# Influencing the homing and differentiation of MNCs in hereditary hemorrhagic telangiectasia

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# 1

# General introduction

## Background

Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber disease is a rare genetic autosomal dominant disorder, known for its endothelial dysplasia causing arteriovenous malformations, severe nose bleeds and internal bleedings1. HHT has an estimated prevalence affecting 1 in 5000 people[1,2]. HHT severity and disease onset are highly variable between patients, and symptoms progressively worsen with age[3]. HHT is officially diagnosed if a patient has 3 out of the 4 criteria, namely epistaxis, telangiectasias, arteriovenous malformations (AVMs) and/or a first degree relative with HHT. As establishing the diagnosis for HHT was often difficult, clinicians treating HHT patients gathered on Curaçao, where they decided on guidelines to improve diagnosis, the Curaçao criteria. The Curaçao criteria are now a standardized set of symptoms and characteristics. When patients are diagnosed, genetic testing is performed to identify the mutation[4,5].

The clinical symptoms remain important in the diagnosis of HHT, as the mutations underlying the disease are sometimes still unknown. So far, several HHT mutations with known target genes have been identified. Although not in every patient a mutation is found, in the majority of patients mutations are found in genes belonging to the TGFβ superfamily, causing a disbalance in the TGFβ signaling pathway by haploinsufficiency of the remaining functional protein[6,7]. HHT type 1 (HHT1) is the most prevalent HHT variant, and its mutation lies in the endoglin gene[8], encoding a protein which functions as co-receptor of TGFβ, and is crucial to neo-angiogenesis and vascular repair[9]. Mutations in the second most prevalent variant is the activin receptor-like kinase 1 (ALK1) and is referred to as HHT2[10]. ALK1 is a TGFβ type I receptor involved in the angiogenic switch; the promotion and inhibition of angiogenesis[11]. HHT3 and HHT4 are caused by mutations in chromosome 5 and 7 respectively, but the genes or proteins affected are not yet identified[12,13]. Mutations in the third gene are in SMAD4, a downstream signaling protein of TGFβ/BMP, causing the combined syndrome of juvenile polyposis, known as JP-HHT[14]. Recently BMP9 (a ligand for ALK1) was identified as a fourth gene responsible for HHT5[15]. The various mouse models available that represent the HHT variants are discussed in more detail in Chapter 2 of this thesis.

### TGFβ signaling and endothelial cells

The vascular defects and impaired angiogenesis in HHT1 are primarily caused by malfunctioning of the activation of ECs. Endoglin is highly expressed on activated endothelial cells (ECs) and it is crucial for angiogenesis, providing signaling involving proliferation, migration and remodeling of the extracellular matrix[9,16]. The effects of endoglin haploinsufficiency are therefore extensively researched in this cell type. Endoglin is a co-receptor for TGFβ, a multifunctional cytokine involved in many cellular processes ranging from proliferation, migration and fibrosis to apoptosis. Upon tissue damage, TGFβ is released by the extracellular matrix, apoptotic cells or platelets, and secreted by fibroblasts, macrophages and T lymphocytes[17,18].

The TGFβ signaling pathway encompasses the TGFβ superfamily of ligands, all binding to a specific type I/II receptor combination (Fig. 1). The TGFβ superfamily of ligands can be divided in two groups, the TGFβ ligands and BMP ligands. Upon binding of the ligand to the type II receptor, a receptor type I is recruited and a tetrameric complex is formed, after which the type II receptor kinase activates the type I receptor. The type I receptor kinase in turn phosphorylates intracellular receptor-regulated SMADs (R-SMADs) proteins. ALK1 can form a complex with the TGFβ or BMP receptor type II in the presence of ALK5 and

the presence of endoglin as co-receptor[19-22]. The TGFβ receptor type I consists of two subtypes, ALK1 signaling via SMADs 1, 5 and 8, and ALK5 signaling via SMAD 2 and 3. In ECs, TGFβ-ALK1 signaling leads to proliferation and migration, whereas TGFβ-ALK5 signaling leads to a quiescent state of the EC. Endoglin mainly stimulates the TGFβ-ALK1 pathway, and suppresses the TGFβ-ALK5 pathway[23]. After forming a complex with the common mediator SMAD or co-SMAD: SMAD4, the R-SMAD and co-SMAD complex translocates to the nucleus where it acts as a transcription factor and modifies target gene expression.

The TGFβ signaling pathway is tightly controlled and involves many activators and inhibitors. Although endothelial cells[24] and pericytes[25] have been the main research focus for HHT, mononuclear cells (MNCs) also play an important role in vascular homeostasis, integrity and repair. We have previously shown that endoglin heterozygosity impairs homing of HHT1-MNCs[26]. Studies in HHT1 patients reported increased occurrences of severe bacterial infections in, such as cerebral, hepatic, muscular and other infections[27,28], possibly due to the impaired MNC response.

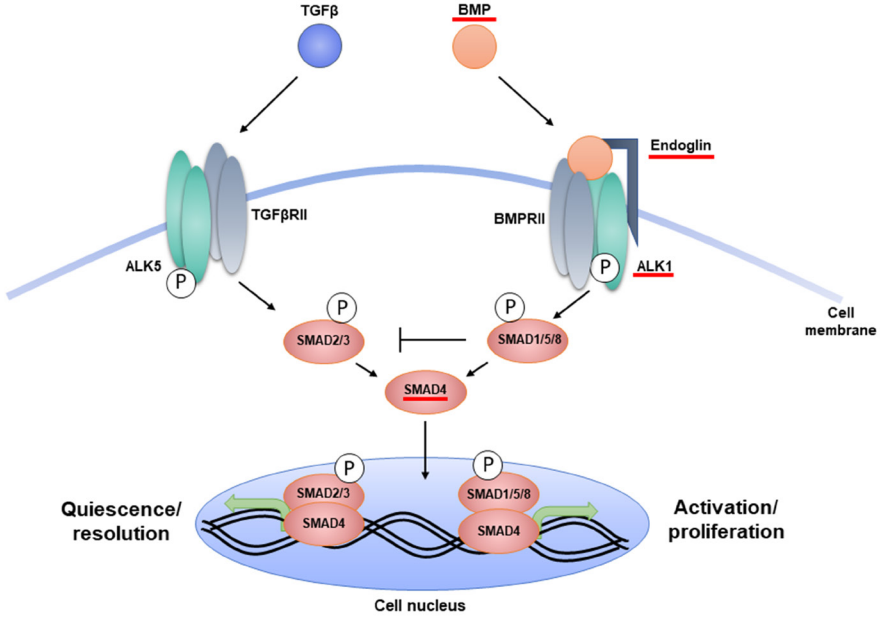


**Fig. 1 Schematic representation of TGFβ signaling. Underlined in red are the proteins affected in various HHT subtypes.**

**MNC trafficking and homing towards tissue damage**

The process by which MNCs are attracted to ischemic, damaged or inflamed tissue is tightly regulated. The first step in the homing process is regulated via the stromal cell derived factor- 1 (SDF1) – CXCR4 axis. MNCs are recruited from the bone marrow and spleen via the circulation to the site of injury. The MNCs are retained within the tissue by high SDF1 levels[29]. Upon ischemia, hypoxia inducible factor 1 alpha (HIF1α) induces expression of SDF1 in the ischemic cells, which is then released into the bloodstream. This increases systemic SDF1 levels and causes MNCs to be released from the bone marrow and spleen, traffic through the circulation and home to the site of injury –the source of SDF1 production– responding to SDF1 upon its binding to the receptor CXCR4[29,30].

There is a delicate balance between SDF1 and CXCR4, and skewing of either one of the proteins or regulators involved in their activation or inhibition will result in impaired homing, a defective inflammatory response and hamper wound healing and tissue repair.

**DPP4 and its inhibition**

Because the MNCs cannot be allowed to migrate indefinitely, there is suppression of the homing signal; dipeptidyl peptidase-4 (DPP4/CD26). By cleaving the first 2 aminoacids, SDF1 is unable to bind to its receptor or reduce the levels of CXCR4[31,32]. DPP4 is a potent proteolytic enzyme[33,34]. It is a 110kd transmembrane protein and is expressed by various cell types, such as endothelial cells, epithelial cells, melanocytes, monocytes and lymphocytes[35,36]. DPP4 is able to enzymatically cleave aminoterminal dipeptides after a proline or an alanine amino acid from specific target proteins. DPP4 inhibition is a well-known therapy currently in use for type 2 diabetes mellitus (T2DM). In T2DM, DPP4 cleaves glucagon-like peptide-1 (GLP1), an incretin hormone which is released upon food intake, stimulating insulin release. DPP4 inhibitors function via the decrease of incretin degradation, thereby preventing low insulin levels.

To summarize, SDF1 is produced in tissues shortly after an ischemic event, and mobilizes MNCs from the bone marrow to the circulation. Subsequent homing of CXCR4+ cells from the blood to the site of injury is mediated by the SDF1 gradient and its receptor CXCR4. DPP4 was also found to co-localize with CXCR4, and both were internalized upon SDF1 binding[37]. DPP4 enzymatically inactivates SDF1, therefore playing a critical role in limiting MNC recruitment to ischemic areas.

## Outline of the thesis

The aim of my thesis is to understand the effect of endoglin heterozygosity on macrophage-mediated wound healing and tissue repair (Overview in Fig. 2). Regulating the levels of DPP4 is essential for MNCs to home towards damaged tissue, and thereby contributes to efficient repair. We have also previously reported that in HHT1, MNC-DPP4 levels are elevated and homing of HHT1-MNCs towards injured tissue is impaired[26]. When we pre-treated the MNCs from HHT1 patients with a DPP4 inhibitor, and injected these into mice with induced myocardial infarction (MI), these cells were now able to home to the site of injury[26]. In previous studies TGFβ has been reported to modulate DPP4 levels on MNCs[38-40]. However, the impact and role of TGFβ signaling and the systemic application of DPP4 inhibition were not yet fully investigated. Therefore we studied the effect of DPP4 inhibition on tissue repair after dermal injury and myocardial infarction in endoglin

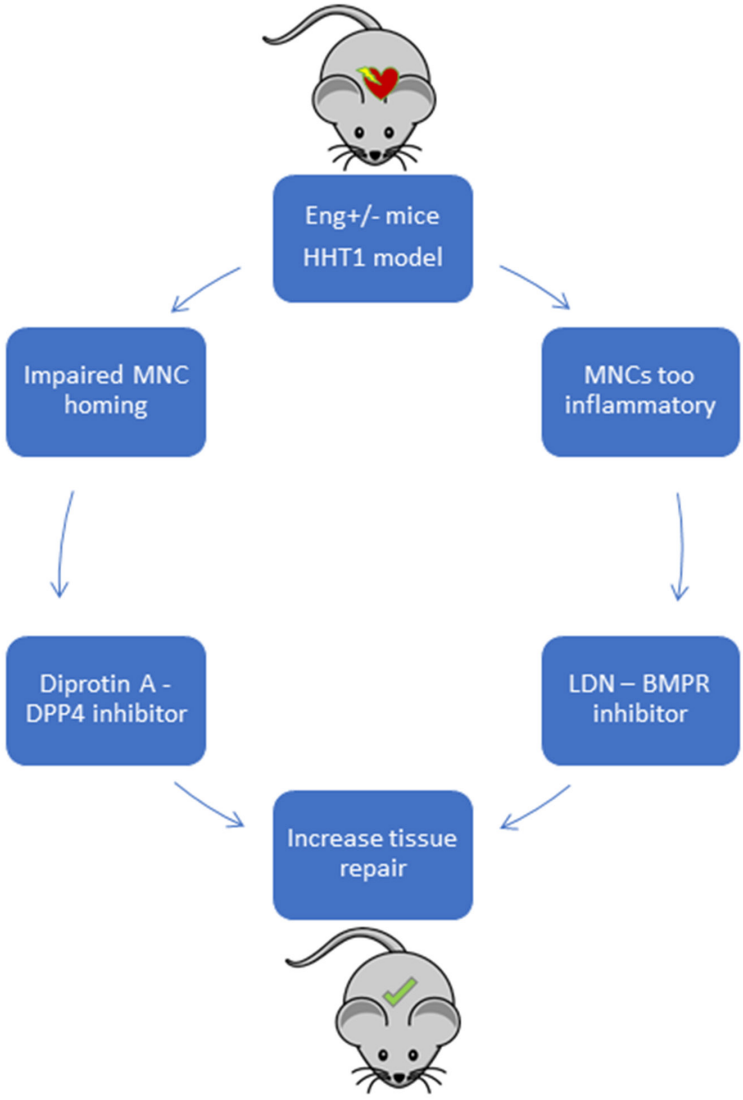


**Fig. 2 Schematic representation of the different aims and approaches to influence HHT1-MNC homing and differentiation to restore tissue repair. Endoglin heterozygous mice were used to model HHT1. In various experimental methods inducing ischemic and/or tissue damage, we aimed to improve tissue repair in the mice via two approaches. First, using DPP4 inhibition, we intended to increase the SDF1-CXCR4 homing mechanism, to restore the impaired homing capacity of the HHT1-MNCs. The second approach was focused on correcting the M1/M2 differentiation in Eng+/- mice. Via use of the BMPR inhibitor LDN we aimed to restore the skewed BMP/TGFβ signaling; stimulating the TGFβ pathway signaling thereby resulting in improved M2 differentiation.**

heterozygous mice, a mouse model of HHT1. We hypothesize that one explanation for the symptoms observed in HHT1 is that the MNCs required for tissue repair and an optimal inflammation response are also affected in their proper functioning. We therefore studied the impaired reparative capacity of macrophages in HHT1 and the deficiency of TGFβ signaling affecting the inflammatory characteristics of macrophages.

## Outline per chapter

HHT1 is caused by mutations in the TGFβ co-receptor endoglin. It was therefore long considered a disorder affecting angiogenesis only. In recent years it has become clear that endoglin heterozygosity disturbs the function of many more cell types and processes. In the following chapters, I will describe several aspects of tissue repair using the mouse model for HHT1, the endoglin heterozygous mouse.

In Chapter 2, a general overview is given on HHT genetics, etiology and signaling, in particular focusing on the role of circulating mononuclear cells, both their impaired homing and contribution to repair in HHT.

In Chapter 3, we show that inhibiting DPP4 in vivo by treating Eng+/- mice after experimentally induced MI, restored homing of MNCs and benefits short term cardiac recovery by reducing fibrosis. Surprisingly, the number of reparative M2 macrophages increased, suggesting that DPP4 inhibition reduces the pro-inflammatory immune response after MI. Furthermore, in Eng+/- mice treated with the DPP4 inhibitor, the number of capillaries present in the infarct border zone increased, whereas number of arteries decreased. This suggests that angiogenesis is stimulated while arteriogenesis is inhibited by DPP4 inhibitor treatment in Eng+/- mice.

In Chapter 4, we explore the use of the small molecule BMPR inhibitor LDN-193189. Endoglin heterozygosity disturbs the BMP/TGFβ signaling balance. In vitro analysis of macrophage differentiation revealed that LDN treatment increased the number of reparative macrophages. By inhibiting BMP signaling using LDN, we aimed to stimulate TGFβ signaling in the Eng+/- animals. Treatment of Eng+/- mice with LDN restored cardiac function and reduced fibrosis after experimentally induced MI. In a second ischemia model, experimentally induced hind limb ischemia, LDN improved blood flow recovery of Eng+/- mice. We found that macrophage signaling via canonical and non-canonical pathways is severely impaired by endoglin heterozygosity.

As macrophage differentiation and tissue repair is impaired in HHT1, in Chapter 5 we studied the effect of DPP4 inhibition in a dermal wounding model in Eng+/- animals, assessing the healing of the lesion. Compared to untreated animals, dermal application of a DPP4 inhibitor increased wound closure speed and increased M2 macrophage numbers in the lesion area. Levels of fibrosis were decreased, signifying a reduction in scarring of the wound site. Furthermore, investigation of intracellular signaling in macrophages showed that in cultured Eng+/- macrophages, non-canonical signaling was severely deregulated.

In Chapter 6 we describe another defect in HHT1 mice: an abnormal epicardial response after myocardial damage. In this study, we analyzed the composition and the behavior of the epicardial layer at different time-points post-MI and found that epicardial thickening is delayed. Furthermore, the epicardium was hyperactive in its response to cardiac ischemic injury when assessed at 14 days post-MI. Systemically treating Eng+/- mice with a DPP4 inhibitor reduced epicardial thickening 14 days post-MI and increased the percentage of

macrophages present in the epicardial infarct border zone.

Finally, in Chapter 7 the results and conclusions of the previous chapters are summarized and discussed in light of future research to understanding HHT.

## References

1. Rozenberg, D., Leek, E. & Faughnan, M.E. Prevalence and nature of dyspnea in patients with hereditary hemorrhagic telangiectasia (HHT). Respir Med 109, 768-777 (2015).

2. Begbie, M.E., Wallace, G.M. & Shovlin, C.L. Hereditary haemorrhagic telangiectasia (Osler-Weber-Rendu syndrome): a view from the 21st century. Postgrad Med J 79, 18-24 (2003).

3. Plauchu, H., de Chadarévian, J.P., Bideau, a. & Robert, J.M. Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. American journal of medical genetics 32, 291-297 (1989).

4. Shovlin, C.L. Hereditary haemorrhagic telangiectasia: pathophysiology, diagnosis and treatment. Blood reviews 24, 203-219 (2010).

5. Shovlin, C.L., et al. Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). American journal of medical genetics 91, 66-67 (2000).

6. Bourdeau, a., et al. Endoglin expression is reduced in normal vessels but still detectable in arteriovenous malformations of patients with hereditary hemorrhagic telangiectasia type 1. The American journal of pathology 156, 911-923 (2000).

7. Bourdeau, A., et al. Potential Role of Modifier Genes Influencing Transforming Growth Factor-β1 Levels in the Development of Vascular Defects in Endoglin Heterozygous Mice with Hereditary Hemorrhagic Telangiectasia. The American Journal of Pathology 158, 2011-2020 (2001).

8. McAllister, K.A., et al. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet 8, 345-351 (1994).

9. van Laake, L.W., et al. Endoglin Has a Crucial Role in Blood Cell-Mediated Vascular Repair. Circulation 114, 2288-2297 (2006).

10. Johnson, D.W., et al. Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. Nat Genet 13, 189-195 (1996).

11. Larrivée, B., et al. ALK1 Signaling Inhibits Angiogenesis by Cooperating with the Notch Pathway. Developmental Cell 22, 489-500 (2012).

12. Cole, S.G., Begbie, M.E., Wallace, G.M.F. & Shovlin, C.L. A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. Journal of medical genetics 42, 577-582 (2005).

13. Bayrak-toydemir, P., et al. Rapid Publication A Fourth Locus for Hereditary Hemorrhagic Telangiectasia Maps to Chromosome 7. American Journal of Medical Genetics 2162, 2155-2162 (2006).

14. Gallione, C.J., et al. SMAD4 mutations found in unselected HHT patients. Journal of medical genetics 43, 793-797 (2006).

15. Wooderchak-Donahue, W.L., et al. BMP9 Mutations Cause a Vascular-Anomaly Syndrome with Phenotypic Overlap with Hereditary Hemorrhagic Telangiectasia. The American Journal of Human Genetics 93, 530-537 (2013).

16. Lebrin, F. & Mummery, C.L. Endoglin-mediated vascular remodeling: mechanisms

underlying hereditary hemorrhagic telangiectasia. Trends in cardiovascular medicine 18, 25-32 (2008).

17. Grainger, D.J., Mosedale, D.E. & Metcalfe, J.C. TGF-β in blood: a complex problem. Cytokine & Growth Factor Reviews 11, 133-145 (2000).

18. Wan, M., et al. Injury-activated transforming growth factor β controls mobilization of mesenchymal stem cells for tissue remodeling. Stem cells 30, 2498-2511 (2012).

19. Lebrin, F., et al. Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. EMBO J 23, 4018-4028 (2004).

20. Goumans, M.J. & Ten Dijke, P. TGF-beta Signaling in Control of Cardiovascular Function. Cold Spring Harb Perspect Biol (2017).

21. Goumans, M.J., Lebrin, F. & Valdimarsdottir, G. Controlling the angiogenic switch: a balance between two distinct TGF-b receptor signaling pathways. Trends Cardiovasc Med 13, 301-307 (2003).

22. Goumans, M.J., Zwijsen, A., Ten Dijke, P. & Bailly, S. Bone Morphogenetic Proteins in Vascular Homeostasis and Disease. Cold Spring Harb Perspect Biol (2017).

23. Goumans, M.J., Liu, Z. & ten Dijke, P. TGF-β signaling in vascular biology and dysfunction. Cell Research 19, 116-127 (2009).

24. Sugden, W.W. & Siekmann, A.F. Endothelial cell biology of Endoglin in hereditary hemorrhagic telangiectasia. Curr Opin Hematol 25, 237-244 (2018).

25. Thalgott, J., Dos-Santos-Luis, D. & Lebrin, F. Pericytes as targets in hereditary hemorrhagic telangiectasia. Frontiers in Genetics 5, 1-16 (2015).

26. Post, S., et al. Impaired recruitment of HHT-1 mononuclear cells to the ischaemic heart is due to an altered CXCR4/CD26 balance. Cardiovascular Research 85, 494-502 (2010).

27. Guilhem, A., Malcus, C., Clarivet, B. & Plauchu, H. Immunological abnormalities associated with hereditary haemorrhagic telangiectasia. 351-362 (2013).

28. Dupuis-Girod, S., et al. Hemorrhagic hereditary telangiectasia (Rendu-Osler disease) and infectious diseases: an underestimated association. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 44, 841-845 (2007).

29. Ceradini, D.J., et al. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nature medicine 10, 858-864 (2004).

30. Cencioni, C., Capogrossi, M.C. & Napolitano, M. The SDF-1/CXCR4 axis in stem cell preconditioning. Cardiovascular research 94, 400-407 (2012).

31. Christopherson, K.W., Hangoc, G. & Broxmeyer, H.E. Cell surface peptidase CD26/dipeptidylpeptidase IV regulates CXCL12/stromal cell-derived factor-1α-mediated chemotaxis of human cord blood CD34+ progenitor cells. The Journal of Immunology 169, 7000 (2002).

32. Yoo, E., et al. Loss of CD26 protease activity in recipient mice during hematopoietic stem cell transplantation results in improved transplant efficiency. Transfusion 53, 878-887 (2013).

33. Lambeir, A.M., et al. Kinetic investigation of chemokine truncation by CD26/dipeptidyl peptidase IV reveals a striking selectivity within the chemokine family. Journal of Biological Chemistry 276, 29839-29845 (2001).

34. 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).

35. Yamada, K., et al. Localization of CD26/DPPIV in nucleus and its nuclear translocation enhanced by anti-CD26 monoclonal antibody with anti-tumor effect. Cancer cell international 9, 17 (2009).

36. Zhong, J., et al. A potential role for dendritic cell/macrophage-expressing DPP4 in obesity-induced visceral inflammation. Diabetes 62, 149-157 (2013).

37. Herrera, C., et al. Comodulation of CXCR4 and CD26 in human lymphocytes. J Biol Chem 276, 19532-19539 (2001).

38. 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).

39. Steinbrecher, a., et al. Targeting dipeptidyl peptidase IV (CD26) suppresses autoimmune encephalomyelitis and up-regulates TGF-beta 1 secretion in vivo. Journal of immunology (Baltimore, Md. : 1950) 166, 2041-2048 (2001).

40. Arndt, M., et al. Dipeptidyl peptidase IV (DP IV/CD26) mRNA expression in PWM-stimulated T-cells is suppressed by specific DP IV inhibition, an effect mediated by TGF-beta(1). Biochemical and biophysical research communications 274, 410-414 (2000).