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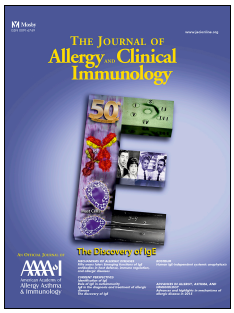

Thymus Transplantation for Complete Digeorge Syndrome: European Experience

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PII: S0091-6749(17)30576-6

DOI: 10.1016/j.jaci.2017.03.020

Reference: YMAI 12738

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 8 July 2016

Revised Date: 3 March 2017

Accepted Date: 15 March 2017

Please cite this article as: Davies EG, Cheung M, Gilmour K, Maimaris J, Curry J, Furmanski A, Sebire N, Halliday N, Mengrelis K, Adams S, Bernatoniene J, Bremner R, Browning M, Devlin B, Erichsen HC, Gaspar HB, Hutchison L, Ip W, Ifversen M, Leahy TR, McCarthy E, Moshous D, Neuling K, Pac M, Papadopol A, Parsley KL, Poliani L, Ricciardelli I, Sansom DM, Voor T, Worth A, Crompton T, Markert ML, Thrasher AJ, Thymus Transplantation for Complete Digeorge Syndrome: European Experience, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.03.020.

THYMUS TRANSPLANTATION FOR COMPLETE DIGEORGE SYNDROME: EUROPEAN EXPERIENCE

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FUNDING:
The research leading to these results has received funding from: European Union Seventh Framework Programme ([FP7/2007-2013] [FP7/2007-2011]) under grant agreement N° 261387; Great Ormond Street Hospital Children's Charity, Mason Medical

Research Fund, Wellcome Trust.The work reported is based on independent research, partly supported by the National Institute for Health Research, Great Ormond Street Hospital Biomedical Research Centre (KG, EGD, AJT). The views expressed in this publication are those of the authors and not necessarily those of the NHS, the National Institute for Health Research or the Department of Health. AJT is a Wellcome Trust Principal Research Fellow.

ABSTRACT

Background: Thymus transplantation is a promising strategy for the treatment of athymic complete DiGeorge syndrome (cDGS).

Methods: Twelve patients with cDGS were transplanted with allogeneic cultured thymus.

Objective: To confirm and extend the results previously obtained in a single centre.

Results: Two patients died of pre-existing viral infections without developing thymopoeisis and one late death occurred from autoimmune thrombocytopaenia. One infant suffered septic shock shortly after transplant resulting in graft loss and the need for a second transplant. Evidence of thymopoeisis developed from 5-6 months after transplantation in ten patients. The median (range) of circulating naïve CD4 counts ($x10^6$/L) were 44(11-440) and 200(5-310) at twelve and twenty-four months post-transplant and T-cell receptor excision circles were 2238 (320-8807) and 4184 (1582 -24596) per10^6 T-cells. Counts did not usually reach normal levels for age but patients were able to clear pre-existing and later-acquired infections. At a median of 49 months (22-80), eight have ceased prophylactic antimicrobials and five immunoglobulin replacement. Histological confirmation of thymopoeisis was seen in seven of eleven patients undergoing biopsy of transplanted tissue including five showing full maturation through to the terminal stage of Hassall body formation. Autoimmune regulator (AIRE) expression was also demonstrated. Autoimmune complications were seen in 7/12 patients. In two, early transient autoimmune haemolysis settled after treatment and did not recur. The other five suffered ongoing autoimmune problems including: thyroiditis (3); haemolysis (1), thrombocytopaenia (4) and neutropenia (1).

Conclusions: This study confirms the previous reports that thymus transplantation can reconstitute T cells in cDGS but with frequent autoimmune complications in survivors.

CLINICAL IMPLICATIONS

Thymus transplantation should be the treatment of choice for infants with cDGS except possibly in those with severe pre-existing viral infections. The risk of autoimmune complications is a significant issue for survivors and further work is needed to understand this better.

CAPSULE SUMMARY

In twelve patients with complete DiGeorge syndrome treated with thymus transplantation, there was a 75% survival with T-cell reconstitution. Autoimmunity, mostly manageable, was a frequent occurrence in survivors.

KEY WORDS

89 DiGeorge syndrome; athymia; thymus transplantation

90

INTRODUCTION

DiGeorge Syndrome with athymia, complete DiGeorge Syndrome (cDGS), results in a state of profound T cell deficiency. The causal associations have been reviewed elsewhere[1]; DGS can be associated with a hemizygous microdeletion at chromosome 22q.11, CHARGE syndrome, mutations in TBX1, deletions at chromosome 10p13-14 or fetal toxin exposure from glucose, ethanol or retinoic acid. Around 1.5 % of children with 22q.11 deletion have the complete form of DiGeorge Syndrome [2] whereas the incidence of the problem in relation to other causes is unknown. The immunological phenotype is either of a profound T-cell lymphopenia or, in atypical cDGS, there may be oligoclonal expansions of memory phenotype T-cells conferring little or no protective immunity and causing inflammatory disease in the form of rashes, enteropathy and lymphadenopathy [3]. cDGS differs from severe combined immunodeficiency (SCID) in that the underlying defect prevents development of the thymus whereas the underlying defect in SCID is a genetic defect in the hematopoietic lineage.. Patients with both cDGS and SCID, have a similar high risk of early death from infection.

Two approaches have been used to correct the immunodeficiency in patients with cDGS. The first is T-cell replete haematopoietic stem cell transplantation (HSCT) but, because of the absence of thymus, this approach can only achieve engraftment of post-thymic T-cells. Whilst there are a number of reports of long lasting survival in patients treated in this way, particularly after matched sibling donor transplantation, the quality of the immune reconstitution achieved is poor [4]. Survival after matched unrelated donor and matched sibling transplants were reported as being 33% and 60% respectively [5]. The alternative approach is to use thymus transplantation, which aims for a more complete reconstitution with ability to produce naïve T cells that show a broad T cell receptor (TCR) repertoire. Postnatal thymus tissue is readily available as it is routinely removed from infants undergoing open heart surgery through median sternotomy. This approach has been used at a single centre in the United States since the mid-1990s. There may have been some patient selection bias in the thymus transplanted group as patients suffering from severe co-morbidities or with serious opportunistic infections were excluded. Nevertheless, the results compare very favourably with the outcome of HSCT with an approximately 75% long term survival in 60 patients[6]. Evidence of thymopoiesis and a diverse repertoire of naïve circulating T-cells, capable of HLA restricted specific antigen responses was seen in survivors. Non–survival in this cohort was mostly associated with pre-transplant morbidity, mainly viral infections and/or chronic lung disease [7]. Autoimmune hypothyroidism was relatively common at just over 20% whilst an additional number of patients developed this problem pre-transplantation [6]. More serious and potentially life threatening autoimmunity including immune cytopaenias and enteropathy was also reported though much less commonly. The reasons for the occurrence of these complications are ill-understood[8].

In order to test whether the technology could be successfully translated from the single centre and to make this treatment approach more readily available in Europe, a centre for thymus transplantation was established in London to provide this treatment for patients in Europe. This report outlines the results of the first 12 patients treated with more than 24 months of follow up.

METHODS

PATIENTS

Patients were recruited between 2009 -2014. In order to qualify for the study, those with typical cDGS had a maximum T-cell count of 50 x10^6/L, no naïve T-cells and absent proliferative response to phytohaemagglutinin (PHA) response. Atypical cDGS patients had less than 5% naïve CD4 cells (CD45 RA^+, $CD27^+$ or CD45 RA^+, $CD62L^+$). In addition there had to be at least one feature of the following: congenital heart disease, hypoparathyroidism, hemizygosity for 22q.11 deletion or CHARGE syndrome. For further patient details see Online Repository.

Patients with typical cDGS, without clonal expansions were not given any immunosuppression. In those with atypical cDGS, CyclosporinA (CSA) was used pre-transplantation to control inflammatory disease and this was continued post-transplantation. These patients were also treated with three doses of rabbit anti-thymocyte globulin (ATG, Genzyme) 2 mg/kg body weight, Methylprednisolone 2 mg/kg intravenously for four days followed by oral prednisolone 1mg/kg for five days.

OBTAINING, CULTURING AND TRANSPLANTING DONOR THYMUSES

For details, including screening of donors, and the transplant procedure which has been described previously [9] see Online Repository. To assess cellular composition changes during the period of culture, separate thymuses were cultured specifically for analysis. For detailed methods see Online Repository

LABORATORY ANALYSIS

Flow cytometric analysis, mitogen responsiveness and measurement of T-cell receptor signal joint excision circle (TREC) levels involved standard methods described in the Online Repository. Testing for possible donor T cell engraftment using short tandem repeats utilised a method previously described [10]

Clonality of T cells was assessed using T-cell receptor V beta chain spectratyping on the CD3 positive population as previously described [11]. Regulatory T cell (Treg) numbers were measured on the CD4 population using CD25 and CD127 and intracellular staining for FoxP3. Spectratyping was also performed on Treg populations purified by cell sorting based on CD4+, CD25 Hi, CD127 – cells and compared to the remaining CD4 cells. For assessment of Treg function, total CD4+ cells were isolated and FoxP3 cells studied for CTLA4 upregulation and transendocytosis of CD80 based on a previously reported method [12] modified by running the assay for a period of 21 rather than 16 hours and by fixing/permeabilising the cells to allow staining for total CTLA4 rather than cycling surface CTLA4.

The frequency of interferon-gamma (IFN-γ)-producing cells in response to either an autologous or third party EBV-transformed lymphoblastoid cell line (LCL)-specific stimulation was assessed on peripheral blood mononuclear cells (PBMC) using an ELISPOT assay, as previously reported [13]

Histological studies were performed on formalin fixed tissue including immunohistochemical analysis by standard methods or as described previously [14] . Details of the antibodies used are given in the Online Repository.

ETHICS

The study was approved by the Institute of Child Health and Great Ormond Street Hospital Research Ethics Committee covering both thymus donation, including screening of the donors, and the transplant procedure in the recipient. Culture of thymus was undertaken under a licence from the UK Human Tissue Authority.

RESULTS

PATIENTS

Details of the patients, including the genetic defect, comorbidities and infections acquired pre-transplantation are shown in Table I. Median age at transplantation was 10 (range 2.5-26) months. In two cases, the molecular basis of the DGS was undefined though, subsequently, in one of these a putative mutation has been found in TBX1 (analysis performed by Prof Klaus Schwartz, University of Ulm, Germany). Neither of these cases was an infant of a diabetic mother. Atypical cDGS cases outnumbered typical in a ratio of 2:1. There was no evidence of Bacillus Calmette Guerin (BCG) -associated disease in the two recipients of this vaccine. Two patients had hypothyroidism before transplantation, the cause of which was not established. Both had negative tests for thyroid peroxisomal antibodies. One had a low TSH suggesting a possible central cause whilst in the other the problem proved to be transient. No patients had clear cut autoimmune disease prior to transplantation.

THYMUS CULTURES

During the period of thymic culture there was progressive lymphoid cell depletion and reciprocal increase in the proportion of EpCam positive TEC cells (Figure 1 a-c). A small fraction of T cells remained with a predominance of single positive CD4 cells (Figure 1 d) which could be induced to activate and to proliferate (Figure 1 e-f). Histological sections of thymus slices before and after culture confirmed lymphoid depletion though some persisting lymphoid cells could be seen. There was preservation of a “network” of epithelium seen on cytokeratin staining with CK5 and CK14, staining predominantly medullary thymic epithelium (mTEC), and with CK8 staining both mTEC and cortical thymic epithelium (cTEC) (Figure E1 in the Online Repository, CK14 data not shown).

CLINICAL OUTCOMES

The surgical procedure was well tolerated in all patients. There were no wound infections or problems with wound healing. The “dose” of thymus transplanted ranged between 8-18 g/m^2 BSA.

Of the eight patients with atypical cDGS, all received CSA but three did not receive ATG because of concerns over potential worsening of pre-existing viral infections. One patient (P11) with atypical cDGS additionally received two courses of Alemtuzumab to control inflammatory features within 3 months prior to transplantation.

Nine of the 12 patients are alive at median follow up time of 49 months (range 21-80 months). Two patients (P7 and P12) died at eight months and two weeks respectively after transplantation from pre-existing viral infections: disseminated cytomegalovirus (CMV) and parainfluenza 3 pneumonitis respectively. ATG had been withheld in both of these. One further patient died of cerebral haemorrhage associated with immune thrombocytopaenia at 23 months post-transplantation. In P1, a first thymus graft failed to survive and she received a second successful graft after 12 months. More clinical detail of this case is given in the Online Repository.

Clinical outcomes in survivors have generally been good with exceptions mainly from autoimmune problems or other non-immunological aspects of DGS (Table II). All developed thymopoeisis as evidenced by detection in the blood of naïve T cells with TRECs, with or without additional evidence from biopsies showing the features of thymopoeisis.

Skin rashes

Three patients, P1 (after 2nd transplant), P2 and P6, developed skin rashes early (3-6 weeks) after transplant. They underwent skin biopsy which showed a non-specific dermatitis similar to the spongiotic dermatitis previously described in these cases. No donor DNA could be detected in the skin or blood in any of these patients.

Infections cleared

Patients were able to clear a range of infectious agents after transplantation including those present before and those acquired after transplantation (Table II). Both cases receiving BCG vaccine prior to transplantation developed a localised severe inflammatory response at the inoculation site and in regional lymph nodes as T cell reconstitution occurred. In patient 3, a primary EBV infection occurred 15 months after transplantation. He was able to clear this infection though low level EB viraemia persisted for 18 months before clearing. P2, on chronic immunosuppression, managed to clear a number of virus infections.

Autoimmunity

Some form of autoimmune complication occurred in seven of the ten patients surviving to 12 months (Table II). This took one of two forms, a very early onset before evidence of T cell immune reconstitution or an onset at or after T cell reconstitution. More detail of the autoimmune/inflammatory complications in each patient are provided in the Online Registry (Table E1). Two cases (P4, P9) were in the early onset category, both with haemolytic anaemia which responded to treatment and did not recur. In five other patients, autoimmune problems, occurring at or after the time of T cell reconstitution, comprised mainly cytopaenias and/or thyroiditis. The latter was associated with the presence of anti-thyroid peroxisomal (TPO) antibodies. A number of other transient autoimmune /inflammatory phenomena also occurred in some patients at or soon after immune reconstitution. It was not possible to identify any association between the development of autoimmunity and any methodological factors including the choice of thymus donor, thymus culture medium used, amount of tissue transplanted or use of ATG conditioning. Six of the ten patients surviving to 12 months had partial HLA matching at 1-5 loci at 4-digit resolution typing (Table EII in Online Repository). The three patients without any autoimmune complications all fell in to this group but three others, also with some matching, developed autoimmunity though in one of these this was just a transient early

haemolysis. All patients without any HLA matching developed autoimmunity (one with transient early haemolysis only). A trend towards less autoimmunity in the presence of some HLA matching was not statistically significant (Fisher's exact test).

IMMUNOLOGICAL TESTING POST TRANSPLANTATION

T cell Immunity

Donor leukocyte engraftment was not detected in any of the patients. Circulating T-cell numbers in surviving patients rose from around 5 months and naïve T cells from around 6-7 months after transplantation (Figure 2). The correlation between naïve cell numbers using different flow cytometric strategies is shown in the Online Registry (Figure E2). Cell numbers achieved, generally, did not reach the normal age-related range (Table E III in the Online Repository). There was a continuing rise in naïve cell numbers up to 24 months and then maintenance at a relatively steady level. Low numbers of T cells in P2 were likely due to immunosuppression. No other patients received long term immunosuppression. Numbers of TRECs showed a similar time course to naïve T cells (Figure 3a). There was a relatively poor correlation between TRECs and naïve CD4 and CD8 cells (Figure E3 in the Online repository). Normal TCR diversity by V beta spectratyping of CD3 cells was achieved in seven patients, including those with atypical cDGS and an abnormal spectratype pre-transplantation (Figure E4 in the Online Repository). An abnormal spectratype persists in three patients (P2 P6 and P9). Further analysis showed a normal CD4 spectratype in P6 whilst both CD4 and CD8 spectratypes were abnormal in P9. Mitogen responsiveness to PHA (Figure 3b) improved in all patients but fell again with the immunosuppression in P2. For unknown reasons, it never normalised in P1. . This patient had good evidence for thymopoeisis on biopsy and blood analysis.Following primary EBV infection, peripheral blood mononuclear cells from Patient 3 showed the ability to produce a good interferon gamma (IFN γ) response against an autologous EBV transformed lymphoblastoid cell line (LCL) but responded significantly less well to a third party LCL (Figure 3c). Phenotyping of circulating cells with markers of Tregs was performed in five patients (P2,P4,P6,P9,P10)and showed these cells to be present in low absolute numbers though when expressed as the proportion of CD4 cells there was no difference to a healthy age- range matched control group (Figure 4a&b and Figure E5 in the Online Repository). In P2, P4, P6, P9 the proportions of CD45RA positive Tregs was 6,32,8 and 44 % respectively, whilst in the controls the median level was 67 (range 27-94). The functional ability of CD4+ Foxp3+ cells in six patients (P2,4,5,6,8,9) in terms of CTLA4 upregulation upon activation and transendocytosis of CD80 was comparable to adult control samples (Figure 4 c &d and Figures E6 &7 in the Online Repository). In P9, spectratyping performed on sorted Treg cells showed a diverse repertoire (Figure E8 in the Online Repository).

There was no correlation found between the level of immunological reconstitution achieved and factors relating to the choice of thymus donor, thymus culture medium used, amount of tissue transplanted or the use of ATG conditioning.

B cell immunity

All patients were on immunoglobulin replacement prior to transplantation. Five patients stopped immunoglobulin at around 24 months post-transplant as per the protocol and have

normal IgG levels. To date, five patients have been immunised against tetanus toxoid and show protective responses. Three received conjugate pneumococcal vaccine and two of these have made good protective responses. One patient failed to respond to this vaccine and is being re-immunised. IgA levels were undetectable before transplant in 11/12 patients and low (0.1g/L) in the other. The levels have normalised after transplant in all survivors with the exception of P2.

B cell numbers remained normal (Figure E9a in the Online Repository) in all patients except those (P2, 4)receiving treatment with anti CD20 monoclonal antibody (rituximab) The proportion of CD19+ B cells which were CD27+ IgD- (class switched memory B, CSMB, cells) was tested in 9 patients. It remained relatively low compared to published age related controls [15] in some patients whereas in others it was within normal limits particularly after two years. (Figure E9b in Online Repository)

THYMIC BIOPSIES

Biopsies of up to four transplanted thymic slices were undertaken on 11 patients (including one after each transplant in P1) at a median time of 4 months (range 2-8 months) after transplantation. Areas of histologically normal thymic tissue were seen in the muscle, including cortico-medullary distinction and Hassall body formation in 5 biopsies. In these biopsies immunohistochemical staining showed abundant T (CD3+) cells with evidence of cortical thymopoeisis, as defined by the expression of TdT, CD1a and Ki67, and of normal maturation to the late medullary thymic epithelial (mTEC) stage defined by the expression of CK5 and CK14, Claudin 4, AIRE and involucrin.Foxp3 staining showed frequent positive cells present (Fig 5 and Figure E10 in Online Repository). Biopsies in a further two cases (P8,P9) showed less well developed thymic architecture but definite evidence of cortical thymopoeisis as defined by the presence of CD1a and Ki67 positive cortical thymocytes (not shown). Biopsies with no evidence of thymopoeisis were found in P1 (first transplant), 2, 5 and 7. In P 2 & 5 it was likely the biopsies “missed” thymus in the muscle as there was later appearance of thymic emigrants in the blood indicating thymopoeisis. In P7 who died of CMV, a biopsy taken at four months showed viable thymic epithelium but very little thymopoeisis (Figure E11a-d in Online Repository)). CMV could not be demonstrated in this thymic tissue (not shown). P12 died very early after transplant and a post mortem examination of transplanted thymus revealed viable epithelium with extensive neovascularisation (Figure E11 e-f in Online Repository).

DISCUSSION

This study shows that transplantation of cultured thymic epithelium can reconstitute T cell immunity in patients with complete DiGeorge syndrome enabling them to control opportunistic infections and to have a quality of life not restricted by susceptibility to infection. This confirms and adds to the results in the previously reported series [6, 7], with the survival rate and the level of immune reconstitution achieved being similar between the two series. The proportion of children suffering autoimmune complications is higher in the present study but as numbers are relatively small it is difficult to know if this difference is

significant. In the present study, novel data documenting changes in the cellular composition of thymus slices during culture are provided as well as data on TREC levels achieved and numbers, phenotype and function of Tregs. There is also detailed histological evidence on thymic biopsies to confirm full maturation of mTEC. Whilst all but one of the patients in this study had a recognised genetic cause for DGS, the previous studies included a number of such cases, including those with maternal diabetes, and showed that such patients have an equivalent outcome.

The levels of T cell reconstitution achieved in surviving patients were not usually normal for age but were sufficient to allow clearance of viral and other infections. In most cases, normal mitogen responsiveness was achieved and a diverse repertoire was demonstrated on TCR spectratyping. Circulating Tregs could be detected in proportions similar to control children though at lower absolute numbers and their CTLA4 mediated function was shown to be normal. . Apart from one case in which an IFN γ response to EBV was shown, antigen specific T cell responses were not assayed in this study. Such responses were studied to tetanus and candida antigens in the previous series and showed positive responses in all but one of the surviving patients [7] Most patients with follow up of more than two years have been able to stop immunoglobulin and, in those tested so far, all show normal antibody responses to tetanus and two of three to conjugated pneumococcal vaccine. IgA deficiency corrected in all but one patient. The numbers of class switched memory B cells remains relatively low in some patients, but in order to assess the significance of this finding longer follow up is needed to see if the proportions rise with time. The reasons for the suboptimal numbers of T cells achieved in most patients is not clear. It could be that insufficient thymus tissue was transplanted but against this is the fact that there was no correlation in this study or in the North American series [8] between the amount of tissue transplanted and the eventual T cell or naïve T cell counts achieved. Neither was there any association between counts and the type of medium used for culture, the use of ATG nor the presence of chance overlap of HLA antigens between donor and recipient.

We have shown here that the cultured thymus loses most of its lymphoid cell populations during culture and is relatively enriched for thymic epithelial cells (TECs). However, viable lymphoid cells capable of proliferation are still present. These cells may be necessary for the maintenance and growth of the TECs[16]. Theoretically, these cells could mediate graft versus host disease but this was not seen and, on blood analysis, engraftment of donor haematopoietic cells was not detected in any patient. One situation where thymopoeisis may not develop is in the context of pre-existing cytomegalovirus (CMV) infection as seen in P7 in this study and in the previous studies [7, 17]. The finding of viable thymic epithelium but no thymopoeisis on biopsy is consistent with the possibility that this virus, the agents used to treat it or both may inhibit the development of thymopoeisis. Children with cDGS complicated by CMV infection did not survive in either this or the previous study.
Biopsy of transplanted thymus has been shown to be helpful in determining whether thymopoeisis is developing[17]. In that report biopsies were done at around two months post-transplantation. The positive ones all showed evidence of cortical thymopoeisis but, in over half, no thymic medulla or Hassalls corpuscles were seen[17]. In the present study, biopsies were done later (median 4 months). In most of those that were positive there was clear cortico-medullary differentiation as well as development of Hassalls corpuscles with

immunohistochemical evidence that differentiation of mTEC proceeds to the terminal stages. It is likely that the difference in timing of the biopsies accounted for these differences between this and the previous series.

In the present and previous series, autoimmune complications were relatively common, predominantly involving thyroiditis and cytopaenias. Some of these complications were of a transient nature which may reflect immune dysregulation during T cell reconstitution sometimes seen in other clinical situations such as after HSCT and in experimental models [18]. Two very early cases of autoimmunity were seen before any T cell emergence and could conceivably have had nothing to do with the transplant.
The reasons for the susceptibility to autoimmune complications are poorly understood. The possibility that inadequate negative selection by non-MHC matched mTEC contributes to the development of autoimmunity was not supported by the finding in this and the previous larger study [8] of no beneficial effect of chance, partial HLA matching.
In conclusion, this study has strengthened the case for thymus transplantation being the corrective treatment of choice for complete DiGeorge syndrome, offering the possibility of immune reconstitution to a degree that will give a quality of life not limited by infection susceptibility. Autoimmunity, a common complication, can often be managed relatively easily but a proportion of children can suffer serious consequences. Further work is required to understand better the pathogenesis of this problem. As newborn screening programmes for SCID expand, more patients may require this treatment. Further work is needed to streamline the labour-intensive process requiring specialised facilities for generating and transplanting thymus. A model of human thymus transplantation into the nude mouse may be useful in further exploring this [19]. Other patients who might benefit from this approach include SCID infants who fail to immune reconstitute after HSCT or gene therapy because of thymic insufficiency.

ACKNOWLEDGMENTS

The following provided technical help in thymus preparation: Margaret Brocklesby; Geoffrey White, Chris Fisher, Catherine Ingram, Gulrukh Ahsan, Patricia Plumbly.
Drs John Hartley, James Soothill and Dr Garth Dixon provided invaluable help in microbiological screening of donors and donor thymuses.
Dr Christine Rivat helped sort cells for spectratyping.
Patricia Cheng provided invaluable help in manuscript preparation.
The following assisted in the clinical care of the patients: Tore Gunnar Abrahamsen, Nathalie Aladjidi, Waseem Qasim, Caroline Laffort, Christine Vaksdal Nilsen, Mari-Anne Vals.

REFERENCES

1. Davies, E.G., *Immunodeficiency in DiGeorge Syndrome and Options for Treating Cases with Complete Athymia.* Front Immunol, 2013. **4**: p. 322.
2. Ryan, A.K., et al., *Spectrum of clinical features associated with interstitial chromosome 22q11 deletions: a European collaborative study.* J.Med.Genet., 1997. **34**(10): p. 798-804.
3. Markert, M.L., et al., *Complete DiGeorge syndrome: development of rash, lymphadenopathy, and oligoclonal T cells in 5 cases.* J Allergy Clin Immunol, 2004. **113**(4): p. 734-41.
4. McGhee, S.A., M.G. Lloret, and E.R. Stiehm, *Immunologic reconstitution in 22q deletion (DiGeorge) syndrome.* Immunol.Res., 2009. **45**(1): p. 37-45.

5. Janda, A., et al., *Multicenter survey on the outcome of transplantation of hematopoietic cells in patients with the complete form of DiGeorge anomaly.* Blood, 2010. **116**(13): p. 2229-2236.
6. Markert, M.L., B.H. Devlin, and E.A. McCarthy, *Thymus transplantation.* Clin.Immunol., 2010. **135**(2): p. 236-246.
7. Markert, M.L., et al., *Review of 54 patients with complete DiGeorge anomaly enrolled in protocols for thymus transplantation: outcome of 44 consecutive transplants.* Blood, 2007. **109**(10): p. 4539-4547.
8. Markert, M.L., et al., *Factors affecting success of thymus transplantation for complete DiGeorge anomaly.* Am.J.Transplant., 2008. **8**(8): p. 1729-1736.
9. Markert, M.L., et al., *Successful formation of a chimeric human thymus allograft following transplantation of cultured postnatal human thymus.* J.Immunol., 1997. **158**(2): p. 998-1005.
10. Hassan, A., et al., *Host natural killer immunity is a key indicator of permissiveness for donor cell engraftment in patients with severe combined immunodeficiency.* J Allergy Clin Immunol, 2014. **133**(6): p. 1660-6.
11. Amrolia, P.J., et al., *Adoptive immunotherapy with allodepleted donor T-cells improves immune reconstitution after haploidentical stem cell transplantation.* Blood, 2006. **108**(6): p. 1797-808.
12. Schubert, D., et al., *Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations.* Nat Med, 2014. **20**(12): p. 1410-6.
13. Yang, J., et al., *Characterization of Epstein-Barr virus-infected B cells in patients with posttransplantation lymphoproliferative disease: disappearance after rituximab therapy does not predict clinical response.* Blood, 2000. **96**(13): p. 4055-63.
14. Rucci, F., et al., *Abnormalities of thymic stroma may contribute to immune dysregulation in murine models of leaky severe combined immunodeficiency.* Front Immunol., 2011. **2**(15).
15. Morbach, H., et al., *Reference values for B cell subpopulations from infancy to adulthood.* Clin Exp Immunol, 2010. **162**(2): p. 271-9.
16. Anderson, G. and E.J. Jenkinson, *Lymphostromal interactions in thymic development and function.* Nat.Rev.Immunol., 2001. **1**(1): p. 31-40.
17. Markert, M.L., et al., *Use of allograft biopsies to assess thymopoiesis after thymus transplantation.* J.Immunol., 2008. **180**(9): p. 6354-6364.
18. King, C., et al., *Homeostatic expansion of T cells during immune insufficiency generates autoimmunity.* Cell, 2004. **117**(2): p. 265-77.
19. Furmanski, A.L., et al., *T-cell reconstitution after thymus xenotransplantation induces hair depigmentation and loss.* J.Invest Dermatol., 2013. **133**(5): p. 1221-1230.
20. Shearer, W.T., et al., *Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study.* J Allergy Clin Immunol, 2003. **112**(5): p. 973-80.

504 **Table I – Patient Characteristics**

505

Patient/Gender/Age at transplant (months)	Diagnosis	CD3 (naïve) x 10^6/L	Other problems & infection at the time of transplantati
1. Female, 14 & 26*****	CHARGE (CHD7)	Typical 20 (0)	Atrioventricular Canal, Hypoparat Recurrent Sepsis, Non-specific e Previous B cell lymphoma HHV6
2.Male, 8	22q.11.2 deletion	Typical 30 (0)	Fallots Tetralogy, Hypoparathyroi *C.difficile*
3. Male, 18	CHARGE (CHD7)	Atypical 1200 (0)	Choanal atresia –Tracheostomy, Bilateral facial nerve palsy, Small ventricular septal defect (cl Chronic lung disease (colonised v
4. Male, 26	CHARGE (CHD7)	Atypical 800 (0)	Truncus arteriosus, Nephrocalcin Chronic lung disease, enteropathy, Rotavirus
5. Male, 9	Undefined	Typical 30 (0)	Truncus arteriosus, Hypoparathyr Not dysmorphic, BCG, Rotavirus
6. Male,10	CHARGE (CHD7)	Atypical 650 (0)	Fallots Tetralogy, Hypoparathyroi Chronic enteropathy, Norovirus
7. Male, 4$^+$	22q.11.2 deletion	Atypical 1470 (0)	Patent ductus, Bronchomalacia, Hypoparathyroidism**,** CMV
8. Male, 5	22q.11.2 deletion	Atypical 350 (0)	Recurrent aspiration, Hypoparath Ventricular septal defect (closed s Patent foramen ovale
9. Male, 16	Undefined (Putative Mutation in TBX1	Atypical (mild) 120 (2)	Recurrent sepsis, Mastoiditis, Hyp Hypothyroidism BCG, Rotavirus,
10. Female 2.5 $^+$	22q.11.2 deletion	Typical 0	Truncus arteriosus Aortic incompe Hypoparathyroidism, Hypothyroid Recurrent pneumonia
11. Male, 5	22q.11.2 deletion	Atypical 1250 (40)	Hypoparathyroidism, Asymptomatic Coronavirus
12. Male, 14 $^+$	22q.11.2 deletion	Atypical 370 (0)	Hypoparathyroidism, chronic lung Parainfluenza 3, Rotavirus

506 **Footnotes: *** two transplants; $^+$ these patients subsequently died after transplantation

507 BCG – Bacillus Calmette Guerin, CMV – Cytomegalovirus, RSV – Respiratory syncytial virus

508 **Table II - Clinical outcome in patients surviving beyond 12 months**
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510

Patient Follow up (months)	Infections cleared (bold if present pre-transplantation)	Autoimmunity	Attending School/ Pre-school	Significant Ongoing Treatments
1. 69 (after second transplant)	**HHV6**	Transient nephritis Thyroiditis	YES	Thyroxine
2. 80	**C. difficile**, RSV Adenovirus^{+} Enterovirus, Varicella Parainfluenza 3 Norovirus,Rhinovirus	Transient colitis Chronic AIHA ITP	YES	Splenectomy. Sirolimus Iron Chelation IG replacemer
3.M, 67	RSV, Parainfluenza 3 Metapneumovirus, EBV (primary)	None	YES	Azithromycin prophylax Tracheostomy decanulat
4.M, 55	**Rotavirus** Parainfluenza 3 Metapneumovirus, RSV, Influenza A	Early transient AIHA	YES	Azithromycin prophylax
5.M, 49 mo	**BCG, Rotavirus** Parainfluenza 3	None	YES	Azithromycin prophylax
6.M, 46	**Norovirus**	None	YES	Ig therapy Cleft lip/palate re
8. M 30	Rhinovirus, RSV	Thyroiditis ITP, Neutropenia	YES	Gastrostomy fee Thyroxine

9. M 25	**BCG, Rotavirus, RSV**	Early transient AIHA	NO	On Ig therapy Thyroxine
10. F 23	HHV6, Adenovirus	ITP – Fatal at 23 months post-transplant	NO	On Ig therapy Thyroxine
11. M 21	**Coronavirus**, *C.difficile* Campylobacter	Thyroiditis ITP Elevated transaminases	YES	On Ig therapy Thyroxine

Footnotes:

$^{+}$ -1x10^{5} copies/ml of blood

AIHA – Autoimmune haemolytic anaemia; BCG – Bacillus Calmette –Guerin; EBV – Epstein Barr virus; HHV6 – Human herpes virus 6; Ig – Immunoglobulin ; ITP – Immune thrombocytopaenia; RSV – Respiratory syncytial virus;

Legends to Figures:

1. Analysis of cellular composition of thymus slices by flow cytometry at different time points during culture. **a.** Dot plots show representative anti-CD45 versus EpCam 1 staining. The percentages of EpCam1+CD45- cells are given in the regions shown. Histograms show anti-HLA DR staining gated on the EpCam1+ CD45- population shown in the dot plots. **b.** Number of live cells recovered showing the overall number of thymocytes and the number ofCD4/CD8 double positive (DP) thymocytes retrieved per mg of tissue. **c.** The percentage of cells that were CD45-EpCam1+HLA-DR+ (as frequency of live gate). **d.** Proportion of cells in each thymocytesubset (SP, single positive; DP, double positive; DN, double negative) based on CD4 and CD8 surface expression. **e.** When stimulated for 5 days, thymocytes from day 15 slices proliferate. **f.** When stimulated for 72hours, CD4 single positive thymocytes from day 22 slices upregulate the activation marker, CD25

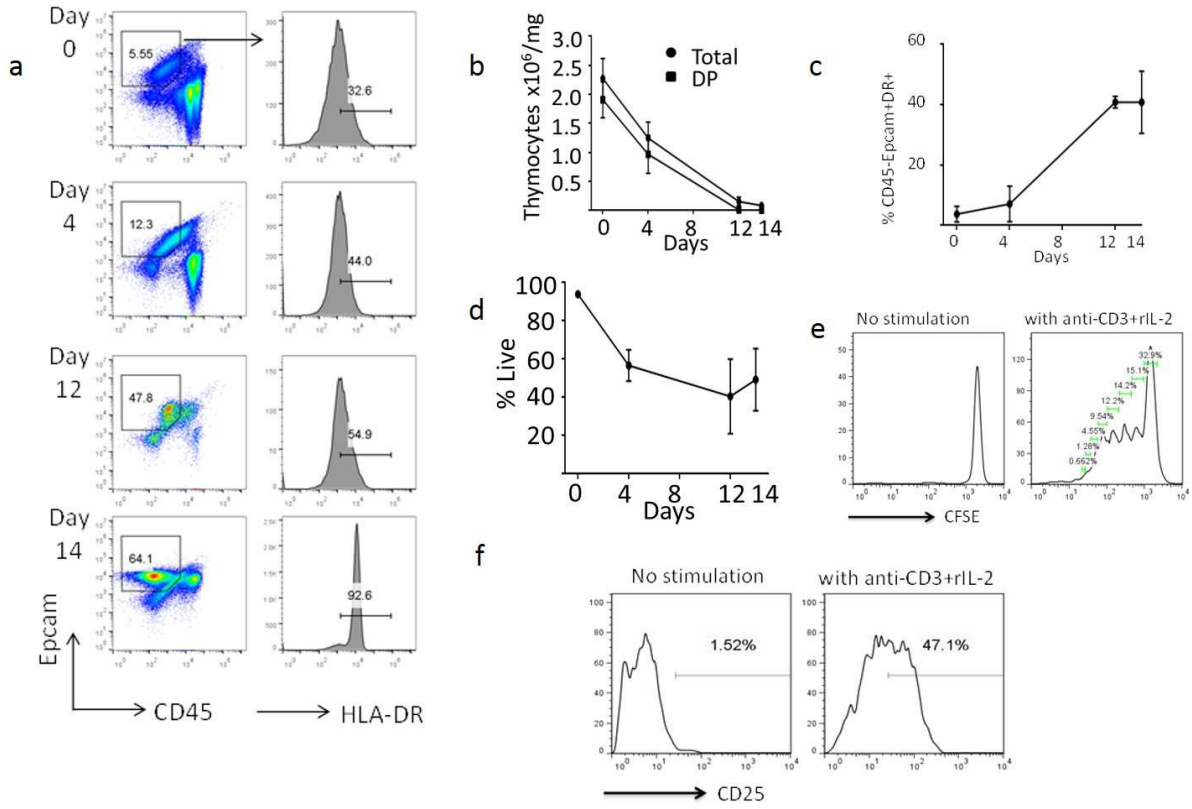
2. T cell reconstitution after transplantation. Dotted lines indicate 10^{th} percentile of published lymphocyte subset counts in normal children aged 1-2 years and 2-5 years [20].

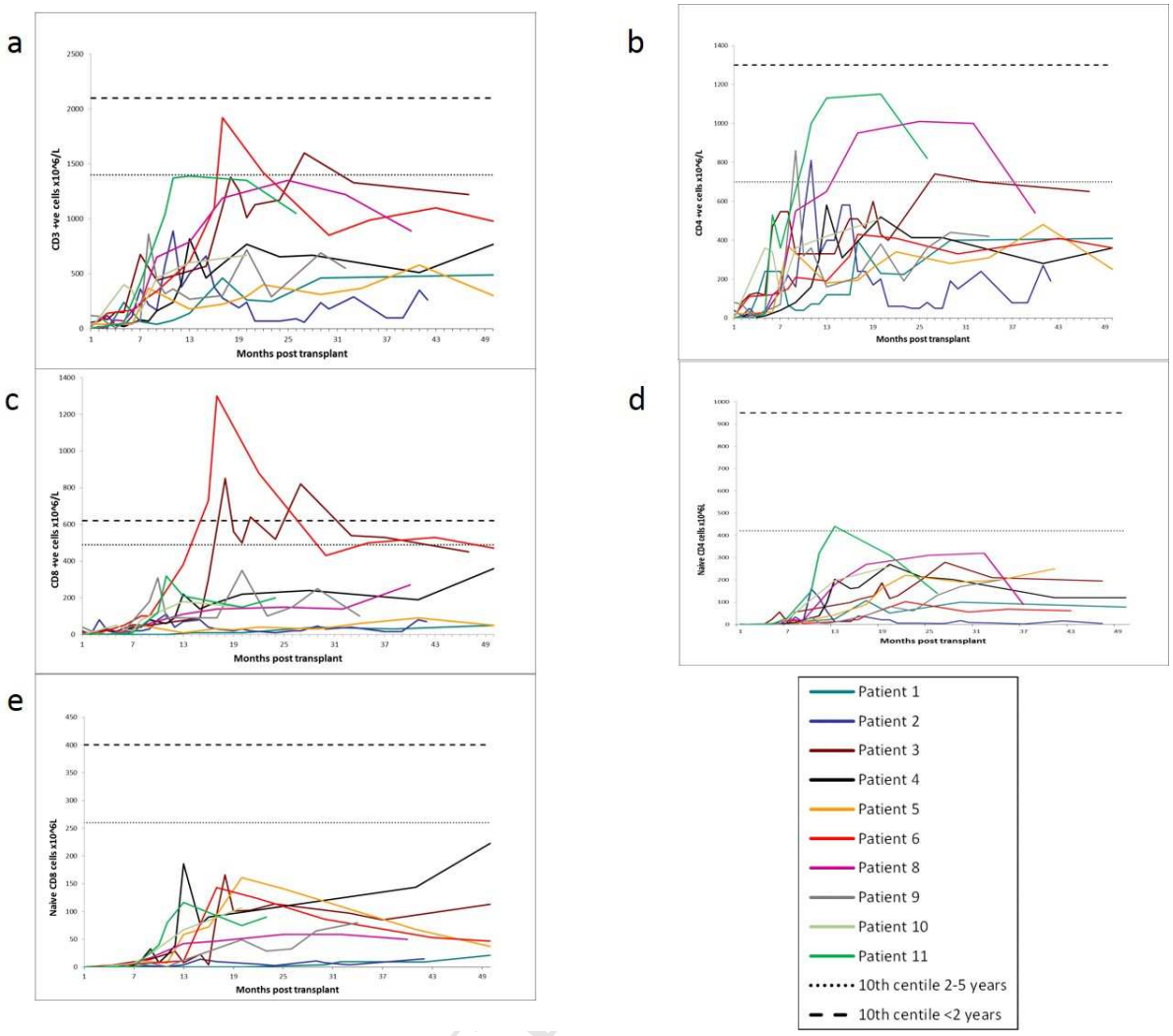
3. a. TREC levels performed on CD3 cells with 10^{th} percentile for *in-house* normal ranges for children of <2 years and 2-5 years. **b.** PHA responses – maximum counts per minute after stimulation of isolated mononuclear cells stimulated with phytohaemagglutinin. Dotted line indicates the 10^{th} percentile for in-house normal adult controls **c.** Frequency of interferon gamma producing cells in patient's peripheral blood mononuclear cells (PBMC) measured by ELISPOT (mean ± SEM) in response to autologous and third party EBV transformed lymphoblastoid cell lines in P3 Following primary Epstein Barr virus infection. Two-tailed Student's t-test for unpaired samples was applied.

1) **4. a & b.** Cells with T regulatory phenotype expressed as percentage of CD4 cells and in absolute numbers in patients (n=5) and an age-range matched control group (n=11). **c. & d.** Transendocytosis assay shows CD4 + FOXP3+ cells in patients (n=6) and controls (n=5) incubated with anti CD3 plus untransfected Chinese Hamster ovary (CHO) cells, or with anti CD3 plus CHO transfected with CD80 with or without anti-CTLA4. **c.** Upregulation of CTLA4 expression (shown as mean fluorescence intensity , MFI, of Tregs normalised to MFI of CTLA4 in that individual's own naïve conventional T cells (as internal negative control). Panel **d.** Relative total fluorescence intensity of CD4+ Foxp3 + cells that have acquired GFP tagged onto CD80 as a result of transendocytosis of CD80. This is derived from MFI of GFP multiplied by the number of GFP positive cells to get total fluorescence intensity, divided by number of Tregs acquired. In both panels, the patients and controls showed equivalent results.

5. Histological appearances of positive thymic biopsies. **a & b.** haematoxylin & eosin showing medullary differentiation and Hassall body formation. Original

magnification 10x and 40x respectively. **c.** expression of Foxp3 within thymic medulla (brown). Original magnification 20x. **d.** Double staining with TdT (brown, nuclear signal) showing immature thymocytes within the cortical area and CD3 (blue, membrane signal) highlighting maturing T lymphocytes within the medulla (Original magnification 40x).**e.** AIRE expressing cells within medullary region (original magnification 20x) **f.** Double staining for AIRE (brown) and Involucrin (blue) that shows co-localization of AIRE expressing cells with fully mature involucrin expressing mTEC (original magnification 40x).





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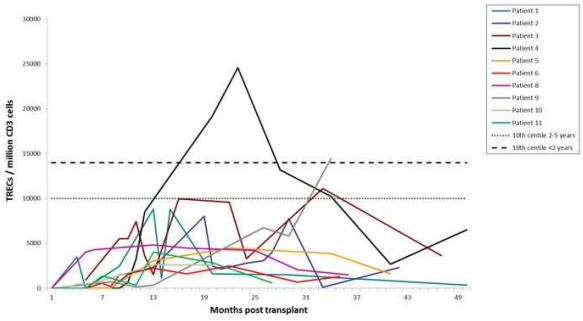

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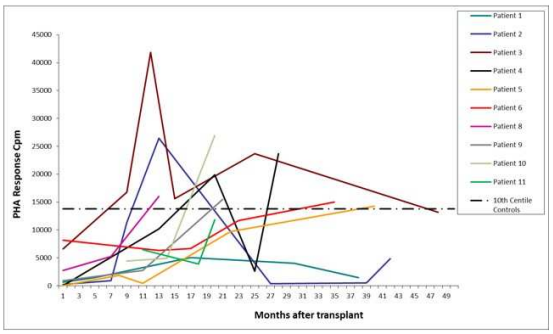

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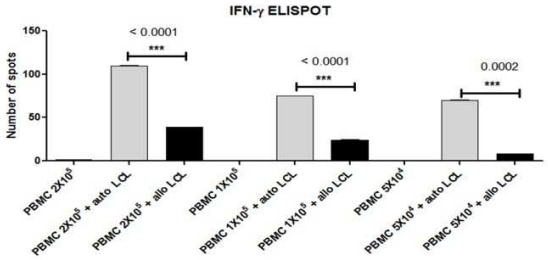

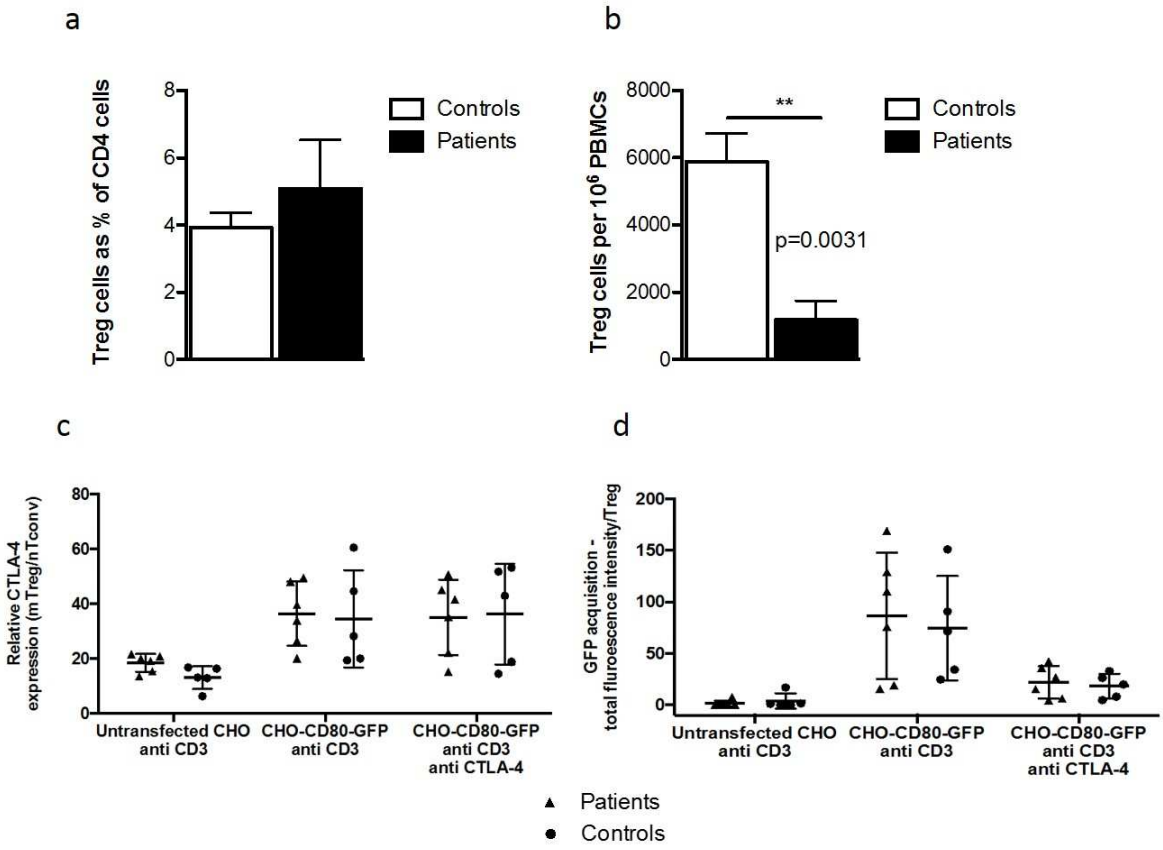

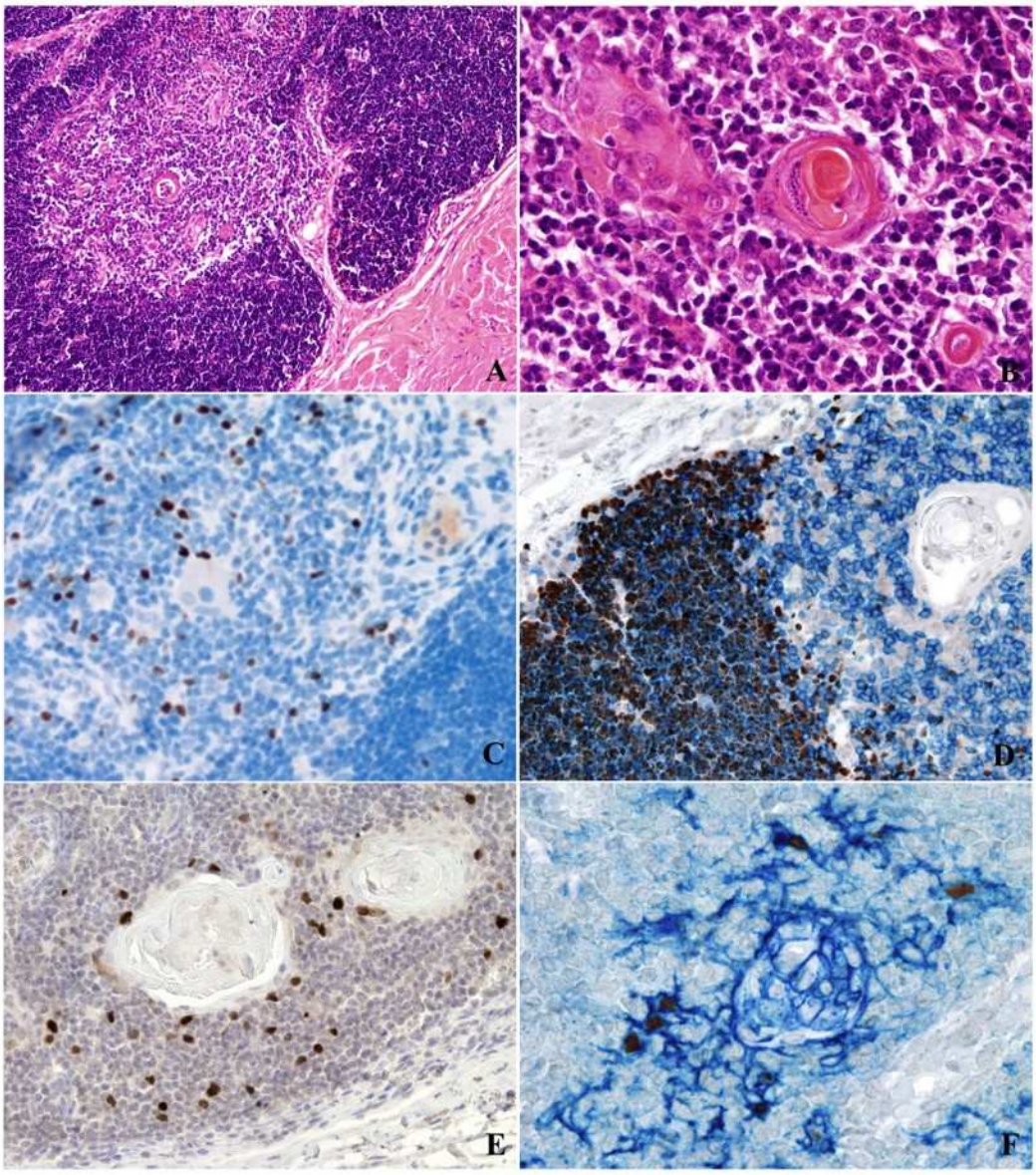

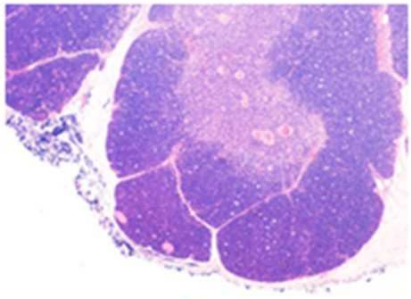

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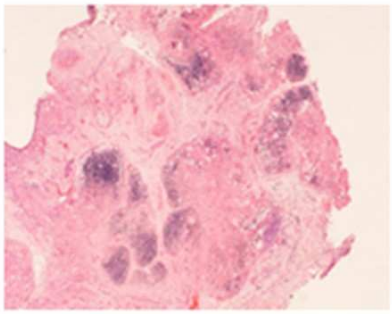

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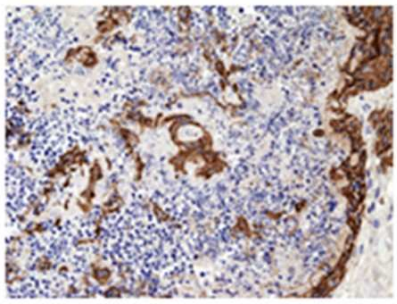

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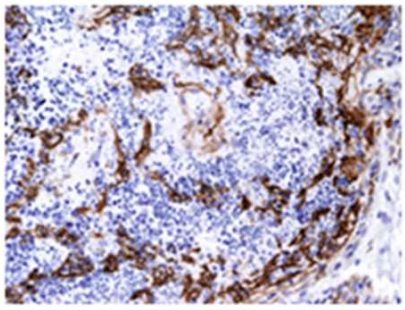

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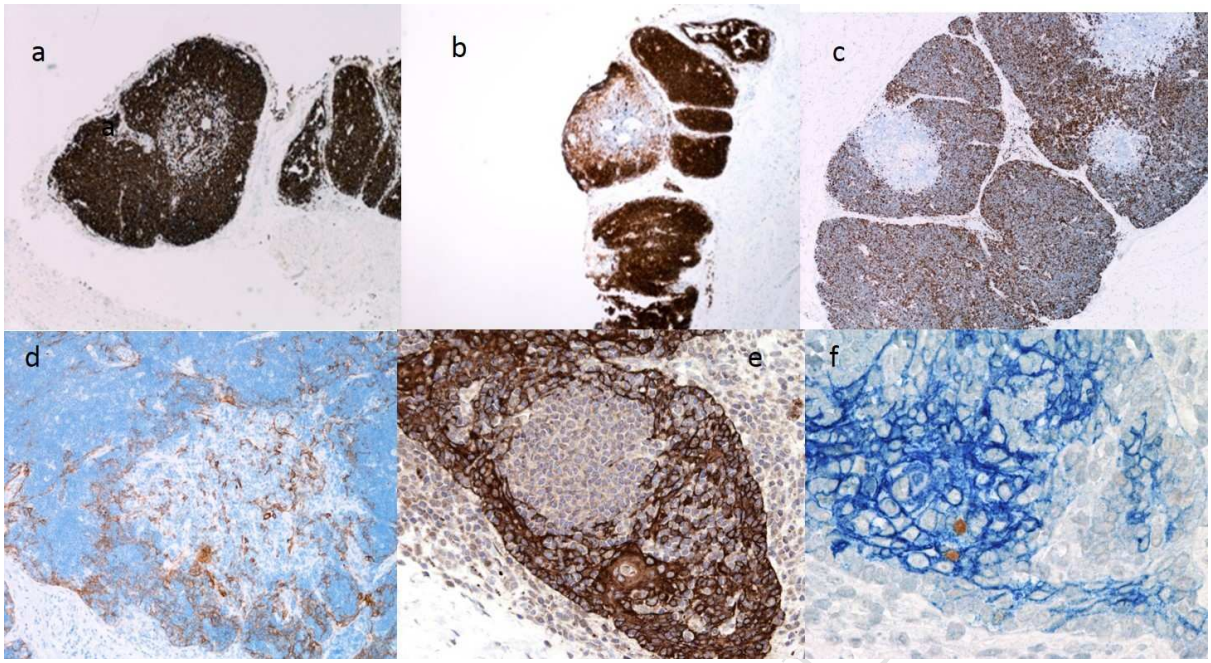

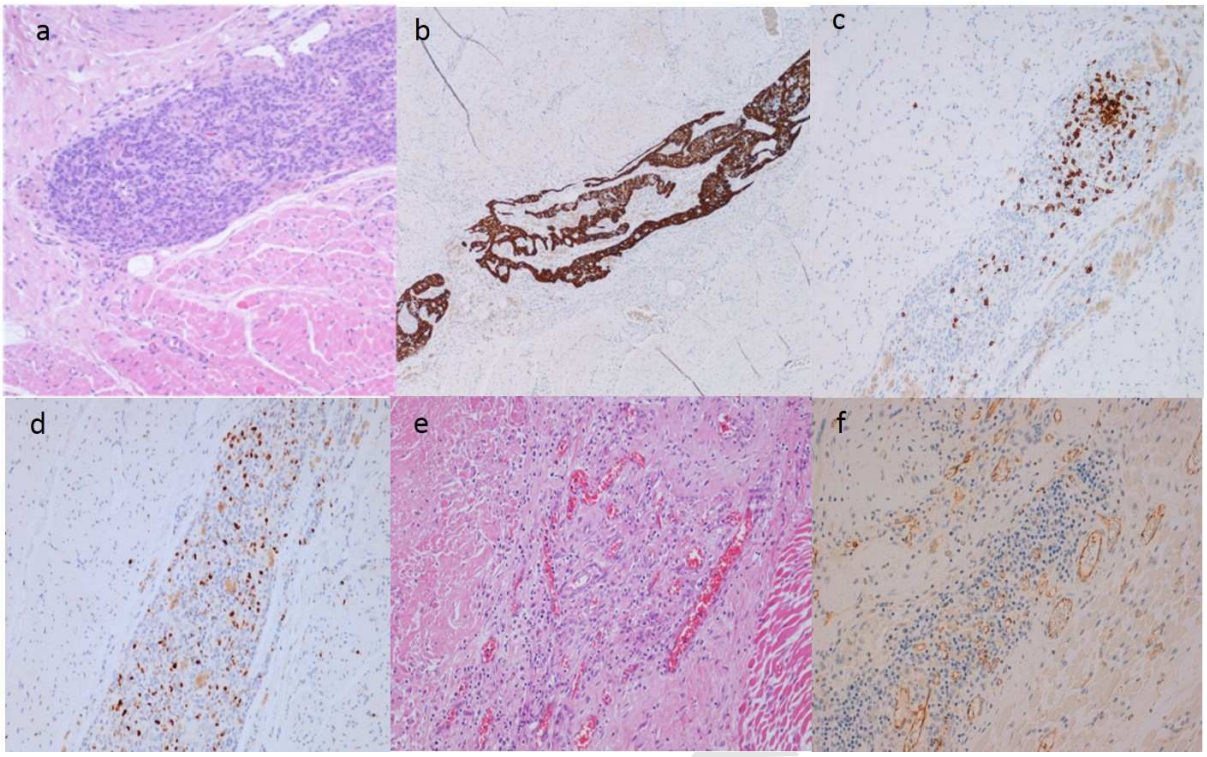

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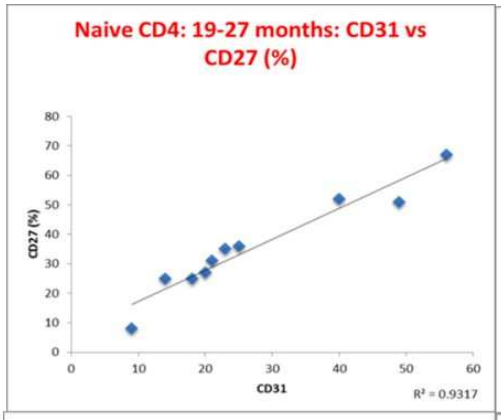

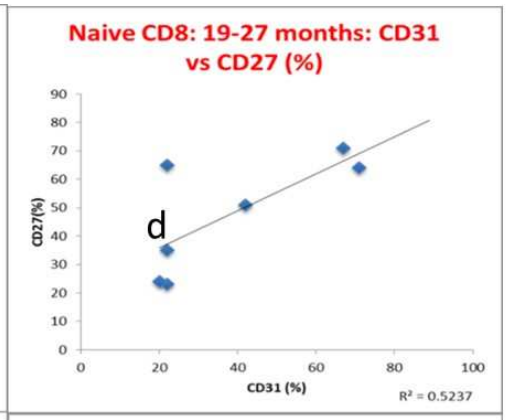

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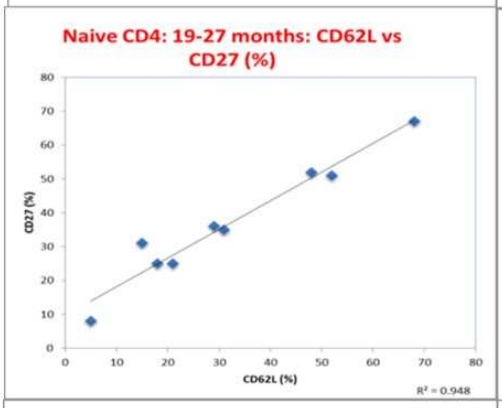

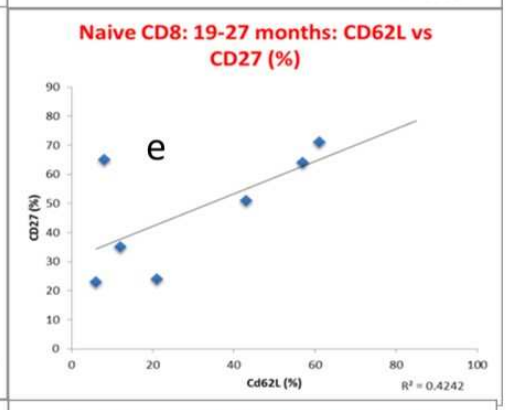

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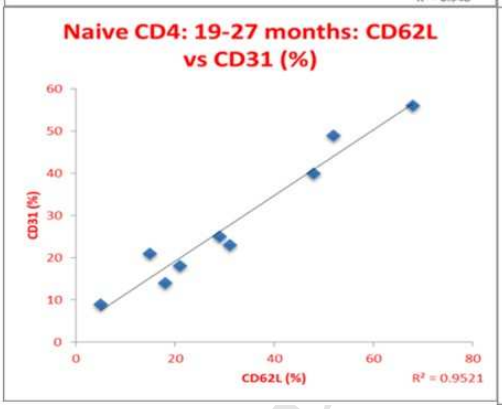

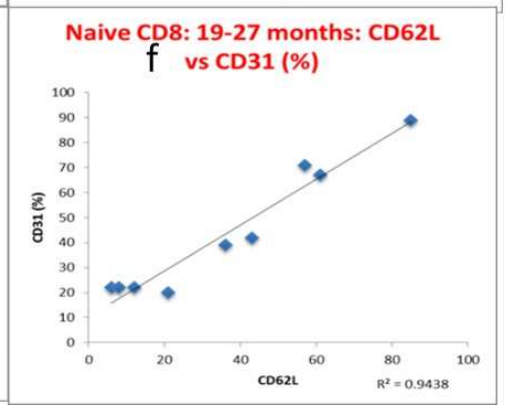

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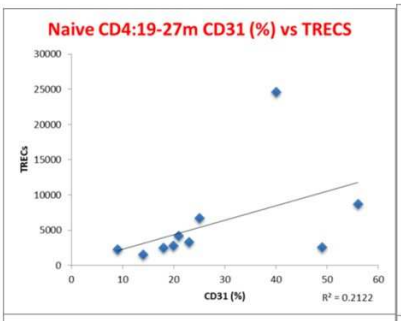

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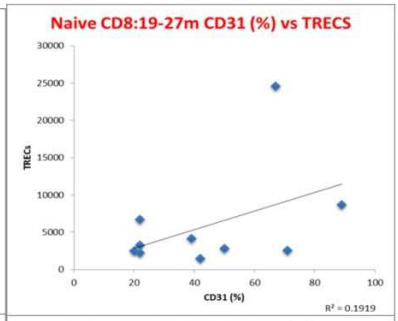

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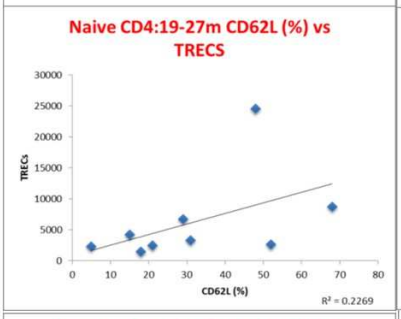

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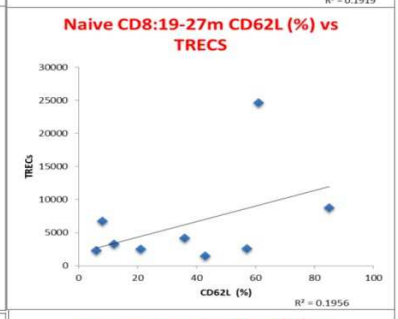

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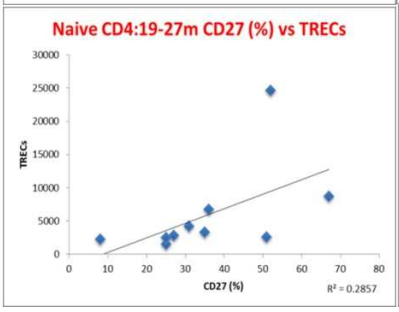

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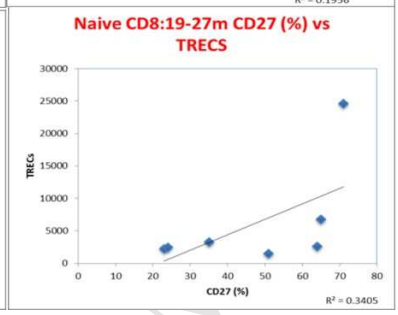

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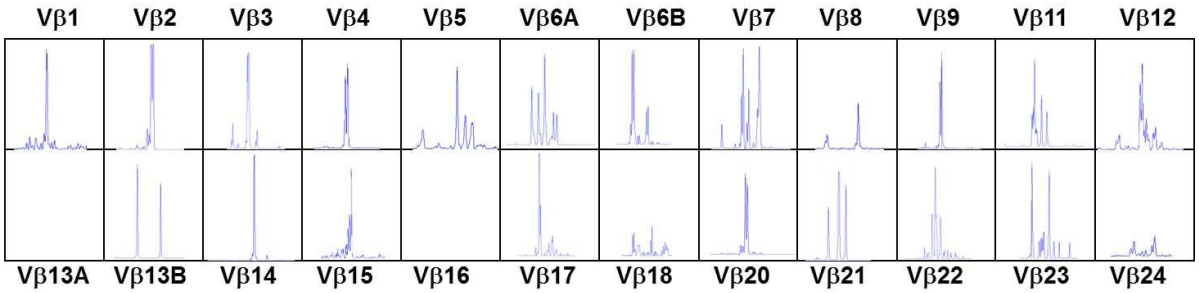

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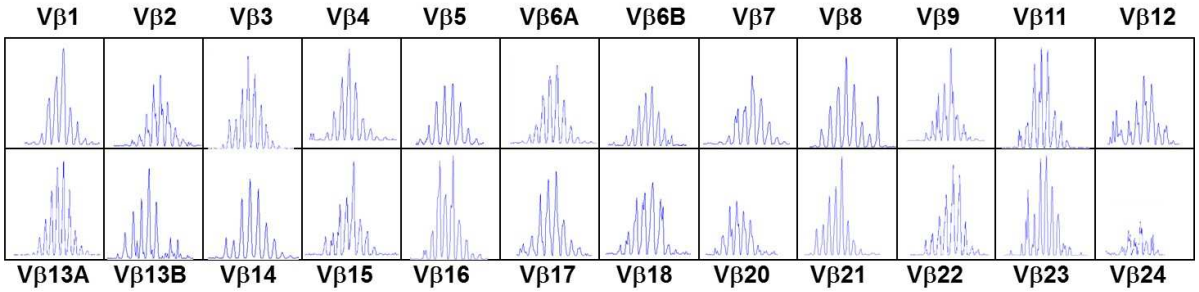

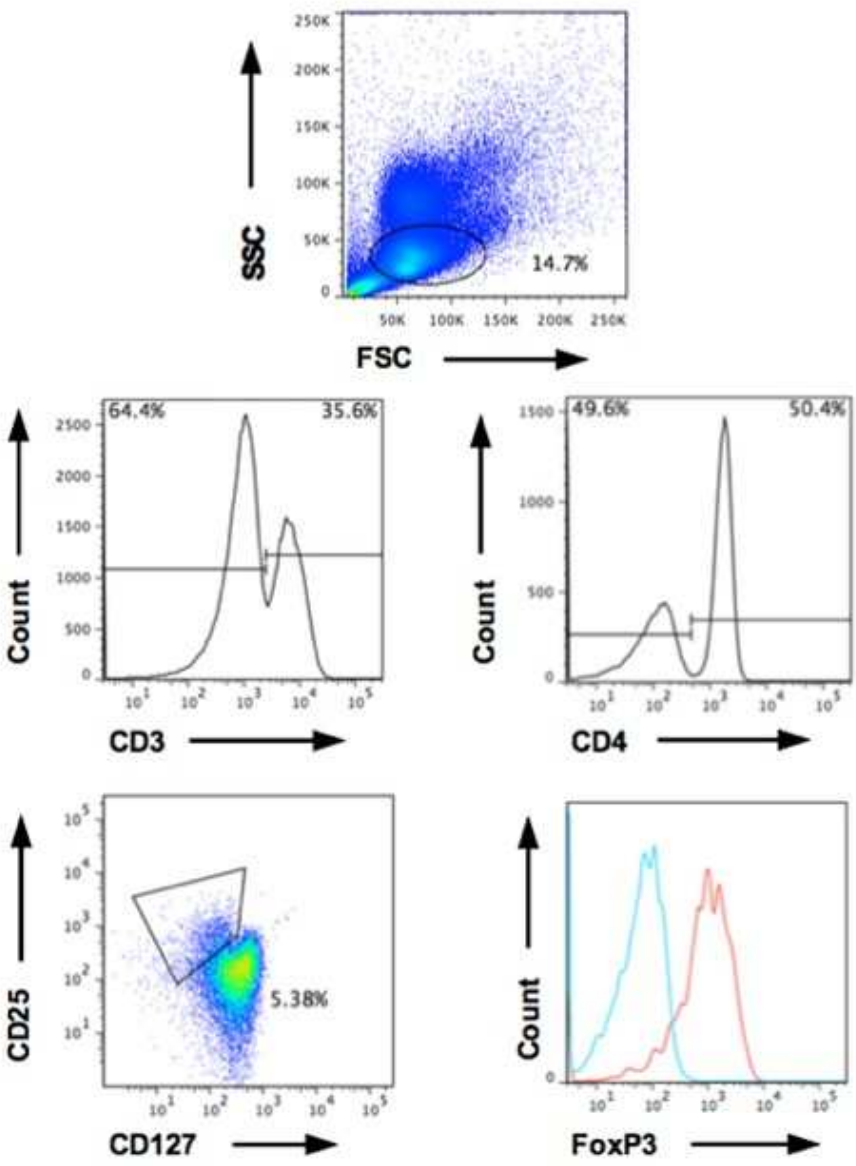

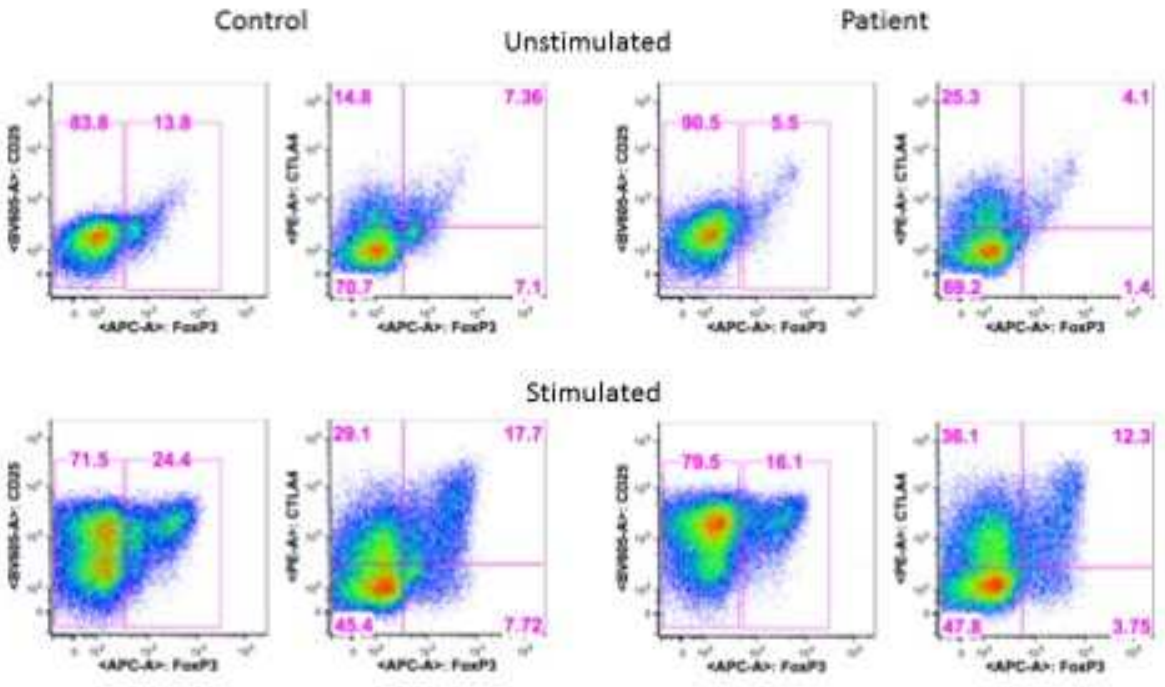

Gated on all CD4+ lymphocytes

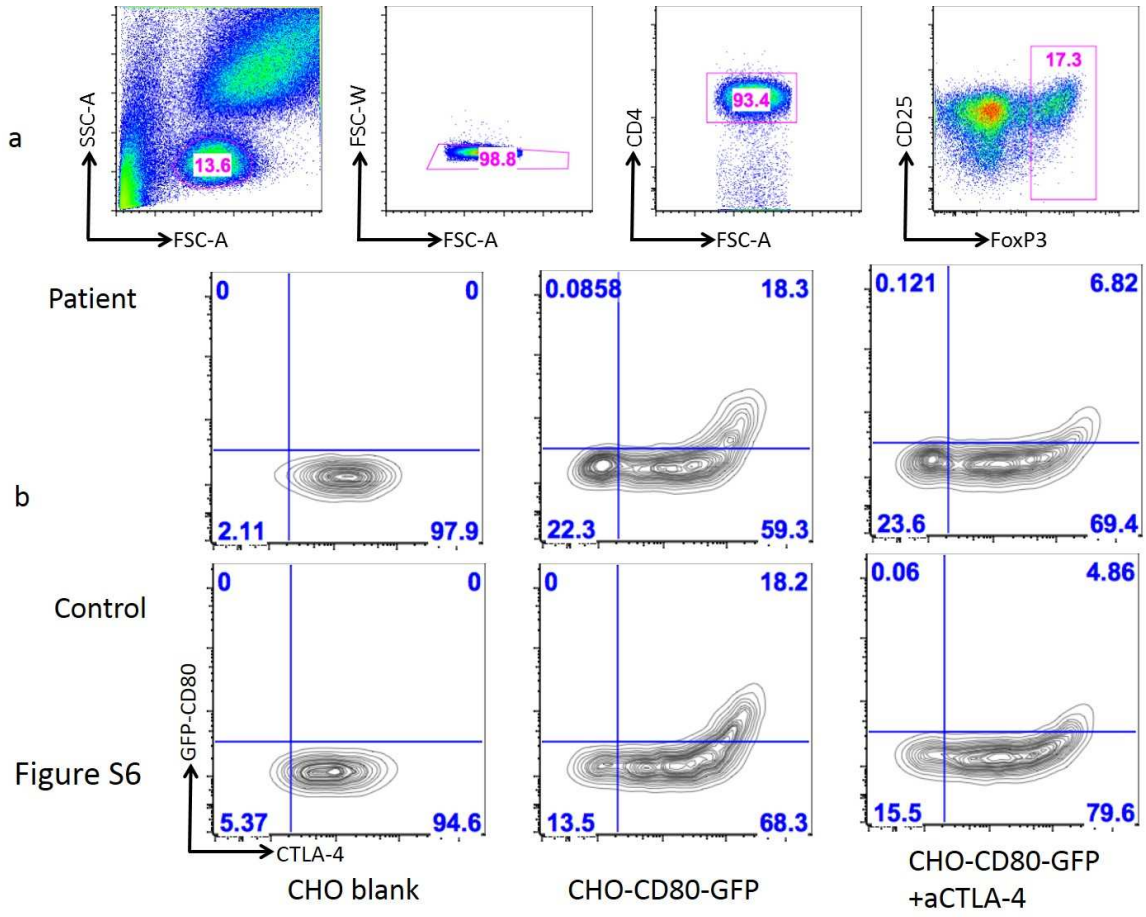

Figure S6

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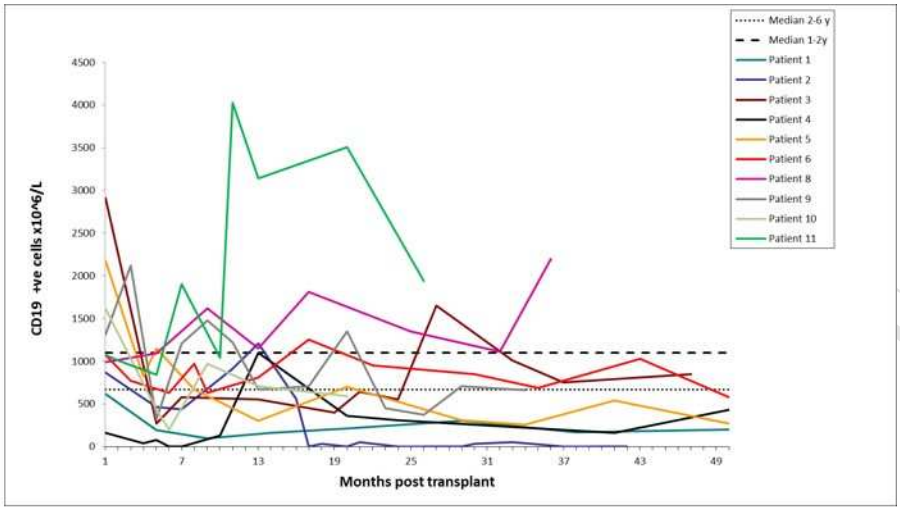

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