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Glucocorticoids mobilize macrophages by transcriptionally up-regulating the exopeptidase DPP4

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ABSTRACT

Glucocorticoids are potent endogenous anti-inflammatory molecules, and their cognate receptor, glucocorticoid receptor (GR), is expressed in nearly all immune cells. Macrophages are heterogeneous immune cells having a central role in both tissue homeostasis and inflammation and also play a role in the pathogenesis of some inflammatory diseases. Paradoxically, glucocorticoids have only a limited efficacy in controlling the resolution of these macrophage-related diseases. Here, we report that the transcriptomes of monocyte-like THP-1 cells and macrophage-like THP-1 cells (THP1-MΦ) have largely conserved gene expression patterns. In contrast, the differentiation to THP1-MΦ significantly altered the sensitivity of gene transcription to glucocorticoids. Among glucocorticoid-regulated genes, we identified the exopeptidase dipeptidyl peptidase-4 (DPP4) as a critical glucocorticoid-responsive gene in THP1-MΦ. We found that GR directly induces DPP4 gene expression by binding to two glucocorticoid-responsive elements (GREs) within the DPP4 promoter. Additionally, we show that glucocorticoid-induced DPP4 expression is blocked by the GR antagonist RU-486 and by GR siRNA transfection and that DPP4 enzyme activity is reduced by DPP4 inhibitors. Of note, glucocorticoids highly stimulated macrophage mobility; unexpectedly, DPP4 mediated the glucocorticoid-induced macrophage migration, and siRNA-mediated knockdowns of GR and DPP4 blocked dexamethasone-induced THP1-MΦ migration. Moreover, glucocorticoid-induced DPP4 activation was also observed in proinflammatory M1-polarized murine macrophages, as well as peritoneal macrophages, and was associated with increased macrophage migration. Our results indicate that glucocorticoids directly up-regulate DPP4 expression and thereby induce migration in macrophages, potentially explaining why glucocorticoid therapy is less effective in controlling macrophage-dominated inflammatory disorders.

Glucocorticoids exert a wide-array of systemic and tissue-specific effects, by signaling through the cognate glucocorticoid receptor (NR3C1; GR) in numerous tissues and cell types to systematically influence development, homeostasis, metabolism and inflammation (1). One of the most important effects of both endogenous and exogenous glucocorticoids is immunomodulation, exerted mainly by suppressing transcription of pro-inflammatory genes and/or induction of anti-inflammatory genes (2). Synthetic glucocorticoids are commonly prescribed anti-inflammatory and

immunomodulatory agents. Their therapeutic activity is substantial in a wide spectrum of diseases, including acute and chronic inflammation, autoimmune disorders (3), organ transplantation (4), and hematological cancers (5).

Contrary to well-known anti-inflammatory effects of glucocorticoids, there is emerging evidence of pro-inflammatory effects during inflammation (6-8). For example, glucocorticoid signaling in macrophages has been reported to upregulate the expression of NLRP3 inflammasome component and to enhance the ATP-dependent secretion of cytokines such as TNF-α and IL-6 (9). These findings suggest that glucocorticoids likely play a dual role regulating the innate and adaptive immune response differentially. These effects may depend on the type of inflammatory stimulus (10) and/or the timing of treatment (11), thus, modulating the balance of the cellular state towards a net pro-inflammatory or anti-inflammatory state (7). These macrophage-intrinsic properties may explain why glucocorticoids are less effective in macrophage-mediated diseases (12), such as chronic obstructive pulmonary disease (COPD) (11), ulcerative colitis (13), systemic lupus erythematosus (14), and rheumatoid arthritis (15).

Macrophages are involved in all phases of the inflammatory response, including alarm, mobilization and resolution phases, and are able to drive either the propagation or resolution of inflammation (12,16). The ontogeny of macrophages is still not fully understood; however, it is accepted that they can be grouped as tissue resident macrophages (established independently of hematopoiesis), or infiltrating macrophages derived from circulating monocytes that are established following an inflammatory response (17-19). Thus, macrophages may contribute to the pathophysiology of several diseases including inflammatory disorders (20,21), cancer (22) and also state of low-grade inflammation such as obesity (23,24).

The migratory capacity of macrophages has been studied in tumor-associated macrophages (TAM), since they have a critical role in different stages of tumor progression (25). In other inflammatory pathologies, such as multiple sclerosis, differences in activation and polarization of macrophages promote their migratory properties towards chemoattractants (26). This migration is associated with cytoskeleton rearrangements and also has been proposed to depend on the levels and type of integrin expression (26). For example, the expression of the chemokine receptor CXCR4 on mouse and human mature macrophages has been associated with migration towards lymph nodes during murine peritonitis resolution (27). Finally, in human macrophages (differentiated from $CD14^+$ monocytes), CXCR4 expression and cell motility (showing a longer distance travelled) was induced upon dexamethasone stimulation. However little attention has been directed to the identification of genes essential for macrophages movement (28).

Dipeptidyl peptidase-4 (DPP4), encoding for a membrane glycoprotein with exopeptidase activity, was recently described being involved in the inflammatory macrophage profile associated with type 2 diabetes (T2D), obesity and atherosclerosis (29,30). This protein is multifunctional with both enzymatic and non-enzymatic activities. The extracellular domain of DPP4 presents the catalytic site, primarily associated to inactivation of incretin hormones, such as glucagon-like peptide (GLP) 1 and 2, and the gastric inhibitory polypeptide (GIP), as well as catalysis of chemokines (CCL2/MIP-1a, CXCL12/SDF-1, and CCL5/Rantes, among others) (31). DPP-4 has also binding sites for adenosine deaminase (ADA) and fibronectin (FN), and their binding are associated with pro-inflammatory responses (32).

DPP4 inhibitors, such as Linagliptin and Sitagliptin (33-36), are useful for the control of blood glucose in T2D patients (33) who do not respond well to metformin (34) and sulphonylureas (35), and patients suffering from the diabetogenic effects of glucocorticoids (36,37).

In this study, using THP-1 cells we show that the monocytes-to-macrophages differentiation was associated with both higher GR levels and greater sensitivity to glucocorticoids on macrophage-like THP-1 cells (THP1-MΦ) compared to monocyte-like THP-1 cells. These changes resulted in modifications of the glucocorticoid-dependent macrophage transcriptome. During the monocyte to THP1-MΦ differentiation cells undergo

chromatin remodeling, thus enhancing GR accessibility to glucocorticoid-response elements (GREs) within the DPP4 promoter. Furthermore, we show that DPP4 is a novel glucocorticoid-responsive gene specifically in human and mouse macrophages, but not regulated in monocytes. *In vitro* migration assay using THP1-MΦ and M1 polarized bone marrow derived-macrophages (BMDMs) reveals that glucocorticoids regulate the macrophages movement via a DPP4-dependent process.

Results

Transcriptome analysis of monocyte-like THP-1 cells and macrophage-like THP-1 cells reveals a high conservation of gene expression between both cell types

We investigated whether the state of cell differentiation among monocytes and macrophages modifies the gene expression, with profiles of monocyte-like THP-1 cells (THP-1) and macrophage-like THP-1 cells (THP1-MΦ) analyzed by genome wide microarray (Supplementary figure 1). Regulated genes were evaluated through the Venn diagram (Figure 1A), which showed that a large number of genes are commonly expressed in THP-1 and THP1-MΦ (6,637 genes, corresponding to 82.4% of the total expressed genes). In contrast, 608 genes were only found in THP-1 while 803 were restricted to THP1-MΦ (Figure 1A). Provocatively, a volcano plot comparing genes expressed in THP1-MΦ with monocyte-like THP-1 cells, revealed that the gene NR3C1/GR was highly expressed in THP1-MΦ (Figure 1B). This data was validated by qRT-PCR and western blot showing a 6-fold increase in the GR mRNA and a 3-fold increase in GR protein in THP1-MΦ compared to undifferentiated monocyte-like THP-1 cells (Figure 1C and 1D, respectively). Interestingly, this phenomenon also was observed in primary murine bone marrow derived macrophages (BMDM) using M-CSF, where we found an increase in GR mRNA in macrophages post-differentiation from bone marrow monocytes (BMM) (Supplementary figure 2).

Monocyte-to-macrophage differentiation enhances their responsiveness to glucocorticoids in macrophages

Due to the different expression levels of GR in monocyte-like THP-1 cells and THP1-MΦ, we investigated whether the state of cellular differentiation alters the sensitivity to glucocorticoids. For these experiments, monocyte-like THP-1 cells and THP1-MΦ were treated with Dex for 6 hours and total isolated mRNA was subsequently analyzed by genome wide microarray. Principal component analysis (PCA) demonstrated considerable separation between treatment (control vs Dex) in both cell types. However, Dex-treated monocyte-like THP-1 cells and THP1-MΦ were dramatically separated indicating differential glucocorticoid-regulated transcriptomes in these cells (Supplementary figure 3A). To identify unique and common genes regulated by Dex among the cell types, the gene list was sorted by Venn diagram, revealing only 741 genes commonly regulated by Dex in monocyte-like THP-1 cells and THP1-MΦ (Figure 2A). Moreover, the number of genes uniquely regulated by Dex in THP1-MΦ (4,222 genes) was 7-fold higher with respect to monocyte-like THP-1 cells (529 genes) (Figure 2A). The most significant genes commonly and uniquely regulated by Dex in monocyte-like THP-1 cells and THP1-MΦ were further plotted on a volcano plot and validated by qRT-PCR (Supplementary figure 3B-G). To elucidate the significance of these findings in both cell types, gene sets were analyzed by IPA software. Provocatively, cellular movement was overrepresented in THP1-MΦ compared to monocyte-like THP-1 cells (Figure 2B). Moreover, cell-mediated immune response, immune cell trafficking, and inflammatory response pathways were enhanced in macrophages compared to monocytes (Figure 2B and supplementary figure 4), suggesting that THP1-MΦ are more responsive than monocyte-like THP-1 cells to signaling by glucocorticoids.

Glucocorticoids enhance macrophage-like THP-1 cells migration

Pathway analysis of the microarray data revealed that cell movement was the top biological function following Dex treatment in both monocyte-like THP-1 cells and THP1-MΦ, although cell movement annotation was more dramatically regulated in Dex-treated THP1-MΦ. The number of genes involved in cell movement

and differentially regulated according to IPA was 1,102 genes, 44.3% of them were induced, while the remaining 63.7% were inhibited in THP1-MΦ. Heat map analysis of the top 25 genes by heat maps revealed that the magnitude of Dex-induced gene expression was greater in THP1-MΦ (Figure 2C). Based on these findings, we evaluated how GR could impact THP1-MΦ migratory properties in a cell migration assay based on the Boyden Chamber principle. Spontaneous cell migration assay with cells seeded on the insert and serum as the chemoattractant at the bottom revealed that Dex treatment did not impact the monocyte-like THP-1 cells migratory properties at 6 and 24 hours (Figure 2D). However, Dex treatment of THP1-MΦ increased their migratory properties after 24 hours (Figure 2D). This effect was blocked by 1-hour pre-treatment with the GR antagonist, RU486 (Figure 2D). This finding was corroborated by knocking down GR expression via siRNA. THP1-MΦ transfected with siRNA GR had approximately 75% GR silenced (Figure 2E) and a significant reduction in Dex-induced cell migration compared to cells transfected with non-targeting control (NTC) siRNA (Figure 2F). These results suggest that glucocorticoids regulate the migration of macrophage-like THP-1 cells.

The DPP4 is a new glucocorticoid-responsive gene regulated in macrophage-like THP-1 cells

Data from our transcriptome analysis suggested that the pathways cell migration and inflammation were highly regulated by glucocorticoids, thus, to substantiate this finding we turned to the use of Nanostring analysis. Among the 594 genes present in the code-set Human Immunology, 194 genes were regulated by Dex, specifically 134 in monocyte-like THP-1 cells and 157 in THP1-MΦ (Figure 3A). The expression pattern of genes related to the immune response sorted through Venn diagram showed that 97 genes were commonly regulated by Dex in THP-1 and THP1-MΦ (corresponding to 50% of the total regulated genes) (Figure 3A). Additionally, the number of genes regulated exclusively by Dex in THP-1 and THP1-MΦ was 37 and 60 genes respectively, indicating that almost twice as many genes were differentially regulated by Dex in THP1-MΦ (Figure 3A). Amongst these genes, *DPP4* was the most upregulated by glucocorticoids in THP1-MΦ and also was part of the 100 genes mostly induced by microarray (Figure 2A and 3B). Interestingly, using a small cohort of RNA samples (n=9) from human monocyte derived macrophages (MDM) (38) that were treated with 100 nM of dexamethasone for 6 hours we found that glucocorticoids significantly induced the mRNA expression of DPP4 in 6 out of 9 samples analyzed (Supplementary figure 5). Furthermore, *DPP4* was not regulated by glucocorticoids in monocyte-like THP-1 cells (Figure 3B). Glucocorticoid-dependent regulation of *DPP4* was confirmed by qRT-PCR, through a dose-response and time-course analysis indicating that high levels of DPP4 mRNA were exclusively upregulated in THP1-MΦ by 10, 100 and 1000 nM Dex (Figure 3C), and maximally induced 12 hours after Dex treatment (Figure 3D). Additionally, Dex-induced DPP4 upregulation was observed at the protein level by western blot and flow cytometry, observing a double immunoreactive band of approximately 100 and 114 kDa (also induced by 10, 100 and 1000 nM Dex) (Figure 3E) and higher fluorescent intensity induced by Dex exclusively in THP1-MΦ (Figure 3F). Finally, the functionality of GR in DPP4 induction was evaluated through pharmacological and genetic inhibition, using a pre-treatment with GR antagonist RU-486 and transfection with GR siRNA, respectively. *DPP4* induction by Dex in THP1-MΦ was blocked in the presence of RU486, both at the transcript level (6 hours) (Figure 3G) and protein level (24 hours) (Figure 3H), and inhibited by GR silencing, at the protein level at 24 hours (50% downregulation with respect to not-targeting control, NTC) (Figure 3I).

Glucocorticoids regulate DPP4 expression by binding to GREs in regulatory regions of DPP4 gene

Through the determination of nascent RNA levels, we observed that glucocorticoids directly regulated the induction of *DPP4*, with an increase within just 30 minutes of treatment (Figure 4A), thus indicating that *DPP4* is a direct transcriptional target of the GR. *In silico* analysis of the human *DPP4* gene revealed the presence of numerous putative GREs located in the

regulatory region between 1.5 and 6.5 kb upstream of the transcription start site (TSS), each one with a score higher than 80% in relation to the consensus GRE sequence (Figure 4B). Therefore, monocyte-like THP-1 cells and THP1-MΦ treated with 100 nM Dex or vehicle during 2 hours were evaluated by chromatin immunoprecipitation coupled to real time PCR (ChIP-qPCR), using anti-GR antibody and IgG isotype as negative control. In THP1-MΦ treated with Dex, an enrichment of GR was observed in GRE -4,200/-4,185 (3.6-fold) and GRE -1,782/-1,767 (3.2-fold) of the regulatory region of *DPP4*, suggesting a direct transcriptional control of the *DPP4* gene by GR (Figure 4D). In Dex-induced monocyte-like THP-1 cells this effect was not observed (Figure 4C). As a control to evaluate whether there is a differential specificity of GR in the binding to the DPP4 promoter, we analyzed the recruitment of liganded GR to the GRE located in the promoter region of the glucocorticoid target gene *GILZ*. We observed an enrichment of GR to the GRE of GILZ in both monocyte-like THP-1 cells and THP1-MΦ following Dex treatment (6 and 13-fold, respectively, Figure 4C and 4D). These data suggest that chromatin structure reorganization occurs during monocytes-to-macrophages transition, thus allowing GR binding, and serving as the mechanism through which DPP4 is differentially regulated in monocyte-like THP-1 cells and THP1-MΦ. To address this question, we evaluated chromatin accessibility using Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE) assay in the same regions of *DPP4* gene where GR occupied sites according to ChiP-qPCR in macrophages. Upon differentiation and Dex treatment, we observed an increase in DNA accessibility in both regions of the *DPP4* gene flanking GRE -4,200/-4,185 and GRE -1,782/-1,767 respectively in THP1-MΦ, while there was limited accessibility of them in monocyte-like THP-1 cells (Figure 4E). As control, DNA accessibility was also observed in the site flanking GRE of the GILZ promoter, without differences between THP1-MΦ and monocyte-like THP-1 cells. The increase in FAIRE enrichment seen at the two GREs of *DPP4* gene suggests that the chromatin remodeling during the monocytes-to-macrophages transition is required for GR to initiate Dex-induced *DPP4* gene transcription.

Glucocorticoids enhance DPP4 enzymatic activity in THP1-MΦ and this effect is blocked by DPP4 inhibitors Sitagliptin and Linagliptin

The effects mediated by DPP4 in different cell types has been associated with both enzymatic and non-enzymatic functions. Based on this classification (and having shown that liganded GR upregulates mRNA and protein DPP4 levels), we evaluated whether glucocorticoids could also modulate the DPP4 enzymatic activity. For these studies, monocyte-like THP-1 cells and THP1-MΦ were stimulated with Dex for 24 hours, or were pre-treated with RU-486, or co-stimulated after 3 hours with two concentrations of specific DPP4 inhibitors, Sitagliptin and Linagliptin. Cell lysates of each experimental condition were evaluated by fluorometric assay, showing no DPP4 activity from monocyte-like THP-1 cells under evaluated conditions (Figure 5A). Conversely, in THP1-MΦ we observed that Dex increased DPP4 enzymatic activity and this effect is mediated by GR since it was blocked by RU486 pre-treatment (Figure 5A). In addition, using Sitagliptin (25 and 50 nM) and Linagliptin (1 and 10 nM), we found that both inhibitors completely block DPP4 enzymatic activity induced by Dex (Figure 5A). Notably, treatment with both DPP4 inhibitors directly blocked DPP4 enzymatic activity without affecting Dex-induced DPP4 protein expression (Figure 5B).

DPP4 promotes macrophage-like THP-1 cells migration

The multifunctionality of DPP4, enzymatic activity to regulate chemokine actions or non-enzymatic via interaction with other proteins, could also regulate, directly or indirectly, macrophage mobility. To investigate this idea, we silenced DPP4 in THP1-MΦ using siRNA or DPP4 inhibitors, evaluating cell migration behind glucocorticoid effects. Expression of DPP4 induced by Dex was decreased by 80% at the protein level in THP1-MΦ transfected with siRNA DPP4 with respect to NTC (Figure 5C). Using this strategy to evaluate GR participation in cell migration, we compared the migratory potential of THP1-MΦ primed with Dex in the presence of DPP4 inhibitors. Cells were seeded in

the insert and medium with serum was added in the lower chamber as chemoattractant. Interestingly, the Dex-mediated increase in the migratory potential was inhibited in presence of either 10 nM Linagliptin or 50 nM Sitagliptin, suggesting that this effect was dependent on DPP4 catalytic function (Figure 5D). Finally, NTC and DPP4 siRNA macrophages, primed or unprimed with Dex for 24 hours, were used to evaluate DPP4 participation in cell migration. An increase in the migratory potential induced by Dex in the NTC was suppressed by DPP4 knockdown (Figure 5D), suggesting DPP4 activity is necessary for enhancing macrophage migration. Interestingly, CXCL12 was able to promote the migration of cells treated with Dex for 24 hours, and this effect was inhibited by both GR and DPP4 knockdown (Figure 5E).

The induction of Dpp4 gene by glucocorticoids is an exclusive mechanism in pro-inflammatory M1 macrophages

Our findings in THP1-MΦ indicate that glucocorticoids promote cell migration in part by inducing DPP4 expression. To examine whether this is a conserved mechanism, we assessed the effects of Dex treatment on mouse BMM and BMDMs. After inducing macrophage differentiation with M-CSF (M0, unstimulated macrophages), cell cultures were activated by treatment with LPS/IFN-γ (M1) or with IL4 (M2). The phenotype of unpolarized (M0) macrophages as well as macrophages polarized to M1 and M2 was evaluated by confocal microscopy, showing the polarization-dependent morphological differences and the "classical" marker CD68 (Figure 6A). Moreover, qRT-PCR analysis revealed that M1 macrophages exhibited the characteristic upregulation of *Nos2/iNOS*, *Ccl5/Rantes* and *Tnf* mRNA, while M2 macrophages exhibited increased expression of *Arg1*, *Retnla* and *Chil3* (Figure 6B). To assess the impact of macrophage activation on glucocorticoid responsiveness, BMM, M0, M1, and M2 macrophage cultures were treated with 100nM Dex for 6 hours. Quantitative RT-PCR revealed that expression of M1-associated genes *Nos2* and *Tnf* were suppressed 6 hours after Dex treatment, whereas expression of M2-associated genes was not affected (Figure 6B). Interestingly, while Dex treatment induced equivalent expression of the classic glucocorticoid target genes *Gilz* in each group and the induction of the chemokine receptor *Cxcr4* in all groups of the macrophage populations, *Dpp4* was exclusively induced only in M1 macrophages (Figure 6C and supplementary figure 6 and 7). Finally, we examined the effects of glucocorticoid treatment on BMDM macrophage migration. For this purpose, M0, M1, and M2 macrophages were treated for 24 hours with Dex and their spontaneous migration was assessed. Consistent with our finding in THP1-MΦ, we found that Dex treatment enhanced spontaneous migration of M1 macrophages, but did not affect the migration of M0 or M2 macrophages (Figure 6D). Interestingly, M1 macrophages treated with Dex during 24 hours and then pre-treated or not during 1 hour with 50nM of Sitagliptin were used to evaluate the CXCL12-induced migration. We observe that the migration of M1 macrophages could also be induced by CXCL12 and this effect was blocked by pre-treatment of Sitagliptin (Figure 6E). Finally, we evaluated glucocorticoid-induced effects in peritoneal macrophages that naturally show an ambulatory or motile phenotype. Together with *Gilz* induction, we observed *Dpp4* induction upon Dex-treatment (Figure 6F) and higher cell migration induced by glucocorticoids (Figure 6G), suggesting that glucocorticoid-induced migration in macrophages is mediated by DPP4.

Discussion

The Glucocorticoid Receptor is expressed in almost all immune cells and mediates the actions of both endogenous or exogenous glucocorticoids, acting as potent regulators of inflammation (39). Interestingly, glucocorticoids have complex and different pleiotropic effects on monocytes and macrophages, but their contribution towards systemic anti-inflammatory effects is not yet fully understood. Here, we evaluated whether the process of monocyte-to macrophage differentiation modified glucocorticoid responsiveness. Transcriptome analysis of monocyte-like THP-1 and macrophage-like THP-1 cells revealed a higher GR expression in macrophage-like THP-1 compared to monocyte-like THP-1 cells. In addition, we report the identification of the pro-diabetic and pro-inflammatory exopeptidase

DPP4 as a new glucocorticoids-responsive gene exclusively regulated in macrophages. Provocatively, DPP4 promotes the migration of macrophages that is induced by glucocorticoids.

Glucocorticoids suppress inflammation through the induction of potent anti-inflammatory effects, and are frequently used to treat chronic inflammatory diseases involving lymphocytes, although they are less effective in suppressing macrophage-mediated diseases (11,13,14). Within macrophages, glucocorticoid action is dependent on the context and the timing, and they also have the capacity to mediate pro-inflammatory activities, such as enhancing leukocytes trafficking and pro-inflammatory cytokines production mainly during the first steps of the immune response (9).

One theory explaining this dual effect on immune cells gene regulation is that glucocorticoids initiate opposing forces simultaneously inducing pro- and anti-inflammatory pathways as a pro-resolutive strategy in order to quickly recover the cellular and tissue homeostasis (7,40). The effect of GR activation is highly gene, cell and stimulus-specific, as it is evident from our transcriptome data. For example, the inhibition of the CSF1 (*Colony Stimulating Factor 1*) and its receptor CSF1R could be one possible reason why glucocorticoids alone would not induce the differentiation of monocytes into macrophages.

The process of differentiation of monocytes to macrophages involves major structural and biochemical changes in the cell. However, transcriptome analysis between monocyte-like THP-1 cells and THP1-MΦ demonstrated high number of genes commonly regulated between both cell types. These associations observed could be due to different gene expression levels, rather than *de novo* transcription of uniquely expressed genes, as is the case for GR. Moreover, higher GR levels observed in macrophages could be related to greater sensitivity towards glucocorticoids post-differentiation of monocytes.

Genes involved in cell movement, trafficking and chemotaxis were overrepresented among up-and down glucocorticoid-regulated genes in THP1-MΦ, with DPP4 induced and differentially regulated by Dex exclusively in THP1-MΦ and primary murine polarized M1 macrophages. DPP4, also known as CD26, was originally described as a marker of T cell differentiation and activation (41). This study provides the first evidence that DPP4 expression is directly regulated by glucocorticoids, making it a promising candidate for glucocorticoids effects in human pro-inflammatory macrophages. Additionally, the presence of a highly conserved GRE motif in the position -4,200/-4,185 in human and mouse indicates possible shared mechanisms between species.

The importance of DPP4 for the medical community lies on the approval of the use of its inhibitors for treatment of type 2 diabetes, as monotherapy, or in combination with other oral anti-diabetes drugs (42), and the benefits in decreased risk of major cardiovascular events (43,44). DPP4 inhibitors have anti-inflammatory effects, playing a critical role in obesity-induced inflammation and insulin resistance limiting macrophage infiltration in chronic inflammatory mouse models and regulating M1/M2 balance by mediating the reversion of one to the other (24). Previously, Zhong *et al*., showed that DPP4 expression was increased during monocyte differentiation into dendritic cells (DC)/macrophages and that non-enzymatic DPP4 function was associated with inflammation during obesity (32).

The exopeptidase DPP4 involved in the regulation of the immune system cleaves dipeptides from the N-terminal region of peptides and proteins (with a residue of Ala or Pro in the penultimate position), as well as various chemokines (45). The loss of two amino acids resulting from DPP4 enzymatic action can cause: 1) increased or reduced biological peptide / protein activity, 2) increased specificity towards the receptor, 3) ligand inactivation or 4) generation of receptor antagonists (46). Therefore, as chemokines direct leukocyte migration under homeostasis and inflammation, DPP4 proteolytic processing could have relevant consequences for correct functioning of the immune response. Our findings in macrophage-like THP-1 cells and murine polarized M1 macrophages indicate that glucocorticoids enhance spontaneous and CXCL12-induced migration in part by inducing DPP4 expression.

Non-catalytic DPP4 functions have also been related to adhesion and migration processes, and interaction with extracellular matrix proteins

(fibronectin and collagen). Moreover, DPP4 inhibitors ameliorate atherosclerosis by preventing monocyte recruitment and chemotaxis via modulation of RAC-1 (21). Additionally, DPP4 in T cells interacts with the chemokine receptor CXCR4 (41), selectively binding the chemokine CXCL12 (45). This binding could promote both receptor internalization, regulating local and temporal CXCL12 activity (41,47). Regarding the non-enzymatic activity of DPP4 regulating the macrophage mobility, Hiromura et al., have shown that DPP4 inhibitors affect DPP4 and Caveolin-1 (CAV-1) interaction, resulting in the suppression of inflammation in mouse and human macrophages (48). In addition to this, it is well-known that CAV-1 activation of GTP binding protein RAC-1 plays a role in cell migration (49,50). Thus, DPP4 inhibitors could block the interaction of DPP4/CXCR4 axis, the activation of CXCR4 by CXCL12 and finally the consequential activation of CAV-1/RAC-1 in the promotion of macrophage mobility. Interestingly, we observed that CXCR4 and CXCL12 genes were enriched in the cell movement and migration pathways by IPA. Our data show that glucocorticoids also increased the expression of these genes in macrophage-like THP-1 cells and primary mouse macrophages, and that they could be another downstream regulator of migratory capacity mediated by GR activation.

Finally, enzymatic DPP4 activity induced by Dex was completely blocked by specific DPP4 inhibitors (Sitagliptin and Linagliptin), suggesting the possibility that synthetic glucocorticoids would present a low efficacy in the resolution of macrophages induced inflammation. Alternatively, these data also may indicate that glucocorticoids, through DPP4 induction, potentiate the retention and egress of macrophages from inflamed tissues, perhaps contributing to their anti-inflammatory properties of glucocorticoids.

Materials and Methods

Reagents

Dexamethasone (Dex) and RU486, were purchased from Steraloids, Inc (Newport, RI, USA). Heat-inactivated fetal calf serum and charcoal-stripped heat-inactivated FBS were purchased from Gemini Bio-Products (West Sacramento, CA, USA). RPMI medium, penicillin/streptomycin, HEPES (pH 7.0) and β-mercaptoethanol were purchased from Invitrogen (ThermoFisher Scientific, Carlsbad, CA, USA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Aldrich (St. Louis, MO, USA). Recombinant human and mouse CXCL12 were purchased from Biolegend (San Diego, CA, USA). Human anti-GR and anti-DPP-4 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). DPP-4 inhibitors, Sitagliptin and Linagliptin were purchased from Selleckchem (Houston, TX, USA). Dharmafect, not targeting control (NTC), siRNA GR and siRNA DPP4 (ON-TARGETplus siRNA) were purchased from Horizon/Dharmacon (Lafayette, CO, USA). TaqMan® RT-PCR primer probes were purchased from Applied Biosystems (Foster City, CA, USA).

Mouse Colony maintenance

All studies were performed with approval by the NIEHS animal care and use committee. The mice used for these studies were C57BL/6J purchased from the Jackson Laboratories (Bar Harbor, ME, USA). Mice were maintained in a pathogen free facility with 12- hour day-night cycles. Standard mouse chow and water were provided ad-libitum.

Cell culture

The human monocytic cell line THP-1 (ATCC®-TIB-202, Manassas, VA, USA) together with their derived macrophages were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units of penicillin/streptomycin, 25 μM of HEPES (pH 7.0) and 50 μM of β-mercaptoethanol (complete medium) at a ratio of 2.5×10^5 cells/mL under conditions of humidity at 5% CO_2 and 37°C. Monocyte-like THP-1 cells were differentiated into macrophage-like THP-1 cells (THP1-MΦ). Briefly, monocyte-like THP-1 cells (2×10^6 cells/well) were activated with 0.5 μM PMA in serum-free medium supplemented with 25 μM of HEPES during 3 hours. Subsequently, adherent cells were washed with PBS and cultured first for 24 hours with recovery medium without PMA (complete RPMI) and then another 24 hours in RPMI supplemented with 10% charcoal-stripped serum before being treated with dexamethasone. The success of the differentiation protocol was

evaluated using phase contrast microscopy and flow cytometry using a fluorophore-conjugated panel of antibodies against markers of monocyte and macrophage lineages (CD15s-BV510, CD11b-PE-Cy7 and CD11c-BV421), and Cell Tracker and 7-AAD for viability (BD Biosciences, San Jose, CA, USA) (Supplementary figure 1). Where indicated, cells were pre-treated with 1 or 10 μM RU-486 for 1 hour, prior to the addition of Dex or pre-treated with Dex for 3 hours prior the addition of DPP4 inhibitors, Linagliptin (1 and 10 nM) or Sitagliptin (25 and 50 nM).

Generation of knockout macrophage-like THP-1 cells by siRNA

For siRNA experiments, 3-day macrophage-like THP-1 cells completely differentiated were seeded in 6 well plates at a density of $1x10^6$ cells/mL and, then transfected with 25nM of NTC or with 25nM of mixture of 4 siRNA provided as a single reagent of siRNA against GR or against DPP4 (ON-TARGETplus siRNA, Dharmacon) and DharmaFECT-1 in a mixture of Optimen and medium without antibiotic. The transfection reaction was maintained at 37°C in 5% CO2 for 24 hours. The transfected cells were recovered in complete medium for another 24 hours and maintained in charcoal-stripped medium for additional 24 hours prior to adding 100nM of Dex for 6 hours for RNA or 24 hours for protein analysis. The efficiency of the transfection was evaluated by western blot and cells with GR or DPP4 silencing were used for functional analysis.

Analysis of gene expression using Micrroarray, Nanostring and qRT-PCR

Cultures of $1x10^6$/mL of monocytes THP-1 and THP1-MΦ were stimulated for 6 hours with vehicle or 100 nM of Dex, for analysis of gene expression by microarray and Nanostring (n = 3 biological replicates / condition), from 2 to 48 hours with 1, 10, 100 and 1000 nM Dex for analysis of time-course and dose-response and from 0.5 to 6 hours with 100 nM Dex for analysis of DPP4 nascent RNA levels (Forward: 5'-GCTTCCCTCTAATTGGACTTGA-3'; Probe: 5'-TTGCAGACACCGTGGAAGGTTCTT-3'; Reverse: 5'-ACGGTGATGATGGTGACAAG-3') by real-time quantitative Reverse Transcription PCR (qRT-PCR). The data from microarray (GSE135130) and Nanostring (GSE135165) were deposited in a GEO database (GSE).

Microarray analysis

Following Dex stimulation, cells were collected and lysed for total RNA extraction using Qiagen RNeasy minikit (Qiagen, Hilden, Germany). Gene expression analysis by Microarray was carried out using Agilent whole Human Genome 4x44 multiplex format oligo arrays (014850) (Agilent Technologies, Santa Clara, CA, USA) following the Agilent 1-color microarray-based gene expression analysis protocol. Starting with 500 ng of total RNA, complementary RNA (cRNA) labeled with the Cy3 probe was synthesized according to the manufacturer's protocol. For each sample, 1.65 μg of Cy3-labeled cRNA was fragmented and hybridized for 17 hours in a rotating hybridization oven. The oligo arrays were washed and then scanned with an Agilent scanner. Data was obtained using the Agilent Feature Extraction software (v12), performing the error modelled, adjusting for additive and multiplicative noise. The resulting data were processed using the OmicSoft Array Studio software (Version 7.0) and visualized by principal component analysis (PCA). To identify the differentially expressed probes and to determine statistical differences between the means of the groups, an analysis of variance (ANOVA) was used. In addition, we used a multiple test correction ANOVA and Benjamini-Hochberg with a value of $p < 0.05$ to reduce the number of false positives.

Nanostring analysis

The analysis of gene expression using the NanoString © platform (www.nanostring.com, Seattle, WA, USA) was carried out using the Human Immunology Codeset (NS_Immunology_C2328) which measures 547 endogenous RNAs and 14 housekeeping genes. 50 ng of each total RNA sample was used according to the manufacturer's instructions. RNA expression was quantified in nCounter Digital Analyzer and raw counts were generated and normalized with nSolver software (v3.0). The data were normalized using the manufacturer's positive and negative control probes, as well as 2 housekeeping genes (HPRT1, PPIA). All samples

passed the initial QA/QC controls of nSolver and the replicates were well correlated (R> 0.98). The raw and normalized compiled data (Log2 of counts) were reanalyzed in Partek for statistical analysis, (finding 159 probes with an average expression of less than 4 counts that were excluded) with 388 probes finally subjected to analysis of variance (ANOVA) in the treatment groups with p value corrected by FDR post-hoc Benjamini-Hochberg for each comparison group.

qRT-PCR analysis

The analysis of the gene expression in dose response and time course using monocyte-like THP-1 cells and THP1-MΦ, human monocytes derived macrophages (38), murine bone marrow monocytes, bone marrow-derived macrophages and peritoneal macrophages by qRT-PCR was carried out using 50 ng of total RNA, the One-Step RT-PCR kit (BioRad, Hercules, CA, USA) together with sets of predesigned and validated TaqMan primer/probes for each analysed transcript (Applied Biosystems). NR3C1(Hs00230813_m1), GILZ/TSC22D3 (Hs00608272_m1), DPP4 (Hs00897391_m1), FKBP5 (Hs01561006_m1), PER1 (Hs00242988_m1), AREG (Hs00950669_m1), NLRP3 (Hs00918082_m1), HSD11B1 (Hs01547870_m1), TNF (Hs99999043_m1), CCL2 (Hs00234140_m1), HIST1H4C (Hs00543883_s1), CCL20 (Hs00355476_m1), CYP19A1 (Hs00903411_m1), CD86 (Hs01567026_m1), NOX1 (Hs00246589_m1), HSD11B2 (Hs00388669_m1) and PPIB (Hs00168719_m1) human genes and *Nr3c1* (Mm00433832_m1), *Gilz/Tsc22d3* (Mm00726417_s1), *Dpp4* (Mm00494549_m1), *Cxcr4* (Mm01996749_s1), *Nos2* (Mm00440502_m1), *Ccl5* (Mm01302427_m1), *Tnf* (Mm00443258_m1), *Arg1* (Mm00475988_m1), *Retnla/Fizz1* (Mm00445109_m1), *Chil3/Ym1* (Mm00657889_mH) and *Ppib* (Mm00478295_m1) for mouse genes. The samples were run in duplicate in the real-time thermocycler model CFX96 from BioRad (Hercules, CA, USA). The Ct values from each transcript were normalized to the housekeeping gene PPIB and expressed relative to the level of the transcript in the unstimulated. As a positive control of the effect of Dex, the glucocorticoid-responsive genes FKBP5 and GILZ were used. Additionally, the activity of GR and levels of each transcript regulated by Dex was evaluated in the presence or absence of RU-486.

Analysis of canonical pathways using Ingenuity Pathway Analysis

The lists of significantly regulated genes were annotated using Ingenuity Pathway Analysis (IPA®). Enrichment or overlap of canonical pathways and the top biological functions were determined by IPA, using Fisher's test ($p < 0.05$). Gene networks involved in the inflammatory response, cell movement and chemotaxis were constructed using the Pathdesigner tool of IPA.

Protein analysis by western blot and flow cytometry

Total proteins were extracted in RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) supplemented with inhibitor cocktail of proteases (Roche, Rotkreuz, Switzerland). Equal amounts of protein were loaded and separated in precast Novex 10% Tris-Glycine mini gels (ThermoFisher Scientific, Waltham, MA, USA), and transferred to nitrocellulose membranes under semi-dry rapid transfer system (BioRad) and blocked with blocking buffer (LI-COR, Lincoln, NE, USA) for 60 minutes at room temperature. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies anti-GR (1:1000 dilution), anti-DPP4 (1:1000 dilution) in 5% skimmed powdered milk in TBS-T and 5% BSA in TBS-T, respectively. Blots were washed and incubated with goat anti-rabbit IRDye680-conjugated secondary antibody (LI-COR) for 1 hour at room temperature and visualized with LICOR Odyssey Imaging scanner system (LI-COR). The obtained immunoreactivity was normalized to β-Actin and/or β-Tubulin proteins as a loading control and was expressed relative to the protein level of the unstimulated condition. To determine the expression at the protein level of activation markers in monocyte-like THP-1 cells and THP1-MΦ by flow cytometry, the cells were stimulated for 24 hours with 100 nM Dex, fixed in paraformaldehyde for 10 minutes at room temperature, and permeabilized according to the surface or intracellular staining evaluated. The

immunostaining process was performed using a panel of antibodies (BD Biosciences, San Jose, CA, USA) conjugated against CD15s-BV510, CD11b-PE-Cy7 and CD26/DPP4-PE, and their respective isotypes according to the manufacturer's specifications, prior to blocking Fc using a commercial blocker. The samples were evaluated in triplicate in the LSR II cytometer (BD Biosciences) and analysed through the software FACSDiva version 6.1.3.

Determination of the enzymatic activity of DPP4 by fluorometric assay

Monocyte-like THP-1 cells and THP1-MΦ were treated with the GR antagonist RU486 before the stimulation of Dex for 24 hours or with Dex during the first 3 hours before add 2 concentrations of the specific inhibitors of DPP4, Sitagliptin and Linagliptin. The cells were collected and lysed with lysis solution according to the manufacturer's instructions using the commercial kit DPP4 Activity Assay (Sigma Aldrich, St. Louis, MO, USA). The results were plotted as pmol/mL/min (microunit/mL), where one unit of DPP4 is the amount of enzyme that hydrolyses the DPP4 substrate to produce 1.0 μmole of AMC per minute at 37 °C.

In silico analysis of GREs in the human DPP4 gene

Analysis in silico using the JASPAR software database revealed the presence of putative GREs in the promoter region. These GREs were mapped and analysed by multiple alignments against the consensus sequence using the STAMP software, demonstrating a likelihood of GR binding in those regions of the DNA. According to this, primers probes flanking each of the GREs found in the promoter region of DPP4 were used to Chromatin immunoprecipitation coupled to real time PCR (ChIP-qPCR) and Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE) analysis.

Chromatin immunoprecipitation coupled to real time PCR (ChIP-qPCR) and Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE) analysis.

Monocyte-like THP-1cells and THP1-MΦ seeded at a density of 1x106 cells / mL stimulated with or without 100 nM Dex for 2 hours were collected and evaluated by chromatin immunoprecipitation using the EZ-Magna ChIP™ A/G Chromatin Immunoprecipitation Kit with immunomagnetic beads (EMD Millipore). For this, centrifuged and pelleted cells were cross-linked using 1% formaldehyde for 10 min at room temperature followed by quenching the reaction with 1X glycine for 5 minutes and then lysed and homogenized with Dounce homogenizer for the isolation of the nuclear fraction in a solution containing cOmplete™ Protease Inhibitor Cocktail (Sigma-Aldrich). The nuclear fraction isolated was sonicated in Bioructor with a controlled temperature high pressure cooling system (Diagenode, Sparta, NJ, USA). A fraction of the fragmented chromatin was used to evaluate the quality of chromatin through agarose gel electrophoresis. DNA fragments sized between 0.2 and 0.5 kb were immunoprecipitated using 3 μg of anti-GR monoclonal antibody (Cell Signaling) or the same concentration of anti-IgG as isotype control (EMD-Millipore). Subsequently, the immunocomplexes were isolated using magnetic beads of protein A/G agarose, washed with solutions of low and high concentration of salts, LiCl solution and TE buffer, treated with RNase, proteinase K and temperature to dissociate them for recovery and elution of the DNA. Aliquots of each DNA sample recovered were purified using columns, analysed by quantitative PCR, using primers-probes flanking the two GREs. GRE -4,200/-4,185 (Forward: 5'-CCTAGTGGAGCTGTGAGAAGA-3'; Probe: 5'-TCCAGTTACACGGAACAAGCTGTCC-3'; Reverse: 5'-CAGGCTGGCGTTGAGTATATG-3') and GRE -1,782/-1,767 (Forward: 5'-GCACAGGGTGTGAAGATATTTG-3'; Probe: 5'-TGCCCTCCAGAGAACAAATTGACCT-3'; Reverse: 5'-GAGGCTGGCTGACATCTAC-3'). The Ct values of each of the samples analysed in triplicate were compared with respect to the initial input and normalized to the IgG isotype values, and expressed as the fold enrichment of the stimulated condition compared to the control. Additionally, GRE located in the promoter of GILZ gene was used as positive control. FAIRE analysis was performed according to Simon *et al*. (51) using the same set of primers-probes previously analysed for ChIP-qPCR.

Experimental setup for mouse peritoneal macrophages and bone marrow derived macrophages

Peritoneal macrophages (PM) and bone marrow derived macrophages (BMDMs) were isolated from 8- to 12-wk-old C57BL/6 mice by flushing the peritoneal cavity with 5 mL ice cold complete medium and flushing the femur and tibia with complete medium, respectively. The bone marrow monocytes (BMM) were purified by negative selection using EasySep™ mouse monocytes Isolation kit (Stemcell Tech, Vancouver, BC, Canada) and resuspended in complete medium supplemented with 100 ng/mL M-CSF (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells a density of $5.0x10^5$ cells/wells were incubated for 6 days at 37 °C and 5% CO_2 with medium change every 3 day. The bone marrow-derived macrophages (BMDMs) phenotype was analyzed by phase contrast microscopy and confocal immunofluorescence using anti-CD68 antibody (Biolegend, San Diego, CA, USA), by flow cytometry of the surface markers Ly6C-PerCP/Cy5.5, CD11b-FITC, F4/80-APC, DPP4-PE and CD80-BV421 and CD206-PE/Cy7, as M1 and M2 markers, respectively (Biolegend) and by the gene expression profile using qRT-PCR. For polarization to M1 and M2, BMDMs unpolarized (M0) were stimulated for 24 hours with 10 ng/mL of murine recombinant IFN-γ (Miltenyi Biotec) and 50 ng/mL of LPS (Sigma) and with 10 ng/mL of murine recombinant IL-4 (Miltenyi Biotec) to the M1 and M2 respectively, in 10% charcoal-stripped serum. For experimental setup, M0, M1 and M2 macrophages were stimulated with 100nM Dex for 6 hours for gene expression profile and until 24 hours for protein analysis.

Analysis of migratory capacity of monocyte-like THP-1 cells, THP-1 derived macrophages, peritoneal macrophages and BMDMs unpolarized and polarized to M1 and M2

THP-1 monocytes, 6-day THP1-MΦ untransfected (mock) and transfected with NTC, siRNA GR and siRNA DPP4 primed or not with 100 nM Dex, 10 μM RU486 or with RU486 plus Dex during 24 hours, PM primed or not with 100 nM Dex or BMDM unpolarized and polarized to M1 and M2, primed or not with Dex, were used to evaluate the migratory properties. Immediately after the stimulation, the supernatant was collected, the cells were washed and detached with fresh and warmed 10mM PBS-EDTA, collected in serum-free medium (without chemoattractant molecules) or in presence of DPP4 inhibitors, counted and reseeded at a density of $4x10^5$ cells/mL in the insert of a QCM™ Chemotaxis 5μm 24-well Migration Assay (with a 5μM pore size for monocytes/macrophages movement) (EMDMillipore, Burlington, MA, USA), while in the lower chamber medium with 10% FBS or 100 ng/mL of human or mouse CXCL12 (SDF-1α) recombinant as a chemoattractant. After 6 and 24 hours, the migratory cells that adhered to the lower surface of the insert in the chamber were detached, lysed, and quantitated by the incorporation of a fluorescent probe CyQUANT® GR Dye in a plate reader, according to the manufacturer's instructions. Each migration assay was repeated three times. The percentage of cells migrated were calculated in relation to unstimulated or untransfected conditions.

Immunofluorescence (IF) staining

M0, M1, and M2 macrophages were grown in glass bottom culture dishes (MatTek corporation). Then, cells were washed with warm PBS, fixed with warm 4% paraformaldehyde for 20 minutes at room temperature, and permeabilized in PBS containing 2% bovine serum albumin and 0.1% Triton X-100 for 30 minutes at room temperature. Cells were then blocked for 1 hour with PBS containing 5% goat serum and 0.1% Triton X-100 at room temperature prior to incubate the specimens at 4 °C o.n. with anti-CD68 (Biolegend, San Diego, CA, USA) antibody. The following morning, samples were washed with 1X PBS containing 0.1% Tween, and incubated with the secondary antibody goat anti-rat AF594 for 1 hour at room temperature. Samples were then washed, air dried and mounted with ProLong gold antifade mountant with DAPI (Thermo scientific). A Zeiss laser scanning confocal microscope (LSM 880; Carl Zeiss) was used to analyze CD68 expression.

Statistical analysis

The GraphPad Prism version 7.0 was used to analyse the data. To determine the statistical significance of the results, the Two-tailed unpaired Student's *t* test and One-or Two-way ANOVA statistical test was performed with the ad/hoc post-test according to the distribution of the data. Those comparisons whose value was $p<0.05$ were considered statistically significant. In all the experiments the samples were analysed in duplicate and each experiment was performed at least 3 times independently.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions
D.D.J., M.A.H., and J.A.C., designed and analysed the experiments. D.D.J., performed most of the experiments; M.G.P., performed the immunofluorescence assays and J.B., performed the experimental setup for peritoneal macrophages; D.D.J., and J.A.C., wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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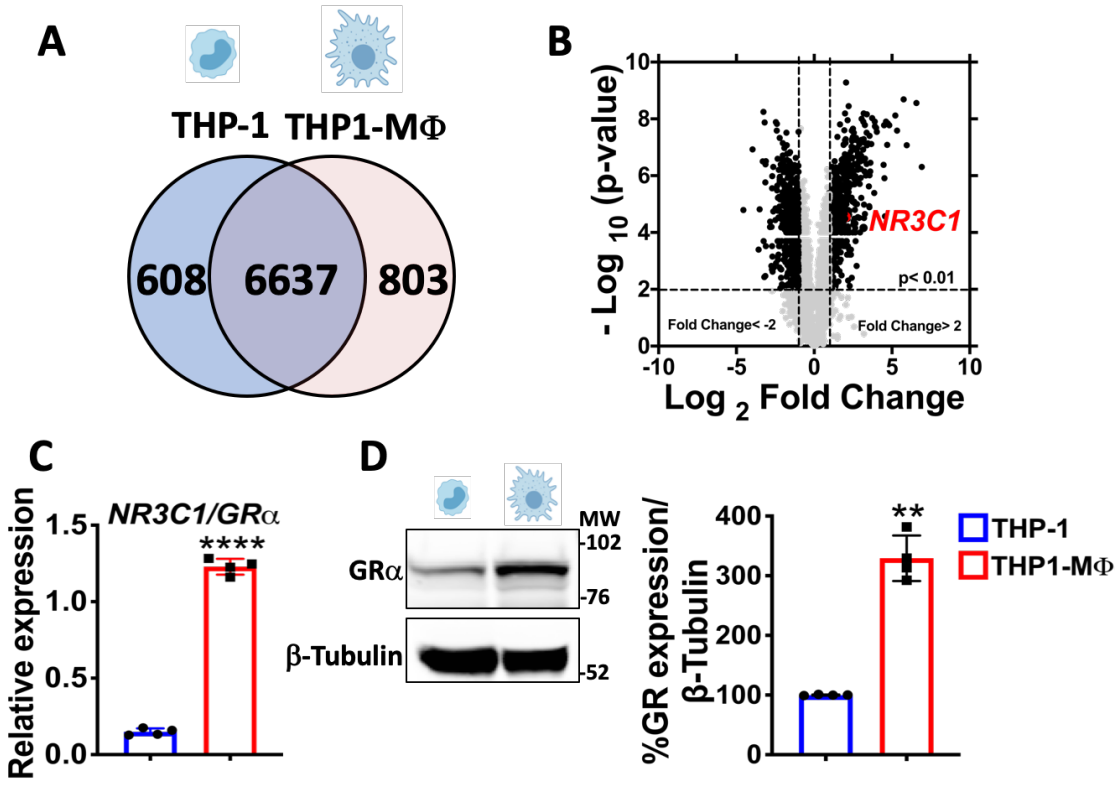

Figure 1: The transcriptome of macrophage-like THP-1 cells shows a higher GR expression compared to monocyte-like THP-1 cells. A) Venn diagram summarizing *microarray* data analysis shows the number of genes commonly expressed by monocyte-like THP-1 (THP-1) and macrophage-like THP-1 (MΦ-THP-1) cells, and uniquely expressed in both cell types. B) Volcano plot showing significantly differentially expressed genes in MΦ-THP-1versus THP-1. In MΦ-THP-1 *GR* gene expression (aka *NR3C1/GR*) is 4-fold higher than THP-1. (C) Validation by qRT-PCR and (D) western blotting of GR expression that results higher in MΦ-THP-1 than THP-1. Data are mean ± SD and are representative of four independent experiments. *P<0.05; two tailed unpaired Student's t test.

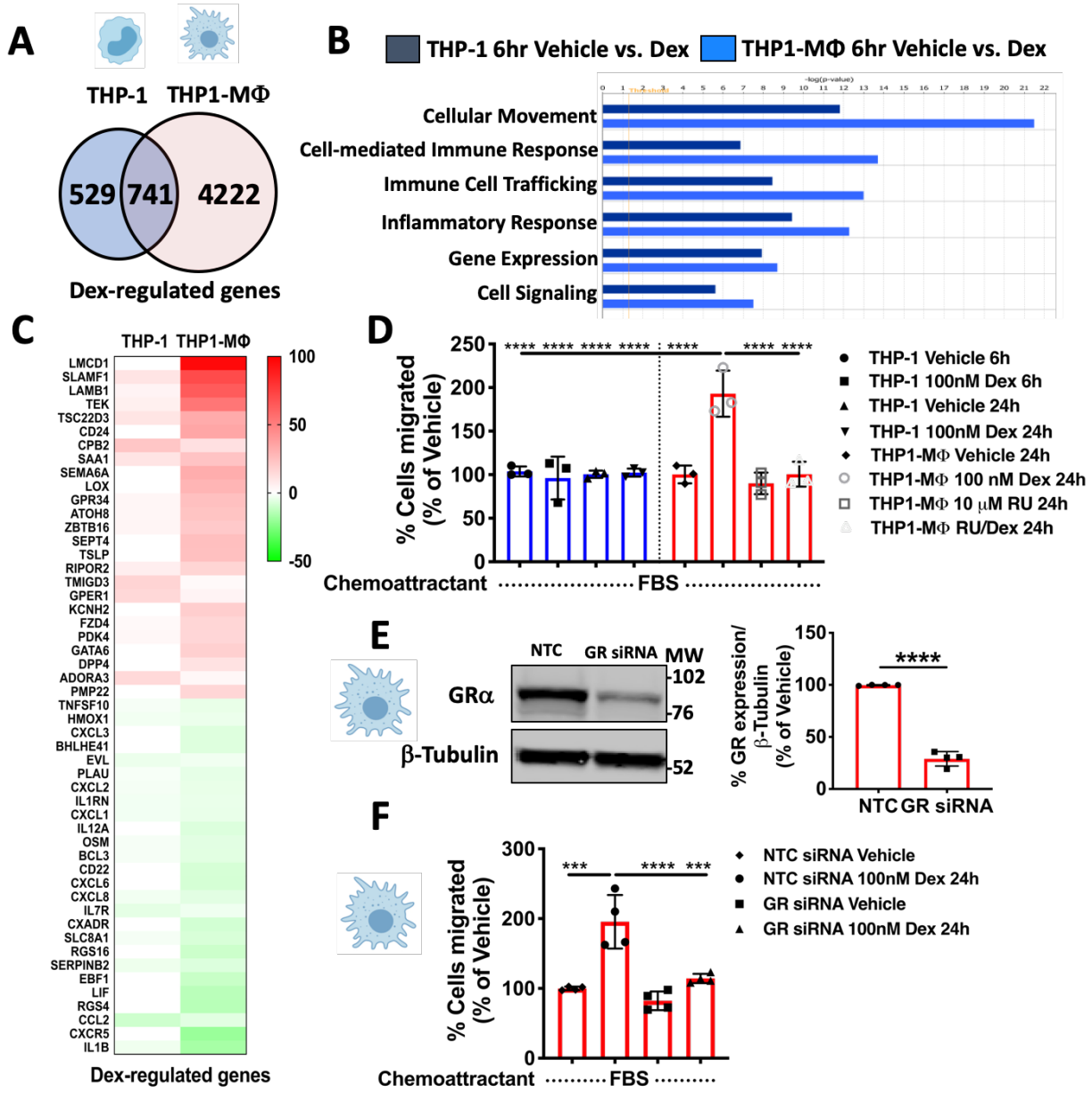

Figure 2: Glucocorticoids regulate the cell migration of macrophage-like THP-1 cells. A) Venn diagram, summarizing *microarray* data analysis, shows the number of genes commonly regulated by Dex in monocyte-like THP-1 cells (THP-1), macrophage-like THP-1 cells (MΦ-THP-1), and uniquely regulated by Dex in both cell types. B) The top 5 biological functions that are differentially regulated in THP-1 and MΦ-THP-1. C) Heatmap representing the top 25 up-regulated and the top 25 down-regulated genes by glucocorticoids in THP-1 and MΦ-THP-1 associated with the cellular movement pathway. D) In vitro transwell assay using 10% of FBS as chemoattractant was used to evaluate spontaneous cell migration of THP-1 and MΦ-THP-1 treated with vehicle, 100 nM Dex, 10 μM RU-486, or RU-486 with Dex. The graph shows that Dex treatment induces migration only in MΦ-THP-1and this phenomenon is reversed by using GR antagonist RU486. E) THP1-MΦ cells were transfected with NTC or GR siRNAs. 24 hours after transfection, GR protein knockdown was evaluated by western blot (75% reduction). On the left, a representative immunoblots of GR and β-tubulin expression is shown. On the right, densitometry analysis of GR normalized to β-tubulin. (F) In vitro migration assay of THP1-MΦ cells transfected with NTC or GR

siRNAs that have been treated for 24 hours with or without 100nM of Dex. The histograms show that GR knockdown abolishes Dex-induced macrophage migration. Cell migration was calculated as percentage relative to vehicle-treated groups. Data are mean ± SD and are representative of three independent experiments. ***P<0.001; ****P<0.0001; two tailed unpaired Student's *t* test (E) and One-way ANOVA statistical test with Tukey's multiple comparisons test (D and F).

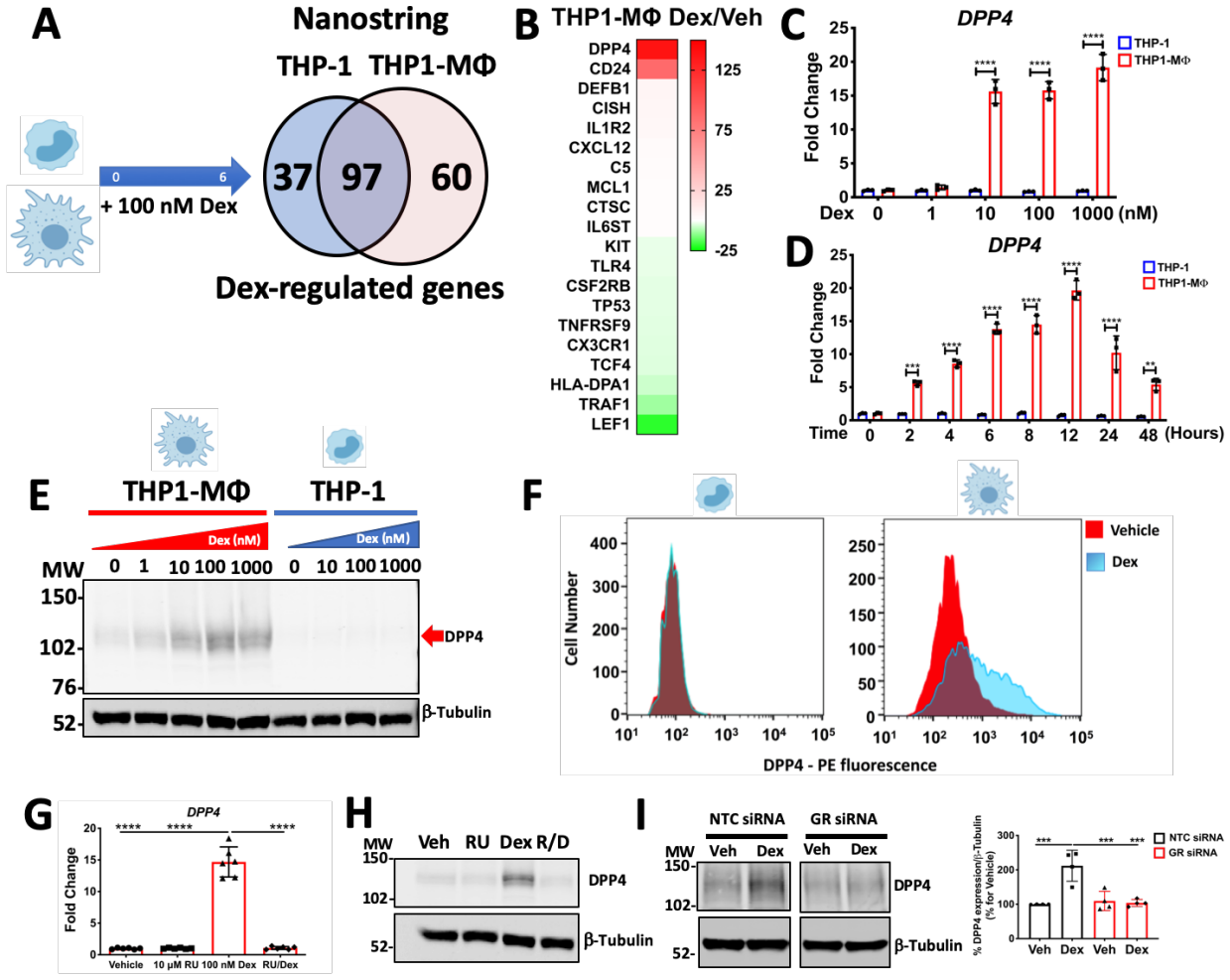

Figure 3: Glucocorticoids up-regulate *DPP4* mRNA and protein levels in macrophage-like THP-1 cells by GR activation. A) Venn diagram, summarizing *Nanostring Immunology codeset data analysis,* shows the number of genes commonly regulated by Dex in monocyte-like THP-1 (THP-1) and macrophage-like THP-1 (MΦ-THP-1) cells, and uniquely regulated by Dex in both cell types. B) Heatmap of the top 10 up- and down-regulated genes by Dex in THP1-MΦ. (C-F) Glucocorticoid-induced *DPP4* levels are uniquely expressed in THP1-MΦ cells. (C) Quantitative RT-PCR analysis of *DPP4* mRNA levels in THP-1 and THP1-MΦ cells in a Dex dose-response and (D) Dex time course experiments. (E) A representative western blot and (F) flow cytometry analysis of DPP4 protein from THP-1 and THP1-MΦ treated with 100nM of Dex for 24h. The immunoreactive band is indicated by red arrow. (G-I) Evaluation of GR participation in DPP4 up-regulation. Pharmacological inhibition of GR with RU-486 and GR knockdown by siRNA abolish Dex-induced expression of *DPP4* at both (G) mRNA and (H-I) protein levels. **P<0.01; ***P<0.001; ****P<0.0001; Two-way ANOVA test with Tukey's multiple comparisons test (C-D) and One-way ANOVA test with Tukey's multiple comparisons test (G and I).

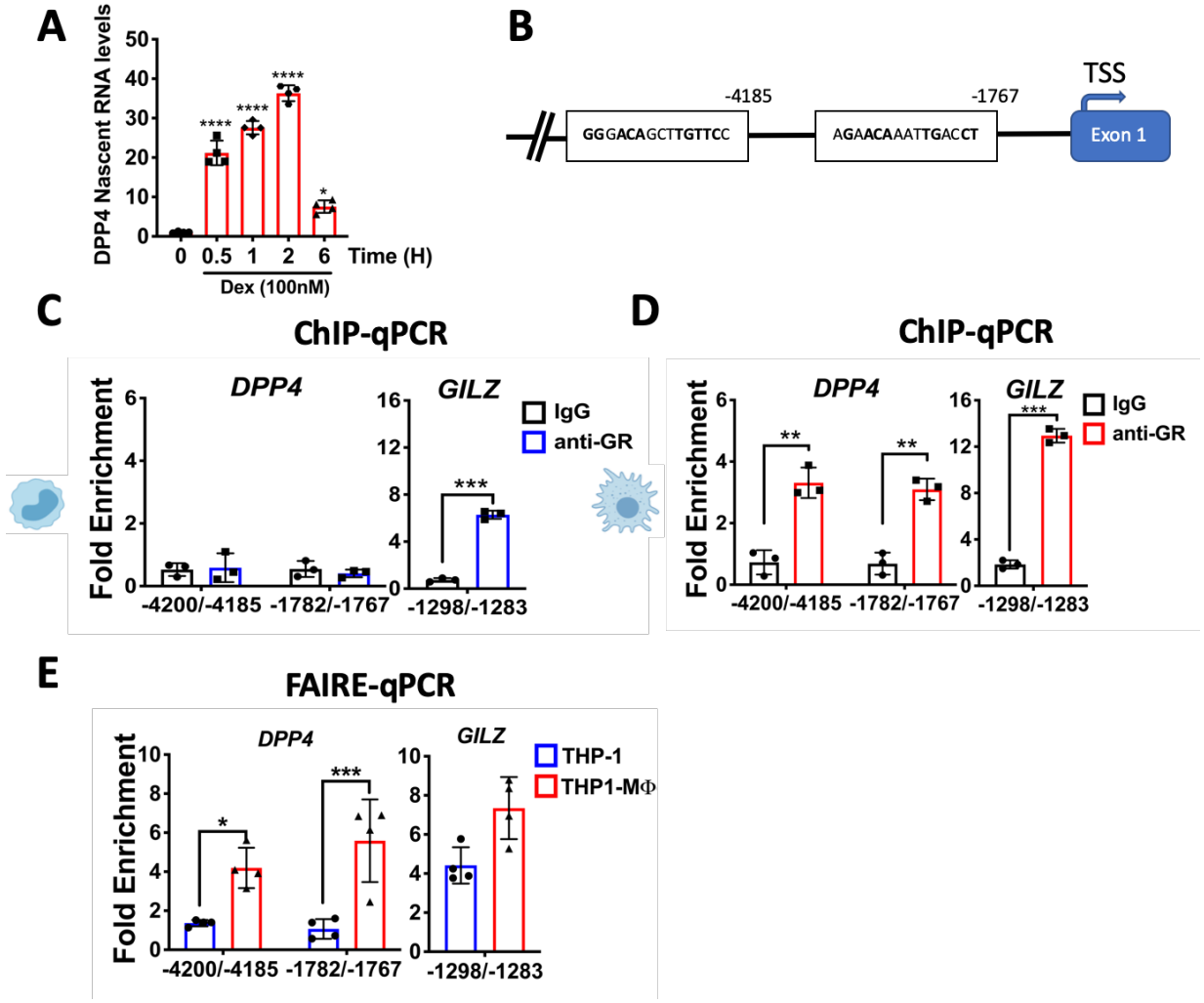

Figure 4: Glucocorticoids regulates *DPP4* expression by binding to GREs in the DPP4 promoter. A) Quantitative real-time PCR analysis of DPP4 nascent mRNA from macrophage-like THP-1 cells (THP1-MΦ) that have been treated with 100 nM Dex for the indicated times. Dex treatment stimulates the transcription of a significant amount of *DPP4* nascent mRNA within 30 minutes following Dex exposure. B) Schematic representation of a fragment of human *DPP4* gene that highlights 2 identified GR-responsive elements (GRE) located at positions -4,200/ -4,185 and -1,782/-1,767, from the transcription star site (TSS). (C) monocyte-like THP-1 cells and (D) THP1-MΦ were treated with or without 100 nM Dex for 2 hours to evaluate by chromatin immunoprecipitation coupled to PCR (ChIP-qPCR) the amount of GR bound to *DPP4* GREs exclusively in THP1-MΦ. GR-ChIP samples were analyzed by quantitative PCR and the Ct values of each experimental group analyzed in triplicate, compared to their respective inputs, and normalized to the IgG isotype control. GR recruitment to *DPP4* GREs is expressed as fold enrichment of Dex- versus vehicle-treated cells. (C-D) ChIP-qPCR of GR bound GRE located in the promoter region of *GILZ* gene was used as positive control. (E) Chromatin remodeling was measured by FAIRE-qPCR to evaluate GR accessibility to the GREs in *DPP4* gene and *GILZ* gene (as control) in response to monocyte-to-macrophage differentiation process. For each GRE, chromatin accessibility was determined and compared to the respective DNA input. Data are mean ± SD and are representative of three independent experiments. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. One-way ANOVA test with Tukey's multiple comparisons test (A) and Two-way ANOVA test with Sidak's multiple comparisons test (C-E).

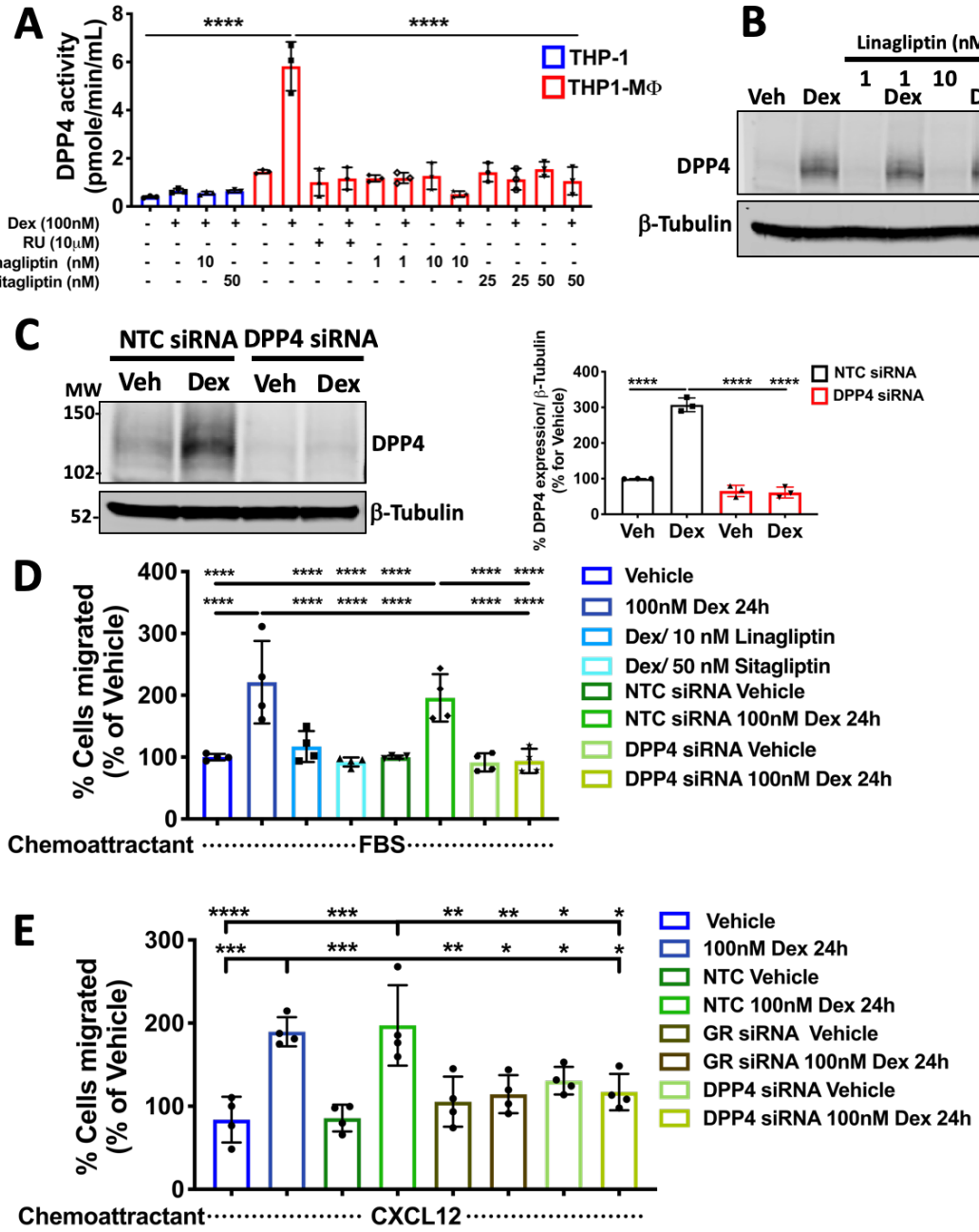

Figure 5: The cell migration induced by dexamethasone in macrophage-like THP-1 cells is mediated through DPP4 activity. (A) The enzymatic activity of DPP4 measured by fluorometric assay and induced by Dex was completely blocked by DPP4 inhibitors Sitagliptin (25 and 50 nM) and Linagliptin (1 and 10 nM) in MΦ-THP-1. (B) Western blotting for DPP4 showing that both DPP4 inhibitors do not affect the Dex-induced DPP4 protein expression. (C) THP1-MΦ cells were transfected with NTC or DPP4 siRNAs. 24 hours after transfection cells were treated with or without Dex for 24h and DPP4 protein knockdown was evaluated by western blot (> 70% reduction). On the left, a representative immunoblots of DPP4 and β-tubulin expression is shown. On the right, densitometry analysis of DPP4 normalized to β-tubulin is shown. (D) Dex-induced THP1-MΦ migration is mediated by DPP4 expression. In vitro migration assay

shows that both pharmacological inhibition of DPP4 enzymatic activity, by Linagliptin and Sitagliptin, and silencing of DPP4 expression block the spontaneous migration of THP1-MΦ induced by Dex treatment. (E) CXCL12-induced THP1-MΦ migration was blocked by GR and DPP4 knockdowns of cells following Dex treatment. Data are mean ± SD and are representative of 3-4 independent experiments. ***P<0.001; ****P<0.0001; One-way ANOVA statistical test with Tukey's multiple comparisons test.

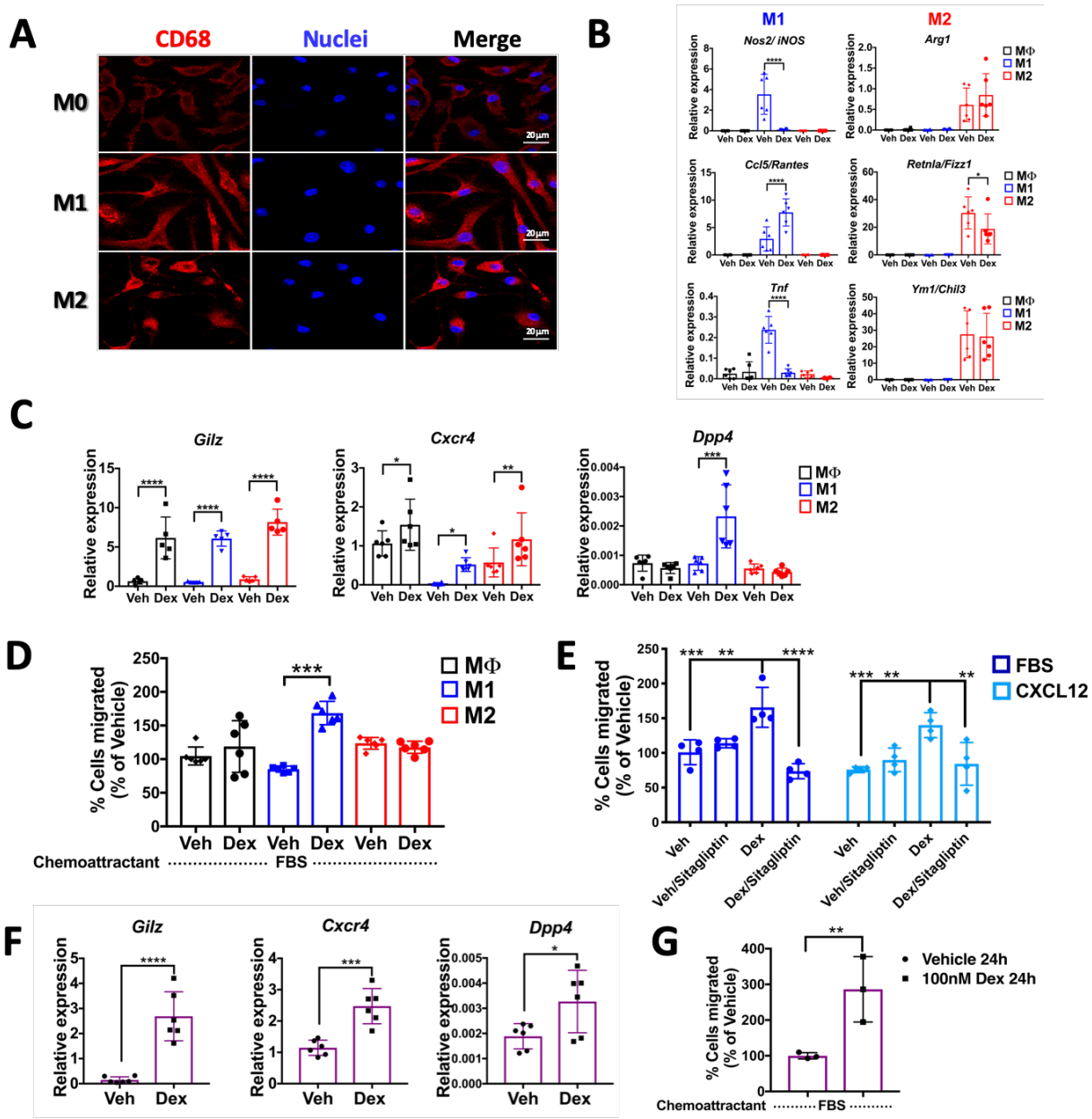

Figure 6: Glucocorticoids up-regulate *Dpp4* expression in mouse bone marrow-derived macrophages (BMDM) polarized to classically activated profile M1 and Peritoneal Macrophages promoting migration. Bone marrow monocytes (BMM) isolated by negative selection and differentiated into BMDM with 100 ug/mL M-CSF were evaluated by immunofluorescence microscopy. (A) Immunofluorescence staining of in vitro cultured mouse M0, M1, and M2 macrophages labelled with the macrophage marker CD68. Scale bar represents 20 µm. (B) Gene expression profiles by qRT-PCR of M1 (*Nos2, Ccl5 and Tnf*) and M2 (*Arg1, Retnla, Ym1*) markers at 6 hours with 100nM of Dex. (C) mRNA levels of *Gilz, Cxcr4,* and *Dpp4* regulated by glucocorticoids in M0, M1, and M2 macrophages. (D) In vitro migration assay, using 10% FBS as chemoattractant, of M0, M1, and M2 macrophages treated with or without dexamethasone. After 24 hours, M1 macrophages show a significant increase in the spontaneous migration upon Dex-treatment. (E) The cell migration of primary M1 macrophages induced by CXCL12 is blocked by pharmacological inhibition of DPP4 following dexamethasone treatment. (F) mRNA levels of *Gilz, Cxcr4* and *Dpp4* regulated by glucocorticoids in mouse peritoneal macrophages (PM). (G) In vitro migration

assay, using 10% FBS as chemoattractant, of PM treated with or without dexamethasone shows an increase in the migratory potential induced by glucocorticoids. Data are mean ± SD and are representative of 3-5 independent experiments. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; Ordinary one-way ANOVA statistical test with Tukey's multiple comparisons test and unpaired two tailed *t* test.

Glucocorticoids mobilize macrophages by transcriptionally up-regulating the exopeptidase DPP4

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