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# The glucocorticoid dexamethasone programs human dendritic cells for enhanced phagocytosis of apoptotic neutrophils and inflammatory response

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## ABSTRACT

**GCs are powerful anti-inflammatory compounds inhibiting inflammatory cell recruitment and production of proinflammatory cytokines. We have recently found that DCs, the key players of T cell priming and polarization, respond to allogeneic apoptotic neutrophils with proinflammatory cytokine release and Th1 cell activation. Here, we show that monocyte-derived human DCs develop their capacity to engulf apoptotic cells by up-regulating a set of apoptophagocytic genes. This gene expression pattern was reprogrammed when differentiation took place in the presence of the synthetic GC Dex, which increased the expression of phagocytosis receptors MERTK and CD14, the bridging molecule C1QA, DNASE2, and ADORA3. The increased phagocytosis was attenuated by the addition of ADORA3 antagonist and could not be observed when bone marrow-derived DCs of ADORA3 KO mice were treated with Dex. The GC-treated human DCs loaded with allogeneic apoptotic neutrophils secreted, in response to LPS and IFN-γ, the inflammatory cytokine TNF-α. Furthermore, the Dex-treated DCs could activate autologous T lymphocytes toward Th1 effector cells, and this was enhanced by their exposure to allogeneic apoptotic neutrophils.**

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## Introduction

Continuous clearance of apoptotic cells is crucial in maintaining the homeostasis of a living organism. Molecular defects in the uptake mechanism of apoptotic bodies lead to altered immune tolerance and autoimmune diseases in mammalian organisms [1, 2]. As part of an inflammatory response, neutrophil granulocytes recruited to the site of infection or injury engulf and kill efficiently the pathogens and damaged cells and then undergo spontaneous apoptosis to prevent the uncontrolled release of toxic cell content [3, 4], and like other apoptotic cells, they release "find-me" and express "eat-me" signals to their environment [5]. Responding to these signals, nonprofessional and professional phagocytes are alarmed to remove dying cells by phagocytosis.

DCs are professional phagocytes and the most potent APCs found all over the body in lymphoid and nonlymphoid organs [6], specialized for the capture, processing, and presentation of antigens to T cells through their MHC molecules [7, 8]. The outcome of DC-mediated immune response (stimulation or tolerance) depends on the type of the pathogens or other antigens, the costimulatory molecules, and cytokines that instruct the polarization of T cells [9–12]. As a result of their fundamental role in initiation of T cell-mediated immunity, DCs are the focus of current immunotherapeutic research [13]. Human DCs can be generated in vitro from $CD34^+$ progenitors in the presence of GM-CSF and TNF-α [14]. DCs with the characteristics of iDCs can also be differentiated from

Abbreviations: ADORA3=adenosine A3 receptor, C1QA=complement component 1, q subcomponent, A chain, C2=complement component 2, CMTMR=5-(and-6)-{[(4- chloromethyl)benzoyl]amino} tetramethylrhodamine, $C_t$=comparative threshold, Dex=dexamethasone, DOCK1=dedicator of cytokinesis 1, FC=fold change, FCGR2B=Fc fragment of IgG, low-affinity IIb, receptor (CD32), GAS6=growth arrest-specific 6, GC=glucocorticoid, iDC=immature DC, IRF=IFN regulatory factor, ITGB=integrin β, KO=knockout, MERTK=c-mer proto-oncogene tyrosine kinase, MFGE8=milk fat globule-EGF factor 8 protein, MRS1220=9-chloro-2-(2-furanyl)-5-([phenylacetyl]amino)(1,2,4)-triazolo[1,5-*c*]quinazoline, NLRP12=nucleotide-binding domain, leucine-rich repeat family, pyrin domain-containing 12, OLR1=oxidized low-density lipoprotein (lectin-like) receptor 1, PPARG=peroxisome proliferator-activated receptor γ, PROS1=protein S (α), SCARB1=scavenger receptor class B, member 1, SE=succinimidyl ester, TGM2=transglutaminase 2, TLDA=Taqman low-density array

*The online version of this paper, found at www.jleukbio.org, includes supplemental information.*

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blood monocytes using GM-CSF and IL-4 [15–17] or IL-13 [18–20]. iDCs are able to internalize exogenous antigens by fluid-phase uptake through macropinocytosis and receptor-mediated endocytosis [21, 22]. Apoptotic cells are taken up by DCs through phagocytosis using the $\alpha_v\beta_5$, the scavenger receptor CD36, and the MFGE8 receptor [23, 24]. The internalized apoptotic cell-associated antigens are selectively presented to $CD4^+$ Th cells or to $CD8^+$ effector T cells, a process known as cross-presentation. The concomitant pro- or anti-inflammatory stimuli [25, 26] and the site of antigen encounter [27, 28] determine the extent and direction of DC-induced T cell stimulation. According to our recent results, human monocyte-derived DCs, in contrast to macrophages, can respond to allogeneic apoptotic neutrophils with a robust, inflammatory response and T lymphocyte activation [29].

GCs are widely used immunosuppressive and anti-inflammatory agents to treat autoimmune, inflammatory, and allergic diseases and to prevent allograft rejection after transplantation [30–32]. GCs are small lipophilic compounds exerting their effect by binding to the intracellular GC receptor, which afterwards, translocates to the nucleus and regulates the transcription of target genes directly or indirectly [33] or acting in a DNA-binding independent way through direct protein–protein interactions [34]. The therapeutic effects of the GCs were initially attributed to their inhibitory effect on T cell immunity [35]. Several studies, however, have demonstrated that they have impact on other immune cells as well. In human monocytes, GCs induce an anti-inflammatory phenotype and augment phagocytosis of apoptotic cells [36, 37]. In DCs, GCs were reported to induce a tolerogenic phenotype and suppress their activation [38, 39]. The differentiation and maturation of DCs were also influenced, and the mannose receptor-mediated endocytosis was up-regulated by GCs [40–42]. However, the effect of GCs on the phagocytosis of apoptotic cells by DCs has not been investigated. Furthermore, knowing from our previous results [29] that long-term interaction of apoptotic neutrophils with DCs renders them prone to proinflammatory cytokine responses and based on reports in the literature that GCs induce a tolerogenic phenotype of DCs, we found it important to investigate whether Dex can suppress the proinflammatory effect of apoptotic neutrophils. According to the presented results, Dex increased the capacity of iDCs to engulf apoptotic cells through up-regulation of several apoptophagocytic genes. Surprisingly, this resulted in an increased inflammatory cytokine production and activation of autologous T cells.

## MATERIALS AND METHODS

### Cell culture and reagents

Human PBMCs were isolated from "buffy coats" of healthy blood donors by density gradient centrifugation through Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden), as described previously [43]. To generate iDCs, monocytes were plated into six-well culture dishes at a density of $2 \times 10^6$ cells/ml and cultured for 2 or 5 days in AIM V medium (Invitrogen, Carlsbad, CA, USA) containing 800 U/ml GM-CSF and 500 U/ml IL-4 (PeproTech EC, London, UK). Medium was supplemented with IL-4 and GM-CSF at Days 0 and 3. For GC-treated samples, Dex (Sigma-Aldrich, St. Louis, MO, USA) was added to the cell culture medium from Day 0 of differentiation. Monocyte-to-DC differentiation was controlled by the phenotypic analysis of untreated and GC-treated cells using anti-CD209, anti-CD14, and anti-CD40 antibodies (all from BD PharMingen, San Diego, CA, USA).

Allogeneic neutrophils were isolated from peripheral blood by density gradient centrifugation using Histopaque 1119 and Histopaque 1077 (Sigma-Aldrich), and cells were cultured for 16 h in IMDM (Invitrogen), supplemented with 10% human AB serum (Sigma-Aldrich) while they underwent spontaneous apoptosis. Autologous lymphocytes (the leftover after magnetic separation of monocytes) were kept at –70°C in cell-freezing medium (FBS:DMSO, 9:1, all from Sigma-Aldrich).

### RNA preparation and TaqMan real-time RT-PCR

Total RNA was isolated from untreated and Dex-treated human monocytes and monocyte-derived DCs using the TRIzol reagent (Invitrogen). To determine the expression level of genes (see Supplemental Table 2), a custom-made 384-well TLDA (Applied Biosystems, Foster City, CA, USA) was used with two replicates/target gene and three biological parallels. 18S rRNA was used as an endogenous control, and gene-expression values were calculated based on the $\Delta\Delta C_t$ method. Relative expressions were determined using the equation, where relative quantity equals $2^{-\Delta\Delta Ct}$. FCs represent the ratio of relative gene expressions (average of three donors) of differentiated DCs and monocytes referred to as "Differentiation" or the ratio of Dex-treated and nontreated, referred to as "Differentiation and Dex."

### Human phagocytosis assays

DCs were stained with CellTracker Orange CMTMR, and the freshly isolated neutrophils were labeled with the yellow-green fluorescent cell tracer dye CFDA-SE. Both dies were purchased from Invitrogen, and stainings were done according to the manufacturer's protocol. Before the assay, the labeled apoptotic neutrophils were washed three times with PBS, and DCs were counted and replated in fresh medium. DCs and apoptotic neutrophils were cocultured for 8 h at a ratio of 1:5 at 37°C and 5% $CO_2$ atmosphere. In the case of Dex-treated cells, the GC was not present during the coincubation. Cells were collected by trypsinization, washed with PBS, and fixed with 1% PFA in PBS (Sigma-Aldrich), and the phagocytosis rate was determined by flow cytometry analysis (FACSCalibur, BD Biosciences, Immunocytometry Systems, San Jose, CA, USA), as percent phagocytic cells that have engulfed the CFDA-labeled apoptotic neutrophils (positive for CMTMR and CFDA).

For the blocking experiments, the replated DCs were preincubated with 10 $\mu$g/ml anti-MERTK (clone 125508, R&D Systems, Minneapolis, MN, USA) or anti CD-14 (Abcam, Cambridge, UK) antibodies for 15 min at 37°C and 5% $CO_2$ atmosphere. The antibodies were present during the phagocytosis assay as well. For testing the effect of ADORA3 antagonist on phagocytosis, DCs were treated with the selective ADORA3 antagonist MRS1220 (Tocris Bioscience, Ellisville, MO, USA) for a period of 1 h, 37°C, and 5% $CO_2$ atmosphere, and the antagonist was present throughout the DC-apoptotic neutrophil coincubation period.

### Cell surface labeling

DCs were blocked with 50% human AB serum (Sigma-Aldrich) for 30 min at 37°C and then washed with PBS-1% BSA and stained for 30 min on ice with anti-MERTK (clone 125518) or anti-CD14 and mouse IgG1 (isotype control from Sigma-Aldrich) mAb at a concentration of 10 $\mu$g/ml, followed by FITC-conjugated anti-mouse antibody (Sigma-Aldrich) used at a ratio of 1:50. Samples were washed with PBS-1% BSA and fixed with 1% PFA in PBS (Sigma-Aldrich), and then stained cells were detected by flow cytometry.

### Immunoblotting

Monocytes and DCs, differentiated in the presence or absence of Dex, were collected and washed with PBS, followed by their lysis in 50 mM Tris-HCl containing 0.1% Triton X-100, 1 mM EDTA, 15 mM 2-ME, and proteinase inhibitors. Insoluble cellular material was removed by centrifugation, and

the lysates were mixed with 5× Laemmli loading buffer and boiled for 10 min, and 15 μg protein of each sample was loaded onto a 10% SDS polyacrylamide gel. Proteins were transferred onto PVDF membranes, followed by blocking with 5% skimmed milk. Membranes were probed by anti-TGM2 mAb (4G3 hybridoma cells were a kind gift of Alexey M. Belkin, University of Maryland School of Medicine, Baltimore, MD, USA), followed by incubation with HRP-conjugated anti-mouse antibody for 1 h at room temperature. Immunoblots were developed with Immobilon Western chemiluminescent substrate (Millipore, Billerica, MA, USA).

### Determination of TNF-α secretion

Differentiated but not stained monocyte-derived DCs (controls and Dex-treated) were cocultured with unstained apoptotic neutrophils for 8 h and then stimulated with 0.1 μg/ml LPS and 10 ng/ml IFN-γ for an additional 16 h. The culture supernatants were harvested and stored for cytokine measurements. The concentrations of released TNF-α were measured by the human TNF-α duo set ELISA kit (R&D Systems), according to the manufacturer's specifications.

### Human IFN-γ ELISPOT assay

DCs, differentiated for 5 days in the presence or absence of a different concentration of Dex, were cocultured with nonlabeled, allogeneic apoptotic neutrophils for 8 h, and then autologous lymphocytes were added at a ratio of 1:25 for 5 days at 37°C in a 5% $CO_2$ atmosphere. Cells were collected on Day 5 and subjected to anti-human IFN-γ Ready-Set-Go ELISPOT assay (eBioscience, San Diego, CA, USA) on MultiScreen$_{HTS}$ PVDF plates (Millipore S.A., Molsheim, France). After 48 h at 37°C, the cells were removed, and the plates were washed with PBS. The cytokine spots were detected by biotinylated anti-IFN-γ antibody, followed by avidin-HRP-conjugated antibody (BD PharMingen). The reaction was stopped by washing with tap water, and the air-dried plates were analyzed by a computer-assisted ELISPOT image analyzer (Series 1 ImmunoSpot analyzer, Version 4.0 Software Academic, Cellular Technology, Shaker Heights, OH, USA). Secreted IFN-γ was measured by the OptiEIA system (BD PharMingen).

### Animal experiments

**Animals.** Experiments were performed according to local ethical guidelines and approved by the Animal Experimental Committee of the University of Debrecen (Hungary): 8- to 12-week-old C57BL/6J and ADORA3 KO (provided by Merck and Co., Rahway, NJ, USA) mice were kept in specific pathogen-free conditions and were killed after isoflurane narcosis by cervical dislocation.

**Isolation and differentiation of mouse bone marrow-derived DCs.** Bone marrow cells were isolated from the femur of mice using a 26G needle and saline. We washed the bone marrow in saline and then resuspended and cultured the cells in 25 mM HEPES-modified RPMI-1640 medium (Sigma-Aldrich), supplemented with 10% FBS, 2 mM glutamine, and penicillin-streptomycin. Bone marrow cells were plated in six-well culture plates ($1.5\times10^6$ cells/ml) and were differentiated to DCs by GM-CSF (20 ng/ml) and IL-4 (20 ng/ml; PeproTech, Rocky Hill, NJ, USA) for 9 days. Every 3rd day, one-half of the old medium was changed to fresh medium containing cytokines, and the cells were treated with Dex from Day 0.

**Isolation of neutrophil granulocytes and phagocytosis assay.** Neutrophil granulocytes were isolated by density gradient centrifugation from mouse bone marrow (C57BL/6J) using Histopaque 1119 and Histopaque 1077 (Sigma-Aldrich). To generate apoptotic neutrophils, the cells were cultured for 24 h in IMDM, supplemented with 10% mouse serum, and stained with CFDA-SE (Invitrogen). The purity of the neutrophils was controlled by May-Grünwald/Giemsa staining (Sigma-Aldrich). On the 9th day of differentiation, mouse DCs were counted, stained with CMTMR (Invitrogen), replated in serum-free medium, and coincubated with washed apoptotic mouse neutrophils for a period of 8 h at a ratio of 1:5 at 37°C and 5% $CO_2$ atmosphere. Samples were collected by trypsinization, washed with PBS, fixed with 1% PFA, and analyzed by FACSCalibur.

### Statistical analysis

Statistical analysis was performed by paired Student's *t* test.

## RESULTS

### Dex increases the phagocytosis of apoptotic neutrophils by human iDCs

Human macrophages have a high capacity to take up apoptotic cells, such as dying neutrophils, and this can be enhanced by the presence of the synthetic GC Dex during their differentiation [43]. When human monocyte-derived iDCs, isolated from buffy coats and differentiated in vitro for 5 days, were exposed to apoptotic neutrophils, it was found that they could also engulf apoptotic neutrophils [29]. iDCs were less effective than macrophages; even after several hours of coincubation, the percentage of DCs with engulfed apoptotic cells was 9–25% varying among donors. Similarly to macrophages, the presence of Dex during the differentiation of monocytes to DCs leads to significantly increased phagocytosis (**Fig. 1**). This Dex-mediated effect showed donor-dependent variability and did not correlate with the concentration of Dex (Supplemental Table 1), although it was observed that the smallest concentration of Dex (10 nM) was the most effective in the majority of donors.

### Gene expression pattern of monocyte-derived DCs differentiated in the absence or presence of Dex

To explore the molecular mechanism of the elevated phagocytosis in DCs upon GC treatment, we studied the expression

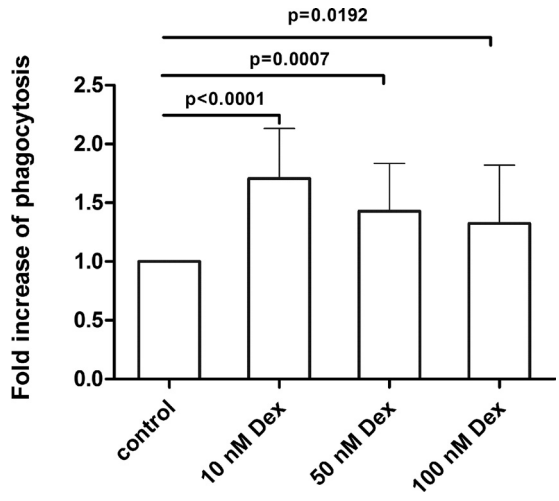


**Figure 1. Increased phagocytosis of apoptotic neutrophils by Dex-treated human iDCs.** Human monocytes were differentiated in the absence (control) and presence of different concentrations of Dex for 5 days, and then a phagocytosis assay was performed ($n$=16), as described in Materials and Methods. The percentage of phagocytic DCs was determined by flow cytometry. Elevated phagocytic capacity of GC-treated cells is presented as fold increase compared with the values of the control iDCs, which were taken as one, and statistical analysis was performed (error bars indicate SD).

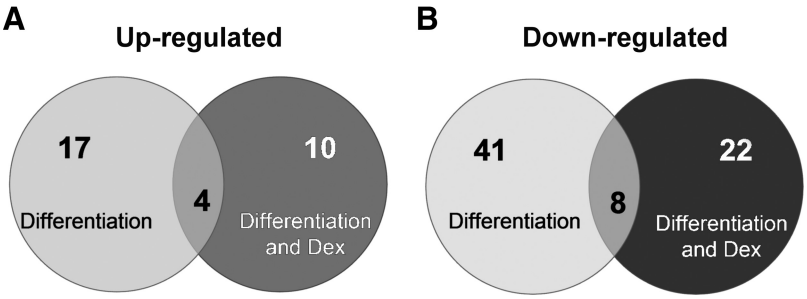


**Figure 2. Changes in the expression of apoptophagocytic genes of human iDCs differentiated in the presence or absence of Dex.** Control and Dex-treated cells of three donors were analyzed by TLDA measurements, and genes with FC > 2 (Up-regulated) or FC < 0.5 (Down-regulated) were selected. Venn diagram showing the number of up-regulated (A) or down-regulated (B) apoptophagocytic genes during differentiation (left circles) or genes changed by Dex treatment (right circles). FCs were calculated using the average relative gene expressions of three donors.

pattern of a panel of 95 apoptophagocytic genes designed previously (Supplemental Table 2). This experiment was performed with the most efficient Dex concentration enhancing phagocytosis by iDCs from three different donors. Changes in gene expression, during differentiation or by Dex treatment during differentiation, were determined based on the average relative expressions (**Fig. 2**). Comparing iDCs with monocytes, 17 genes were up-regulated during the 5 days of differentiation, whereas the addition of Dex to differentiating iDCs resulted in the up-regulation of only 10 genes, and there were only four overlaps (Fig. 2A).

**Table 1** lists the up-regulated genes and their FCs. Among the genes up-regulated during the 5 days of differentiation, there are six, which show more than a tenfold increase in their relative expression (FC>10) compared with the monocytes: DOCK1, FCGR2B, GAS6, IRF4, PROS1, and PPARG. However, except FCGR2B, these are not the ones that are further, and most up-regulated by Dex; DOCK1, IRF4, and PPARG are even down-regulated by the GC treatment (Supplemental Table 3). Only four of the genes up-regulated during differentiation, namely: ADORA3, C1QA, C2, and FCGR2B are further induced by Dex (Table 1). The apoptophagocytic

**TABLE 1. List of Up-Regulated Apoptophagocytic Genes in DCs Differentiated in the Absence or Presence of Dex ($n = 3$)**

| Up-regulated apoptophagocytic genes | | | |
|---|---|---|---|
| HUGO gene symbol[a] | Gene description | FC (differentiation) | FC (differentiation and Dex) |
| ADORA1 | adenosine A1 receptor | 5.889 | 0.103 |
| ***ADORA3*** | ***adenosine A3 receptor*** | ***5.340*** | ***6.985*** |
| ***C1QA*** | ***complement component 1, q subcomponent, A chain*** | ***4.642*** | ***4.625*** |
| ***C2*** | ***complement component 2*** | ***2.250*** | ***3.053*** |
| CD14 | CD14 molecule | 0.005 | 7.474 |
| DNASE2 | deoxyribonuclease II, lysosomal | 0.106 | 4.540 |
| DOCK1 | dedicator of cytokinesis 1 | 13.403 | 0.439 |
| ***FCGR2B*** | ***Fc fragment of IgG, low-affinity IIb, receptor (CD32)*** | ***17.886*** | ***2.705*** |
| GAS6 | growth arrest-specific 6 | 16.290 | 1.263 |
| IL-10 | interleukin 10 | 0.323 | 3.143 |
| IRF4 | IFN regulatory factor 4 | 32.347 | 0.170 |
| ITGAM | integrin, $\alpha$ M (complement component 3 receptor 3 subunit) | 7.181 | 0.192 |
| ITGB5 | integrin, $\beta$ 5 | 7.890 | 0.603 |
| MERTK | c-mer proto-oncogene tyrosine kinase | 0.023 | 15.197 |
| MFGE8 | milk fat globule-EGF factor 8 protein | 3.971 | 0.315 |
| MSR1 | macrophage scavenger receptor 1 | 3.890 | 0.280 |
| NLRP12 | NLR family, pyrin domain-containing 12 | 0.003 | 2.446 |
| OLR1 | oxidized low-density lipoprotein (lectin-like) receptor 1 | 4.133 | 0.053 |
| PPARG | peroxisome proliferator-activated receptor $\gamma$ | 41.123 | 0.293 |
| PROS1 | protein S ($\alpha$) | 142.906 | 0.815 |
| SCARB1 | scavenger receptor class B, member 1 | 0.997 | 2.288 |
| TGFBR1 | TGF-BR1 | 3.973 | 0.618 |
| TGM2 | transglutaminase 2 (C polypeptide, protein-glutamine-$\gamma$-glutamyltransferase) | 5.771 | 0.112 |

[a]Gene symbol defined by Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC; http://www.genenames.org). Monocytes of three donors were differentiated and treated with Dex for 5 days. Controls and treated samples were used to analyze changes of gene expression of 95 preselected apoptphagocytic genes by TLDA technique. Genes were normalized to the level of 18S rRNA, and FCs were calculated using the average relative expression of three donors versus monocytes (differentiation) and versus the appropriate nontreated controls (differentiation and dex). The genes represented show the up-regulated genes (FC>2) during the differentiation or by Dex treatment. Those that were induced further by Dex are shown in bold and italics. NLR, Nucleotide-binding domain, leucine-rich repeat.

genes that were highly up-regulated in all donors by GC treatment are: the phagocytosis receptors MERTK and CD14, the adenosine receptor ADORA3, C1QA (which can form a bridge between dying cells and phagocytes), and the digestive enzyme DNASE2. Examples of relative gene expression values for the mentioned genes are shown in **Fig. 3**. Control experiment revealed that the presence of GC allows but skews DC differentiation to a $CD1a^{-}CD14^{+}$ subtype, whereas it does not influence expression of CD209 (data not shown). In the case of MERTK and CD14, flow cytometry data showed that both could be detected only on the surface of Dex-treated iDCs (Fig. 3A and B).

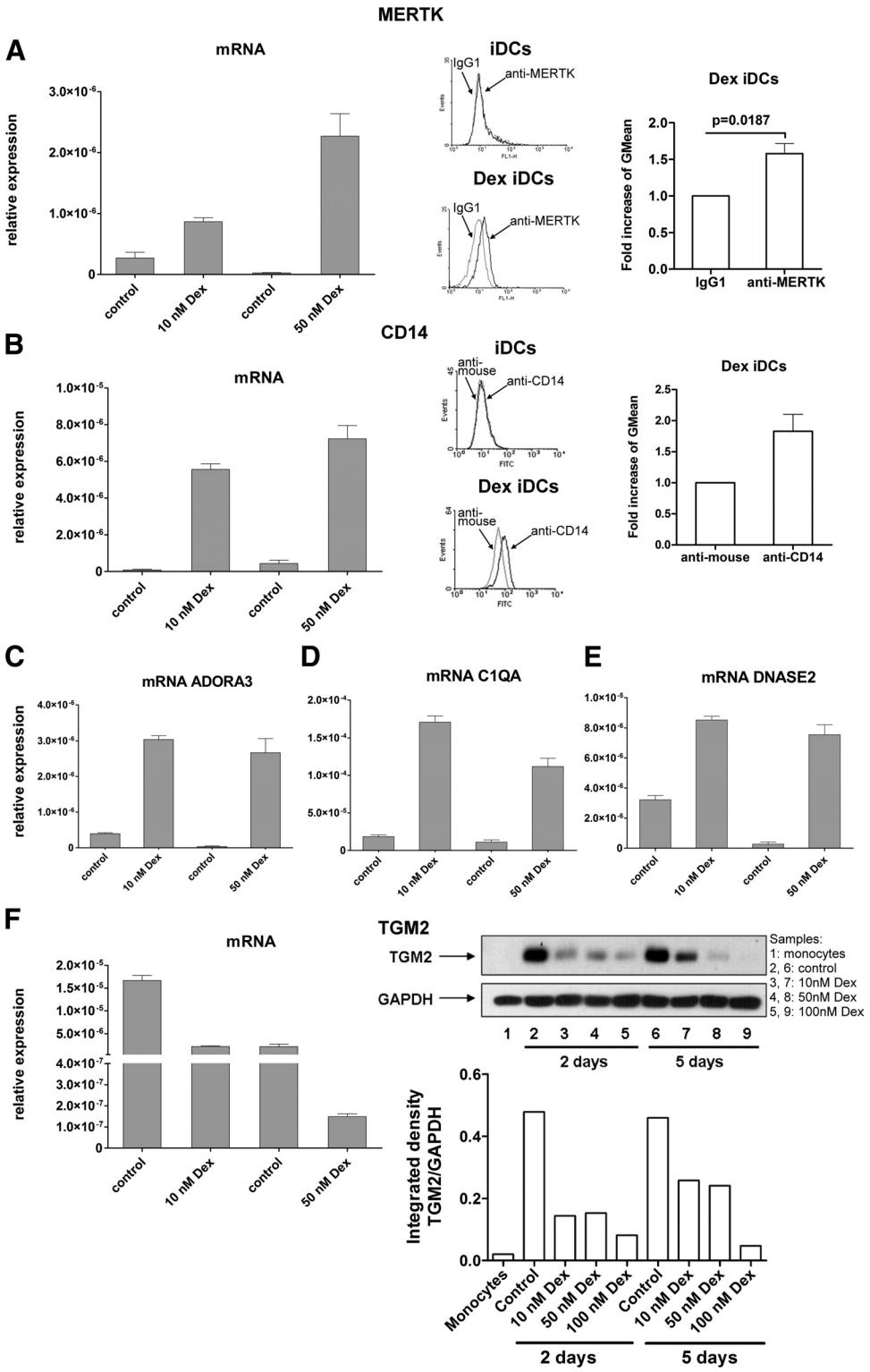


**Figure 3. Changes of the expression of apoptophagocytic genes by Dex in human DCs with increased phagocytosis of apoptotic cells.** Control and Dex-treated DCs were differentiated for 5 days, and then samples with the most effective GC concentration for increasing phagocytosis were selected for the TLDA analysis. The relative expression level of genes highly up-regulated in all donors, with a FC $>$ 2 in each sample, is shown for two donors (A–F). Error bars indicate SD. In the case of MERTK, one representative example of cell surface labeling is shown, and the fold increase of GMean values of Dex-treated iDCs compared with the isotype control (IgG1) is presented for three donors (A). In the case of CD14, one example of flow cytometry data shows that the cell surface labeling and the fold increase of GMean values for Dex-treated cells compared with the control antibody (anti-mouse) are presented for two donors (B). Relative expression of TGM2 in control and Dex-treated human DCs is shown. The Western blot analysis and the quantification by densitometry represent one example of three donors (F).

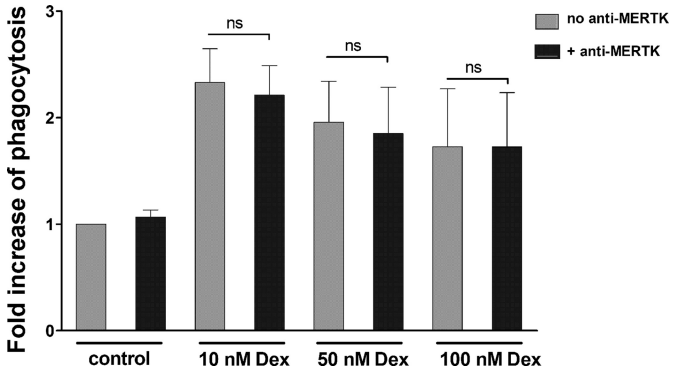


**Figure 4. Effect of blocking antibody specific for MERTK on the phagocytosis of apoptotic neutrophils by human DCs differentiated in the presence of Dex.** DCs on Day 5 of differentiation were preincubated with 10 $\mu$g/ml anti-MERTK antibody and then loaded with labeled apoptotic neutrophils. Data were evaluated by flow cytometry, and an increase of phagocytosis was calculated compared with the Dex-nontreated samples, which were taken as one. Statistical analysis showed no significant change in phagocytosis of antibody-preincubated samples compared with their controls. Bars represent the mean ± SD ($n$=3).

The number of down-regulated genes was 41 during differentiation and 22 when Dex was also present (Fig. 2B), and only eight overlapping genes were found (Supplemental Table 3). From the most down-regulated four genes—ADORA1, ICAM3, OLR1, and TGM2—the latter was tested further. We have shown previously that TGM2 plays an important role in the phagocytosis of apoptotic cells by macrophages [44], and it is also up-regulated during differentiation of DCs [45]. However, it is strongly down-regulated upon GC treatment at mRNA and protein levels from the early stage of DC differentiation and even by the lowest Dex concentration (Fig. 3F).

## Effect of anti-MERTK antibody on the increased phagocytosis of apoptotic neutrophils induced by Dex

As MERTK was up-regulated in Dex-treated samples, we wanted to see whether binding of a blocking anti-MERTK antibody to iDCs [46, 47] during their coincubation with dying neutrophils could prevent the observed enhancement of phagocytosis. We performed phagocytosis assays with Dex-treated iDCs preincubated with the antibody, but there was no inhibition of apoptotic cell uptake (**Fig. 4**). A similar experiment with the anti CD-14 antibody also has been carried out, but no decrease in engulfment of apoptotic neutrophils by Dex-treated iDCs was observed (data not shown).

## Role of ADORA3 in Dex-induced increase of phagocytosis

ADORA3 was one of the genes that was up-regulated most in GC-treated iDCs, so we tested the effect of an ADORA3 antagonist on Dex-mediated enhancement of apoptotic neutrophil uptake. iDCs were preincubated with the highly selective ADORA3 antagonist MRS1220 [48] (referred to as MRS in the figure) and then loaded with apoptotic neutrophils. The antagonist could decrease Dex-induced enhancement of phagocytosis (**Fig. 5A**).

The involvement of ADORA3 in the Dex effect was tested in a mouse system as well. DCs differentiated in the absence or presence of Dex from bone marrow progenitors of WT and ADORA3 KO mice were coincubated with mouse apoptotic neutrophils. Although in the case of WT DCs, Dex increased the uptake of apoptotic cells, lack of ADORA3 resulted in the unresponsiveness of DCs to Dex treatment, and there was even less phagocytosis by them upon Dex treatment (Fig. 5B). It was also noted that WT DCs were somewhat less efficient in engulfing apoptotic neutrophils than the KO ones (data not shown).

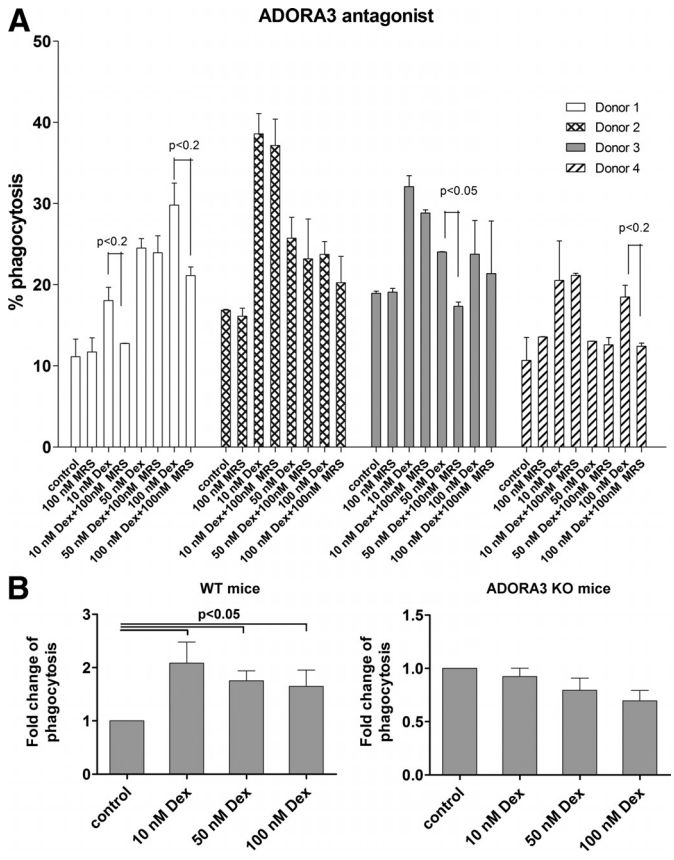


**Figure 5. The effect of pharmacological inhibition and genetic ablation of ADORA3 on apoptotic neutrophil phagocytosis by DCs.** Human DCs (control and Dex-treated) of four donors were harvested on Day 5 of differentiation and then incubated with 100 nM human ADORA3 antagonist [MRS1220 (MRS)] for 1 h, 37°C. The phagocytosis assay was performed as described previously, and samples were analyzed by flow cytometry (A). WT and ADORA3 KO mouse bone marrow progenitors were differentiated to DCs, as described in Materials and Methods, in the presence of Dex. On the 9th day, cells were incubated with mouse apoptotic neutrophils, and then flow cytometry analysis was performed, and the FCs were calculated compared with the controls, which were taken as one. Bars represent the mean ± SD of four independent experiments (B).

### Enhanced apoptotic cell phagocytosis by Dex-treated DCs results in proinflammatory cytokine secretion and T lymphocyte activation

To assess the T cell-polarizing potential of apoptotic, cell-loaded DCs, we cocultured them with autologous T lymphocytes for an additional 5 days and detected IFN-γ-secreting T cells by ELISPOT assay. Despite the high variability of the cellular response among donors, we could detect higher numbers of IFN-γ-secreting T cells in all cases when Dex-treated DCs were exposed to apoptotic neutrophils. Dex by itself was able to potentiate iDCs for T cell activation, but this effect was more pronounced in neutrophil-loaded DCs. Again, the most effective Dex concentrations were donor-dependent (**Fig. 6A**).

It was shown previously that monocyte-derived human DCs activated by LPS and IFN-γ respond to allogeneic apoptotic neutrophils with inflammatory cytokine production, and the engulfment of apoptotic neutrophils leads to the activation of T cells [29]. To test the effects of Dex on DC-mediated inflammatory responses, we differentiated monocytes to iDCs in the presence or absence of Dex and then loaded the cells with allogeneic apoptotic neutrophils. After 8 h coincubation, the cells were stimulated with LPS and IFN-γ for an additional 16 h before measuring the concentration of secreted TNF-α in the culture supernatants. Surprisingly, iDCs, differentiated in the presence of Dex, responded with increased TNF-α secretion, and when these cells were fed by apoptotic neutrophils, there was even higher cytokine release as compared with controls (Fig. 6B). The most effective Dex concentration leading to this response varied among individuals.

## DISCUSSION

In this study, we have examined the effect of GC on the uptake of apoptotic cells by human monocyte-derived iDCs and showed significantly increased phagocytosis upon Dex treatment, which resulted from a reprogrammed expression pattern of apoptophagocytic genes, and the engulfed allogeneic apoptotic neutrophils can provoke the Dex-treated DCs to activate autologous T cells.

The exogenous (therapeutic) GCs have an impact on nearly every cell type of the immune system [49], particularly on macrophages and DCs. Early exposure of differentiating monocytes to GCs induces a distinct, functional, proresolution phenotype with increased phagocytosis of apoptotic neutrophils [36], a phenomenon observed in human macrophages as well [46]. GCs may act at the very first step of the immune response by modulating DC differentiation, maturation, and function [50], freezing the cells at an immature stage and enhancing mannose receptor-mediated and fluid-phase endocytosis [41, 42]. When we tested the effect of GCs on human monocyte-derived DCs, which resemble tissue resident, migratory DCs [7], we also observed the morphological changes (less adherent cells with abundant cytoplasm) reported earlier [41]. Phagocytosis of apoptotic cells by these cells, investigated with an in vitro phagocytic system established previously [43] to mimic events at in vivo inflammation sites, was increased in GC-treated DCs. The dosage of Dex required for and the extent of the enhancement of phagocytosis varied among individual donors. The varying sensitivity to Dex treatment is very

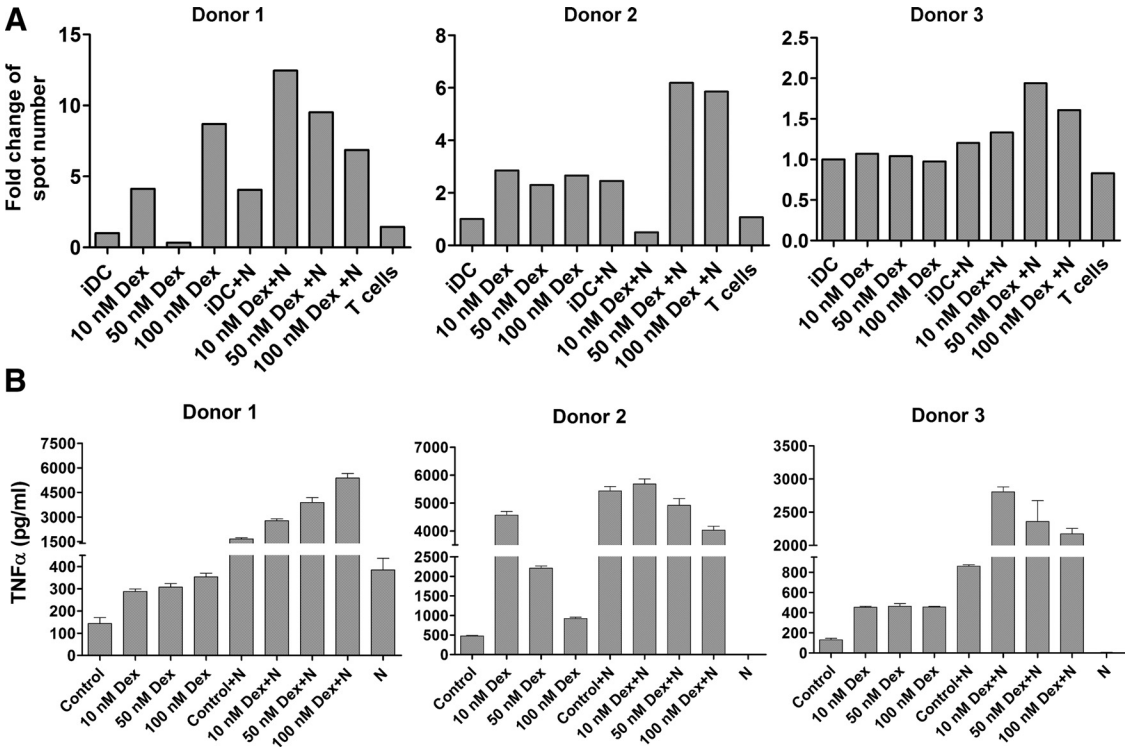


**Figure 6. Effect of Dex on apoptotic neutrophil-loaded, DC-induced T cell activation.** iDCs and nonactivated DCs were incubated with apoptotic neutrophils and cocultured with autologous lymphocytes at a ratio 1:25 for 5 days. The nonadherent lymphocytes were harvested and subjected to a 48-h IFN-γ ELISPOT assay. Data were analyzed by a computer-assisted ELISPOT image analyzer, and FCs were calculated compared with iDC controls (A). The secretion of TNF-α cytokine of control and Dex-treated iDCs from the same donors incubated with apoptotic neutrophils (N) was measured after the 8-h coculture period, followed by 16 h further activation with LPS and IFN-γ. The supernatants were collected, and the concentration of TNF-α was measured by ELISA. Bars represent the mean ± SD of pg/ml cytokine concentration (B). The concentration of basal TNF-α secretion of nonstimulated iDCs was 18.88 ± 15.33 pg/ml (range 3.33–33.99 pg/ml).

likely a result of the molecular heterogeneity and polymorphism of the GC receptor generating diversity in the sensitivity of a GC response [51].

To study the mechanism behind the GC-induced increase in phagocytosis of human DCs, we examined the gene expression changes of an apoptophagocytic gene panel in Dex-treated DCs. The TLDA used for this purpose contained genes involved in the apoptophagocytic process, which can be grouped in the following categories: receptors (integrins, scavenger receptors, adenosine receptors, tyrosine kinases, etc.), bridging molecules, signal generators, effectors, cytokines, nuclear receptors, engulfment genes, autophagy genes, IRF genes (Supplemental Table 2). The 10 genes found up-regulated by Dex during the differentiation to iDCs are members of cell surface molecules (ADORA3, FCGR2B), bridging molecules (C1QA, C2), phagocytosis-tethering/tickling receptors (CD14, MERTK, SCARB1), effectors (DNASE2), cytokines (IL-10), and inflammatory regulators (NLRP12). There is a significant reprogramming of phagocytic function-related gene expression by Dex, as the 5-day differentiation process itself in the absence of Dex results in the induction of 17 apoptophagocytic genes, and only four of them overlapped with the Dex up-regulated genes, namely ADORA3, C1QA, C2, and FCGR2B.

The extensive change in the gene expression pattern is also manifested in the large number of down-regulated apoptophagocytic genes. One may presume that the product of these genes has additional functions that are unrelated to phagocytosis but do not fit or would even block GC-mediated molecular events and should be switched off for optimal GC response, whereas others are up-regulated to replace them. For example, one of the down-regulated genes is TGM2, a multifunctional enzyme that belongs to the family of calcium-dependent transamidating acyltransferases [52]. We reported previously that the loss of TGM2 leads to delayed phagocytosis of apoptotic cells by macrophages and to development of autoimmunity in mice [44]. It was also proposed that TGM2 takes part in the formation of phagocytic portals by interacting with ITGB3 and MFGE8. In the absence of the enzyme, the formation of the engulfing portals is less efficient, and the uptake of apoptotic cells becomes slow and random [53]. In human DCs, TGM2 is highly induced during the 5 days of differentiation at mRNA and protein levels as well. Although TGM2 is strongly down-regulated by Dex, iDCs have increased phagocytic capacity. This means that alternative pathways up-regulated by Dex can replace the apoptophagocytic action of TGM2, whereas other functions of this protein, which might not be compatible with the GC-mediated response of DCs—e.g., facilitation of cellular attachment, signaling as a G protein, protein cross-linking—are not manifested.

Galon et al. [54] used DNA microarray analysis to investigate the effect of Dex on human PBMCs. They found that Dex could regulate genes with critical roles in innate and adaptive immune responses. Furthermore, they pointed to the role of GC, not only as an immunosuppressant but also as a major immunopermissive and immunoenhancing agent. Ehrchen et al. [37] described the GC-dependent regulation of 133 genes in human monocytes by microarray technology. Among the GC-up-regulated genes described in their study, there are four, namely ADORA3, MERTK, C1QA, and IL-10, which were also induced in our study and overlap with up-regulated genes in monocyte-derived DCs, where ADORA3, the phagocytosis receptors MERTK and CD14, the bridging molecule C1QA, and the effector DNASE2 were the most up-regulated genes.

MERTK, a member of the TAM (TYRO3, AXL, MER) receptor protein tyrosine kinase family with an important role in innate immunity, is up-regulated by Dex in human macrophages [47] and plays a key role in the GC-induced phagocytosis enhancement [46]. We show here that also in monocyte-derived DCs, Dex up-regulates the expression of MERTK, leading to its increased amount on the cell surface. However, by applying blocking antibodies against MERTK, we could not diminish the GC-induced enhancement of phagocytosis by DCs. It cannot be excluded that the binding of the anti-MERTK antibody was not strong enough to block the phagocytosis. It is equally possible that the role of this surface molecule in DCs is not pronounced enough in mediating the enhancement of phagocytosis by Dex so that their blocking would make a significant difference in phagocytosis efficiency.

ADORA1 and ADORA3 receptors are expressed on myeloid and plasmocytoid DCs, and their activation induces chemotaxis as a result of mobilization of intracellular calcium and reorganization of the cytoskeleton in iDCs [55, 56]. In human monocytes, ADORA3 was found to be up-regulated by GC [37]. Our results show that also in human monocyte-derived DCs, Dex up-regulates the expression of ADORA3. Using a potent and highly selective antagonist of the human ADORA3 receptor (MRS1220), we could attenuate the Dex-induced enhancement phagocytosis of apoptotic neutrophils. In the case of ADORA3 KO mice, Dex was ineffective to increase phagocytosis of apoptotic neutrophils.

It was shown previously that stimulation of ADORA3 inhibits adenylyl cyclase activation via the $G_i$ protein, resulting in decreased cAMP levels [57], and cAMP in macrophages could specifically inhibit phagocytosis of apoptotic neutrophils [58], suggesting that a similar regulatory process can take place in DCs. Adenosine seems to be a soluble mediator, probably released in low concentrations during phagocytosis of apoptotic neutrophils from iDCs or dying neutrophils, and activates ADORA3. Thus, ADORA3 contributes to Dex-induced phagocytosis, possibly by lowering intracellular cAMP levels.

It was a surprising finding that Dex, which is generally considered one the most effective immune-suppressor agents, promoted a proinflammatory response in iDCs reacting to allogeneic apoptotic human neutrophils, followed by their exposure to autologous T cells. We showed previously that in contrary to human macrophages, DCs respond to these stimuli in an unexpected way by secreting proinflammatory cytokines and inducing T cell activation [29]. In Dex-treated DCs, we could detect even a higher number of IFN-γ-producing T cells under such conditions, which may be attributed to the increased phagocytosis of apoptotic neutrophils. We even detected elevated IFN-γ secretion in some samples treated with different concentrations of Dex but not loaded with the dying neutrophils. Following stimulation with LPS + IFN-γ, a high level of the proinflammatory cytokine TNF-α was measured from the supernatant of the DCs exposed to apoptotic cells, which was

enhanced further by Dex. Furthermore, Dex itself could increase TNF-α production of DCs, which possibly could be attributed to the up-regulation of TLR4 and TLR2 by GCs, as it was shown in PBMCs [54]. These results demonstrate that the GC effect is more complex than it has been described so far and may drive inflammatory and immune-suppressive responses in a context-dependent manner. In endothelial cells, Dex enhances inflammation initiated by ATP [59], which is another example of when GCs are not uniformly immunosuppressive, and their effect depends on cell type, dose, and timing of exposure.

The interaction of the host's DC subpopulations with allogeneic apoptotic neutrophils may occur in transplanted organs as a result of early tissue injury causing infiltration of donor neutrophil granulocytes. The strong proinflammatory effect of these allogeneic apoptotic neutrophils is one of the possible reasons of allograft rejection. Based on the results presented here, it should be strongly considered that Dex, which is often used as an immunosuppressive agent treating transplantation rejection, may have an undesired effect promoting activation of autologous T lymphocytes toward Th1 effector cells via DCs, engulfing an increased number of apoptotic neutrophils, thereby facilitating rather than delaying immune rejection.

## AUTHORSHIP

J.H. designed and performed the experiments, analyzed the results, and wrote the paper. G.M. designed the experiments and wrote the paper. Z.D. and G.Z. performed experiments and analyzed the data. A.P. designed the animal experiments. E.R and L.F. designed the research and wrote the paper.

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