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The Inverse Agonist DG172 Triggers a PPARβ/δ-Independent Myeloid Lineage Shift and Promotes GM-CSF/IL-4-Induced Dendritic Cell Differentiation

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ABSTRACT

The stilbene derivative (*Z*)-2-(2-bromophenyl)-3-{[4-(1-methylpiperazine) amino]phenyl}acrylonitrile (DG172) was developed as a highly selective inhibitory peroxisome proliferator-activated receptor (PPAR)β/δ ligand. Here, we describe a novel PPARβ/δ-independent, yet highly specific, effect of DG172 on the differentiation of bone marrow cells (BMCs). DG172 strongly augmented granulocyte-macrophage-colony-stimulating factor (GM-CSF)-induced differentiation of primary BMCs from *Ppard* null mice into two specific populations, characterized as mature ($CD11c^{hi}MHCII^{hi}$) and immature ($CD11c^{hi}MHCII^{lo}$) dendritic cells (DCs). IL-4 synergized with DG172 to shift the differentiation from $MHCII^{lo}$ cells to mature DCs in vitro. The promotion of DC differentiation occurred at the expense of differentiation to granulocytic $Gr1^{+}Ly6B^{+}$ cells. In agreement with these findings, transcriptome analyses showed a strong DG172-mediated repression of genes encoding neutrophilic markers in both differentiating wild-type and *Ppard* null cells, while macrophage/DC marker genes were up-regulated. DG172 also inhibited the expression of transcription factors driving granulocytic differentiation (*Cebpe*, *Gfi1*, and *Klf5*), and increased the levels of transcription factors promoting macrophage/DC differentiation (*Irf4*, *Irf8*, *Spib*, and *Spic*). DG172 exerted these effects only at an early stage of BMC differentiation induced by GM-CSF, did not affect macrophage-colony-stimulating factor–triggered differentiation to macrophages and had no detectable PPARβ/δ-independent effect on other cell types tested. Structure-function analyses demonstrated that the 4-methylpiperazine moiety in DG172 is required for its effect on DC differentiation, but is dispensable for PPARβ/δ binding. Based on these data we developed a new compound, (*Z*)-2-(4-chlorophenyl)-3-[4-(4-methylpiperazine-1-yl)phenyl]acrylonitrile (DG228), which enhances DC differentiation in the absence of significant PPARβ/δ binding.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that function as ligand-inducible transcription factors in lipid metabolism and immune regulation (Kostadinova et al., 2005; Yang et al., 2010; Wahli and Michalik, 2012). Consistent with their physiologic functions PPARs are associated with major human diseases, including hyperlipidemia, diabetes, arteriosclerosis, inflammatory disorders, and cancer (Desvergne et al., 2006; Peters et al., 2012; Wahli and Michalik, 2012). Consequently, their potential as therapeutic targets has led to the development of subtype-selective, high-affinity ligands (Peraza et al., 2006).

PPARβ/δ serves as a receptor for a broad range of natural agonistic ligands with functions in inflammatory processes, including unsaturated fatty acids (Xu et al., 1999), prostaglandin I_2 (prostacyclin) (Lim et al., 1999), and 15-hydroxyeicosatetraenoic acid (15-HETE) (Naruhn et al., 2010). Different laboratories and companies have developed a number of PPARβ/δ-specific agonistic ligands (Peraza et al., 2006), several of which are well characterized and have been used in numerous experimental studies. Synthetic antagonistic ligands for PPARβ/δ have been explored to a much lesser extent, but several inhibitory compounds have been described over the past years. These include the irreversible inhibitor and partial PPARγ agonist

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ABBREVIATIONS: BMC, bone marrow cell; DC, dendritic cell; DG172, (*Z*)-2-(2-bromophenyl)-3-{[4-(1-methylpiperazine)amino]phenyl}acrylonitrile; DG228, (*Z*)-2-(4-chlorophenyl)-3-[4-(4-methylpiperazine-1-yl)phenyl]acrylonitrile; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage-colony-stimulating factor; GW501516, {2-methyl-4-[({4-methyl-2-[4-(trifluoromethyl)phenyl]-5-thiazolyl}methyl)thio]phenoxy} acetic acid; LPS, lipopolysaccharide; M-CSF, macrophage-colony-stimulating factor; MFI, mean fluorescence intensity; PPAR, peroxisome proliferator-activated receptor; RT-qPCR, real-time quantitative polymerase chain reaction; ST247, methyl 3-{*N*-[4-(hexylamino)-2-methoxyphenyl]sulfamoyl}thiophene-2-carboxylate; TR-FRET, time-resolved fluorescence resonance energy transfer; WT, wild-type.

GSK3787 (Palkar et al., 2010; Shearer et al., 2010), the PPARβ/δ-specific GSK0660 (Shearer et al., 2008) and its improved derivative methyl 3-{*N*-[4-(hexylamino)-2-methoxyphenyl]sulfamoyl} thiophene-2-carboxylate (ST247) (Naruhn et al., 2011; Toth et al., 2012), and the stilbene (*Z*)-2-(2-bromophenyl)-3-{[4-(1-methylpiperazine)amino]phenyl}acrylonitrile (DG172) (Lieber et al., 2012). These ligands act as inverse agonists, as indicated by their inhibitory effect on the basal expression of PPARβ/δ target genes and an increased recruitment of transcriptional corepressors (Naruhn et al., 2011). DG172 is a PPARβ/δ-selective compound characterized by high affinity and potent repressive effects on PPARβ/δ target genes (Lieber et al., 2012).

There is a large body of evidence implicating PPARβ/δ in inflammation-associated processes (Kostadinova et al., 2005; Yang et al., 2010; Wahli and Michalik, 2012), including T-helper cell function (Kanakasabai et al., 2010) and macrophage polarization (Kang et al., 2008; Odegaard et al., 2008). Nevertheless, the precise role of PPARβ/δ in immune cell differentiation and regulation is still poorly understood. Therefore, we sought to analyze the effect of PPARβ/δ ligands on differentiating bone marrow cells (BMCs) from wild-type (WT) and *Ppard* null mice. At an early stage of this study it became evident that DG172 strongly influenced BMC differentiation induced by the granulocyte-macrophage-colony-stimulating factor (GM-CSF), whereas the genetic disruption of *Ppard*, the agonist {2-methyl-4-[({4-methyl-2-[4-(trifluoromethyl)phenyl]-5-thiazolyl}methyl) thio]phenoxy} acetic acid (GW501516) and the inverse agonist ST247 affected differentiation only to a marginal extent, indicating a PPARβ/δ-independent mechanism.

Exposure of mouse BMCs to GM-CSF as the only growth factor or cytokine results in a mixed population of adherent and non-adherent cells consisting of macrophages, dendritic cells (DCs), and neutrophils (Inaba et al., 1992). While the numbers of non-adherent granulocytic cells decrease in these cultures within a few days, loosely adhering immature DCs and strongly adherent macrophages increase. Inclusion of IL-4 strongly shifts the balance toward the differentiation to immature DCs (Schuler et al., 1999), while the addition of the macrophage-colony-stimulating factor [macrophage-colony-stimulating factor (M-CSF); CSF-1] instead of GM-CSF produces a basically pure population of macrophages (Weischenfeldt and Porse, 2008). The different myeloid cell types can be identified by selectively expressed surface markers, such as Gr1 (Ly6G) on neutrophils and MHCII, CD11c, and F4/80 on DCs and macrophages (Inaba et al., 1992; Schuler et al., 1999; León et al., 2004; Weischenfeldt and Porse, 2008; Lee et al., 2013). Lineage specification is determined by key transcription factors that drive differentiation along a specific path, such as C/EBPε and Gfi1 for neutrophils or Spi1 (PU.1) and Irf8 for monocytic cells (Rosenbauer and Tenen, 2007). We used this experimental system in the present study to investigate in detail the DG172-induced lineage shift in BMC differentiation.

Materials and Methods

Cell Culture. BMCs were isolated from mice as described (Resnitzky et al., 1986) and cultured in RPMI 1640 supplemented with 10% fetal calf serum, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and recombinant GM-CSF (20 ng/ml) (PeproTech, Hamburg, Germany) for 6 days, if not indicated otherwise. In some experiments IL-4 (5 or 200 ng/ml, as indicated) (PeproTech) and/or lipopolysaccharide (LPS; 100 ng/ml) (Sigma-Aldrich, Taufkirchen, Germany) were added, or M-CSF (Biomol, Hamburg, Germany; 20 ng/ml) was used instead of GM-CSF. Thioglycollate-elicited macrophages were obtained as described (Naruhn et al., 2011). NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium, complemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in a humidified incubator at 37°C and 5% CO_2.

Ligands. DG172, its derivatives, and ST247 were synthesized as previously described (Lieber et al., 2012; Toth et al., 2012). GW501516 was purchased from Axxora (Lörrach, Germany). Synthesis and experimental

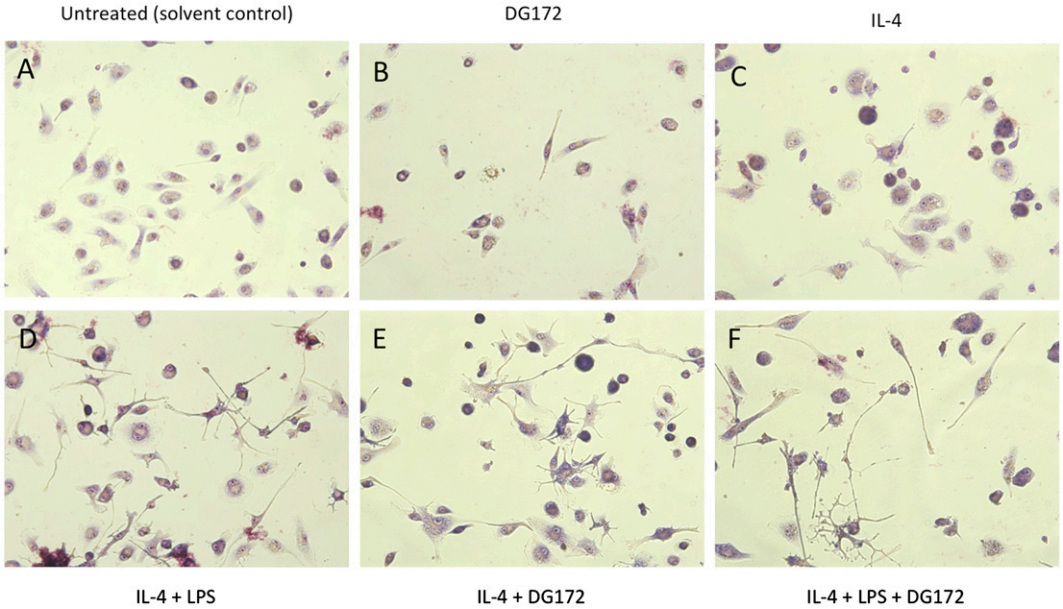

Fig. 1. Effect of DG172 on the morphology of BMCs differentiated in vitro. BMCs were differentiated for 9 days in the presence of GM-CSF. IL-4 (200 ng/ml) and/or DG172 (1 μM) were added as indicated. Loosely attached and floating cells were collected, cultured for another 3 days under the same conditions. In panels (E) and (F), LPS (100 ng/ml) was added for the last 2 days of culture.

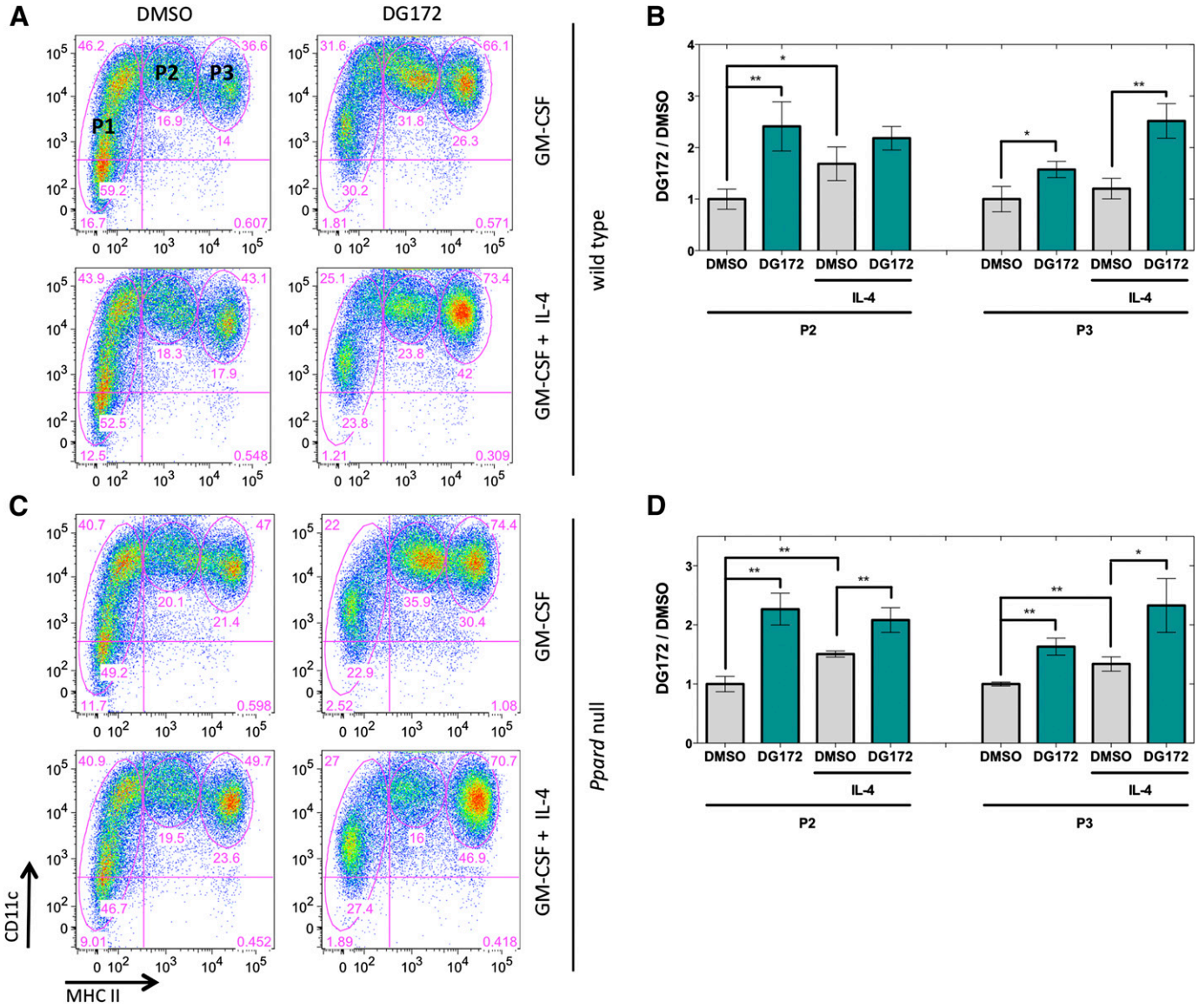

Fig. 2. Effect of DG172 on the DC surface markers CD11c and MHCII by differentiating BMCs. BMCs from WT (A, B) and *Ppard* null (C, D) mice were differentiated with GM-CSF ± IL-4 (1 ng/ml) in the absence or presence of DG172 for 6 days. Surface expression of CD11c and MHCII was determined by FACS in non-adherent cells. Three cell populations showing distinct expression patterns were identified (P1, P2, and P3) and the fractions of these cells relative to the total population are indicated (%). Panels (A) and (C) show representative experiments and panels (B) and (D) show the data from three independent experiments (average ± S.D.). $^{*}P < 0.05$; $^{**}P < 0.01$ by t test.

details for (*Z*)-2-(2-chlorophenyl)-3-[4-(4-methylpiperazine-1-yl)phenyl] acrylonitrile and (*Z*)-2-(4-chlorophenyl)-3-[4-(4-methylpiperazine-1-yl) phenyl]acrylonitrile (DG228) are described in the Supplemental Material.

Mice. C57Bl6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). *Ppard* null (epiblast-specific disruption of *Ppard*) and WT mice were generated by crossing floxed *Ppard* mice (Barak et al., 2002) and Sox2-Cre mice (Hayashi et al., 2002) as described (Scholtysek et al., 2013). The floxed *Ppard* mice were kindly provided by Dr. R. Evans (Salk Institute, La Jolla, CA). Sox2-Cre mice were obtained from Jackson Laboratory. Genotyping was performed with the following primers: *Ppard* intron 3 (forward: GGC TGG GTC ACA AGA GCT ATT GTC TC); *Ppard* exon 4 (forward: GGC GTG GGG ATT TGC CTG CTT CA); *Ppard* intron 4 (reverse: GAG CCG CCT CTC GCC ATC CTT TCA G; fragment sizes: *Ppard* WT: 360 bp; *Ppard* floxed: 400 bp; and *Ppard* ko: 240 bp); and *Cre* (forward: CCT GGA AAA TGC TTC TGT CCG; reverse: CAG GGT GTT ATA AGC AAT CCC; fragment size: 390 bp).

Fluorescence-Activated Cell Sorting (FACS) Analyses. Cells were washed with phosphate-buffered saline incubated with 10 μg/ml TruStain fcX (BioLegend, San Diego, CA) for 10 minute at 4°C to block unspecific Fc-binding, and subsequently stained with the following antibodies for 30 minute at 4°C: fluorescein isothiocyanate (FITC)-labeled anti-mouse CD14 (Sa14-2), APC/Cy7-labeled anti-mouse F4/80 (BM8), allophycocyanin (APC)-labeled anti-mouse MHCII (I-A/I-E) (M5/114.15.2), Pe-Cy7-labeled anti-mouse CD11c (N418), Pacific blue-labeled anti-mouse Ly-6G (1A8), PerCP-Cy5.5-labeled anti-mouse CD14 (Sa2-8), PE-labeled anti-mouse F4/80 (BM8) (BioLegend), and FITC-labeled anti-mouse Ly-6B.2 (7/4) (Biozol, Eching, Germany). Isotype control antibodies were as follows: FITC-labeled rat IgG2α,κ, APC/Cy7-labeled rat IgG2β,κ, APC-labeled rat IgG2β,κ, PeCy7-labeled Hamster IgG, Pacific blue-labeled rat IgG2α,κ, PerCP-Cy5.5-labeled rat IgGα,κ, PE-labeled rat IgG2β,κ, and FITC-labeled rat IgG2α (BioLegend). Cells were analyzed using a FACSCanto flow cytometer and FlowJo 9.5.1 software (BD Biosciences, Heidelberg, Germany). Data were plotted using biexponential transformation.

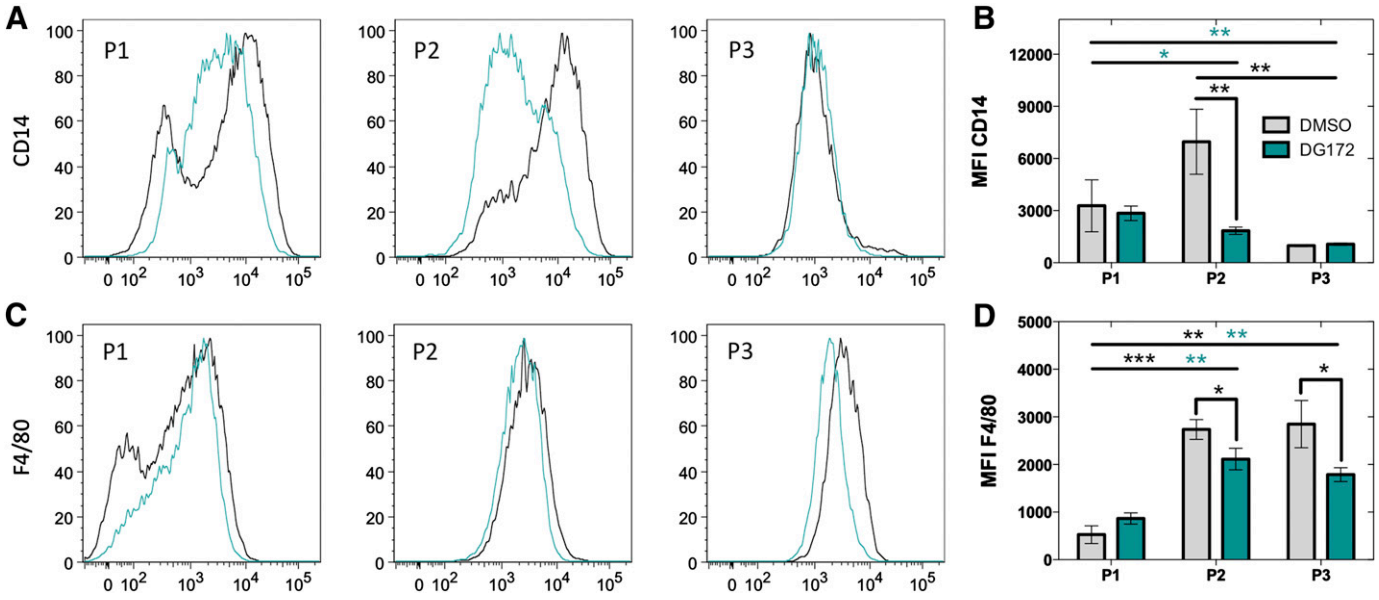

Fig. 3. CD14 and F4/80 levels on subpopulations of differentiating BMCs differing in dendritic surface marker expression. Surface expression of CD14 (A, B) and F4/80 (C, D) on differentiating non-adherent BMCs (GM-CSF) from *Ppard* null mice was determined by FACS and gated to the P1, P2, and P3 populations defined in Fig. 2. The data are presented as histograms of CD14 and F4/80 surface expression levels. Numbers represent MFI values. Panels (A) and (C) show representative experiments and panels (B) and (D) show the data from three independent experiments (average ± S.D.). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by *t* test.

Immunoblotting of S100A8. Cells were lysed in 60 mM TrisHCl, pH 7.5, 30 mM NaCl, 0.1 mM EGTA, 1% Triton X-100, and a Roche protease inhibitor mix. Cell lysates were subjected to SDS-PAGE on 20% gels, and immunoblotting was performed with the Trans-Blot Turbo Transfer System (BioRad, München, Germany) using the optimized protocol for low-mol.-wt. proteins, a rat anti-mouse monoclonal antibody against S100a8/Mrp8 (Biozol), and a horseradish peroxidase–labeled second antibody (Cell Signaling Technology, Leiden, Netherlands). Bands were visualized by the ChemiDoc MP Imaging System and quantified using Image Laboratory 5.0 software (BioRad).

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). cDNA was synthesized from 0.1 to 1 μg of RNA using oligo(dT) and random primers and the iScript kit (Biorad, Germany). qPCR was performed in a Mx3000P real-time polymerase chain reaction system (Stratagene, La Jolla, CA) for 40 cycles at an annealing temperature of 60°C. Polymerase chain reactions were carried out using the Absolute QPCR SYBR Green Mix (Abgene, Hamburg, Germany) and a primer concentration of 0.2 μM following the manufacturer's instructions. *L27* was used as the normalizer. Comparative expression analyses were statistically analyzed by Student's *t* test (two-tailed, equal variance) and corrected for multiple hypothesis testing via the Bonferroni method. The RT-qPCR primer sequences are listed in Supplemental Table 1.

Microarrays. Mouse Agilent 4-plex Array 44K, design id 028005, was used for the analysis of the gene expression of the different samples in a reference-design assay as previously published (Kaddatz et al., 2010). Raw microarray data were normalized using the loess method implemented within the limma package of R/Bioconductor (Smyth, 2005). Probes were assigned to genes as described (Adhikary et al., 2011) using Ensembl Release 70 (http://jan2013.archive.ensembl.org/Mus_musculus/Info/Index). Probes were considered regulated if they had a minimum intensity value of 5 and a comparison specific change as specified in the Results. Raw and normalized microarray data from this publication have been submitted to the EBI ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) and assigned the identifier [accession: E-MTAB-2628vi]. All data are minimum information about a microarray experiment compliant.

Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) assay. Ligand binding was determined by TR-FRET in vitro using the Lanthascreen TR-FRET PPARβ/δ competitive binding assay (Life Technologies, Darmstadt, Germany) as described (Naruhn et al., 2011).

Results

DG172 Promotes the Differentiation of DCs from GM-CSF-Induced Mouse BMCs and Reduces Ly6b^{+}/Gr1^{+} Granulocytic Cells. After differentiation of BMCs for 9 days in the presence of GM-CSF, IL-4, and/or DG172, the loosely attached and floating cells were collected and cultured for another 3 days under the same conditions. Compared with cells with GM-CSF only (Fig. 1A), cells showed morphologic alterations upon co-treatment with DG172 (more spindle-shaped cells; Fig. 1B) or IL-4 (larger, rounded cells; Fig. 1C). Addition of LPS to the latter triggered a mature DC morphology (Fig. 1D), as described (Dearman et al., 2009). A very similar effect was observed when DG172 was used instead of LPS (Fig. 1E), while no further morphologic changes were seen when both DG172 and IL-4 were added (Fig. 1F). These observations suggested that DG172 synergizes with IL-4 to promote the differentiation into mature DC.

FACS analysis of DC surface markers CD11c and MHCII confirmed the morphologic observations. Fig. 2 shows three distinct populations: MHCII^{-}, CD11c^{hi}/MHCII^{lo}, and CD11c^{hi}/MHCII^{hi}, subsequently referred to as P1, P2, and P3, respectively (Fig. 2A). DG172 increased both P2 and P3. This effect was observed in both WT (Fig. 2, A and B) and *Ppard* null cultures (Fig. 2, C and D) and was therefore independent of PPARβ/δ. IL-4 at a low concentration of 1 ng/ml synergized with DG172 by further increasing P3 (Fig. 2D).

These data support the view that DG172 promotes DC differentiation, which was further investigated by additional FACS phenotyping using the myeloid surface markers CD14 and F4/80. As shown in Fig. 3, A and B, P3 cells exhibited lower mean fluorescence intensity (MFI) for CD14 than did P1

and P2 cells. In P2 cells the CD14 MFI level decreased further upon DG172 treatment, consistent with the promotion of their differentiation to DCs (Mahnke et al., 1997). In contrast, the MFI measured for F4/80 was higher on P2 and P3 compared with P1 cells, but was reduced by DG172 in both P2 and P3 (Fig. 3, C and D). Decreasing F4/80 surface expression has previously been reported for differentiating DCs (León et al., 2004). The P2 and P3 populations thus likely comprise $CD11c^{hi}/MHCII^{lo}$ immature and $CD11c^{hi}/MHCII^{hi}$ mature DCs, respectively. These are clearly distinguished from the P1 population, which is composed of $MHCII^{-}$ cells and presumably represent cells at an early stage of differentiation. The described effects were specific for GM-CSF-induced DC differentiation, since no DG172 effects were observed on differentiation to macrophages triggered by M-CSF (Supplemental Fig. 1).

To analyze the fate of granulocytic cells we determined the surface markers Ly6B and Gr1 (LY6C) in the same samples. FACS analysis identified three distinct subpopulations in cells treated with DG172 (days 1–6): $Ly6B^{-}Gr1^{-}$, $Ly6B^{+}Gr1^{-}$, and $Ly6B^{+}Gr1^{+}$, defined as populations A, B, and C (PA, PB, and PC) in Fig. 4A, with PC cells representing differentiated neutrophils. Gating for these subpopulations showed that only the double-negative PA cells were positive for CD11c and MHCII expression, which is in agreement with the conclusion that the P3 cells defined in Fig. 2 represent mature DCs.

Granulocytic cells decreased in GM-CSF-induced BMC cultures after 48 hours, as shown by the shrinking number of $Ly6b^{+}/Gr1^{+}$ cells (Fig. 4B, top) (Lee et al., 2013), an effect that was clearly enhanced by DG172 (Fig. 4B, bottom). These observations are consistent with the conclusion that DG172 promotes DC differentiation at the expense of granulocytes.

DG172-Induced Transcriptome Changes in GM-CSF-Induced Mouse BMCs. To gain further insight into the DG172-triggered alterations to BMC differentiation we performed microarray analyses of cells exposed to GM-CSF in the presence or absence of the ligand (5 days incubation; sample subsequently referred to as d1-6). To be able to identify PPARβ/δ-independent effects of DG172 in this system we included in this study the inverse PPARβ/δ agonist ST247 and the PPARβ/δ agonist GW501516. As shown by the Venn

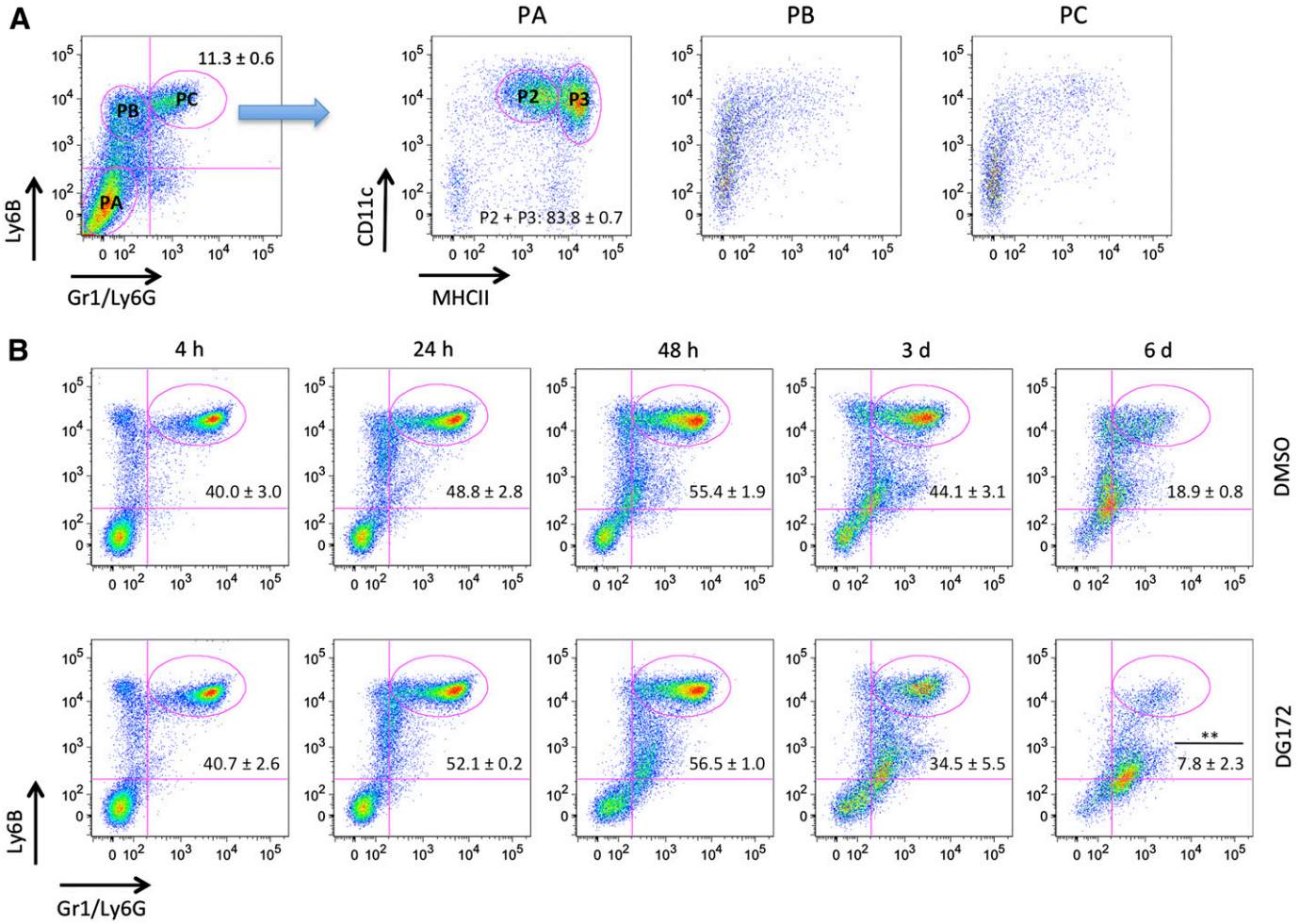

Fig. 4. Effect of DG172 on the granulocytic surface markers Ly6B and Gr1 (Ly6G) on differentiating BMCs. (A) CD11c and MHCII levels in relation to Ly6B and Gr1 surface expression. BMCs treated with GM-CSF and DG172 (days 1–6; combined adherent and floating cells) were gated for the PA, PB, and PC subpopulations defined in the left panel and analyzed for surface expression of CD11c and MHCII. (B) BMCs were exposed to in GM-CSF for 1 day, followed by cultivation in GM-CSF ± DG172 for the indicated times. Surface expression of Ly6B and Gr1 on non-adherent cells was determined by FACS. The dotted plots show the results of a representative experiment; numbers next to the PC area are the average of three independent experiments (± S.D.). $^{**}P < 0.01$ by t test between dimethylsulfoxide (DMSO)- and DG172-treated cells.

diagram in Fig. 5A, only a small fraction (n = 66; threshold twofold) of all DG172-repressed genes (n = 598) was also repressed by ST247, and an even smaller number (n = 19) was activated by GW501516. In addition, we compared the effect of DG172 on BMCs from both WT and *Ppard* null mice and found a substantial number of genes to be repressed by DG172 irrespective of the *Ppard* status (n = 227). An analogous situation was found with DG172-activated genes (n = 702; Fig. 5B). Of these genes, only a small fraction was also activated by ST247 (n = 40) or repressed by GW501516 (n = 31). Furthermore, a large fraction of genes (n = 162) was induced by DG172 in a PPARβ/δ-independent fashion. Supplemental Datasets 1A and 1B list all genes repressed or activated by DG172 in cells from *Ppard* null mice.

To gain further insights into the effect of DG172 on differentiation, we performed microarray analyses on BMC cultures that were exposed to DG172 for only 1 day, either from day 2 to 3 (d2-3) or from day 5 to 6 (d5-6), and then harvested for microarray analysis. The data in Fig. 5C and Supplemental Datasets 2 and 3 clearly suggest a stage-specific effect of DG172: while more than 20% (n = 108) of all genes (n = 511 in total in the d1-6 sample) were repressed in the d2-3 sample, only a marginal number of genes (n = 15) in the d5-6 sample coincided with those in the d1-6 sample (n = 389 in total).

Both the DG172-repressed and DG172-activated genes (d2-3 *Ppard* null BMCs) were functionally annotated according to functions and diseases using ingenuity pathway analysis (Fig. 5, D and E). The top categories according to the P values were inflammatory response, cellular movement, hematological system development and function, and immune cell trafficking, associated with the functions listed in Fig. 5, D and E. These data clearly connect the DG172-regulated genes to the observed effect on GM-CSF-induced BMC differentiation.

This conclusion is clearly supported when the regulated genes are analyzed according to their functions in cells of the two major myeloid lineages. The summary of microarray data in Fig. 6A shows a strong down-regulation of genes selectively expressed by neutrophils, such as *S100A8*, *S100A9*, and *Ltf* (lactoferrin); *Mpo* (myeloperoxidase); and *Hp* (haptoglobin). In contrast, genes characteristic of the antigen-presenting cells were up-regulated, including five different H2 MHC genes, *CD80* (B7-1), *CD86* (B7-2), and *CD209* (DC-SIGN). These

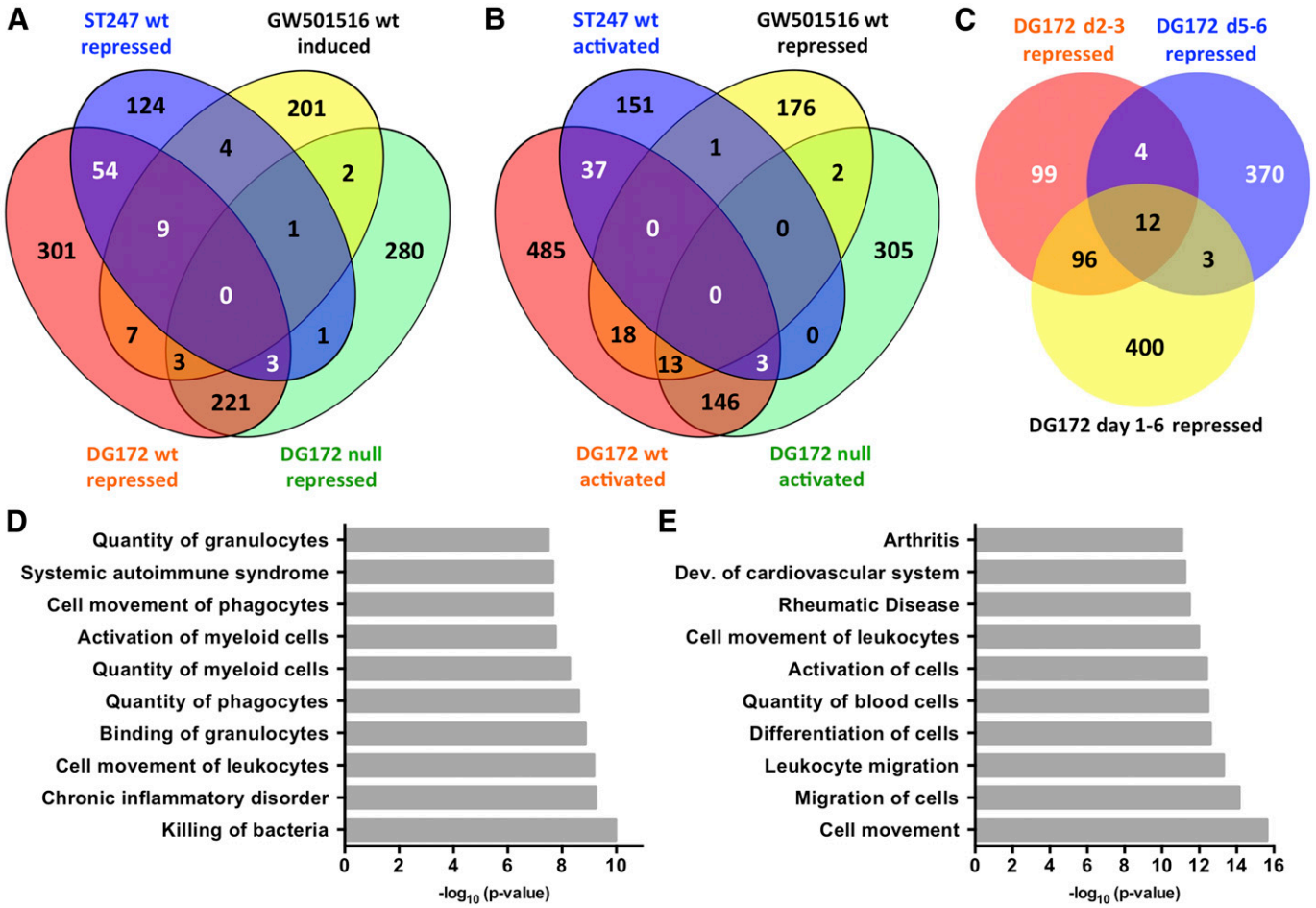

Fig. 5. Effect of PPARβ/δ ligands on the transcriptome of GM-CSF differentiated BMCs from WT and *Ppard* null mice. (A) BMCs from WT and *Ppard* null mice were differentiated with GM-CSF for 6 days in the presence of the agonist GW501516 or the inverse agonists DG172 or ST247. The Venn diagram shows the overlap of genes induced by GW501516 and repressed by DG172 or ST247. (B) Venn diagram as in panel (A), except that the directions of regulation are opposite. (C) Venn diagram showing the stage-specific effect of DG172. BMCs from *Ppard* null mice were differentiated with GM-CSF for 3 days (top left) or 6 days (top right, bottom) in the presence of DG172 from day 2 to 3, day 5 to 6 or during the entire culture period (days 1–6). (D) Annotation of DG172-repressed genes (days 2–3; ≥ twofold) according to functions and diseases using ingenuity pathway analysis. (E) Annotation of DG172-acivated genes (days 2–3; ≥ 1.5-fold).

results were confirmed by RT-qPCR in all instances tested (Fig. 6B). We also detected a strong PPARβ/δ-independent DG172-mediated repression of S100A8 protein expression by immunoblot analysis ($\geq$ 10-fold; Fig. 6C).

Myeloid differentiation is governed by several key transcription factors (Rosenbauer and Tenen, 2007). Therefore, we analyzed the regulation of the corresponding genes by DG172 in our experimental system. While *Klf5*, *Gfi1*, and *Cebpe*, which are selective for the granulocytic lineage, were down-regulated in both d0-6 and d2-3 DG172-exposed BMC cultures, the macrophage/DC-associated genes *Spib*, *Spic*, *Irf4*, and *Irf8* were up-regulated (Fig. 7A). The fact that these genes were not regulated in d5-6 cells indicates that this DG172 effect is restricted to an early stage of differentiation. Several of these transcription factors indeed represent master switches for lineage determination (see Fig. 7B). The microarray data could also be confirmed by RT-qPCR (Fig. 7C). Thus, these results strongly confirm the conclusion that DG172 switches the GM-CSF-induced differentiation of BMCs in favor of APCs.

DG172 Acts at a Specific Stage of GM-CSF-Induced Differentiation. We next sought to identify the critical stage of differentiation affected by DG172. The expression data in Figs. 5 and 7 strongly suggested that the effect of DG172 on BMC differentiation is restricted to an early stage around day 2. FACS analyses of CD11c and MHCII on day 6 BMCs exposed to DG172 at different times after initiating GM-CSF-induced differentiation confirmed this conclusion. As shown in Fig. 8, the DG172-induced increase in CD11c^{hi}MHCII^{hi} cells was observed only, when DG was added prior to day 4. In the same experimental setting, a clearly stage-dependent effect was also seen on the repression of granulocytic marker genes *S100a8*, *S100a9*, and *Mmp9* (Fig. 9A, top).

Differentiation of BMCs with M-CSF to macrophages had no significant effect on *S100a8*, although the canonical PPARβ/δ target gene *Adrp* was strongly repressed (Fig. 9B). Consistent with this result, no DG172 effect on *S100a8* was observed with primary macrophages obtained from either WT and *Ppard* null mice (Fig. 9C, left panel) in the presence of a strong repression of the PPARβ/δ target gene *Angptl4* (Fig. 9C, right panel). Likewise, *Angptl4*, but not *S100a8*, was repressed by DG172 in NIH3T3 fibroblasts (Fig. 9C, right-hand-side bars). Taken together these results clearly demonstrate that the PPARβ/δ-independent effect of DG172 is both cell type and differentiation stage specific.

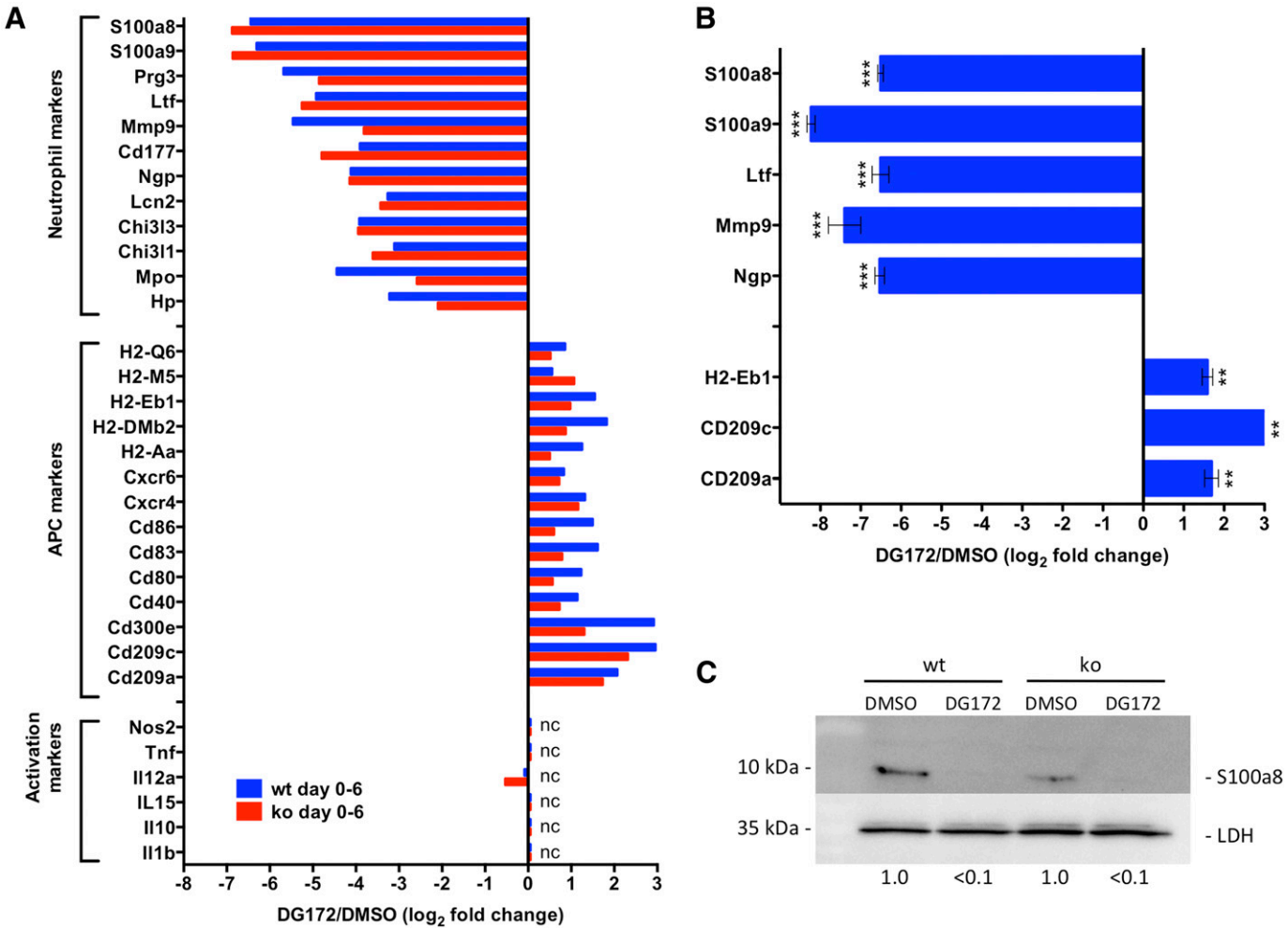

Fig. 6. Effect of DG172 on specific target genes in GM-CSF-treated BMCs. (A) Summary bar plot of microarray data for neutrophil markers, APC markers, and activation markers (nc, no change). (B) RT-qPCR validation for individual genes. Values are the average of triplicates; error bars represent the S.D. $^{*}P < 0.05$ by t test relative to solvent control; $^{**}P < 0.01$; $^{***}P < 0.001$. (C) Validation of S100A8 protein down-regulation by DG172. Quantitation of the data is shown below the immunoblot (normalized to 1.0 for untreated WT or null cells).

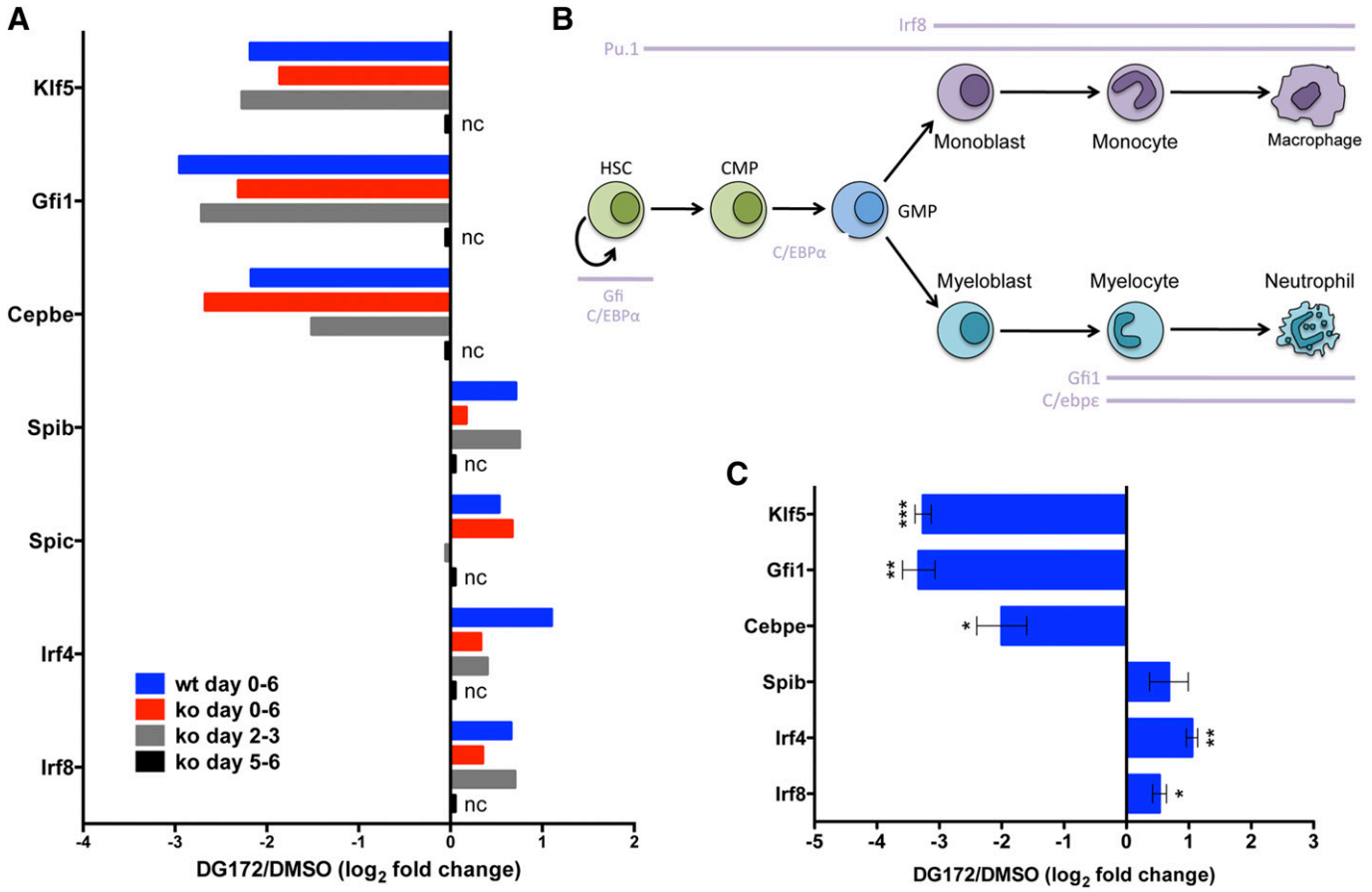

Fig. 7. Effect of DG172 on genes encoding myeloid transcription factors in GM-CSF-treated BMCs. (A) Summary bar plot of microarray data (nc, no change). (B) Schematic representation of the role of transcription factors in myeloid differentiation pathways. (C) RT-qPCR validation for individual genes in d2-3 cells. Values are the average of triplicates; error bars represent the S.D. *$P < 0.05$ by t test relative to solvent control; **$P < 0.01$; ***$P < 0.001$.

Differential Effects of Structural Derivatives of DG172 on DC Differentiation and PPARβ/δ Binding. Finally, we investigated whether the PPARβ/δ-dependent and PPARβ/δ-independent effects of DG172 could be associated with specific structurally features, and might thus be potentially separable. To address this issue we synthesized six derivatives of DG172 (highlighted in blue in Fig. 10) and analyzed the potential of these compounds (1) to promote GM-CSF-induced BMC

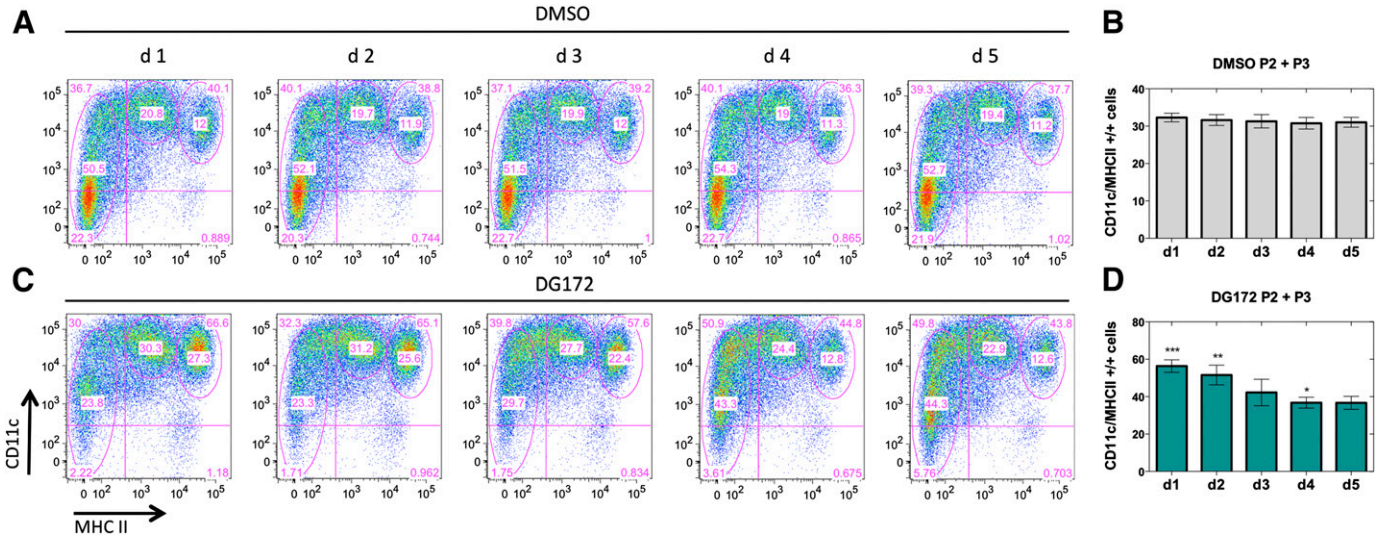

Fig. 8. Stage-dependent effect of DG172 on DC surface markers on differentiating BMCs. BMCs from WT mice were differentiated with GM-CSF for 6 days and solvent [dimethylsulfoxide (DMSO); panels (A) and (B)] or DG172 [panels (C) and (D)] was added at the indicated times after initiating GM-CSF treatment. Surface expression of CD11c and MHCII on non-adherent cells was determined by FACS. Subpopulations were defined as in Fig. 2. Panels (A) and (C) show representative experiments and panels (B) and (D) show the data from three independent experiments (average ± S.D.). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by t test between DMSO- and DG172-treated cells.

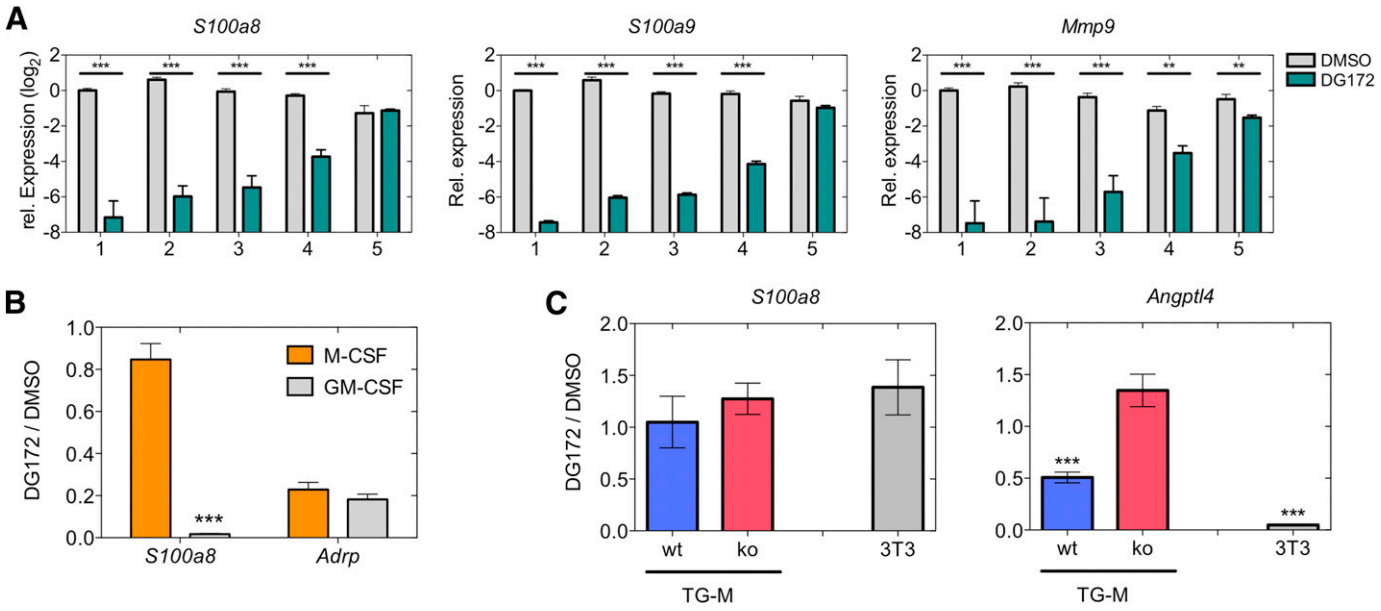

Fig. 9. Stage- and cell-type-specific effects of DG172 on transcription of myeloid marker genes but not on PPARβ/δ target genes. (A) BMCs from WT mice were differentiated with GM-CSF for 6 days and DG172 was added at the indicated times after initiating GM-CSF treatment. Expression of the granulocytic marker genes *S100a8*, *S100a9*, and *Mmp9* was determined by RT-qPCR and normalized to *L27* [relative expression = 1.0 for dimethylsulfoxide (DMSO) on day 1]. (B) Repression of *S100a8* expression in BMCs differentiated with GM-CSF, but not after M-CSF-induced differentiation to macrophages, while the direct PPARβ/δ target gene *Adrp* is repressed in both conditions. (C) Repression of *Angptl4*, but not *S100a8*, in thioglycollate-elicited peritoneal macrophages from WT or *Ppard* null mice and in NIH3T3 fibroblasts. Data in (B) and (C) are represented as the ratio of expression in DG172- and DMSO-treated cells (average of triplicate). Error bars indicate the standard deviation. $^{**}P < 0.01$; $^{***}P < 0.001$ by *t* test.

differentiation (FACS analysis of CD11c and MHCII expression) and (2) to interact with the PPARβ/δ ligand binding domain in vitro (competitive TR-FRET). The data in Fig. 10 indicate that the *N*-methylpiperazine residue is required for the enhanced differentiation into P2 and P3 cells, since a significant effect was observed only with those three compounds carrying this moiety, i.e., DG132, DG172, and DG195 [(*Z*)-2-(2-chlorophenyl)-3-[4-(4-methylpiperazine-1-yl)phenyl] acrylonitrile]. In contrast, PPARβ/δ binding was affected to a considerably lesser extent, as long as a halogen atom was introduced in the ortho, i.e., DG138 and DG195, or meta (DG208) position of the opposing phenyl substituent. Consequently, DG139, bearing a para chloro and a *N*-dimethylamino substituent, not only lacked the effect on BMC differentiation, but also failed to interact with PPARβ/δ. These results suggested that a separation of the two activities exerted by DG172 is possible. Therefore, we replaced the *N*-diemthylamino moiety in DG139 with *N*-methylpiperazine residue yielding DG228 (red in Fig. 10). In agreement with our prediction, DG228 had a strong effect on BMC differentiation, but only weakly bound to PPARβ/δ (IC50 $>$ 10 μM compared with the parent compound 27 nM for DG172). Collectively, these data indicate that the *N*-methylpiperazine residue is essential for the PPARβ/δ-independent effect of DG172 on BMC differentiation, while the position of the halogen atom in the phenyl substituent is crucial for PPARβ/δ binding.

Discussion

Our FACS data show that DG172 strongly augments CD11c^{hi}MHCII^{hi} cells in GM-CSF-induced BMC cultures, in particular in the presence of IL-4 (population P3; Fig. 2). Furthermore, exposure of GM-CSF/IL-4-treated cultures to DG172 induced tightly adherent cells displaying the typical morphology of mature DCs (Fig. 1). DG172 also induced a second population in GM-CSF-induced BMC cultures, which we characterized as CD11c^{hi}MHCII^{lo} cells (population P2; Figs. 2 and 3). MHCII^{lo} cells have previously been described in early GM-CSF-induced bone marrow cultures (Masurier et al., 1999). It is likely that this population comprises immature DCs, as suggested by their apparent conversion to P3 cells by IL-4 (Fig. 2). This may also explain the synergistic action of the two mediators: DG172 promotes the differentiation from P1 to P2 cells, and from P2 to P3 cells, with the latter further promoted by IL-4. However, adherent cells represent a substantial fraction of the P2 population (data not shown), suggesting that these are macrophages rather than undifferentiated DCs. Therefore, it is likely that P2 cells present a mixed population of committed monocytic cells with the potential to differentiate to DCs, as previously suggested by others (Masurier et al., 1999).

DG172 treatment also led to a reduction of granulocytic cells in GM-CSF-induced BMC cultures (Figs. 4B and 6), indicating that DG172 induces a lineage switch by favoring the DC lineage at the expense of granulocytic differentiation. In contrast to macrophages and DCs, neutrophils are present in freshly isolated BMCs (see the early time points in Fig. 4B) and are partly replaced by BMCs differentiating along the granulocytic lineage after a few days of culture (Inaba et al., 1992). This replenishment by new granulocytes is apparently prevented by DG172, which can be explained by at least two different models. DG172 either pushes cells around the stage of the granulocyte macrophage progenitor into the monocytic lineage, thereby depleting the progenitor pool for granulocytic differentiation, or alternatively, DG172 actively blocks differentiation to granulocytes, thus favoring monocytic/DC differentiation. Because DC differentiation is promoted by pro-inflammatory stimuli (Dearman et al., 2009), it is important to note that we did not observe any effect on the expression of genes

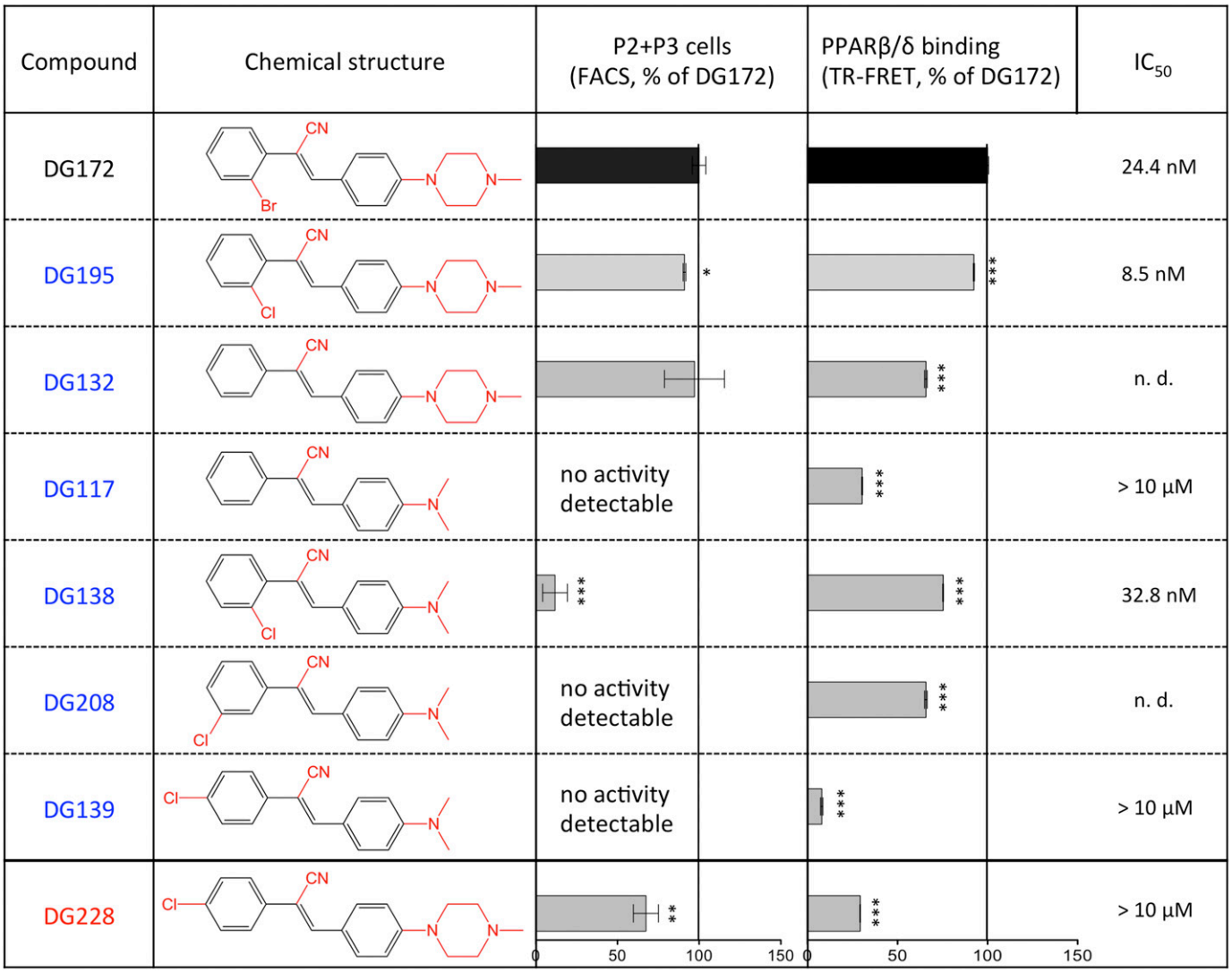

Fig. 10. Differential effects of structural alterations to DG172 on GM-CSF-induced BMC differentiation and PPARβ/δ binding. The DG172 derivatives indicated on the left were tested for their effects to promote BMC differentiation to P2 and P3 cells ($CD11c^{hi}MHCII^{lo}$ and $CD11c^{hi}MHCII^{hi}$ cells) and interaction with the PPARβ/δ ligand binding domain in vitro (competitive TR-FRET). All values represented by bars were calculated relative to the effect of DG172 [DG172 value/dimethylsulfoxide (DMSO) value normalized to 100%] at a concentration of 1 μM for all compounds. IC_{50} values were determined by titration over a range of 0.1 nM–10 μM (competitive TR-FRET) as previously described (Lieber et al., 2012) (n.d., not determined). Data represent the average of triplicates. Error bars indicate the S.D. $^{*}P < 0.05$ by t test; $^{**}P < 0.01$; $^{***}P < 0.001$ relative to DG172. DG117: (Z)-3-(4-(dimethylamino)phenyl)-2-phenylacrylonitrile; DG132: (Z)-3-(4-(4-methylpiperazin-1-yl)phenyl)-2-phenylacrylonitrile; DG138: (Z)-2-(2-chlorophenyl)-3-(4-(dimethylamino)phenyl)acrylonitrile; DG139: (Z)-2-(4-chlorophenyl)-3-(4-(dimethylamino)phenyl)acrylonitrile; DG195, (*Z*)-2-(2-chlorophenyl)-3-[4-(4-methylpiperazine-1-yl)phenyl]acrylonitrile; DG208: (Z)-2-(3-chlorophenyl)-3-(4-(dimethylamino)phenyl)acrylonitrile; DG228: (Z)-2-(4-chlorophenyl)-3-(4-(4-methylpiperazin-1-yl)phenyl)acrylonitrile.

encoding pro-inflammatory cytokines, including *Tnf* and *Il1b* (Fig. 6A).

DG172 acts at a relatively early stage of differentiation, i.e., during the first 2 days of exposing BMCs to GM-CSF, as shown by its effect on the expression of genes coding for myeloid transcriptional regulators (Fig. 7) and the DC surface markers CD11c and MHCII (Fig. 8). While the granulocytic transcription factor genes *Cepbe*, *Gfi1*, and *Klf5* were down-regulated by DG172 on day 2, factors associated with the macrophage/DC lineage, such as *Spib*, *Spic*, *Irf4*, and *Irf8*, were up-regulated (Yamanaka et al., 1997; Schotte et al., 2004; Tamura et al., 2005;Rosenbauer and Tenen, 2007; Halene et al., 2010; Diakiw et al., 2012). Several of these transcriptional regulators have lineage determining functions. For instance, Gfi1 is not only indispensable for granulocyte differentiation (Hock et al., 2003), but also inhibits macrophage differentiation by repressing the activity of Spi1 (Pu.1) (Dahl et al., 2007); vice versa, high levels of Spi1 inhibit the transcription of *Gfi1* by inducing the repressors Egr2 and Nab2, thereby blocking neutrophil differentiation (Laslo et al., 2006). RT-qPCR showed only a weak DG172 effect on *Spi1* (data not shown; *Spi1* is not represented in the microarray). However, the partial redundancy of Spi subtypes suggests that SpiB and SpiC have similarly crucial functions in myeloid differentiation (Garrett-Sinha et al., 2001).

Other examples are C/EBPε, whose different isoforms are endowed with the ability to specifically reprogram myeoid lineage commitment (Bedi et al., 2009; Halene et al., 2010) and Irf8, which extinguishes neutrophil production and promotes DC lineage commitment (Becker et al., 2012). These and other studies have clearly shown that hematopoietic cell fate is dependent on several key transcription factors, and

that the dosage of each of these factors and their expression relative to each other plays a pivotal role (Mak et al., 2011). The fact that DG172 influences the expression of these transcription factors is consistent with its presumed action at an early stage of GM-CSF-induced differentiation, perhaps around the stage of the granulocyte macrophage progenitor, which would also provide a likely explanation for its profound effect on myeloid lineage determination. Ingenuity upstream regulator analysis (Supplemental Fig. 2) identified serum response factor (SRF) and its coactivators MKL1 and MKL2 and the transcription factors C/EBPα and C/EBPε as the most significantly affected pathways in DG172-treated cells (d2-3). Although the latter finding is consistent with our data showing a strong repression of the *Cebpe* gene by DG172, a potential involvement of SRF is difficult to judge at present, since this transcription factor has previously not been associated with myeloid differentiation.

An important issue is the open question of which protein is targeted by DG172 to achieve its effect on BMC differentiation. Since DG172 is a stilbene and thus bears some structural resemblance to tamoxifen, we tested its binding to the estrogen receptor in vitro using a competitive TR-FRET assay, but were unable to detect any interaction (data not shown). Likewise, no binding was measurable to PPARα, PPARγ, and RARα in analogous assays. We also questioned whether DG172 might be a ligand for AhR because the structurally similar stilbene 4-hydroxytamoxifen can induce AhR target genes (DuSell et al., 2010), and AhR is required for DC differentiation in mice (Nguyen et al., 2010; Vogel et al., 2013). However, we did not see any agonistic or antagonistic effect of DG172 on *Cyp1a1*, one of the major AhR target genes, in cells with functional AhR signaling (data not shown). Furthermore, AhR ligands are known to regulate AhR target genes in differentiated macrophages (Bessede et al., 2014), which does not apply to DG172 (Fig. 9).

The nuclear receptor Nur77 (Nr4a1) plays an essential role in myeloid differentiation in mice (Hanna et al., 2011), and its target genes overlap with those identified in our microarray analyses (Figs. 5–7). However, DG172 had no detectable effect on Nur77 target genes in cell types other than GM-CSF-induced BMCs, although these genes responded to the Nur77 ligand DIM-C-pPhOCH(3) (Cho et al., 2008) (data not shown). We also tested the possibility that DG172 is an antagonist of Nur77 by applying DG172 together with DIM-C-pPhOCH(3), but could not detect any effect. Therefore, it is unlikely that Nur77 is a target of DG172.

It is also possible that the PPARβ/δ-independent function of DG172 is not mediated by a nuclear receptor, as has been reported for the regulation of AMPK by PPAR ligands (Lee and Kim, 2010). A systematic approach to identify the relevant DG172 target(s) will require a cellular system that is amenable to genome-wide RNA interference experiments or the biochemical purification of drug-protein complexes, which due to the highly selective nature of DG172's effect on myeloid differentiation is currently not available.

Since DG172 is orally bioavailable we also tested its potential effect in mice, but were unable to detect any alterations to the composition of the bone marrow by FACS analysis using the same markers as in Figs. 2–4 (data not shown). Therefore, it is possible that the effects seen in BMC cultures occur in vivo only in specific conditions, e.g., certain disease-associated processes. The use of DG172 in mouse models of inflammation, infection, or cancer may shed some light on this question in the future. Notwithstanding these open questions pertaining to its effects in vivo, DG172 (or its novel more selective derivative DG228; Fig. 10) may also prove useful to improve the generation of DCs from human BMCs or monocytes; for instance, for therapeutic applications.

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Authorship Contributions

Participated in research design: Diederich, Brendel, Müller-Brüsselbach, Müller.

Conducted experiments: Lieber, Scheer, Meissner, Giehl.

Contributed new reagents or analytic tools: Diederich, Brendel.

Performed data analysis: Finkernagel, Müller-Brüsselbach, Müller.

Wrote or contributed to the writing of the manuscript: Lieber, Müller-Brüsselbach, Müller.

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