IFN-gamma gene polymorphisms associate with development of EBV-positive Lymphoproliferative Disease in hu PBL SCID mice

Running Title: IFN-γ gene polymorphisms and LPD development

Julie E. Dierksheide*, Robert A. Baiocchi#,†, Amy K. Ferketich‡, Sameek Roychowdhury¶, Ronald P. Pelletier§, Charles F. Eisenbeis#,†, Michael A. Caligiuri#,‡, Anne M. VanBuskirk*,†

* Division of Surgical Oncology, Department of Surgery
#Division of Hematology and Oncology, Department of Internal Medicine
† Ohio State University Comprehensive Cancer Center
‡Division of Epidemiology and Biometrics
¶Department of Medical Microbiology, Virology, Immunology, and Medical Genetics
§ Division of Transplantation, Department of Surgery
The Ohio State University, Columbus, OH 43210.

1 Supported in part by grants from the Roche Organ Transplant Research Foundation, the OSU Comprehensive Cancer Center- R.J. Solove Research Institute Seed Grant program, the Department of Surgery Medical Research Development Fund and the National Institutes of Health: P30 CA16058 (R.A.B., C.F.E., M.A.C., A.M.V.), R29 AI-40909 and R03 AI-54383 (A.M.V.), K08 CA93518 (C.F.E.) and T32 CA 09338 (R.A.B.). AMV is the 2002 recipient of the American Society for Transplantation Women’s and Minority Faculty Grant.

2 Address correspondence to:
Anne M. VanBuskirk, Ph.D.
Surgical Oncology
Ohio State University
1017 Wiseman Hall
400 West 12th Avenue
Columbus, OH 43210
Email: vanbuskirk.1@osu.edu

Scientific Section: Immunobiology

Word Count: abstract (200), text (4972)
Abstract:

Post-transplant lymphoproliferative disorder (PTLD) is a devastating post-transplant complication often associated with Epstein-Barr virus. While type and length of immunosuppression are risk factors, a patient’s inherent immune capacity also likely contributes to this disorder. This report utilizes severe combined-immunodeficient mice injected with human peripheral blood leukocytes (hu PBL-SCID mice) to test the hypothesis that cytokine genotype associates with development of EBV-associated lymphoproliferative disease (LPD). We observed that the A/A genotype for base +874 of the IFN-γ gene was significantly more prevalent in PBL producing rapid, high penetrance LPD in hu PBL-SCID mice, compared to PBL producing late, low penetrance LPD or no LPD. In examining the relationship between genotype and cytolytic T-lymphocyte (CTL) function, TGF-β inhibited re-stimulation of CTL in PBL with adenosine at IFN-γ base +874, but not in PBL homozygous for thymidine. Importantly, neutralization of TGF-β in hu PBL-SCID mice injected with A/A genotype PBL resulted in reduced LPD development and expanded human CD8+ cells. Thus, our data show that TGF-β may promote tumor development by inhibiting CTL restimulation and expansion. Further, our data indicate that IFN-γ genotype may provide valuable information for both identifying transplant recipients at greater risk for PTLD, and developing preventative and curative strategies.
Introduction:

Post-transplant lymphoproliferative disorder (PTLD) is a devastating complication of solid organ and stem cell transplantation that can have 70-80% mortality \(^1\). PTLD is often associated with Epstein-Barr virus (EBV), a ubiquitous herpes virus that establishes latent infection in the majority of healthy adults. Primary infection with EBV and elevated viral load in the post-transplant period are significant risk factors for developing PTLD, particularly in pediatric patients \(^2\)-\(^4\). However, the majority of adults are EBV sero-positive, so additional risk factors for PTLD have been difficult to determine. The incidence of PTLD varies according to the organ transplanted, as well as the intensity and duration of immunosuppression. In renal transplant recipients PTLD occurs in 1-2% of patients, but the incidence is as high as 20% in bone marrow and in lung transplant recipients \(^1\). There is no accepted standard of therapy for PTLD.

CTL activity is crucial for prevention and/or sustained recovery from PTLD\(^5\)-\(^8\). It is thought that immunosuppression inhibits the EBV-specific cellular immunity that normally prevents the progression of EBV-driven transformation of latently infected cells. Reduction of immunosuppression is effective in treating some, but not all PTLD patients \(^1\),\(^8\), and increases the likelihood of developing acute rejection episodes that can result in graft loss. Current clinical trials for treatment of PTLD include cellular and monoclonal antibody therapies; however, recurrences may occur after these treatments \(^9\),\(^10\).

IFN-\(\gamma\) is an important cytokine in cellular immunity and CTL function. One polymorphism in the IFN-\(\gamma\) gene is a single nucleotide polymorphism at position +874 containing either a thymidine (T) or an adenosine (A). The presence of the thymidine at +874 correlates with microsatellite repeats associated with high cytokine production and creates an NF-\(\kappa B\) binding site \(^11\)-\(^13\). The T/T genotype is often referred to as a “high producer” and A/A genotype as “low producer”\(^13\), although in vitro cytokine production can only be reliably inferred from genotype if T cells are maximally stimulated \(^14\) or a large
DIERKSHEIDE, et.al.  

IFN-\( \gamma \) GENE POLYMORPHISMS AND DEVELOPMENT OF PTLD

Sample size is used. In general, the relationship between IFN-\( \gamma \) polymorphisms and disease states has not been extensively studied, although there are reported links to GvHD, chronic hepatitis infection, and tuberculosis. We recently observed in a small study that a majority of PTLD patients exhibit the A/A cytokine genotype for IFN-\( \gamma \), and that this is significantly increased compared to the frequency seen in non-PTLD renal transplant patients.

TGF-\( \beta \) is a ubiquitous, pluripotent cytokine that can suppress multiple T cell and antigen presenting cell (APC) functions (reviewed in), including EBV-specific CTL effector function. TGF-\( \beta \) has been linked to EBV activation and replication, and increased transformation. These data suggest that TGF-\( \beta \) may be important in EBV-driven cancers. By activating viral replication and suppressing viral-specific CTL function, the increased viremia and B cell transformation combined with simultaneously suppressed T cell responses could promote PTLD. TGF-\( \beta \) and IFN-\( \gamma \) are antagonistic and counter-regulatory (reviewed in), in that both in vitro and in vivo studies have shown that IFN-\( \gamma \) can inhibit TGF-\( \beta \) activity, and vice versa. Thus, high levels of IFN-\( \gamma \) may protect against the immunosuppressive effects of TGF-\( \beta \), or reducing TGF-\( \beta \) may allow increased IFN-\( \gamma \) activity.

The hu PBL-SCID mouse, in which human (hu) peripheral blood leukocytes (PBL) from healthy EBV sero-positive donors are injected into SCID mice, is a reproducible model of spontaneous EBV-driven lymphoproliferative disease (LPD). EBV-positive B cell tumors spontaneously arising in hu PBL-SCID mice are phenotypically and genotypically very similar to PTLD. Previous reports indicated donor-derived variability in LPD development in the hu PBL-SCID mouse, however, factors contributing to this variability are largely undefined. We hypothesized that the low producer A/A IFN-\( \gamma \) genotype would associate with rapid LPD in the hu-PBL SCID mouse model. Here we demonstrate that PBL from individuals with specific IFN-\( \gamma \) genotypes differ in their ability to rapidly develop high penetrance LPD in the hu PBL-SCID mouse. In examining the relationship between IFN-\( \gamma \) genotype and CTL function, we demonstrate that restimulation of CTL from donors of the A/A or A/T IFN-\( \gamma \)
gene genotypes is inhibited following exposure to TGF-β in vitro. To further assess the contribution of TGF-β to the LPD process, we neutralized TGF-β in hu PBL-SCID mice. Effective TGF-β neutralization resulted in protection from LPD and an expansion of human CD8+ cells. Given that CTL activity is crucial for prevention and/or sustained recovery from PTLD, our data suggest that increased susceptibility to TGF-β may provide an explanation for the relationship between IFN-γ genotype and development of PTLD.

**Materials and Methods:**

**PBL:** PBL were obtained from American Red Cross leukopacks, or from volunteers using institutional review board approved protocols. PBL were isolated by ficoll-hypaque according to standard methods. Donors were tested for EBV reactivity by ELISA (Meridian, Cincinnati, OH) and EBV-reactive trans vivo DTH assays prior to injection into SCID mice or use in CTL restimulation cultures. Approval was obtained from the Ohio State University institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki.

**Genotyping Assays:** Genomic DNA was isolated from PBL using Qiagen (Valencia, CA) DNA extraction kits. HLA analysis was done using Pel-Freez Clinical Systems AB/DR PCR-SSP unitrays (Brown Deer, WI). Cytokine genotyping for TGF-β, TNF-α, IL-6, IL-10, and IFN-γ was accomplished using Cytgen cytokine genotyping trays from One Lambda (Canoga Park, CA). PCR products were run on 2% agarose gels and visualized with ethidium bromide. Banding patterns were interpreted using manufacture’s templates and compared to internal controls in each lane.

**hu PBL-SCID Model:** Female Balb/c or CB.17 scid/scid (SCID) mice were purchased from Charles River or Taconic. Mice were housed and treated in accordance with NIH and institutionally approved
DIERKSHEIDE, et.al.                  IFN-γ GENE POLYMORPHISMS AND DEVELOPMENT OF PTLD guidelines. Mice received 50 x 10^6 human PBL intraperitoneally in saline. PBL from each donor were injected into three to five separate mice. Human PBL engraftment was monitored with bi-weekly ELISAs for the presence of human IgG in mouse serum, as previously described 40. Mice included in this study had >750 µg/ml of human IgG, which increased to >1mg/ml when tumors were detected. Latency was defined as the time after injection until mice became moribund or died 39. All animals were inspected at death for the presence of tumors, and these tumors confirmed to be of human B cell origin using flow cytometry. Only mice with confirmed human tumors were considered to have LPD.

Flow Cytometry: Splenocytes and tumor cells from hu PBL SCID mice were analyzed via flow cytometry to assess CD8+ T cell levels and T cell activation. All antibodies and isotype control antibodies were directly conjugated and obtained from BD Pharmingen (San Diego, CA). Samples were read on a FACScan (BD) and analyzed using Cell Quest software.

In vivo TGF-β neutralization: hu PBL-SCID mice received 125µg anti-TGF-β monoclonal antibody (A411, obtained from Dr. P. Heeger, Clevland Clinic Foundation, Cleveland, OH) intraperitoneally three times per week or 100µg every other day for 8 weeks. Control animals received PBS or 100µg mouse IgG. Neutralization of endogenous TGF-β was confirmed by TGF-β ELISA of serum from hu-PBL SCID mice tested at 4,6 and 8 weeks post cell injection. In the survival trial, animals received 100µg anti-TGF-β (1D11, Genzyme, Cambridge, MA), isotype control antibody or PBS intraperitoneally every other day for the duration of the study.

LCL lines and culture: EBV-transformed lymphoblastoid cell lines (LCL) were derived from hu PBL-SCID tumors or by in vitro infection with EBV-containing supernatant of the B89.5 cell line using
standard protocols. LCL with defined HLA molecules was the kind gift of Dr. Hildebrand (B62→221, Oklahoma Medical Research Foundation, Oklahoma City, OK).

**CTL Restimulation.** PBL were depleted of natural killer cells (NK) cells using Miltenyi Biotech (Auburn, CA) anti-CD56 magnetic beads and LD columns. PBL were plated at 2 x 10^6 cells/ml. LCL tumor lines used as stimulator cells were γ-irradiated at a dose of 20,000 rads or treated with mitomycin C (5ug/ million cells). There were no observed differences in CTL function using either irradiated or mitomycin-treated LCL as stimulator cells. LCL were plated at 1 x 10^5 cells/ml. PBL and LCL were co-cultured with cytokine for five days and viable PBL were recovered and used in cytolysis assays. After 5 days, restimulation cultures contain a mix of CD3^+CD4^+ and CD3^+CD8^+ cells, with no CD3^+CD56^+ lymphokine-activated killer cells detectable. The HLA-typing of the donors and LCL stimulators are shown in Table 1. PBL were stimulated with autologous LCL, except for donor 6J and donors 1P, 2A, 3M and 5C. Donor 6J was stimulated with LCL expressing only B62 and DR 1, while donors 1P, 2A, 3M and 5C were stimulated with LCL expressing only A1, B8, DR3. Thus, LCL stimulators did not express HLA-A, -B molecules different from the PBL donor.

**Cytokines:** Recombinant human TGF-β was purchased from R&D Systems (Minneapolis, MN) or Leinco (St. Louis, MO) and used at 10 ng/ml.

**Cytolysis Assays:** Standard non-radioactive cytotoxicity assays were set up using PBL from 5-7-day re-stimulation cultures and either HLA-matched or mismatched LCL lines at various effector-to-target ratios, with target cells plated at 5 x 10^4- 1 x 10^5 cells/ml. All samples were plated in triplicate. Alamar blue (Biosource, Carmillo, CA) was used at a dilution of 1:10. Cells were cultured for 24 hours, and read on a Cytofluor II fluorescent multi-well plate reader (Perspective Biosystems) at an excitation
wavelength of 530 nm and an emission wavelength of 590 nm. Percent lysis was determined as follows: \( \frac{\text{targets alone} - ((E+T) - (E \text{ alone}))}{\text{targets alone}} \). Lytic units (LU)\(^3\) are arbitrarily defined as the number of lymphocytes required to yield the selected lysis value (in this case, 30%). To define LU, all curves must pass through this lysis value, and it must be in the linear portion of the curve. The number of LU per million cells is calculated using the following formula: \( \text{LU}_{30} \text{ per million cells} = \frac{10^6}{(\# \text{ effectors/percent lysis}) \times (30)} \).

**Growth inhibition assays:** Long-term tumor growth inhibition assays were performed similarly to Wilson et al\(^{44}\). Briefly, LCL targets were plated at 1 x 10\(^5\) cells/ml into 96-well plates and serially diluted 1:2. PBLs were harvested from 5-day re-stimulation cultures and plated at 1 x 10\(^4\) cells/well. On day 14, alamar blue was added, and plates were read as described above. Percent inhibition of proliferation was calculated for each effector cell population and compared to the proliferation of LCL targets alone as follows: \( \frac{[(\text{proliferation of effectors} + \text{targets})/ (\text{proliferation of targets})] \times 100}{} \). As an additional control, viable cells were subjected to flow cytometry and shown to be CD19/CD20\(^+\), demonstrating outgrowth of LCL rather than CTL.

**Cytokine ELISAs for IFN-\(\gamma\) and TGF-\(\beta\).** Levels of IFN-\(\gamma\) in culture supernatants (SN) were measured by ELISA using antibody pairs from Endogen (Rockford, IL), and TGF-\(\beta\) in serum was measured using antibody pairs from BD Pharmingen or a TGF-\(\beta\) ELISA kit (R&D sytems) according to manufacturer’s instructions. Briefly, microtiter plates were coated with anti-IFN-\(\gamma\) or anti-TGF-\(\beta\) (2\(\mu\)g/ml) overnight and blocked with borate-buffered saline (BBS) buffer containing 5% BSA and 1% normal goat serum for 2 hours. Cytokine standards or SN were added at titrated dilutions for 4 hours at room temperature. Detecting antibody (0.5\(\mu\)g/ml for IFN-\(\gamma\), 2\(\mu\)g/ml for TGF-\(\beta\)) was added for 2 hours. Streptavidin-horseradish peroxidase (1:1,000) was added for 30 minutes. After washing, 2,2’-azino-bis(3-ethylbenz-
thiazoline-6-sulfonic acid) substrate was added for 20 minutes. Optical Density was measured at 405nm using a Biorad ELISA reader. IFN-γ and TGF-β concentrations were determined by comparing the optical density of dilutions in the linear phase of the curve to the standard curve run on the same plate.

**Statistical methods:** The genotypes of rapid tumor producers for specific cytokines was compared to other subjects using Fisher’s exact test. For TNF-α, the presence of the A allele at base –308 (high TNF-α) was compared to other genotypes. For IL-10, the absence of the G allele at base –1082 (low IL-10) was compared to the other genotypes. In the IL-6 gene, homozygosity for the C allele at base –174 (low IL-6) was compared to other genotypes. For TGF-β, the genotype associated low TGF-β production (changes in the leader sequence resulting in proline residues at codons 10 and 25) was compared to high/intermediate TGF-β genotypes. Finally, for IFN-γ, homozygosity for the A allele at base 874, as well as the presence of the A allele were compared to other genotypes for the three LPD groups (rapid vs. late vs. no LPD). Median time to LPD onset (latency) and median penetrance (percentage of mice developing human tumors) were compared using the exact Wilcoxon Rank Sum test. CTL activity was compared using Student’s t-test. Survival times were compared using the Log Rank test. For all statistical tests, p<.05 was considered significant.
Results:

Heterogeneity in LPD development in hu-PBL SCID mice

PBL from each of forty-nine EBV-reactive donors were injected into 3-5 SCID mice per donor. Recipient mice were monitored for up to 6 months for engraftment by human cells (as evidenced by human IgG in the serum) and development of LPD (human CD45+ CD19/CD20+ tumors infiltrated with small numbers of CD45+CD3+ cells). As shown in Table 2, PBL from 47% (23 of 49) of the donors produced no LPD after 20 weeks, while 24% (12 of 49) developed LPD tumor rapidly (median time to LPD, 8 weeks) and with high penetrance (median 100%, range 80-100%). PBL from the remainder of the donors (29%, 14 of 49) produced LPD later (median 12 weeks), and in fewer mice (median penetrance 55%, range 33-100%). As determined by the exact Wilcoxon Rank Sum test, the differences in latency and penetrance between the rapid and intermediate/late groups are statistically significant (p<.0001).

Cytokine genotype and the development of LPD in hu PBL- SCID mice. To test our hypothesis that cytokine genotypes correlate with LPD development, we assessed the distribution of cytokine genotypes for IFN-γ, TNF-α, IL-6, IL-10 and TGF-β in the PBL used to produce EBV-LPD in hu PBL-SCID mice.

We compared rapid, high penetrance LPD producers with intermediate /late LPD producers and with donors whose PBL did not produce LPD (as determined in Table 2). Table 3 demonstrates that analysis of the distribution of polymorphisms for IFN-γ demonstrated statistically significant differences between rapid LPD producers and the other two groups. Of the 12 rapid LPD producers, none were of the T/T genotype, 5 were T/A genotype (41.7%), and 7 were A/A genotype (58.3%). In contrast, donors whose PBL produced intermediate/late LPD or not at all, exhibited a more heterogeneous distribution of genotypes (14 T/T, 37.8%; 15 T/A, 43.3% and 8 A/A, 18.9%). Statistical analyses of these data indicate that the A/A genotype was significantly more frequent in the rapid LPD producers compared to the
intermediate/late LPD producers and the no LPD producers (p = .0144). The absence of the T/T genotype among the rapid LPD producers was also striking, suggesting that the presence of the T allele correlated with a lack of LPD development in hu PBL-SCID mice. All (12 of 12) of the rapid LPD producers had at least one A allele present, contrasted to the intermediate/late LPD producers (8 of 14) and no LPD producers, where 15 of 23 donors had at least one A allele present. This is a statistically significant difference between the three groups (p = .0257). When the cytokine polymorphism distributions for TNF-α, IL-6, TGF-β and IL-10 were analyzed, no statistical differences were observed between the groups of donors. Similar to the reported distributions for TGF-β genotypes, the majority of our donors exhibited genotypes for high TGF-β production. Indeed, 48 of the 49 PBL donors, and all of those producing rapid LPD had genotypes linked to high TGF-β production.

*TGF-β inhibition of CTL activity is associated with IFN-γ genotype.* To further examine the relationship between IFN-γ genotype and CTL function, we next tested whether TGF-β could inhibit re-stimulation of CTL activity in vitro. PBL were cultured with irradiated HLA-matched LCL stimulators in the presence or absence of TGF-β for 5 days. CTL activity was assessed using standard CTL assays. Figure 1A shows that PBL from individuals with the A/A or A/T IFN-γ genotype had an impaired CTL response if TGF-β was added to the re-stimulation cultures. TGF-β-treated cultures for these donors had 25-70% inhibition of cytolysis compared to control cultures. In contrast, TGF-β had no effect on CTL restimulation of T/T genotype PBL. Data are shown as the mean percent control lysis, determined using LU. The difference between the A/A + T/A genotype cultures and the T/T genotype cultures was significant (p = .015). In Figure 1B we verified the inhibition of CTL re-stimulation using two-week LCL growth inhibition assays, similar to those described by Wilson et al. Growth inhibition assays assess the ability of a set number of re-stimulated CTL to lyse a titrated number of LCL under more stringent conditions than regular CTL assays. LCL not killed by the CTL will proliferate and detectable
DIERKSHEIDE, et.al.      IFN-γ GENE POLYMORPHISMS AND DEVELOPMENT OF PTLD
differences in metabolic activity are seen after two weeks. Figure 1B shows that CTL inhibited long
term growth of matched but not mismatched LCL, and that A/A or A/T genotype CTL (n=3 donors) re-
stimulated in the presence of TGF-β did not inhibit growth of their matched LCL targets. In contrast,
T/T genotype CTL re-stimulated in the presence of TGF-β (n=3 donors) inhibited LCL growth similarly
to control CTL. Thus, TGF-β can inhibit CTL restimulation in A/A or A/T genotype PBL, but not T/T
genotype PBL. Given that A/A and A/T genotype PBL are more likely to produce LPD in hu PBL SCID
mice, we next examined the influence of TGF-β in the in vivo system.

In vivo treatment with anti-TGF-β improves survival of hu PBL SCID mice. Like the majority of the
general population, all of the rapid LPD donors exhibited genotypes linked to high TGF-β production.
Given our in vitro data indicating that TGF-β could inhibit CTL restimulation, we investigated whether
treatment with anti-TGF-β would prolong survival of hu PBL SCID mice. hu PBL SCID mice were
injected intraperitoneally with 100µg of PBS, isotype control antibody or a commercially available anti-
TGF-β antibody (Genzyme) three times per week for the duration of the experiment. All animals were
engrafted, as evidenced by >750µg/ml human IgG in the sera at 4 weeks post injection (not shown). As
shown in Figure 2, animals treated with either PBS or isotype control antibody had a mean survival of
60 days. In contrast, animals treated with anti-TGF-β survived greater than 80 days. Thus, anti-TGF-β
treatment significantly enhanced survival of hu PBL SCID mice (p<.002).

In vivo Neutralization of TGF-β reduces LPD and results in CD8+ expansion and activation. To
investigate the mechanism by which in vivo treatment with anti-TGF-β antibody prolonged survival, and
to assess the utility of anti-TGF-β treatment, we performed a second experiment using the A411 anti-
TGF-β antibody and a second PBL donor. In the second trial we assessed neutralization of TGF-β, LPD
development and CD8 T cell expansion. We initially injected hu PBL-SCID mice with anti-TGF-β
antibody three times per week and monitored human Ig levels, serum TGF-β and LDP development. All animals were engrafted, as evidenced by >750µg/ml human IgG in the sera at 4 weeks post injection (not shown). Also at week 4, hu PBL-SCID mice routinely exhibited circulating levels of 9000 pg/ml TGF-β. Treatment of the animals with anti-TGF-β significantly reduced that level to less than 4000 pg/ml (p<.001) (Figure 3A). Animals were sacrificed at 9 weeks, at which point 100% of the control animals had developed human B cell tumors. In contrast, only 20% (1 of 5) of the animals receiving 125µg anti-TGF-β developed LPD (Figure 3B). Flow cytometric analysis of the spleens and tumors indicated that human CD8+ cells had dramatically expanded in the anti-TGF-β treated mice. Control mice had a median of 0% CD8+ cells in their spleens. These mice rarely had human non-B cells in the spleens. In contrast, animals receiving 125µg anti-TGF-β had a median of 17.5% CD8+ cells in their spleens. The one treated animal that developed a B cell tumor had significant numbers of B cells in the spleen (25%), as well as significant numbers of CD8+ cells (25%). Importantly, CD8+ T cells were also expanded in the tumor of the one tumor-positive anti-TGF-β treated animal.

To further examine the effects of anti-TGF-β, an additional study using a third donor was performed. Hu PBL-SCID mice were treated with 100µg anti-TGF-β antibody every other day for 9 weeks (Figure 4). Anti-TGF-β treatment effectively neutralized TGF-β in the sera of these animals (not shown). Flow cytometry was used to assess the expansion human cells in the tumors (Figure 4A) and spleens (Figure 4B). Tumors from control IgG- treated mice contained human B cells and very few CD3+ CD8+ T cells. Likewise, spleens from these animals contained B cells but very few, if any, T cells. In contrast, tumors and spleens from anti-TGF-β treated mice exhibited large numbers of CD3+ T cells that were memory cells expressing CD45RO. In the tumors, 20% of the CD3+ cells also expressed CD25, indicating that they were activated. However, only 9% of the CD3+ cells in the spleens expressed CD25.

**Discussion:**
PTLD can be a devastating post-transplant complication, even in adult patients with previously established anti-EBV immunity. Known risk factors include the type of transplanted organ, EBV viral load, as well as the type and duration of immunosuppression administered to prevent graft rejection. While most transplant patients at a particular transplant center receive similar immunosuppressive regimens, only a portion of these patients develop PTLD. This suggests that the inherent immune capacity of a transplant patient contributes to the development of PTLD. Our original observation with 9 PTLD patients at The Ohio State University Medical Center suggested an impressive skewing of the IFN-γ genotype distributions, with 80% of PTLD patients exhibiting the A/A genotype, compared to 27% of 135 non-PTLD renal transplant patients. We have since extended our study to include 12 PTLD patients. The proportion of patients with the A/A genotype for the IFN-γ gene is higher in PTLD patients than in 135 non-PTLD transplant patients at the same transplant center (58% versus 27%, p=.02). When we assessed the genotype distributions for TGF-β, IL-6, IL-10 and TNF-α, we did not observe any statistically significant differences between PTLD and non-PTLD patients.

Our preliminary clinical observation is strengthened by the current prospective study using the hu PBL-SCID mouse model of spontaneous EBV-LPD. Fifty-three percent of the EBV-seropositive donors produced LPD in the hu PBL-SCID mice within 6 months. Of donors producing LPD, 12 rapidly produced LPD (median time to LPD, 8 weeks) with high penetrance (median 100%). The other LPD producer phenotype developed LPD later (median time 12 weeks) and with lower penetrance (median 55%). While the heterogeneity of LPD production among PBL donors in this animal model is well established, the factors leading to this heterogeneity have not been extensively studied. EBV strain and atopic status have been implicated as factors. Our prior work had identified the IFN-γ A/A genotype as a risk factor in PTLD. Therefore, we performed a more complete evaluation of the relationship between cytokine genotype and LPD development in the hu PBL SCID mouse model using
49 donors. We did not collect data on either the EBV strain infecting the different donors, or the donors’ atopic status, and so cannot address these issues in our donor pool. Murine NK cells are also known to influence LPD development, as are murine macrophages, and it is possible that differential ability to activate murine NK cells could account for some heterogeneity in LPD development. We purposefully did not deplete or neutralize NK cells in our study, reasoning that this made the model more stringent. Thus, any observed association of cytokine polymorphism and LPD would be more likely to be a strong association.

Importantly, we observed a striking association of the A allele for IFN-γ at base +874 with LPD production. Of the rapid, high penetrance LPD donors, 58% were homozygous for the A allele (A/A), while 42% were heterozygous (T/A). None of the rapid, high frequency LPD producers were homozygous for the T allele. In contrast, all genotypes were represented in the groups of donors who produced LPD late or not at all. The frequency of the A/A genotype among the rapid LPD producers was significantly different compared to the intermediate/late LPD producers, and the no LPD donors (p=0.0144). Also significant (p=0.0257) is the presence of the A allele in rapid LPD producers compared to the other 2 LPD groups. These data mirror our clinical observations, suggesting that the IFN-γ genotype association with LPD production in hu PBL-SCID mice may have clinical relevance.

The A/A, T/A and T/T IFN-γ genotypes for base +874 have been reported to correspond to low, intermediate and high in vitro cytokine production, respectively. We observed a clear-cut association of genotype with cytokine production only when HLA-A, -B matched donors were tested using the same EBV-LCL, thereby providing the same antigenic stimulus. Of the four donors that met these criteria, the A/A genotype donor produced the least IFN-γ, with the 2 A/T genotype donors producing an intermediate amount of cytokine and the 1 T/T genotype donor producing the most IFN-γ
IFN-γ is a critical regulatory cytokine in cellular immunity that is important in immune surveillance of EBV-related LPD. TGF-β is antagonistic to IFN-γ and has been implicated in EBV reactivation, suppressing T cell effector function or otherwise inhibiting immune surveillance (reviewed in 21). Strong cellular immunity and CTL activity is crucial to preventing or clearing PTLD or LPD in hu PBL-SCID mice. Our data indicate that the low producer IFN-γ genotype is associated with both PTLD in patients and LPD in hu PBL-SCID mice. In trying to understand the relationship between IFN-γ genotype and TGF-β in LPD development, we focused on whether TGF-β could differentially influence CTL restimulation in vitro. Detecting CTL activity against EBV antigens requires a 5-12 day restimulation culture. We determined that CTL were restimulated efficiently in vitro regardless of the IFN-γ genotype (not shown), indicating that a lack of CTL precursors or a generalized defect in CTL restimulation could not explain the association of the A/A genotype with LPD development. However, when TGF-β was present, CTL restimulation was significantly reduced in A/A or A/T, but not T/T, genotype PBL.

Given that all of the donors whose cells produced LPD rapidly and with high penetrance in hu PBL-SCID mice exhibited genotypes consistent with high TGF-β production, and that TGF-β could inhibit CTL restimulation in vitro, we then examined whether reducing TGF-β in hu PBL-SCID mice would prolong survival of hu PBL-SCID mice. A survival trial using a commercially available anti-TGF-β antibody resulted in 100% survival greater than 80 days in the anti-TGF-β treated mice. In contrast, all
Dierksheide, et al. IFN-γ GENE POLYMORPHISMS AND DEVELOPMENT OF PTLD

Control animals died within 70 days. These data support an important role of TGF-β in LPD development.

To determine the mechanism by which anti-TGF-β prolonged survival, additional experiments were performed with both a different antibody and using additional PBL donors. Antibody treatment resulted in a significant reduction of circulating TGF-β. Serum TGF-β levels can be variable and often correlate with platelet numbers, as platelets both release latent TGF-β and activate it. Although we were unable to control for platelet activation in our samples, sera from anti-TGF-β treated animals consistently had less TGF-β than control treated animals within the same experiment. Control-treated mice had B cell tumors with very few (<5%) infiltrating CD8+ T cells. Spleens of these animals had B cell infiltration but no CD8+ T cell infiltration. In contrast, anti-TGF-β treatment resulted in a dramatic expansion of human CD3+ CD8+ cells in the tumors. These CD3+ cells were CD45RO and 20% also expressed CD25+, indicating they were activated memory cells. A large number of CD45RO+ CD3+ T cells also infiltrated the spleens of these mice, but fewer expressed CD25.

Whether the CD8+ T cell expansion is limited to EBV-reactive T cells, or is non-specific will be tested in future experiments. It is possible that in anti-TGF-β treated mice, CD8+ cells could be expanded non-specifically and result in graft-versus-host disease (GVHD). GVHD in the hu PBL-SCID model can present with CD8+ cell infiltration in the spleen. Although we observed general increases in CD8+ cells in the spleens of anti-TGF-β treated animals, only one animal in the 9-week trial and none of the survival trial animals, exhibited overt GVHD with cachexia.

In summary, we demonstrate a significant association of the A/A genotype for IFN-γ with rapid lymphoproliferative disease production in hu PBL-SCID mice. In contrast, the T/T genotype is absent in rapid LPD producing donors and is uncommon among PTLD patients. We hypothesize the following...
mechanism by which genotype could influence LPD development: in A/A and T/A genotype individuals, TGF-β acts to inhibit memory CTL re-stimulation, allowing EBV transformation of B cells and uncontrolled growth of the transformed cells. Strong cellular immunity is critical for the control of EBV lymphoproliferative disease 5-8, and IFN-γ is an important cytokine in cellular immunity. TGF-β is important in EBV-associated disease 27,28,59,60 and can contribute to viral activation 23-26. Further, TGF-β can inhibit T cell and APC functions 20,22. Our data show an association of IFN-γ cytokine gene polymorphisms with both LPD development and functional differences in CTL restimulation cultures in response to TGF-β. In addition, our data demonstrate that antibody neutralization of TGF-β can prevent LPD and result in expansion of activated, memory CD8+ cells in hu PBL-SCID mice. Thus, we propose that the association of the A/A genotype with LPD production in hu PBL-SCID mice and with PTLD in humans may reflect sensitivity in these individuals to TGF-β -mediated inhibition of CTL re-stimulation. The subsequent reduction in CTL activity could then predispose these individuals to EBV-driven LPD.
Acknowledgements

The authors thank Drs. Burlingham, Ledbetter, Moeschberger, Orosz, and Porcu for helpful discussions. Tyler Hoppes, Kelly Johnson, Vanita Malik and Navneet Cheema provided excellent technical assistance, and Donna Bucci provided expert administrative assistance. The authors wish to express their appreciation to the members of the OSU Tissue Typing Laboratory for assistance with blood draws and HLA-typing.
<table>
<thead>
<tr>
<th>Donor</th>
<th>HLA-typing of Donor</th>
<th>HLA-typing of LCL Stimulator</th>
</tr>
</thead>
<tbody>
<tr>
<td>1P</td>
<td>A1, 24 B8,51 DR2,5</td>
<td>A1, B8, DR3</td>
</tr>
<tr>
<td>2A</td>
<td>A1,2, B8, DR3,13</td>
<td>A1, B8, DR3</td>
</tr>
<tr>
<td>3M</td>
<td>A1,24 B7,8 DR15,17**</td>
<td>A1, B8, DR3</td>
</tr>
<tr>
<td>4D</td>
<td>A30,32 B8,18 DR4,7</td>
<td>A30,32 B8,18 DR4,7</td>
</tr>
<tr>
<td>5C</td>
<td>A1,24 B8,27 DR3,4</td>
<td>A1, B8, DR3</td>
</tr>
<tr>
<td>6J</td>
<td>A1,2 B17,62 DR1,7</td>
<td>A(null), B62, DR1</td>
</tr>
<tr>
<td>7AO</td>
<td>A23,30 B27,44 DR1,7</td>
<td>A23,30 B27,44 DR1,7</td>
</tr>
<tr>
<td>8G</td>
<td>A2,31 B50,60 DR4</td>
<td>A2,31 B50,60 DR4</td>
</tr>
<tr>
<td>9J</td>
<td>A23,32 B37,49 DR1,15</td>
<td>A23,32 B37,49 DR1,15</td>
</tr>
<tr>
<td>10E</td>
<td>A1,31 B18,27 DR11</td>
<td>A1,31 B18,27 DR11</td>
</tr>
<tr>
<td>11T</td>
<td>A2,11 B7,71 DR13,15</td>
<td>A2,11 B7,71 DR13,15</td>
</tr>
<tr>
<td>12G</td>
<td>A2, B 7,38</td>
<td>A2, B 7,38</td>
</tr>
</tbody>
</table>

*Matching HLA are shown in bold.

**DR17 is a split of DR3
### Table 2. LPD development and penetrance.

<table>
<thead>
<tr>
<th>Donor Group</th>
<th>Median onset (weeks)</th>
<th>Range (weeks to LPD)</th>
<th>Median LPD Penetrance</th>
<th>Range (LPD Penetrance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid LPD (n=12)</td>
<td>8 *</td>
<td>6-10</td>
<td>100**</td>
<td>80-100%</td>
</tr>
<tr>
<td>Late LPD (n=14)</td>
<td>12</td>
<td>10-18</td>
<td>55</td>
<td>33-100%</td>
</tr>
<tr>
<td>No LPD (n=23)</td>
<td>&gt;20</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>

- *p < .0001 compared to late LPD group
- **p< .0001 compared to late LPD group
### Table 3. IFN-γ genotypes and LPD development in hu PBL-SCID mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Rapid LPD</th>
<th>Intermediate/Late LPD</th>
<th>No LPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/A*</td>
<td>58.3%</td>
<td>7.1%</td>
<td>26.1%</td>
</tr>
<tr>
<td>T/A</td>
<td>41.7%</td>
<td>50.0%</td>
<td>39.1%</td>
</tr>
<tr>
<td>T/T**</td>
<td>0%</td>
<td>42.9%</td>
<td>34.8%</td>
</tr>
</tbody>
</table>

- 3-5 SCID mice were injected per PBL donor, and all mice were engrafted, as evidenced by >750 µg/ml human IgG in the sera.
- * A/A genotype is significantly more prevalent in the Rapid LPD group, p=0.0144.
- ** the presence of the A allele (A/A + T/A) is significantly more prevalent in the Rapid LPD group, p=.0257
Figure Legends

**Figure 1a. IFN-γ genotype associates with TGF-β-mediated inhibition of CTL activity.** PBL were cultured with HLA-A, -B matched LCL in the absence or presence of 10ng/ml TGF-β for 5 days. Viable cells were washed three times to remove any exogenous TGF-β and CTL activity assessed using standard lysis assays as described in Materials and Methods. Data are shown as percent control lysis of PBL cultured with LCL in the absence of TGF-β. For each donor, multiple effector to target ratios were tested in triplicate, and lytic units determined from the linear portions of the curves. The percent inhibition was calculated using lytic units from control versus TGF-β treated cultures. The results shown are the mean and standard deviation for the triplicates from representative experiments for each donor. When analyzed by t-test, the CTL activity in A/A and T/A PBL restimulated in the presence of TGF-β is significantly different from either control CTL activity or the CTL activity in T/T PBL after culture with TGF-β (p=.015).

**Figure 1b. The ability of CTL to prevent matched LCL growth is inhibited by CTL re-stimulation in the presence of TGF-β.** CTL were re-stimulated in the presence or absence of 10ng/ml TGF-β. At the end of 5 days, CTL activity was assessed by standard CTL assays as in panel A. In addition, a portion of the re-stimulated cells (10^4/well) were cultured with titrated numbers of HLA-A, -B matched or mis-matched LCL for 2 weeks. Data are shown as the mean percent LCL growth ± SD in wells containing both CTL and LCL compared to growth in wells containing only LCL as determined by alamar blue. Data are combined for 3 donors of each genotype at an 8:1 effector to target ratio. Solid bars: control CTL re-stimulated in the absence of TGF-β. Open bars: CTL re-stimulated in the presence of TGF-β.
Figure 2. **In vivo treatment with anti-TGF-β prevents death from LPD.** SCID mice were injected with 50 million PBL as described in Materials and Methods. Animals received either PBS (n=3), isotype 100µg control antibody (n=5) or 100µg anti-TGF-β (n=5) every other day for the duration of the experiment. Animals were confirmed to be engrafted by the presence of >750 µg/ml human IgG in their sera, and were monitored for LPD development. Survival time was determined for each group. When animals died or became moribund, flow cytometry was performed to confirm the development of LPD. As shown, all control animals (PBS or isotype control antibody) died within 70 days, while animals treated with anti-TGF-β antibody survived greater than 80 days. The differences in survival were highly significant (p=.004 for PBS vs. anti-TGF-β and p=.002 for Isotype control vs. anti-TGF-β).

Figure 3a. **Anti-TGF-β neutralizes TGF-β in vivo.** Hu PBL-SCID mice were injected with 125µg anti-TGF-β antibody (A411) or PBS three times per week. Serum samples were tested at week 6 for the presence of TGF-β by ELISA. Data are shown as mean pg/ml of TGF-β derived from triplicate determinations, 5 mice per group.

Figure 3b. **Anti-TGF-β reduces incidence of LPD in a dose dependent manner.** Hu PBL-SCID mice were treated with 100µg or 125 µg anti-TGF-β antibody A411 or mouse IgG three times per week for 9 weeks. At harvest, the presence of B cell tumors was assessed visually and confirmed by flow cytometry.

Figure 4a. **Flow cytometric analysis of tumors in anti-TGF-β and control treated hu PBL-SCID mice.** hu PBL-SCID mice were injected with 100µg anti-TGF-β (A411) or mouse IgG every other day for 9 weeks. At harvest, tumors were analyzed by flow cytometry for the presence of human B cells and T cell expansion and activation. Data are shown from a representative animal in each group (n=5 mice per group).
Figure 4b. Flow cytometric analysis of spleens from anti-TGF-β and control treated hu PBL-SCID mice. hu PBL-SCID mice were injected with 100µg anti-TGF-β (A411) or mouse IgG every other day for 9 weeks. At harvest, spleens were analyzed by flow cytometry for the presence of human B cells and T cell expansion and activation. Data are shown from a representative animal in each group (n=5 mice per group).
B.

![Graph showing matched and mis-matched LCL growth](image)

- **Matched LCL**
  - A/A-A/T
  - T/T

- **Mis-matched LCL**
  - A/A-A/T
  - T/T

**Percent Control LCL Growth**

---

**Control LCL**
DIERKSHEIDE, et al. IFN-γ GENE POLYMORPHISMS AND DEVELOPMENT OF PTLD
References


33. Soto P, Price-Schiavi SA, Carraway KL. SMAD2 and SMAD7 Involvement in the Post-translational Regulation of Muc4 via the Transforming Growth Factor-{beta} and Interferon-{gamma} Pathways in Rat Mammary Epithelial Cells. J Biol Chem. 2003;278:20338-20344


51. Lacerda JF, Ladanyi M, Louie DC, Fernandez JM, Papadopoulos EB, O'Reilly RJ. Human Epstein-Barr Virus (EBV)-specific cytotoxic T lymphocytes home preferentially to and


IFN-gamma gene polymorphisms associate with development of EBV-positive Lymphoproliferative Disease in hu PBL SCID mice

Julie E Dierksheide, Robert A Baiocchi, Amy K Ferketich, Sameek Roychowdhury, Ronald P Pelletier, Charles F Eisenbeis, Michael A Caligiuri and Anne M VanBuskirk