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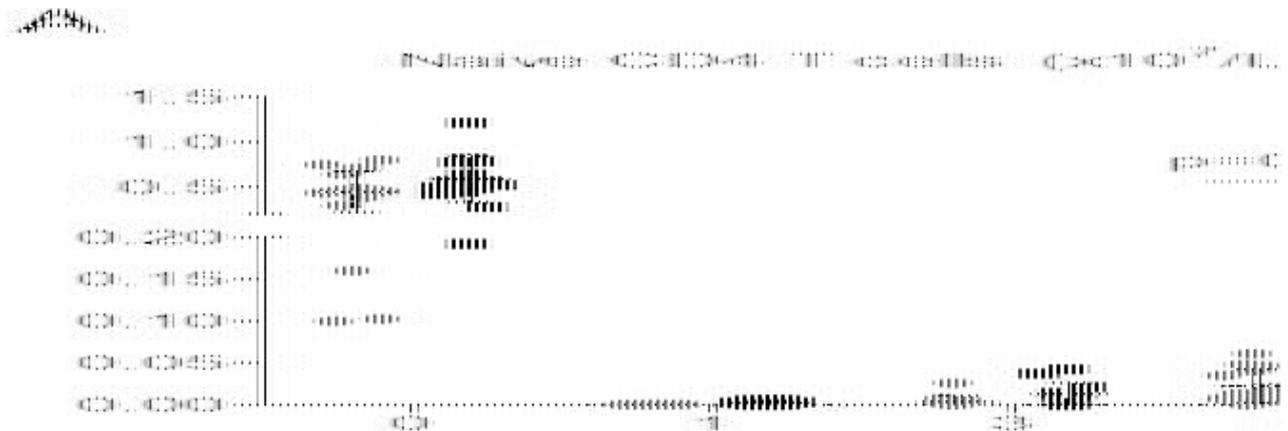
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Keratinocyte growth factor impairs human thymic recovery from lymphopenia

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Conflict of interests: AJC reports that, prior to September 2017, he received speaker honoraria and consulting fees from Genzyme Sanofi; RSB reports receiving speaker honoraria from Amgen, Novartis, MSD, Bristol-Myers Squibb, AstraZeneca, Roche, and Celgene, and consulting fees from MSD, Bristol-Myers Squibb and AstraZeneca. DJ has received research grant and consulting fees from Roche/Genentech and consulting fees from GSK and Chemocentryx. JLJ reports receiving speaker and honoraria and consulting fees from Genzyme Sanofi. All other authors declare no competing interests.

Abstract

Background: The lymphocyte-depleting antibody alemtuzumab is a highly effective treatment of relapsing-remitting multiple sclerosis (RRMS); however 50% of patients develop novel autoimmunity post-treatment. Most at risk are individuals who reconstitute their T-cell pool by proliferating residual cells, rather than producing new T-cells in the thymus; raising the possibility that autoimmunity might be prevented by increasing thymopoiesis. Keratinocyte growth factor (palifermin) promotes thymopoiesis in non-human primates.

Methods: Following a dose-tolerability sub-study, individuals with RRMS (duration ≤ 10 years; expanded disability status scale ≤ 5.0 ; with ≥ 2 relapses in the previous 2 years) were randomised to placebo or 180mcg/kg/day palifermin, given for 3 days immediately prior to and after each cycle of alemtuzumab, with repeat doses at M1 and M3. The interim primary endpoint was naïve CD4+ T-cell count at M6. Exploratory endpoints included: number of recent thymic-emigrants (RTEs) and signal-joint T-cell receptor excision circles (sjTRECs)/mL of blood. The trial primary endpoint was incidence of autoimmunity at M30.

Findings: At M6, individuals receiving palifermin had fewer naïve CD4+T-cells ($2.229 \times 10^7/L$ vs. $7.733 \times 10^7/L$; $p=0.007$), RTEs (16% vs. 34%) and sjTRECs/mL (1100 vs. 3396), leading to protocol-defined termination of recruitment. No difference was observed in the rate of autoimmunity between the two groups

Conclusion: In contrast to animal studies, palifermin reduced thymopoiesis in our patients. These results offer a note of caution to those using palifermin to promote thymopoiesis in other settings, particularly in the oncology/haematology setting where alemtuzumab is often used as part of the conditioning regime.

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Introduction

T-cell lymphopenia is strongly associated with autoimmunity (1-5). A striking example is autoimmunity following treatment of relapsing remitting multiple sclerosis (RRMS) with the lymphocyte-depleting humanised anti-CD52 monoclonal antibody alemtuzumab (Lemtrada). Two short courses of alemtuzumab given 12 months apart effectively suppress RRMS for many years (6-11) however between 6 months and five years after treatment 40% of patients develop thyroid autoimmunity (typically Graves' disease). A further 2% of individuals develop idiopathic thrombocytopenic purpura (ITP), 0.1% anti-glomerular basement membrane (GBBM) disease and rare cases of autoimmune haemolytic anaemia, autoimmune neutropenia and autoimmune pancytopenia have been reported. An additional 20% of patients develop novel autoantibodies without clinical symptoms (6-8, 12-14).

We have previously shown that, while B-cell reconstitution after alemtuzumab is rapid, via the generation of new cells from the bone marrow (15), CD4 and CD8 T-cells take 35 and 20 months respectively to reach normal range (16). Furthermore, and paradoxically, for at least nine months after treatment, thymopoiesis (determined by measuring naïve T-cell production, recent thymic emigrants and T-cell receptor excision circles) is reduced (17). Instead, T-cell reconstitution occurs by the proliferation of cells that have escaped depletion. As a result the post-treatment T-cell pool is dominated by "memory-like cells" with a restricted T-cell receptor (TCR) repertoire (17). In keeping with animal studies demonstrating the pro-autoimmune nature of lymphopenia-induced T-cell proliferation, (2-4, 18) we have shown that individuals with the least thymic function and most restricted TCR repertoire after alemtuzumab are at greatest risk of developing autoimmune complications (17). These observations raised the possibility that autoimmunity after alemtuzumab might be reduced if thymic function could be restored.

Keratinocyte growth factor (KGF) promotes thymopoiesis through its trophic effects on thymic epithelial cells (TECs). TECs play a pivotal role in T-cell development, providing essential growth factors and presenting self-antigen to developing thymocytes. When administered to mice undergoing bone marrow transplantation (BMT) or experimental graft-versus-host disease (GvHD) KGF enhanced thymopoiesis (19, 20). In rhesus macaques, KGF enhanced thymic naive T cell production and reduced lymphopenia-induced T-cell proliferation after myeloablation and peripheral blood progenitor cell autologous transplantation (21). In this model KGF (given as palifermin at a dose of 250mcg/kg per day for three days before and after transplantation) was well tolerated, and its positive effects on

the thymus were maintained for up to 12 months. In 2005 palifermin was licensed (as Kepivance) to prevent mucositis induced by chemotherapy. In its pivotal trial 60mcg/kg of palifermin was given for three days prior to conditioning, then for three days after haematopoietic stem cell transplantation (HSCT) (22); this regimen was well tolerated. Later, a trial of three doses of palifermin (60mcg/Kg) before conditioning and up to nine doses after allogeneic HSCT was shown to be safe, although it had no impact on the incidence of acute GVHD (23) or absolute lymphocyte count recovery (24). Although thymic function was not directly studied in these patients, the result suggested that higher doses of palifermin might be required to see positive immunological effects.

Therefore, we designed a study to explore the tolerability of higher doses of palifermin (90, 120 and 180 mcg/kg/day, given for three days prior to and after alemtuzumab with further doses at months 1 and 3); and then test the efficacy of the highest tolerated dose in a placebo-controlled trial (CAMTHY) aimed at testing two hypotheses: (i) that palifermin increases thymic T-cell reconstitution after alemtuzumab and (ii) thereby reducing the risk of alemtuzumab induced autoimmunity. Here we report the unexpected results of a pre-planned interim analysis (aimed at testing hypothesis one) which led to protocol-defined termination of recruitment.

Results

Clinical trial participants: Between June 2013 and February 2015 28 patients were enrolled and randomized to 1 of 2 treatment groups (palifermin, n=14; placebo, n=14) then followed for 30 months (Figure 1). Their baseline characteristics are shown in Table 1. The pre-planned interim analysis was conducted by independent statisticians and the unblinded results reported first to the Trial Steering Committee who took the decision to recommend early termination of the trial, as per protocol; no further patients were recruited and no further palifermin was given. All enrolled patients completed the study, and all analyses were completed with blinding intact, after which one investigator (AC) was unblinded.

Palifermin significantly reduced thymic T-cell reconstitution in our patient cohort: As we have previously reported (17) thymic function (assessed by measuring naïve T-cells, recent thymic emigrants (RTE) and TRECs/mL) was significantly reduced following treatment with alemtuzumab. However unexpectedly, this was further impaired in patients receiving palifermin. The interim analysis

endpoint, mean naïve (CCR7+ CD45RA+) CD4+ T cell count at month 6, was reduced in the palifermin group: $2.23 \times 10^7/L$ (SD 2.0) versus $7.73 \times 10^7/L$ (SD 5.74) in those receiving placebo, $p=0.007$ (Figure 2A, Supplemental Table 1), even after adjusting for baseline naïve CD4 T-cell counts, total palifermin dose and age (adjusted treatment group $p=0.007$, Supplemental Table 2). This difference was also evident at months 1 and 3 post treatment: $0.036 \times 10^7/L$ (SD 0.025) versus $0.341 \times 10^7/L$ (SD 0.25), and $0.387 \times 10^7/L$ (SD 0.68) versus $1.326 \times 10^7/L$ (SD 1.29) respectively (Supplemental Table 1). The difference in naïve CD4 T-cell numbers was greatest at month 1 suggesting that palifermin's negative effect on thymic function occurred early. This was not due to globally reduced T-cell numbers but due to a specific reduction in naïve T-cells (Supplemental Table 1).

Palifermin also reduced the mean proportion of recent thymic emigrants (RTEs) in the CD4+ T-cell pool (month 1: 2.94% (SD 2.77) versus 7.93% (SD 8.71); month 3: 4.83% (SD 7.88) versus 13.29% (SD 11.75); month 6: 16.05% (SD 13.21) versus 33.95% (SD 18.68) (Figure 2B, Supplemental Table 1). TRECs/mL were also lower in the palifermin group at months 3 and 6; median values: 54.64 and 130.42 versus 162.93 and 2900.99 respectively (Figure 2C, Supplemental Table 3). In keeping with reduced thymopoiesis, there was a trend towards more restricted CD4+ and CD8+ TCR repertoires after palifermin; for instance, Shannon's entropy was 12.7 versus 13.3 in the placebo group and the mean CD4 clonality score was 0.102 versus 0.067. Palifermin reduced the number of unique clones per mcg of DNA (75,111 versus 84,017; Supplemental Table 4 and 5). As per our previous reports, the CD8+ TCR repertoire was more restricted than the CD4+ TCR repertoire at baseline, becoming increasingly restricted after treatment, particularly in the palifermin treated group (Supplemental Table 4 and 5).

Following alemtuzumab, mean proportions of T effector RA (TEMRA) and in particular effector memory (EM) cells were increased in the CD4+ T-cell pool, particularly in the palifermin arm (Supplemental Table 1). Similar changes were seen in the CD8+ T-cell pool (Supplemental Table 6). Palifermin had no effect on the usual rise in the relative number of CD4+ T regulatory cells (CD4+CD25hiCD127lo) after alemtuzumab (Supplemental Table 7). No difference was seen in thymic size or density between the two arms of the study (Supplemental Table 8).

In view of the unexpected negative effects of palifermin on thymopoiesis, we retrospectively assessed thymic function in patients treated on the dose-escalation sub-study. Naïve CD4+ T-cells and

TRECs/mL at 6 months were lower in the 90mcg/Kg arm of the dose-escalation (n=3) compared to placebo, and lower still in the three patients on 120mcg/Kg or 180mcg/Kg of palifermin (Supplemental Table 9 and 10). Given the variation in TRECs/mL between individuals prior to treatment, we normalised TRECs/mL at 6 months to baseline levels (Supplemental Table 10); this was lowest - at 9.64% of baseline- in the 180mcg/Kg group versus 13.57% after 120mcg/Kg and 28.89% following 90mcg/Kg palifermin.

Adverse events: were common in both arms of the study (Table 2). In keeping with the chemotherapy experience, palifermin caused an infusion syndrome comprising: an erythematous rash, oedema of the hands and face, oral symptoms (sensory and/or altered taste) and discolouration of the tongue. Unexpectedly 10/14 patients treated with palifermin developed transient hair thinning (lasting weeks to months) after treatment, in one individual this was marked. Mild to moderate upper respiratory tract infections were also more common in the palifermin group occurring in 8/14 patients vs 4/14 patients in the control arm. Palifermin administration before alemtuzumab did not alter the latter's well reported infusion-associated symptoms, except that chest tightness was reported less commonly. At the interim analysis cut off (M6 for each participant) no SAEs/SUSARs were reported.

Autoimmunity was not increased by Palifermin: Although the protocol-defined early termination of the trial meant it was underpowered to detect an effect of palifermin on the development of autoimmunity, patients were categorised at month 30 into those who had developed a clinical autoimmune disease during the trial, those who had developed novel autoantibodies (measured on at least two occasions six months apart) without developing clinical symptoms and those with no expression of autoimmunity. There were no differences between the groups. 4/14 palifermin patients developed a clinical autoimmune disease, compared to 5/13 on placebo (one patient was lost to follow-up in the placebo group; Fisher's exact test, two-sided, p=0.69). 5/14 patients on palifermin developed either clinical autoimmunity or de novo autoantibodies, compared to 8/13 on placebo (p=0.26).

Human and murine TECs express CD52

Recently published RNASeq data has shown that murine cortical and medullary TECs (cTECs and mTECs) express CD52, alemtuzumab's target molecule, at least at the level of mRNA (25, 26). Given this, we analysed bulk RNAseq data, generated from sorted human cortical and medullary TECs

collected in the Hollander Lab in Oxford (see methods), and found that CD52 is also highly expressed by human TECs (figure 3; GEO ID: GSE127209). *CD52* expression was within the top 10% of detectable transcripts in all TEC subsets and was significantly higher in cTEC than mTEC^{lo} (3.8-fold change, adjusted $p = 0.02$) but not between cTEC and mTEC^{hi} or mTEC^{hi} and mTEC^{lo} (adjusted $p > 0.05$ in both).

Discussion

Here we report the unexpected finding that palifermin (keratinocyte growth factor) exacerbates alemtuzumab's negative impact on thymopoiesis. We have demonstrated this by three independent techniques: naïve CD4+ T-cell count (the primary interim outcome measure), circulating numbers of recent thymic emigrants and T-cell receptor excision circles (TRECs)/mL. Since the overall aim of the trial was predicated on palifermin's ability to boost thymopoiesis to reduce autoimmunity after alemtuzumab, in accordance with the trial protocol, recruitment to the study was halted and further dosing of palifermin suspended following a planned interim analysis.

Our results contradict palifermin's ability to enhance thymopoiesis in murine and non-human primate models (19-21). Although a species difference is possible, the fact that the KGF receptor (FGFR2IIIb) is expressed on human epithelial cells makes this unlikely. We also do not believe that this is a dose effect. Our decision to test the efficacy of the highest tolerated dose of palifermin was based on our interpretation of results from a trial of palifermin in preventing GvHD following allogeneic HSCT. In that study, three 60mcg/Kg daily doses of palifermin before conditioning and up to 9 doses after transplant did not accelerate total lymphocyte recovery, nor reduce the incidence of acute GVHD. Whilst the absence of detailed immune phenotyping data and lack of information on TRECs and TCR repertoire makes it difficult to distinguish palifermin's effect on the thymus versus lymphopenia-induced proliferation, the result suggested to us that 60mcg/Kg was unlikely to have a positive effect on thymopoiesis. Our suspicion was confirmed by the results of another trial of palifermin, published during the course of this study, which demonstrated that up to 3 doses of 60mcg/kg of palifermin neither increased CD4 T cell counts, nor thymic function (assessed by measuring naïve CD4 cells, RTEs and thymic size on CT scan) in HIV-infected patients with persistent CD4 T cell lymphopenia despite virologically effective antiretroviral treatment (27); suboptimal dosing was postulated as a

cause for their negative result. Importantly, no previous study has reported a reduction in thymic function with palifermin. In our own study, none of the doses tested in the tolerability sub-study had a positive effect on thymopoiesis. With the caveat that only 3 individuals were treated at each dose level, all doses (from 90 to 180mcg/Kg/day) impaired thymic function after alemtuzumab, with an apparent dose effect.

By analysing publically available (25, 26) and in house generated RNASeq datasets (GEO ID: GSE127209) we have shown that murine and human TECs express high levels of CD52 mRNA, with *CD52* appearing in the top 10% of all detectable transcripts in all TEC subsets. Interestingly, in the human thymus cTEC express the highest level of *CD52* of the TEC subsets, whereas in mouse thymus cTEC express the lowest level of *Cd52* (25). Species differences are therefore clearly important when considering the potential effects of anti-CD52 treatment on thymopoiesis. High levels of CD52 mRNA in TECs raises the possibility that alemtuzumab impairs thymic function by damaging CD52 expressing TECs. Studies using the human CD52 transgenic mouse model (28) confirm that alemtuzumab can penetrate the thymus causing depletion of CD52 expressing single-positive and double-positive thymocytes (TECs were not analysed in this study). However, thymic T-cell depletion was significantly less than in the periphery (50% of thymocytes at a dose of 10mg/Kg, vs. 100% T-cell depletion in the circulation at 0.5-1mg/kg) suggesting that antibody penetrance is incomplete.

Given this, we hypothesise that palifermin may worsen alemtuzumab's negative impact on thymic function by causing TECs to upregulate CD52 expression, so making them more susceptible to damage. Although density of CD52 expression is not the only factor that determines susceptibility to alemtuzumab-induced depletion, it is a critical factor (29, 30). In support of this, in our study, palifermin's negative effect on thymic function was most marked at the earliest time points, at the point of co-administration with alemtuzumab. For example, the biggest difference in the number of naïve CD4 T-cells between the two arms of the study was at month 1, where there was a 9.5 fold difference compared to a 3.4 fold difference at month 3, and a 2.6 fold difference at month 6. A similar effect was seen in the TREC/mL data where the biggest difference between the two arms of the study was at month 3 (the earliest point measured; a 13 fold difference vs. a 3 fold difference at month 6). These data suggest that whilst the initial doses of palifermin exaggerate alemtuzumab-induced thymic damage, later doses may be protective.

We have previously reported that reduced thymic function and consequent lymphopenia-induced T-cell proliferation is greatest in those who develop autoimmunity after alemtuzumab (17). Our new finding of TEC *CD52* expression also raises the possibility that alemtuzumab leads to autoimmune complications due to a direct effect on central tolerance, as has been reported in a murine model of acute GvHD, where GvHD-induced mTEC^{hi} loss resulted in reduced tissue-restricted self-antigen (TRA) expression and *de novo* generation of autoreactive T cells (31).

Although palifermin significantly reduced thymopoiesis in our patients, there was no evidence that it increased the risk of developing autoimmunity at 30 months of follow up. However autoimmunity can occur for up to 5 years after alemtuzumab so we will continue to monitor these patients clinically and immunologically. 8/14 patients treated with palifermin developed mild to moderate upper respiratory tracts infection compared to 4/14 patients in the control arm, perhaps reflecting an increased susceptibility to infective complications. However urinary tract infections were equal in both groups (2/14) and no serious infections occurred in either arm of the study. Furthermore infections were not more common in those receiving the higher dose of palifermin in the open-label dose escalation study compared to the other doses - 2/3 vs 0/3 vs 1/3 in the low middle and high dose groups respectively (Supplemental Table 11).

In conclusion we have shown that palifermin (180mcg/kg/day given over 12 days) worsens thymic function following alemtuzumab treatment of RRMS, and therefore should not be used to promote T cell reconstitution in this setting. Whilst we hypothesise that palifermin's negative impact on thymopoiesis is due to co-administration with alemtuzumab, our trial serves as a note of caution to those performing or contemplating trials of palifermin in other settings, particularly in the oncology/haematology setting where alemtuzumab is often used as part of the conditioning regime. We also note that palifermin worsened clinical outcome in the KARE study (32) a trial of KGF in the treatment of acute respiratory distress syndrome (ARDS), despite encouraging results in animal studies. Together, KARE and CAMTHY serve as a reminder to be cautious when translating efficacy data from animal studies to humans, and when co-administering drugs that may interact. It remains to be seen if alemtuzumab induced autoimmunity can be reduced by preserving thymic function.

Methods

Participants: Participants were aged 18-50 years with: relapsing-remitting multiple sclerosis (33); disease duration of 10 years or less; at least two relapses in the previous 2 years with at least one in the previous 12 months (untreated or on beta interferon or glatiramer acetate) and an expanded disability status scale (EDSS) score of 5.0 or less. Exclusion criteria included: progressive forms of multiple sclerosis; previous thymectomy; previous treatment with alemtuzumab, natalizumab, mitoxantrone, cyclophosphamide, cladribine, rituximab or any other immunosuppressant or cytotoxic therapy; a history of malignancy, or a history of clinically significant autoimmunity other than multiple sclerosis.

Randomisation and masking Participants were randomised (1:1) to receive palifermin or placebo using an online randomisation service. Because palifermin's known adverse effects (skin reddening and tongue discolouration) may compromise blinding, samples for immunological assays were recoded with a randomly generated identifier for each participant-visit and were analysed blind in batches. Radiological assessments of thymic size and density were performed by masked assessors outside of the core trial team.

Drug treatments: All patients received 12mg/day alemtuzumab for 5 consecutive days at baseline, followed by 12mg/day for 3 consecutive days at month 12, with methylprednisolone pre-treatment on days 1, 2 and 3 of each cycle. As is standard practice, all patients were given 200mg oral acyclovir twice a day for 28 days after each cycle of alemtuzumab to reduce the risk of oral herpes simplex.

For the open label dose escalation tolerability sub-study, 3 individuals were treated at each of the following palifermin doses: 90mcg/kg/day, 120mcg/kg/day and 180mcg/kg/day given as an intravenous bolus injection on days -5, -4 and -3 prior to each cycle of alemtuzumab and on days 8, 9 and 10. Three further doses were given at month 1 (+/- 7 days) and month 3 (+/- 2 weeks) after each cycle of alemtuzumab. Each dose level was separated by a minimum of 10 days (from the day 10 dose) and escalation between doses only occurred if no adverse events greater than grade 2 occurred. As all doses were equally tolerated (Supplemental Table 11), for the subsequent placebo-controlled study, participants received 180mcg/Kg/day of palifermin, or an equivalent volume of normal saline.

Clinical and laboratory assessments: In addition to standard alemtuzumab safety monitoring, at each three-monthly visit for 30 months of follow-up, participants were assessed clinically and their blood assayed for markers of thymic function by immune-phenotyping, signal joint T cell receptor excision circles (sjTRECs) in whole blood quantification (34), and T cell receptor beta chain (TCRB) sequencing. In addition, to assess thymic size and density, a non-contrast low dose chest computer tomography (CT) was performed at baseline and at month 6. Details of these methods are as follows:

Immunophenotyping: Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised blood by density centrifugation on Ficoll. For surface staining, washed cells were re-suspended in FACS buffer (PBS, 2mM EDTA, 0.01% sodium azide) containing 2% mouse serum for 20 minutes to reduce non-specific binding, incubated at 4 degrees for 30 minutes with different mAb combinations against surface targets, washed twice in FACS buffer, and then fixed for 20 minutes in 2% formaldehyde before acquisition. The FoxP3/Transcription Factor Staining Buffer set (eBioscience) was used for intracellular staining of FoxP3 and Ki67. Cells were fixed and made permeable for 40 mins at room temperature, and then stained for 30 mins at room temp in permeabilisation buffer. The following fluorescent-labelled antibodies (all purchased from BD Bioscience) were used in various combinations (catalog numbers are given in brackets): CD3-V450 (560365), CD3-APCCy7 (557832), CD4-V500 (560768), CD8-APC (555369), CCR7-FITC (561271), CD45RA-PECy5 (555490), CD45RA-PECy7 (337186), CD25-PE (555432), Ki67-PE (556027), CD127-V450 (560823), CD31-V450 (561653), FoxP3-AF647 (560045). Data were acquired on a Canto II and analysed using FlowJo v7.6.5 (Tree Star Inc); the gating strategy is outlined in Supplemental Figure 1. Cell counts for the different CD4 and CD8 subpopulations were calculated based on CD4 and CD8 counts determined by FACS performed at the Department of Immunology, Addenbrooke's Hospital, Cambridge - a clinical laboratory approved for good clinical practice. Where CD4 or CD8 counts were below the laboratory detectable limit, the value was replaced in the analysis with the lower limit of detection divided by the square root of 2 ($LLD/\sqrt{2}$).

SjTREC/mL Quantification: Thymic function was also estimated by quantification of signal joint T cell receptor excision circles (sjTRECs) in whole blood as described by Lorenzi et al (2008) (34). In brief, DNA was extracted directly from 300ul of fresh blood collected in EDTA using the Wizard Genomic DNA purification kit (Promega). The TREC content of each sample was determined by qPCR using a standard curve derived from plasmid constructs encoding the sjTREC sequence. Standards were

diluted over the range 10^7 - 10^1 , and a curve run in each experiment alongside positive and negative controls. Taqman technology was used in a 25ul reaction mixture containing 700nM of each primer CACATCCCTTTCAACCATGCT and GCCAGCTGCAGGGTTTAGG, 150 nM Taqman hydrolysis probe (6-FAM-ACACCTCTGGTTTTTGTAAAGGTGCCCACT-TAMRA) and 12.5 μ l JumpStart™ Taq ReadyMix (SIGMA), supplemented with MgCl₂ at a final concentration of 5mM. Each reaction contained 500ng DNA. Samples were run in triplicate and the replicate average taken as the sample result. Cycling conditions were: 94 °C 2 min, 40 cycles at 94 °C 30 s / 60 °C 15 s / 72 °C 2 min, 72 °C 5 min. The number of sjTRECs/mL was calculated as: total DNA (ug) in 300ul whole blood/ DNA (ug) in PCR reaction (0.5ug) x No of TRECs (derived from the standard curve) x 1000/300. Primers and probes were all from SIGMA-Aldrich.

TCR repertoire analysis: Genomic DNA from magnetically sorted CD4 and CD8 cells was extracted using the Qiagen Allprep method, according to the manufacturer's instructions. Samples were quantified and diluted for library preparation. Amplification and sequencing of CDR3 regions in rearranged TCR β -chains was performed using the immunoSEQ Assay (Adaptive Biotechnologies). The immunoSEQ assay combines multiplex PCR with high-throughput sequencing and a sophisticated bioinformatics pipeline for TCR β CDR3 region analysis. Sequencing data were analysed using the immunoSEQ analyser (<https://clients.adaptivebiotech.com/login>). Sample diversity or "richness" was calculated by Shannon's Entropy defined as $H = -\sum_{i=1}^N P_i \log_2 P_i$ where N is the number of unique clones and P_i the frequency of the clones i . Entropy ranges from 0, in a sample with only one clone to $H_{\max} = \log_2 N$ for polyclonal highly diverse samples. Clonality was defined as 1-Pielou's evenness metric and was calculated by $1-H/\ln(N)$. Clonality describes the shape of the distribution of proportional abundances and ranges from 0 to 1; values near to one indicate an increasingly asymmetric division in which a few clones are present at high frequencies (35, 36).

Thymic imaging: To assess thymic size and density, a non-contrast low dose chest CT was performed at baseline and at six months on a Siemens Emotion 16 (2007) scanner, with contiguous 1mm sections. Measurement of thymus height, width, depth (cm), volume (cm³) of the thymus and average density (measured in Hounsfield Units [HU]) were made on the soft tissue window reconstructions using Siemens Syngo via multimodality reading solution imaging software. A region of interest volume tool was used which involved manual contouring of the thymus on several trans-axial levels, with semi-automated propagation (Supplemental Figure 2). Transaxial measurements were made using a

calliper-measuring tool. All measurements were performed by one of two radiologists, one of who is a thoracic radiology consultant (10 years' experience) and the other a specialty registrar who had received training. Ten studies were dual read by both radiologists together to ensure consistency in method of measurement, and there was good inter-observer agreement. Both radiologists were blinded to the treatment status of the patients and the same radiologist interpreted both the baseline and repeat CT on each patient.

Analysis of CD52 expression by human TECs: Thymus tissue was removed from patients (n = 4) aged between 11 days and 3 months old and prepared as described with some modifications (37). In brief, tissue was dissected and physically dissociated in sterile PBS. A single cell suspension was prepared using three rounds of mechanical and enzymatic digestion with Liberase TM/DNAseI as detailed in Stoeckle *et al.* The subsequent antigen presenting cell-enriched single cell suspension was enriched for CD4^{lo/neg} cells using magnetic-activated cell sorting. cTEC were isolated as EpCAM^{lo} CDR2⁺ and mTEC as EpCAM^{hi} CDR2⁻. mTEC were subsequently separated into MHC high (mTEC^{hi}) and MHC low (mTEC^{lo}). For three patients, 30,000 - 50,000 cells were sorted from each TEC subset. For one patient three replicates of 7,575 cells were sorted from each TEC subset. The study of human thymus tissue has been granted ethical approval and is publicly listed (IRAS ID 156910, CPMS ID 19587).

RNA was extracted from sorted TEC subsets using a Qiagen Plus RNeasy Micro Kit. Smarter-seq was used to generate transcriptomic libraries, which were subsequently sequenced on an Illumina HiSeq 4000 (38). Adapters were trimmed from reads using Trimmomatic (39). Reads were aligned against the Ensembl human reference genome (GRCh38) using two-pass mapping with STAR (version 2.5.3a) (40). Aligned reads were assigned to genes using HTSeq (version 0.5.4) (41). EdgeR trimmed mean of M-values (TMM) was used to adjust counts data prior to log₂ FPKM calculation and differential expression analysis (42). P-values were adjusted for multiple hypothesis testing using Benjamini-Hochberg correction. The vioplot package in R was used to generate plots.

Statistics: A "stop-go" interim analysis, testing the effect of palifermin on naïve T-cell reconstitution (as a read-out of thymic function), was planned when 28 patients reached month 6. An independent trial steering committee adjudicated the results of the interim analysis.

The pre-planned efficacy threshold for the interim analysis was a statistically significant increase in the number of peripheral naïve (CCR7+CD45RA+) CD4+ T-cells in the palifermin group, by at least 50%, compared to placebo at month 6 post-alemtuzumab (as an indicator of thymopoiesis). We believed this to be a conservative estimate as palifermin increases naïve CD4+ T cell numbers threefold in rhesus macaques and twofold in mice (maximal at 3-9 months in macaques and 30-80 days in mice). Power calculations suggested that 28 patients (14 placebo, 14 palifermin) had 80% power to detect this increase. A p value of <0.05 was considered significant.

Multivariate linear regression was used to model naïve CD4+ T cell count at 6 months with explanatory variables of treatment group, age, baseline naïve CD4+ T count and total dose of palifermin received. To aid interpretation of the model intercept, the continuous variables were median-centered. An unpaired two-tailed t-test and Mann-Whitney U test were also performed on naïve CD4+ T cell count at 6 months, comparing palifermin versus placebo. For exploratory end-points, summary statistics were calculated by treatment arm; no formal statistical tests were applied (exploratory end-point *P*-values reported in the text are given for descriptive purposes only). Continuous variables were summarised using *n* (non-missing sample size), mean, standard deviation, median, maximum and minimum. Categorical variables were reported as frequency and percentages (based on the non-missing sample size) of observed levels. For any laboratory tests where the measurement made was considered to be less than the detectable limit, the value was replaced in the analysis with the lower limit of detection divided by the square root of 2 ($LLD/\sqrt{2}$).

If the interim analysis were successful, 80 patients would have been recruited to the trial which would have given 78% power to detect a relative risk reduction of 50% of autoimmunity after alemtuzumab, using a 2-sided 5% significance level.

Study approval: CAMTHY was conducted in accordance with the International Conference on Harmonisation Guidelines for Good Clinical Practice and the principles of the Declaration of Helsinki, and was approved by NRES Committee London – Hampstead (Rec: 12/LO/0393). All participants gave written informed consent.

Author Contributions

AJC and JLJ conceived the trial. JLJ was chief investigator; AJC, LA, OKE, WLB and EN were sub-investigators and alongside JLJ were responsible for the day-to-day running of the trial. HKM, SJT, LJ, JD, SH, ZGG, SM, IR, MD, AEH and GH performed and analysed the laboratory assays. JB and TJS performed and analysed the thoracic CT scans. SD was the trial statistician. DD and RSB gave advice on trial design. With the help of DD, RSB and DJ, JDI led the adjudication of the interim analysis. All authors revised the manuscript. All authors read and approved the final version.

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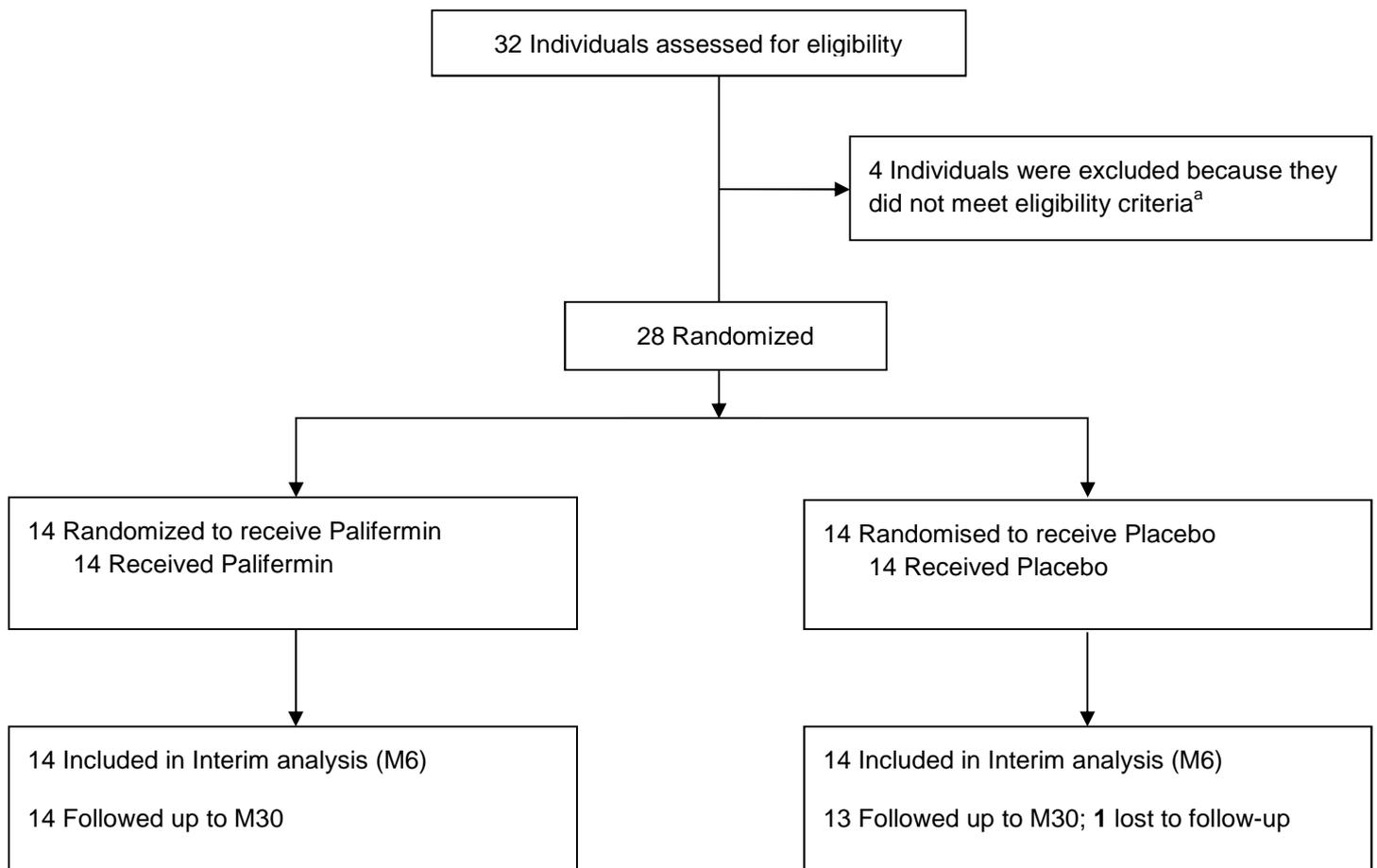


Figure 1: Flow of Participants Randomized to Palifermin (rhKGF) vs. Placebo on CAMTHY

^a One individual was excluded because their RRMS disease activity was in sufficient to warrant treatment with alemtuzumab; two individuals were excluded due to abnormal liver function tests; one individual was excluded due to abnormal thyroid function tests.

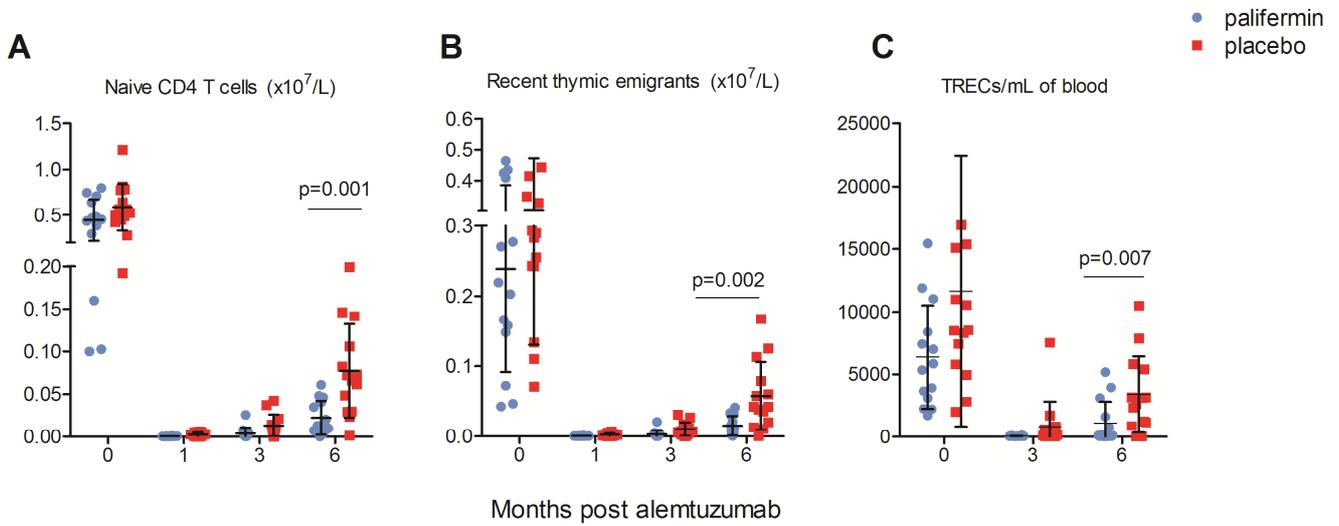


Figure 2: Thymic function is significantly reduced in patients treated with palifermin (n=14) vs. placebo (n=14). Graphs represent: A) Numbers of circulating naïve (CCR7+CD45RA+) CD4+ T-cells; B) Numbers of circulating CD4+ recent thymic emigrants (RTEs), defined as naïve CD4+ T-cells co-expressing CD31, and C) T-cell receptor excision circles (TRECs) per mL of blood, at baseline (0) and months 3 and 6 post-alemtuzumab. The data shown are the mean +/- SD; p values are shown for the month 6 data and were calculated using Mann-Whitney non parametric tests. Naïve CD4 cell count at month 6 was the predefined interim primary outcome measure. P values for RTEs and TRECs are shown for descriptive purposes only.

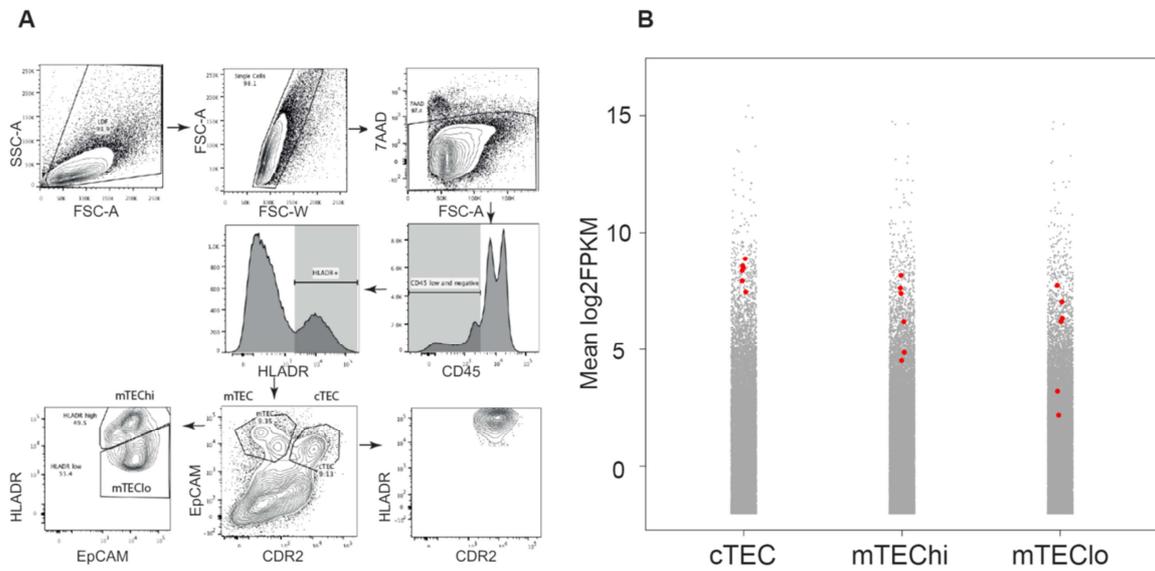


Figure 3 Analysis of *CD52* expression in human thymus. A) FACS strategy for isolating TEC subsets. cTEC are EpCAM^{lo} CDR2⁺; mTEC^{lo} as EpCAM^{hi} CDR2⁻ MHC^{low}; and mTEC^{hi} as EpCAM^{hi} CDR2⁻ MHC^{high}. B) *CD52* expression in RNA-seq datasets from human thymus (n = 6). Violin plots show the distribution of gene expression in cTEC, mTEC^{hi} and mTEC^{lo}. Points show mean log₂ FPKM expression of *CD52* and error bars show 95% confidence intervals.

Variable	Statistic	Placebo (n=14)	Palifermin (n=14)
Age (years)	Mean (SD)	30·8 (7·80)	32·1 (8·22)
	Median	30·5	32·5
	Min, Max	19, 49	19, 48
Gender	%	14·3 M; 85·7 F	42·9 M; 57·1 F
Weight (kg)	Mean (SD)	77·4 (16·0)	75·8 (19·8)
	Median	77·2	71·9
	Min, Max	56,2, 112·2	44·6, 116·8
Dose (Palifermin or equivalent volume of placebo)	Mean (SD)	165 (34·8)	160 (41·7)
	Median	159·6	155·16
	Min, Max	121·44, 242·4	96·18, 252·12

Table 1: Baseline characteristics Data are: mean (SD), median, minimum and maximum values (range) or percentage. The baseline characteristics did not differ significantly between the groups.

		Placebo	Palifermin
Infusion-associated symptoms occurring at baseline	Erythematous skin rash	5 (36%)	14 (100%)
	Oral symptoms	2 (14%)	11 (79%)
	Facial oedema	0 (0%)	8 (57%)
	Urticarial skin rash	8 (57%)	6 (43%)
	Oedema of hands	0 (0%)	6 (43%)
	Discoloured tongue	0 (0%)	6 (43%)
	Pyrexia	4 (29%)	4 (29%)
	Headache	3 (21%)	4 (29%)
	Skin sensitivity	2 (14%)	4 (29%)
	Fatigue	0 (0%)	3 (21%)
	Chills	3 (21%)	3 (21%)
	Hair thinning	0 (0%)	2 (14%)
	Chest tightness	7 (50%)	1 (7%)
	Infusion-associated symptoms occurring with IMP at month 1 and month 3	Erythematous skin rash	1 (7%)
Oral symptoms		1 (7%)	8 (57%)
Facial oedema		0 (0%)	7 (50%)
Oedema of hands		0 (0%)	6 (43%)
Discoloured tongue		0 (0%)	5 (36%)
Skin sensitivity		0 (0%)	5 (36%)
Peeling skin		0 (0%)	2 (14%)
AEs unrelated to infusions	Hair loss	2 (14%)	10 (71%)
	Skin rash	4 (29%)	8 (57%)
	Upper respiratory tract infection	4 (29%)	8 (57%)
	Urinary tract infection	2 (14%)	2 (14%)
	Neutropenia	1 (7%)	1 (7%)
	Fatigue	2 (14%)	1 (7%)

Table 2: Summary of adverse events (AEs) that occurred in patients randomised to receive Palifermin (n=14) vs. Placebo (n=14). Data shown are: incidence in patients, n (%); all AEs were grade 1 or 2