Influencing the homing and differentiation of MNCs in hereditary hemorrhagic telangiectasia

Dingenouts, C.K.E.

7

General Discussion

HHT1 is caused by mutations in endoglin, the TGFβ co-receptor. Its main symptoms include severe epistaxis and hemorrhages, and as a result HHT1 was long considered a disorder affecting angiogenesis only. As it became clear that endoglin heterozygosity disturbs the function of many more cell types and processes, such as wound repair, the aim of my thesis was to understand the role of immune cells on tissue repair in the context of endoglin heterozygosity. We therefore investigated the impact of increased TGFβ signaling and the systemic application of DPP4 inhibition in endoglin heterozygous mouse models.

DPP4 regulation is essential for the controlled manner in which MNCs to home towards damaged tissue and contribute to repair. MNC homing in endoglin heterozygous mice is impaired, therefore we studied the effect of DPP4 inhibition in order to increase homing and thus enhance tissue repair. Furthermore, endoglin heterozygosity causes the TGFβ and BMP signaling balance to be disturbed. To investigate the effect of skewing the BMP/TGFβ signaling pathways towards increased TGFβ signaling, we inhibited BMP signaling using BMPRI inhibitor LDN. We investigated the effects of LDN and DPP4 inhibitor treatments in various ischemic and wounding models: MI, HLI and wound healing of the dermis, for WT and *Eng*$^{+/-}$ mice, both at functional and molecular level. Below the findings and their implications are discussed in more detail.

Inhibition of DPP4 increases MNC homing and drives differentiation of macrophages

Cardiovascular disease is a major health issue in the western world. Understanding and improving tissue repair in HHT1 setting could help improve treatment for other patients with tissue damage as well. For tissue repair, the initial immune response to damage is similar to that of inflammation caused by pathogens. A normal inflammatory response clears away cell debris and initiates fibrosis and angiogenesis. HHT1 is hallmarked by impaired angiogenesis and subsequent decreased maturation of the vasculature formed. In addition, an elevated immune response adds to the impaired tissue repair. As we discuss in Chapter 3, we were able to restore MNC homing and short term improvement of cardiac function after MI in a murine model of HHT1. However, cardiac function did not show any major long term improvement. So although fibrotic scarring was significantly reduced and angiogenesis increased, it became apparent that this is not sufficient for tissue repair. In the DPP4 treated *Eng*$^{+/-}$ mice, we also found that arteriogenesis was reduced, indicating maturation of vessels is still not optimal. Furthermore, an effect on macrophage differentiation was observed. Both HHT1 patients and *Eng*$^{+/-}$ mice have elevated numbers of M1 inflammatory macrophages, and after treating with a DPP4 inhibitor, the number of M1 in the infarct border zone significantly increased. Looking at the inflammation (14 days post-MI) in normal, healthy animals the inflammation was resolved, and only a few macrophages were still present in the infarct border zone, however in *Eng*$^{+/-}$ animals, these numbers were highly increased. This long term increased macrophage presence points out the imbalance in the HHT1 immune response, underlining the defect in inflammation and an intrinsic problem regarding macrophage function.

Interestingly, DPP4 inhibitor treatment increased the number of reparative macrophages (M2) in the infarct border zones of the *Eng*$^{+/-}$ mice. DPP4 inhibition seems therefore able to either recruit more M2-like macrophages, or induce their differentiation. The increase of M2 macrophages was not observed in wild type (WT) mice, therefore I hypothesize that either the defect in TGFβ signaling affects macrophage differentiation and/or function, and another possibility is that the differentiation in 'healthy' mice is already optimal, so therefore we do not detect any differences when treating WT mice with DPP4 inhibitor. The variation in stimuli-response between WT and *Eng*$^{+/-}$ mice was also apparent when we treated macrophages *in*

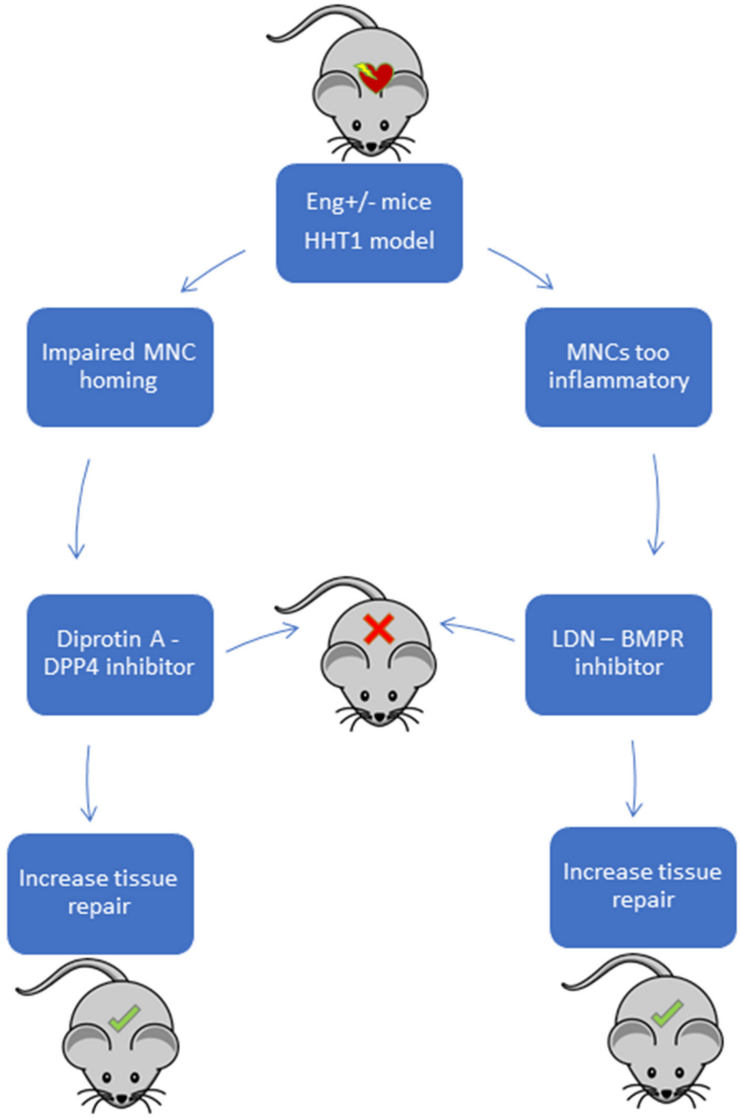

Fig. 1 Influencing the homing and differentiation of MNCs in HHT1. Depicted is a schematic representation of the thesis subjects. Endoglin heterozygous mice were used to model HHT1. In various experimental methods inducing ischemic and/or tissue damage, we improved tissue repair in the mice via two approaches. First, using DPP4 inhibition, via increase of the SDF1-CXCR4 homing mechanism, we restored the impaired homing capacity of HHT1-MNCs, and also increased reparative M2 macrophage numbers post-MI. The second treatment approach focused on stimulating TGFβ signaling and M2 differentiation via use of the BMPR inhibitor LDN. In vitro studies showed LDN increased M2 differentiation. In vivo, we observed increased tissue repair in several experimental models, however we did not find an effect on macrophage differentiation. Combining the DPP4 inhibitor and LDN treatment together did not result in a positive outcome on repair after induction of MI. Thus, the stand-alone treatments improved tissue repair in Eng+/- mice, but the combined treatments did not.

vitro with TGFβ in Chapter 4. In this chapter we applied TGFβ to macrophage cultures, and we observed that M2 macrophages were induced in wild type cells, which did not happen in the *Eng*$^{+/-}$ cells. Only upon combining TGFβ with ALK1/2/3 inhibition using LDN did these macrophages differentiate towards M2. Furthermore, LDN treatment reduced fibrosis after MI, increased heart function, and increased blood flow recovery after HLI – however in the heart LDN treatment did not have any effect on macrophage M1/M2 differentiation or numbers present (Fig. 2), suggesting perhaps other effects of LDN that cause the increased tissue repair. Indeed we found that non-SMAD signaling in *Eng*$^{+/-}$ macrophages was severely blunted, and although LDN treatment did not affect these non-SMAD responses, SMAD2 phosphorylation was significantly increased in *Eng*$^{+/-}$ macrophages compared to WT upon treatment with LDN. Furthermore, the effects on signaling described were performed on cultured macrophages, of course as LDN is given systemically to the mice *in vivo*, we cannot exclude effects on other cell types, such as fibroblasts or endothelial cells.

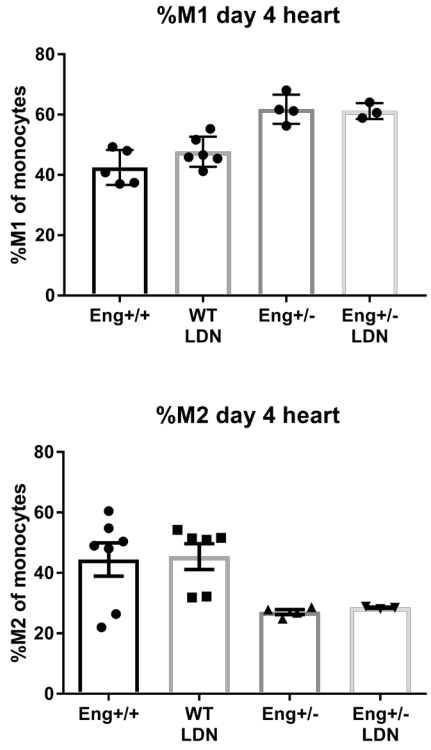

Fig. 2 LDN treatment had no effect on in vivo macrophage differentiation in the heart. Flow cytometric analysis of MNCs isolated from the left cardiac ventricle. Measurement 4 days post-MI. Ly6Chigh =M1 Ly6Clow =M2.

The separate beneficial results by DPP4 inhibition and BMPR inhibition on tissue repair in *Eng*$^{+/-}$ mice made us question if combined therapy with the two compounds would have a synergistic effect on cardiac repair post-MI. In Chapter 3, we obtained improved MNC homing and M1/M2 balance via DPP4 inhibition using Diprotin A, and in Chapter 4, ischemic damage was restored back to WT levels by BMP inhibition, using LDN. Furthermore, the reduction of infarct size and increased angio- and arteriogenesis by both individual treatments implied that a combination of treatments could be promising. We therefore induced MI in WT and *Eng*$^{+/-}$ mice, and treated the mice with both the DPP4 inhibitor Diprotin A (DipA) and the BMPR inhibitor LDN, and monitored their cardiac function over time using ultrasound. Usually, about 10-20% of the mice perish because of cardiac rupture during the first week after MI. Unexpectedly, in the first week after myocardial infarction, 60% of the WT animals treated with the DipA/LDN combination therapy died of acute cardiac rupture, while only 20% of *Eng*$^{+/-}$ mice died when receiving the same treatment (Fig. 3).

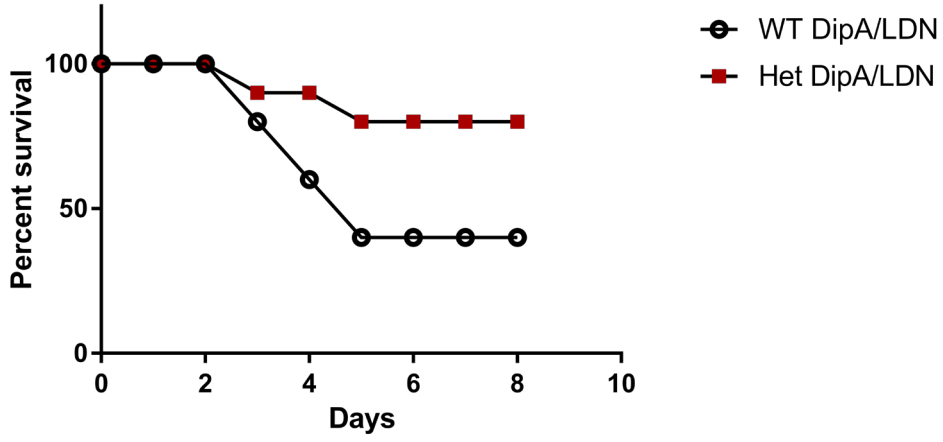

Fig. 3 DipA/LDN co-treatment causes acute cardiac rupture in WT mice. Survival graph: black = WT, red= Eng+/- animals. All animals were treated with the DipA/LDN combination therapy. N=10 mice per group.

Mice that died of acute rupture were assessed for infarct size, and measurements showed that the relative infarct size was larger compared to the surviving mice (Fig. 4). This suggests animals with large infarct sizes, and in particular WT animals, are negatively affected by treatment with DipA/LDN, causing cardiac ruptures. We hypothesize that because all ruptures were in the first week post-MI; this coincides with the acute phase of tissue repair, where MNCs infiltrate the damaged tissue. In WT mice post-MI, this process is optimal, and treatment with DipA/LDN could actually be interfering with this process. I hypothesize that where DipA stimulates homing and influences differentiation, and LDN reduces the level of fibrosis; the cardiac tissue is not able to handle the influx of cells and consequently ruptures.

Of the surviving mice, DipA/LDN treatment did not show a short or long term beneficial effect (Fig. 5), confirming the combined treatment has no positive effect on cardiac recovery, even with smaller infarct sizes.

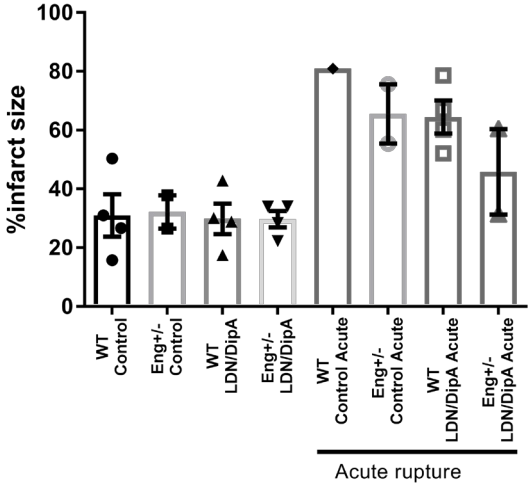

Fig. 4 Infarct size. On the left side of the graphs: animals that survived up to 3 months post-MI, right side of the graph; animals that died of acute cardiac ruptures (minus 2 mice where the heart was not isolated). DipA/LDN co-treatment. N=1-4 per group. Hematoxylin and Eosin staining of cardiac tissue, percentage infarct size area of the left ventricle.

Endoglin heterozygosity blunts intracellular TGFβ signaling in macrophages

Further investigating macrophage function and signaling in *Eng*$^{+/-}$ mice, we found that while the SMAD response was not severely affected, the non-canonical pathways were highly unresponsive to any TGFβ stimulation or inhibition.

In Chapter 5 the baseline levels of several non-canonical signaling proteins were compared. The *Eng*$^{+/-}$ levels for pERK1/2 and p-p38 were significantly higher than the wild types. ERK and p38 are involved in inflammation, cell survival and apoptosis[1-4], moreover p38 is also involved in M2 differentiation and activation[5,6]. The varying levels of these signaling pathways indicate that the macrophage responses to stress are affected. Therefore we hypothesized that there could be a direct link between endoglin/TGFβ signaling and DPP4. DPP4 is a transmembrane protein, found on multiple cell types and as discussed previously, regulates MNC homing. It was reported that DPP4 has stimulatory functions regarding T cell activation[7-9], indicating there are possibly more unknown functions of DPP4 involving the immune system. We found that DPP4 inhibition decreases phosphorylation of several non-canonical TGFβ signaling molecules involved in the pro-inflammatory response: ERK1/2 and AKT (Chapter 5). Other studies showed that TGFβ downregulates DPP4 expression[10] and in another study DPP4 inhibition was reported to increase TGFβ secretion in MNCs[11]. The decrease in ERK1/2 and AKT activation, together with the direct link between TGFβ decreasing DPP4 expression suggests that DPP4 can have either direct and/or indirect effects on TGFβ signaling. At the least for inflammatory conditions, my study in Chapter 5 and other studies show that DPP4 inhibition has immunosuppressive actions[12]; phosphorylation of pro-inflammatory NFκB-related proteins such as p65, IκBα and JNK were found to be decreased when macrophages were stimulated with LPS and treated with a DPP4 inhibitor.

In Chapter 6 we observed that DPP4 inhibition increased the percentage of macrophages present in the epicardium of *Eng*$^{+/-}$ mice after MI. We conclude here that endoglin is important for a fully functional immune system and a change in the immune response may be the cause the decreased epicardial repair in *Eng*$^{+/-}$ mice after MI. We demonstrated that

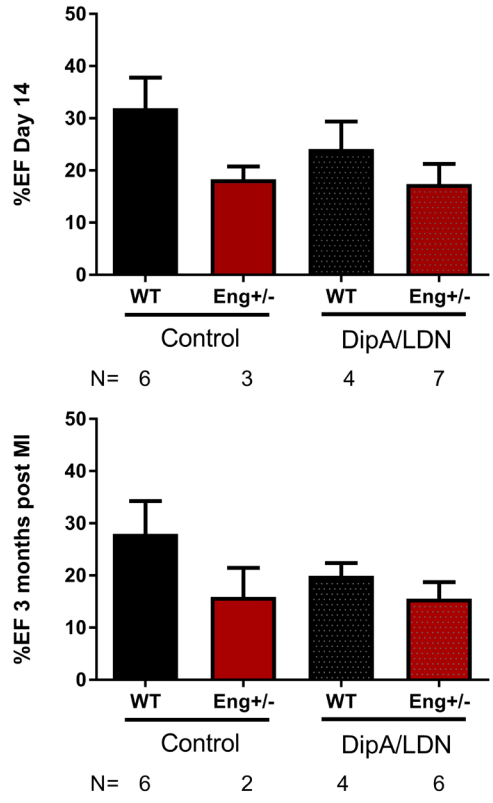

Fig. 5 Long term cardiac function does not improve with DipA/LDN combination treatment. A. Percent ejection fraction 14 days post-MI. Control versus DipA/LDN co-treatment in mice. Measurements taken via cardiac ultrasound of the left ventricle. B. Percent ejection fraction 14 days post-MI. Control versus DipA/LDN co-treatment in mice. Measurements taken via cardiac ultrasound of the left ventricle.

where *Eng*$^{+/-}$ mice after MI typically show increased epicardial thickening, short term DPP4 inhibitor treatment normalizes epicardial thinning to WT levels.
In conclusion, DPP4 inhibitors can affect macrophage polarization, as is also reported in a murine model of obesity where DPP4 inhibition resulted in M2 polarization in the liver and adipose tissues, reducing the obesity-induced inflammation response and resistance to insulin[13]. This M2 shift we too observed in the myocardial infarct border zone and in dermal tissue treated with a DPP4 inhibitor (Chapter 3 and 5 respectively), suggesting DPP4 inhibitors are anti-inflammatory and can modulate macrophage differentiation toward the reparative M2 macrophages.

Modulation of DPP4-mediated processes
An interesting point of discussion is that DPP4 can be either membrane-bound or shedded/cleaved to a soluble form. It is therefore interesting to take into account what the differences in signaling and mechanistic effects of these two types of the protein are. Solubilization of DPP4 is a process not yet completely understood, but in hypoxic conditions is mediated by several MMPs, resulting in the shedding of DPP4[14]. An important source for soluble

DPP4 are the kidneys, although recent research also points towards the leukocytes and macrophages[15]. The intracellular and transmembrane parts of DPP4 are absent in the soluble form, but its enzymatic function remains intact[16,17]. Soluble DPP4 was shown to induce endothelial constriction, possibly contributing to exacerbation of cardiovascular disease[18]. In disease setting, soluble DPP4 can either have good or bad prognostic values when measured as a biomarker in the serum; high plasma levels DPP4 activity were found associated with increased occurrence of coronary artery disease[19], the progression of atherosclerosis in mice[20] and was indicative of colorectal metastases in patients[21].
Another point of discussion is that when DPP4 cleaves SDF1, GLP1 or any of its other ligands, we can question whether or not these cleaved products are completely inactive[22]. DPP4 inhibition may therefore modulate ligand activity in subtle or unexpected ways, and this can vary for every type of DPP4 inhibitor molecule available[23]. For example, Linagliptin is known to block EndoMT, whereas Sitagliptin does not[23,24]. Further research into these inhibitors is therefore necessary, especially in HHT1 context, where endoglin heterozygosity influences more functions and processes in the immune system and macrophages that we currently know of. For example, endoglin was shown to have direct cross-talk with the Hippo pathway (regulating cell proliferation and apoptosis), resulting in altered monocyte chemotactic protein-1 (MCP1/CCL2) expression[25]. The interactions of DPP4 and endoglin thus remain interesting targets for immune modulation.

Clinical applications of DPP4 inhibitors and future perspectives

Many DPP4 inhibitors have been developed and are currently used in the clinic. The different DPP4 inhibitors have various mechanisms of action[23]; for example because of permanent or reversible binding to their targets. Therefore the differences between the various DPP4 inhibitors need to be extensively researched and documented, as the disease type, organ and cell type can severely affect results[23,26,27]. The protein DPP4 itself is able to interact, degrade or bind to many other proteins, such as SDF1, GLP1, NPY, ADA, fibronectin and collagen[28]. Therefore, DPP4 inhibition could be used in several clinical applications other than improving the MNC homing in HHT1 or decreasing the fibrotic response in tissue repair. DPP4 inhibitors are known to regulate microRNA levels, possibly useful in decreasing kidney disease progression in chronic renal disease and diabetes mellitus type 2 (DMT2)[27,29]. Also in atherosclerosis, DPP4 inhibition led to decreased vascular smooth muscle cell proliferation, decreased macrophage inflammation status and reduced foam cell induction[30,31]. In accordance with our findings for DPP4, inhibiting DPP8 and 9 (which are highly expressed in AS plaques) *in vitro* reduced inflammation status of murine macrophages[32], confirming the immunomodulatory role of DPP4 and its related proteins.
DPP4 inhibitors have possible applications in many fields of medicine. In anti-tumor therapy, DPP4 inhibition was able to improve T cell migration *in vivo*, and resulted in improved reactions to immunotherapy[33,34]. DPP4 has even been linked to stress and depression disorders; soluble DPP4 was found decreased in patients suffering from depression and could be reversed by anti-depressive treatment[35]. Contrastingly, in another patient cohort plasma DPP4 activity was found increased[36], confirming more research is necessary, and already suggesting possible population and disorder/treatment differences exist for DPP4 plasma activity.
DPP4 is highly expressed on lymphocytes[28] and high membrane DPP4 was shown to decrease recovery of cardiac function in CVD patients[37]. Inhibition of DPP4 could therefore possibly be a suitable treatment in cardiovascular disease patients. DPP4 inhibition in clinical trials is well tolerated and despite some reports about adverse effects[38-41], its

overall use seems to be either beneficial[42-45] or results show treatment had no effect on cardiovascular outcome or risk[46-48]. In future research, DPP4 inhibition is best used in concert with other drugs or therapies that stimulate cardiac repair, like anti-coagulants or cell therapy.
More research is needed to understand the beneficial impact of DPP4 inhibition on the HHT1 immune system and tissue repair, and the research presented in this thesis aimed to improve insight on the mechanisms involved in endoglin heterozygosity, in order to improve treatment in not only HHT1 patients, but also patients with ischemic injury.

References

1. Jung, Y.C., *et al.* Anti-inflammatory effects of galangin on lipopolysaccharide-activated macrophages via ERK and NF-kappaB pathway regulation. *Immunopharmacol Immunotoxicol* **36**, 426-432 (2014).

2. Wang, C., *et al.* Microgravity activates p38 MAPK-C/EBPbeta pathway to regulate the expression of arginase and inflammatory cytokines in macrophages. *Inflamm Res* **64**, 303-311 (2015).

3. Monick, M.M., *et al.* Constitutive ERK MAPK activity regulates macrophage ATP production and mitochondrial integrity. *Journal of immunology (Baltimore, Md. : 1950)* **180**, 7485-7496 (2008).

4. Shao, Q., Han, F., Peng, S. & He, B. Nur77 inhibits oxLDL induced apoptosis of macrophages via the p38 MAPK signaling pathway. *Biochem Biophys Res Commun* **471**, 633-638 (2016).

5. Jimenez-Garcia, L., Herranz, S., Luque, A. & Hortelano, S. Critical role of p38 MAPK in IL-4-induced alternative activation of peritoneal macrophages. *Eur J Immunol* **45**, 273-286 (2015).

6. Zhang, O. & Zhang, J. Atorvastatin promotes human monocyte differentiation toward alternative M2 macrophages through p38 mitogen-activated protein kinase-dependent peroxisome proliferator-activated receptor gamma activation. *Int Immunopharmacol* **26**, 58-64 (2015).

7. Thompson, C. Fiery entrance for the HIV co-receptor. *Lancet* **343**, 49 (1994).

8. Gorrell, M.D., Gysbers, V. & McCaughan, G.W. CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes. *Scandinavian journal of immunology* **54**, 249-264 (2001).

9. Hildebrandt, M., *et al.* Apheresis-related enrichment of CD26++ T lymphocytes: phenotypic characterization and correlation with unfavorable outcome in autologous hematopoietic progenitor cell transplantation. *Transplantation and cellula therapy* **52**, 765-776 (2012).

10. Uematsu, T., Tanaka, H., Yamaoka, M. & Furasawa, K. Effects of Oral Squamous Cell Carcinoma-derived TGF-β1 on CD26/DPPIV Expression in T Cells. *Anticancer research* **24**, 619-624 (2004).

11. Reinhold, D., *et al.* Inhibitors of dipeptidyl peptidase IV induce secretion of transforming growth factor beta 1 in PWM-stimulated PBMC and T cells. *Immunology* **91**, 354-360 (1997).

12. Shinjo, T., *et al*. DPP-IV inhibitor anagliptin exerts anti-inflammatory effects on macrophages, adipocytes, and mouse livers by suppressing NF-kappaB activation. *Am J Physiol Endocrinol Metab* **309**, E214-223 (2015).

13. Zhuge, F., *et al.* DPP-4 Inhibition by Linagliptin Attenuates Obesity-Related Inflammation and Insulin Resistance by Regulating M1/M2 Macrophage Polarization. *Diabetes* **65**, 2966-2979

(2016).

14. Röhrborn, D., Wronkowitz, N. & Eckel, J. DPP4 in diabetes. *Frontiers in Immunology* **6**, 1-20 (2015).

15. Wang, Z.*, et al.* Soluble DPP4 originates in part from bone marrow cells and not from the kidney. *Peptides* **57**, 109-117 (2014).

16. Lambeir, A.-M., Durinx, C., Scharpé, S. & De Meester, I. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. *Critical reviews in clinical laboratory sciences* **40**, 209-294 (2003).

17. Waumans, Y.*, et al.* The dipeptidyl peptidase family , prolyl oligopeptidase , and prolyl carboxypeptidase in the immune system and inflammatory disease , including atherosclerosis Reviewed by :. **6**, 1-18 (2015).

18. Romacho, T.*, et al.* Soluble dipeptidyl peptidase-4 induces microvascular endothelial dysfunction through proteinase-activated receptor-2 and thromboxane A2 release. *J Hypertens* **34**, 869-876 (2016).

19. Yang, G.*, et al.* Increased Plasma Dipeptidyl Peptidase-4 Activities in Patients with Coronary Artery Disease. *PLoS One* **11**, e0163027 (2016).

20. Ervinna, N.*, et al.* Anagliptin, a DPP-4 Inhibitor, Suppresses Proliferation of Vascular Smooth Muscles and Monocyte Inflammatory Reaction and Attenuates Atherosclerosis in Male apo E-Deficient Mice. *Endocrinology* **154**, 1260-1270 (2013).

21. De Chiara, L.*, et al.* Postoperative serum levels of sCD26 for surveillance in colorectal cancer patients. *PLoS One* **9**, e107470 (2014).

22. Cantini, G., Di Franco, A., Mannucci, E. & Luconi, M. Is cleaved glucagon-like peptide 1 really inactive? Effects of GLP-1(9-36) on human adipose stem cells. *Mol Cell Endocrinol* **439**, 10-15 (2017).

23. Shi, S., Kanasaki, K. & Koya, D. Linagliptin but not Sitagliptin inhibited transforming growth factor-β2-induced endothelial DPP-4 activity and the endothelial-mesenchymal transition. *Biochemical and Biophysical Research Communications* **471**, 184-190 (2016).

24. Kaji, K.*, et al.* Dipeptidyl peptidase-4 inhibitor attenuates hepatic fibrosis via suppression of activated hepatic stellate cell in rats. *Journal of Gastroenterology* **49**, 481-491 (2014).

25. Young, K.*, et al.* BMP9 Crosstalk with the Hippo Pathway Regulates Endothelial Cell Matricellular and Chemokine Responses. *PloS one* **10**, e0122892 (2015).

26. Dushenko, S.*, et al.* Gate-Tunable Spin-Charge Conversion and the Role of Spin-Orbit Interaction in Graphene. *Phys Rev Lett* **116**, 166102 (2016).

27. Shi, S., Koya, D. & Kanasaki, K. Dipeptidyl peptidase-4 and kidney fibrosis in diabetes. *Fibrogenesis & tissue repair* **9**, 1 (2016).

28. Klemann, C., Wagner, L., Stephan, M. & von Horsten, S. Cut to the chase: a review of CD26/dipeptidyl peptidase-4's (DPP4) entanglement in the immune system. *Clin Exp Immunol* **185**, 1-21 (2016).

29. Kanasaki, K.*, et al.* Linagliptin-Mediated DPP-4 Inhibition Ameliorates Kidney Fibrosis in Streptozotocin-Induced Diabetic Mice by Inhibiting Endothelial-to-Mesenchymal Transition in a

Therapeutic Regimen. *Diabetes* **63**, 2120-2131 (2014).

30. Brenner, C.*, et al.* DPP-4 inhibition ameliorates atherosclerosis by priming monocytes into M2 macrophages. *International Journal of Cardiology* **199**, 163-169 (2015).

31. Yang, C.-J., Fan, Z.-X., Yang, J. & Yang, J. DPP-4 inhibitors: A potential promising therapeutic target in prevention of atherosclerosis. *International Journal of Cardiology* **202**, 797-798 (2016).

32. Waumans, Y.*, et al.* The Dipeptidyl Peptidases 4, 8, and 9 in Mouse Monocytes and Macrophages: DPP8/9 Inhibition Attenuates M1 Macrophage Activation in Mice. *Inflammation* **39**, 413-424 (2016).

33. Barreira da Silva, R.*, et al.* Dipeptidylpeptidase 4 inhibition enhances lymphocyte trafficking, improving both naturally occurring tumor immunity and immunotherapy. *Nat Immunol* **16**, 850-858 (2015).

34. Ohnuma, K., Hatano, R. & Morimoto, C. DPP4 in anti-tumor immunity: going beyond the enzyme. *Nature immunology* **16**, 791-792 (2015).

35. Wagner, L.*, et al.* Identifying neuropeptide Y (NPY) as the main stress-related substrate of dipeptidyl peptidase 4 (DPP4) in blood circulation. *Neuropeptides* **57**, 21-34 (2016).

36. Zheng, T.*, et al.* Increased Dipeptidyl Peptidase-4 Activity Is Associated With High Prevalence of Depression in Middle-Aged and Older Adults: A Cross-Sectional Study. *J Clin Psychiatry* **77**, e1248-e1255 (2016).

37. Post, S.*, et al.* Reduced CD26 expression is associated with improved cardiac function after acute myocardial infarction. *Journal of Molecular and Cellular Cardiology* **53**, 899-905 (2012).

38. Monami, M., Dicembrini, I. & Mannucci, E. Dipeptidyl peptidase-4 inhibitors and heart failure: A meta-analysis of randomized clinical trials. *Nutrition, Metabolism and Cardiovascular Diseases* **24**, 689-697 (2014).

39. Savarese, G.*, et al.* Cardiovascular effects of dipeptidyl peptidase-4 inhibitors in diabetic patients: A meta-analysis. *International Journal of Cardiology* **181**, 239-244 (2015).

40. Kannan, S.*, et al.* Risk of overall mortality and cardiovascular events in patients with type 2 diabetes on dual drug therapy including metformin: A large database study from the Cleveland Clinic. *J Diabetes* **8**, 279-285 (2016).

41. Tagaya, Y., Okada, S., Hisada, T., Niijima, Y. & Yamada, M. Interstitial pneumonia during administration of dipeptidyl peptidase-4 inhibitors. *J Diabetes* **8**, 442 (2016).

42. Zhou, J.B., Bai, L., Wang, Y. & Yang, J.K. The benefits and risks of DPP4-inhibitors vs. sulfonylureas for patients with type 2 diabetes: accumulated evidence from randomised controlled trial. *Int J Clin Pract* **70**, 132-141 (2016).

43. Foroutan, N., Muratov, S. & Levine, M. Safety and efficacy of dipeptidyl peptidase-4 inhibitors vs sulfonylurea in metformin-based combination therapy for type 2 diabetes mellitus: Systematic review and meta-analysis. *Clin Invest Med* **39**, E48-62 (2016).

44. Monami, M., Ahrén, B., Dicembrini, I. & Mannucci, E. Dipeptidyl peptidase-4 inhibitors and cardiovascular risk: a meta-analysis of randomized clinical trials. *Diabetes, Obesity and Metabolism* **15**, 112-120 (2013).

45. Ou, S.M.*, et al.* Dipeptidyl peptidase-4 inhibitors and cardiovascular risks in patients with

pre-existing heart failure. *Heart* **103**, 414-420 (2017).

46. Koska, J., Sands, M., Burciu, C. & Reaven, P. Cardiovascular effects of dipeptidyl peptidase-4 inhibitors in patients with type 2 diabetes. *Diab Vasc Dis Res* **12**, 154-163 (2015).

47. Theiss, H.D.*, et al.* Safety and efficacy of SITAgliptin plus GRanulocyte-colony-stimulating factor in patients suffering from Acute Myocardial Infarction (SITAGRAMI-Trial)—Rationale, design and first interim analysis. *International journal of cardiology* **145**, 282-284 (2010).

48. Zhong, J., Maiseyeu, A., Davis, S.N. & Rajagopalan, S. DPP4 in Cardiometabolic Disease: Recent Insights From the Laboratory and Clinical Trials of DPP4 Inhibition. *Circulation Research* **116**, 1491-1504 (2015).