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Environmental and Genetic Risk Factors for Post-Transplant Lymphoproliferative Disease

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Dedicated to my father and to the memory of my mother

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Résume
Les lymphomes post-transplantation (PTLD) sont les complications malignes les plus fréquentes de l'immunosuppression (IS) après les cancers cutanés, et présentent la première cause de mortalité et de perte du greffon due aux cancers chez les transplantés. De plus, les lymphomes post-transplantation peuvent se comporter de manière plus agressive avec un plus mauvais pronostic. L'EBV joue un rôle clef dans la physiopathologie de la majorité des PTLDs. Chez l'hôte immunocompétent, les lymphocytes T cytotoxiques (LTC) spécifiques de l'EBV empêchent la croissance des lymphocytes B porteurs de l'EBV, mais cette immunovigilance est abaissée lors d'une immunosuppression, qu'elle soit thérapeutique ou pathologique. Les études s'intéressant aux facteurs de risque des PTLD ont été effectuées sur des données issues de larges registres de transplantation et qui sont par nature insuffisamment détaillées pour étudier l'influence du maintien d'un traitement IS au cours de temps sur le risque de PTLD. Les études s'intéressant aux risques génétiques des PTLDs ont été focalisées sur les polymorphismes des gènes des cytokines, mais elles étaient limitées par l'effectif réduit et/ou l'hétérogénéité des populations cas et témoins. Etant donnée l'étiologie virale des PTLDs et le rôle important du système immunologique, nous avons émis l'hypothèse qu'une hypoactivité spontanée des protéines cibles et des éléments de leurs voies de signalisation, ou une hypersensibilité de ces protéines en réponse à l'effet des IS seraient à l'origine d'une immunosuppression trop intense et par conséquent favoriseraient la lymphoma-génèse. De plus, ce travail tente de répondre à la question suivante: est-ce que certains IS ont une influence particulière sur la survenue des PTLD, indépendamment de leur effet sur le système immunitaire? L'effet des inhibiteurs de la calcineurine (ICN) sur la transformation des cellules sanguines mononuclées (PBMC) par l'EBV a été exploré avec deux panels de 10 marquages par cytométrie en flux : l'un pour caractériser les lymphoblastes émergents et l'autre pour caractériser la réponse T. L'effet de l'administration chronique de la ciclosporine a été exploré sur un modèle de lymphome spontanée chez la souris, exprimant le transgène CD40/LMP1. Nous montrons que les ICN ne semblent pas avoir de rôle autre que l'inhibition des LTC. Chez les souris CD40/LMP1, la ciclosporine produit une augmentation des lymphocytes B activés dans la rate. Le profil des infiltrations des lymphocytes T dans la rate ne semble pas être expliqué seulement par une inhibition de LTC par la ciclosporine. Finalement, une étude pharmacogénétique clinique de type cas-témoins a été effectuée, et montre que des polymorphismes des gènes *IL10* et *IL2* sont associés à la pathologie, ainsi que l'administration d'azathioprine, un médicament immunosuppresseur heureusement aujourd'hui largement abandonné en transplantation.
Mots-clés : lymphomes post-transplantation, PTLD, immunosuppresseurs, inhibiteurs de la calcineurine, pharmacogénétique, cyclosporine, tacrolimus

Abstract
Post-transplant lymphoproliferative disorders (PTLDs) represent a serious complication in solid organ transplantation and are the first cause of cancer related mortality in this population. Furtehrmore, lymphomatous PTLD in this setting is frequently extranodal, can behave more aggressively and tends to have a worse prognosis. Previous work addressing risk factors for PTLD have been based on data from large registries, lacking the detail required to address the role of individual maintenance immunosuppressant drugs, taken over time. Studies looking at genetic risk factors for PTLD have focused on cytokine gene polymorphisms, but have been limited by small size, heterogeneous case/control populations, or both. Given a viral aetiology and a key role of the immune system, we hypothesise that a susceptibility to immunosuppression might favour lymphomagenesis. Moreover, we raise the question whether individual IS drugs have a role on lymphomagenesis additional to imuunosuppression. The effect of the calcineurin inhibitors on the transformation of peripheral blood mononuclear cells (PBMCs) was explored using two panels of 10 markers and flow cytometry: one panel to characterise the emerging lymphoblasts and the other to characterise the T cell response. Chronic administration was explored in a model of spontaneously occurring lymphoma model in transgenic mice expressing CD40/LMP1. We show that in the early steps of lymphomagenesis, chronic exposure to the CNIs does not appear to have a role outside of inhibiting the T response. In the CD40/LMP1 mouse model, there is evidence that

chronic cyclosporine treatment enhances lymphomagenesis. A T response in this model cannot be ruled out, however the profile of T lymphocyte splenic infiltrates does not appear to be explained solely T cell inhibition by cyclosporine. Finally, we performed a case control study with the aim to study the pharmacogenetics of drug response and showed polymorphisms in *IL10* and *IL2* associate with PTLD, as well as azathioprine, whose use as an immunosuppressant in transplantation has fortunately largely been replaced by newer antimetabolite.
Keywords: post transplant lymphoma, PTLD, immunospuppressants, calcineurin inhibitors, pharmacogenetics, cyclosporine, tacrolimus

Selected Abbreviations and Special Terms

A20	tumor necrosis factor, alpha-induced protein 3
AIDS	acquired immunodeficiency syndrome
AZA	azathioprine
bcl-2	B cell lymphoma 2 protein
BCL-6	B cell lymphoma 6 protein
B-16	melanoma cell line
B95-8	marmoset B-lymphoblastoid cell line, producer of cell free virus
BST1	bone marrow stromal cell antigen-1, CD157
CC531	colon carcinoma line
CLL	chronic lymphocytic leukemia
CMV	Cytomegalovirus
c-MYC	Myc protein, transcription factor
CNI	calcineurin inhibitor
CsA	cyclosporine A
CT-26	mouse colon adenocarcinoma
CTL	cytotoxic T lymphocyte
CTS	Collaborative Transplant Study
DLBCL	diffuse large B-cell lymphoma
EBNA	EBV nuclear antigens
EBV	Epstein Barr virus
FKBP12	member of the FK506 binding protein family, binds tacrolimus
FTY720	fingalimod
HBV/HCV	hepatitis virus B/C
HIV	Human immunodeficiency virus (HIV)
HL	Hodgkin's lymphoma
IC	immune competent
ICAM1	intracellular adhesion molecule1, CD54
Ig	Immunoglobulin
IL2RB	IL-2 receptor blocker/antagonist
IFN-γ	interferon gamma
GC	germinal centre
HLA	human leukocyte antigen
KLN-205	murine non-small cell lung cancer
(S)LCL	(spontaneous) lymphoblastoid cell line
LMP	latent membrane proteins
LPD	lymphoproliferative disease
LPS	lipopolysaccharide (endotoxin)
MHC	major histocompatibility complex
mTOR	mammalian target of rapamycin

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFAT	nuclear factor of activated T-cells
NHEK	normal human epidermal keratinocytes
NHL	Non-Hodgkin lymphoma
NK	Natural Killer cells
NLC	nurse like cells
NMSC	non-melanoma skin cancer
OPTN	Organ Procurement and Transplantation Network
p53	tumor protein 53, a tumour suppressor protein
PBMC	peripheral blood mononuclear cells
PT	post-transplant
PTLD	post-transplant lymphoproliferative disorder/disease
Renca	murine renal cell adenocarcinoma
SCID	severe combined immunodeficiency
SIP	shpingosine-1-phosphate
T24	human bladder transitional carcinoma
TAC	tacrolimus
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
UNOS	United Network for Organ Sharing
VCAM1	vascular cell adhesion molecule 1, CD106
VCA	viral capsid antigen
VEGF	vascular endothelial growth factor
vs	versus

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Introduction

Post-transplant lymphoproliferative disorders (PTLDs) represent a serious complication in solid organ transplantation and are the first cause of cancer related mortality in this population. Pre-transplant Epstein Barr Virus seronegativity and receipt of T cell depleting agents for induction or severe/refractory rejection are known risk factors, but they primarily impact early occurring disease. On the other hand, late occurring disease, which has typically not correlated with the above or other specific risk factors, has been shown to be associated with older recipient age and prolonged receipt of calcineurin inhibitors. Furthermore, recent data has contributed to, and in some instances shed light on, previous debate concerning the role of viruses other than EBV and the level of HLA mismatches as risk factors for PTLD. Gene association studies focusing on key cytokines and their receptors have identified several polymorphisms that may prove useful to identify patients at risk, with distinction for early and late occurring disease. Determining the influence of individual maintenance immunosuppressive agents on lymphomagenesis has been limited by the complexity of the multi-drug regimens used, absence of measures of drug exposure and consideration of drugs used as time-dependent covariates in multivariable analyses.

The CNIs (cyclosporine and tacrolimus) remain the pillars of modern maintenance immunosuppressive regimens that comprise multi-drug combinations (an antimetabolite, predominantly mycophenolate, and oral corticosteroids). Newer drugs, such as the mTOR inhibitors, which have demonstrated positive effects in preventing or treating a variety of tumours including lymphomas, have a less well defined place in immunosuppressive regimens, but may be used as calcineurin-sparing first-line drugs *de novo*, or after the first few months following transplantation, through the post-transplant period.

That the spectrum of malignancies observed post-transplantation includes a majority with a viral aetiology, or a possible viral aetiology, suggests a key role of the immune system and an immune-surveillance of these types of tumours. Although there is evidence that some immunosuppressants, namely the calcineurin inhibitors, may adversely influence tumourigenesis, it is exceedingly difficult to separate possible direct effects, or tertiary effects (contributing to a favourable tumour microenvironement) from the known pharmacological action to inhibit activation in T lymphocytes. In the case of EBV-driven lymphomagenesis, it is an added challenge to differentiate from the well-characterised predominant influence of EBV.

The aim of this work was to evaluate the effects of chronic exposure to calcineurin inhibitors in two stages of the lymphomagenetic process: in early EBV infection and transformation and latter effects in an established lymphoma model. The effect on EBV infection and transformation was assessed by employing two sets of 10-marker panels coupled with flow cytometry: one panel characterising the emerging lymphoblastoid clone and the other the T lymphocyte response. The LMP1/CD40 transgenic mouse is a model of insidious spontaneously occurring lymphoma. Mice aged 5 months and older were treated with cyclosporine for three months: the effects on splenic B cells and ex vivo additional cyclosporine treatment were explored.

Finally, a case control study was designed to address some of the deficiencies in published work exploring genetic risk factors to date, namely small and/or heterogeneous case and control populations. The principal aim of this epidemiological work was to test polymorphisms in genes related to the calcineurin pathway with the hypothesis that these might influence the degree of immunosuppression, and consequently phenotypes related to over immunosuppression, such as post transplant lymphoproliferative disease.

Part One

Calcineurin Inhibitor Effects on Tumourigenesis and Lymphomagenesis

Experimental work:

The effects of chronic calcineurin inhibitor exposure on early lymphoma events *(EBV infection and transformation in vitro)* and late lymphoma events *(in vivo, established lymphoma model in LMP1-CD40 transgenic mice)*

Calcineurin Inhibitor Effects on Tumorigenesis and Lymphomagenesis

Calcineurin inhibitor effects in tumour models

Evidence exists that the CNIs can produce cell-autonomous, tumour-promoting effects. Cyclosporine (CsA) treated (1000ng/mL) human lung adenocarcinoma cells (A-549), normally non-invasive *in vitro*, exhibited morphological and functional changes related to increased invasiveness, an effect that was shown to be due to CsA-induced expression of, and the cell's inherent responsiveness to, TGF-β (Hojo et al., 1999). This was shown to occur in anchorage dependent culture conditions, but not anchorage independent, and is therefore relevant to solid tumours. Similar phenotypic effects resulted following cyclosporine treatment in mink lung epithelial cells (CCL-64), mouse mammary gland epithelial cells (NMuMG) and murine renal adenocarcinoma cells (Renca). *In vivo*, significantly higher numbers of lung metastases were observed in CsA treated immunodeficient SCID-beige mice (CsA 20mg/kg, every second day) inoculated with either Renca, murine Lewis lung carcinoma cells (LLC) or human bladder transitional carcinoma cells (T24) via the tail vein. Simultaneous treatment with anti-TGF-β antibody, presumably of mice treated with Renca (not revealed in text), produced similar results to controls (Hojo et al., 1999). Increases in lung metastases have been demonstrated for tacrolimus (significant for 4mg/kg/ second daily, numerically higher but not statistically significant for 2mg/kg/second daily) in BALB/c or SCID-beige mice inoculated with Renca (Maluccio et al., 2003).

This frequently cited finding is often reported without reference to survival, or outcomes other than numbers of lung metastases in mice models. In deed, subsequent work by the same group that focused on the tumour arresting effects of mTOR

inhibitors confirmed the increased lung metastases with cyclosporine following tail vein inoculation with Renca in BALB/c (Luan et al., 2002) and SCID mice (Luan et al., 2003). However, tumour volume following subcutaneous injection of the same tumour line in BALBc mice (Luan et al., 2002), and murine non-small cell lung cancer cells (KLN-205) in DBA/2 mice (Boffa et al., 2004), was no different to control mice. Despite the increased number of lung metastases in models using Renca in BALB/c (Luan et al., 2002), or human (RCC 786-O) or murine renal cell carcinoma in SCID mice (Luan et al., 2002, 2003), survival was not different to controls. There were fewer mice with metastases DBA/2 mice subcutaneously implanted with KLN-205 (4/6 CsA treated vs 8/8 controls), although this is probably not significantly different (Boffa et al., 2004). A similar dosing schedule was used in all reports (CsA 20mg/kg, every second day). Nevertheless, the increased metastatic burden in the majority of reports, along with the *in vitro* data showing enhanced motility mediated by TGF-β and likely other mechanisms, is noteworthy and has been demonstrated with short dosing schedules. Four days cyclosporine treatment (20mg/kg/day) resulted in increased tumour load at the site and an increased number of metastases in a model employing the weakly antigenic colon carcinoma line (CC531) implanted under the testis fat flap in male WAG/RIT rats (Van de Vrie et al., 1997). Of note is that *in vitro* there was no growth enhancement, and short term *in vivo* growth assays were no different to control systems.

The TGF-β mediated morphological changes by cyclosporine were challenged by one group that found no elevation of TGF-β following *in vitro* cyclosporine (1000ng/mL) treatment of T24 and Renca cells, despite observing similar increases in pseudopodia formation and increased motility (Tanaka et al., 2002). Interestingly, treatment with fingolimod (FTY720), an agent presently used to arrest lymphocytes from ganglionar migration in multiple sclerosis, inhibited cyclosporine mediated changes. FTY720 becomes phosphorylated *in vivo* and inhibits at least one of the sphingosine-1-phosphate receptors (SIP), a family of receptors implicated in mediating cell-to-cell adhesion. The *in vivo* implications of this finding are uncertain as T cell mobilisation of effector immune functions are likewise dependent on SIP receptor signalling, the observed mechanism of action of fingolimod. In deed, SIP2 -/-

mice develop clonal diffuse large B-cell lymphoma with age (Cattoretti et al., 2009). However, the inhibition of cyclosporine mediated morphological alterations outside of TGF-β mediated signalling are of interest. There is a growing body of evidence implicating cyclosporine in cytoskeletal changes that have been both related to and external to its well known action on inhibiting NFAT (Descazeaud et al., 2012).

It has been raised that induction of VEGF by CNIs may also play a role in promoting tumours (Guba et al., 2004). VEGF serum expression in CsA treated (10mg/kg/day) BALB/c tumour-bearing mice (intra-portal injection of mouse colon adenocarcinoma, CT-26) was significantly higher than in controls. CsA treated mice had elevated tumour area and significantly increased tumour volume compared to controls when the same line was implanted in dorsal skin fold chambers, and while vascular area as a proportion of tumour area was similar to control, absolute microvascular density was significantly higher with CsA. Similar results were achieved by the same group in a murine heart-transplant model: subcutaneous tumour implantation (CT-26 was implanted in Balb/c mice and B-16 melanoma cells in C57BL/6J mice) was followed by transplant and immunosuppressant treatment 7 days later. In this model, a higher dose of cyclosporine was necessary in order to prevent graft rejection (40mg/kg/day). As in the previous model, cyclosporine increased tumour volume compared to controls (Koehl et al., 2004) but here survival was difficult to gauge, as it could not be dissociated from graft loss. It is difficult to conclude that cyclosporine was the cause for elevated serum VEGF in the above report, and that VEGF was the mechanistic cause for the cyclosporine induced angiogenesis and enhancement of tumours found by this group, given that mice were immune competent and that the developing tumours might be the source of VEGF. *In vitro*, similar VEGF production was found in cyclosporine treated CT-26 line compared to the control (Guba et al., 2002). In a different model, serum VEGF levels were similar to controls in cyclosporine treated (20mg/kg/second daily) SCID mice injected with human renal cell carcinoma via the tail vain (Luan et al., 2002). On the other hand, elevated VEGF mRNA and protein expression was observed in the renal cortex of CsA treated (7.5mg/kg/day), tumour-free immune competent adult male Sprague-Dawley rats (days 7 and 28 mRNA, day 28 only for protein expression)

(Shihab et al., 2003): the effect could be cell-type specific and manifest locally, or depend on tumour development and tumour type. A low dose of cyclosporine (100ng/mL) enhanced *in vitro* vascular growth measured by an aortic ring assay, (thoracic aortae from male Wistar rats, Harlan Sprague-Dawley, assay to measure de novo vascular growth) (Koehl et al., 2004). This was inhibited by concomitant mouse anti-TGF-β antibody suggesting that cyclosporine induced TGF-β secretion might be the primary mechanism. On the other hand, recent work has shown that cyclosporine treatment (5.0μg/mL) enhanced VEGF mRNA stability in 786-0 and Caki-1 renal cancer cells suggesting a mechanism whereby the drug contributes to enhance levels in systems where VEGF is already being produced (Basu et al., 2010).

Regarding experimental models of EBV-driven lymphoma, independent effects to those on the immune system are difficult to demonstrate. Furthermore, it is an added challenge to differentiate from the well-characterised predominant influence of EBV. Nevertheless, the above mechanistic considerations may be of potential relevance to lymphoproliferative disease.

Evidence for direct effects: EBV transformation of fractionated B cells

As already mentioned, CsA treated fractionated B cells (with active removal of monocytes and T-cells), infected by EBV resulted in a greater number of wells positive for B cell outgrowth in a 96 well plate. The difference between cyclosporine treated (1000ng/mL) and untreated conditions was most apparent when B cells were seeded at lower cell number (10 and 100 cells per well, compared to 1000 cells per well where there was no difference between treatment and control given that a majority of wells resulted in outgrowth) (Tanner and Menezes, 1994). In other words, for seeding at low cell numbers, cyclosporine increased the likelihood of outgrowth. This effect was dose dependent and maximal at 500-1000ng/mL of CSA, depending on the PBMC donor. There was a small (17%), probably not significant, increase in

IL-6 activity in cyclosporine treated, fractionated cells (assayed by co-incubating the supernatant with 7TD1 cells, an IL6 sensitive cell line).

CsA potentiated EBV transformation in fractionated B cells from healthy donor PBMCs or splenocytes (~80% purity, 500ng/mL CsA), assessed by colony counts, cell counts, [^{3}H] thymidine incorporation and LMP1 antibody assays (Chen et al., 2001, 2003, 2008, 2009; Ranjan et al., 1998). Cyclosporine-enhanced transformation at four weeks was similarly enhanced by 10 min of H_2O_2 treatment immediately after EBV infection (Chen 2001, 2003). Addition of α-tocopherol (40μM) reversed the effects of H_2O_2 and cyclosporine. Cultures were of B cells fractionated from human PBMCs (Chen et al., 2001) or splenocytes (Chen et al., 2003), infected by EBV for 12 hours and put to culture at 125,000 cells per well in 96 well plates or in flasks at $3x10^6$ cells in 5mL, respectfully. Weekly replenishment of culture medium included cyclosporine or α-tocopherol in the first two weeks. Despite longer-term cultures with cyclosporine, the oxidative effects of cyclosporine occurred maximally at 10 min of culture (Chen et al., 2008). However, in vitro work has shown that calcineurin is sensitive to extracellular oxidants, which might inhibit phosphatase activity by altering the oxidation state of the iron ion in the active site: in this way H_2O_2 is effectively an inhibitor of calcineurin activity (Carballo et al., 1999; Furuke et al., 1999; Reiter et al., 1999). CSA-induced oxidative stress *in vitro* is a well noted phenomenon whose clinical implications have been difficult to demonstrate. Furthermore, clinical use of antioxidants has not translated the benefits observed in experimental systems. In all reports, supernatant of B95-8 representing lytic cell-free virus was used to infect cells (Chen et al., 2001, 2003, 2008, 2009; Tanner and Menezes, 1994).

It is difficult to draw conclusions from these findings that cyclosporine enhances transformation of isolated B cells in the presence of lytic virus. The hu-PBMC/SCID mouse model is frequently used to model lymphomas arising in the immunosuppressed, and tumours bear phenotypic similarities to PTLDs. SCID mice do not have functional B and T cells for the reason that they have defunct DNA repair

capacity, precluding functional V(D)J gene-rearrangement and leading to inadequate B and T antigen receptors in mouse lymphocytes, however Natural Killer (NK) cell populations are expected to be unaltered. Human PBMCs injected intaperitoneally into SCID mice may give rise to spontaneous tumours, although incidence and onset varies between PBMC donors such that donors are classified as producers of high-, intermediate/low- incidence or no incidence/penetrance (Dierksheide et al., 2005; Johannessen et al., 2000; Mosier et al., 1992a, 1992b; Picchio et al., 1992). These profiles have been found to be reproducible (Johannessen et al., 1998). Human Ig is detected and rises in mice with the developing tumour. After T cell and monocyte depletion, B cells isolated from, EBV-positive PBMC donors and injected into SCID mice fail to produce tumours (Johannessen et al., 2000; Veronese et al., 1992). Fractionated B cells give rise to tumours only when additional, lytic virus is injected, and the rate of onset is particularly rapid (Veronesi et al., 1994). Similarly, in beige/nude/xid mice that reject human T cell xenografts, but accept B cell grafts, tumours do not arise unless additional lytic virus, even at small doses, is injected (Dosch et al., 1991). In the same vein, tumours derived from “high-incidence”, otherwise normal, donors frequently exhibit evidence of viral replication with multiple episomal forms and are polyclonal, while intermediate/low incidence tumours infrequently exhibit replication which, when present, is a single episomal form, and tumours are typically monoclonal (Mosier et al., 1992a, 1992b; Picchio et al., 1992).

The first report demonstrating that T cells are necessary for tumourigenesis in hu-PBMC SCID mice model also showed that cyclosporine treatment reduced tumour incidence (2/10 vs 31/38), and tumours appeared in the ‘intermediate onset’ time frame (Veronese et al., 1992). However, it was not specified whether the same donors were used for the initial and cyclosporine treated experiments, and the report pre-dates the donor-type distinction. Human Ig levels in CsA treated mice were detected, as opposed to when fractionated B cells were used, but Ig levels in CsA treated mice were a fraction of the normal PBMC condition (about one-fifth 40 days following inoculation).

Evidence for direct effects: effects on established lymphoblastoid cell lines

The effect of calcineurin inhibitors on viral reactivation is of interest given the profile of high incidence, fast-onset tumours in the hu-PBMC SCID model. CsA was capable of inducing the viral lytic cycle from latency III in some recently EBV transformed lymphoblastoid cell lines (LCLs) (n= 4 of 11 LCLs, 3-fold increase of the number of VCA positive cells compared to controls): no induction was demonstrated in established lymphoproliferative disease (LPD)-LCLs (n=3). The authors put forward that induction of the lytic cycle may not be possible in long-term established LCLs as EBV likely exists in a more tightly restricted pattern (Tanner and Menezes, 1994).

Direct growth promoting effects by the CNIs have also been demonstrated. Spontaneous lymphoblastoid cell lines (SLCLs) derived from the PBMCs of transplant patients with an active EBV infection, presumably arising spontaneously *in vitro*, were used in a series of experiments testing the effects of the CNIs on cell viability. CsA (1000ng/ml) and TAC (10ng/ml) treated cells exhibited slightly increased cell growth, measured by MTT assay (two cell lines) and [^{3}H] thymidine incorporation (six cell lines), an effect that exhibited a dose-dependence and was maximal at the aforementioned doses (measured by [^{3}H] thymidine incorporation) (toxic beyond these doses). CsA and TAC treatment did not affect cell division (measured by CFSE fluorescence in three cell lines) but did enhance cell viability (counts following tryptan blue dye exclusion). The degree of growth enhancement was reported to be 20% or more. Although the CNIs did not affect Fas expression (measured by immunofluorescent staining in three cell lines), CsA and TAC both produced a 20% relative reduction in anti-Fas apoptosis (activation induced apoptosis): this was highly reproducible, and statistically significant with only CsA

tested (observed in six experiments, APO™-BrdU TUNEL Assay) (Beatty et al., 1998). These findings are not surprising given that caclineurin is implicated in programed cell death in B and T cell physiology in processes involving signalling via elevated intracellular Ca2+ (Bonnefoy-Berard et al., 1994; Farcasanu et al., 1995; Genestier et al., 1994; Zhao et al., 1995) and CNIs inhibit this proces (Fruman et al., 1995). It is interesting that the antiapoptotic protein bcl-2 binds calcineurin and sequesters it in the cell membrane, thus inhibiting its cytoplasmic activity (Shibasaki et al., 1997)

Finally, cyclosporine exposed EBV-transformed B cells were rendered resistant to cytolysis by T cells, NK cells and complement. The effect was dose and time dependent, was reversible, and was accompanied by inhibited cell growth as measured by thymidine incorporation. (Hudnall, 1991) [Abstract].

Evidence for indirect effects: full PBMC systems

It is well known that CNIs enhance transformation of PBMCs by lytic EBV *in vitro*, and cyclosporine is used routinely to this end. This is presumed to be due to the inhibitory effect on cytotoxic T cells, however indirect factors might contribute and are likely to be of importance given that without T cells spontaneous tumours do not form *in vivo* in the hu-PBMC SCID model.

IL-6 activates B cells, is a growth factor for EBV immortalised cells and, in *in vitro* PBMC cultures, its primary source is monocytes activated by viral antigen (Tanner and Menezes, 1994). EBV immortalised cells also secrete IL-6 but its contribution to autocrine growth is partial compared to other factors (Tosato et al., 1990). EBV positive B cells (ROHA-9MC3) transfected with episomal constructs designed to secrete high levels of human IL-6 formed colonies in soft agar, a property

consistent with a malignant phenotype, and were tumorigenic in immuno-compromised nude mice (control cells did not produce tumours) (Scala et al., 1990). Expression of the IL-6 receptor was additionally found to be up-regulated in this model. A similar expression system in an EBV-immortalised B-cell line achieved by a different group likewise resulted in enhanced tumorigenicity in athymic mice, however lack of growth advantage compared to non-expressing immortalised cells *in vitro* led the authours to propose IL-6 inhibition of cytotoxicity, presumably due to natural killer cells, was responsible for the observed *in vivo* result (Tanner and Tosato, 1991).

High circulating IL-6 levels have been found in PTLD patients and have been used for diagnostic purposes, while anti-IL6 antibody was used in a phase I-II trial to treat PTLD in twelve cases resulting in five complete and three partial responses (Haddad et al., 2001). Cell separation studies have revealed that EBV infected endothelial cells are a principle source of IL-6 in PTLD tumours and secrete high levels of the cytokine in *in vitro* culture (Jones et al., 1995; Tosato et al., 1993, reviewed in Tosato et al. 1998).

Cyclosporine stimulates IL-6 production in fractionated T cells (800ng/mL) (Tanner and Menezes, 1994) and cyclosporine treatment of uninfected PBMCs exhibits increased IL-6 (200-800ng/mL) (Tanner and Menezes, 1994; Walz et al., 1990). Although there appears to be a synergistic effect compared to CsA or EBV alone when PBMCs were infected with EBV in the presence of CSA (800ng/mL), results reported for a similar experiment elsewhere in the same paper, but using CSA 500ng/mL, did not show a large difference in IL-6 activity between EBV infection alone and EBV with concomitant CSA (Tanner and Menezes, 1994). Others have found that IL-6 expression is enhanced by CNIs in monocytes under certain conditions. CsA and TAC pre-treatment (but not post-treatment) enhanced the number of IL-6-producing, LPS-stimulated monocytes, cultured at a single cell level (avidin-biotin-peroxidase complex method). The effect was observed at lower LPS concentrations (up to 10µg/mL) and was dose-dependent for the CNIs at LPS

10ng/mL. The authors noted large inter-subject variation in base percentage of IL-6 producing cells, and in response to the two drugs (Murayama et al., 1994).

TGF-β normally inhibits the growth of B cells, however appears to lose this ability once immortalisation has occurred (Tosato et al., 1998). This has been attributed to down-regulation of its receptor and interference of intracellular signalling by LMP1 (Altiok et al., 1994; Kumar et al., 1991; reviewed in Tosato et al., 1998). TGF-β can trigger EBV viral replication (Fahmi et al., 2000; Liang et al., 2002; di Renzo et al., 1994; Schuster et al., 1991), inhibit cytotoxic effector T cells in the tumour microenvironment and promote angiogenesis. Cyclosporine enhances TGF-β expression as has been demonstrated *in vivo* in end-stage renal failure subjects pre-treated with the drug prior to transplantation (Shin et al., 1998).

In the absence of cyclosporine, anti-TGF-β antibody treatment (100μg three times a week) prolonged survival beyond 80 days in SCID mice injected with PBMCs from a high penetrance donor, while the two groups of control mice died within 60 days (PBMC engraftment was confirmed by elevated hu IgG) (Dierksheide et al., 2005). Furthermore, 9 weeks of treatment reduced the incidence of tumours in a dose dependent manner, using PBMCs from a different high penetrance donor (placebo treated 100%, 100μg 75%, 125μg 20%). Anti- TGF-β treatment resulted in expansion of human CD8+ T cells in the spleen and tumours (when relevant), whereas there were low levels in control animals. High penetrance PBMC donors were all carriers of the INFG +874 A allele, associated with reduced INF-γ production. Co-incubation with TGF-β reduced cytolysis in carriers of the A allele in re-stimulation cultures using HLA matched LCLs, while there was no effect in T homozygotes, leading the group to propose that the low INF-γ production led to enhanced TGF-β sensitivity and the observed reduction of cytolysis. The implications of this finding in SCID mice cannot easily be extrapolated to consider what might happen under CNI treatment given that suppression of cytotoxic T cells is a direct pharmacological activity of the drugs. However the potential role of TGF-β is multifaceted and the initial finding that

cyclosporine inhibits LPD in SCID mice requires re-evaluation using a definitive high penetrance PBMC donor.

Tumorigenesis: effects on DNA Repair

Much of the previously discussed work involved potentially additional contribution by the CNIs to the already well-known, primary growth enhancement by EBV. Acquired genetic abnormalities likely contribute to malignancy, and might be of particular relevance for EBV negative PTLD. Aberrant somatic hypermutation resulting in altered expression of oncogenes or in creating proto-oncogenes is associated with lesions in immunocompetent individuals, and is frequently found amongst lesions in AIDs-NHLs (Gaidano et al., 2003) and PTLDs (Capello et al., 2009). A comparison of high-density genome wide analyses of DLBCLs from immune competent (IC, n=105), HIV (n=28) and transplanted (PT, n=44) individuals, revealed features that were common to immune deficiency related disease and features specific to PT-DLBCLs presumably due to iatrogenic immunosuppression (Rinaldi et al., 2010). The mutator phenotype, characterised by microsatellite instability and faulty mismatch repair, was found amongst immunodeficiency-associated non-Hodgkin lymphomas (9/111, 8.1% of PTLD and 3/128, 2.3% of HIV-associated lymphomas) but not amongst NHL tumours derived from immunocompetent individuals (0/364) (Duval et al., 2004). Importantly, it was found amongst both EBV positive and negative tumours. The approximately four-fold apparent relative risk increase in transplant recipients vs HIV positive individuals suggests that immunosuppressive agents might play a role over and above permitting these typically immunogenic alterations to propagate.

DNA repair, induced by UV irradiation (254nm) of PBMCs and measured by [^{3}H] thymidine incorporation, was higher in transplant patients receiving triple therapy (including CsA) vs double therapy (azathioprine and corticosteroid only); n=7

in both groups. The triple therapy group had a significantly shorter mean time since transplant, which corresponds to a shorter exposure period to IS. The effect that the degree of immune suppression has on DNA repair is uncertain as triple therapy provides a more potent degree of immune suppression, hence no conclusion can be made with respect to drug used. *In vitro*, CsA had a dose dependent effect on DNA repair in PBMCs from healthy donors. Although CsA (5000ng/mL, 4.16μmol/L), AZA (27.7 μg/mL, 100 μmol/L) and prednisolone (0.36 μg/mL, 1 μmol/L) all reduced DNA repair *in vitro*, the reduction by CsA alone was lower than the other two drugs alone or in combination. Furthermore, any combination containing cyclosporine (double and triple combinations) resulted in the largest suppression of DNA repair, however this was not always significantly different compared to CsA alone (Herman et al., 2001). UVB (315-380nm) results in a dose dependent (J/m^3) nuclear translocation of NFAT in normal human epidermal keratinocytes (NHEK), and this is inhibited by cyclosporine and ascomycin, a calcineurin inhibitor that like tacrolimus binds FKBP12 (Yarosh et al., 2005). This group found both drugs inhibited removal of cyclobutane pyrimidine dimers (assessed by the dot-blot method) in NHEK and immortalised keratinocytes (HaCaT) (100-1000ng/mL for cyclosporine). Similar findings were shown for artificial models of skin (Canning et al., 2006). Cyclosporine also inhibited apoptosis in these systems (Canning et al., 2006).

Mechanistic considerations and conclusions

The above review summarises all data that implicates a contribution by the calcineurin inhibitors on tumorigenesis/lymphomagenesis beyond their immuno-suppressive action. However, for the most part these are speculative and it remains difficult to prove that effects other than immunosuppression are relevant. The direct action on fractionated B cells might simply be due to a rapid oxidative effect, and this could be a primary reason for results observed generally *in vitro*. IL-6 may be promoted by cyclosporine, and thus contribute to enhance tumourogenicity/ more aggressive phenotype, but whether enhanced IL-6 production occurs *in vivo* at clinically relevant CNI doses remains to be determined. TGF-β is enhanced by the

calcineurin inhibitors and can contribute to tumourigenesis in a number of ways other than by inhibiting cytotoxic T cells. TGF-β can induce the viral lytic cycle and may be the mechanism for the reactivation in recently transformed cells observed by Tanner et al. TGF-β enhances angiogenesis, and this might be the primary cause of cyclosporine enhanced vascularity observed by some authors, an effect they attribute to VEGF: enhanced VEGF has not been consistently attributed to the CNIs. Finally TGF-β is implicated in morphological changes leading to enhanced motility in certain tumours, but that other authors did not corroborate a TGF- β mediated action suggests that cyclosporine might cause this through an alternative possibly direct action on the cytoskeleton. Both cyclosporine and tacrolimus enhanced the survival of EBV-transformed cells, and reduced activation induced apoptosis, however, despite being reproducible, these effects were quite minor. Finally, cyclosporine and a tacrolimus-like drug decrease DNA repair in the context of UV damage, a process that involves NFAT. Exploration into the effects on other aspects of DNA repair could prove revealing.

Experimental work 1A : The effects of chronic calcineurin inhibitor exposure on early lymphoma events (EBV infection and transformation of B lymphocytes *in vitro*): treatment with cyclosporine and tacrolimus

EBV infection and transformation of primary B cells was explored *in vitro* employing two 10-colour flow cytometry antibody panels, one to characterise the emerging lymphoblastoid cells and the other the immune response, with the aim to gain a qualitative insight into the effects on this process by the calcineurin inhibitors, cyclosporine and tacrolimus.

Methods

Peripheral blood from healthy volunteers was collected into EDTA treated tubes. Peripheral blood mononuclear cells (PBMCs) were obtained following Ficoll-Hypaque density gradient separation. Institutional approval was granted by the Limoges University Hospital. All participants gave informed consent before donating blood samples.

For culturing, cells were seeded at 1.0 x 10^6 per millilitre in growth medium comprising 50% complete culture medium and 50% EBV containing culture supernatant (EBV producing marmoset cell line, strain B95-8, cultured in complete culture medium over at least 3 weeks). Complete culture medium comprised RPMI 1640 medium (Gibco BRL-Life Technologies) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine (Gibco BRL-Life Technologies) and 20% heat-inactivated foetal calf serum (Invitrogen, Gibco BRL-

Life Technologies). Seeded PBMCs were separated into three conditions: vehicle (96% ethanol 1/100 %v/v), 100nM cyclosporine (Novartis Pharma AG, Switzerland) 5nM tacrolimus (Astellas Pharma, Levallois-Perret, France). These low doses were chosen from estimates of whole blood concentrations for these drugs in kidney transplant recipients (typical trough concentrations in whole blood for cyclosporine <400ng/mL ~ 300nM, and tacrolimus <15ng/mL ~ 18nM) and to account for there being no haemocytes in these culture systems (estimated to account for 65% of the determined levels in whole blood). These were cultured over 28 days at 37°C in a humidified, 5% CO2 atmosphere: no additions or supplements were made to the culture medium over this period.

Two 10-colour FCM panels were selected to characterise the T cell response during EBV immortalisation (T panel) and to characterise the emerging LCL (B panel, both depicted in Table 2.1) under the three conditions. Conjugated monoclonal antibodies were purchased from Beckman Coulter (France). These had been previously tested for anti-body interaction and steric hindrance. PBMC sampling (5 x 10^5 cells per panel) was conducted on day 0 (representing recently extracted PBMCs, without EBV supernatant) and days 3, 7, 14, 28 (each of the three conditions in on-going culture). Acquisition (5 x 10^4 cells) was performed using a Navios™ flow cytometer, and analyses conducted using Kaluza Software® v 1.2 (both Beckman Coulter).

Table 3 Flurochromes and associated antibodies in B and T 10-colour panels

Panel	FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PB	KO
T	CD4	HLA-DR	CD8	CD38	CD25	CD56	CD127	CD3	CD16	CD45
B	CD54	CD138	CD19	CD38	CD11c	CD79b	CD23	CD43	CD25	CD45

For both panels, an initial gate for living cells was constructed, and counts for each cell type were expressed in 'percentage of living cells'. For the T cell panel, a lymphocytes gate was located over CD45+/low SS. In the lymphocyte gate, CD8+ T

cells were CD3+/CD4-/CD8+; CD4+ T cells were CD3+/CD4+/CD8-; Natural Killer (NK) cells were CD3-/CD56+/CD16+; T regulatory cells were CD3+/CD4+/ CD25+/CD127 negative. Activated CD4+ T cells were CD38+/HLA-DR+/ CD3+/CD4+; activated CD8+ T cells were CD38+/ HLA-DR+/CD3+/CD8+; activated NK cells were CD38+/ HLA-DR+/CD3-/CD56+/CD16+. From the B panel, monocytes were CD11c+/CD25- on day 0 analyses, and CD54+/CD11c++ following activation (analyses on days 3-28). Primary B cells were CD19+/low SS; emerging lymphoblastoid cells (LCL) were CD19+/range in SS. A second type of EBV positive population (titled 'mystery') could not be characterised and was gated on low CD45/negative CD54. Representative gates are depicted in Appendix 1.

Results

The calcineurin inhibitors potently inhibited activated CD4+ T cells, activated CD8+ T cells and T regulatory cells, showing that the low doses of CNI used were effective (Figure 1). The degree of inhibition was similar between the two agents, suggesting equipotency at the doses employed. There was some inhibition of activated NK cells in the presence of CNIs (kinetic data presented for subjects A-I as CD56 staining was impaired for days 0-14 in subjects J-O). Monocytes were not significantly impaired.

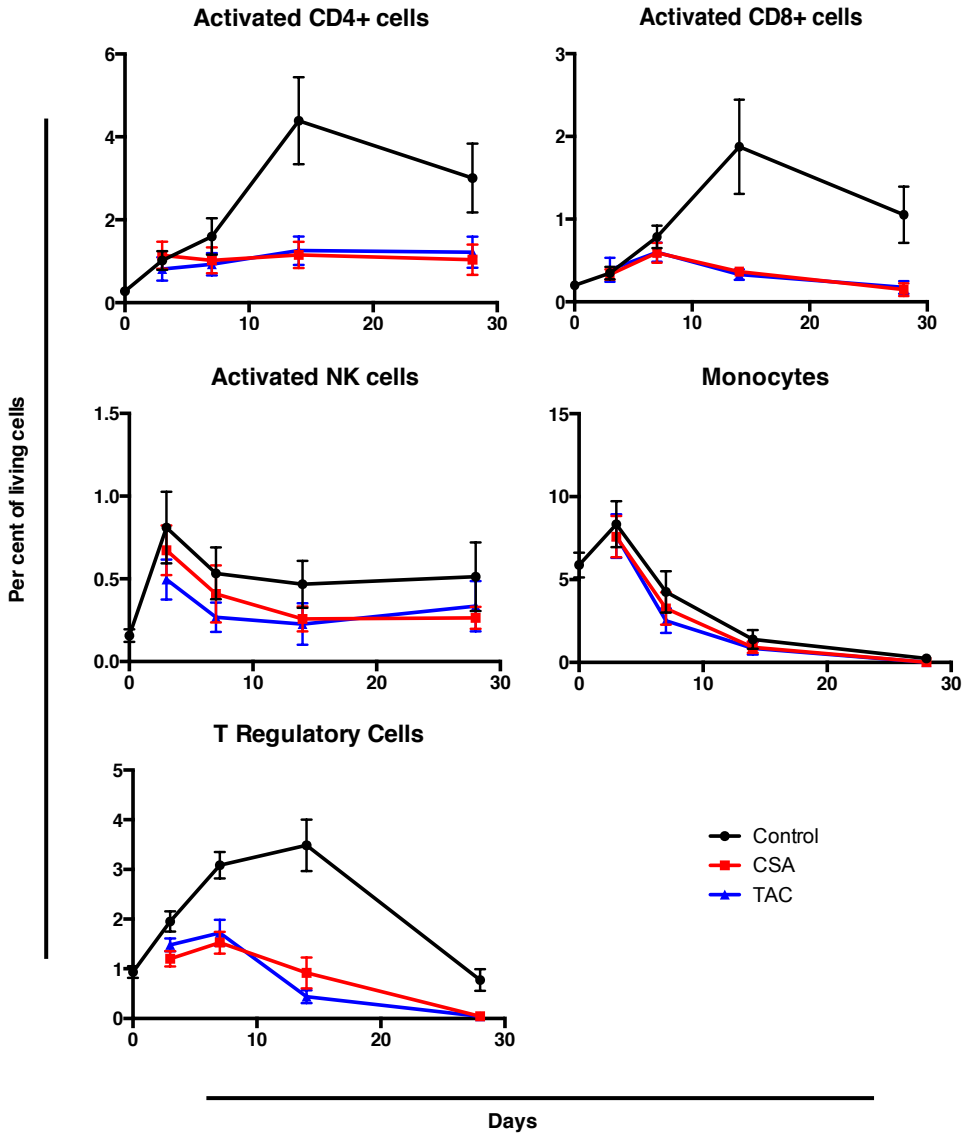

Figure 1 The effect of calcineurin inhibitors on lymphocyte populations and sub-populations. Kinetic profiles exhibit means and S.E. for each time point

The CD4+ and CD8+ sub-populations in the lymphocyte gate were similar between the three conditions, and all followed a similar kinetic profile (Figure 2). Primary B lymphocytes rapidly reduced by day 14. The observed increase on day 14 is due to the entry of the expanding population of CD19 LCLs into this gate. Primary B cells displayed a range in positivity for CD79b (beta component of the B-cell antigen receptor).

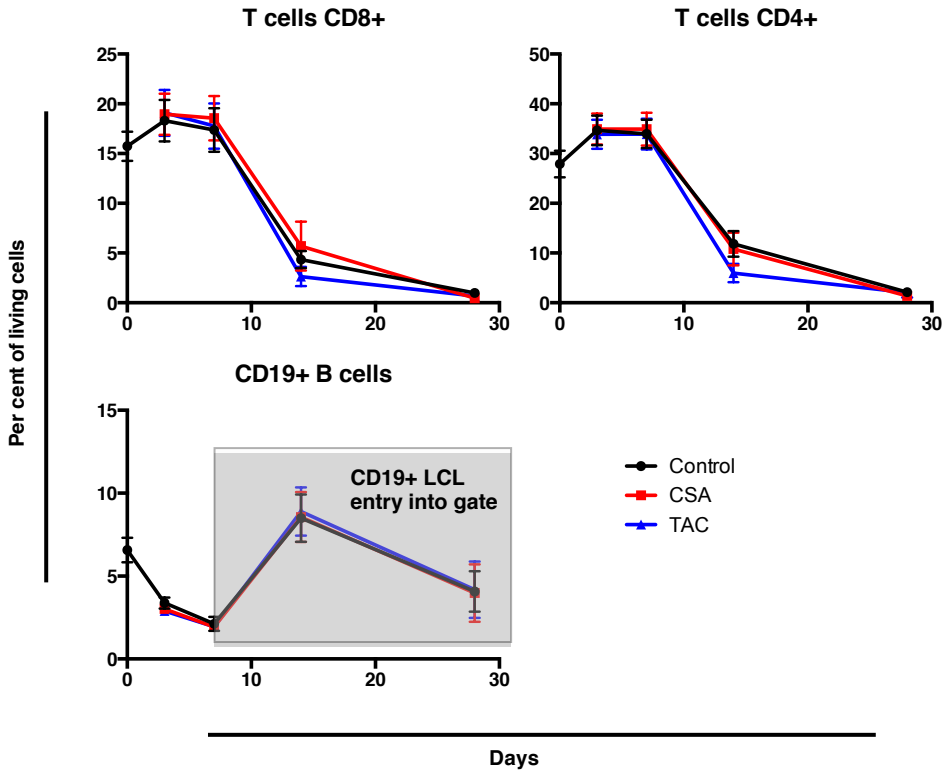

Figure 2 Calcineurin inhibitors have no effect on global B and T cell populations. Kinetic profiles exhibit means and S.E. for each time point

Two distinct populations emerged after 28 days of culture: one a highly activated, CD19+ (CD19 LCL), and the other with no defining characteristics in the B panel ('mystery' population). CD19 LCL was positive for markers of adhesion and activation (CD54, CD23, CD43, CD38). The mystery population was negative or low for all extracellular markers in the B panel. The two populations differed in CD45 positivity: CD19 LCL was $CD45^{high}$ while the mystery population was $CD45^{low}$. There were two small sub-populations, likewise $CD45^{low}$, that were CD19 or CD138 positive (each roughly 0.01% of the mystery line). A separate CD45 intermediate population was often present alongside the mystery population, however it was not possible to gate without interference. This population was associated with CD79b positive sub-population (again, roughly 0.01% of the mystery line).

Table 4 summarises outcomes for all subjects and Figure 5 exhibits all profiles. The CD19 LCL population gave rise to successful lines that exhibited exponential growth, became established and survived freezing and thawing. When the mystery population was present, cells survived for at least 6 weeks beyond day 28, but did not double in growth.

Table 4 Summary of outcomes for all subjects

EBV B95-8 Stock	Sampling Date	Subject ID	Sex	Age	Comment	Outgrowth
1	9/12/11	A	F	29	Insufficient stock CSA	N
2	19/12/11	B	F	42	Insufficient stock CONT	Y (CNIs)
3	20/12/11	C	F	26		Y: CNIs>CONT
4	30/12/11	D	M	27		Y: CNIs>CONT
5	9/01/12	E	F	34		Y: CNIs>CONT
5	10/01/12	F	F	29		Y: CNIs>CONT
6	30/01/12	H*	M	27	Suspected contaminant	-
6	31/01/12	I	F	24	Suspected contaminant	-
7	10/04/12	J	M	25	Day 28 reading unavailable	N
7	10/04/12	K*	M	27	Day 28 reading unavailable	N
7	16/04/12	L	M	43		N
7	16/04/12	M	M	29		N
7	20/04/12	N	F	25		N
7	20/04/12	O	F	24		N

*H and K are the same subject

Notably outcomes were similar for series of experiments performed together (C-F, H-I, J-O). For the series J-O, B95-8 had been recently thawed, cultured for one or two weeks and put to age for 2-3 weeks. In all cases, there was aging for at least 2 weeks (usually 3 or more), but when longer periods of culture/ageing were allowed the outcomes appeared to be better. One subject was profiled twice (K and H): the profiles, however, retain similarity between batches. We did not quantify viral load in the supernatant prior to infection. These batch effects might be due to qualitative or quantitative properties of the supernatant resulting from period spent in culture or period spent in ageing, or possibly due to improved ficoll technique improving the yield of functional NK cells.

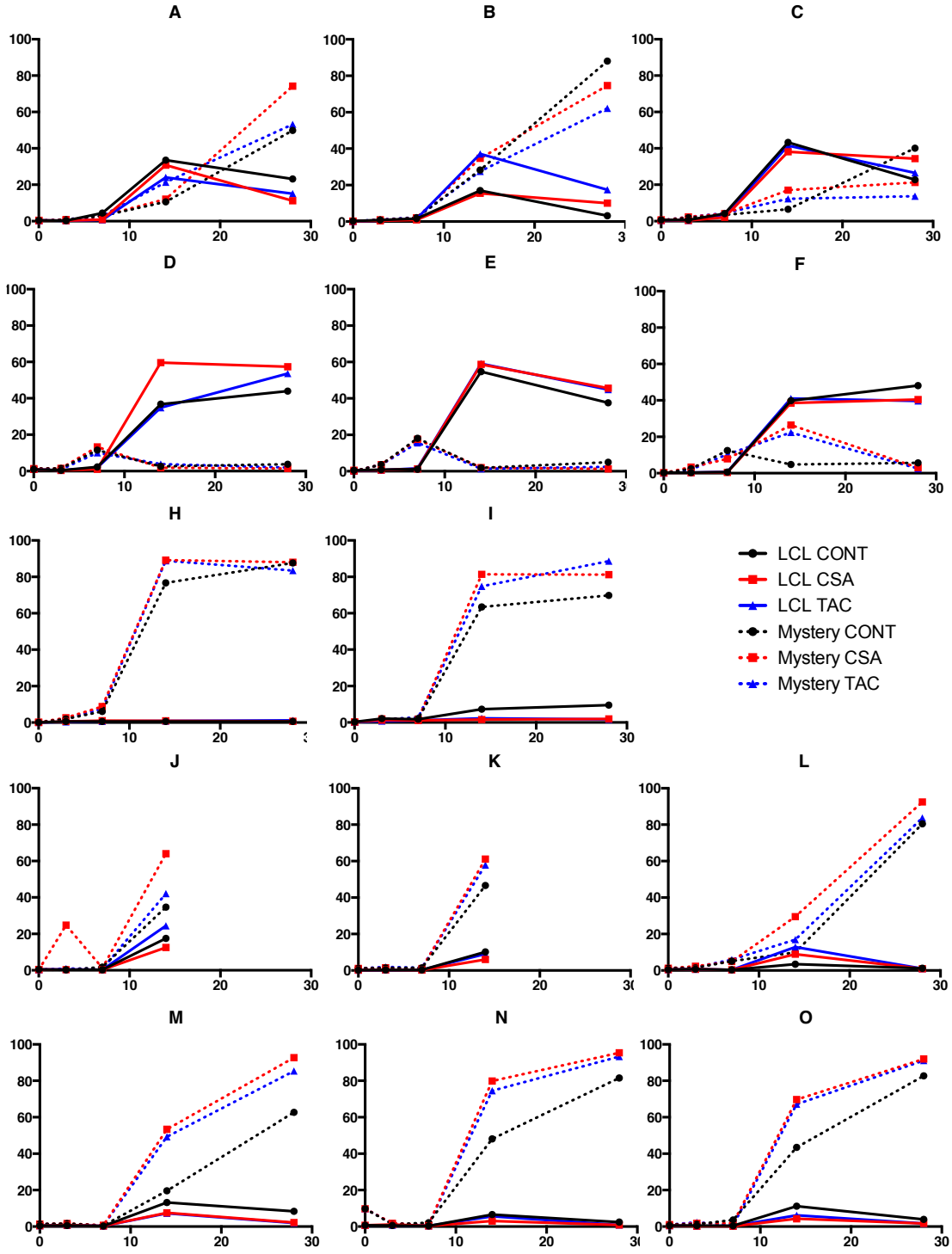

Figure 3 Kinetic profiles for individual subjects depicting CD19LCL and mystery populations on days 0, 3, 7, 14 and 28.

Subjects C, D, E, F were selected to represent summarised data when LCLs emerged and L, M, N and O were selected to represent data for profiles where mystery population predominated and no cell line emerged. There was no difference between the control or treated conditions with respect to CD19 LCL and the mystery line for all subjects combined (Figure 6 IA, IIA, IIIA). The mystery population was negatively associated with the emergence of LCLs. In the series of subjects in which the mystery population predominated there was a greater proportion of viable cells in the calcineurin inhibitor treated systems, with no difference between drugs (Figure 6 IIIC). Although there was no difference between treatment and control systems when CD19 LCL emerged in terms of viable cells (Figure 6 IIB), there was more growth in CNI treated systems as assessed by total cell counts (Figure 6 IB).

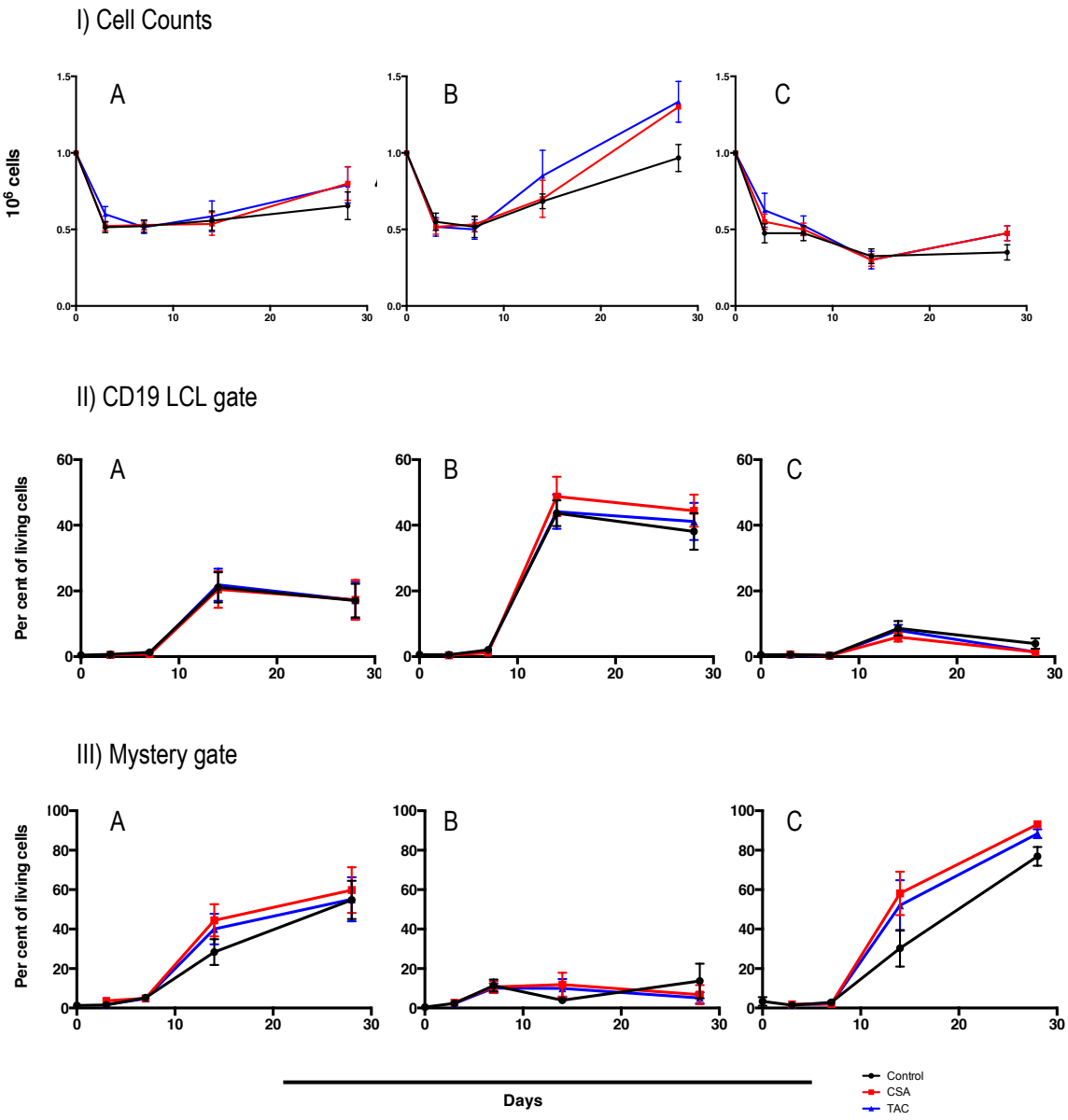

Figure 4 Cell counts (I) and proportions of viable cells for CD19 LCL (II) and mystery populations (III) for all subjects (A, n=14), lines with a predominantly CD19 LCL population (B, n=4), lines with a predominantly mystery population (C, n=4). Kinetic profiles exhibit means and S.E. for each time point.

In the T cell panel, the mystery population exhibited a range in HLA-DR positivity, and a subpopulation was CD56+, but did not express CD16, on day 28 (9-20% of the mystery population): a majority of these were activated, CD25+. These cells were greater in calcineurin treated conditions (Figure 3 A and B). There was likewise a CD8+, which did not exhibit CD3+ (approximately 1% of the mystery

population). The majority of the mystery population, however, was not accounted for by any of the markers of either panel.

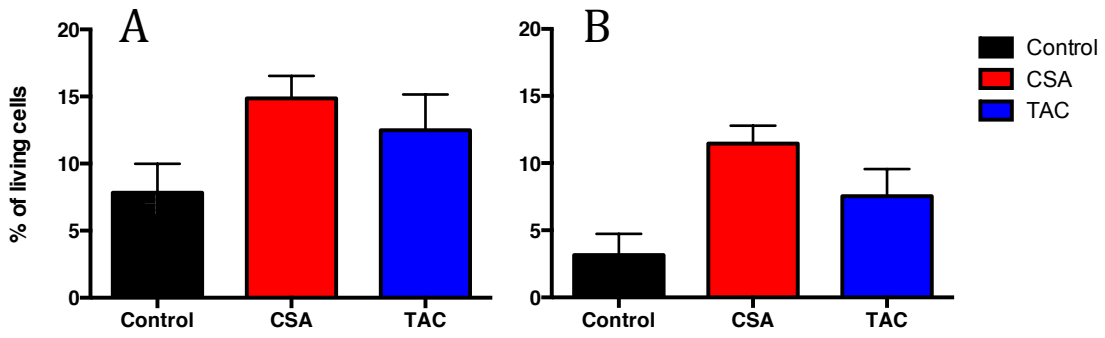

Figure 5 A) CD56+ cells gated on the mystery population; B) CD56+ CD25+ cells gated on the mystery population. Subjects L-O, mean percentage of living cells on day 28, with S.E.

Dry cell pellets were collected following cytometric acquisitions over the entire period of the protocol for several of the lines in which the mystery line predominated (lines K, M, N, O, all three treatment conditions) and were tested for the presence of genomic EBV DNA by quantitative PCR (S. Rogez and M. Al Jawhari, Laboratoire de Bactériologie-Virologie-Hygiène, Limoges University Hospital). Viral levels in the cell pellets increased at day 14 in treated cultures, but returned to be similar to controls at day 28. Levels in supernatants remained similar over the 28-day period.

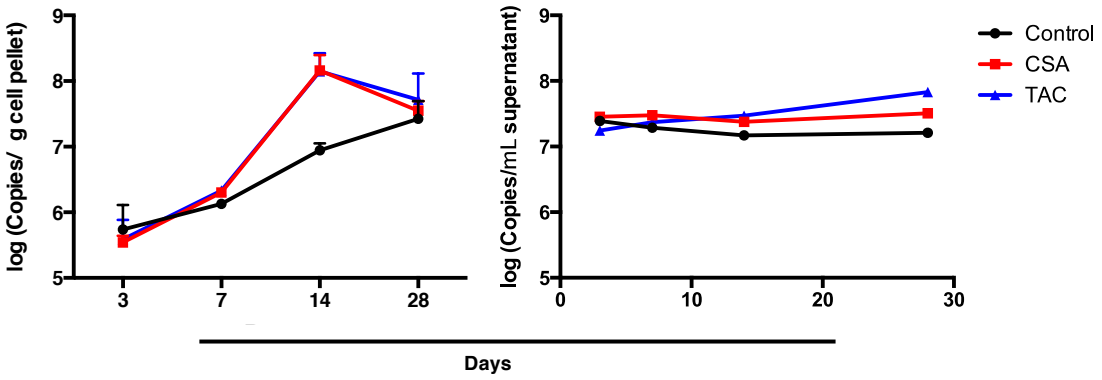

Figure 6 EBV viral DNA in mystery lines for subjects K, M, N and O

Amount of each calcineurin inhibitor was determined by Liquid Chromatography – tandem Mass Spectometry in culture supernatants and cell pellets recovered following cytometric acquisitions on days 3 and 28, for mystery population for line 'J' (F. Saint Marcoux, Laboratory of Pharmacology and Toxicology, Limoges University Hospital). The calibration for these analyses were based on control supernatant for the former, while cells were mixed with 100µL of whole, drug-free blood and compared to routine calibration curves. Drug levels in supernatant decreased by half over the period 3-28 days, while absolute amounts in cell pellets were greater on day 28 compared to day 3. Cell counts for cells retained following cytometry were approximately $8x10^5$ (accounting for cells from both panels, 2 x $5x10^5$, minus cells acquired during acquisition, 2 x $5x10^4$). It was not possible to sample especially to determine drug concentrations, as there was limited culture material.

Being unusual that a large proportion of the mystery line exhibited no fluorescence markers, subsequent tests were undertaken with the aim to characterise its lineage. In particular, lack of CD19 made questionable the origin of these cells. A representative established CD19 LCL (line E, CSA treated) and representative mystery line (line M, CSA treated) were subjected to flow-cytometry using a different system (FACSCalibur flow cytometer, CellQuest Pro software, both Becton Dickinson) and a simplified antibody marker panel (CD45, CD19, CD3, CD11b,

CD16, CD25). An expert engineer performed acquisitions and analyses (C. Ouk, Cytométrie, Imagerie et Mathématiques platform, Limoges University Hospital). Whole blood was used as a control. The CD19 LCL was CD19+ and negative for CD3 and CD16, with some CD11b and CD25 positivity, while the mystery line was not positive for any marker. Furthermore, similar to Navios/Kaluza analyses, CD19 LCLs were CD45+, while the mystery line expressed CD45 at a low level. Representative analyses are depicted in Appendix 1 (Figures 31 and 32).

Discussion

In conclusion, it was confirmed that the observations determined by Navios/Kaluza cytometer and analysis software, using a 10-colour panel were correct: the mystery population did not express CD19, CD3 nor the monocyte marker CD11b. All the data put together suggest that the mystery population had an inhibitory presence on the emergence of a lymphoblastoid cell line. CD56 and CD8 positive subpopulations suggest NK and cytotoxic cells respectively, the former having a very strong presence. The lack of CD3 on CD8+ cells gated on the mystery population, the appearance of activated CD4+ and CD8+ T lymphocytes outside of the lymphocyte gate, the low level CD45 and spread of these populations across FS and SS suggests that we did not achieve a single cell suspension, and that our observations are of active physical cell-to-cell interactions and not random agglomeration. Consistent with this is the presence of the small CD19, CD79b and CD138 sub-populations. Similar results were achieved by an experienced engineer, and cytometric profiles in published reports exhibit similarities to our work (Chen et al., 2003), implying that it is a property of the experimental model, although there may be strategies to overcome this. EBV DNA increased on day 14 in treated cultures, but decreased to levels similar to control on day 28 (Figure 4): this appears to be due to the control of outgrowth by NK cells (Figure 3). Cyclosporine has distinct effects on natural killer cell subpopulations: while $CD56^{dim}$ cells are inhibited, growth and cytotoxic activity in $CD56^{bright}$ cells remains in tact following treatment (Eissens et al., 2010; Wang et al., 2007).

We considered the possibility that the cells that failed to exhibit markers, but survived for at least 6 weeks in cultures, could be nurse-like cells. Nurse-like cells (NLCs) have been found to stimulate and enhance the survival of B lymphocytes under certain conditions associated with specific human pathologies. NLCs were derived from the bone marrow and synovial fluid of individuals with rheumatoid arthritis (RA) (Shimaoka et al., 1998). NLCs of a different phenotype were found to differentiate in a subset of PBMCs from individuals with chronic lymphocytic leukemia (CLL), and it was subsequently shown that PBMCs from normal donors develop NLCs when incubated with CLL cells (Burger et al., 2000; Tsukada et al., 2002). To date, no other nurse-like cells that support B lymphocyte viability associated with other pathologies have been described (PubMed search).

RA-associated nurse-like cells increased CD40 and MHCII production in normal B cells, enhancing their survival for greater than four weeks (Shimaoka et al., 1998). Furthermore, EBV transformed outgrowth was observed following co-culture with NLCs, but not in isolation or culture with fibroblasts. RA-NLCs expressed CD29, CD49c, CD54 (ICAM1), CD106 (VCAM1) and MHCI. CD106 and CD157 (BST1) antibody blocked B cell survival.

CLL-associated nurse-like cells were necessary for the *in vitro* survival of CLL, functioning by blocking apoptosis, and were confirmed to be present in the spleens of individuals with the disease (Burger et al., 2000; Tsukada et al., 2002). CLL-NLCs are large adherent cells and express vimentin, SDF-1, CD14, CD11b, CD33, CD40, CD45RO, CD68, CD80, CD86, HLA-DQ and HLA-DR, but not CD1a, CD2, CD3, CD11c, CD19, CD45RA, CD83, CD106 nor CD154. These cells originate from CD14 cells and fail to arise when CD14+ cells are removed from PBMCs. Compared to monocytes, macrophages and monocyte derived dendritic cells, nurse-like cells expressed CD68 to a higher degree. Antibody to CD106 did not block the

enhanced survival of CLL cells, so CLL-NLCs appear to function differently to RA-NLCs.

Our mystery cells share a number of features with CLL-associated NLCs: the cells were large and had a complex structure (similar profile on the FS SS scatter plot), express low level CD45 and high level HLA-DR (observed in the T cell panel), and do not express CD3, CD11c nor CD19. However, unlike CLL-NLCs, mystery cells did not express CD11b (cytometry work by C. Ouk). In contrast to the CLL work where CLL cells were small and could be observed crowding around a NLC, it would have been difficult to differentiate the large, round NLCs from EBV immortalised B-lymphoblastoid cells under the microscope. It is possible that a different type of nurse-like cell is stimulated form PBMCs by EBV-infected B cells, and this would be a novel finding. Similar to RA-associated NLCs, cultures in which outgrowth did not occur persisted for beyond 4 weeks (up to 10). However, it is unusual that despite incubation with EBV lytic virus in our work, no outgrowth eventuated (six individuals) whereas when observed in the work concerning RA-NLCs, outgrowth was spontaneous.

Lymphoblastoid cell lines became established or were inhibited similarly across the three conditions. There was faster growth of LCLs in calcineurin inhibitor treated systems. When mystery cells were present, there was growth of CD19 LCLs until day 14, and a concomitant increase in EBV viral DNA in calcineurin inhibitor treated systems. On day 28 these returned to levels similar to control, but a greater NK cell activity was apparent in treated systems. Cytometric expression profiles exhibited similar features across the three conditions. There was no qualitative difference between cyclosporine and tacrolimus, or calcineurin inhibitor treated systems compared to controls using the marker panels we employed. This work suggests that the calcineurin inhibitors do not influence the EBV infection and immortalisation of primary B lymphocytes outside of their inhibitory activity of T lymphocytes.

Experimental Work 1B: The effects of chronic cyclosporine exposure on late lymphoma events (*in vivo*, in an established lymphoma model in LMP1-CD40 transgenic mice)

Background

It is a challenge to investigate the effect of cyclosporine on lymphomagenesis in models of EBV-lymphoma given the difficulty in disassociating its inhibitory effect on IL-2 mediated cytotoxic T cell activation by these tumours. Transgenic mice expressing the LMP1-CD40 chimeric protein, a constitutively activated form of CD40, develop insidious B cell lymphomas beyond 12 months of age (Hömig-Hölzel et al., 2008). The observed B activation is independent as T cell dependent immune responses are impaired in these mice. The LMP1/CD40 mouse is an attractive model to test the effects of cyclosporine on lymphomagenesis, given that the observed lymphomas develop spontaneously and over a prolonged period in otherwise immune competent mice.

Latent Membrane Protein 1 (LMP1) is essential for EBV transformation of B cells (Kaye et al., 1993) and alone is oncogenic (Wang et al., 1985). It is expressed in most human malignancies associated with EBV, including post-transplant lymphomas, EBV associated Hodgkin's disease, nasopharyngeal carcinoma, and AIDS-related lymphomas. It is a functional mimic of CD40 signalling, which in B cells is critical for T-cell dependent humoural immunity. LMP1 comprises six trans-membrane domains that permit ligand-independent aggregation into membrane patches, and a cytoplasmic carboxyl-terminal tail that interacts with TNFR-associated factors (TRAFs 1, 2, 3, 5, and 6): this ability to form aggregates permits a chronically activated state. CD40, on the other hand, self-oligomerises when activated by its

principal ligand CD154 (also referred to as CD40L) transiently expressed on activated CD4+ T cells.

Besides the differences due to being transiently or chronically activated, CD40 and LMP1 exhibit some differences in engagement of TNFR-associated factors by their cytoplasmic domains, and this may explain some of their divergent characteristics, and physiological versus pathogenic effects (Graham et al., 2010). For the most part, however, gross phenotypic similarities have been demonstrated using transgenic mouse strains, and LMP1 is considered to be a functional mimic of CD40.

Transgenic mice expressing LMP1 under the control of the immunoglobulin heavy chain (IgH) enhancer/promoter, under a background of CD40+/+, had enlarged spleens and produced lymphomas in older mice (Kulwichit et al., 1998; Uchida et al., 1999). Ratios of mature and immature B cells in the spleen were similar to control mice, but there were more activated B cells in expressor mice. In both CD40+/+ and CD40-/- backgrounds, LMP1 expressing mice produced IgM and IgG1 to T-cell dependent antigen, however only mice on a CD40+/+ background produced the high affinity receptor. Transgenic mice of both backgrounds failed to form functional germinal centres (GCs), indicating extra follicular activation.

Two independent groups produced transgenic mice models of a chimeric protein coupling the extracellular mouse CD40 (mCD40) domain with the LMP1 cytoplasmic domain, both on CD40+/+ backgrounds, both exhibiting essentially normal or heightened T cell dependent antigen responses and forming germinal centres. In one model expression was under the control of the MHCII promoter resulting in B cells, macrophages and dendritic cells (Stunz et al., 2004), and in the other using CD19 Cre-mediated recombination, therefore expression of the chimeric protein was in B cells only (Rastelli et al., 2008). In the former model, unimmunised

mice exhibited spontaneous GC formation, splenomegaly and lymphadenopathy, elevated serum IL-6 and expanded immature and GC B cells (Stunz et al., 2004).

A third distinct transgenic mouse model, which is the model employed in the present work, involves a chimeric protein composed of an LMP1 transmembrane domain and a CD40 intracellular domain. Similar to LMP1 transgenic mice, LMP1-CD40 expression in B cells resulted in suppressed GC formation (Hömig-Hölzel et al., 2008; Panagopoulos et al., 2004), defective T cell dependent responses (Panagopoulos et al., 2004), and in B lymphoma in older mice (Hömig-Hölzel et al., 2008): these effects may thus be attributed to a chronically activated state, and any differences that might exist due to the distinct intracellular domains do not affect the outcomes in question.

Lymphomas arising in the present model share the main characteristics of EBV-associated post-transplant lymphomas that express LMP1, namely continuous NF-kappa B activation and continuous B-cell activation, making this model a good candidate for exploring the effects of cyclosporine, and other immunosuppressive drugs.

This work was performed in collaboration with the group "Mécanismes Moléculaires de la Lymphomagenèse", UMR CNRS 7276, Limoges. N. Faumont and A. David performed animal sacrifice, histology, cytometry and *in vitro* cultures.

Methods

Three pairs of mice of the same age (5, 6 and 13 months at the start of treatment) were injected daily with intra-peritoneal cyclosporine injections (10mg/

kg/day), or vehicle (controls), over a three-month period. The younger mice were female pairs, while in the oldest pair the female was treated and male was the control. Cyclosporine dilutions for injection were prepared second daily (V. Ratsimbazafy, Department of Pharmacy, Limoges University Hospital). Animals were maintained in a pathogen-free environment. All experiments were performed in compliance with local animal welfare protocols and under institutional approval from the University of Limoges.

Flow cytometry was performed on cell suspensions prepared from various lymphoid organs (spleen, inguinal and cervical lymph nodes, and bone marrow) to characterise B- and T- cells and sub-populations, macrophages and granulocytes, as previously described. Antibodies were purchased from BD Biosciences. Acquisitions were performed by FACSCalibur and results were analysed using CellQuest software (both Beckman Dickinson). Splenocytes from control and treated mice were cultured *ex vivo* for up to five days, with or without additional cyclosporine (1000nM, Novartis Pharma AG, Switzerland), and viability was assessed by staining and analysis with FACSCalibur or tryptan blue dye exclusion, as previously described (Hömig-Hölzel et al., 2008).

Results

The spleens of cyclosporine treated mice were larger for the 5- and 6-month old mice (Figure 7, A and C). The 13-month old control was male and had a larger spleen, however the 13-month old cyclosporine treated female presented with a tumour in the cervical lymph node (Figure 7B) while there were no nodal tumours in the placebo treated control.

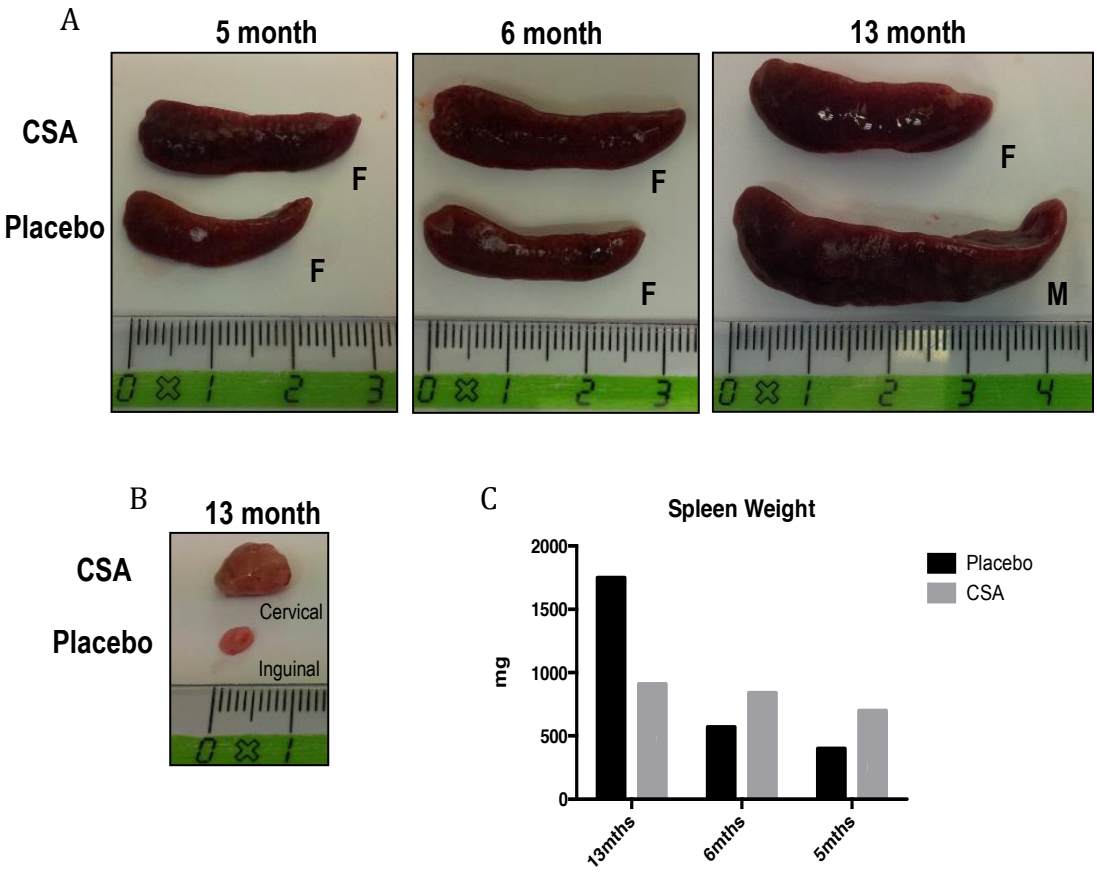

Figure 7 A) Spleens of 5mth, 6mth and 13mth LMP1/CD40 mice after 3 months of i.p. injections with cyclosporine, 10mg/kg, or placebo. B) The 13mth old cyclosporine treated LMP1/CD40 mouse presented with a cervical tumour. C) Spleenic weight in LMP1/CD40 mice following three months of cyclosporine or placebo treatment.

Similar proportions of B and T cells between treated and control mice were observed in the spleen (Figure 8, A and B). Treated mice universally exhibited a greater proportion of activated B cells (CD86+) in the spleen (Figure 9 A). In the 13-month old pair, a greater proportion of activated B cells in the inguinal lymph node was observed in the cyclosporine treated mouse (Figure 9 B).

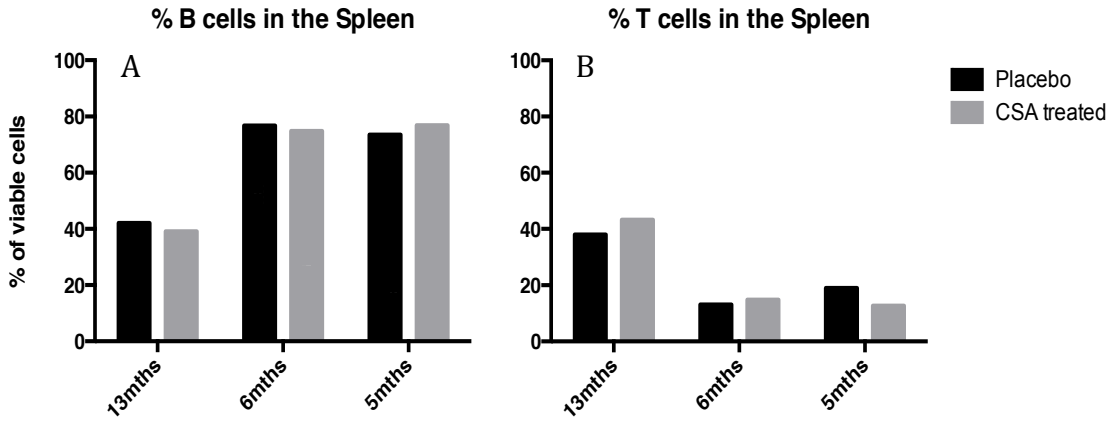

Figure 8. Proportion of B cells (A) and T cells (B) in the spleens of cyclosporine and placebo treated LMP1/CD40 mice.

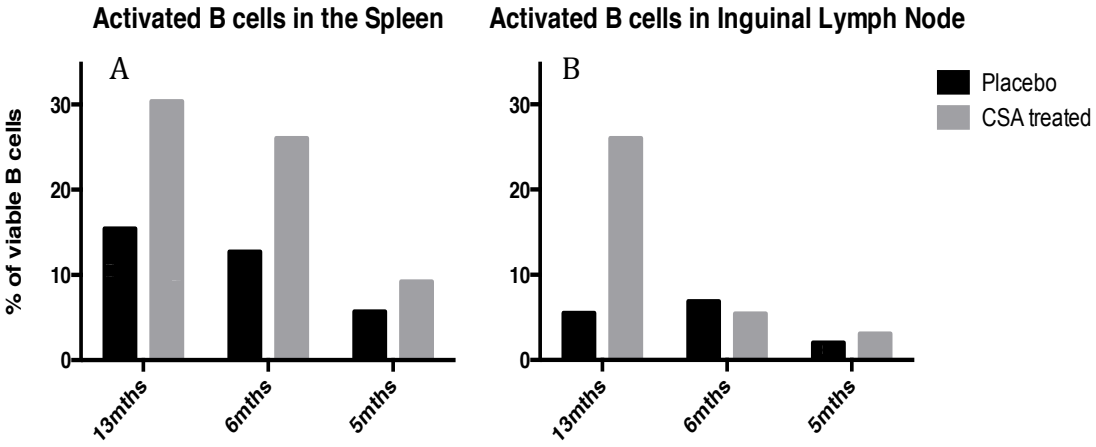

Figure 9. Activated B cells as a proportion of total B cells in the spleens (A) and inguinal lymph node (B) of cyclosporine and placebo treated LMP1/CD40 mice.

Increased T cell infiltration was observed in the pair of older mice, resulting in a roughly equal contribution of B and T cells on splenic weight (Figure 8, A and B): this is largely accounted for by CD4+ T cells (Figure 10 A). Consistent with this is the elevated CD4:CD8 ratio in older mice, which was augmented in the cyclosporine treated mouse compared to control (Figure 10 B). The 6month and 5 month pairs of mice had a similar CD4:CD8 ratio: the ratio is expected to be the similar to young LMP1/CD40 non-expressing mice, as has been demonstrated by other authors (Hömig-Hölzel et al., 2008). There was no trend for an effect by cyclosporine on the

T cell sub-populations. There was no effect by cyclosporine on pro–, pre– and immature B cells in the bone marrow.

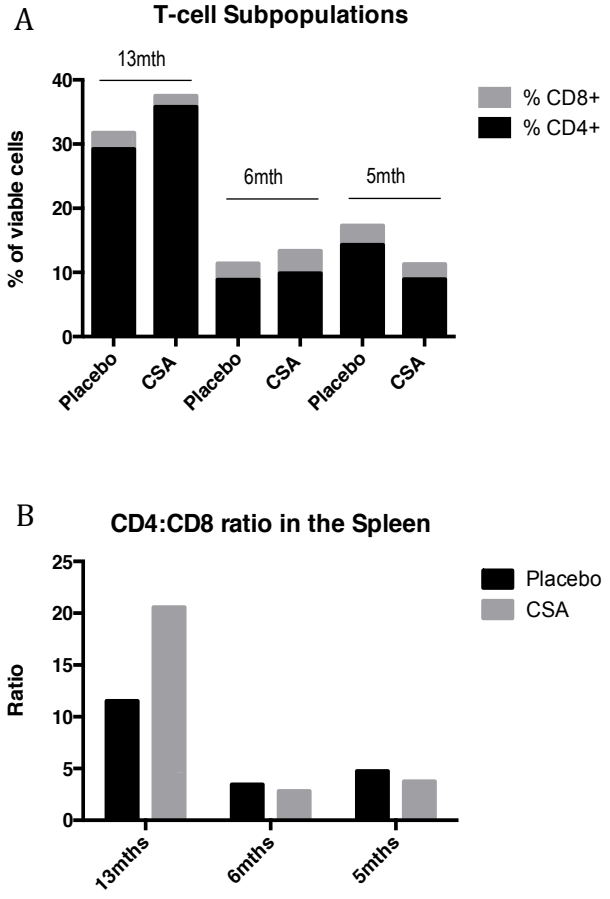

Figure 10 A) Proportions of CD4+ (black) and CD8+ (grey) populations in the spleens of cyclosporine and placebo treated LMP1/CD40 mice. B) CD4/CD8 ratios of splenic T lymphocytes in treated and control mice

Splenocytes from cyclosporine treated mice tended to have an enhanced cellular viability (5 and 13 month mice, grey vs black lines, Figure 11). Cyclosporine treatment in *in vitro* culture universally decreased splenocyte viability (dashed vs full lines, Figure 11).

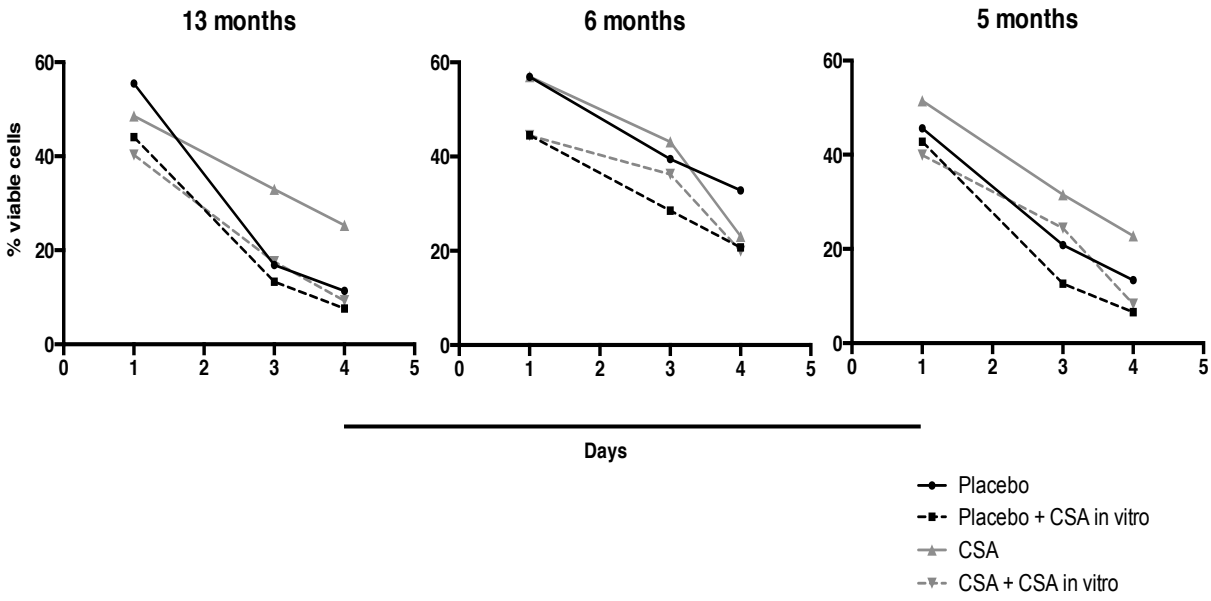

Figure 11 Viability of *ex vivo* cultured splenocytes from placebo (black lines) and cyclosporine treated (grey lines) LMP1/CD40 mice, cultured with (dashed lines) and without (full lines) additional cyclosporine (1000nM) added *in vitro*

Discussion

The LMP1/CD40 mouse model is a model of spontaneously occurring lymphoma of insidious onset that results from constitutive CD40 signalling in B cells. Cyclosporine treatment resulted in accelerated expansion of activated B lymphocytes *in vivo*. There was no appreciable trend for an effect on T cell subpopulations in the spleen between cyclosporine treated mice and controls. *Ex vivo*, spontaneous survival of splenocytes from CSA treated mice was enhanced. CSA inhibited the viability of splenocytes in culture from both cyclosporine treated and untreated mice. These preliminary results suggest that the present model is useful to evaluate the effects of chronic treatment with cyclosporine, as well as other calcineurin inhibitors and other classes of immunosuppressive drugs in the complex tumour microenvironment, and thus permit a more detailed exploration of the mechanisms in future work.

The late onset of tumours in aged mice and heterogeneity of tumours (distinct surface markers and engagement of intracellular signalling pathways) in the work by Hömig-Hölzel et al. implicate secondary oncogenic hits for transformation. An otherwise competent immune system keeps such tumours in check (cytotoxic T cells, natural killer cells, cell-mediated effector responses) and tumours probably arise in aged mice at least in part due to immune senescence. Tumour cells do not exhibit significant up-regulation of LMP1/CD40 expression compared with premalignant cells .

The relationship between activated B cells and T cells in this model is of interest and may provide the key to uncovering the role that cyclosporine plays in permitting the expansion of activated B cells. It is difficult to know if the B cells activated by LMP1/CD40 chimera pose an antigenic source in these mice. Therefore we cannot conclude that cyclosporine treatment contributed to B cell activation by a tertiary mechanism outside of its well-known role in inhibiting T cell activation by antigen. It is interesting that mice expressing chimeric proteins (LMP1/CD40 with a CD40 cytoplasmic domain, and mCD40-LMP1 with an LMP1 cytoplasmic domain) exhibit signs of autoimmunity (Panagopoulos et al., 2004; Stunz et al., 2004). This was not reported in work involving LMP1 transgenic mice models. Hömig-Hölzel et al. attribute activated T cell splenic infiltrates, which occur from a young age (8 weeks), to non-specific bystander activation due to constitutive CD40 signalling (Bangs et al., 2006; Ehl et al., 1997; Koschella et al., 2004; Taraban et al., 2004) and confirm a lack of expression of the LMP1/CD40 transgene in T cells. Furthermore, the expanded T lymphocytes in LMP1-CD40 mice exhibited a similar CD4:CD8 ratio compared to the T population in the spleens non-expressor mice, suggesting that cytotoxic T lymphocytes did not predominate. Future work to examine the activated CD4+ and CD8+ subpopulations in control and cyclosporine treated mice could prove revealing. While the negative effect of cyclosporine in *in vitro* splenocyte cultures suggests that there is no direct activation by cyclosporine, this will need to be confirmed in *ex vivo* cultures using fractionated splenic B cells.

Several lines of evidence suggest that T lymphocyte infiltrates might contribute to pathogenesis in the present model. Firstly, tumours fail to form in hu-SCID mice when B cells are injected in isolation, whereas spontaneous tumours form when T lymphocytes, particularly CD4+ cells, are present (Johannessen et al., 2000; Veronese et al., 1992). Secondly, normal T lymphocytes are commonly coincident in clinical PTLD B-lymphoma specimens, and the spectrum observed is similar to the present model: memory cells are largely implicated (Perera et al., 1998; Thomas et al., 1990). Lastly, the T lymphocyte infiltrate is exaggerated in older LMP1/CD40 transgenic mice, which coincides with the age at which tumours start to appear in these mice.

T cell depleted human PBMCs injected intraperitoneally in SCID mice fail to produce tumours or result in a very low incidence of tumours when derived from high penetrance donors (Johannessen et al., 2000; Veronese et al., 1992). Monocyte depletion has no bearing on the outcome. Depletion of T cell subsets suggests CD4+ are of higher relative importance: some authors show depletion of this subset results in slower tumour progression (Veronese et al., 1992), while others show very low tumour incidence compared to when CD8+ T cells are depleted (Johannessen et al., 2000). In addition, *ex vivo* stimulation to produce an activated T phenotype prior to injection results in an increased incidence and shorter time of onset of tumours, particularly when the CD4+ subpopulation remains present (Johannessen et al., 2000).

The tumours that result in hu-PBMC/SCID mice, like lymphomas in the immunosuppressed, frequently occur in extra nodal sites. However, when excised, tumours are essentially comprised of B-cells. This has led some authors to postulate that initial T cell derived paracrine help is essential, but the developing tumour becomes independent as autocrine growth factors achieve sufficient levels and take over (Amadori et al., 1996; Johannessen and Crawford, 1999). The frequent T cell infiltration in human PTLDs, even severe monoclonal disease, suggests the finding in the hu-PBMC SCID mice might be unique to this model. A possible explanation is that despite an initial graft versus host reaction by human T lymphocytes, they

subsequently become anergic (Tary-Lehmann and Saxon, 1992; Tary-Lehmann et al., 1994).

LMP1/CD40 mice displayed more memory T cells (CD44+) in the spleens of young mice, with both central and effector phenotypes (CD62L high and low expression, respectfully), more notably in the CD4+ sub-population, although evident for CD8+ cells as well (Hömig-Hölzel et al., 2008). In clinical PTLD specimens, small lymphocyte infiltrates primarily exhibited a memory/helper phenotype (TCR alpha/beta +, CD3+, CD4+, CD45RO+), while cytolytic CD8+ were rare (TCR alpha/beta +, CD3 +, CD8 +, Tia-1 +) (Perera et al., 1998). Although B cells in these tumours were CD80+ and CD86+, co receptor CD28 expression in T cells was absent.

While preliminary, the present work suggests that the CD40/LMP1 transgenic mouse model is a good candidate for exploring the effects of the calcineurin inhibitors and other immunosuppressive drugs on lymphomagenesis.

Part Two

Positionining post-transplant lymphoproliferative disease as a pharmacogenetic problem

Clinical epidemiology work

PTLD: A case-control study to determine pharmacological and pharmacogenetic risk factors of post-transplant lymphoproliferative disease

Positioning PTLD as a Pharmacogenetic Problem: literature review of the functional consequences of tested variants from genes involved in the calcineurin inhibitor pathway

Despite great improvements in first year graft survival following kidney transplantation, latter-year graft survival and mortality rates continue stagnant. The challenge in the modern era of medical management in transplantation has been to mediate the consequences of long-term immunosuppression. Cardiovascular disease and calcineurin-associated nephrotoxicity represent direct toxicities primarily due to the calcineurin inhibitors. The spectrums of infections and cancer are similar to those observed in other immunosuppressed states (such as HIV and congenital conditions), suggestive that these are consequences of over-immunosuppression.

Promising work in which pharmacodynamic monitoring based on calcineurin gene expression resulted in mediation of skin cancers in a set of patients implicates the calcineurin pathway as a source for candidate genes related to drug response. It is important to note that despite there being a cocktail of immunosuppressants, the pathway related to perhaps the most important drug class within this cocktail has emerged as pertinent in this method of monitoring. Given the challenges in implementing pharmacodynamic monitoring of immunosuppressants, genetic biomarkers are an attractive option in the aim to predict inter-patient variability in response to immunosuppressants.

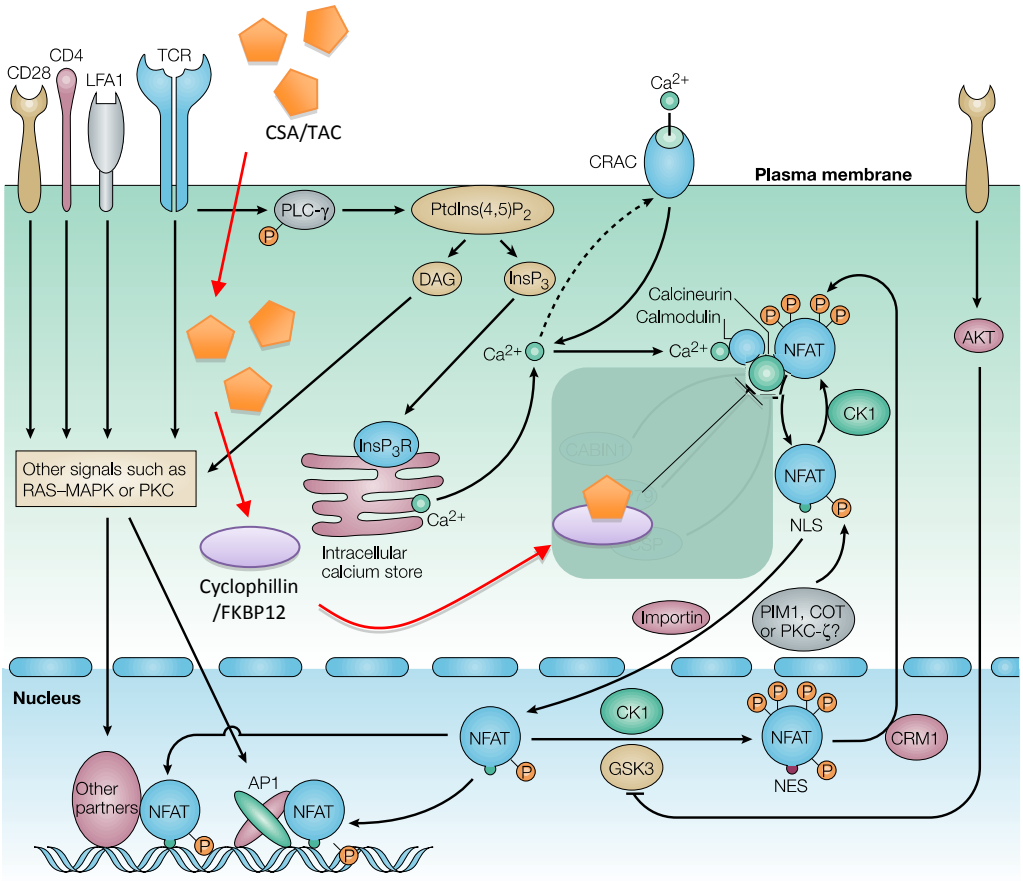

Figure 12 Adapted from (Macian, 2005). Proteins part of homeostatic control of cytoplasmic calcineurin have been hidden for clarity.

Antigen-related activation via the T cell receptor triggers a signaling cascade that results in elevated intra-cellular calcium. Ca^{2+} binding calmodulin allows the protein to bind to calcineurin's catalytic subunit. In addition, Ca^{2+} binding calcineurin's regulatory subunit causes a conformational change that allows calcium-calmodulin to bind. This results in disinhibition by an autoregulatory element that hinders catalytic activity. Activated calcineurin de-phosphorylates NFAT, allowing it to translocate to the nucleus where it acts as transcription factor. The co-factor AP-1 is a Fos-Jun complex, each element of the dimer having been activated in previous steps by different signaling cascades resulting from activation via the T cell receptor (for example the RAS-MAPK or PKC pathways).

Cyclosporin and tacrolimus bind distinct intracellular immunophilins to effectuate calcineurin inhibition, the former binding to cyclophillins and the latter to FKBP12. While cyclopsporin binds two similar but distinct cyclophillins, inhibitory action is attributed to cytoplasmic cyclophillin A (Handschumacher et al., 1984). Cyclophillin B, on the other hand, is located in the endoplastic reticulum (Price et al., 1991). The drug-immunophillin complexes bind calcineurin at a site overlapping the regulatory and catalytic subunits (Huai et al., 2002). While a majority of residues are similarly bound by the CsA-cyclophillin TAC-FKBP12 complexes, there are several differences, most notably an Arg-112 in the catalytic site, which is bound by the CsA complex, but not TAC-FKBP12 (Huai et al., 2002).

There are three calcineurin catalytic subunits, calcineurin Aα, calcineurin Aβ and calcineurin Aγ, and two regulatory subunits calcineurin B1, calcineurin B2. The CnAγ and CnB2 subunits exhibit testes specific expression (Rusnak and Mertz, 2000). CnAβ shows a predominant cellular distribution in B and T lymphocytes in rats. (Jiang et al., 1997). The opposite relative distribution is found in the kidney and tissues of the central nervous system where Aα predominates (Jiang et al., 1997; Kuno et al., 1992). Gene knock-out studies have confirmed that the CnAβ subunit has a crucial role in response to antigen (Bueno et al., 2002). While lymphocytes from CnAα deficient mice exhibited essentially normal responses in vitro, in vivo exposure to antigen and subsequent restimulation assays demonstrated deficient responses (Zhang et al., 1996), however some authors have interpreted this to be potentially due to anergy (Liu, 2009).

The work in our laboratory has focused on genes encoding the following key proteins associated with response to calcineurin inhibition by cyclosporine and tacrolimus: cyclophillin A, FKBP12, CnAα, CnAβ, CnB1, calmodulin, the principle NFAT isoforms, c-Fos and C-jun. Due to there being limited data available concerning some of these genes in the HapMap database, a tagging approach was deemed to provide limited coverage. Furthermore, such an approach would require Next Generation Sequencing for genes with very long sequence and complex

haplotypic structure, such as PPP3CA. At the time of executing genotyping for the present study it was apparent that subject inclusion would continue beyond September 2012. At this point approximately 100 case control pairs had been included. At the same time, parallel work in the unit (POLYCIS, 3-PIGREF: M. Koitka, L. Pouché, O. Noceti, N. Picard) was being conducted in systematically profiling the variation within these genes, and determining their functional significance by way of intracellular tagging and cytometric detection, and mRNA quantification. To guard statistical power and increase the prior probability of working with functional variants a decision was made to a) limit the number of genes included at this round of testing; and b) to work with variants that appear to have a functional effect as determined by literature review, or a predicted consequence on the protein (e.g. aminoacid substitution, frame-shift mutation). Thus the initial list of fifteen protein targets and their genes was reduced to ***PPIA*** (cyclophillin A); ***FKBP1A*** (FKBP12); ***PPP3CA*****,** ***PPP3CB*** and ***PPP3R1*** (calcineurin sub-units CnAα, CnAβ and CnB1, respectively); and ***IL2***. Furthermore, candidate SNPs that could not be genotyped and that would require sequencing to be profiled were set aside for functional confirmation in POLYCIS.

The following strategy was employed in choosing polymorphisms:

– Reported minor allele frequency (MAF) of >10% (based on power calculations, see methods below)

– Convincing functional effect reported: variants were selected if phenotypic associations were supported with expression studies, functional experimental or *in silico* investigations, or if multiple studies reported an effect in the same direction for a given variant

– If a tagging approach had been used in a report, and no functional data were available, the variant was considered only if the population tested was Caucasian

It is important to bear in mind how exhaustively genes have been profiled, and which variants were considered in analyses of functional consequences. Table 5 lists the most notable variants reported to date and details the work done to test functionality, while table 6 describes the sequencing work done to date. On the whole, these genes appear to be highly conserved, with very few polymorphisms reported within coding regions, which, when reported, were rare. A number of common variants in the putative promoter region and introns have been found to have possible functional consequences. In deed, some work discussed below were the first time variants were reported, and led to the initial designation of an accession number, highlighting the importance of latter generation sequencing work. NCBI's SNP database (dbSNP) is certainly improving, especially with large-scale deep sequencing efforts such as the 1000 genomes project and continued contributions by individual labs. As a consequence, profiling of variants is coming close to being exhaustive. However the number of reports without validation and without information for the minor allele frequency in specific populations is high, necessitating sequencing in a population of interest for a true profile of common variants and estimates of allelic frequency. The strategy for POLYCIS has been to focus on the proximal promoter region in these genes (approximately 600 bp), given the potential consequences to altering expression. While not all of these SNPs were tested in the present study, it is hoped that the profiling of the genes presented here might be used in future systematic approaches, especially for ***PPIA***, ***PPP3CA, PPP3CB*** and ***PPP3R1*** which are relatively unexploited genes but whose proteins have roles in various physiological and pathological scenarios (Musson et al., 2012).

Table 5 Notable variants referred to in the review below. Variants marked by an asterisk are included in the PTLD case-control study

Ref SNP / dbSNP	Ref	Postition	Association	MAF Caucasians	MAF 1000 Genomes	Polycis (promoter)
FKBP1A **- FKBP12**						
rs139306132	NCBI dbSNP	exon 4 missense		NA	NA	
rs146341033	NCBI dbSNP	exon 4 missense		NA	NA	
PPIA **- peptidylprolyl isomerase A (cyclophilin A)**						
SNP 3 (no accession number)	An 2007	408 bp upstream from rs8177826	LD	NA	NA	
rs8177826*	An 2007 Rits 2008 Palacin 2008 Moscoso-Solorzano 2008	promoter	**Y**	0.033 (HM CEU)	0.045/98	0.13
rs6850	Bleiber 2005 An 2007 Moscoso-Solorzano 2008	5' UTR	N - no support from functional studies	0.5 (HM CEU - n=2)	0.382/835	0.16
rs3735481	An 2007	intronic tag SNP	N - genotyped, no association	0.173 (HM CEU)	0.460/1004	
rs8177828	Palacin 2008	exon 4 (codon 46 C/T, synonymous)	NT	0.5 (HM CEU - n=2)	0.010/21	
PPP3CA **- protein phosphatase 3, catalytic subunit, alpha isozyme**						
rs2850328	Chiocco 2010 Gabrovska 2011	5' putative promoter region	N - genotyped, no association	0.358 (HM CEU)	0.477/1042	
rs149278688	Poirier 2003	5' putative promoter region	N	NA	0.013/29	0.04
rs45441997 **STR variation**	Chiocco 2010 Poirier 2003	5'UTR	**Y**-Chiocco N-Poirier	GGC9 (0.018) (NIDA CEU)	NA	CGG8 (0.28)
rs3730251	Poirier 2003	exon 2 A83A	N	0.008 (HM CEU)	0.050/108	
rs78927351	Poirier 2003	exon 10 L365L	N	0.008 (HM CEU)	0.005/11	
rs2851060	Chiocco 2012	intronic	N - genotyped, no association	0.23	0.397/868	
rs713455	Chiocco 2012	intronic	N - genotyped, no association	0.29	0.426/930	
rs7840	Chiocco 2011	3'UTR	N - genotyped, no association	0.5 (HM CEU - n=2)	0.126/274	
rs1395475	Liu 2005 Chiocco 2010	intronic	N - genotyped, no association	0.092 (HM CEU)	0.283/618	
rs45497591 STR variation	Chiocco 2010	intron 1	NT: novel report			
rs45625639 **STR variation**	Chiocco 2010	intron 3	NT: novel report			
rs2850965	He 2010a	intron 1	Y - Han Chinese, no functional studies	0.124 (HM CEU)	0.079/172	
PPP3CB **- protein phosphatase 3, catalytic subunit, beta isozyme**						
rs3729702	Poirier 2003	intron 7, +10	N	0.008 (HM CEU)	0.020/43	
rs41306522	Poirier 2003	3' UTR, +81	N	0.025 (HM CEU)	0.010/22	
rs3763679*	He 2010b, He 2011	intron 3	**Y** - Han Chinese, function studied	0.066 (HM CEU)	0.207/452	
PPP3R1 **- protein phosphatase 3, regulatory subunit B, alpha**						

Genbank accession no. NT022184-12#	Tang 2005	promoter 5bp deletion, possibly intron rs3039851	**Y**	0.076 (CEU Tang)	
rs4671887	He 2010	intron 1	Y - Han Chinese, no functional studies	0.442	0.275/601
rs1868402*	Cruchaga 2010	intron 5	**Y**	0.301 (HM CEU)	0.247/540
rs13009282*	NCBI dbSNP	coding - downstream missense mutation	NT	0.451(HM CEU)	0.173/379
rs2044693*	NCBI dbSNP	coding - downstream missense mutation	NT	0.467 (HM CEU)	0.277/604
***IL2*- interleukin 2**					
rs2069762*	various	5'UTR	**Y**	0.232 (HM CEU	0.246/538
rs2069763	various	synonymous, exon 1, Leu38Leu	**Y**	0.34 (CEU Geno)	0.344/752
rs6822844*	various	upstream	**Y**	0.146 (HM CEU)	0.067/147

no longer available, * (gray shading) SNPs included in PTLD case-control study. Y- association found; N- no association; NT- Not tested; HM- Hap Map; CEU- Caucasian European; NIDA- National Institute for Drug Abuse

Table 6 Sequencing work done to date concerning *FKBP1A*, PPIA, *PPP3CA*, *PPP3CB* and *PPP3R1*

Gene	Protein	Common name	Study	Sequencing work summary	Population
FKBP1A	FKBP12	FKBP506-binding protein	Xing 2006	exons amplified and sequenced	79 cases (left ventricular noncompaction)
PPIA	peptidylprolyl isomerase A	Cyclophilin A	An 2007	entire gene (with exception of small part near exon 1 due to high GC content) and putative promoter region	92 European American and 92 African American, representing extremes of distribution for rapid and slow progression to AIDS and HREU
			Palacin 2008	five exons with at least 10bp of the flanking intron regions, 250 bp of the promoter region	250 male patient swith MI, age less than 60 (Spanish)
			Moscoso-Solor-zano 2008	five exons, 290 bp of the promoter region	136 kidney transplant patients (Spanish)
PPP3CA	protein phosphatase 3, catalytic subunit, alpha isozyme	Calcineurin subunit A-alpha	Chiocco 2010	amplification and re-assembly over the *PPP3CA* locus (15 exons)	a sample from European American (597) and African Americans (324), NIDA cohort
			Poirier 2003	fragments of <300bp spanning each exon, as well as their promoter and the intronic sequences flanking exons	95 individuals from the Glasgow Heart Scan Study, with age-adjusted LVM was in the upper decile
PPP3CB	protein phosphatase 3, catalytic subunit, beta isozyme	Calcineurin subunit A-beta	Poirier 2003	as above	as above
			He 2010a, 2010b, 2011	no sequencing, three tagging SNPs in PP3CB (Han Chinese population)	
PPP3R1	protein phosphatase 3, regulatory subunit B, alpha	Calcineurin subunit B-alpha	Tang 2005	resequenced 5'flanking region	25 unelated subjects of mixed ethnicity

FKBP1A

Figure 13 *FKBP1A* gene: 53,392 bp. Exons are in black, 5' and 3' UTR are in white. 5' end is left, scale bar for 100 bp is in the top right corner.

All exons of *FKBP1A* were sequenced in a series of Japanese cases with myopathy (n=79) with no variants found (Xing et al., 2006). We sequenced the promoter and 5'UTR (636 bp) and a the start of exon 4 containing two variants with missense mutations reported without frequency and validation data in NCBI db SNP (584 bp) (n= 46 healthy subjects): no variants were found.

Table 7 Primers and experimental conditions used in sequencing relevant sections of *FKBP1A* (promoter, exon 4)

Region	Forward Primer	Reverse Primer	Hybridization Temperature
Promoter/5'UTR	CTGGATTCTGATTCCGTTGG	GTCTCCTGGGGAGATGGTTT	55
Exon 4	GAGACAGTTGGGGCATGTGT	TTTTTCCCCCTCTAGCTTCT	55

PPIA

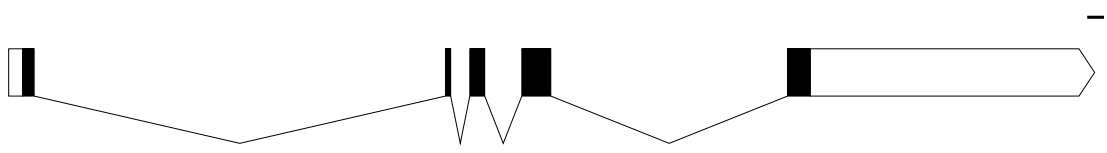

Figure 14 *PPIA* gene: 6,475 bp. Exons are in black, 5' and 3' UTR are in white. 5' end is left, scale bar for 100 bp is in the top right corner.

Sequencing that has included the promoter, coding and flanking regions has revealed most notably the promoter (*PPIA*-rs8177826) and a 5'UTR (*PPIA*-rs6850) polymorphisms (An et al., 2007; Moscoso- Solorzano et al., 2008; Palacín et al., 2008). A rare variant in exon 4, rs8177828 (synonymous) was found in a Spanish population, but was not tested in association analyses (Palacín et al., 2008). An et al. report eleven SNPs in *PPIA* after sequencing 92 African Americans (AA) and 92 European Americans (EA), five in the promoter region. Notably, they sequenced far upstream from the promoter: two SNPs farthest away from the transcription start site they exclude from analyses for failing HWE or rarity. The third, situated 408 bp up-stream from *PPIA*-rs8177826, they entitle SNP3 C>G, was a novel report, and to date does not have an accession number in dbSNP NCBI corresponding to the purported position. SNP3 and *PPIA*-rs8177826 were in perfect linkage disequilibrium (An et al., 2007).

No association was found with MI and neither *PPIA*-rs8177826 C>G nor *PPIA*-rs6850 A>G (Palacín et al., 2008). *PPIA* is part of the HIV intracellular life cycle and associations with HIV susceptibility (An et al., 2007; Rits et al., 2008), disease progression defined as time to CD4 <200 (An et al., 2007; Bleiber et al., 2005; Rits et al., 2008), time to AIDS clinical diseases (An et al., 2007; Rits et al., 2008) or viral RNA load above $10^{4.5}$ copies per ml (Rits et al., 2008) have either conflicted between studies, between sub-populations within studies (for example between Caucasians and Africans (An et al., 2007; Rits et al., 2008)) or were not significant depending on the phenotype. A Genome Wide Association study (GWAS) in a large cohort of HIV-1 infected Caucasians was designed to have sufficient power to detect 1.3% of variation in viral load at set point, and added previously identified polymorphisms derived from a variety of previous candidate gene/SNP studies, thus including *PPIA*-rs8177826 and *PPIA*-rs6850 (Fellay et al., 2009). This group failed to find an association with these SNPs under their stringent definition for significance. Furthermore, investigations in *ex vivo* models of viral replication did not uncover a significant genotype effect for neither *PPIA*-rs8177826 (Rits et al., 2008) nor *PPIA*-rs6850 (Bleiber et al., 2005; Rits et al., 2008), further negating that variations in cyclophilin-A expression may be ultimately significant to disease outcomes in HIV

infection. Moscoso- Solorzano et al. found genotypes with the *PPIA*-rs8177826 G allele to be present in a greater proportion of individuals with calcineurin inhibitor associated nephrotoxicity defined as serum creatinine >30mg/dL that responded to dose reduction.

In vitro functional exploration suggests *PPIA*-rs8177826 possibly has a functional consequence, while *PPIA*-rs6850 does not appear to do so. SNP3 in the upstream promoter has also been investigated, and is an interesting part of the story concerning *PPIA*-rs8177826, with which it exists in linkage disequilibrium. Mobility shift assays confirmed that SNP3 C>G and *PPIA*-rs8177826 C>G influenced protein binding with nuclear extracts from stimulated Th1 human T lymphocytes, minor alleles causing a 4-fold decrease or 2.8-fold increase respectfully in band densities. *In silico* analysis (using TESS software) predicted association with SP1, with loss of binding for the SNP3 G allele, and enhanced binding *PPIA*-rs8177826 G allele. No effect was found for *PPIA*-rs6850 G in either mobility shift or *in silico* analyses. Luciferase reporter assays have demonstrated higher activity for constructs with the *PPIA*-rs8177826 G allele (Moscoso- Solorzano et al., 2008; Palacín et al., 2008). However, considering cyclophilin-A mRNA levels in peripheral blood mononuclear cells, Rits et al. found significantly lower expression in rs8177826 G carriers. No difference in effect on cyclophilin-A mRNA levels was found between *PPIA*-rs6850 and its minor allele. Given the difference in the direction of the minor alleles of SNP3 and *PPIA*-rs8177826 in binding analyses, and the difference in direction of the effect observed between luciferase reporter assays and measured cyclophilin-A mRNA levels, it is difficult to draw any conclusions about the *in vivo* functionality of this SNP as a biomarker of activity.

PPP3CA - Calcineurin A-alpha

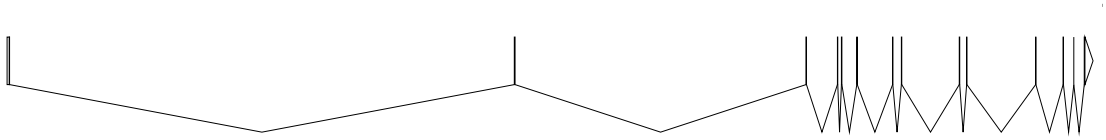

Figure 15 *PPP3CA* gene: 324,041bp. Exons are in black, 5' and 3' UTR are in white. 5' end is left, scale bar for 100 bp is in the top right corner (barely visible).

Fine mapping over the *PPP3CA* locus revealed a number of previously unreported short tandem repeat (microsatellite) variations and a promoter SNP in a large cohort of European and African Americans of elicit drug users, and non-user controls (Chiocco et al., 2010). The group was interested in this gene after *PPP3CA*-rs1395475 emerged as one of 38 SNPs in a large GWAS meeting reproducibility criteria for a significant association with substance abuse (Liu et al., 2005a, 2005b). The new variants and several other *PPP3CA* primarily intronic SNPs were genotyped in African American and European American abuser and control populations. A significant association was found for the promoter *PPP3CA*- rs45441997 but only amongst African Americans: carriers of the 9 GGC repeats were found more prevalent amongst drug abusers (after Bonferroni correction, allelic analyses). In the same direction, homozygous carriers of 10 GGC repeats were found to be more frequent amongst controls (this finding is short of significance following Bonferroni correction, genotype analyses). Subsequent investigations uncovered a significant difference in *PPP3CA* cDNA expression in 13 medial temporal gyrus samples between carriers of 8 vs 10 GGC repeats. Earlier sequencing work did not find any associations with cardiac hypertrophy and dilated cardiomyopathy, however this group report additional noteworthy rare SNPs (MAF 2-3% in a French population): one in the promoter (*PPP3CA*-rs149278688) and two synonymous coding SNPs (*PPP3CA*-rs3730251, *PPP3CA*-rs78927351) (Poirier et al., 2003) (Table 4.1). Finally, the promoter *PPP3CA*-rs2850328 was tested for association with aggressive

breast cancer in a candidate gene study: no association was found amongst 182 breast cancer cases and 180 controls (Gabrovska et al., 2011).

PPP3CB - Calcineurin A-beta

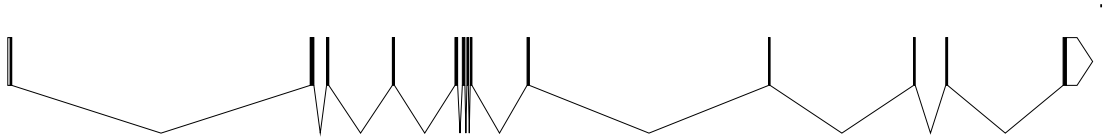

Figure 16 *PPP3CB* gene: 59,219 bp. Exons are in black, 5' and 3' UTR are in white. 5' end is left, scale bar for 100 bp is in the top right corner (barely visible).

Sequencing work around the promoter, untranslated and flanking regions revealed just two rare variants in a French population: these did not associate with cardiac hypertrophy or dilated cardiomyopathy (Poirier et al., 2003).

He et al. report a number of associations between variants in the five calcineurin sub-unit genes with a number of 'endurance' phenotypes (He et al., 2010a), cardiac responsiveness to training (He et al., 2010b) and athletic status (He et al., 2011) in Han Chinese cohorts. A panel of 55 SNPs, all located within introns, untranslated or 3' regions, with a MAF>10% were selected from HapMap (Han Chinese). Limiting our focus to outcomes in *PPP3CA*, *PPP3CB* and *PPP3R1*, four SNPs were found to be significantly associated with at least one of the three phenotypes (Table 4.1). However, without functional effect testing or replication, *PPP3CA*-rs2850965 and *PPP3R1*-rs4671887 (He et al., 2011) failed our updated selection criteria. Luciferase reporter assays were performed for *PPP3CA*- rs3804358 G>C and *PPP3CB*-rs3763679 C>T, both in intron 3 of their respective genes: for the former the minor allele conferred significantly greater luciferase activity, while for the latter significantly lower activity was found. These activities go in the same direction as the associations found in Chinese athletes whereby a greater proportion of mixed-sex elite athletes were carriers of the *PPP3CA*-rs3804358C greater activity allele and a lower proportion were carriers of the *PPP3CB*-rs3763679 T lower

activity. Only *PPP3CB*-rs3763679 is polymorphic in Europeans and was hence retained in our selection. The observed findings were not replicated in a male Spanish Caucasian elite athlete and control cohort (He et al., 2011).

PPP3R1 - Calcineurin B-alpha

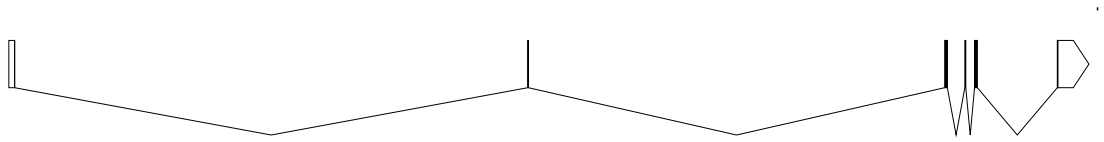

Figure 17 *PPP3R1* gene: 73,662 bp. Exons are in black, 5' and 3' UTR are in white. 5' end is left, scale bar for 100 bp is in the top right corner (barely visible).

Tang et al. sequenced the 5' flanking region of *PPP3R1* and report a novel insertion (-TTAAA-) /deletion (Tang et al., 2005). An association was found between the deletion and the presence of inappropriately high left ventricular mass, but not left ventricular hypertrophy in African Americans only. Although in isolation this association is not convincing of a functional consequence, *in silico* analyses of the alternative sequences using the TRANSFAC transcription factor database and the program MatInspector predicted that the deletion eliminated a consensus Nkx-2 transcription factor binding site. Others have reported significant or near significant associations with an 'endurance' athlete phenotype in studies with questionable methodologies (Ahmetov et al., 2009). Initially this SNP could not be located on NCBI db SNP using the reported Genbank accession no. NT022184-12. However, we recently noted that this variant appears to correspond to *PPP3R1*-rs3039851 -TTAAT-/deletion, which is located in intron 1, which, although not -1059 to -1063 bp relative to the transcription start site is located this distance away from a motif that could be taken to be a start site.

In a candidate-gene endophenotype approach, Cruchaga et al. found a strong association between *PPP3R1*-rs1868402 in intron 5 and phosphorylation of tau protein (CSF ptau181), a biomarker for Alzheimer's disease (Cruchaga et al., 2010).

The genes of 34 proteins known to be involved in tau phosphorylation were considered as candidates, including *PPP3CA* and *PPP3R1*. A tag SNP approach was used to screen the candidate genes (based on HapMap, Caucasian), affording a total of 384 SNPs (355 passing quality control). This SNP was the only one to pass replication in subsequent population data sets. It associated with Alzheimer disease progression, but not risk or age of onset: bibliographic data was discussed to provide mechanistic support for this. Gene expression studies (mRNA extracts from the parietal lobe of AD cases) confirmed lower expression of *PPP3R1* in carriers of the minor allele.

Two additional common SNPs (MAF>10%) from db SNP in NCBI, whose variants potentially give rise to downstream missense sequences were also included: *PPP3R1*-rs13009282, *PPP3R1*-rs2044693.

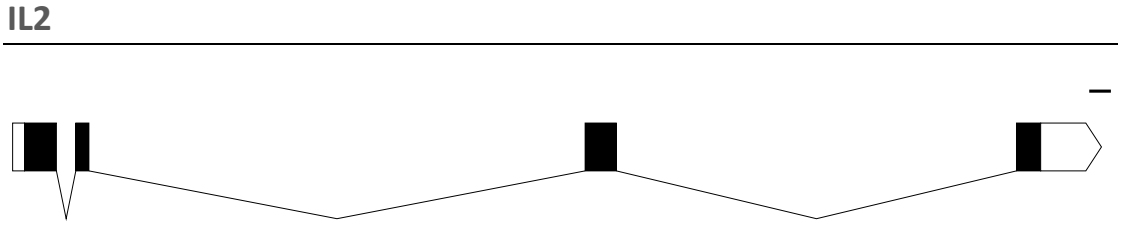

Figure 18 *IL2* gene: 5,025 bp. Exons are in black, 5' and 3' UTR are in white. 5' end is left, scale bar for 100 bp is in the top right corner.

There are only two common SNPs in *IL2*, *IL2*-rs2069762 T>G (often designated *IL2* -330) and exon 1 *IL2*-rs2069763 T>G, (synonymous, receiving a variety of designations). Both have been tested widely and found to associate with a number of outcomes, however phenotypes have varied across the board. Concerning direct measures of activity, greater expression was found for the minor allele *IL2*-rs2069762G in a luciferase reporter assay (Matesanz et al., 2004). However, the same group found lower *IL2* mRNA expression from activated peripheral blood lymphocytes in GG carriers compared to GT or TT. Others have found greater IL-2

production (measured by ELISA) in *ex vivo* cultured PBLs from GG carriers following stimulation (Hoffmann et al., 2001): the widely referred to phenotype pertains to this work. *In silico* work suggests that the minor allele G of *IL2*-rs2069762 would reduce the binding affinity at a putative transcription-factor binding site for two transcription factors (TCF-11 and MafG). Similar work suggests that the minor allele *IL2*-rs2069763T would influence binding of the splicing factors SC35, SRp40 and SRp55 in a way that for certain factors binding is enhanced (SRp55), and for others it is eliminated or decreased (SC35, SRp40): the final consequence is difficult to conclude (Christensen et al., 2005).

Although not strictly in the *IL2* locus, *IL2*-rs6822844 lies far upstream of *IL2* and downstream of *IL12*. A GWAS hit in a type 1 diabetes association study led to fine sequencing of the region in a very large cohort, but no obvious coding, splice or regulatory variants emerged as causative (Burton et al., 2007; Todd et al., 2007). The SNP appeared in the region 4q27, within a gene cluster containing four genes: *KIAA1109*, a protein of unknown function with ubiquitous expression; *TENR*, testis nuclear RNA-binding protein, primarily expressed in the testis; *IL2*; and *IL21*. The region exhibits a high degree of linkage disequilibrium in Caucasians. *IL2* and *IL21* exist in separate clusters in Han Chinese individuals (Shi et al., 2011). Subsequent tagging SNP approaches in a variety of autoimmune/inflammatory conditions implicate *IL2*-rs6822844, where the minor allele appears to confer protection for these conditions (Table 8). Between these studies a variety of additional tagging SNPs were often included, some resulting in significant findings. However, *IL2*-rs6822844 was almost invariably significant, and often shown to be in linkage disequilibrium with other significantly associated SNPs (Table 9). We included this SNP in preference of the other SNPs reported in Table 9 for the reason that it was most frequently associated significantly, and if not functionally significant, appears to serve as a consistent marker.

Table 8 Disease states and associations reported concerning *IL2*-rs6822844

Inflammatory/Autoimmune Disease	Association with rs6822844		
	Significant	Trend	Not Significant
type 1 diabetes mellitus	(Maiti et al., 2010; Zhernakova et al., 2007)	(Espino-Paisán et al., 2011)	
rheumatoid arthritis	(Daha et al., 2009; Hollis-Moffatt et al., 2010a; Maiti et al., 2010; Zhernakova et al., 2007)	(Teixeira et al., 2009)	
psoriasis	(Liu et al., 2008; Warren et al., 2011)		
psoriatic arthritis	(Liu et al., 2008)		
coeliac disease	(Adamovic et al., 2008)		(Maiti et al., 2010)
ulcerative colitis	(Festen et al., 2009; Glas et al., 2009; Márquez et al., 2009)		(Hollis-Moffatt et al., 2010b)
Chron's disease	(Hollis-Moffatt et al., 2010b; Márquez et al., 2009)		(Maiti et al., 2010)
irritable bowel disease	(Márquez et al., 2009)		(Maiti et al., 2010)
juvenile idiopathic arthritis	(Albers et al., 2009)		
multiple sclerosis	(Cavanillas et al., 2010)		(Fedetz et al., 2009)
systemic lupus erythmatosis	(Maiti et al., 2010)		
Sjörgen's syndrome	(Maiti et al., 2010)		
primary sclerosing cholangitis	(Janse et al., 2011)		

Table 9 Summary of genotyping work in 4q27 region, implicating *IL2*-rs6822844 in associations with autoimmune/inflammatory conditions

1. (Todd et al., 2007)
2. (Van Heel et al., 2007)
3. (Zhernakova et al., 2007)
4. (Adamovic et al., 2008)
5. (Liu et al., 2008)
6. (Glas et al., 2009)
7. (Festen et al., 2009)
8. (Márquez et al., 2009)
9. (Daha et al., 2009)
10. (Albers et al., 2009)
11. (Teixeira et al., 2009)
12. (Hollis-Moffatt et al., 2010a)
13. (Hollis-Moffatt et al., 2010b)
14. (Tindall et al., 2010)
15. (Cavanillas et al., 2010)
16. (Maiti et al., 2010)
17. (Espino-Paisán et al., 2011)
18. (Warren et al., 2011)
19. (Stallhofer et al., 2011)
20. (Fedetz et al., 2009)
21. (Janse et al., 2011)

4q27 SNPs Accession No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
rs11732095			*								*										
rs11938795								sig UC							NS						
rs12642902						NS															
rs13119723		sig		sig		sig	sig							sig					sig		
rs13151961		sig			sig	NS	sig												sig		
rs13277113																					
rs1398553			*								*										
rs17388568	sig							NS				NS			NS		trend				
rs2055979																					
rs2069762																				NS	
rs2069763																					
rs2069778																		sig			
rs2736340																					
rs3136534	sig																			NS	
rs4492018			*								*										
rs4505848			*								NS(B)										
rs4833248																					
rs6534347																					
rs6822844		sig	sig	sig	sig	sig	sig	sig CD, sig UC	sig	sig	trend	sig	sig CD NS UC		sig trend	sig	trend	sig	sig	NS	sig
rs6836189	sig																				
rs6840978		sig			sig	sig	sig												sig		
rs907715												NS									

PTLD: A case-control study to determine pharmacological and pharmacogenetic risk factors of Post-transplant Lymphoproliferative Disease

Previous work addressing risk factors for PTLD have been based on data from large registries, lacking the detail required to address the role of individual maintenance immunosuppressant drugs, taken over time. Studies looking at genetic risk factors for PTLD have focused on cytokine gene polymorphisms, but have been limited by small size, heterogeneous case/control populations, or both. The primary aim of this work was to determine the effect of candidate genetic polymorphisms, selected for their potential effect on the response to calcineurin inhibitors, on the occurrence of post-transplant lymphoproliferative diseases. The secondary objectives were to explore the risks of individual immunosuppressant drugs, of their combinations and of surrogate measures of drug exposure.

Methods

Fifteen French transplant centres performing kidney transplantation participated in the present study. The primary source for identifying cases was the French PTLD Registry whose inclusion criteria and population have been previously described (Caillard et al., 2006). Briefly, all 35 kidney transplant centres in France participated in completing the registry, prospectively reporting histologically confirmed or strongly clinically suspected cases of PTLD in adults over the age of 18 years, during the period January 1, 1998 to December 31, 2007. Additional cases, primarily those occurring outside the inclusion period of the registry, were identified via CRISTAL, the French transplant database, and via the participating centres. PTLD occurring during graft patency (prior to graft loss) was considered. Cases were adults

over the age of 18 years at the time of PTLD diagnosis, thus may have been younger at the time of transplant.

The study population was restricted to individuals receiving a kidney-only transplant, with no previous non-kidney transplant and with no HIV infection. Inclusion into the present study was limited to individuals receiving transplants after January 1st 1990, who continued at the same transplant centre during the entire follow up period. Controls were considered only if they were living during the enrolment period and had maintained their kidney graft for at least 3 months.

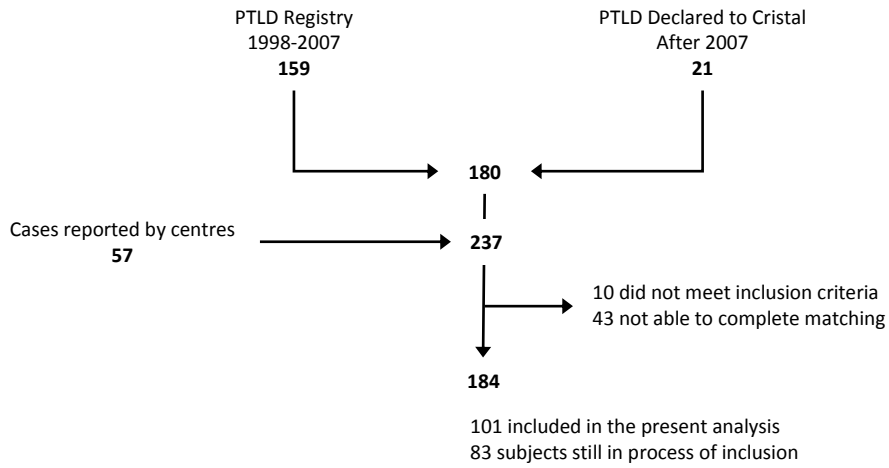

Figure 16 Inclusions into the PTLD case-control study

Cases and controls were matched one-to-one on: transplant centre; sex; age at transplant (+/- 5 years); year of transplant (+/- 1 year); graft order (ie. matched on whether it was the 1st, 2nd or 3rd transplant); and pre-transplant EBV status. Of note is that EBV status was not systematically recorded in CRISTAL for transplants prior to 1997, and this information was sought from centres. Cases with missing EBV statuses were excluded; potential controls with missing EBV statuses were likewise excluded. To approximate time-to-event analysis, controls were selected to have the same time-length of immunosuppression as the period transplant-to-PTLD in the case. In other

words, controls were selected if their graft was patent over this period. All effort was made to match on the same transplant centre, however this was not possible for a small set of cases, maintaining respect for all other matching criteria (n=1 pair in the present analysis). The strategy taken was to complete the match on all criteria less transplant centre, and target inclusions at the centre with the most candidates. All participants gave informed consent to participate in the study. PTLD cases that were deceased at the debut of the study were included if they had a biological sample stored in a dedicated bio-bank for which prior consent had been granted. The institutional review board of the Limoges University Hospital approved all data collection, sample collection and analyses.

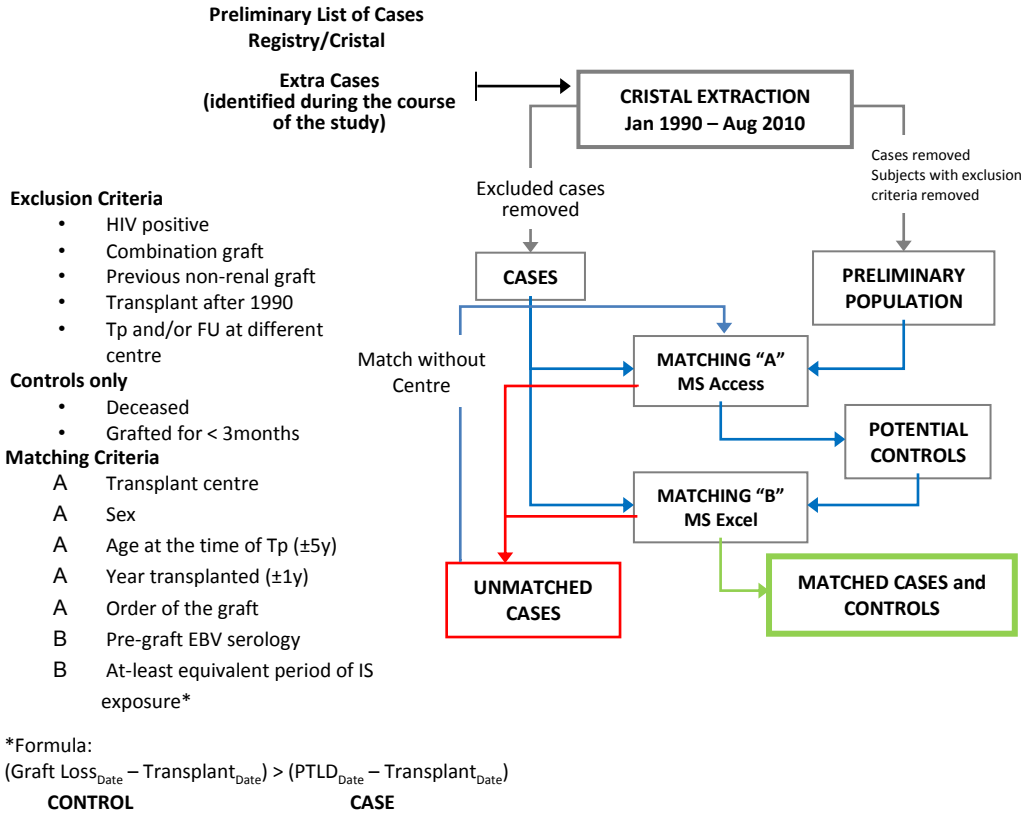

Figure 19 Schematic depicting the matching strategy

The primary source of data necessary for matching was CRISTAL, with supplements from the PTLD registry and records at individual centres (primarily for pre-transplant EBV status). A CRISTAL data base extract for individuals transplanted

between 1990 to the day of extraction (13/08/2010) was obtained (pre-graft and follow-up data for graft receivers), with consent from representative nephrologist collaborators for each centre. Clinical research assistants collected data on-site at each transplant centre from individual medical records (S. Alcolea, Clinical Investigation Centre, CIC Limoges, for Amiens, Bordeaux, Caen, Nancy, Poitiers, Reims, Rennes, Rouen, Saint-Etienne, Tours; S. Ponsard, Nephrology and Pharmacology departments, CHU Limoges for Limoges, Toulouse). For each subject, longitudinal data were collected with respect to all immunosuppressant drugs used in terms of brand name and formulation (day 15 post-transplant, months 1, 3,6, six-monthly until year 5, and yearly thereafter). Where available, dose was recorded (daily dose in mg) as well as blood levels for the calcineurin inhibitors (trough level, C0, for both cyclosporine and tacrolimus, and/or the level at 2 hours post dose, C2, for cyclosporine). Missing doses were imputed by carrying forward the last recorded dose. In addition, data were collected concerning all treated acute rejection episodes (suspected and confirmed), CMV infections and their treatment, lymphoma characteristics and confirmation of pre-transplant EBV and CMV serologies. All data corresponded to the transplant preceding PTLD in the cases and the corresponding period, and same graft in question (ie. 1st, 2nd or 3rd) in controls.

DNA extraction from whole blood, serum and non-malignant tissues contained in non-degrading fixatives was performed using QIAamp® DNA Midi/Maxi kits (Qiagen). Genetic variants related to the calcineurin-signaling pathway were selected from literature reports (full rationale described above). Additional variants in cytokine genes with previous associations with PTLD were also included (Table 1, indicted by an asterisk). Of note is that Genotyping was performed using TaqMan® SNP genotyping assays (Applied Biosystems). Proprietary assays were used for a majority of variants; the assay for *PPIA*-rs8177826 was designed using the Custom TaqMan® Assay Design Tool, and passed Applied Biosystem's quality control procedures. Assay IDs and context sequences for primers are detailed in Appendix 2 (Table 19). Assays for four cytokine SNPs failed and are thus candidates for sequencing when the cohort is completed (Appendix 2, Table 20). The QIAgility® instrument was used to automate distribution of PCR reactants, which together with the Rota-Gene Q®

thermocycler (both Qiagen) permitted reducing the reaction volume by half compared to alternative thermocycler systems (7µl). The final reaction contained TaqMan® genotyping master mix (Applied Biosystems); DNA diluted to 2ng/µl; TaqMan® SNP genotyping assay and Type-it® Fast SNP Probe PCR Kit (Qiagen), the latter used to reduce thermocycling times and enhance allelic discrimination.

The degree of immunosuppression in individuals would be expected to be a potential confounding factor for genetic associations to PTLD, and ideally analyses would be adjusted for this. Due to there being no standard measure for degree of immunosuppression implemented widely in routine clinical practice, we purposed a score (IS score) to reflect total immunosuppression over the entire follow up period. The strategy for this is detailed in Appendix 3.

The Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) was used to estimate the minor allele frequency (MAF) and case sample size required for detecting an association between a single variant and PTLD at 80% power and at an alpha-value of 0.05. PTLD prevalence was estimated at 0.02 given a five-year cumulative incidence of 1.18% in the French renal-transplant cohort (Caillard et al., 2006). A MAF of 0.1 was estimated to detect an association, using an allelic analysis, in approximately 150 cases, matched 1-to-1. Hardy-Weinberg equilibrium was tested by Fisher's exact test using the package 'SNPassoc' in R. Each polymorphism was tested for association with PTLD using logistic regression under appropriate models for the mode of inheritance (recessive, dominant, additive, multiplicative), likewise using 'SNPassoc'. Linkage disequilibrium was tested for all polymorphisms within the same candidate gene or nearby region using the R package 'genetics'. Haplotypes were inferred and tested for association with PTLD where relevant.

The primary risk factors for PTLD are pre-transplant EBV sero-negativity and polyclonal depleting agents for early PTLD, and increasing age and calcineurin

inhibitor use for late PTLD. EBV status and age were accounted for by the matching strategy. The covariates considered in univariate analyses were: maintenance immunosuppressive drugs, considered as a score and ever-taken; induction agents, IL-2 receptor blockers (IL2RB) and polyclonal depleting agents, considered as ever-taken; suspected and confirmed acute rejection episodes (yes/no); treatment of acute rejection with T cell depleting agents (yes/no); suspected and confirmed CMV infections (yes/no). All covariates with an alpha-value less than 0.05 were included in an intermediate multivariable model. Those with $p<0.2$ in the intermediate model were included in the final model. Backwards-stepwise regression based on the likelihood ratio test was performed using the package 'epicalc' in R. The selection of the final model was confirmed by internal validation using bootstrap resampling (package 'bootStepAIC').

Results

A total of 101 case-control pairs were available for the present analysis. Inclusions into the study continue and will be the subject of future analyses, and validation of the findings presented here. Definitions of early PTLD vary in the literature (1 to 2 years post transplant). Here, early PTLD was considered as occurring in the first post-transplant year, as suggested by the frequency histogram for time to PTLD (Figure 20).

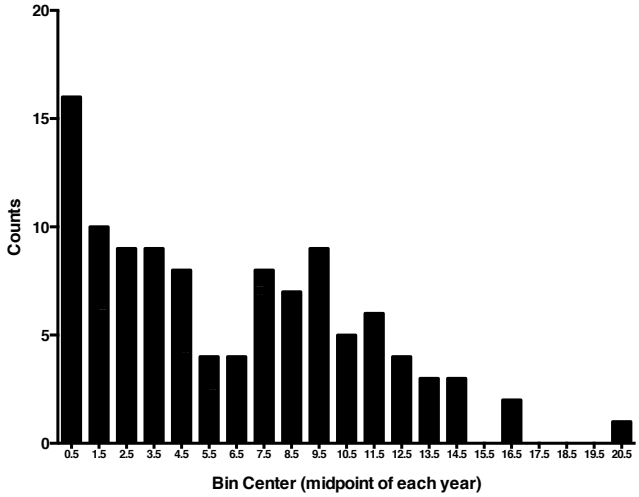

Figure 20 Frequency distribution of time-to-PTLD in the cohort of included cases

Cases complying with initial inclusion criteria (kidney-only transplant, follow-up at one centre) but who were missing data for matching (primarily EBV status); missing a biosample for genotyping or had an incomplete or missing medical record are compared here with included and matched cases (Table 10). Excluded cases had similar demographic characteristics to included cases: similar sex ratio of roughly two-thirds male, similar median age at transplantation of approximately 50 years. This is comparable to cases from the complete French cohort (Caillard et al., 2012). A major reason for non-inclusion was missing pre-transplant EBV status. The greater proportion of early PTLD amongst the excluded cases (37% vs. 16%) suggests the possibility that a greater proportion of unknown statuses reflect EBV-negative individuals. Another major reason for non-inclusion was lack of suitable sample for DNA extraction and genotyping. Accordingly, a greater proportion of deceased cases is observed amongst excluded cases (33% vs. 67%). This is important to note with respect to association tests with genetic variants as case representatives of the phenotype 'PTLD' disproportionately represent survivors, thus the phenotype may be skewed towards 'kidney transplant recipients who developed PTLD and survived'. Mortality in the French cohort is estimated at 61% at five years post-diagnosis (Caillard et al., 2006). Finally, a greater proportion of excluded cases were transplanted at an earlier period of transplantation (median year of transplantation 1994 vs. 1999, excluded and included cases, respectively), reflecting that EBV status

was often missing prior to 1997 and consistent with a greater mortality amongst cases diagnosed in the early 1990s, looking back retrospectively from the present.

Table 10 Characteristics of matched and non-matched PTLD cases

	Cases	Non-matched Cases
Total	101	43
Median age at transplant years (range)	47.9 (19.9 - 78.7)	51.3 (27.7 - 69.6)
Median year of transplant (range)	1999 (1990 - 2009)	1994 (1990 - 2009)
Sex (%)		
Male	65 (64)	24 (56)
Female	36 (36)	17 (40)
Unknown	-	2 (5)
EBV status (%)		
Positive	94 (93)	15 (35)
Negative	7 (7)	3 (7)
Unknown	-	25 (58)
Median age at PTLD diagnosis, years (range)	53.2 (23.9 - 82.32)	57.1 (30.5 - 76.14)
Median interval transplant to PTLD diagnosis, years (range)	5.2 (0.1 - 20.2)	2.76 (0.08 - 19.2)
Early PTLD | Late PTLD (%)		
Early PTLD	16 (16)	16 (37)
Late PTLD	85 (84)	19 (44)
Unknown (date of diagnostic missing)	-	8 (19)
Deceased (%)	33 (32)	30 (70)

Table 11 Case characteristics: Pre-transplant EBV sero-status, graft order (both identical in controls) and Lymphoma types

Cases (n=101)	
EBV sero-status Early PTLD	**n (% of Early PTLD)**
Positive	12 (75)
Negative	4 (25)
EBV sero-status Late PTLD	**n (% of Late PTLD)**
Positive	82 (96)
Negative	3 (4)
Order of graft	**n (% of total)**
1st	94 (93)
2nd	6 (6)
3rd	1 (1)
Lymphoma type	
Unspecified	16
Lymphoid hyperplasia	2
Polymorphic	17
Monomorphic:	
DLBCL	19
Burkitt	4
Hodgkin/Hodgkin like	8
NHL - Small Cell	2
T cell	2
MALT	5
NHL - unspecified	26

Lymphoma types were not discriminated at study entry and a vast heterogeneity is observed (Table 11). Hodgkins lymphoma (Caillard et al., 2011) and MALT have an increased incidence amongst the immunosuppressed. Burkitt's lymphoma is likewise dependent on immune factors, and behaves more aggressively in the immunosuppressed (Picarsic et al., 2011). Polymorphic lesions have been argued to be the same disease as frank Non-Hodgkin lymphoma, caught at an earlier stage (Vakiani et al., 2008). For these reasons, all cases were included despite heterogeneity on the common grounds of representing lymphoproliferative disease arising primarily due to suppressed immunity.

Categorical variables amongst the matching criteria (transplant centre, sex, pre-transplant EBV status, graft order) were identically distributed in controls (Table 11 for cases). Continuous variables (age at transplant, year of transplant) were

comparable (Table 12). A notable difference is that all controls were living at the time of inclusion while 32% of cases were deceased.

Table 12 Cases and Controls: descriptive statistics

	Cases	Controls
Total	101	101
Continuous variables in the matching criteria		
Median age at transplant years (range)	47.9 (19.9 - 78.7)	46.8 (20.6 - 75.8)
Median year of transplant (range)	1999 (1990 - 2009)	1999 (1990 - 2008)
Immunosuppression (ever-taken)		
Class		
Calcineurin Inhibitor	99	98
Calcineurin inhibitor switch	19	14
Antimetabolite	97	97
Antimetabolite switch	14	12
mTOR Inhibitor	12	12
Individual maintenance Immunosuppressant drugs (ever-taken)		
Cyclosporine	76	72
Tacrolimus	42	40
Azathioprine	51	37
Mycophenolate	60	72
Everolimus	3	4
Sirolimus	9	8
Corticosteroid	98	100
Belatacept	2	1
Leflunomide	1	1
Antibody Induction (yes/no)		
IL2 receptor blocker	23	29
Polyclonal Depleting Agents	47	49
Events (yes/no)		
CMV Infection	23	36
Acute Rejection	27	29
Rejection Treatment: T cell depleting agents	5	5

There were no significant differences in immunosuppression scores between cases and controls with respect to PTLD risk (Table 13). A composite score (Composite 1) was calculated for each visit by adding the scores for the CNI group, antimetabolite group, corticosteroid group and other. CNIs were considered by dose or by C0, giving rise to two composite scores. A second composite score (Composite 2) was calculated similarly considering corticosteroids as taken yes/no (1/0), the reason being the likely lower impact of corticosteroids to the final outcome, and also the high degree of missing doses that impacted the total score. Distributions of scores were explored visually (Figure 21) and appeared balanced between cases and controls

Table 13 The impact of immunosuppressant scores on PTLD risk

Normalised AUC	OR	CI	P (LR-test)	No of obs	No of events
Antimetabolites	1.14	0.7-1.88	0.59	198	100
CNIs (dose)	1.25	0.68-2.31	0.46	197	99
CNIs (C0)	0.68	0.43-1.08	0.09	191	95
Corticosteroids	1.58	0.82-3.02	1.58	179	90
Composite score 1 (with CNI dose)	0.91	0.77-1.07	0.24	190	95
Composite score 1 (with CNI C0)	0.9	0.76-1.07	0.24	171	86
Composite score 2 (with CNI dose)	1.08	0.76-1.55	0.66	197	99
Composite score 2 (with CNI C0)	1.11	0.7-1.78	0.65	179	90

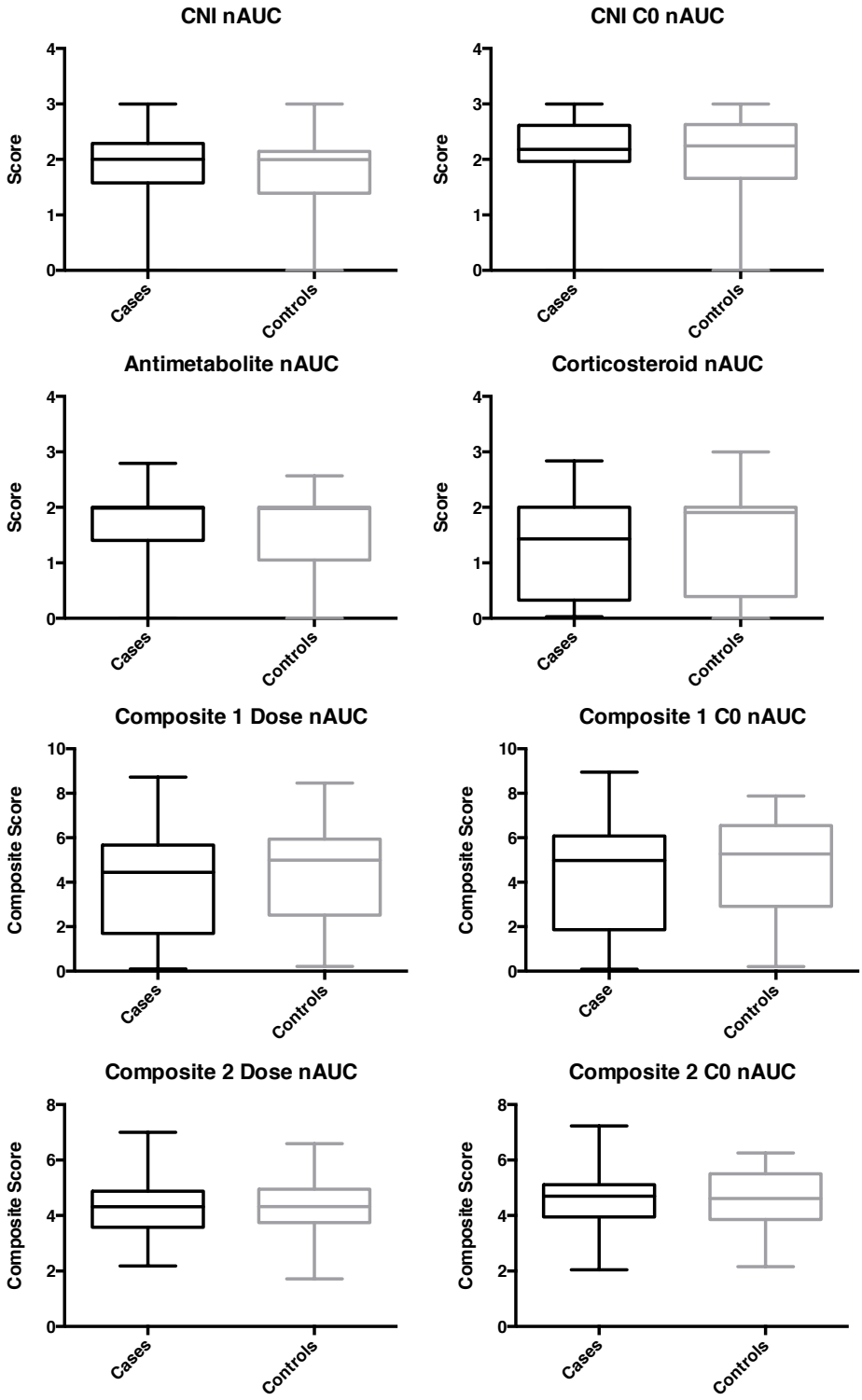

Figure 21 comparison of the distribution of the normalized AUC (nAUC) scores in cases and controls. Composite score 1 includes the corticosteroid nAUC, while composite score 2 only considers corticosteroids as a dichotomic variable (1/0).

All genetic variants were consistent with Hardy-Weinberg equilibrium (Appendix 2). For each variant, the genetic model with the lowest value Akaike Information Criterion (AIC) is displayed in Table 13: variants under the corresponding genetic-model that were included in the intermediate risk-model are indicated with an asterisk. Minor allele frequencies for *PPIA*-rs8177826 and *PPP3CB*-rs3763679 were below 10%, therefore lack of association due to insufficient power cannot be discounted. Furthermore, for the cyclophilin SNP, *PPIA*-rs8177826, this precluded further testing in a sub-group of cyclosporine users (n=60 pairs, considering cyclosporine as ever-taken). Of note, there were no homozygous carriers of the minor allele for this variant hence the odds ratio corresponds to GG vs. GC carriers. Linkage disequilibrium was tested for *IL2* and *IL10* variants, and haplotype analyses were warranted for the *IL10* variants. These were not found to associate with PTLD in allelic analyses.

Table 14 Univariate analyses for PTLD risk concerning cytokine and calcineurin inhibitor related single nucleotide polymorphisms: most likely genetic model presented.

Gene	rs Accession #	PM	MAF	Model	Genotype tested	OR	Confidence Interval	p-value
IL10	rs1800871	C>T	0.25	Recessive	CC-CT TT	1 1.33	(0.5 - 3.52)	0.562
IL10	rs1800872	C>A	0.25	Dominant	CC CA-AA	1 0.79	(0.46 - 1.38)	0.416
IL10	rs1800896	A>G	0.46	Recessive	AA-GA GG	1 2.8	(1.38 - 5.68)	0.003*
TGFB1	rs1800470	T>C	0.38	Multiplicative (allelic)	C vs T	1.64	(1.1 - 2.44)	0.014*
TNF	rs1799964	T>C	0.25	Recessive	TT-TC CC	1 0.83	(0.29 - 2.38)	0.726
TNF	rs1800629	G>A	0.11	Dominant	GG GA-AA	1 0.69	(0.35 - 1.37)	0.289
TNFRSF1A	rs4149570	G>T	0.39	Multiplicative (allelic)	T vs G	1.41	(0.93 - 2.12)	0.103
IL2	rs2069762	T>G	0.26	Multiplicative (allelic)	G vs T	1.59	(1.03 - 2.47)	0.034*
IL2	rs6822844	G>T	0.13	Recessive	GG-TG TT	1 2.08	(0.19 - 23.29)	0.541
PPIA	rs8177826	C>G	0.07	-	GG CG	1 0.58	(0.26 - 1.29)	0.177
PPP3CB	rs3763679	C>T	0.05	Recessive	CC-TC TT	1 0		0.495
PPP3R1	rs13009282	T>C	0.24	Dominant	TT TC-CC	1 0.72	(0.4 - 1.29)	0.271
PPP3R1	rs2044693	C>T	0.34	Dominant	CC CT-TT	1 0.69	(0.4 - 1.2)	0.190
PPP3R1	rs1868402	T>C	0.28	Dominant	TT CT -TT	1 0.45	(0.26 - 0.78)	0.004*

Table 15 Univariate analyses for PTLD risk

	OR	95%CI	P(LR-test)
All cases n=101 pairs			
Azathioprine ever-taken	3.8	1.42 - 10.18	0.003*
Mycophenolic acid ever-taken	0.33	0.13 - 0.84	0.012*
Induction: IL2RB	0.67	0.32 - 1.38	0.272
Induction: Polyclonal Depleting Agents	0.87	0.41 - 1.82	0.705
CMV Infection	1.93	1.01 - 3.68	0.041*
Acute Rejection	0.9	0.49 - 1.68	0.752
Rejection Treatment: T cell depleting agents	1.2	0.36 - 3.96	0.763

Use of T cell depleting agents, a known risk factor but particular for early PTLD, was not found to influence PTLD risk in univariate analyses (Table 14). Sub-group analyses in early PTLD likewise showed no association, but this analysis was liable to being underpowered (n=16 case-control pairs, data not shown). Azathioprine and mycophenolate, considered as ever-taken, were associated with an increased and decreased risk of PTLD respectively. The effect was of similar amplitude in either direction depending on the drug, thus it could not be dissociated whether use of either drug, non-use, or an independent effect by both drugs in opposite directions (an increased risk for azathioprine and protective effect for mycophenolate) was responsible, given that both are of the same class and only one of the two is ever taken at a time, and that virtually all subjects received an antimetabolite.

Table 15 displays use of immunosuppressants as taken immediately post-transplantation (at discharge) and taken at PTLD diagnostic (with the corresponding period in controls). Further information concerning the antimetabolites is presented in Table 16. Notwithstanding that distributions for each variable/scenario cannot be tested directly in a matched case-control design (by Chi-squared test or similar) given that each pair contributes a single stratum associated with the confounders in the matching criteria (Mantel and Haenzel, 1959), interesting trends can be observed, and serve to illustrate an effect related to azathioprine rather than mycophenolate. Amongst azathioprine treated subjects, it is notable that there is a higher distribution of cases in the scenarios drug taken at PTLD diagnostic; drug taken from start to PTLD diagnostic and antimetabolite switch from mycophenolate to azathioprine. In

all scenarios, the distribution of cases is approximately double controls. Furthermore, there is no notable difference in distributions for an azathioprine-to-mycophenolate switch, consistent with there being no protective effect by mycophenolate. Consequently, only azathioprine use was tested as a covariate in multivariate analyses.

Table 16 Maintenance Immunosuppressants taken at discharge post-graft and at PTLD diagnostic (with the corresponding time point in controls)

	Cases	Controls
Total	101	101
Immunosuppressant at discharge		
Cyclosporine	65	70
Tacrolimus	25	22
Azathioprine	39	31
Mycophenolate	48	57
Immunosuppressant at PTLD diagnostic*		
Cyclosporine	56	57
Tacrolimus	40	36
Azathioprine	29	14
Mycophenolate	49	66
Combination therapy at PTLD diagnostic*		
CNI protocol		
CsA + AZA	17	9
CsA + MPA	25	37
TAC + AZA	12	5
TAC + MPA	20	25
CSA	13	12
TAC	8	6
CSA + EVR	1	0
Other		
belatacept protocol	2	1
SRL + MMF	1	3
SRL	0	1
MMF	3	1

***corresponding time-point for controls**

Table 17 Antimetabolite: exploration of switching and stopping during post-transplant follow-up period

	Azathioprine		Mycophenolate	
	Cases	Controls	Cases	Controls
Ever-taken	51	37	60	72
At discharge	39	31	48	57
At PTLD diagnosis	**29**	**14**	49	66
Both at start and finish	**17**	**8**	37	51
switched to alternative antimetabolite	12	15	**11**	**6**
stopped antimetabolite	10	8	0	0

Table 18 Multivariate analysis for PTLD risk

	Adjusted OR	95% CI	P value (log-ratio test)
All cases n=96 pairs, 5 pairs excluded due to missing genotyping data			
Azathioprine ever-taken	4.56	1.45 - 14.32	0.004
IL2 promoter rs2069762 G vs T	1.89	1.03 - 3.48	0.031
IL10 promoter rs1800896 AA-AG vs GG	0.33	0.14 - 0.78	0.007
Late PTLD n=82 pairs, 3 pairs excluded due to missing genotyping data			
Azathioprine ever-taken	4.50	1.43 - 14.14	0.0101
IL2 promoter rs2069762 G vs T	2.09	1.07 - 4.08	0.0297
IL10 promoter rs1800896 AA-AG vs GG	0.37	0.14 - 0.97	0.0441

Under the rare disease assumption, the odds ratio approximates relative risk. The final multivariable model estimates a four-fold increased risk of PTLD due to azathioprine use, an almost doubled risk for minor allele carriers of the IL2 promoter polymorphism, and a 3-fold protective effect of GG carriers of the IL10 promoter polymorphism (Table 18).

Discussion

The main achievement in the present work has been to create a large, heterogeneous case-control cohort with sufficient power to evaluate the effect of common genetic variants on post-transplant lymphoproliferative disease. Many of the known or implicated risk factors were part of the matching criteria and therefore could not be formally tested, however, this was not our intention. The end result is a cohort predominantly representing late occurring PTLD. Pre-transplant EBV serology was a covariate that we considered important *a priori*, which was a major obstacle and the rate-limiting step in completing inclusions, ultimately leading to the exclusion of a large number of cases. A majority of individuals without a sero-status available presented with early PTLD: this served to affirm that our rather restrictive strategy to

systematically seek out EBV statuses. Had we assumed that a majority of individuals with missing EBV sero-statuses were positive, as is frequently done, analyses would have been muddied. Consequently, our findings primarily represent risk factors for late PTLD. Therefore, it is of no surprise that we did not see associations with risk factors traditionally associated generally with PTLD but that have been shown to be related to early occurring disease, namely use of polyclonal T cell depleting agents and aetiologies associated with their use (acute rejections). The suggestion of a protective effect due to CMV infections in univariate analyses disappeared in the intermediate model and hence this variable was excluded in the final multivariate model.

We found *IL10*-rs1800896 AA or AG genotypes, associated with lower IL-10 secretion, more frequent amongst controls. Authors that did not find an association with PTLD focused their work on early PTLD (VanBuskirk et al., 2001), or had mixed case cohorts, and heterogeneous cases and controls (McAulay et al., 2009; Stern et al., 2010). Stern et al. had the largest case cohort, however cases and controls differed with respect to treatment, most notably all controls came from a single centre in Switzerland, were transplanted in a recent era (median year of transplant 2005 vs 1993), thus receiving predominantly IL2RB based induction, and likely more carefully dosed maintenance regimens. Furthermore, cases came from centres participating in the CTS registry with an available DNA sample, although the authors claim cases and controls were ethnically matched: population substructure is nevertheless likely to be significantly more heterogenous in the predominantly Caucasian case cohort, compared to the solely Swiss controls.

IL-10 is implicated in EBV induced B cell growth. Viral IL-10 is produced during lytic infection, and may be necessary for transformation, however LMP1 induces host IL-10 in the latent state. It is thought to have an immunoregulatory role in the microenvironment surrounding transformed B cells, hampering cytotoxic T cell effector mechanisms and down regulating antigen presentation and cytokine production by monocytes (Martinez and De Gruijl, 2008). Furthermore, IL-10

enhances B cell survival, proliferation and antibody production, and has been shown to be a crucial autocrine growth factor for transformed SLCLs (Beatty et al., 1997). Detectable hIL-10 levels have coincided with PTLD amongst 81% of individuals with EBV positive disease and 36% with EBV negative disease (Muti et al., 2003). Our finding goes in the same direction as theses mechanisms.

IL10-rs1800896 GG was more common amongst immune competent NHL cases (Lan et al., 2006; Purdue et al., 2007), but both groups found *IL10*-rs1800890 (-3575T>A) to have the stronger association with the low-secretor genotype (AA) associating with disease. *IL10*-rs1800872 (-592) CC, thought to result in high IL-10 secretion was associated with increased risk for AIDs B-NHL, although no association was found for *IL10* rs1800896 GG (Breen et al., 2003). In this cohort, IL-10 was detected prior to diagnosis in 21% of cases: there was no difference in distributions of the *IL10* rs1800872 CC genotype amongst cases in whom IL-10 was detected and those in whom it was not, nor was there an association between genotype and degree of IL-10 secretion when it was detected (Breen et al., 2003). It is surprising, therefore, that Babel et al. find the genotype associated with a high IL-10 secretion predominantly amongst controls compared to PTLD cases. Bebel et al. speculate that IL-10 in the B-lymphocyte microenvironment might affect IL-6 production in monocytes, or that higher IL-10 might enhance a humoural response to EBV reactivation in controls. Purdue et al. likewise note the apparent inconsistencies in literature reports, and purport that an IL-10 deregulation in general might be responsible for the observed effects (Purdue et al., 2007).

IL2-rs2069762G was the only calcineurin pathway SNP that we found to associate with PTLD. Previous work did not find an association with PTLD in a small paediatric cohort (Lee et al., 2006). Once again this is unexpected, as this polymorphism is thought to result in elevated IL-2 secretion, which would presumably result in decreased sensitivity to calcineurin inhibitors. Although the report most frequently cited concerning this variant shows an increased secretion associated with the GG genotype (Hoffmann et al., 2001), some authors have described decreased IL-2 mRNA in PBMCs of carriers (Moscoso-Solorzano et al.,

2008). It is not known what the effect of this polymorphism would be in the context of antigenic stimulation under calcineurin inhibition. Morgun et al. report an association between the *IL2*-rs2069762TT genotype and acute rejection episodes in kidney transplant recipients, but not in heart transplant recipients (Morgun et al., 2003): this finding likewise goes in the opposite direction of the expected outcome. This variant has not associated with rejection related outcomes in transplantation in other work (Park et al., 2011) (poster abstract, Holweg AJT).

We are confident the effects in opposite direction observed in the antimetabolites is due to azathioprine based on the analyses performed. The large effect by azathioprine, considered as ever-taken, is plausible for a number of reasons. Azathioprine has been implicated in defective mismatch repair: 6-thioguanine, one of the ultimate active metabolites of azathioprine, once incorporated into DNA is methylated and binds the thymines during replication. These S6-methylguanine-thimine pairs are substrates of mismatch repair, and are thought to be the mechanism for delayed cytotoxicity (Karran and Attard, 2008; Swann et al., 1996). Escape from mismatch repair confers a growth advantage, but comes at a cost of genetic instability. These azathioprine related mechanisms have been implicated in acute myeloid leukemia (Offman et al., 2004) and sebaceous carcinoma (Harwood et al., 2001). Azathioprine was a part of most regimens in cases exhibiting mismatch repair deficiency, assessed by the high microsatellite instability phenotype (Borie et al., 2009; Duval et al., 2004), amongst immune deficiency related NHLs and were particularly pertinent for T cell lymphomas.

Azathioprine may have been a factor in previous work that implicated a protective effect by mycophenolate (Cherikh et al., 2003; Robson et al., 2005). Cherikh et al. found mycophenolate based discharge regimens conferred a risk reduction compared to regimens based on azathioprine (RR 0.64, CI 0.46-0.87). Robson et al. found a similar effect (again considering discharge regimens) comparing mycophenolate to non-mycophenolate regimens using data from the CTS registry (European and Canadian centres, RR 0.4, CI 0.17-0.94) but not for the

OPTN/UNOS registry (United States). Post-hoc analysis of OPTN/UNOS registry data considering switches to mycophenolate found a significant risk reduction when this occurred (RR 0.73, CI 0.58–0.91). Bustami et al. found no effect comparing mycophenolate to azathioprine based discharge regimens in groups that received induction, or not, however no numbers are presented for the reader to gauge case distributions (Bustami et al., 2004). All afore mentioned studies were of a cohort design. Funch et al. performed a case-control study, matching 1:4 on centre, age category (adult/child), date of transplant, and at least equivalent survival corresponding to the period transplant to PTLD diagnostic in the case (Funch et al., 2005b). They did not find any difference between mycophenolate containing versus mycophenolate-free triple regimens in univariate or multivariate analyses. Both discharge and maintenance immunosuppression were recorded in data collection, thus presumably analyses were as ever-taken. Triple regimens without mycophenolate could have included a number of different agents and therefore a specific effect by azathioprine cannot be inferred. Caillard et al. recently performed a series of analyses suggesting azathioprine and cyclosporine used in discharge regimens influenced graft localised disease, while mycophenolate use appeared protective (Caillard et al., 2012). These analyses appeared to be univartiate and performed per drug: that azathioprine exhibited a trend for increased risk of unstratified PTLD in the multivariate model, and would have been significant if simultaneous kidney pancreas transplants in this cohort were not included, suggests the effects observed for graft localised disease is attributable to azathioprine (and hence probably azathioprine containing cyclosporine regimens). Stratification for site of disease appears to be worth exploring in future analyses. A final point to make is that a majority of the above studies separated early from late PTLD, although Caillard et al. did not find an association with late PTLD in univariate analyses for azathioprine based discharge regimens. Cases exhibiting mismatch repair deficiency were typically late lymphomas, and almost all were combinations with cyclosporine (Borie et al., 2009; Duval et al., 2004). Our cohort is representative of late PTLD, and might be the reason for the larger effect size observed compared to those reported by Cherikh et al. and Robson et al., although our confidence range is wide.

We considered degree of immunosuppression would be a confounding factor to any analyses involving variants of the calcineurin pathway. Given that there are no pharmacodynamic biomarkers in widespread clinical use, we attempted to do this by considering all immunosuppressants used by constructing a series of scores based on doses or calcineurin trough levels. The use of such scores appears to have been an ambitious attempt to summarise overall degree of immunosuppression into a single value (score) for use in a study design of the type case-control. Although likely not a robust measure, these scores suggest that cases and controls were roughly matched with respect to degree of immunosuppression. Cases and controls were matched on transplant centre, where protocols are more uniformly applied, and year of transplant (+/- 1 year), reflecting the therapeutic targets aimed for in a given transplant era, knowing the trend has been for reductions in targets with time to achieve an optimal risk-benefit balance. Furthermore, order of graft was matched for, and HIV positive individual and concomitant non-kidney transplants were excluded. Overall, it appears the matching strategy was a sufficient means to control potential confounding due to degree of immunosuppression.

These results presented here are preliminary: the list of variants included was a short-list chosen for likely functional effect in order to enhance prior probability for a true effect. A number of the variants discussed in the review above were not included in the present iteration owing to challenges in genotyping and a lack of certitude concerning functional effects. The most recent work in POLYCIS (L. Pouché) has revealed that a number of the above variants are in deed candidates of interest. Furthermore polymorphisms in the genes encoding calmodulin, the principle NFAT isoforms, c-Fos and C-jun will also be included. Moreover, a subsequent iteration will allow external validation of the results presented here.

Conclusion

This work represents an in depth exploration of the influence of the immunosuppressant drugs on lymphomagenesis. Our experimental work shows that in the early steps of lymphomagenis, that is during infection and transformation by EBV, chronic exposure to the CNIs does not appear to have a role outside of inhibiting the T response. In the CD40/LMP1 mouse model, a model of insidious spontaneously occurring lymphoma, there is evidence that chronic cyclosporine treatment enhances lymphomagenesis, as evidenced by increased activated B cells in the spleen. A T response in this model cannot be ruled out, however the profile of T lymphocyte splenic infiltrates does not appear to be explained solely by this possibility. Finally, we performed a case control study with the aim to study the pharmacogenetics of drug response and showed polymorphisms in *IL10* and *IL2* associate with PTLD, as well as azathioprine, whose use as an immunosuppressant in transplantation has fortunately largely been replaced by newer antimetabolites.

Appendix 1: Representative gating

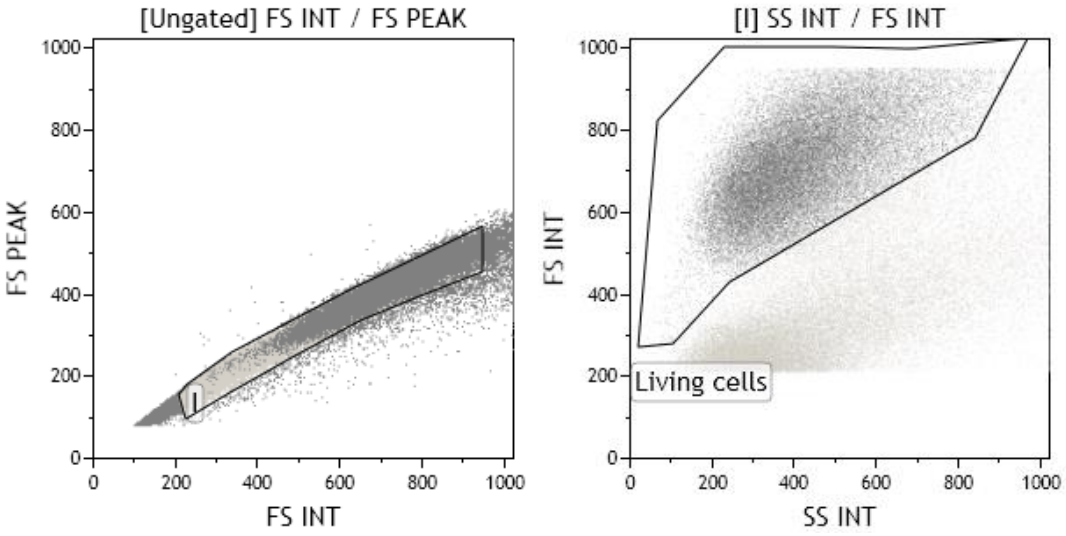

Figure 22 Representative gating for living cells in B panel. Day 28, tacrolimus, subject O depicted

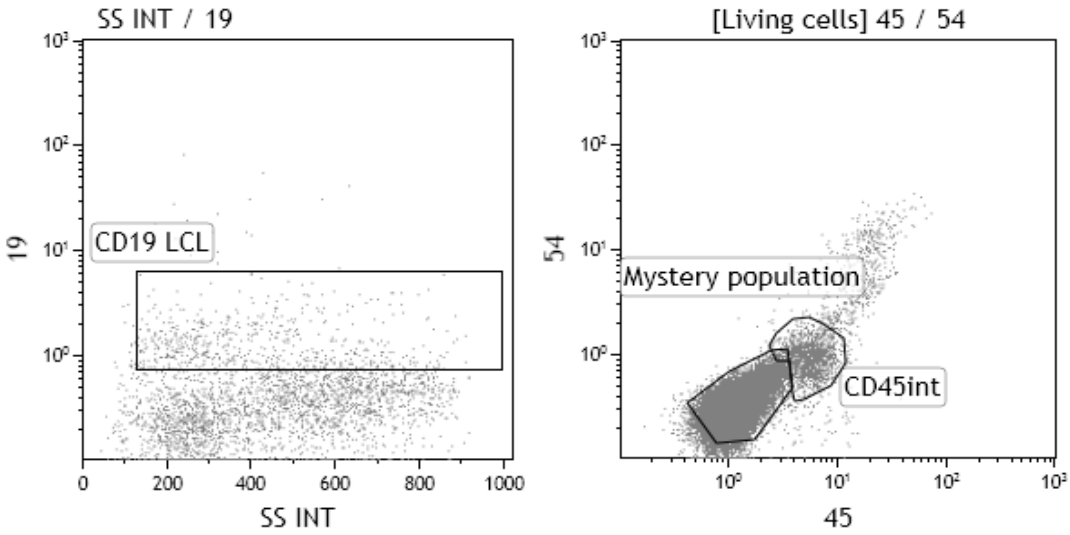

Figure 23 Representative gating for CD19 LCL (left) and mystery population (right). Day 28, tacrolimus, subject O depicted

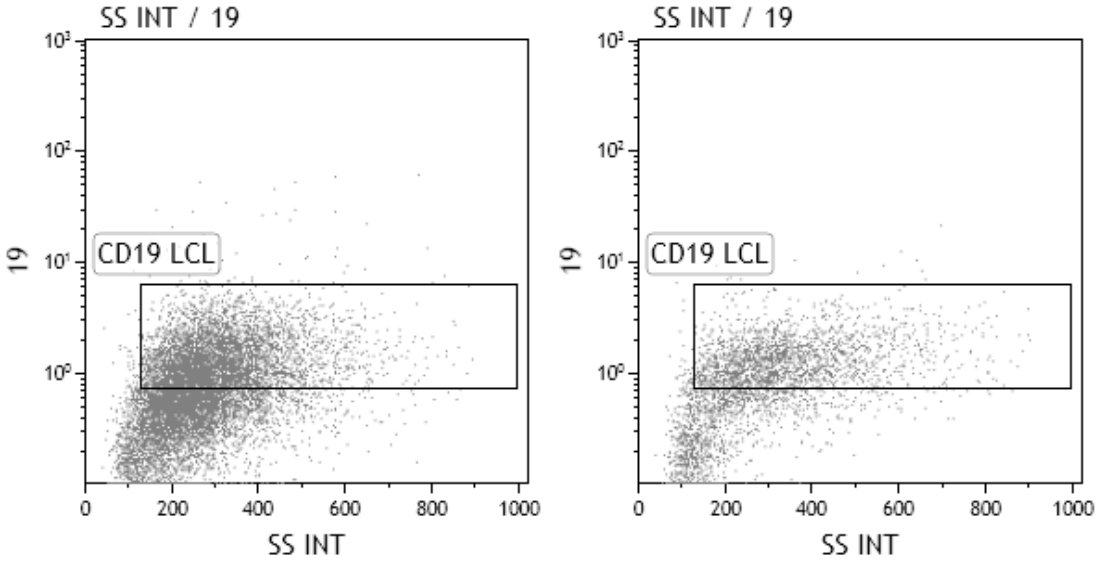

Figure 24 Representative gating for CD19 LCL gating when LCL emerged. Cyclosporin treated, subject D, day 14 (left) and day 28 (right) depicted.

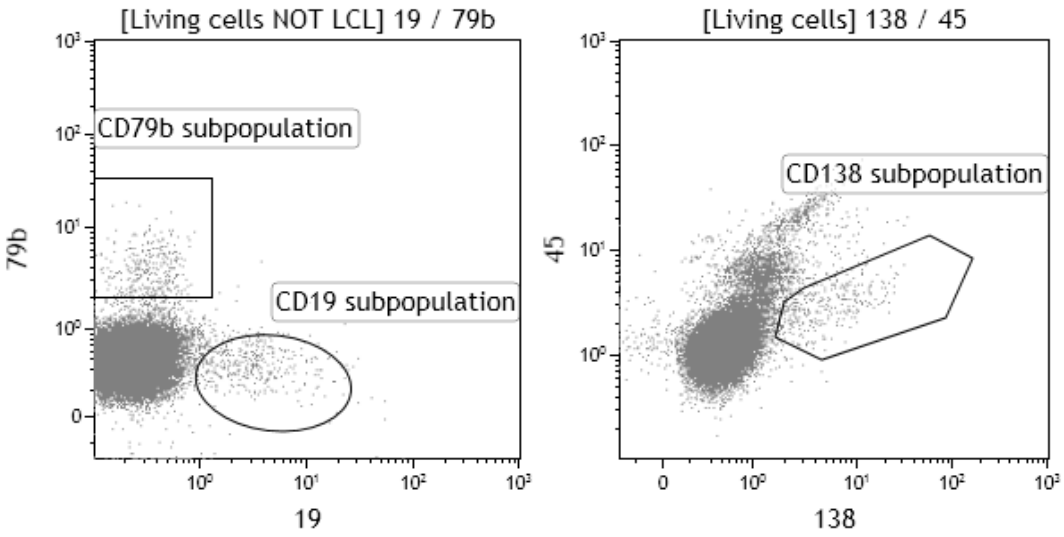

Figure 25 Representative gating for CD19+, CD79b+ and CD138+ subpopulations, only present when the mystery population predominated. Day 28, tacrolimus treated, subject O depicted

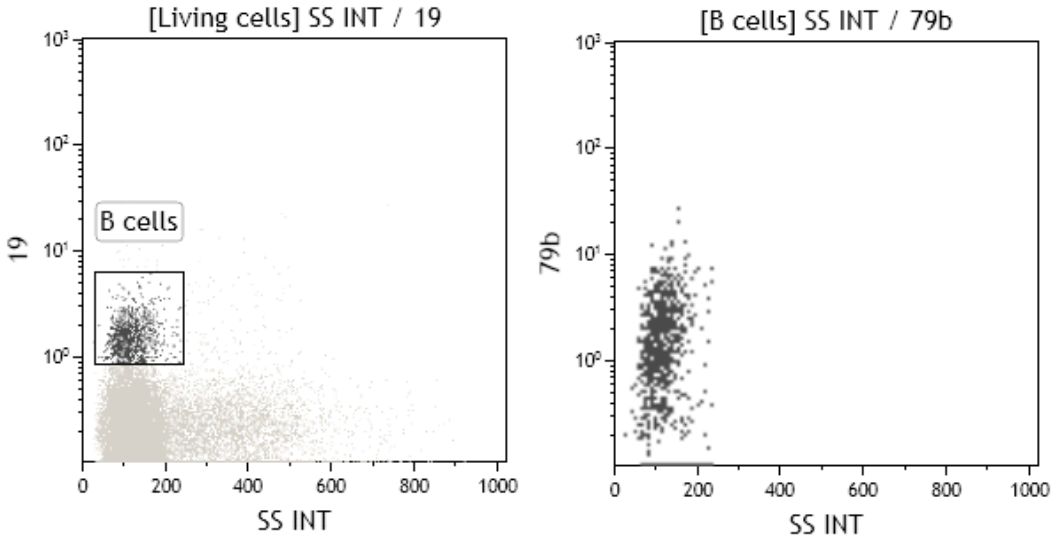

Figure 26 Representative gating for the B cell gate. Day 0, control, subject L depicted

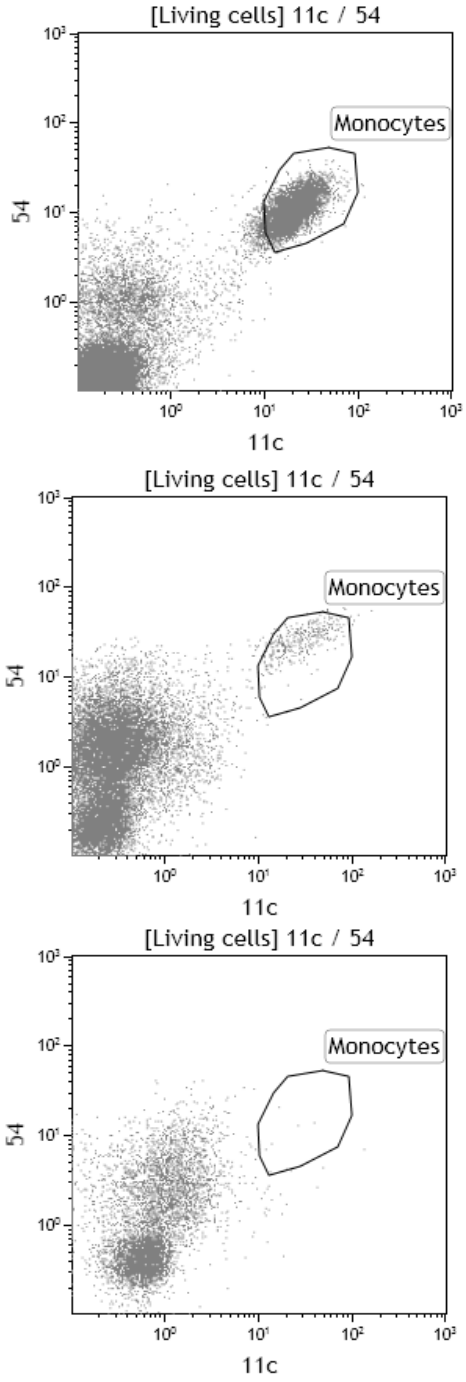

Figure 27 Representative gating for monocytes. Control, subject A depicted, day 0 (top), day 14 (middle) and day 28 depicted to show no interference with emerging LCL.

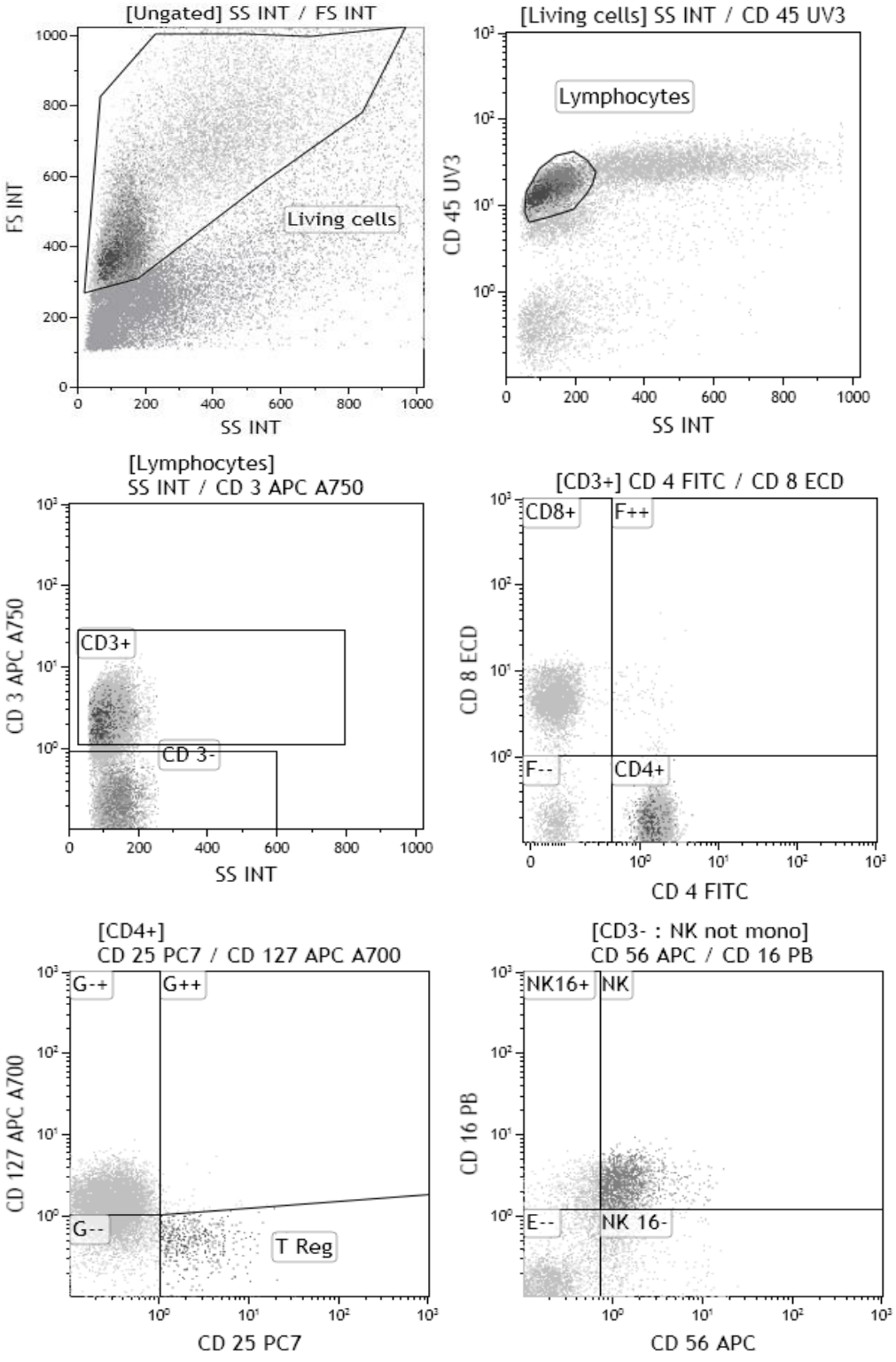

Figure 28 Representative gating for CD4+, CD8+, T regulatory (T Reg) and Natural Killer cell (NK) populations. Day 3, control, subject C depicted.

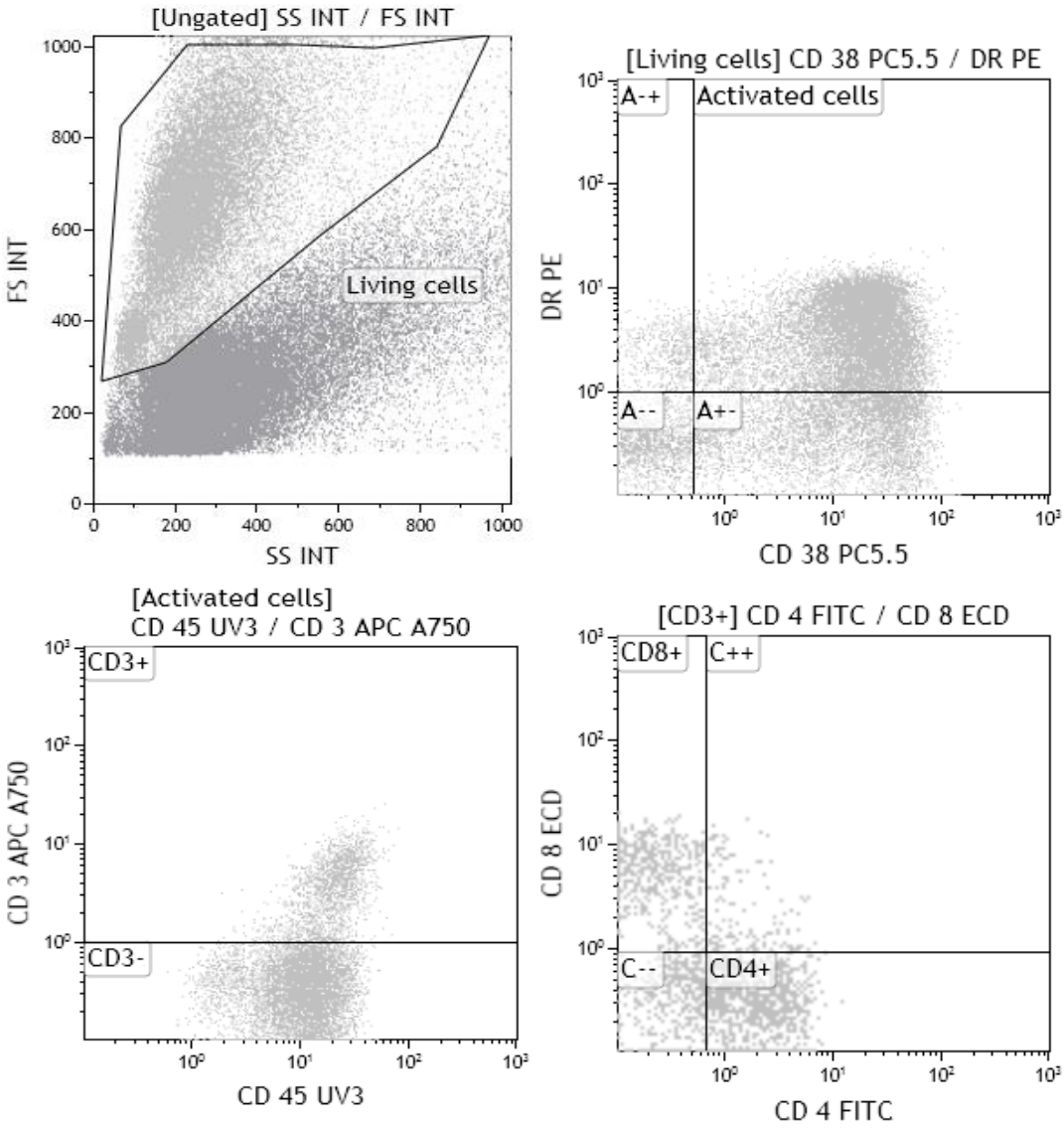

Figure 29 Representative gating for activated T cell populations. Day 14, control, subject C depicted.

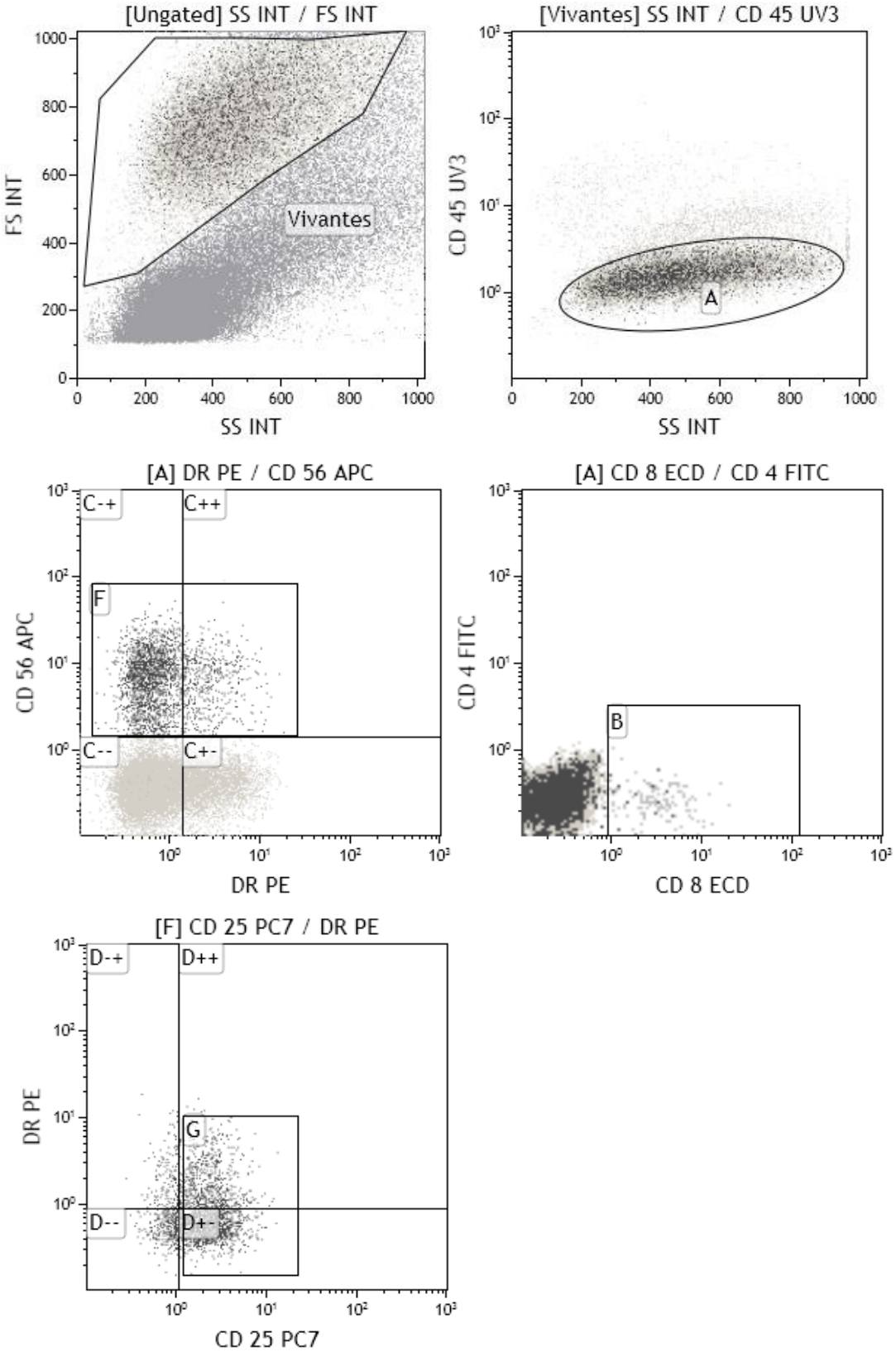

Figure 30 Representative gating for CD56+ positive cells [presumed Natural Killer cells, (F)], CD8+ [presumed cytotoxic killer cells, (B)], and CD25+CD56+ cells (G). Day 28, cyclosporine, subject O depicted

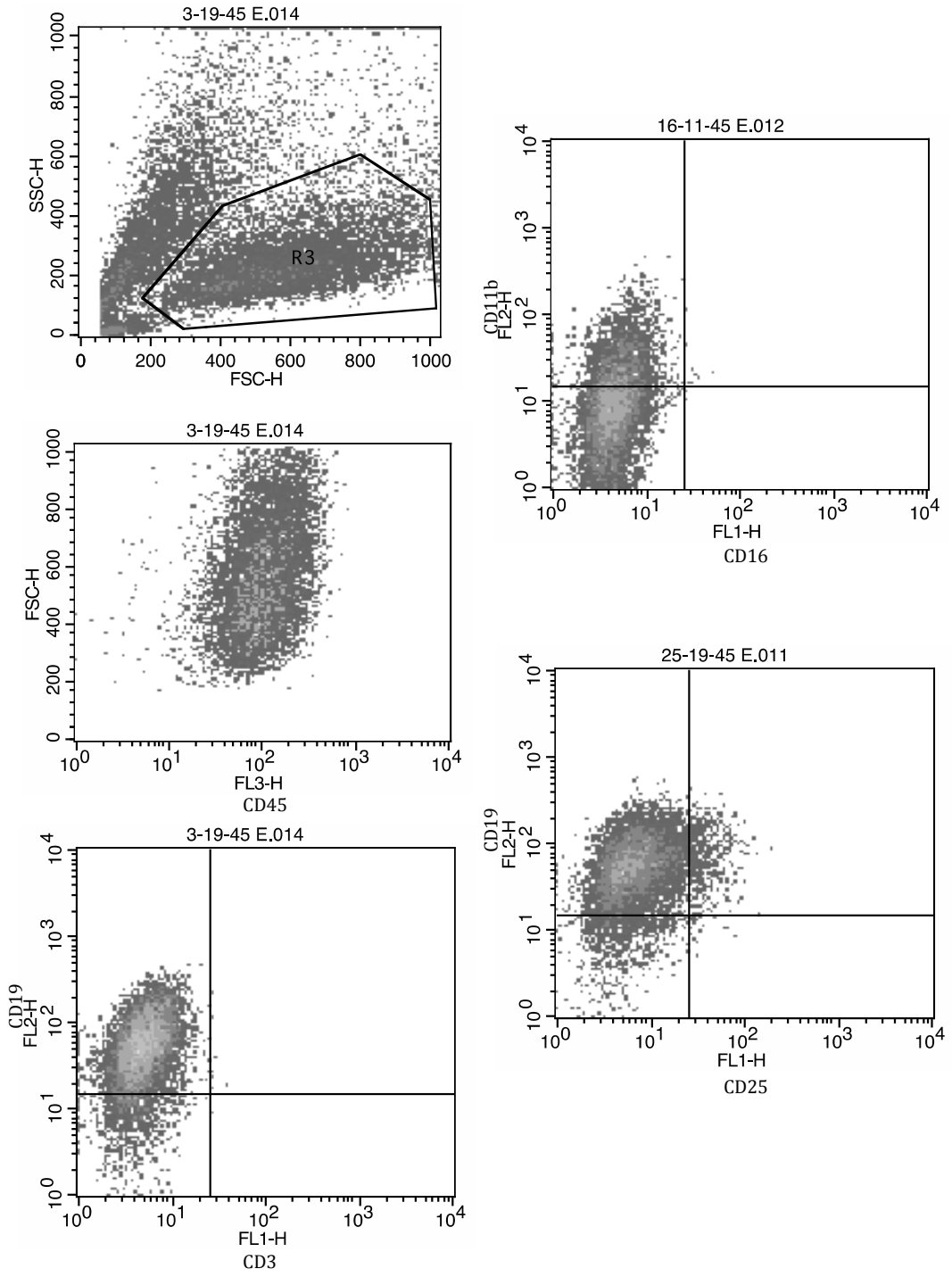

Figure 31 Cytometric profiles using a simplified antibody panel (FACSCalibur flow cytometer, CellQuest Pro software). LCL was line E that had been cyclosproine treated.

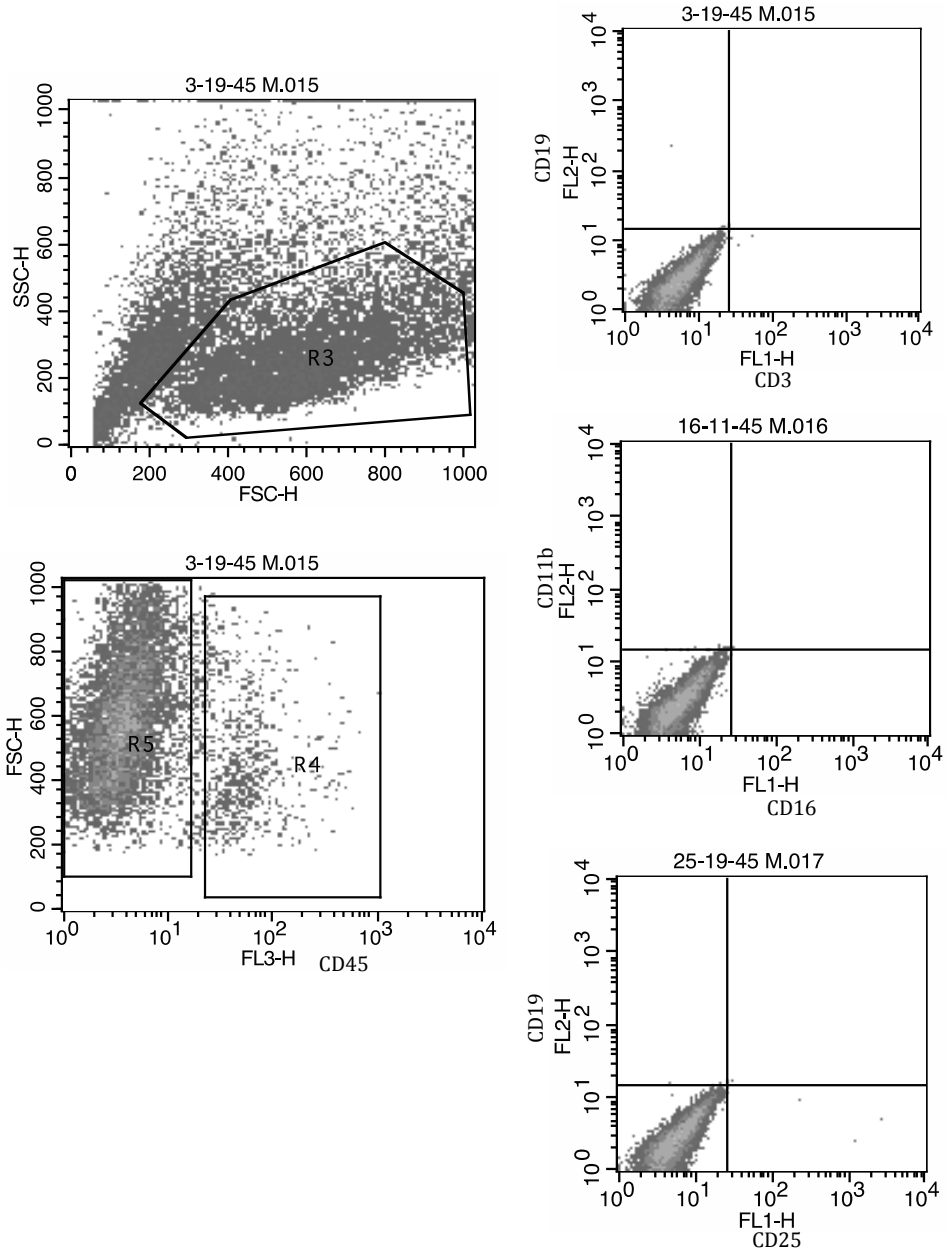

Figure 32 Cytometric profiles using a simplified antibody panel (FACSCalibur flow cytometer, CellQuest Pro software). Mystery line was line M that had been cyclosproine treated.

Appendix 2: Genotyping data

Table 19 Hardey Weinberg Equilibrium and assay details for single nucleotide polymorphisms included in the present study

Gene	rs Accession #	Polymorphism	MAF	HWE p-value	Assay ID/Part No.	Context Sequence
IL10	rs1800871	C>T	0.25	0.13	C___1747362_10	AGTGAGCAAACTGAGGCACAGAGAT**[A/G]**TTACATCACCTGTACAAGGGTACAC
IL10	rs1800872	C>A	0.25	0.09	C___1747363_10	CTTTCCAGAGACTGGCTTCCTACAG**[T/G]**ACAGGCGGGGTCACAGGATGTGTTC
IL10	rs1800896	A>G	0.46	0.38	C___1747360_10	TCCTCTTACCTATCCCTACTTCCCC**[T/C]**TCCCAAAGAAGCCTTAGTAGTGTTG
TGFB1	rs1800470	T>C	0.38	0.76	C__22272997_10	TAGCCACAGCAGCGGTAGCAGCAGC**[A/G]**GCAGCAGCCGCAGCCCGGAGGGCGG
TNF	rs1799964	T>C	0.25	0.25	C___7514871_10	GGAAGCAAAGGAGAAGCTGAGAAGA**[C/T]**GAAGGAAAAGTCAGGGTCTGGAGGG
TNF	rs1800629	G>A	0.11	1.00	C___7514879_10	GAGGCAATAGGTTTTGAGGGGCATG**[A/G]**GGACGGGGTTCAGCCTCCAGGGTCC
TNFRSF1A	rs4149570	G>T	0.39	0.65	C___2645704_10	TGAACTTCTCAGACACATAACTGAA**[A/C]**CTGTCTGGATCTGTTTTCCAATTTG
IL2	rs2069762	T>G	0.26	0.47	C__15859930_10	AGTAACTCAGAAAATTTTCTTTGTC**[C/A]**TAAAACTACACTGAACATGTGAATA
IL2	rs6822844	G>T	0.13	1.00	C__28983601_10	CCTGTCTCGCTCTCCATAGCAAAAA**[G/T]**AGAGGACTCTTTTCATGTTGCCACT
PPIA	rs8177826	C>G	0.07	0.61	AH0I3SE/4331349	AGCCT**[G/C]**GCCTCCC
PPP3CB	rs3763679	C>T	0.05	1.00	C__25474809_10	ATTTTTCTAGTGAATTTATCTTGTA**[C/T]**TTTTGTTTAATGAAGCTGCTTGATC
PPP3R1	rs13009282	T>C	0.24	0.04	C___1282239_10	CCTTTGGTTGGATGCTGTGTTCTCA**[C/T]**GTCAAAAACATGGAACTTTCCTTCC
PPP3R1	rs2044693	C>T	0.34	0.64	C__12044275_10	ATCCGAAATGGAAACGCAGAGCGCC**[A/G]**GGGCAGAGGAGGGCTTTACCCAGGT
PPP3R1	rs1868402	T>C	0.28	0.73	C__12044272_10	TCAGTGAATACTGACATGGTAATGT**[A/G]**TAAGTGTAGTATCTGTATCATTTGG

Table 20 Failed assays and details: candidates for sequencing in the full PTLD cohort

Accession No.	Location	Assay ID	Comment
rs1800471	TGFB1 codon 25 +915 G>C	AHMSNWL	Custom ordered - failed AB QC
rs2430561	IFNG intron 1 +874 A>T	AHN1L2T	Literature reported assay (Lee et al., 2006) Ordered- failed AB QC
rs1800630	TNF promoter -863 C/A	AHGJW6H	Problematic allelic discrimination
rs1800692	TNFRSF1A promoter -1135 T/C	C___8921230_20	Problematic allelic discrimination

Appendix 3: Calculation of a global immunosuppressant score

The treatment period was considered up to the visit prior to the recorded date of PTLD diagnosis, the reason for this being that visits corresponding to the period following diagnostic reflected changes in the drug regimen (namely reduction in immunosuppression). When two drugs of the same class were recorded for a given visit, this invariably reflected a change to a different drug and the subsequent continuation of that drug: in these situations the new drug and dose were selected to ensure the contribution of only one to the total score for a given visit. Corticosteroid dose was considered in prednisolone equivalents. Myfortic® doses were converted to CellCept® equivalent doses. For a given visit, dose quartiles were determined by calculating the 25^{th} percentile and 75^{th} percentile of all recorded doses. Similar percentiles were calculated for trough concentrations (C0s) in the case of the calcineurin inhibitors (CNIs). Table 21 illustrates the quartile calculations for cyclosporine dose.

For a given visit, a score of 1 was assigned for doses/C0s less than the 25^{th} percentile, 2 for doses/C0s in the range 25 – 75%, and 3 for values greater than the 75^{th} percentile. Table 22 illustrates the assigning of scores for cyclosporine dose in two subjects. Scores were combined into groups by drug class: CSA and TAC were combined for a final CNI score, and MPA and azathioprine were combined for a final antimetabolite score: there was never an occasion where both drugs within a drug class were contributed simultaneously to the score as all switches were accounted for (see above).

Table 21 Quartile Calculations for cyclosporine dose per visit

Visit	25th Percentile for Dose (mg/d)	75th Percentile for Dose (mg/d)	Total Contributions
D15	300	463.75	128
M1	300	420	113
M3	245	325	131
M6	205	300	119
Y1	200	300	112
Y1.5	200	300	103
Y2	200	270*	104
Y2.5	200	270*	96
Y3	200	270*	95
Y3.5	175*	250*	86
Y4	175*	250*	83
Y4.5	175*	250*	75
Y5	175*	250*	74
Y6	175*	250*	66
Y7	175*	250*	60
Y8	150	200*	45
Y9	150	200	40
Y10	150	200	29
Y11	150	200	20
Y12	150	200	17
Y13	150	180	12

*doses were harmonised to closest appropriate dose

Table 22 Assigning of the CSA group score

Subject	Visit	CSA Dose (mg/d)	25th percentile for Dose (mg/d)	75th percentile for Dose (mg/d)	CSA group
1	D15	400	300	463.75	2
1	M1	375	300	420	2
1	M3	350	250	325	3
1	M6	300	200	300	2
1	Y1	175	200	300	1
1	Y1.5	NA	200	300	NA
1	Y2	NA	200	275	NA
1	Y2.5	NA	200	270	NA
2	M1	600	300	463.75	3
2	M3	525	300	420	3
2	M6	300	250	325	2
2	Y1	300	200	300	2
2	Y1.5	275	200	300	2
2	Y2	250	200	300	2
2	Y2.5	250	200	275	2
2	Y3	250	200	270	2
2	Y3.5	250	200	270	2
2	Y4	250	176.25	250	2
2	Y4.5	250	175	250	2

A category “other” consisted of all other maintenance drugs: mTOR inhibitors, leflunomide, belatecept, fingolimod (used in clinical trials as a candidate immunosuppressant that was finally abandoned in transplantation). A score was assigned to these: belatacept and fingolimod were assigned 3, leflunomide 2. In the present cohort there was only one single dose of fingolimod given which was accounted for in the score, but not discussed in the results above. The mTOR inhibitors exhibited doses and C0s (when available) within the range corresponding to typical treatment regimens and therapeutic targets, and as such all entries were assigned a score of 2. A small number of subjects were treated with more than one drug within the “other” category for a given visit (namely belatecept together with leflunomide, with no other drug classes used simultaneously), thus a total “other” score was calculated for each visit in these subjects.

Missing doses were considered as dose missing, but drug taken (drug recorded, no dose recorded in the data collection form, score = NA), or drug not taken at all (drug not recorded in the data collection form, score = 0). A score of zero in one of the three main group scores (CNIs, antimetabolites and corticosteroids) reflected an immunosuppressant regimen of double therapy, while two zeros reflected mono-therapy. The majority of individuals were receiving a typical triple therapy regimen over the entire follow up period.

A composite score was calculated for each visit by adding the scores for the CNI group, antimetabolite group, corticosteroid group and other. CNIs were considered by dose or by C0, giving rise to two composite scores. Table A2.3 below demonstrates the calculation of the final score for illustrative purposes. A second composite score (Composite 2) was calculated similarly considering corticosteroids as taken yes/no (1/0), the reason being the likely lower impact of corticosteroids to the final outcome, and also the high degree of missing doses that resulted in NA for the total score.

An Area Under the Curve (AUC) of the scores over the follow-up period was calculated: the calculation was performed in R using the function 'AUC' in the package 'epicalc'. In the event that doses were missing, the last recorded dose was extrapolated to the next recorded dose. The AUCs were normalized by dividing the total AUCs obtained by the period corresponding to the time between first and the last recorded dose. This gave a single final variable per subject, a type of global immunosuppressant score.

$$\text{Normalized AUC of scores} = \frac{\text{AUC of scores calculated over the treatment period}}{\begin{array}{c}\text{Period of recorded follow-up} \\ \text{(last recorded visit – first recorded visit)}\end{array}}$$

Table 23 Calculation of the final composite score 1 for dose and C0

Subject	Visit	Group						
		CNI	Antimetab.	Corticosteroid	CNI C0	Other	Comp. (dose)	Comp. (C0)
1	D15	3	1	1	3	0	5	5
1	M1	3	1	1	3	0	5	5
1	M3	2	2	2	2	0	6	6
1	M6	2	2	2	2	0	6	6
1	Y1	2	1	3	2	0	6	6
1	Y1.5	2	1	2	2	0	5	5
1	Y2	2	1	2	1	0	5	4
1	Y2.5	2	1	2	1	0	5	4
1	Y3	2	1	2	1	0	5	4
1	Y3.5	2	1	2	1	0	5	4
1	Y4	2	1	2	1	0	5	4
1	Y4.5	2	1	2	1	0	5	4
1	Y5	2	2	2	1	0	6	5
1	Y6	2	2	2	2	0	6	6
1	Y7	2	2	2	2	0	6	6
1	Y8	0	2	3	0	2	7	7
1	Y9	0	2	3	0	2	7	7
1	Y10	0	0	3	0	2	5	5
2	D15	1	2	2	2	0	5	6
2	M1	1	0	2	1	2	5	5
2	M3	1	2	2	1	0	5	5
2	M6	2	2	3	3	0	7	8
2	Y1	2	2	3	2	0	7	7
2	Y1.5	2	2	2	2	0	6	6
2	Y2	2	2	NA	1	0	NA	NA
2	Y2.5	2	2	NA	2	0	NA	NA
2	Y3	2	2	NA	2	0	NA	NA
3	D15	1	2	2	2	0	5	6
3	M1	1	2	2	2	0	5	6
3	M3	1	2	1	3	0	4	6
3	M6	1	2	2	3	0	5	7
3	Y1	1	2	2	3	0	5	7
3	Y1.5	1	2	2	2	0	5	6
3	Y2	1	2	2	NA	0	5	NA
3	Y2.5	1	2	2	3	0	5	7
3	Y3	1	2	2	2	0	5	6
3	Y3.5	1	2	1	3	0	4	6

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