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# Resistance of LPS-activated bone marrow derived macrophages to apoptosis mediated by dexamethasone

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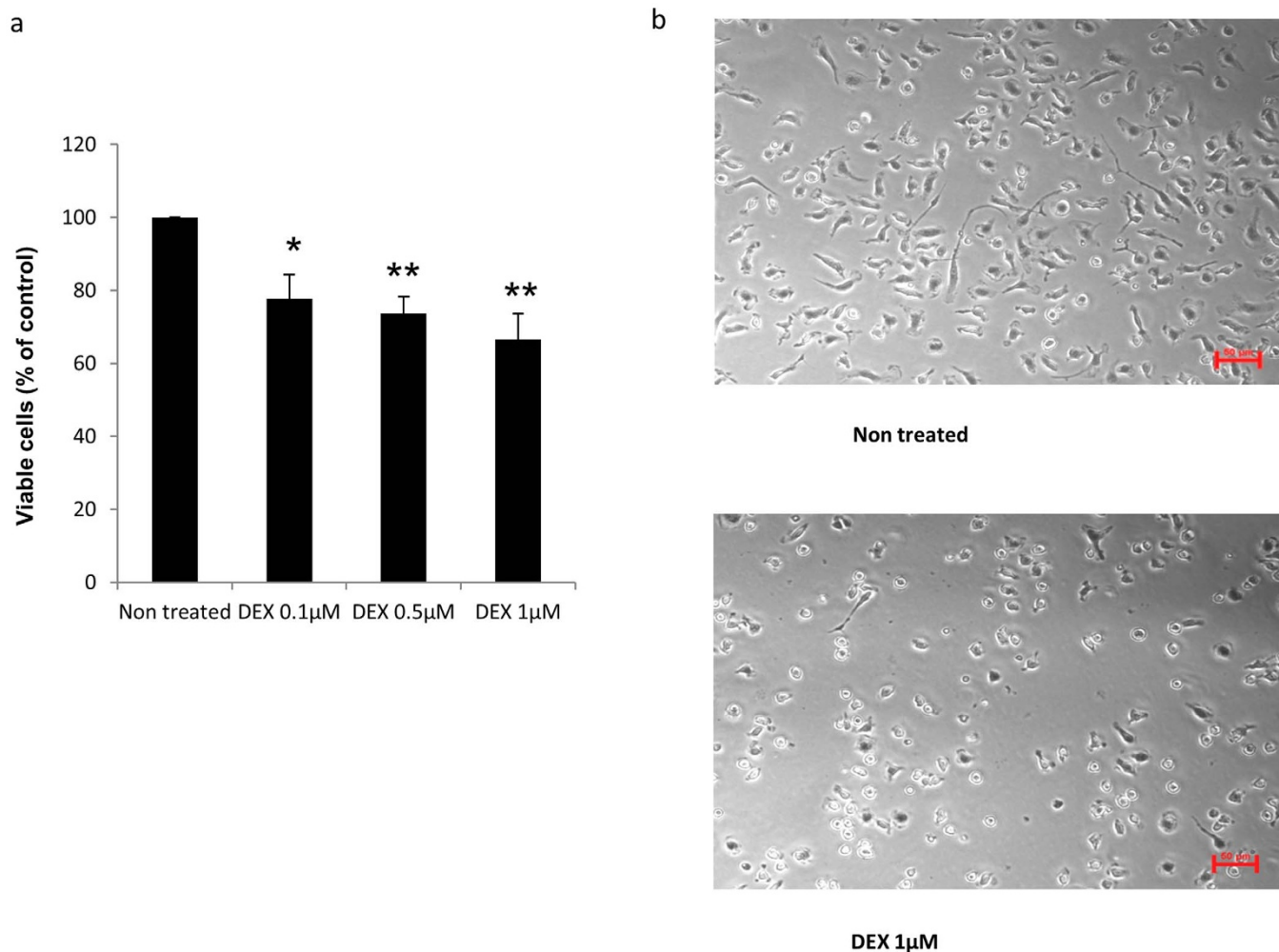
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Glucocorticoids (GC) display pleiotropic effects on the immune system. Macrophages are a major target for GC action. Here we show that dexamethasone (DEX), a synthetic GC, decreased viability of naïve bone marrow-derived macrophages (BMDM), involving an apoptotic mechanism. Administration of DEX together with lipopolysaccharide (LPS) protected BMDM against DEX-mediated cell death, suggesting that activated BMDM respond to DEX differently than naïve BMDM. An insight to the molecular basis of LPS actions was provided by a 7 fold increase in mRNA levels of glucocorticoid receptor beta (GR $\beta$ ), a GR dominant-negative splice variant which inhibits GR $\alpha$ 's transcriptional activity. LPS did not inhibit all DEX-mediated effects on BMDM; DEX significantly reduced the percentage of BMDM expressing high levels of the cell surface markers F4/80 and CD11b and led to a decrease in macrophage inflammatory protein 1 alpha (MIP1- $\alpha$ ) mRNA and protein levels. These two DEX-mediated effects were not prevented by LPS. Our finding that LPS did not reduce the DEX-induced elevation of glucocorticoid-induced leucine zipper (GILZ), a mediator of GCs anti-inflammatory actions, may provide an underlying mechanism. These findings enable a better understanding of clinical states, such as sepsis, in which macrophages are activated by endotoxins and treatment by GCs is considered.

Glucocorticoids (GCs), the major effector hormones of the stress system, influence almost all aspects of mammalian physiology. These steroids exert their effects on a large network of primary, secondary, and tertiary target genes, encompassing up to 20% of the expressed genome in a tissue<sup>1</sup>. The endogenous GC is cortisol and it is produced in the adrenal glands. Corticosteroid-binding globulin binds cortisol with high affinity and facilitates its transport in the blood<sup>2</sup>. For over 50 years, synthetic analogues of the endogenous human GCs, cortisol and its oxidation product cortisone, were the mainstay of second line therapy for a wide range of inflammatory disorders<sup>3</sup>. Yet, the exact mechanisms responsible for their immunosuppressive properties are still not fully understood<sup>4</sup>. GCs affect almost every cell of the immune system regarding apoptosis, adhesion, cellular motility, chemotaxis, phagocytosis and reactive oxygen metabolism<sup>5</sup>. In the absence of GCs, the glucocorticoid receptor (GR) resides in the cytoplasm in an inactive state. Upon GC binding, GR undergoes conformational change and translocates into the nucleus, where it binds to specific DNA sequences, leading to the inhibition of several inflammatory signaling cascades. For instance, active GR interferes with the transcriptional activity of several transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and activator protein 1 (AP-1)<sup>4,6</sup>. As for clinical states, GCs are well recognized as an effective therapy in a wide range of diseases, including autoimmune diseases (e.g. multiple sclerosis<sup>7</sup>), allergies<sup>8</sup> and hematological malignancies<sup>9</sup>.

Macrophages are central components of the innate immune system<sup>10</sup>. They secrete specific compounds that mediate the death of pathogens and they facilitate the recognition of foreign pathogens by the adaptive immune system<sup>11</sup>. Tissue specific macrophages are derived from circulating monocytes which originate from bone marrow progenitors<sup>12</sup>.

Lipopolysaccharide (LPS), the main component of gram-negative bacterial cell wall, is a powerful activator of macrophages<sup>13</sup>. LPS has a pro-inflammatory action on a wide range of cells due to its activation of the Toll like receptor 4 (TLR4)<sup>14</sup>. This endotoxin is recognized as the most potent microbial mediator and its macrophage-inducing activation is implicated in the pathogenesis of sepsis and septic shock<sup>15</sup>; both are major causes of



**Figure 1 | DEX reduces viability of BMDM.** BMDM were incubated with DEX 0.1, 0.5 or 1  $\mu\text{M}$  for 24 hours. Viability was determined by the MTT method (a) and by microscopic observation (b). (a) Cumulative results of 3 independent experiments performed in triplicates. The percentage of viable cells after 24 hours incubation without treatment is considered as 100% (the average O.D value of the non-treated cells is 0.2) and all other values are normalized according to it. \*  $P < 0.05$ , \*\*  $P < 0.01$ , compared to non-treated cells after 24 hours incubation. Values depict mean  $\pm$  SEM. (b) Light microscope pictures of non-treated BMDM and BMDM treated with DEX 1  $\mu\text{M}$  after 24 hours incubation.

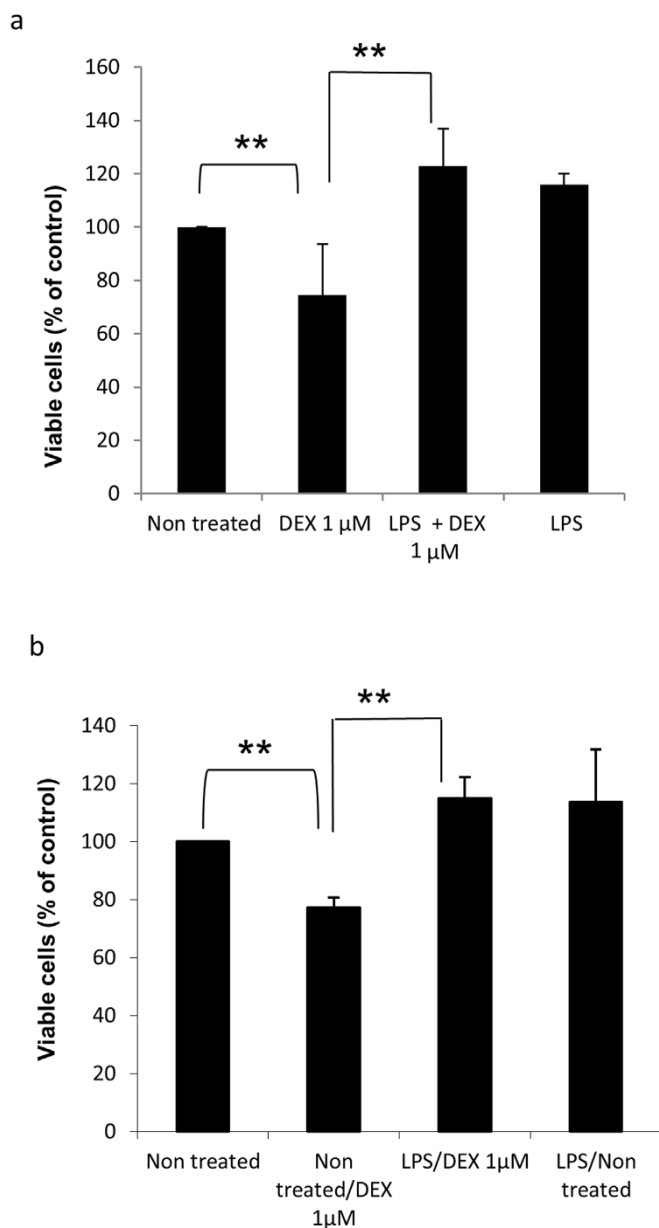
mortality in intensive care units<sup>16</sup>. During sepsis, endogenous GCs are released from the adrenal glands and are a key component of the host response<sup>17</sup>. Compromised production of GCs impairs the survival of sepsis patients<sup>18</sup>. GCs improve the clinical outcome of septic shock by several mechanisms: their effects on macrophages<sup>19</sup>, their impact on endothelial dysfunction<sup>20</sup> and their inhibition of the humoral response during sepsis<sup>21</sup>.

In a murine model of LPS-induced septic shock, it was shown that GR actions in macrophages play an important role in GCs protective effect during this shock. For example, treatment of macrophages with GCs induced a decrease in the levels of secreted tumor necrosis factor alpha (TNF- $\alpha$ ), a cytokine with a central role in the development of septic shock<sup>19</sup>.

Over the years, enormous efforts have been directed to develop a plethora of synthetic GCs, characterized by improved pharmacokinetic and pharmacodynamic properties<sup>3</sup>. Dexamethasone (DEX) is a synthetic GC, characterized by a 20–30 fold higher immunosuppressive potency compared to cortisone<sup>22</sup>. Application of DEX to macrophages of different sources resulted in a wide range of responses, including apoptosis of macrophages in the nervous system<sup>23</sup> and on the other hand, enhanced viability of murine RAW 264.7 macrophages<sup>24</sup> and blood monocyte-derived macrophages<sup>10</sup>. Since the

mechanisms of the immunosuppressive properties of GCs are yet to be clarified<sup>4</sup> and bone marrow monocytes are the source for resident macrophages throughout the body<sup>12</sup>, we utilized bone marrow-derived macrophages (BMDM) to address the question of whether and how DEX affects naïve and LPS-activated BMDM concerning their viability and phenotype.

In the present study, we found that LPS-activated BMDM exhibited resistance to some of the effects that DEX had on naïve BMDM. DEX induced the apoptotic death of naïve BMDM, while LPS-activated BMDM were protected from DEX-mediated death. Nevertheless, LPS-activated BMDM were not protected from DEX-induced reduction of the expression of the surface markers F4/80 and CD11b, indicating that the anti-inflammatory properties of DEX differ according to the activation status of the macrophages. We also found that pre-incubation of BMDM with LPS, prior to DEX treatment, resulted in an increase in the mRNA levels of a dominant-negative splice variant of GR, GR $\beta$  and in a decrease in the mRNA levels of the active GR $\alpha$ . However, the mRNA levels of the mediator of GCs anti-inflammatory actions, glucocorticoid-induced leucine zipper (GILZ)<sup>25</sup>, were elevated in cells treated with LPS before their incubation with DEX. These results may provide an explanation for the partial resistance to DEX that LPS-activated BMDM exhibit.



**Figure 2 | LPS prevents DEX-mediated reduction of BMDM viability.** (a) BMDM were incubated for 6 hours in DMEM culture medium alone. Then, the cells were cultured overnight with DEX 1  $\mu$ M, DEX 1  $\mu$ M + LPS 0.05  $\mu$ g/ml, LPS 0.05  $\mu$ g/ml or DMEM alone, as a control (non-treated). Viability was determined by the MTT method. The percentage of the non-treated viable cells is considered as 100% (the average O.D value of the non-treated cells is 0.171) and all other values are normalized according to it. \*\*  $P < 0.01$ , values depict mean  $\pm$  SEM of 4 independent experiments performed in triplicates. (b) BMDM were incubated with DMEM culture medium alone or with LPS 0.05  $\mu$ g/ml for 6 hours. Then, the medium was replenished and the cells were incubated overnight with DEX 1  $\mu$ M or with DMEM alone, as a control (non-treated). Viability was determined by the MTT method. The percentage of the non-treated viable cells is considered as 100% (the average O.D value of the non-treated cells is 0.171) and all other values are normalized according to it. \*\*  $P < 0.01$ , values depict mean  $\pm$  SEM of 5 independent experiments performed in triplicates.

## Results

**DEX decreases viability of naïve BMDM.** To address the response of macrophages to GCs in normal and in infectious disease states, (e.g. sepsis), we chose to focus on DEX effects on naïve and LPS-activated BMDM. To determine whether BMDM viability is

modified by DEX, we first assessed the effect of DEX on naïve BMDM, by subjecting BMDM to a range of DEX concentrations for 24 hours and assessed the percentage of viable cells, using the colorimetric MTT assay (Figure 1). The results show that incubation of the cells with the different DEX concentrations for 24 hours decreased viability of naïve BMDM. Namely, incubation with 0.1, 0.5 and 1  $\mu$ M DEX led to a significant reduction in the viability of BMDM by 22, 26 and 34%, respectively, compared with corresponding untreated cells (Figure 1a). A phase contrast microscopy image of control and DEX-treated BMDM (Figure 1b), visualized the effect of a 24 hour treatment by DEX (1  $\mu$ M) on cell morphology: while control cells were numerous, elongated and spread, the DEX-treated cells were less in number and lost their elongated form.

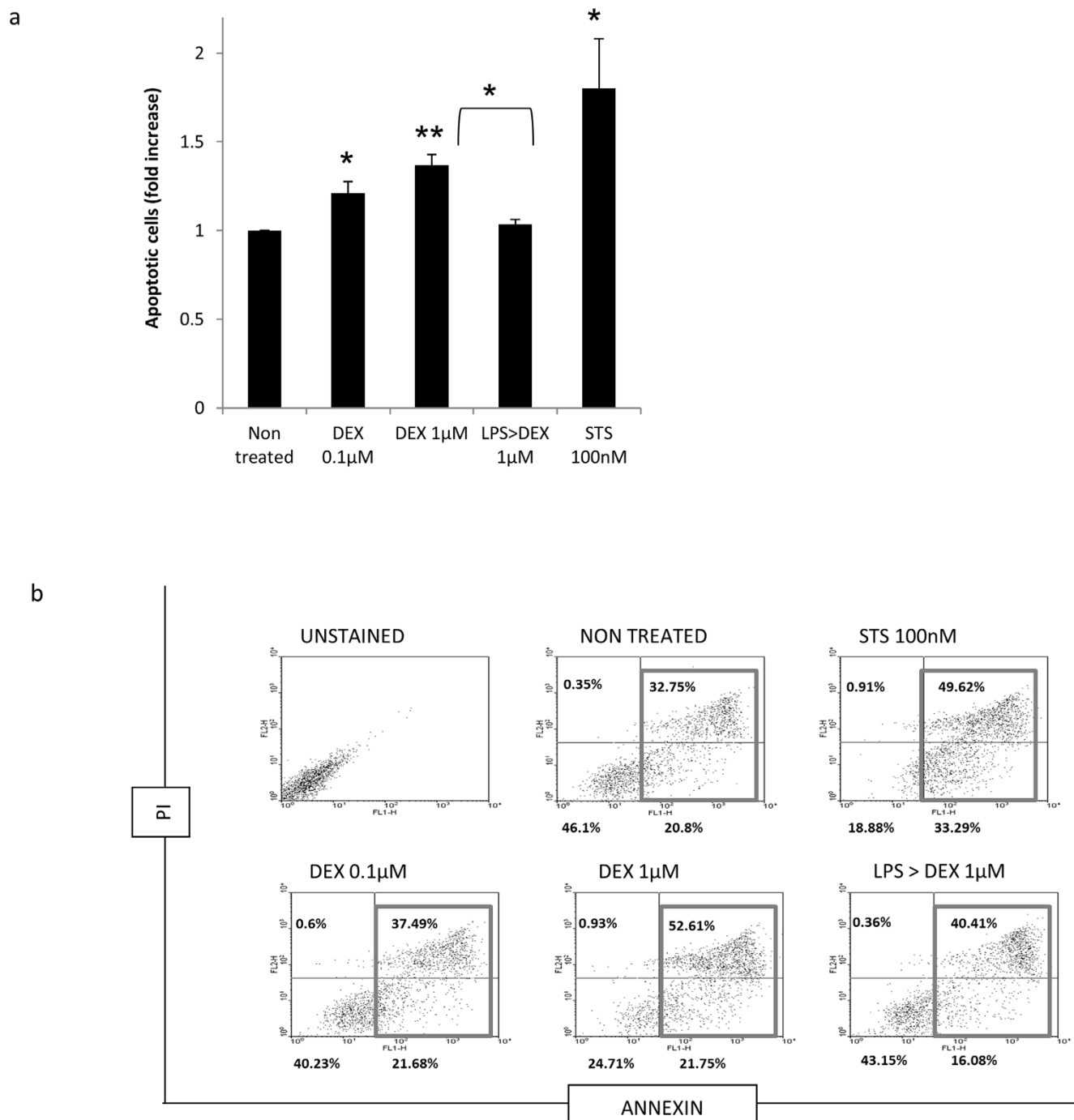
**LPS prevents DEX-mediated reduction of BMDM viability.** We next raised the question of whether the viability of LPS-activated BMDM is affected by DEX in a similar manner to naïve BMDM. For that, BMDM were incubated with LPS 0.05  $\mu$ g/ml together with DEX 1  $\mu$ M for 24 hours and subjected to the colorimetric MTT assay as described above. Whereas treatment with LPS alone induced a non-significant increase in the viability of BMDM, addition of LPS to the DEX-treated cultures protected BMDM from cell death (Figure 2a). Namely, the percentage of viable cells (non-treated cells were considered as 100%) in the LPS-treated cultures was similar to that of cells treated by a combination of LPS and DEX (128 and 130%, respectively). These values were significantly higher than that of cells treated with DEX alone (76%) (Figure 2a). Addition of DEX 1  $\mu$ M to cells incubated in medium for 6 hours resulted in 76% cell viability while the percentage of viable cells in cultures treated with LPS 0.05  $\mu$ g/ml for 6 hours, followed by DEX 1  $\mu$ M was 117% (Figure 2b). Notably, LPS treatment alone for 6 hours, followed by medium, resulted in cell viability similar to that of non-treated cells (there was a non-significant increase in the viability of BMDM, induced by LPS, Figure 2b). It thus appears that LPS-activated BMDM are protected from DEX-induced cell death even when exposure to LPS precedes treatment by DEX.

## LPS induces resistance of BMDM to apoptosis mediated by DEX.

We next questioned whether DEX affects the viability of BMDM by inducing apoptotic cell death, utilizing the Annexin-PI staining method. Non-treated BMDM undergo “natural” apoptosis, as they are cultured in the absence of the necessary growth factor M-CSF (macrophage colony stimulating factor). Nonetheless, we demonstrate a dose dependent increase of 20 and 37% in the fraction of apoptotic BMDM in the presence of 0.1 and 1  $\mu$ M DEX, respectively. However, incubation of BMDM with LPS 0.05  $\mu$ g/ml prior to the addition of DEX 1  $\mu$ M protected the cells from DEX-mediated apoptosis (Figure 3a). As shown in Figures 2a and 2b, LPS alone did not increase the percent of viable BMDM, compared to the non-treated cells. Staurosporine (STS), an effective protein kinase C inhibitor, frequently applied *in-vitro* as an inducer of apoptosis<sup>26</sup> was used as a positive control. Indeed, STS-treated cultures displayed an 80% elevation in the fraction of apoptotic BMDM (Figure 3a). A representative experiment is depicted in Figure 3b.

## LPS does not prevent DEX-induced decrease in BMDM expressing high levels of F4/80 and CD11b.

The notion that GCs exert anti-inflammatory effects on activated macrophages during sepsis<sup>19</sup>, coupled to the lack of DEX effect on the viability of LPS-activated macrophages (Figures 2, 3), raises the need to delineate the underlying action of DEX on activated macrophages. F4/80 and CD11b are activation and differentiation markers of macrophages<sup>12,27</sup>. It has been previously reported that GCs influence CD11b expression<sup>28</sup>, yet we have observed, by FACS analysis, that the main effect of DEX treatment on the expression of F4/80 and CD11b by BMDM was confined to the macrophage population expressing high levels of these markers. A 24 hour incubation with 0.1 and 0.5  $\mu$ M DEX led to

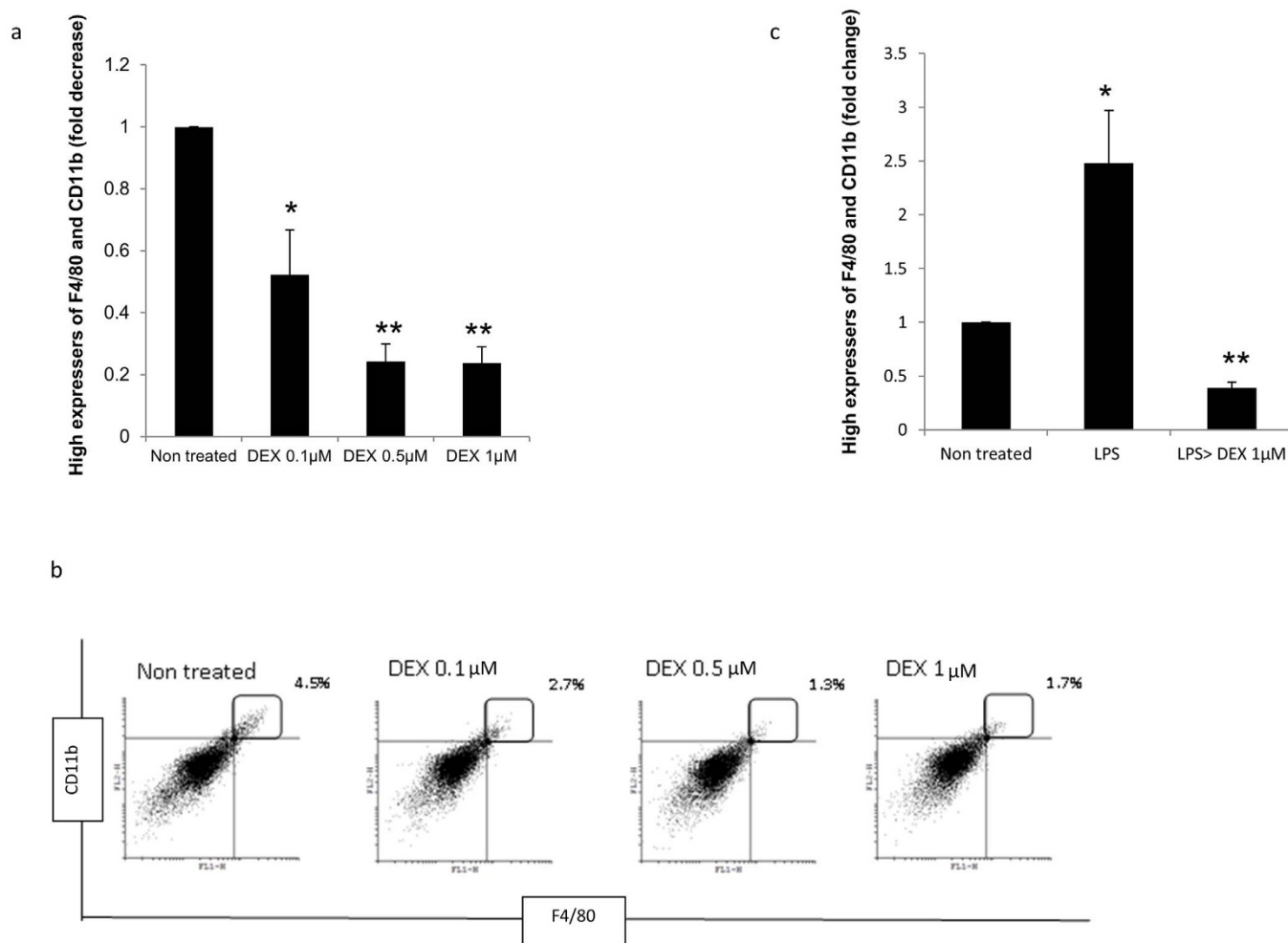


**Figure 3 | LPS induces resistance of BMDM to apoptosis mediated by DEX.** (a) BMDM were incubated for 4 hours with DEX 0.1 or 1 µM, STS 100 nM (positive control) or DMEM alone (non-treated) or with LPS 0.05 µg/ml overnight followed by DEX 1 µM for 4 hours (LPS > DEX 1 µM). The cells were then subjected to Annexin – PI staining and flow cytometry analysis. The graph demonstrates the fold increase in the apoptotic rate of BMDM under each treatment, normalized to the non-treated cells (the average apoptotic rate of the non-treated cells is 42.9%). \*  $P < 0.05$ , \*\*  $P < 0.01$ . Values depict mean  $\pm$  SEM of 3 independent experiments. (b) Annexin – PI analysis of a representative experiment.

a dose dependent reduction of 48 and 76%, respectively, in the fraction of BMDM which are high expressers of F4/80 and CD11b (Figure 4a and b). Activation of BMDM with LPS 0.05 µg/ml prior to DEX 1 µM treatment did not protect the cells from the above mentioned DEX effect (Figure 4c). Namely, although LPS alone increased the fraction of high expressers of F4/80 and CD11b cells, the subsequent addition of DEX reduced this fraction to similar levels as displayed by cells treated by DEX alone. These data are in line with the premise that GCs (e.g. DEX), convey their anti-inflammatory effect on activated macrophages by reducing their overall levels of

activation and differentiation markers, impairing their ability to perform adhesive interactions.

**DEX-mediated reduction in mRNA and protein levels of the activation-associated chemokine, MIP-1 $\alpha$ .** The above findings of DEX-associated decrease in the fraction of naïve and LPS-activated high expressers of F4/80 and CD11b raised the question of whether and how DEX affects the extent of BMDM activation. We therefore determined the levels of secreted MIP-1 $\alpha$  in naïve and activated BMDM. MIP-1 $\alpha$  is a chemokine secreted from activated

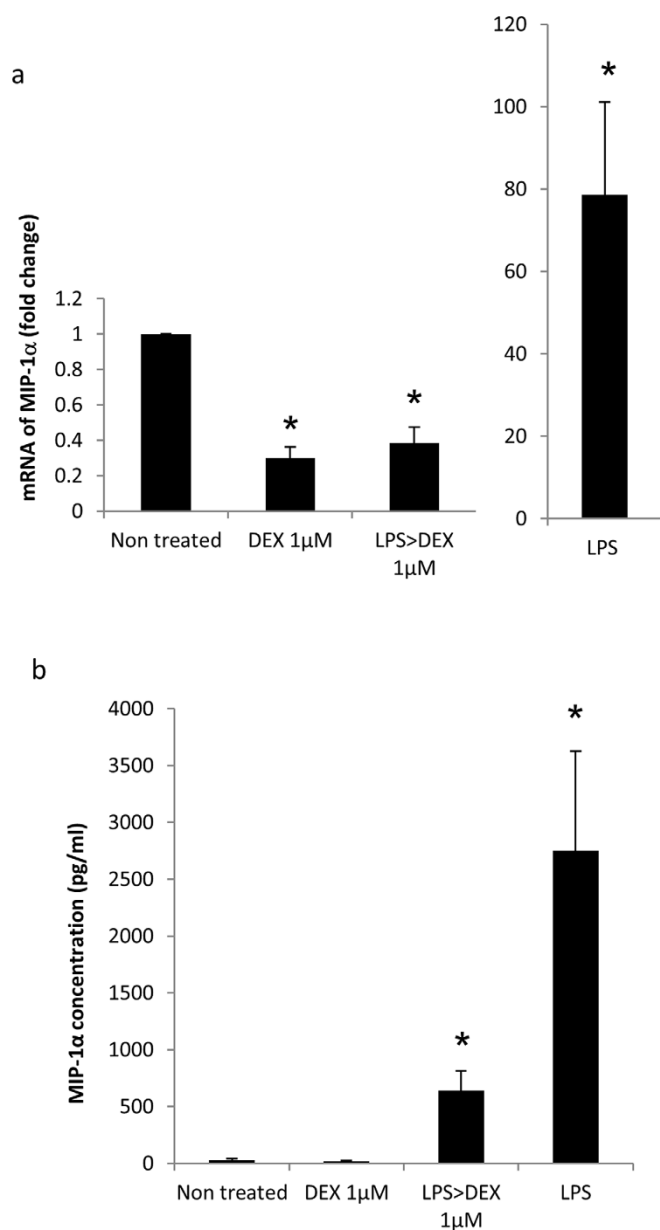


**Figure 4 | LPS does not prevent DEX-induced decrease in BMDM expressing high levels of F4/80 and CD11b.** (a) BMDM were incubated with DEX 0.1, 0.5 or 1  $\mu\text{M}$  for 24 hours and were subjected to FACS analysis (on live cells only) for detection of F4/80 and CD11b expression. The graph demonstrates the fraction of cells expressing both markers at a high level, normalized to the non-treated cells. \*  $P < 0.05$ , \*\*  $P < 0.01$ . Values depict mean  $\pm$  SEM of 4 independent experiments. (b) Analysis of a representative experiment. The boxed regions represent F4/80 and CD11b high expressers. (c) BMDM were incubated with culture medium alone or with LPS 0.05  $\mu\text{g}/\text{ml}$  for 3 hours. Then, the medium was replenished and DEX 1  $\mu\text{M}$  was added to the cells that were pre-incubated with LPS. LPS 0.05  $\mu\text{g}/\text{ml}$  or DMEM alone (non-treated) were added to the other plates. After 24 hours, the cells were subjected to FACS analysis for detection of F4/80 and CD11b expression. The graph demonstrates the fraction of cells expressing both markers at a high level, normalized to the non-treated cells. \*  $P < 0.05$ , \*\*  $P < 0.01$ . Values depict mean  $\pm$  SEM of 4 independent experiments.

macrophages<sup>29</sup>. Indeed, LPS-treated BMDM show an  $\sim 80$  fold elevation in the mRNA levels of MIP-1 $\alpha$  (Figure 5a). Incubation of BMDM with DEX 1  $\mu\text{M}$  reduced basal mRNA levels of MIP-1 $\alpha$  by 70%, yet treatment of BMDM with LPS prior to DEX did not prevent the DEX-associated reduction in MIP-1 $\alpha$  mRNA levels (Figure 5a). In addition to its effect on MIP-1 $\alpha$  mRNA levels, DEX also led to an accompanied reduction in the levels of MIP-1 $\alpha$  protein secreted from BMDM. Treatment of the cells with 1  $\mu\text{M}$  DEX resulted in MIP-1 $\alpha$  protein concentration of 17.9  $\text{pg}/\text{ml}$ , secreted by BMDM, while LPS treatment led to secreted levels of 2749  $\text{pg}/\text{ml}$  (Figure 5b). Incubation of BMDM with LPS, followed by DEX 1  $\mu\text{M}$  treatment, gave rise to levels of MIP-1 $\alpha$  secreted protein that were 4.3 times lower than those secreted after LPS treatment alone (Figure 5b). Therefore, pre-incubation of BMDM with LPS did not fully prevent the DEX effect on MIP-1 $\alpha$  protein levels. These data may suggest that the anti-inflammatory effects of GCs on activated macrophages include a reduction of the levels of secreted proteins, such as MIP-1 $\alpha$ , that take part in the generation and amplification of inflammation<sup>29</sup>.

**LPS may exert its protective activity against DEX-mediated death via GR $\beta$ .** The ability of LPS to prevent DEX-induced BMDM death

(Figures 2–3) prompted us to identify potential molecular players in this cascade. Binding of GC (e.g. DEX) to its intracellular receptor causes the translocation of GR $\alpha$  to the nucleus where it binds to specific DNA sequences. GR $\beta$  is a dominant-negative isoform of GR that regulates the activity of GR $\alpha$ <sup>30</sup>. Incubation of BMDM with DEX 1  $\mu\text{M}$  culminated in a 50% reduction in GR $\beta$  mRNA levels, suggesting a positive feedback mechanism of DEX on its own effects. On the other hand, a 6 hour treatment of BMDM with LPS 0.05  $\mu\text{g}/\text{ml}$  resulted in a 10.7 fold increase in GR $\beta$  mRNA levels as compared to non-treated cells (Figure 6a) and pre-treatment of BMDM with LPS overnight, prior to DEX 1  $\mu\text{M}$  treatment, gave rise to a 7.2 fold increase in GR $\beta$  mRNA levels. We also analyzed GR $\alpha$  mRNA levels under DEX and LPS treatments and our results showed that LPS 0.05  $\mu\text{g}/\text{ml}$  alone, induced a 5.8 increase in GR $\alpha$  levels, yet incubation with LPS followed by DEX 1  $\mu\text{M}$  treatment culminated in a 70% decrease in GR $\alpha$  levels, compared to the non-treated cells (Figure 6b). The ratio of GR $\beta$  to GR $\alpha$  under each treatment is presented by Figure 6c. These data suggest that LPS may protect BMDM from DEX-mediated death by affecting the levels of GR isoforms; inducing an increase in the dominant-negative GR $\beta$  as opposed to a decrease in the active GR $\alpha$  levels.



**Figure 5 | DEX-mediated reduction in mRNA and protein levels of the activation-associated chemokine, MIP-1 $\alpha$ .** BMDM were incubated for 6 hours with DEX at a concentration of 1  $\mu$ M, LPS 0.05  $\mu$ g/ml, LPS 0.05  $\mu$ g/ml overnight followed by 6 hours treatment with DEX 1  $\mu$ M or with DMEM alone, as a control (non-treated). (a) Levels of MIP-1 $\alpha$  mRNA were measured after the different treatments by q-PCR, relative to the expression of reference gene HPrt1. Data from 3 independent experiments was averaged and shown as normalized gene expression  $\pm$  SEM.

\*  $P < 0.05$ . (b) Supernatants from the cultures were collected and analyzed (in duplicates) for MIP-1 $\alpha$  protein levels by ELISA. The graph represents data from 5 independent experiments and values depict mean  $\pm$  SEM.

\*  $P < 0.05$ .

**Pre-incubation of BMDM with LPS, prior to DEX treatment, results in an increase in GILZ mRNA levels.** GILZ is a marker for GCs' activity, as its expression is most frequently upregulated by GCs. GILZ mediates some of the actions of GCs, in particular, those associated with anti-inflammation. In that respect, increased GILZ mRNA levels decrease macrophage sensitivity to LPS and to pro-inflammatory cytokines<sup>25</sup>. As a mediator of GCs' anti-inflammatory actions in BMDM, elevation of GILZ by DEX may explain the apoptotic death of these cells, induced by DEX.

Moreover, decreased levels of GILZ in BMDM incubated with LPS, prior to DEX treatment, may point to the mechanism by which pre-incubation with LPS prevents DEX-associated cell death. We thus questioned whether treatment of BMDM with DEX and LPS affects GILZ mRNA levels in these cells. Indeed, incubation of BMDM with DEX 1  $\mu$ M, led to a 10 fold increase in GILZ mRNA. Treatment of the cells with LPS 0.05  $\mu$ g/ml, led to a reduction of 92% in GILZ levels (Figure 7). However, pre-incubation of BMDM with LPS 0.05  $\mu$ g/ml overnight, prior to DEX treatment, attenuated the DEX-induced increase in GILZ mRNA, but still resulted in a 6 fold increase compared to the non-treated cells (Figure 7). As LPS did not prevent the DEX-mediated rise in GILZ, this may explain the inability of LPS to prevent certain DEX-mediated anti-inflammatory effects (Figure 8).

Taken together, LPS protective action on BMDM from DEX-induced death may operate by affecting the balance between the GR isoforms, and not by preventing the elevation in GILZ, mediated by DEX.

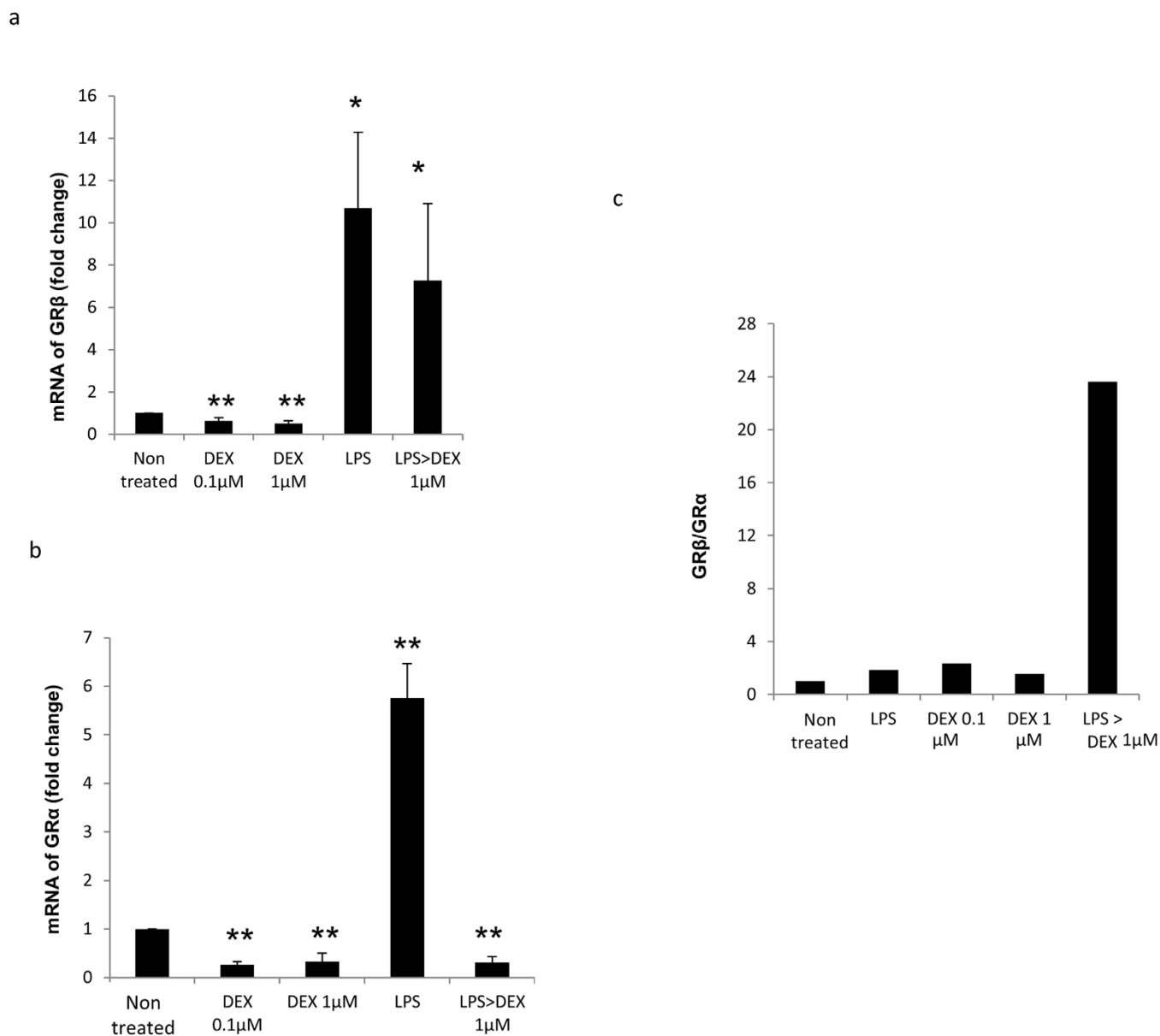
## Discussion

GCs exhibit a remarkably broad spectrum of activities and are known for their anti-inflammatory effects<sup>3</sup>. The earlier idea that GCs have uniform activities in tissues turned out to be simplistic and it is now well known that their effects depend on various contexts, including the location, specialization and the state of specific cells<sup>1</sup>. GCs, endogenous or exogenous, are important factors in sepsis<sup>18</sup>, multiple myeloma<sup>31</sup> and other malignant diseases<sup>32</sup>; all of these states are influenced by the status of macrophages responding to the disease process<sup>19,33,34</sup>.

In this study, we chose to assess the effects of DEX on naïve and activated macrophages in order to obtain a better understanding of GCs effects in various clinical states, characterized by both the activation of macrophages and their responsiveness to GCs. We focused on BMDM since bone marrow progenitors are considered to be the source for resident macrophages throughout the body<sup>12</sup>, although recent findings have called into question this dogma<sup>35</sup>. We show that LPS-activated BMDM respond to DEX differently than naïve BMDM and demonstrate resistance to some of DEX effects. Of note, in order to simplify interpretation of the results, we chose to perform the experiments in the absence of CSF-1, a known activator of macrophages, despite higher cell death, in order to highlight the differences between naïve and activated bone marrow-derived macrophages in their response to DEX. Activated BMDM do not go through apoptosis in the presence of DEX, unlike naïve BMDM. Yet, they respond to DEX by a decrease in the fraction of F4/80-CD11b high expressers and by a decrease in MIP-1 $\alpha$  mRNA and protein levels, similarly to naïve BMDM. These effects of DEX on LPS-activated BMDM cannot be attributed to the DEX-induced death of these cells because BMDM treated by LPS prior to DEX, are as viable as non-treated cells (Figures 2b and 3).

There are two major isoforms of GR: GR $\alpha$  and GR $\beta$ . Most data point to GR $\beta$  as an inhibitor of GR $\alpha$  activity, either through competition for coregulators or through formation of inactive  $\alpha/\beta$  heterodimers. GR $\beta$  resides in the nucleus of most cells, whereas GR $\alpha$  resides in the cytoplasm, undergoing nuclear translocation in response to GCs<sup>30</sup>. Thus, resistance to GCs could be explained by either loss of function mutations in the GR $\alpha$  gene or by increased expression of GR $\beta$ , which acts as a dominant-negative inhibitor of GR $\alpha$ <sup>30</sup>. Our results suggest that LPS-activated BMDM are resistant to some of DEX effects mainly by the second mechanism, since activation of BMDM with LPS results in elevation of GR $\beta$  mRNA in these macrophages (see Figure 8).

Although we show elevation of GR $\beta$  upon combined LPS and DEX treatment, it is not necessarily the only driving cause for the resistance of LPS-activated BMDM to DEX and we cannot rule out the participation of other factors in this effect. LPS and GCs trigger different, sometimes opposite, signaling pathways. Activated GR



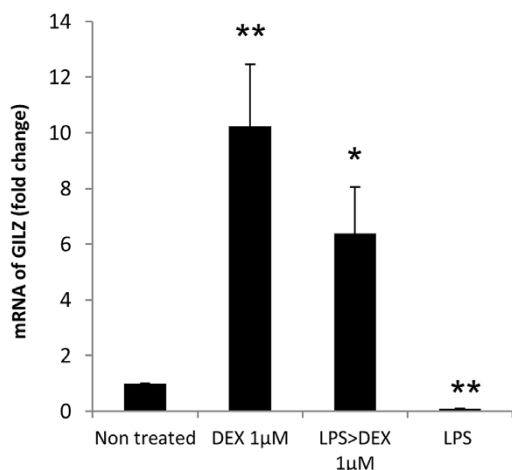
**Figure 6 | LPS affects the levels of GR isoforms.** BMDM were incubated for 6 hours with DEX at a concentration of 0.1 or 1  $\mu$ M, LPS 0.05  $\mu$ g/ml, DMEM culture medium alone, as a control (non-treated) or LPS 0.05  $\mu$ g/ml overnight followed by 6 hours treatment with DEX 1  $\mu$ M. mRNA levels of GR $\beta$  (a) or GR $\alpha$  (b) were measured after the different treatments by q-PCR, relative to the expression of reference gene HPrt1. Data from 4–6 independent experiments was averaged and shown as normalized gene expression  $\pm$  SEM. \*  $P < 0.05$ , \*\*  $P < 0.01$ . (c) A graph representing the ratio GR $\beta$ /GR $\alpha$  under each treatment.

interferes with the transcriptional activation function of nuclear factor kappa b (NF $\kappa$ B)<sup>36</sup>, while LPS induces the phosphorylation and translocation of NF $\kappa$ B to the nucleus<sup>37</sup>. GCs activate a phosphatase, responsible for the de-activation of mitogen activated protein kinase (MAPK) pathway<sup>38</sup> while LPS activates the MAPK pathway<sup>39</sup>. In addition, activated members of the MAPK pathway, such as c-Jun N-terminal kinase (JNK) phosphorylate GR at a specific site, ensuring the rapid inhibition of GR-dependent gene expression<sup>40</sup>. These molecular pathways were not examined by us in LPS-activated BMDM, incubated with DEX, and warrant further investigation.

Previous studies have shown that elevation of GR $\beta$  results in hypo-responsiveness of certain cells to GCs, yet only in rare cases there is no response at all<sup>41</sup>. Here, we demonstrate hypo-responsiveness of LPS-activated BMDM to DEX. It seems that different concentrations of DEX are needed in order to cause an identical percent of inhibition in naïve BMDM; 1  $\mu$ M DEX induces the death of 34% of naïve

BMDM, while at the same time period of 24 hours, the same concentration of DEX causes a reduction of 76% in the fraction of F4/80-CD11b high expressers and a decrease of 70% in MIP-1 $\alpha$  mRNA levels. These results imply that different phenotypes of BMDM demonstrate a variable sensitivity to DEX, and may also display a differential response to elevation of GR $\beta$ . This partial responsiveness of activated BMDM to DEX is also demonstrated in this study by the increase of GILZ mRNA levels despite the pre-incubation of BMDM with LPS, prior to DEX treatment. GILZ is considered to be the mediator of some of the anti-inflammatory actions of GCs<sup>25</sup> and thus its elevation may explain the DEX-mediated effects that were not prevented by activation of BMDM with LPS.

Certain patients develop resistance to GCs with time. For instance, clinical reports in pediatric inflammatory bowel disease patients have shown that the percent of ulcerative colitis patients, resistant to GCs treatment, increased from 21% to 29% in one year<sup>42</sup>. Such patients



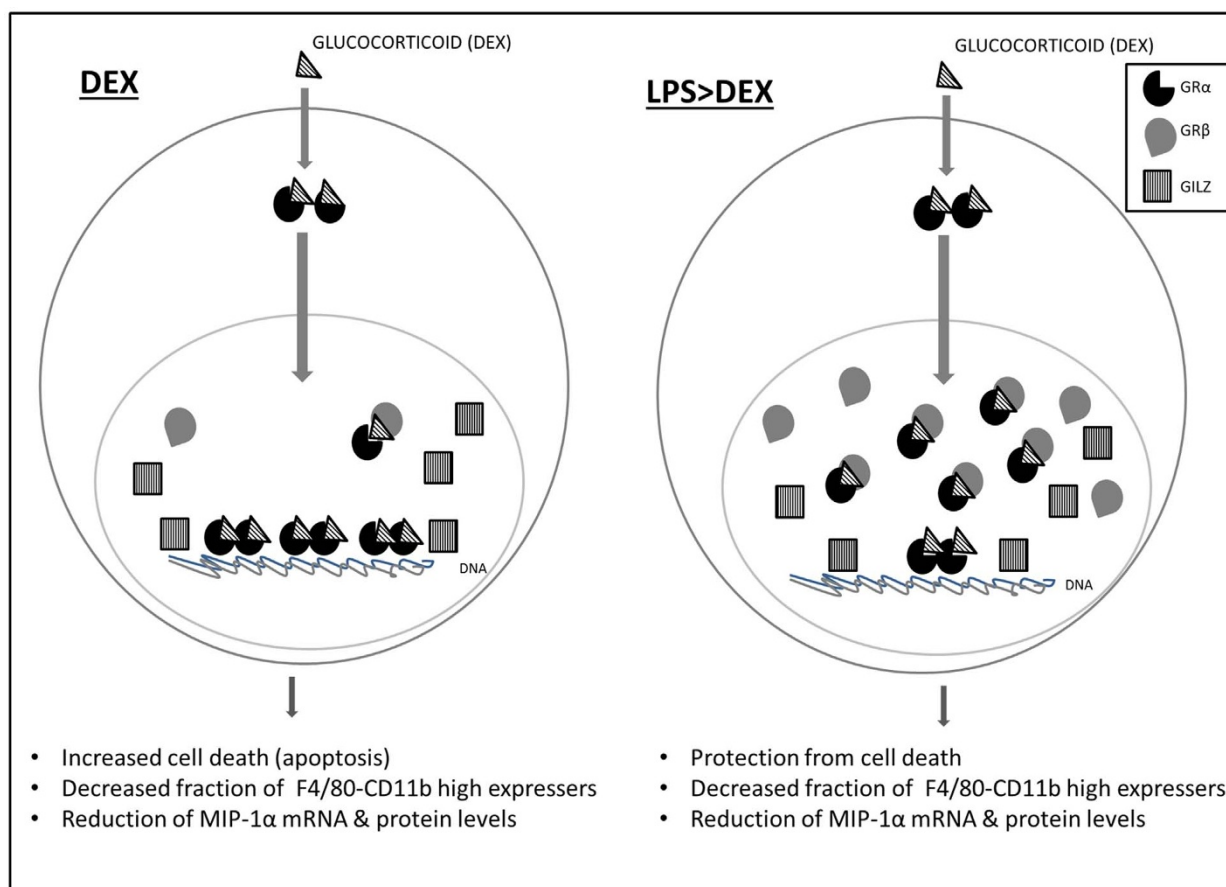
**Figure 7** | Pre-incubation of BMDM with LPS, prior to DEX treatment, results in an increase in GILZ mRNA levels. BMDM were incubated for 6 hours with DEX at a concentration of 1  $\mu$ M, LPS 0.05  $\mu$ g/ml, LPS 0.05  $\mu$ g/ml overnight followed by 6 hours treatment with DEX 1  $\mu$ M or with DMEM alone, as a control (non-treated). Levels of GILZ were measured after the different treatments by q-PCR, relative to the expression of reference gene HPrt1. Data from 4 independent experiments was averaged and shown as normalized gene expression  $\pm$  SEM. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

may benefit from the inhibition of GR $\beta$ , a potential inducer of this resistance. In that respect, it was shown that silencing GR $\beta$  by siRNA enables a more robust effect of GCs on human T cells<sup>43</sup>.

Our novel finding that LPS-activated BMDM express elevated GR $\beta$  mRNA levels paves the way for a better understanding of the mechanisms by which activation of macrophages renders these cells less responsive to DEX, as demonstrated by our study. These results may have implications in clinical states, characterized by the activation of macrophages, as the source of the symptoms, and by the choice of GCs as the principal treatment. For example, macrophages participate in the inflammation and joint destruction underlying rheumatoid arthritis and are among the most active cells in the patients' synovium<sup>22</sup>. Targeting GR $\beta$  in macrophages of rheumatoid arthritis patients might enable them to respond better to GCs and control the disease. In addition, certain patients with severe asthma respond poorly to GCs. Managing these patients is difficult as GCs are considered to be an effective therapy for asthma<sup>44</sup>. Asthmatic patients that show low response to GCs might thus benefit from GR $\beta$  inhibition in alveolar macrophages.

Targeting LPS-induced increase of GR $\beta$  in activated macrophages will not only improve patients' response to GCs but will also allow administration of lower GCs doses and by that, experience fewer side effects.

Addressing the response of macrophages to LPS, prior to DEX, in GR $\beta$  knock down conditions, is thus warranted. Future clinical



**Figure 8** | A model depicting LPS effect on the expression of GR isoforms and protective effects against DEX-mediated cell death. GR $\alpha$ , the active form of GR, resides in the cytoplasm. Interaction of GR $\alpha$  with its ligand induces its homodimerization, translocation to the nucleus and the subsequent binding to specific DNA sequences. Pre-incubation of BMDM with LPS, prior to DEX treatment, results in decreased levels of GR $\alpha$  and increased levels of GR $\beta$ , the nuclear dominant-negative isoform of GR. In that case, incubation of BMDM with DEX causes the translocation of GR $\alpha$  to the nucleus; however the LPS-induced relative high levels of GR $\beta$  and low levels of GR $\alpha$  lead to lower numbers of GR $\alpha$  homodimers and augment the formation of the non-active GR $\alpha$ -GR $\beta$  heterodimers. By this molecular cascade, LPS prevents some of DEX effects on BMDM but not the DEX-mediated increase in GILZ mRNA levels.





Table 1 | Primer sequences used for PCR amplification

Gene	Upstream primer	Downstream primer
GR $\beta$	5'-AAA GAG CTA GGA AAA GCC ATT GTC-3'	5'-CTG TCT TTG GGC TTT TGA GAT AGG-3'
GR $\alpha$	5'-GGC AGC GGT TTT ATC AAC TG-3'	5'-TCA ATA CTC ATG GAC TTA TCC AAA AA-3'
MIP-1 $\alpha$	5'-CCC AGC CAG GTG TCA TTT-3'	5'-CTG CCT CCA AGA CTC TCA GG-3'
HPrt1	5'-TCC TCC TCA GAC CGC TTT T-3'	5'-CCT GGT TCA TCA TCG CTA ATC-3'
GILZ	TaqMan gene expression assay	

studies should be considered to investigate the benefit of GR $\beta$  targeting in patients showing only a partial response to GCs.

## Methods

**Mice.** Female C57BL/6 and BALB/c mice, aged 8–12 weeks, were obtained from the Tel-Aviv University Animal Breeding Center and were used for the generation of BMDM. Mouse handling and the experimental procedures were approved by the Institutional Animal Care and Use Committee of the Tel-Aviv University (permit numbers: M-11-040 and M-10-044) and were performed in accordance with the approved guidelines.

**Reagents.** DEX powder, suitable for cell culture (Sigma, Rehovot, Israel) was reconstituted in 100% ethanol and kept at  $-20^{\circ}\text{C}$  at a stock concentration of 0.1 mM. LPS from the E. coli strain 0127:B8 (Sigma) was reconstituted in sterile double distilled water (DDW) at 1 mg/ml stock solution and kept at  $-20^{\circ}\text{C}$ . Staurosporine (STS) from Streptomyces sp. (Sigma) was purchased as a ready-made solution of 1 mM STS in dimethyl sulfoxide (DMSO) and was stored at  $-20^{\circ}\text{C}$ .

**Generation of BMDM.** Preparation of bone marrow cell cultures was based on the protocol described by Boltz-Nitulescu *et al.*<sup>45</sup>. Briefly, bone marrow cells were isolated from femurs and tibias. Cells were then incubated for 7 days, the period required for bone marrow cells to differentiate to macrophages<sup>45</sup> in Dulbecco's Modified Eagle medium (DMEM), containing 10% heat-inactivated fetal bovine serum and supplemented with 30% L-conditioned medium. The medium was replenished on the 4th day. The non-adherent cells were purged and the macrophages (adherent cells) were collected by treatment with 15 mM EDTA in PBS for 10 minutes at  $4^{\circ}\text{C}$ . The population of macrophages (BMDM), as assessed by expression of the F4/80 surface molecule by FACS analysis, was typically 97% pure. The BMDM were washed, and cultured for 24–72 hours in DMEM containing the different reagents as indicated (i.e. DEX, LPS, and STS).

**Cell viability assays.** BMDM were seeded in triplicate into 96-well, flat-bottom culture plates ( $2 \times 10^4$  per well, as described by<sup>46</sup>) and cultured in the presence of DEX 0.1–1  $\mu\text{M}$ , LPS 0.05  $\mu\text{g}/\text{ml}$  or the combination of both as indicated. Untreated cells served as control. After incubation with DEX and LPS, cell viability was determined using the colorimetric methylthiazolotetrazolium bromide (MTT) assay<sup>47</sup>. Briefly, MTT was added to a final concentration of 5  $\mu\text{g}/\text{ml}$  in each well and cells were further incubated for 3 hours at  $37^{\circ}\text{C}$ . After complete solubilization of the dye by acid/alcohol, plates were read at 570 nm in an ELISA reader (SpectraMAX 190 microplate reader).

For visualization of the cells, BMDM ( $1.2 \times 10^6$  cells in a 60 mm Falcon culture plate) were incubated with DEX 1  $\mu\text{M}$  or DMEM alone for 24 hours. The cells were then captured using bright-field microscopy (Nikon eclipse Ti-S microscope and the integrated NIS-Elements imaging software).

**Annexin-PI staining.** Annexin-PI staining of BMDM was performed using an apoptosis kit (MEBCYTO® Apoptosis kit, MBL, Massachusetts, USA) as described by the manufacturer. Briefly, BMDM ( $1.2 \times 10^6$  cells in a 60 mm Falcon culture plate) were incubated overnight with LPS 0.05  $\mu\text{g}/\text{ml}$  or in medium only and then treated with STS 100 nM, DEX 0.1–1  $\mu\text{M}$  or LPS 0.05  $\mu\text{g}/\text{ml}$  for 4 hours, after which the adherent and non-adherent cells were collected and centrifuged. Fluorescent-labeled antibodies were added to each test tube and the analysis of fluorescence intensity was accomplished by flow cytometry (FACSort flow cytometer, Becton-Dickinson, New Jersey, USA). Results were analyzed using Cyflog software.

**Detection of surface molecule expression.** The following fluorescence-conjugated antibodies directed to cell surface markers were used: FITC conjugated anti-F4/80 (eBioscience, San Diego, USA), PE conjugated anti-CD11b (Milteny Biotec, San Diego, USA) and the relevant isotype controls (eBioscience).  $1.2 \times 10^6$  cells per treatment were seeded in 60 mm Falcon culture plates for 24 hours in the presence or absence of DEX 0.1–1  $\mu\text{M}$  or LPS 0.05  $\mu\text{g}/\text{ml}$ . BMDM were then collected with 15 mM EDTA and incubated, protected from light, with the fluorescence-conjugated antibodies for 30 minutes at  $4^{\circ}\text{C}$ . Cells were then analyzed by a FACSort flow cytometer (Becton-Dickinson). Ten thousand total events were collected and gated by the expression of F4/80 and CD11b. Results were analyzed using Cyflog software.

**Real-time RT PCR.** To quantify mRNA expression levels of macrophage inflammatory protein 1 alpha (MIP-1 $\alpha$ ), GILZ, GR $\alpha$  and GR $\beta$ , we used quantitative

reverse transcription PCR. After incubation of BMDM at the different treatment conditions ( $3 \times 10^6$  cells per treatment in 60 mm Falcon culture plates), total RNA from the cells was isolated using 5 prime (Maryland, USA) PerfectPure RNA tissue kit according to the manufacturer's protocol. cDNA was prepared using high capacity cDNA reverse transcription kit (Applied Biosystems, California, USA). Real-time quantitative PCR was performed using the StepOnePlus Real-time PCR system and the SYBR Green PCR Master Mix (Agentek, Tel-Aviv, Israel) or the TaqMan gene expression assay (Applied Biosystems). HPrt1 was used as an inner standardization control. Primer sequences used for amplification are depicted in table 1.

**ELISA.** BMDM ( $10^5$  cells in 500  $\mu\text{l}$  DMEM per well) were seeded in 24-well, flat-bottom culture plates and cultured for 6 hours in the presence of DEX 0.1–1  $\mu\text{M}$ , LPS 0.05  $\mu\text{g}/\text{ml}$  or LPS 0.05  $\mu\text{g}/\text{ml}$  overnight followed by DEX 1  $\mu\text{M}$  for 6 hours.

Supernatants from the cultures were then collected and analyzed for MIP-1 $\alpha$  protein levels. Protein levels were determined by ELISA kit (Peprotech, Rehovot, Israel), according to the manufacturer's instructions. Plates were read at 570 nm in an ELISA reader (SpectraMAX 190 microplate reader).

**Statistical analysis.** Since we considered each treatment as a distinct sample and the response of the cells to each treatment was independent of their response to other treatments and because the distributions of the variable of interest are normal, comparisons between data of the treated samples versus controls were performed using Student's t-test. P values less than 0.05 were considered statistically significant.

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## Author contributions

D.N. was the principal investigator; Y.O.H. planned and performed the research, analyzed the data and wrote the paper; N.D.U. set up the BMDM culture system and interpreted data; M.C.S. analyzed data, planned experiments and reviewed the manuscript; M.M. provided clinical input.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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