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Yannick Waumans[1], Gwendolyn Vliegen[1], Lynn Maes[1], Miche Rombouts[2], Ken Declerck[3], Pieter Van Der Veken[4], Wim Vanden Berghe[3], Guido R Y De Meyer[2], Dorien Schrijvers[2], Ingrid De Meester[1]

[1]Laboratory of Medical Biochemistry, [2]Laboratory of Physiopharmacology, [3]Laboratory of Protein Chemistry, Proteomics and Epigenetic Signaling (PPES), [4]Laboratory of Medicinal Chemistry; Department of Pharmaceutical Sciences, University of Antwerp, Belgium

Correspondence: Ingrid De Meester, Laboratory of Medical Biochemistry, Universiteitsplein 1, 2610 Antwerp, Belgium. Phone: +32 (0) 3 265 27 41; e-mail: ingrid.demeester@uantwerpen.be

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Abbreviations: DPP, dipeptidyl peptidase; wt, wild-type; ApoE$^{-/-}$, apolipoprotein E-deficient; BMDMo, bone marrow-derived monocytes; BMDMφ, bone marrow-derived macrophage; IFN, interferon; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor

Abstract

Atherosclerosis remains the leading cause of death in Western countries. Dipeptidyl peptidase (DPP) 4 has emerged as a novel target for the prevention and treatment of atherosclerosis. Family members DPP8 and 9 are abundantly present in macrophage-rich regions of atherosclerotic plaques and DPP9 inhibition attenuates activation of human M1 macrophages *in vitro*. Studying this family in a mouse model for atherosclerosis would greatly advance our knowledge regarding their potential as therapeutic targets.

We found that DPP4 is downregulated during mouse monocyte-to-macrophage differentiation. DPP8 and 9 expression seems relatively low in mouse monocytes and macrophages. Viability of primary mouse macrophages is unaffected by DPP4 or DPP8/9 inhibition. Importantly, DPP8/9 inhibition attenuates macrophage activation as IL-6 secretion is significantly decreased.

Mouse macrophages respond similarly to DPP inhibition, compared to human macrophages. This shows that the mouse could become a valid model species for the study of DPPs as therapeutic targets in atherosclerosis.

Introduction

Over the past decades atherosclerosis has placed an enormous medical and economic burden on the Western world. Atherosclerosis is an inflammatory disorder of the arterial wall which can ultimately lead to cardiovascular morbidity and mortality. As such, it remains the leading cause of death in Western countries [1]. Even though anti-inflammatory and lipid-lowering treatments exist which can reduce complications, therapeutic strategies aiming to prevent plaque destabilization remain elusive [2]. Pro-inflammatory M1 macrophages play a major role in the development of atherosclerosis, plaque rupture and subsequent infarction [3]. Therefore, therapies which could shift the balance away from pro-inflammatory towards anti-inflammatory macrophages may prevent atherosclerosis or plaque rupture [4].

The dipeptidyl peptidases (DPPs) are a family of proline-specific serine proteases capable of cleaving off a dipeptide from the amino-terminus. DPP4 is the best characterized peptidase in the family. Its involvement in glucose homeostasis through the cleavage of glucagon-like peptide 1 has made it a validated therapeutic target for the treatment of type 2 diabetes [5]. Vildagliptin, sitagliptin, linagliptin, saxagliptin and alogliptin are all DPP4 inhibitors which have been approved for therapeutic use [6]. Many other substrates are known, such as glucose-dependent insulinotropic peptide [7], glucagon-like peptide-2 [8], substance P [9], b-type natriuretic peptide [10], stromal-cell derived factor (SDF)-1α/β [11], granulocyte/macrophage-colony stimulating factor [12], high mobility group box 1 [13] and eotaxin [14]. Peptidomic research has shown that it is also most likely involved in collagen turnover [15]. Lastly, in the immune system, DPP4 can be found as a marker of T-cell activation where it functions as a co-stimulatory molecule [16].

DPP8 and 9 are two very similar family members whose functions, however, are much less understood. Due to their near-identity within the active centre, no specific inhibitors are

available at the moment [17]. Despite this setback, they have been implicated in apoptosis [18, 19], ROS-sensing [20], spermatogenesis [21], neuropeptide Y signaling [22] and DPP9 was recently discovered to be allosterically activated through a SUMO1 interaction [23]. Even more important is the increasing body of evidence pointing towards a role of DPP8 and 9 in inflammation and the immune system. First, DPP8 and 9 are upregulated in experimentally induced asthma in rats and DPP8 seems to be upregulated during experimentally induced colitis in mice [24, 25]. Second, differential expression and activity patterns during ischemia-triggered inflammation might indicate their involvement [26]. Finally, upregulation of DPP8 and 9 upon stimulation of Jurkat T-cells and Raji B-cells may indicate biological roles in lymphocyte activation [27].

DPP 8 and 9 are abundantly present in macrophage-rich regions of atherosclerotic plaques and abolishment of DPP9 activity attenuates activation of human M1 macrophages *in vitro* [28], providing interesting therapeutic prospects in reducing atherosclerosis or preventing plaque rupture. Moreover, in recent years, DPP4 inhibitors have stirred some interest as they might improve atherosclerotic disease [29], although recent reports suggest DPP4-class-inhibitors may increase the risk of heart failure [30, 31]. In order to characterize DPPs as therapeutic targets in atherosclerosis an adequate animal model is essential. The mouse is perfectly suited due to its relatively low maintenance cost and the existence of well-established models for a number of inflammatory disorders, including atherosclerosis. However, results obtained from an animal model should ideally reflect human biology. Therefore, we have characterized the expression and function of DPP4, 8 and 9 in J774 mouse macrophages, wild-type (wt) and, as a model for atherosclerosis, Western diet-fed apolipoprotein E-deficient ($ApoE^{-/-}$) mouse monocytes and macrophages [32]. We compare our findings with what is known about DPPs in human monocytes and macrophages to assess

the applicability of the mouse as a model for the study of these enzymes as therapeutic targets in atherosclerosis.

Materials and Methods

Inhibitors and Antibodies

DPP4 inhibitor sitagliptin was extracted from Januvia tablets (Merck). DPP8/9 inhibitor 1G244 was synthesized at the laboratory of Medicinal Chemistry of the University of Antwerp, which is a partner of the Antwerp Drug Discovery Network (ADDN, www.addn.be). DPP4 antibody ab28340 and DPP8 antibody ab42075 were purchased from Abcam. DPP9 antibody ta504307 was purchased from Origene.

Cell Culture

The mouse macrophage cell line J774 was obtained from the American Type Culture Collection and grown in DMEM-GlutaMax medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Life Technologies, Ghent, Belgium) at 37 °C under 5% CO_2.

Monocytes and macrophages were derived from the bone marrow of female wt (C57BL/6) and ApoE$^{-/-}$ (C57BL/6J-Apoe$^{-/-}$) mice, purchased from Jackson laboratories. The ApoE$^{-/-}$ mice were fed on a cholesterol-rich diet (AB diets, 0.2% cholesterol) and all mice were sacrificed between 4 and 6 months of age. A protocol for the differentiation of bone marrow cells into monocytes and macrophages was adapted from Francke et al. [33]. After sacrifice, bone marrow was flushed from femurs and tibias in RPMI-1640 (Gibco, Life Technologies, Ghent, Belgium) supplemented with 2% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 U/ml heparin (Sigma, Diegem, Belgium). Cells were passed through a cell strainer and washed twice in HEPES-buffered RPMI-1640. Cells were seeded at 500 000 cells/well in non-tissue coated or tissue coated 12-well plates (Greiner Bio-One, Wemmel, Belgium) for the differentiation into monocytes and macrophages respectively, and grown in

RPMI-1640 supplemented with 10% fetal calf serum, 15% L-cell conditioned medium, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C under 5% CO_2. Medium was replaced every four days. Monocytes (BMDMos) were harvested at day 5 and resting macrophages (M0 BMDMφs) at day 6. M0 BMDMφs were further differentiated into M1 BMDMφs and M2 BMDMφs through classical interferon (IFN) γ/lipopolysaccharide (LPS) activation [100 U/ml IFNγ (Immunotools, Friesoythe, Germany) and 100 ng/ml LPS (Sigma, Diegem, Belgium); 24 h] and alternative interleukin (IL)-4 activation [25 ng/ml IL-4 (Immunotools, Friesoythe, Germany); 24 h] respectively. M0 BMDMφs were pre-incubated with DPP4 inhibitor (sitagliptin, 10 μM) or DPP8/9 inhibitor (1G244, 8 μM) for 1 h before IFNγ/LPS activation and inhibitors remained present throughout activation.

Activity Measurements

For activity measurements cells were lysed in a 50-mM Tris buffer (pH 8.3) with 1% octylglucoside, 10 mM EDTA and 70 μg/ml aprotinin. DPP4 and DPP8/9 activity was measured kinetically in these lysates using the fluorogenic substrate glycyl-prolyl-4-methoxy-β-naphthylamide at pH 8.3 and 37 °C, as reported earlier [34, 35]. One unit of activity is defined as the amount of enzyme necessary to catalyze the conversion of 1 μmol of substrate in 1 minute under reaction conditions. DPP4 and DPP8/9 activities can be distinguished by adding a selective DPP4 inhibitor (sitagliptin, 10 μM) [36] or a selective DPP8/9 inhibitor (1G244, 10 μM) [37] as described for a colorimetric enzyme activity assay [21]. The used controls were Tris buffer and DMSO in Tris-buffer for the DPP4- and DPP8/9-inhibitor, respectively. Specific activity was defined as the activity in units per gram of cell lysate as determined in a Bradford protein assay.

Gene Expression analysis

RNA was isolated with the High Pure RNA Isolation Kit (Roche) according to manufacturer's instructions. Nucleic acids were retained on a filter tube. DNA was degraded *in situ* with DNase I. RNA was then eluted from the filter tube in RNase free eppendorf tubes. cDNA was generated by mixing RNA and oligo dTs. Mixtures were incubated at 70 °C for 5 min to remove secondary structure RNAs. The complexes were mixed with 0.25 mM dNTPs, M-MLV reverse transciptase (Promega, Leiden, Netherlands), RT buffer (Promega), and RNase IN (Invitrogen, Merelbeke, Belgium). Samples were incubated at 42 °C for 60 min, 75 °C for 15 min and stored at 4 °C. DPP4 gene expression was analyzed using a SYBR green gene expression assay [SensiMix SYBR Hi-ROX Mastermix (Bioline, Sint-Pieters-Woluwe, Belgium)]. Primers used for DPP4 were forward: 5'-GCGCTTGTCACCATCATCAC-3', and reverse: 5'-GCCGCTTCATCTTTGCTCAG-3'; for GAPDH forward: 5'-AGGTCGGTGTGAACGGATTTG-3', and reverse: 5'-GGGGTCGTTGATGGCAACA-3'; for β-actin forward: 5'-GGCTGTATTCCCCTCCATCG-3', and reverse: 5'-CCAGTTGGTAACAATGCCATGT-3'. DPP8 and 9 gene expression were analyzed with the Taqman probe gene expression assay (Eurogentec reaction buffer) according to manufacturer's instructions. Taqman primers used for DPP8 were Mm00547049_m1 and for DPP9 Mm00841122_m1. All samples were run in triplicate in the Applied Biosystems 7300 Real-time PCR system.

Western Blotting

For western blotting, cells were lysed in a 50-mM Tris buffer pH 7.6 with 0.1% Triton-X-100, 150 mM NaCl and cOmplete Protease Inhibitor Cocktail (Roche, Vilvoorde, Belgium), dissolved as per manufacturer's instructions. Cell lysates were separated through SDS-PAGE (7.5%) and transferred to a nitrocellulose membrane by electroblotting. Membranes were

blocked during 1 h in a 50-mM Tris buffer with 5% non-fat dry milk, 0.1% Tween-20 and 150 mM NaCl, after which they were incubated overnight with primary antibody at 4 °C. Subsequently, membranes were incubated for 2 h with secondary antibodies. Antibodies were detected with Supersignal West Femto Chemiluminescent Substrate (Thermo Scientific, Erembodegem, Belgium) and visualized using the OptiGo (Isogen, Sint-Pieters-Leeuw, Belgium). Images were quantified with the TotalLab software (Isogen).

Cell Viability

Viability of J774 cells and of M0 BMDMφs was determined using PrestoBlue® Cell Viability Reagent (Life Technologies, Ghent, Belgium), according to manufacturer's instructions. J774 cells or M0 BMDMφs were seeded at 500 000 cells/ml in a 96-well plate and incubated in the presence or absence of DPP4 inhibitor (sitagliptin, 10 μM) or DPP8/9 inhibitor (1G244, 8 μM). The controls used here are medium for the DPP4-inhibitor and DMSO in medium for the DPP8/9-inhibitor. PrestoBlue® reagent was then added to the cells at a 1:10 ratio, followed by an incubation of 30 min at 37 °C. Fluorescence was then measured in an Infinite^{TM} 200 reader (Tecan Benelux, Mechelen, Belgium).

ELISA

Tumor necrosis factor (TNF) α and interleukin (IL)-6 levels were determined in the supernatant of M1 BMDMφs after differentiation in the presence or absence of DPP4 inhibitor (sitagliptin, 10 μM) or DPP8/9 inhibitor (1G244, 8 μM) with the murine TNF-α mini ELISA development kit and the murine IL-6 mini ELISA development kit respectively (Peprotech, London, United Kingdom), according to manufacturer's instructions. Absorbances were measured in a Versamax Microplate Reader (Molecular Devices, Berkshire, United Kingdom).

Griess assay

Nitrite concentrations were determined in the supernatant of M1 BMDMφs as a measure of NO secretion in a modified Griess assay [38]. A 45-µl aliquot of supernatant was first added to 40 µl of water in a 96-well plate to which then 5 µl of sulfanilic acid (0.6% in 5% phosphorous acid) was added. This was incubated for 15 min at room temperature on a plate shaker, after which 5 µl 0.06% N-(1-naphthyl)ethylenediamine dihydrochloride was added. This was again incubated for 15 min at room temperature on a plate shaker. Absorbance was then measured at 548 nm in an Infinite^{TM} 200 reader (Tecan Benelux, Mechelen, Belgium).

Statistical Analysis

All data are presented as the mean ± SEM. Differences in DPP activity and gene expression between wt and ApoE$^{-/-}$ cell subtypes were modelled using linear mixed model analysis. A stepwise-backwards strategy was used to model the fixed effects starting from a model with all fixed effects and their pairwise interactions. The significance of the fixed effects was tested using an F-test. The difference in DPP activity between J774 macrophages and primary wt cell subtypes was tested in a one-way ANOVA with Bonferroni as a post-hoc test. DPP protein expression levels were compared between wt and ApoE$^{-/-}$ cell subtypes in Kruskal-Wallis tests. Finally, cell viability and TNFα, IL-6 and NO levels were analyzed with Student T-tests. All statistical analyses were performed with the IBM SPSS statistics 20.0 software.

Results

DPP activity in J774 macrophages and mouse BMDMos and BMDMφs

Cells isolated from the bone marrow of mice were differentiated into monocytes, M0, M1 and M2 macrophages. These cells were then harvested for the measurement of DPP activities.

Due to the absence of specific substrates or inhibitors which can distinguish between DPP8 and DPP9 activity, the combined DPP8/9 activity was measured.

DPP8/9 activity was about six-fold higher in J774 macrophages compared to BMDMφs, whereas DPP4 activity was comparable between both cell types (figure 1A and B).

DPP8/9 activity remained unchanged in all primary monocyte and macrophage subtypes (figure 1B) (n = 4-6, $P > 0.05$). DPP4 activity, on the other hand, significantly decreased upon differentiation of BMDMos into BMDMφs (figure 1A) (n = 4-6, $P \leq 0.05$). No differences were observed after further differentiation into M1 or M2 BMDMφs (figure 1A). DPP activities in the primary monocyte and macrophage subtypes were comparable between wt and ApoE$^{-/-}$ mice (figure 1A and B).

DPP gene expression in mouse BMDMos and BMDMφs

The gene expression of DPP4, DPP8 and DPP9 were also analysed through qPCR. DPP4 gene expression was concordant with data from the enzyme activity assay. Expression was highest in BMDMos and significantly decreased upon differentiation into BMDMφs (figure 2A, $P \leq 0.001$, n = 4 - 6), with no further significant decrease upon M1 or M2 differentiation. DPP8 expression remained constant between different cell types (figure 2B, $P > 0.05$, n = 4 – 6). DPP9 expression was also similar throughout different cell types, although expression in M1 BMDMφs was slightly but significantly decreased versus BMDMos (figure 2C, $P \leq 0.05$, n = 4 - 6). No significant differences were found between wt and ApoE$^{-/-}$ mice ($P > 0.05$, n = 4 - 6).

DPP protein expression in mouse BMDMos and BMDMφs

No DPP4 could be detected in monocytes or macrophages on western blot (data not shown). Currently, no antibodies targeted specifically at mouse DPP4 are commercially available. Therefore, it is highly likely that the failure to detect DPP4 stems from poor antibody specificity. DPP8 and DPP9 protein could be detected in monocytes and all subtypes of macrophages (figure 3A). There tended to be an upregulation of DPP8 and 9 upon monocyte-to-macrophage differentiation, with DPP8 expression seemingly highest in M2 macrophages. However, none of these differences were significant after quantification (figure 3B, $n = 3$ -6, $P > 0.05$).

Effect of DPP inhibition on cell viability of mouse macrophages

In order to exclude that any effects of DPP inhibitors on the monocyte/macrophage activation were due to cell death, we examined cell viability of M0 BMDMφs and J774 macrophages in the presence of DPP4 inhibitor sitagliptin and DPP8/9 inhibitor 1G244.

No significant effects of the inhibitors could be seen on the viability of the primary M0 BMDMφs of wt mice (figure 4B) ($n = 4$, $P > 0.05$). Similar results were obtained in $ApoE^{-/-}$ mice (data not shown). However, DPP8/9 inhibitor 1G244 had a dramatic effect on the viability of J774 macrophages (figure 4A). Cell viability of these cells was significantly reduced after only 5 hours of incubation ($n = 3$, $P \leq 0.05$) and viability continued to decrease until after 24 h practically no cells remained alive ($n = 3$, $P \leq 0.05$). We therefore decided to exclude J774 macrophages from further experiments.

Effect of DPP inhibition on macrophage activation

M0 BMDMφs were pre-incubated for 1 hour with a DPP8/9 inhibitor or a DPP4 inhibitor and subsequently activated and differentiated into M1 BMDMφs stimulation in the presence

of these inhibitors. We then examined the effect of DPP inhibition on TNFα, IL-6 and NO secretion after 24 hours (figure 5). Stimulation of M0 BMDMφ with IFNγ/LPS caused a significant increase in TNFα, IL-6 and NO into the medium (data not shown). DPP4 inhibition had no effect on TNFα secretion (n = 8, $P > 0.05$). Small differences in IL-6 and NO secretion could be observed, but these were not significant (n = 8, $P > 0.05$). DPP8/9 inhibition diminished the secretion of TNFα, IL-6 and NO, and this was statistically significant for IL-6 secretion (n = 8, $P \leq 0.05$).

Discussion

DPP4 inhibition has recently emerged as a therapeutic strategy for the treatment of atherosclerosis as a number of studies have shown its benefits in ApoE$^{-/-}$ mice [29, 39–43]. We recently reported that DPP8 and 9, but not DPP4, are associated with macrophage-rich regions in human atherosclerotic plaques and that DPP9 inhibition attenuates pro-inflammatory IFNγ/LPS activation in human M1 macrophages [28]. It would be interesting to study the potential of inhibiting DPP9 in order to prevent macrophage inflammation and plaque development in an *in vivo* mouse model. Ideally, experimental animal models should not only model the pathology in humans, but also the biology of the target. However, as of yet, hardly any data concerning the expression or function of DPP4, 8 or 9 in mouse monocytes or macrophages have been published. Therefore, we have characterized DPP4, 8 and 9 in monocytes and macrophages of wt and Western diet-fed ApoE$^{-/-}$ to assess the applicability of an experimental mouse model for the study of DPPs as therapeutic targets in atherosclerosis.

Although, DPP4 surface expression has been shown on human monocytes and macrophages [44], our group previously showed that DPP4 activity is actually negligible in

human monocytes and macrophages [28]. By contrast, we found higher DPP4 activity and gene expression in primary mouse monocytes which then decreased upon differentiation into macrophages. Shah et al. showed that long-term inhibition of DPP4 with alogliptin in ApoE$^{-/-}$ mice exerts anti-atherosclerotic effects, possibly through inhibition of monocyte migration and activation [40]. In the present study, we confirmed that mouse monocytes show DPP4 activity whereas, as mentioned, DPP4 in human monocytes is negligible [28]. As such, DPP4 inhibitors would probably not exert any monocyte-related anti-atherogenic actions in humans. However, other mechanisms that explain the effects of DPP4 inhibitors in atherosclerosis have been proposed. For instance, one study showed that des-fluoro-sitagliptin improves endothelial dysfunction in ApoE$^{-/-}$, probably through the preservation of GLP-1 [29]. Another study found that anagliptin can suppress proliferation of vascular smooth muscle cells [43]. In other words, even though DPP4 inhibitors could have beneficial effects on atherosclerosis in humans similar to those observed in ApoE$^{-/-}$ mice, the underlying mechanisms might be different. Accordingly, a preliminary report shows that both vildagliptin and sitagliptin treatment reduce intima media thickness, a surrogate marker for early atherosclerosis [45].

DPP8/9 activity and gene expression was similarly low in all wt and ApoE$^{-/-}$ BMDMos and BMDMφs subtypes examined. DPP8 and 9 protein expression was more variable with a tendency towards upregulation in macrophages vs monocytes, but quantification did not show statistically significant differences. These findings could indicate posttranscriptional expression and/or activity regulation, but more likely reflect the experimental variability of western blotting.

Furthermore, our data indicate that the J774 cell line is not applicable for the study of DPPs in healthy macrophages. DPP8/9 activity was up to ten times higher in J774 cells than in primary mouse BMDMφs, and, unlike primary BMDMφs J774 cells were extremely

sensitive to DPP8/9 inhibition. Indeed, DPP8 and 9 have been shown to be involved in apoptosis [18, 19, 22, 28]. Only the *transformed* J774 macrophages are sensitive to inhibition, whereas healthy primary macrophages remain viable. This offers interesting perspectives with regard to the treatment of neoplastic disease where specifically targeting malignant cells without affecting healthy tissue is of the essence. One study found that vildagliptin synergizes with parthenolide in the treatment of leukemia through inhibition of DPP8 activity and DPP9 activity, and not of DPP4 [46]. This combination reduced the viability and clonogenic growth of cells from acute myeloid leukemia patients while leaving normal cells from healthy controls unharmed. The underlying mechanism by which vildagliptin enhances parthenolides effects remain unclear. However, vildagliptin does not synergize with other cytotoxic therapeutics, therefore making it likely that vildagliptin (i.e. in this case DPP8 and 9 inhibition) influences one or several of the pathways with which parthenolide interferes [46]. Parthenolide inhibits the NFκB pathway and STAT3 phosphorylation, increases the production of reactive oxygen species and activates p53 [47–51]. One could argue that DPP8/9 inhibition could affect one of these pathways in the J774 cells as well, leading to the spectacular decrease in viability observed in our experiments. Despite its possible significance in leukemia, the observed differences are not practical when extrapolating to healthy primary macrophages. Therefore, we decided to further exclude J774 macrophages from this study and would advise against the use of these cells for the study of DPP biology in healthy macrophages. Only the *transformed* J774 macrophages are sensitive to inhibition, whereas healthy primary macrophages remain viable. This offers interesting perspectives with regard to the treatment of neoplastic disease where specifically targeting malignant cells without affecting healthy tissue is of the essence. One study found that vildagliptin synergizes with parthenolide in the treatment of leukemia through inhibition of

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Finally, we examined the effect of DPP inhibition on the secretion of pro-inflammatory mediators by M1 BMDMφs. We observed a trend towards a decrease in secretion of TNFα, IL-6 and NO after DPP4 inhibition. The limited effect on secretion might be due to the inherently low DPP4 activity present in the macrophages. DPP8/9 inhibition, on the other hand, significantly decreased IL-6 secretion. There was also a trend towards a decrease in TNFα and NO secretion. These latter results are in line with findings for humans in the study by Matheeussen et al. [28], where DPP8/9 inhibition was found to attenuate the secretion of pro-inflammatory cytokines by primary M1 macrophages.

Table 1 compares our data in mice with published data of DPPs in primary human monocytes and macrophages (Table 1). Mainly due to differences in the expression pattern of the DPPs between mice and humans, we would advise caution when extrapolating data obtained from mouse models to human pathophysiology regarding mechanisms involving monocytes or macrophages. On the other hand, similar effects of DPP8/9 inhibition could be seen on the pro-inflammatory response of mouse M1 BMDMφs, meaning that the $ApoE^{-/-}$ mouse model could be useful for the study of DPPs as therapeutic targets in atherosclerosis. Unexplored in the present study, fibroblast activation protein α, another member of this family of proline-specific peptidases, has also been shown to contribute to plaque instability [52]. Such results show the potential of inhibiting multiple members of the DPP family as a therapeutic strategy for the treatment of atherosclerosis and is discussed in more detail in a recent review on the DPP family in the immune system [53]. In summary, this study contributes to the growing amount of data on the DPP family in atherosclerosis and provides further insight into the applicability of the mouse to study the DPPs in this disease.

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Conflict of Interest

The authors have no potential conflicts of interest.

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Legends

Figure 1. (A) J774 macrophages (n = 3) show similarly low DPP4 activities compared to bone marrow-derived macrophages (BMDMφ) from wild-type (wt, white, *n* = 6) and apolipoprotein E-deficient ($ApoE^{-/-}$, grey, *n* = 4) mice. DPPIV activity decreases significantly upon differentiation of bone marrow derived monocytes (BMDMo) to resting M0 BMDMφs. M1 or M2 activation does not further alter DPP4 activity. (B) DPP8/9 activity in J774 macrophages (n = 3) is significantly higher than in BMDMos or BMDMφs of wt mice (white, *n* = 6) or $ApoE^{-/-}$ mice (grey, *n* = 4). Specific activities were determined in an enzymatic assay with the substrate glycyl-prolyl-4-methoxy-β-naphthylamide and distinguished through the addition of a specific DPP4 inhibitor (sitagliptin, 10 μM) or a specific DPP8/9 inhibitor (1G244, 10 μM). The data are presented as the mean ± SEM. ***$P \leq 0.001$

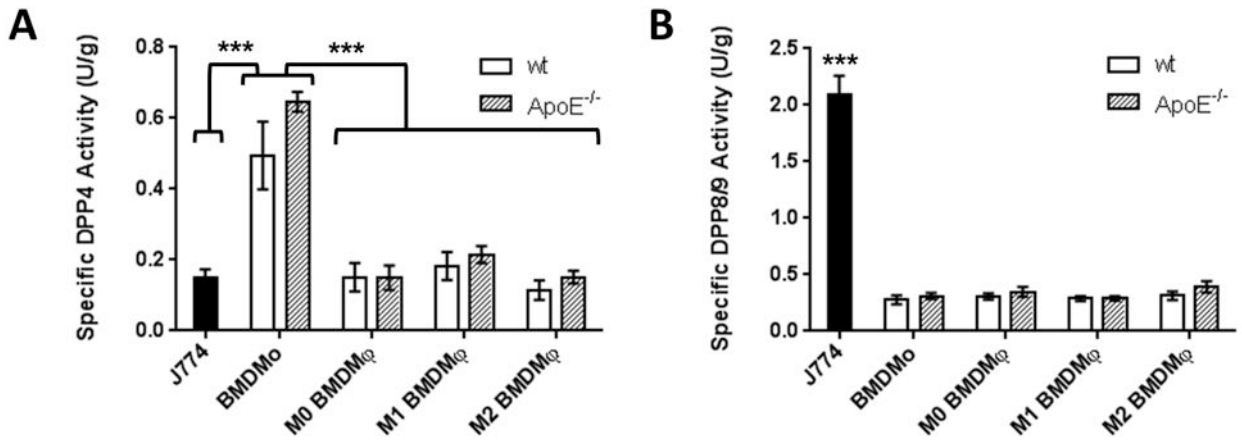
Figure 2. DPP4, 8 and 9 gene expression in primary mouse monocytes and macrophages. qPCR analysis of DPP4, 8 and 9 in lysates of bone marrow-derived monocytes (BMDMo) and M0, M1 and M2 bone marrow-derived macrophages (BMDMφ) from wt (n = 4 - 6) and $ApoE^{-/-}$ mice (n = 4). All values were normalized to β-Actin and GADPH as housekeeping genes (HKGs) and expressed as the mean ± SEM. *$P \leq 0.05$, *** $P \leq 0.001$

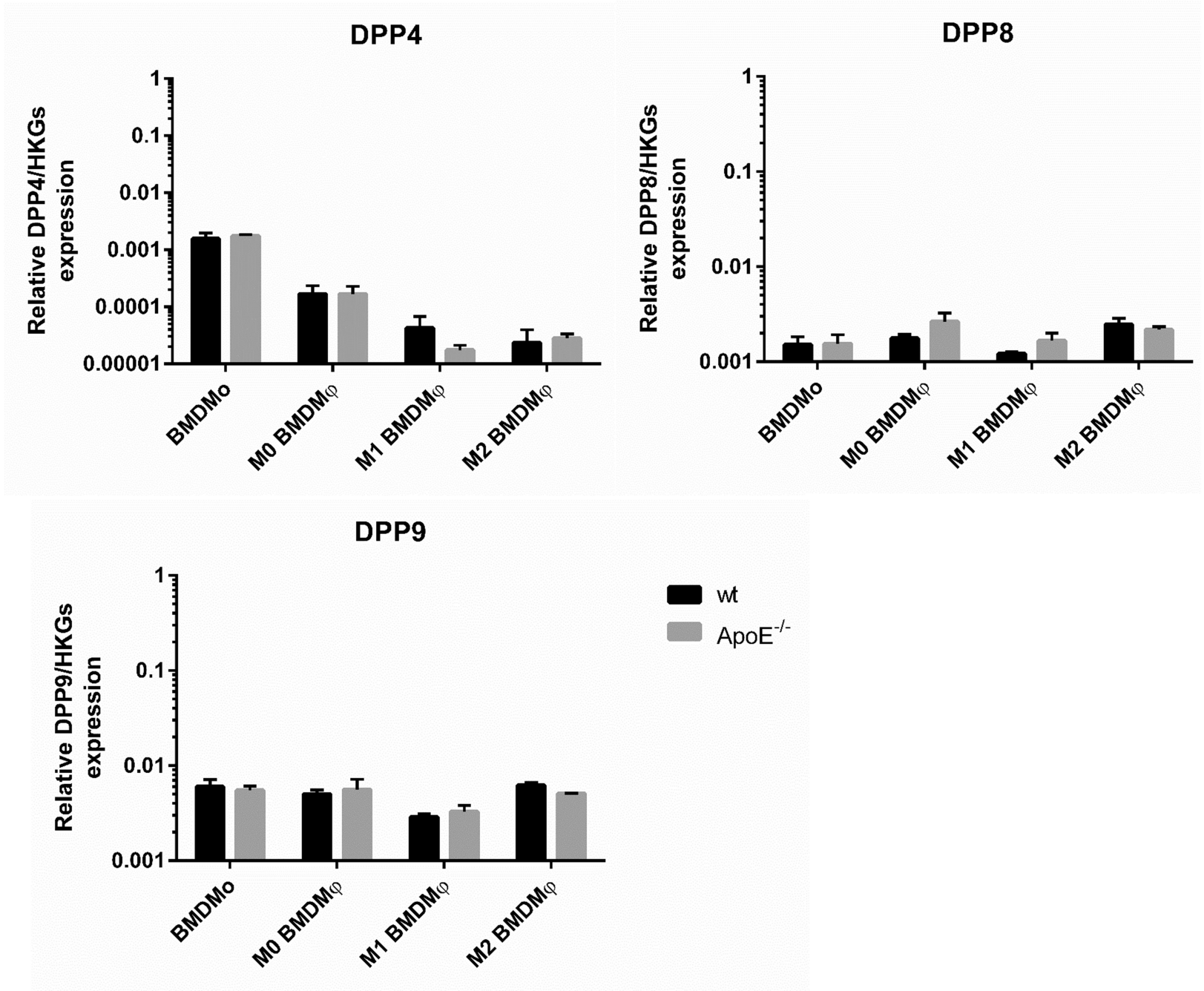
Figure 3. DPP8 and DPP9 protein expression is highly variable over bone marrow-derived monocytes (BMDMo) and bone marrow-derived macrophages (BMDMφ) subtypes of wild-type (*n* = 6) or apolipoprotein E-deficient ($ApoE^{-/-}$, *n* = 3-4) mice, with expression seemingly higher in BMDMφs. (A) Representative Western blots of DPP8 and DPP9. (B) Western blots were analyzed through densitometric quantification and the expression versus β-actin is

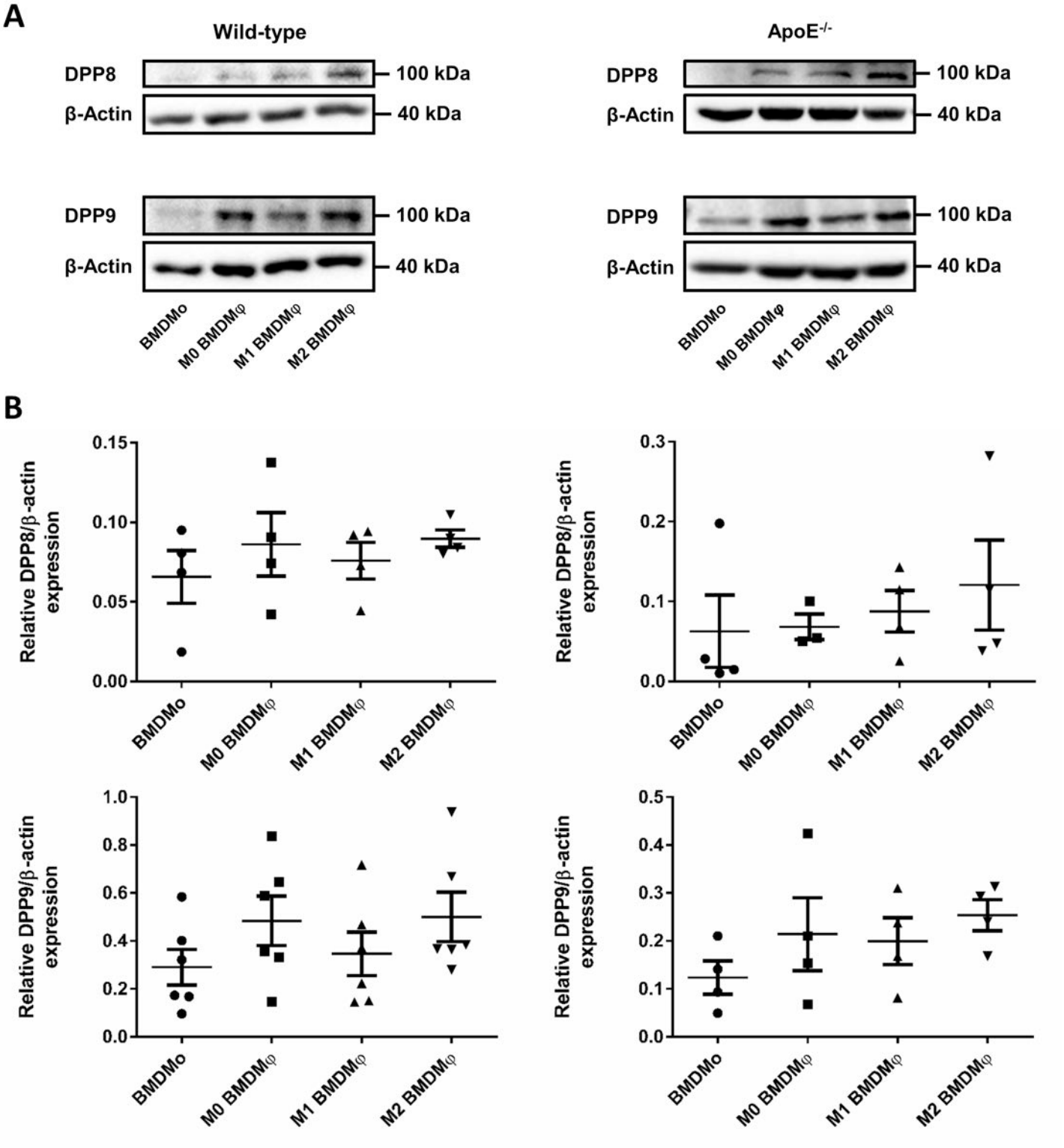
shown for each band. No significant differences were observed. Data are presented as the mean ± SEM.

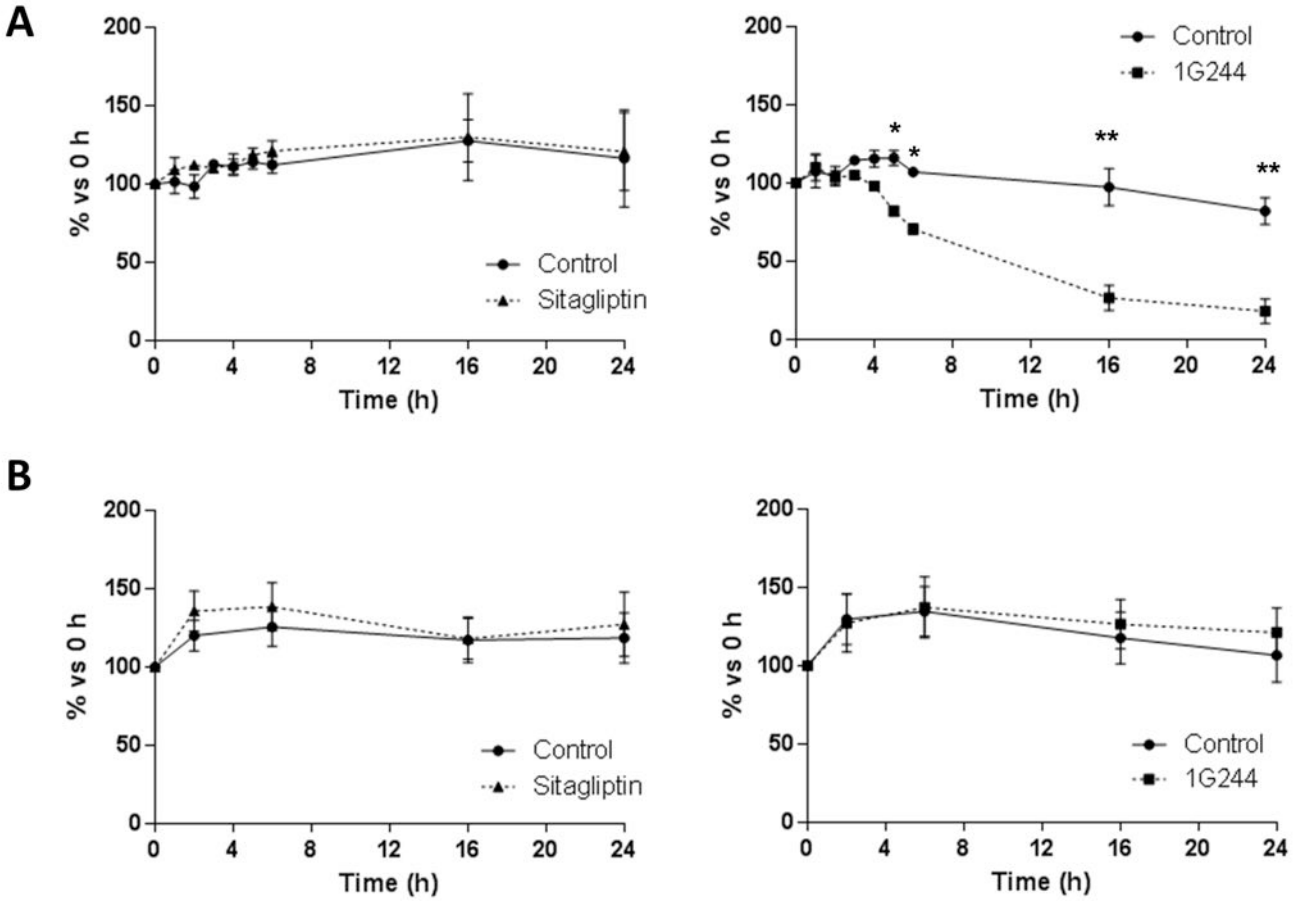
Figure 4. (A) J774 macrophages remain viable after being exposed to the DPP4 inhibitor sitagliptin (10 μM) for up to 24 hours. Exposure to DPP8/9 inhibitor 1G244 (8 μM) results in a significant reduction in J774 macrophages after an exposure time of 5 hours (n = 3). (B) Bone marrow-derived macrophages remain viable after exposure to sitagliptin or 1G244 for up to 24 hours (n = 4). Cell viability in the presence of DPP4 and DPP8/9 inhibitor was assayed using PrestoBlue Cell Viability Reagent. The data are presented as the mean ± SEM. *$P \leq 0.05$ **$P \leq 0.01$

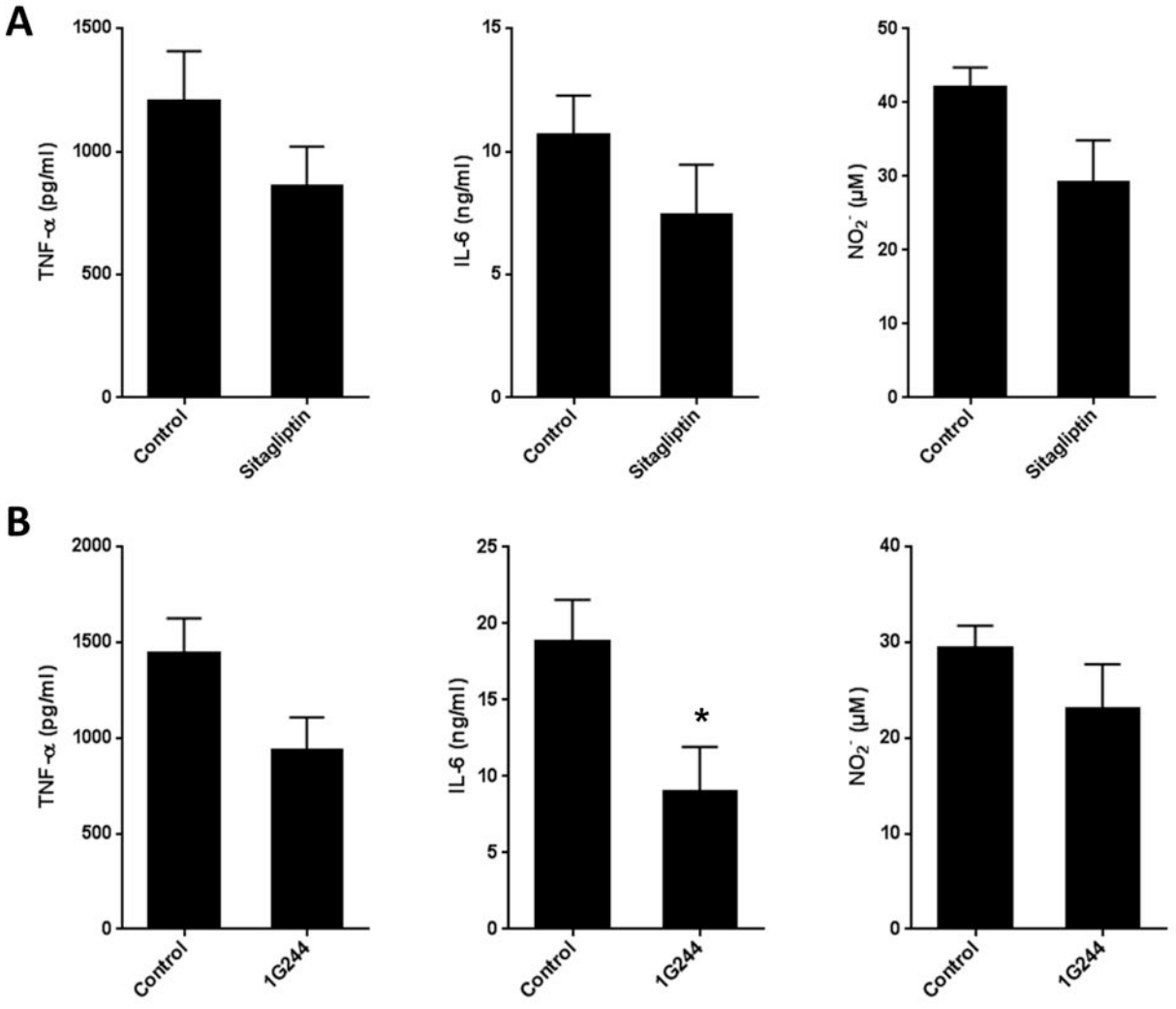
Figure 5. DPP4 and DPP8/9 inhibition show a trend towards reduction of secretion of tumour necrosis factor (TNF) α, interleukin (IL)-6 and NO by M1 bone marrow-derived macrophages (BMDMφs) of wild-type mice. IL-6 is significantly reduced after DPP8/9 inhibition. The concentrations of TNFα, IL-6 and NO_2^- were determined in the supernatant of M1 BMDMφs. M0 BMDMφs were, prior to activation, incubated for 1 hour with (A) DPP4 inhibitor sitagliptin (10 μM), or (B) DPP8/9 inhibitor 1G244 (8 μM) and subsequently differentiated and activated to M1 BMDMφs with 100 U/ml interferon γ and 100 ng/ml lipopolysaccharide. TNFα and IL-6 concentration were measured with the murine TNFα and IL-6 mini ELISA development kit according to the manufacturer's instructions (Peprotech, London, United Kingdom). NO_2^- was determined as a measure for NO secretion in an adapted Griess assay. The data are presented as the mean ± SEM (n = 8). *$P \leq 0.05$


A
Specific DPP4 Activity (U/g)
0.8
0.6
0.4
0.2
0.0
***
***
wt
ApoE-/-
J774
BMDMo
M0 BMDMφ
M1 BMDMφ
M2 BMDMφ
B
Specific DPP8/9 Activity (U/g)
2.5
2.0
1.5
1.0
0.5
0.0
***
wt
ApoE-/-
J774
BMDMo
M0 BMDMφ
M1 BMDMφ
M2 BMDMφ


DPP4
Relative DPP4/HKGs expression
1
0.1
0.01
0.001
0.0001
0.00001
BMDMo
M0 BMDMφ
M1 BMDMφ
M2 BMDMφ
DPP8
Relative DPP8/HKGs expression
1
0.1
0.01
0.001
BMDMo
M0 BMDMφ
M1 BMDMφ
M2 BMDMφ
DPP9
Relative DPP9/HKGs expression
1
0.1
0.01
0.001
BMDMo
M0 BMDMφ
M1 BMDMφ
M2 BMDMφ
wt
ApoE-/-


A
Wild-type
ApoE-/-
DPP8
β-Actin
100 kDa
40 kDa
DPP9
BMDMo
M0 BMDMφ
M1 BMDMφ
M2 BMDMφ
B
Relative DPP8/β-actin expression
Relative DPP9/β-actin expression
0.15
0.10
0.05
0.00
0.3
0.2
0.1
0.0
1.0
0.8
0.6
0.4
0.2
0.5


A
B
% vs 0 h
Time (h)
Control
Sitagliptin
1G244
*
**
0
50
100
150
200
0
4
8
12
16
20
24


A
TNF-α (pg/ml)
1500
1000
500
0
Control
Sitagliptin
IL-6 (ng/ml)
15
10
5
0
Control
Sitagliptin
NO2- (μM)
50
40
30
20
10
0
Control
Sitagliptin
B
TNF-α (pg/ml)
2000
1500
1000
500
0
Control
1G244
IL-6 (ng/ml)
25
20
15
10
5
0
*
Control
1G244
NO2- (μM)
40
30
20
10
0
Control
1G244