BRIEF REPORT

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Angiogenic T cells are decreased in people with type 2 diabetes mellitus and recruited by the dipeptidyl peptidase-4 inhibitor Linagliptin: A subanalysis from a randomized, placebo-controlled trial (RELEASE study)

Stefanie A. de Boer MD, PhD[1] | Melanie Reijrink BSc[1] |
Wayel H. Abdulahad PhD[2,3] | Elisa S. Hoekstra BSc[2] | Riemer H. J. A. Slart MD[4,5] |
Hiddo J. L. Heerspink PhD[6] | Johanna Westra PhD[2] | Douwe J. Mulder MD[1]

[1]Department of Internal Medicine, Division of Vascular Medicine, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands

[2]Department of Rheumatology and Clinical Immunology, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands

[3]Department of Pathology and Medical Biology, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands

[4]Department of Nuclear Medicine and Molecular Imaging, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands

[5]Department of Biomedical Photonic Imaging (BMPI), University of Twente, Enschede, The Netherlands

[6]Department of Clinical Pharmacy and Pharmacology, University of Groningen, University Medical Centre Groningen, Groningen, Netherlands

Correspondence
Douwe J. Mulder, Department of Internal Medicine, Division of Vascular Medicine, University of Groningen, University Medical Centre Groningen, Groningen, Netherlands, HP AA41, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.
Email: d.j.mulder@umcg.nl

Abstract

Angiogenic T (Tang) cells are mediators of vascular repair, and are characterized by surface expression of CXCR4. This receptor for stromal cell-derived factor-1α (SDF-1α) is cleaved by dipeptidyl peptidase-4 (DPP-4). Tang cell levels were investigated in people with type 2 diabetes mellitus (T2DM) compared with matched healthy controls and after treatment with the DPP-4 inhibitor Linagliptin. People with T2DM were randomized to 5 mg/day Linagliptin (n = 20) or placebo (n = 21) for 26 weeks. Tang cell frequency was identified in peripheral blood mononuclear cells (CD3$^+$CD31$^+$CXCR4$^+$) and levels of endothelial progenitor cells (EPCs) (CD34$^+$CD133$^+$KDR$^+$) were also assessed in whole blood. Circulating Tang cell levels were significantly lower in people with T2DM compared with the healthy control group. SDF-1α levels increased significantly in Linagliptin-treated people with T2DM compared to placebo, and a trend was observed in change of Tang cell levels, while EPC count did not change. In conclusion, circulating Tang cell levels were considerably lower in people with T2DM, while a trend was observed in recruitment of Tang cells after 26 weeks of treatment with Linagliptin. These data suggest that DPP-4 inhibitors may potentially exert beneficial effects on bone marrow-driven vascular repair.

KEYWORDS
angiogenic T cells, dipeptidyl peptidase-4 (DPP-4) inhibitors, Linagliptin, stromal cell-derived factor-1α (SDF-1α), type 2 diabetes mellitus

1 | INTRODUCTION

Type 2 diabetes mellitus (T2DM) is associated with an increased cardiovascular disease (CVD) risk. The underlying pathogenesis of CVD in T2DM is incompletely understood. Recent data suggest that the enzyme dipeptidyl peptidase-4 (DPP-4) constitutes a link between T2DM and cardiovascular (CV) risk.[1]

Several pathways involved in CVD may be targeted by inhibiting DPP-4. This changes the proportion of lymphocyte subpopulations[2,3] and degrades multiple chemokines, such as stromal cell-derived factor 1α (SDF-1α, also known as CXCL12) and chemokine (C-C motif) ligand 5 (CCL5), and blocks binding to their major receptors CXCR4 and CCR5, respectively.[4] These chemokines are involved in the production of progenitor cells in the bone marrow, including endothelial progenitor cells (EPCs) that facilitate endothelial cell function.[5] Additionally, a recently discovered subpopulation of T cells, angiogenic T (Tang) cells, constitute the central cell cluster of EPC colonies[6] and cooperate with EPCs to facilitate vascular repair.[7] Tang cells enhance the differentiation of early EPCs and promote neovascularization and endothelial repair, possibly through the secretion of proangiogenic cytokines, such as vascular endothelial growth factor (VEGF), interleukin (IL)-8 and matrix metalloproteinases.[6] Tang cells are characterized by co-expression of CD31 and CXCR4, and are heterogeneous, since they may express CD4 or CD8, and can co-express naïve and memory markers.[7]

Since CXCR4 is the receptor for SDF-1α, we hypothesized that DPP-4 inhibition may increase the frequency of circulating Tang cells. Therefore, we determined the frequency of circulating Tang cells in treatment-naive people with T2DM compared with healthy controls, and assessed the effect of 26 weeks of DPP-4 inhibition on the frequency of Tang cells and EPCs. The effect of DPP-4 inhibition on circulating levels of SDF-1α, VEGF and IL-8 was also determined.

2 | METHODS

2.1 | Study design and population

Samples included in this study were from the RELEASE trial (NCT02015299). The study design and selection of participants have been discussed previously.[8] Participants were randomized in a 1:1 ratio to Linagliptin 5 mg once daily or a matching placebo for 26 weeks, stratified by age (30–49 vs. 50–70 years). Additionally, a group of 19 healthy controls was included for measurement of Tang cells. Healthy controls did not have diabetes, obesity or CVD and were of similar age. Following the direct assessment of standard clinical laboratory variables, peripheral blood mononuclear cells (PBMCs) were isolated and stored in liquid nitrogen for determination of Tang cell levels, and plasma was obtained from heparinized blood.

2.2 | Tang cell analysis at inclusion and at 26 weeks

Frequencies of CD4+ and CD8+ Tang cell populations were analysed in thawed PBMCs by flow cytometry. Briefly, cells were incubated with anti-CD3-PerCP, anti-CD8-AF700, anti-CD25-PE, anti-CD45Ro-FITC, anti-CCR7PE-Cy7, anti-CD31-PB and anti-CXCR4-BV605 (Biolegend, San Diego and BD Biosciences, Vianen, Netherlands). Samples were analysed on a BD™ LSR II flow cytometer and plotted using Kaluza v1.2 (Beckman Coulter). Tang cells were expressed as percentages of total T cells (CD3+) or as percentage of total CD4+ (gated as CD3+CD8− population) or of total CD8+ cells (see Figure S1 for gating strategy). Based on the expression of the surface molecules CCR7 and CD45Ro, a distinction was made in naïve, central memory (CM), effector memory (EM) and terminally differentiated (TD) cell populations for both CD4+ and CD8+ subsets.

2.3 | EPC analysis at inclusion and at 26 weeks

For the EPC analysis 200 μL lithium-heparin blood was incubated with anti-CD34-PE (clone 581; IQproducts), anti-CD133-APC (clone AC133; Miltenyi Biotec) and anti-KDR/VEGFR-FITC (KDR-FITC; R&D systems) or appropriate isotype controls (see Figure S1 for gating strategy). Flow cytometry measurements were performed on a BD Bioscience FACS Calibur flow cytometer, and analysed using Kaluza analysis software.

2.4 | Enzyme-linked immunosorbent assay

Circulating levels of C-reactive protein, IL-8, VEGF and SDF-1α were measured in plasma samples with Duosets enzyme-linked immunosorbent assays (R&D Systems) according to the manufacturer's instructions.

2.5 | Statistical methods

Means or medians were compared with independent sample Student t-tests or Mann–Whitney U-tests, as appropriate, and discrete variables using chi-squared or Fisher's exact tests. An ANCOVA model was constructed with change from baseline (ie, Tang cell percentages or EPC levels) as a dependent variable, randomization group as fixed factor and baseline levels as covariate. Variables were log transformed when distribution was non-parametric. All analyses were performed with SPSS (2013. IBM SPSS Statistics, Version 22.0). P values of <0.05 were taken to indicate statistical significance.

3 | RESULTS

Forty-five of the 50 screened participants with T2DM were randomized, 44 started treatment, and cell isolation was possible in 41

participants (20 Linagliptin, 21 placebo). The median [interquartile range (IQR)] age was 63 [56–67] years, median [IQR] BMI was 30.4 [27.4–37.2] kg/m^2, median [IQR] diabetes duration was 1 [0–3.5] years and the mean (SD) glycated haemoglobin (HbA1c) level was 45 (±4.8) mmol/mol. Baseline characteristics were well balanced between groups. More baseline characteristics can be found in the paper by De Boer et al.[8] The median [IQR] age in the healthy control group (n = 19, 63% male) was 56 [44–67] years.

Circulating Tang cell levels were significantly lower in people with T2DM (median [IQR] 11.5 [9.4–17.8]%) compared with healthy controls (median [IQR] 20.4 [16.6–27.3]%; $P < 0.001$ [Figure 1A]). This was also seen in CD4$^+$ T-cell subsets (median [IQR] 7.7 [4.3–10.8]%) versus healthy controls (median [IQR] 13.2 [8.7–20.2]%; $P < 0.01$) and in CD8$^+$ T-cell subsets (median [IQR] 26.7 [19.4–38.1]% vs. 46.5 [32.1–55.2]%; $P < 0.001$ [Figure 1B,C]). There were no differences between the T2DM and the healthy control group regarding percentages CD4$^+$-naive, EM and TD populations, while percentage of CM cells was higher in people with T2DM ($P = 0.02$; Table 1). There were significantly lower numbers of CD8$^+$-naive cells in people with T2DM

compared to healthy controls ($P = 0.005$). Tang cells were specifically present in naive fractions of both CD4$^+$ cells and CD8$^+$ cells. In people with T2DM, percentages of Tang cells were reduced in the EM fraction of both CD4$^+$ cells and CD8$^+$ cells (Table 1).

The frequency of total Tang cells (CD3$^+$) increased in the Linagliptin-treated ($P = 0.029$) but remained stable in the placebo-treated participants (Figure 1D,E). The change from baseline differed significantly between Linagliptin and placebo (median [IQR] +1.58 [–0.36 to 2.63]% vs. –0.24 [–0.74 to 0.52]%; $P = 0.016$). After adjustment for baseline total Tang cells, the trend remained present ($P = 0.098$). A similar increasing trend was seen for CD8$^+$ Tang cells ($P = 0.052$), but not for CD4$^+$ Tang cells ($P = 0.41$). There were no significant changes in T-cell subsets or Tang cells within these subsets after Linagliptin treatment and placebo (data not shown). The change in the frequency of Tang cells in Linagliptin-treated people with T2DM correlated negatively with change in HbA1c ($\rho = 0.39$, $P = 0.020$); this was not observed with placebo. Concomitantly, a rise in SDF-1α levels (median [IQR] 180 [126–238] pg/mL vs. 0 [0–2] pg/mL; $P < 0.001$ compared to
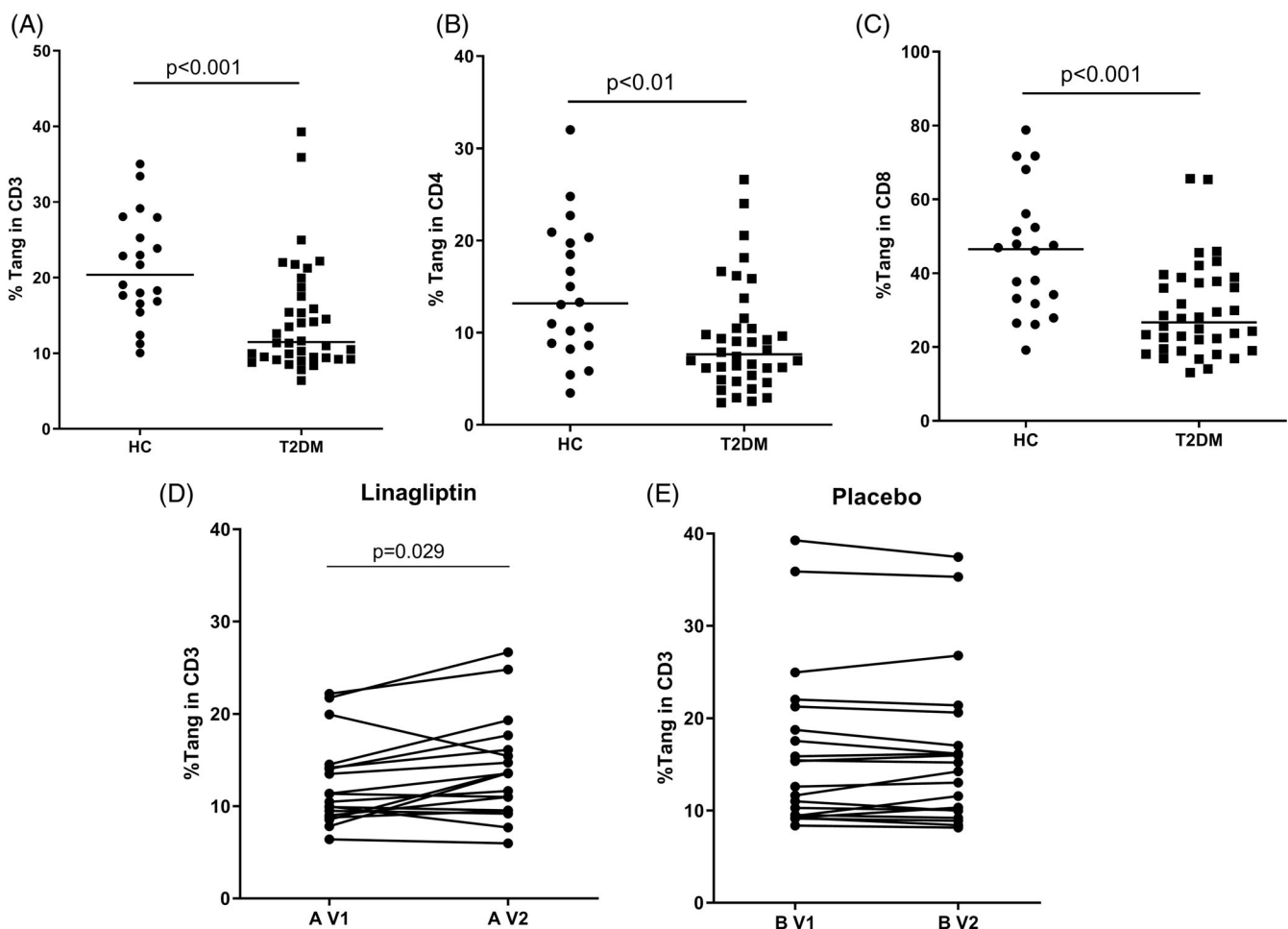

FIGURE 1 Frequencies of circulating Tang cells (CD31$^+$, CXCR4$^+$) in healthy controls (HC) and people with type 2 diabetes mellitus (T2DM) in A, CD3$^+$ cells and in B, CD4$^+$ and C, CD8$^+$ subsets measured by flow cytometry. Frequencies of circulating CD3$^+$ Tang cells in people with type 2 diabetes mellitus (T2DM) before and after 26-week treatment with Linagliptin in D or with placebo in E. V1 = visit 1, baseline; V2 = visit 2, week 26

TABLE 1 Percentages of T cells, CD4^{+} and CD8^{+} cells, subsets within CD4^{+} cells and CD8^{+} cells, and percentages of angiogenic T cells within these subsets

		Healthy control group, % Median [IQR]	T2DM group, % Median [IQR]	P
T cells		73.3 [63.3–75.9]	69.8 [62.3–77.3]	0.878
CD4^{+} cells		50.0 [42.1–57.3]	47.8 [41.0–53.0]	0.378
CD4^{+}	Naive	39.2 [29.5–49.3]	36.0 [23.6–44.4]	0.242
	CM	23.2 [17.1–32.7]	30.8 [22.8–38.4]	0.020
	EM	26.7 [19.2–37.9]	25.2 [18.1–38.4]	0.612
	TD	4.9 [2.8–7.3]	3.8 [2.2–6.3]	0.343
CD8^{+} cells		14.2 [11.4–20.4]	17.2 [11.7–24.9]	0.325
CD8^{+}	Naive	32.0 [21.5–41.6]	17.6 [11.1–27.8]	0.005
	CM	4.8 [3.0–6.0]	6.8 [4.3–9.3]	0.112
	EM	29.3 [23.0–37.1]	33.0 [25.3–41.6]	0.167
	TD	27.2 [19.0–40.8]	36.5 [22.0–43.0]	0.172
Tang cells		20.4 [16.6–27.3]	11.2 [9.4–17.8]	<0.001
CD4^{+} cells		13.2 [8.7–20.2]	7.7 [5.3–10.8]	<0.01
CD4^{+}	Naive	10.4 [4.8–30.9]	15.8 [9.2–28.2]	0.409
	CM	3.4 [2.5–8.0]	3.7 [2.3–5.9]	0.437
	EM	4.8 [2.0–20.0]	1.6 [1.0–2.7]	0.0002
	TD	9.5 [6.6–14.1]	8.2 [3.7–11.2]	0.143
CD8^{+} cells		46.5 [32.1–55.2]	27.7 [19.4–38.1]	<0.001
CD8^{+}	Naive	53.2 [28.4–85.6]	66.7 [51.1–78.4]	0.437
	CM	21.9 [12.1–30.7]	18.2 [12.6–25.8]	0.387
	EM	19.7 [12.9–65.2]	6.9 [4.8–11.5]	<0.0001
	TD	10.6 [8.4–12.7]	5.4 [3.3–7.4]	0.0003

Abbreviations: CM, central memory; EM, effector memory; IQR, interquartile range; TD, terminally differentiated; Tang cells, angiogenic T-cells; T2DM, type 2 diabetes mellitus.

placebo) was seen in Linagliptin- compared to placebo-treated participants (Figure S2). No differences in levels of IL-8 and VEGF were observed after Linagliptin or placebo treatment (data not shown).

A non-significant change after Linagliptin treatment was seen in levels of EPCs (CD34^{+}CD133^{+}KDR^{+}; P = 0.089) but was not seen for placebo (Figure S3A). There was no correlation with Tang levels at baseline (data not shown). EPC numbers from people with T2DM at baseline correlated negatively with plasma levels of IL-8 (Spearman ρ = −0.407, P = 0.008), and VEGF (ρ = −0.412, P = 0.008), and C-reactive protein (ρ = −0.319, P = 0.043; Figure S3B).

4 | DISCUSSION

In the present study we showed that levels of Tang cells were significantly reduced in treatment-naive people with T2DM compared to age- and sex-matched healthy controls. Tang cells are particularly found in naive subsets of both CD4^{+} cells and CD8^{+} cells. After 26 weeks of treatment with the DPP-4 inhibitor Linagliptin, a remarkable rise in levels of SDF-1α was observed, coinciding with a significant increase in CD3^{+} Tang cells and a trend for both CD4^{+} and CD8^{+} Tang cells, while Tang cells remained unchanged in placebo.

Tang cells are required for colony formation and differentiation of early EPCs[6] and, therefore, play an important role in endothelial repair. Tang cells were found to be reduced in hypertension-related cerebral small vessel disease[9] and in people with active rheumatoid arthritis.[7] By contrast, levels were not reduced in people with systemic lupus erythematosus, only in the disease controls, that is, people with rheumatoid arthritis and people with CV risk factors,[10] and were increased in lupus nephritis in a later study.[11] Miao et al [12] did observe increased CD8^{+} Tang cell levels in people with systemic lupus erythematosus, but not CD4^{+} Tang or total CD3^{+} Tang cells. These data underline the complexity of Tang cells and suggest that several diseases affect Tang cell levels.

Interestingly, we did not observe a correlation between levels of Tang cells and EPCs. The loss of association between these two angiogenic bone marrow-derived cells may indicate a disruption of angiogenic repair by multiple disease-related processes, such as oxidative stress and hyperglycaemia.[13]

The only other intervention study on Tang cell levels was in people with rheumatoid arthritis,[14] in which Tang cells increased significantly after TNF-α blockade treatment, especially in those people responding well to therapy.[14] Our results, although obtained with a completely different intervention, seem to be in line. Tang cell levels

increased following Linagliptin treatment and their rise correlated negatively with HbA1c reduction. This supports the hypothesis that interventions on disease control stimulate bone-marrow Tang cell recruitment, which may potentially have a beneficial effect on endothelial repair.

The effects of DPP-4 inhibitors on EPC levels have been investigated previously. A 4-day study with Linagliptin increased EPC levels in people with T2DM,[15] while 12-week treatment did not affect EPC levels.[16] In the first study, EPCs were defined as CD34^{+} CD133^{+} or CD34^{+} KDR^{+}, while in the second study EPCs were defined as CD34^{+} CD133^{+}KDR^{+}, as in the present study. Consequently, the duration, as well as gating strategy could have played a role in the neutral effect of Linagliptin in the present study and underlines the importance of measuring EPCs by standardized markers. As mentioned, IL-8 and VEGF levels were negatively correlated to EPC levels at baseline in people with T2DM, and both IL-8 and VEGF levels were not changed after Linagliptin treatment as well. We did see an increase in SDF-1α levels in the Linagliptin-treated participants.

The present study has some limitations. First, the duration of treatment was 6 months, so whether long-term changes would be sustained or even be of a larger magnitude is unknown. Additionally, it is unclear whether an increase in Tang cell levels would also result in a significant reduction of CV events. Second, the trial lacked an active control group, so whether Tang cell levels would increase after treatment with other anti-hyperglycaemic drugs is also unclear. Furthermore, we included recently diagnosed people with T2DM so it might not be possible to extrapolate the findings to those with a longer diabetes duration or with advanced atherosclerotic disease.

In conclusion, Tang cell levels are decreased in people with T2DM, and Linagliptin stimulates recruitment of these cells. This might indicate the value of DPP-4 inhibitor treatment in the course of T2DM in repairing endothelial damage of the micro- and microvasculature.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

D.J.M. analysed and interpreted data, conceived and designed the study and wrote the manuscript. S.A.d.B. and E.S.H. collected, analysed and interpreted the data, and wrote the manuscript. M.R. interpreted the data and wrote the manuscript. R.H.J.A.S. analysed the data, conceived and designed the study and reviewed the manuscript critically for intellectual content. W.H.A. analysed and interpreted the data, conceived the study and reviewed the manuscript critically for intellectual content. H.J.L.H. conceived and designed the study and reviewed the manuscript critically for intellectual content. J. W. collected, analysed and interpreted data, conceived and designed the study and reviewed the manuscript critically for intellectual content. D.J.M. and J.W. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final version.

ORCID

Stefanie A. de Boer https://orcid.org/0000-0002-7532-2536
Melanie Reijrink https://orcid.org/0000-0001-9447-7333
Hiddo J. L. Heerspink https://orcid.org/0000-0002-3126-3730
Johanna Westra https://orcid.org/0000-0002-6581-6508

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SUPPORTING INFORMATION

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