CD23, CD45,  $\kappa$  light chain,  $\lambda$  light chain, (all of them from DAKO, Denmark). Anti-mouse CD45 (IBL-5/25, developed in our laboratory), a-LMP 1 and MIB-1 (both from DAKO, Denmark) antibodies were also used.

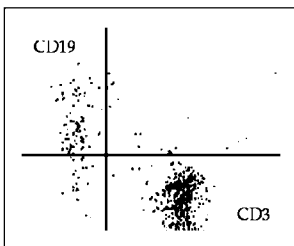


Figure 1. Site of inoculation prior to LPD, FACS analysis

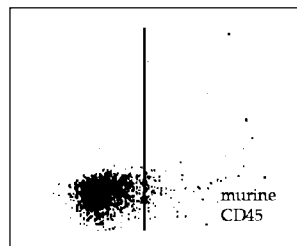


Figure 2. Origin of periportal mass of cells, FACS analysis

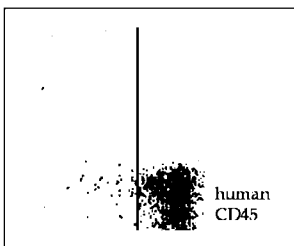


Figure 3. Origin of periportal mass of cells, FACS analysis

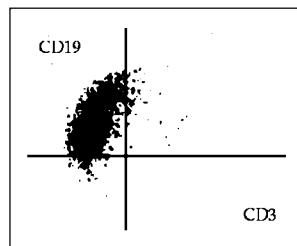


Figure 4. Phenotype of periportal mass of cells, FACS analysis

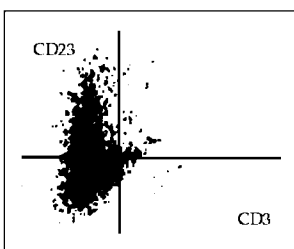


Figure 5. Phenotype of periportal mass of cells, FACS analysis



Figure 6. Phenotype of periportal mass of cells, FACS analysis

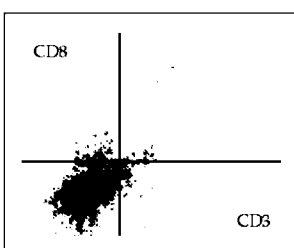


Figure 7. Phenotype of periportal mass of cells, FACS analysis

### Immunohistochemistry

Histologic examinations were performed on the intraperitoneal lymphoid mass and organs infiltrated by LPD (liver, spleen) from mice bearing clinical signs of malignant disease. Cryostat slides of frozen organs were fixed in acetone for 5 minutes then left to air-dry for 2 minutes. Further reactions were performed in a humidity chamber. After rehydration with PBS the inhibition of endogenous peroxidase activity was performed by using phenylhydrazin-hydrochloride (1 mg/ml) for 20 minutes. Saturation of the unspecific binding sites with 5% FCS albumin was completed. Antibodies were incubated for 45 minutes each on the slides including the streptavidin-peroxidase conjugated antibody. Signals were developed in a citrate buffer containing  $H_2O_2$  and 3-amino-9-ethyl carbasol (AEC). After 15 minutes development was halted by PBS solution. Hematoxylin nuclear counter staining was used. First classical hematoxylin-eosin stain made from all the samples for microscopic classification of the pathologic events. Normal human lymphoid tissues and monoclonal antibodies with irrelevant specificity were used as positive and negative controls, respectively.

### Flow cytometric analysis

FACS phenotype analysis was performed on cells forming the intraperitoneal mass within LPD bearing mice and on cells cultured *in vitro* later on. Centrifugation (4°C, 5 minutes, 1000/min) following the homogenising of the intraperitoneal mass resulted in a suspension of cells in PBS solution. Cells were incubated with FITC (fluorescein-isothiocyanat) or PE (phycoerythrin) labelled antibodies for 30 minutes over ice. Measurements were validated by carefully selected positive and negative controls. FACSCalibur type (Becton Dickinson, USA) flow cytometer, and the CellQuest software (Becton Dickinson, USA) were used for analysis.

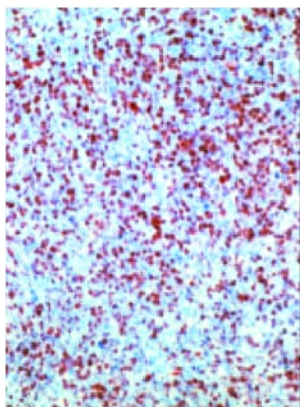
### In vitro culturing

Following sterile biopsy from the affected organs cells were mechanically dispersed to perform *in vitro* culturing over continuous control in DMEM medium containing 10% FCS in a tissue culture thermostat (Forma Scientific, USA) (37°C, 5%  $CO_2$  saturated humidity).

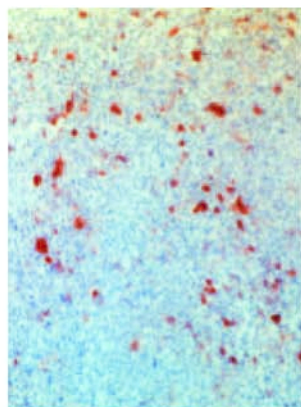
### Results

#### Engraftment of human cells

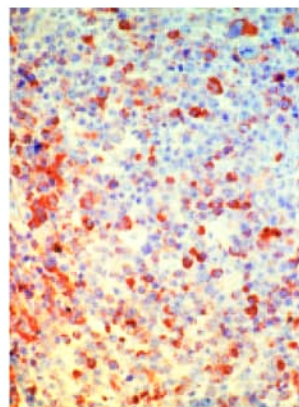
Using the original protocol we created altogether 10 hu-PBL-SCID xenogeneic chimeras with PBL from three different human blood donors (Table 1). PBL from two donors could develop LPD in the mice. In case of the



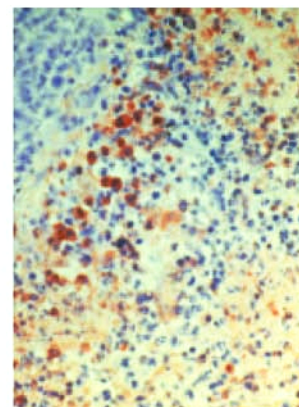
**Figure 8.** Intra-peritoneal mass of cells, MIB-1 immunohistology, 40x



**Figure 9.** Intra-peritoneal mass of cells, a-LMP-1 immunohistology, 40x



**Figure 10.** Intra-peritoneal mass of cells, a-kappa light chain immunohistology, 40x



**Figure 11.** Intra-peritoneal mass of cells, a-lambda light chain immunohistology, 40x

third donor no LPD occurred since the person was EBV seronegative.

One week after cell transfer at the site of inoculation T-lymphocytes were in majority compared to B-lymphocytes among human cells, similarly to the phenotype profile of physiologic human peripheral lymphocytes. FACS analysis showed the dominance of CD3<sup>+</sup> cells compared to CD19<sup>+</sup> cells (*Figure 1*) isolated from the peritoneal mass.

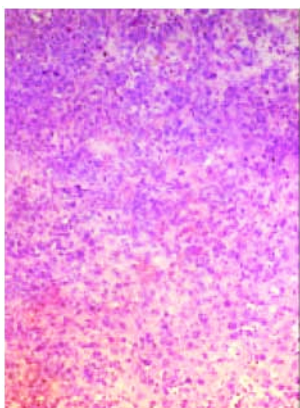
#### *LPD formation in the peritoneum*

The first sign of the developing LPD was a loosely structured tissue like mass of cells in the peritoneal cavity, in our case it was located periportally. The cells prepared from the peritoneal "tumor-like" mass shared human CD45<sup>+</sup> phenotype according to flow cytometry (*Figures 2,3*). At this stage of the disease B cells began to proliferate and formed a dominant subpopulation. Among B cells some were also CD23<sup>+</sup> activated intermediate B cells. The

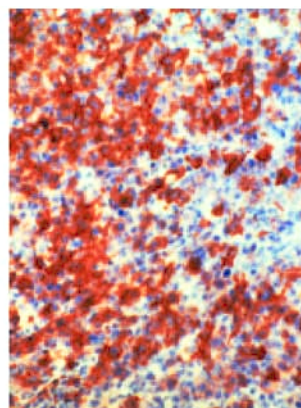
T cell subpopulation that is in minority by this time is probably rather CD8<sup>+</sup> (*Figures 4-7*). Immunohistochemistry revealed further precious data. The proliferative capacity of cells that formed the intra-peritoneal mass could be demonstrated by their MIB-1 positivity. Many cells in a diffuse pattern showed such phenotype indicating the increased rate of growth (*Figure 8*). The majority of tumor forming cells were light chain positive while a minority showed light chain positive phenotype (*Figures 9,10*). The presence of the transforming EBV was proved by the expression of LMP-1 (latent membrane protein type 1) in some human B cells (*Figure 11*).

#### *Host invasion by LPD*

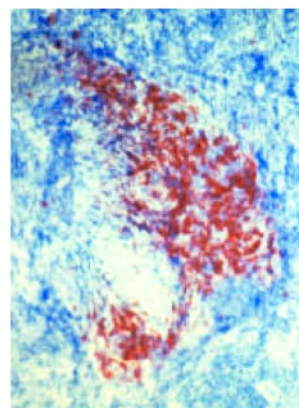
Dissemination of the human lymphatic tissue occurred during the next stage of LPD in the mouse model. Besides the lymphoid mass of proliferating cells located in the hilus of the liver and the spleen macroscopic haemorrhagic infarcts appeared on the surface of the liver in the mice.



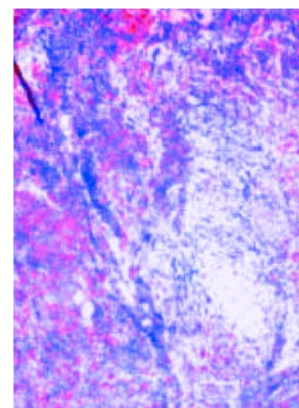
**Figure 12.** Infiltration of the liver, hematoxylin-eosin staining, 40x



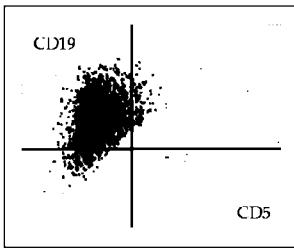
**Figure 13.** Infiltration of the spleen, a-CD20 immunohistology, 100x



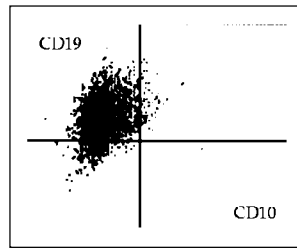
**Figure 14.** Infiltration of the spleen, a-CD3 immunohistology, 20x



**Figure 15.** Infiltration of the spleen, a-CD20 immunohistology, 20x



**Figure 16.** Phenotype of *in vitro* culture, FACS analysis



**Figure 17.** Phenotype of *in vitro* culture, FACS analysis

Tissue samples of the liver and the spleen were analysed histologically. Proliferating lymphocytes infiltrating the tissue regardless of hepatic lobuli were easy to distinguish from the hepatocellular background after hematoxylin-eosin staining during microscopic examination (**Figure 12**). Immunohistochemistry revealed characteristic localisation of human lymphocyte subsets. The CD3+ T cells preferred periarteriolar lymphatic sheaths (PALS) in the spleen, while the CD20+ B cells favoured lymphatic follicles (**Figures 13-15**) similarly to the normal anatomic location. LPD was lethal to mice in 46-67 days, agony was accompanied by palpable abdominal tumor, weakness, hypokinesia and ruffled hair.

#### *In vitro* culturing of tumor cells

Transformed cells removed from the host animals could be cultured *in vitro* for approx. 6 weeks. At small magnification colonies formed large spherical groups with many fission. Based on the findings of flow cytometry besides a CD19+ phenotype tumor cells also shared and CD5- and CD10- phenotype meaning that they were B cells but did not belong to the B1 or centroblast subgroups (**Figures 16,17**). No dominant clone developed, light chain profile still favoured the positive population to the positive population at a ratio of approx. 3:1 (**Figure 18**).

#### Reproducing LPD by using the modified protocol

Using the modified protocol we created altogether 6 chimeras with PBL from three different human blood donors. Activation by the xenogeneic environment increased the susceptibility of human lymphocytes to EBV. In each pair of mice only one was treated with subsequent *in vivo* superinfection. An extra SCID mouse was intraperitoneally injected 1.5 ml of B95-8 supernatant as negative control (**Table 2**). Note that neither the transfer of a smaller amount of PBL from EBV seropositive donors nor the *in vivo* administration of B95-8 supernatant by itself caused the emergence of LPD. Only the constellation of the two mentioned circumstances could create the disease. In chimeras that received subsequent *in vivo* superinfection LPD developed and proved to be lethal in 41-43

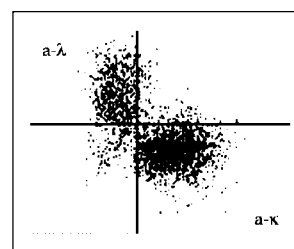
days. Palpable abdominal tumor, weakness, hypokinesia and ruffled hair accompanied their agony. At autopsy of affected mice an intraperitoneal loosely structured mass of cells in some ascitic fluid and enlarged, unusually pale liver and spleen were observed. Flow cytometric analysis of the intraperitoneal mass of cells showed mainly human CD45+ phenotype and the dominance of CD19+ B cells compared to CD3+ T cells (**Figures 19-20**). The double negative population contained murine cells (figures not shown). *In vitro* cultures of cells removed from the peritoneal cavity of moribund LPD hosts showed high capacity for proliferation for over a month before fission halted. At small magnification colonies formed large spherical groups of cells adhered to each other after mitosis.

#### Discussion

There is always uncertainty in creating a hu-PBL-SCID xenogeneic chimera model. Reproducibility is a major problem concerning experiments involving biological systems. According to some literature data, since biological systems are very complex and sensitive to starting conditions and non-linear formulas rule them, at the level of present measurement accuracy results can not be forecasted even if starting parameters are clear. The adherence of the graft depends on many factors that are not clearly understood or cannot be interfered properly<sup>6</sup> (E.g. the graft versus host reaction can influence the biologic response of the recipient).

The suggested minimal amount of cells required for successful transfer, the protocol of preparing a cell suspension and the treatment of SCID mice prior to transplantation almost differs in each laboratory. The likelihood of graft acceptance can be raised with an increase of input cell quantity and careful protocols that spare cell functions. If the original protocol is followed for creating hu-PBL-SCID models the minimally required amount of cells is approx.  $5 \times 10^7$  cells per inoculation, smaller quantities usually fail to successfully repopulate the host.<sup>8</sup> Yet we must admit that increased cell input quantity raises not only the chances of host repopulation but also points of variation that lead to unspecific side effects of grafting and unpredictable survival of chimeras.

Since in SCID mice myelopoietic cells develop adequately, intact innate immunity including granulocytes, monocytes, macrophages and NK cells decrease the prob-



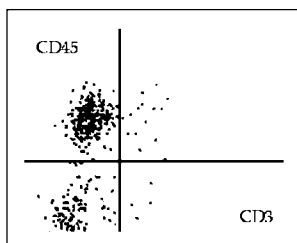
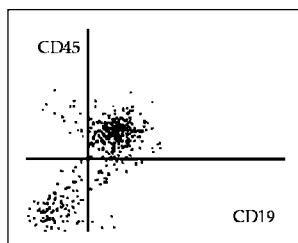
**Figure 18.** Clonality of *in vitro* culture, FACS analysis

**Table 2. Chimeras created following the modified protocol, mouse No. 7 was negative control**

No. of mouse	No. of donor	In vivo infection	LPD emergence	Survival (days)
1.	1.	-	-	>74
2.	1.	+	+	41
3.	2.	-	-	>74
4.	2.	+	+	42
5.	3.	-	-	>74
6.	3.	+	+	43
7.	-	+	-	>74

ability of graft adherence and influences LPD as well. Against murine NK cells an antibody known as anti-asialo  $G_{M1}$  or a sublethal irradiation of 1-4 Gy can be useful.<sup>10</sup> Unfortunately SCID mice are sensitive to irradiation and T cell lymphomas may emerge.

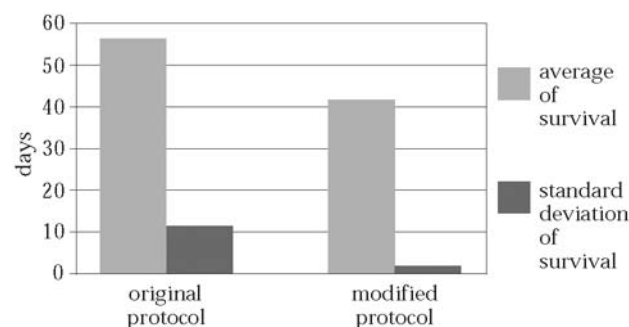
According to some experiments not only murine NK cells, but simultaneously transferred human NK cells can also decrease the activity of autologous B cells.<sup>4</sup> *In vitro* activation of human cells prior to transfer may also be helpful. This can help lymphocyte homing processes too.<sup>14</sup> PBL donors can be divided into at least two groups based on the incidence of LPD. The ability of donors to create LPD is constant with time and it is characteristic to donors. Approx. 40% of donors belong to the high incidence group and 60% to the low-intermediate incidence group. It is an interesting observation that the number of clones is greater in LPD developing from PBL of high incidence donors.<sup>16</sup> The incidence of LPD in the two groups shows no direct relation with the concentration of latently infected B cells in PBL. The frequency of latently infected cells among B-lymphocytes in the peripheral blood is individual and varies considerably. In a population of healthy carriers it is between  $5-500 \times 10^{-7}$  with a mean of  $50 \times 10^{-7}$  and is also characteristic to donors. This low number of latently infected cells is due to the fact that it concerns only a fraction of memory B cells and can also explain the minimum amount of cells required for successful repopulation. Immunosuppression during human allograft transplantation protocols causes an average 40-50 fold increase of latently infected B cells in the peripheral blood of patients

**Figure 19. Phenotype of peritoneal LPD, FACS analysis****Figure 20. Phenotype of peritoneal LPD, FACS analysis**

as it may perturb mechanisms that regulate the absolute numbers. Memory cells are unlikely to be under direct immunosurveillance therefore their increased numbers must be due to the prolonged survival or increased production of precursors that are under direct cytotoxic control.<sup>17</sup> Donors possess different Th1/Th2 cytokine profiles that influence both graft adherence, B cell proliferation and the development of graft versus host reactions.<sup>1</sup> The prevalence of CD8+ cells within the graft is various. Therefore inadequate cytotoxic immunosurveillance may also play a part in lymphoproliferation.

EBV subtypes differ in their ability to create LPD, "type A" EBV is more potent than "type B" EBV.<sup>5</sup> The B95-8 marmoset cell line secretes "type A" EBV. Genetic differences i.e. deletions within the EBNA-2 gene can considerably decrease transforming capacity since almost all viral promoters contain EBNA-2 dependent elements. The protein product of the EBNA-2 gene is considered to be a functional homologue of Notch IC and results in differentiation arrest and continued proliferation. Obviously it is also a main target of cytotoxic activity.<sup>17</sup>

Besides the mentioned uncertainties of creating chimera models, the exact timespan necessary for LPD emergence can not be forecasted either. In case of an EBV+ PBL donor the chances of developing LPD in the system with-

**Figure 21. Differences between the protocols in the average of survival and the standard deviation of survival**

in 60-110 days is about 80-100%. The matter is complex and that means variation in results.

The original method for inducing posttransplant LPD in SCID mice was modified during our experiments to standardise and increase the efficiency of B cell tumor occurrence with the reduction of unspecific factors. We transferred a smaller quantity of human PBL compared to the original protocol and administered EBV from the B95-8 cell line as subsequent *in vivo* superinfection. These alterations apparently circumvented some points of variation. The well-documented human LPD developed in the animals except for some valuable differences. A fraction of the formerly used cell number of EBV seropositive donors was sufficient to develop the disease in a slightly shorter

and in a more narrow time-span regardless of other donor characteristics. That is following the modified protocol the intraperitoneal transfer of  $1-2 \times 10^7$  lymphocytes was sufficient to create LPD, average survival decreased from 56 to 42 days and more importantly standard deviation of survival fell from 10.5 days to 1 day for all three donors. (*Figure 21*). Standard deviation describes the reproducibility of individual measurements and the number of observations does not influence its value. It is therefore possible to state that subsequent *in vivo* superinfection can be a useful alteration to fine-tune the EBV+ hu-PBL-SCID model and our modified *in vivo* model of human LPD can be helpful in detailing the steps of lymphoproliferation and in testing new tactics of treating the disease.

### Acknowledgements

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