INHIBITORY EFFECTS OF GLUCOCORTICOID ON IMMUNOCYTES

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ABSTRACT

Effective anti-inflammatory and immunosuppressive drugs, glucocorticoids (GCs) influence both innate and adaptive immunity via the intracellular glucocorticoid receptor (GR). Although the suppressive effects on immune cells have been hypothesized, they have not been rigorously investigated. In this study, we analyzed how dexamethasone (DEX) treatment suppressed lymphoid and myeloid cell proliferation in Balb/c mice. High dosages of DEX were shown to reduce the number of immunocytes and to dampen their activity. Magnificently, high doses of DEX lowered the percentages of macrophages, DC cells, and monocytes by over 90%, while it reduced the number of B cells by around 70%. Furthermore, we found that DEX inhibits immune cells by binding to GR with great affinity. We conclude that DEX therapies affect a variety of immune cells, both lymphoid and myeloid cells, either by depleting them or by down-regulating their immune function, presumably via the GR signaling pathway. This discovery may increase the clinical value of DEX for achieving temporary immune insufficiency.

INTRODUCTION

Immunity suppression by glucocorticoids (GCs) is well established. The hypothalamic-pituitary-adrenal (HPA) axis regulates GH synthesis and secretion in circadian and ultradian rhythms (1). Researchers have found that GCs have powerful anti-inflammatory and immunosuppressive effects (2–5). In the therapy of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis, and eczema, glucocorticoids, both natural and synthetic, continue to play a pivotal role. They're also employed in the treatment of certain forms of leukemia and to mitigate the negative effects of chemotherapy and immunosuppressive drugs after an organ transplant (4, 6, 7). GCs cause systemic lymphodepletion by primarily attaching to the glucocorticoid receptor (GR) of immunocytes, which is different from the targeted depletion induced by antibodies. To accomplish this, GCs create a complex with the intracellular GR, inducing a conformational alteration in the receptor that permits translocation to the nucleus, where it binds to chromatin. The immunocytes are suppressed as a result of the activation of several genomic and non-genomic processes that either promote or inhibit gene transcription (8–10). GCs regulate signaling pathways and elicit immediate effects by interacting with enzymes and other cell proteins (11). There have been a number of studies on the anti-inflammatory and immunomodulatory effects of GCs in the 70 years since their discovery by Philip Hench and Edward Kendall, but the molecular processes are still not fully understood. Despite widespread application, the mechanism of action on immune cells of the steroid dexamethasone (DEX) remains elusive. A decrease in major histocompatibility

complex (MHC) class II and costimulatory molecules in DC after exposure to DEX lowers their ability to activate T cells (12), and an inhibition of the T-cell receptor (TCR)-mediated signal transmission pathway follows (13). However, the effects of GCs on B cells have not been thoroughly studied, despite their widespread usage in B cell-dependent illnesses. Some studies showed that GCs regulate apoptosis via lowering levels of the B-cell lymphoma/leukemia-2 (Bcl-2) protein (14). Studies on the effects of GCs on B cells have yielded mixed results in the past, especially on the dosage relationship between GCs and B cell activity and antibody synthesis or levels (15). Another function of GCs is to dampen the activity of innate immunity cells; for example, dexamethasone suppresses both immature and adult dendritic cells (DCs) by interfering with their development. By modulating monocyte-macrophages, GCs can control overactive immune cells that arise during inflammation (16). Curiously, research has shown that glucocorticoids can increase neutrophil survival and growth by promoting the expression of anti-apoptotic proteins like Bcl-2 family members (17).

The coronavirus disease pandemic of 2019 (COVID-19) has recently threatened global public health. Up to 12% of COVID-19 hospitalized patients may require invasive mechanical ventilation (18), with acute respiratory distress syndrome (ARDS) being the most common diagnosis (19). Furthermore, in these people, COVID-19 frequently manifests as an uncontrolled inflammatory state, which can lead to multiple organ failure (20). The severity of this reaction may be lessened by corticosteroids (21). The outcomes of a randomized clinical study involving patients

with COVID-19 showed that dexamethasone use reduced mortality in hospitalized patients who required additional oxygen or mechanical ventilation (22). However, in other viral studies, GCs' immunosuppressive properties appear to cause patients' viral loads to increase (23, 24). Therefore, the use of corticosteroids for severe cases of COVID-19 must be carefully considered, as there is evidence to suggest that they may have both beneficial and detrimental effects on the patient's condition. As such, further studies are needed to better understand the impact of corticosteroids on viral infections and their efficacy in treating COVID-19.

The effects of GCs on the immune system's regulation are nuanced. DEX, a synthetic glucocorticoid, was chosen to evaluate the impact on several types of immune cells in order to systematically investigate the immunosuppressive pathways. Our results demonstrated that DEX's effects varied among the various types of cells that constitute the immune system. The effects on B cells and macrophages were dose-dependent, and we showed that DEX had a high affinity for GR in the afflicted immune cells, providing a mechanism for its immunosuppressive features.

MATERIALS AND METHODS

Mice and cell lines

Female Balb/c mice were housed and treated according to the guidelines of the Institutional Animal Care and Use Committee of Fudan University in China. All mice were 6–8 weeks old and age-matched. The Committee on Experimental Animals of SHMC approved all experiments with the reference number 202012037S.

Reagents

Dexamethasone (DEX) was purchased from Xi’an Reyphon Pharmaceutical Co., Ltd. (Xi'an, China). Mifepristone (RU 486) was purchased from MCE (NJ, USA). BioLegend (CA, USA) provided fluorescently labeled antibodies against CD45 (30-F11), CD4 (GK1.5), CD8 (53-6.7), CD25 (3C7), CD11b (M1/70), F4/80, Ly6C (HK1.4), CD49b (DX5), and B220 (RA3-6B2). BD Bioscience (CA, USA) provided CD11c (HL3) and Ly6G (1A8).

Treatments

The mice received intraperitoneal injections of either 100 micrograms of DEX dissolved in phosphate-buffered saline (PBS) into each animal or a vehicle for a total of three days in a row. The collection of peripheral blood mononuclear cells (PBMC) took place 24 hours following the final injection of DEX. In order to determine the effect of dexamethasone dosage on immune cells, the doses per mouse were increased to 5 mg/kg for the high dose and 1.25 mg/kg for the low dose. Eight micrograms of RU486 were dissolved in 20 microliters of dimethyl sulfoxide (DMSO), then 200 microliters of corn oil (MCE, NJ, USA) were added, and the mixture was vortexed. Mice were given an intraperitoneal injection of maize oil containing 400 mg of RU486 per kg of body weight every 12 hours before each DEX dosage. PBMC were collected 72 hours after those treatments for FCM analysis (Supplementary 1A).

Flow cytometry

Whole blood that had been pretreated with an anticoagulant was then treated with red cell lysis buffer for flow cytometry (FCM) analysis. The viability dye eflour780 was added to phosphate-buffered saline (PBS) for 15 minutes at room temperature, and the

single-cell suspensions were washed twice with PBS containing 2% fetal bovine serum. Cells were fixed with a commercial transcription factor staining buffer solution and then stained for 15 minutes at room temperature with fluorochrome-tagged antibodies to identify cell surface antigens (eBioscience, Oregon, USA). All stained samples were examined on an LSR Fortessa and analyzed with FlowJo software (BD, CA, USA).

Statistical analysis

The student's t-test was performed in order to compare continuous variables. The data were analyzed using one-way ANOVA for single-factor comparisons across groups and two-way ANOVA for comparisons involving multiple dimensions. The data are shown either as the mean plus standard error or as a percentage using Prism 7 (GraphPad Software). P values of less than 0.05 were considered statistically significant.

RESULTS

Immunosuppressive Effects of Dexamethasone on Lymphoid and Myeloid Cells

To examine the immunosuppressive effects of DEX on myeloid-derived innate immune cells, Balb/c mice were intraperitoneally (ip) administered 5 mg/kg DEX or a vehicle (PBS) daily for 3 days (Fig. 1A). FACS examination of PBMC taken on the fourth day (sFig. 1A and sFig. 2A) demonstrated that the numbers of these cells were significantly decreased, with the exception of neutrophils (Figs. 1B, 1C, and s1B). Further analysis revealed that DEX triggered the greatest decreases in the proportions of macrophages (95%), dendritic cells (90%), and monocytes (86%), with a lesser

reduction in the proportions of NK and NKT cells (Fig. 1D). In contrast, the number of neutrophils expanded, indicating a distinct regulating mechanism.

Due to the short half-life of DEX in circulation (two to three days), we sought to see if its effects might persist beyond cessation. The administrations were stopped after 21 days of daily administration, and the PBMC were examined. The proportions of these affected myeloid-derived innate immune cells went back to normal 5 days after the medication was stopped (Figs. 2A–2F).

Immunosuppressive Effects of Dexamethasone on Lymphoid-Derived Cells

The immunosuppressive effects of DEX on lymphoid-derived cells, namely T and B cells, were then evaluated. Balb/c mice were treated as depicted in Figure 1A, and FACS analysis was done to determine if CD3+, CD4+, CD8+, CD4+CD25+ T cells, and B220+ B cells were altered (sFigs 1A and 2A). All studied cells were shown to be inhibited by DEX (Fig. 3A and sFig. 2B) with 72%, 40%, 45%, 40%, and 60% reductions, respectively (Fig. 3B). In addition, we determined the persistence of the inhibitory impact on each of these cell types following drug removal. Unlike myeloid-derived cells, lymphoid-derived cells took up to 30 days to get back to normal after the medication was stopped (Figs. 4A–4E).

Immunosuppressive Effects Are Related to the Dose of Dexamethasone

DEX is used in a variety of dosages to minimize the negative effects of chemotherapy in advanced cancer patients (7). Balb/c mice were administered high (5 mg/kg) and low (1.25 mg/kg) dosages of DEX in order to investigate the immunoregulatory effects of DEX on the immune system. We noticed that a smaller dosage of DEX

lowered immune cell numbers less than a higher dose (Figs. 5A, 5D, and sFig. 3A). Nonetheless, macrophages and NKT cells expanded in response to a modest dosage of DEX (Figs. 5B-5D, sFigs. 3A, and 3B).

Dexamethasone suppresses the immune system by strongly binding to GR.

Since glucocorticoid-mediated immune regulation is attributed to glucocorticoid receptor-induced changes in gene expressions, we examined whether the immunoregulatory effects of DEX were connected to GR signaling. Mifepristone (RU486), a progesterone receptor (PR) and GR antagonist with low affinity for GR, was given intraperitoneally 12 hours before each DEX injection. PBMC were obtained 72 hours after treatment (sFigs. 3A, 3B, and 4A). As a GR-blocker, we noticed that RU486 eliminated the immunological modulation by low-dose DEX. Normal levels of T and NK cells have been restored (Fig. 6A). When a greater dosage of DEX, 5 mg/kg, was administered, a more powerful immunosuppressive effect was observed, and the efficacy of RU486 was diminished (Fig. 6B). These results demonstrated that DEX was competitively bound to GR with RU486 and that the affinity increased with increasing dosage.

DISCUSSION

Both lymphoid and myeloid cell subsets in peripheral blood mononuclear cells (PBMC) were downregulated by DEX's immunoregulatory actions, as shown by our findings. Our study utilized dosages of DEX that approximate those used in clinical practice (18), whose effects on DCs, monocytes, macrophages, NK cells, T cells, and

B cells were profoundly suppressive.

Glucocorticoids transmit signals through the glucocorticoid receptor (GR), a member of the superfamily of nuclear receptors that affects genomic and non-genomic regulatory pathways in almost every mammalian tissue. Non-genomic regulation produces apoptosis by an emergent mechanism, while genomic regulation is receptor-dependent (19, 20). It has been shown that glucocorticoids might produce variable biological effects in dendritic cells dependent on GR isoforms to inhibit DC maturation and activity in one manner (21) and enhance the development of "tolerogenic" DCs in another. Tolerance results from the downregulation of MHC class II, costimulatory molecules (such as CD80 and CD86), and pro-inflammatory cytokines (such as IL12 and TNF), which induce Treg (22). Glucocorticoids also stop T cells from becoming active by interfering with TCR signaling and by stopping T cells from making interleukin (IL)-2 by binding to GR (23).

Concerning B cells, immature B cells are more susceptible to glucocorticoid-induced apoptosis than adult B cells (14, 24), although further information is required about the effects of GCs on humoral immunity. Our study demonstrates that a high-affinity binding of GR by DEX may suppress immune cells, but it also demonstrates that using RU486 to inhibit the GR can reverse this effect and restore immune cells to a normal level.

This work has concentrated on the landscape of immunosuppression as contributed by crucial immune cell types, especially those with involvement in adaptive immunity, despite the fact that there have been numerous studies and clinical reports of

glucocorticoid effects on immunity during the past 60 years. Surprisingly, we found that modest dosages of DEX might enhance the ratio of macrophages, whereas large doses of DEX could decrease the number of macrophages. At low concentrations, GCs were reported to positively regulate NLRP3, a component of the inflammasome complex in macrophages, and thereby augment the pro-inflammatory response. This double-edged sword effect of GCs on macrophages had not been reported previously and suggests a complex regulation via GR signaling (25). Nanomolar quantities of GC may have an immunostimulatory impact on many macrophage processes such as adhesion, chemotaxis, phagocytosis, and cytokine generation, providing an alternate explanation (26). A macrophage's pattern-recognition receptors, such as Toll-like receptors, recognize infectious pathogens and damaging signals, triggering the inflammasome complex and the release of inflammatory cytokines, making it an important link in the chain connecting innate and adaptive immune activation (27). The purpose of this chain reaction is to stimulate adaptive immune responses. When the body detects an infection, especially one triggered by bacteria, the first line of defense is an inflammatory reaction, which is initiated by neutrophils. In contrast to the other immune cells, we discovered that neutrophils were expanded by the DEX administration. Recent investigations have shown that neutrophils are the cells least responsive to GCs and most resistant to DEX-induced apoptosis, a phenomenon that may be explained by the overexpression of anti-apoptotic proteins such as Mcl-1 and XIAP in neutrophils (28).

Overall, our results suggest that high-dose DEX therapy has a deleterious effect on the

populations of myeloid and lymphoid cells other than neutrophils. Even though myeloid cells were back to normal after 5 days of stopping DEX treatment, lymphoid cells were adversely impacted by the drug and needed much longer to recover. These results will allow for a deeper appreciation for the nuanced ways in which GCs affect immunological responses and a more cost-effective use of the medicine to accomplish systemic inhibition of the immune system.

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Conflict of interest statement

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

Bin Wang and Shuting Wu conceived, directed the experiments with input from all authors. Material preparation, data collection and analysis were performed by Shuting Wu. Shuting Wu, Shushu Zhao, Gan Zhao and Yiwei Zhong performed data analysis and interpretation. The first draft of the manuscript was written by Shuting Wu and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The data generated in this study are available upon request from the corresponding author.

Ethics approval

This study was performed in line with the guidelines of the Institutional Animal Care and Use Committee of the Fudan University in China. All experiments were approved by the Committee of Experimental Animals of SHMC with the reference number 202012037S.

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FIGURE LEGENDS

Figure 1. DEX's immunoregulatory effects on myeloid-derived innate immune cells

(A) Diagram of DEX treatment in mice. PBMC were isolated at 96 h after treatment with DEX and analyzed by flow cytometry (**B, C)**. Antibodies to Ly6G, CD11c, F4/80, Ly6C, and CD49b were used to identify neutrophils, DC, macrophages, monocytes, and NK cells. Antibodies to CD3 and CD49b were used to identify NKT cells. The results are presented in percentages of $CD11b^+$ cells (**B**) and $CD45^+$cells (**C**); (**D**) shows the proportion of the decline in each innate immune cell type. SEM is indicated by the error bars, **** P 0.0001, and n = 5. Statistical significance was determined with an unpaired, two-tailed T-test. The data are representative of two independent experiments.

Figure 2. Recovery curves of innate immune cells after drug withdrawal

PBMC were isolated on days 5, 9, 15, 20, and 30 after DEX withdrawal and analyzed by flow cytometry. Antibodies to Ly6G, CD11c, F4/80, Ly6C, and CD49b were used to identify neutrophils, DC cells, macrophages, monocytes, and NK cells; antibodies to CD3 and CD49b were used to identify NKT cells. Results from the DEX treatment group are shown in blue, and results from the PBS vehicle group are shown in red. Shown are the percentages of $CD11b^+$ neutrophil cells (**A**), $CD11b^+$ DC cells (**B**), $CD11b^+$ macrophages (**C**), $CD11b^+$ monocytes (**D**), $CD45^+$ NK cells (**E**), and $CD45^+$ NKT cells (**F**) SEM is indicated by the error bars, and **** P 0.0001.

Statistical significance was determined with an unpaired, two-tailed T-test. The data are representative of two independent experiments.

Figure 3. DEX's immunoregulatory effects on lymphatic cells

PBMC were isolated 96 hours after treatment with 5 mg/kg DEX daily and analyzed by flow cytometry. B cells were identified using an anti-B220 antibody. The results are presented as percentages of $CD45^+$ cells (**A**) and the percentage of the decline in each lymphatic immune cell type (**B**). SEM is indicated by the error bars, **** P 0.0001, and n = 5. Statistical significance was determined with an unpaired, two-tailed T-test. The data are representative of two independent experiments.

Figure 4: Lymphatic cell recovery curves after drug withdrawal.

PBMC were isolated on days 9, 20, and 30 after DEX withdrawal, and T cell subsets in $CD45^+$ cells were analyzed by flow cytometry. B cells were identified using an anti-B220 antibody. Results from the DEX treatment group are shown in blue, and results from the PBS vehicle group are shown in red. Shown are the percentages of $CD3^+$T cells (**A)**, $CD4^+$T cells (**B)**, $CD4^+CD25^+$T T cells (C**)**, $CD8^+$T T cells (D**)**, and $B220^+$B cells (**E)**. The results are presented as percentages of $CD45^+$ cells. SEM is indicated by the error bars, and **** P 0.0001. Statistical significance was determined with an unpaired, two-tailed T-test. The data are representative of two independent experiments.

Figure 5. Immunosuppressive effects under different doses of DEX

PBMC were isolated on day 8 after treatment with different doses of DEX and analyzed by flow cytometry. Antibodies to Ly6G, CD11c, F4/80, Ly6C, CD49b, CD3, Foxp3, and B220 were used to identify neutrophils, DC cells, macrophages, monocytes, NK, T, Treg, and B cells, respectively. Antibodies to CD3 and CD49b were used to identify NKT cells. The results are presented as percentages of $CD11b^{+}$cells (**A**) and $CD45^{+}$ cells (**B, C),** and the proportion of the decline in each immune cell type (D). SEM is indicated by the error bars, and **** P 0.0001. Statistical significance was determined with an unpaired one-way ANOVA test.

Figure 6. The high-affinity binding of DEX to GR reduces immune cells.

PBMC were isolated and analyzed by flow cytometry 3 days after treatment with 1.25 mg/kg DEX (**A**) or 5 mg/kg DEX (**B**). Antibodies to B220, Foxp3, and CD49b were used to identify B, Treg, and NK cells. Antibodies to CD3 and CD49b were used to identify NKT cells. The results are presented as percentages of $CD45^{+}$ cells. SEM is indicated by the error bars, and **** P 0.0001. Statistical significance was determined with an unpaired one-way ANOVA test.

Figure 1

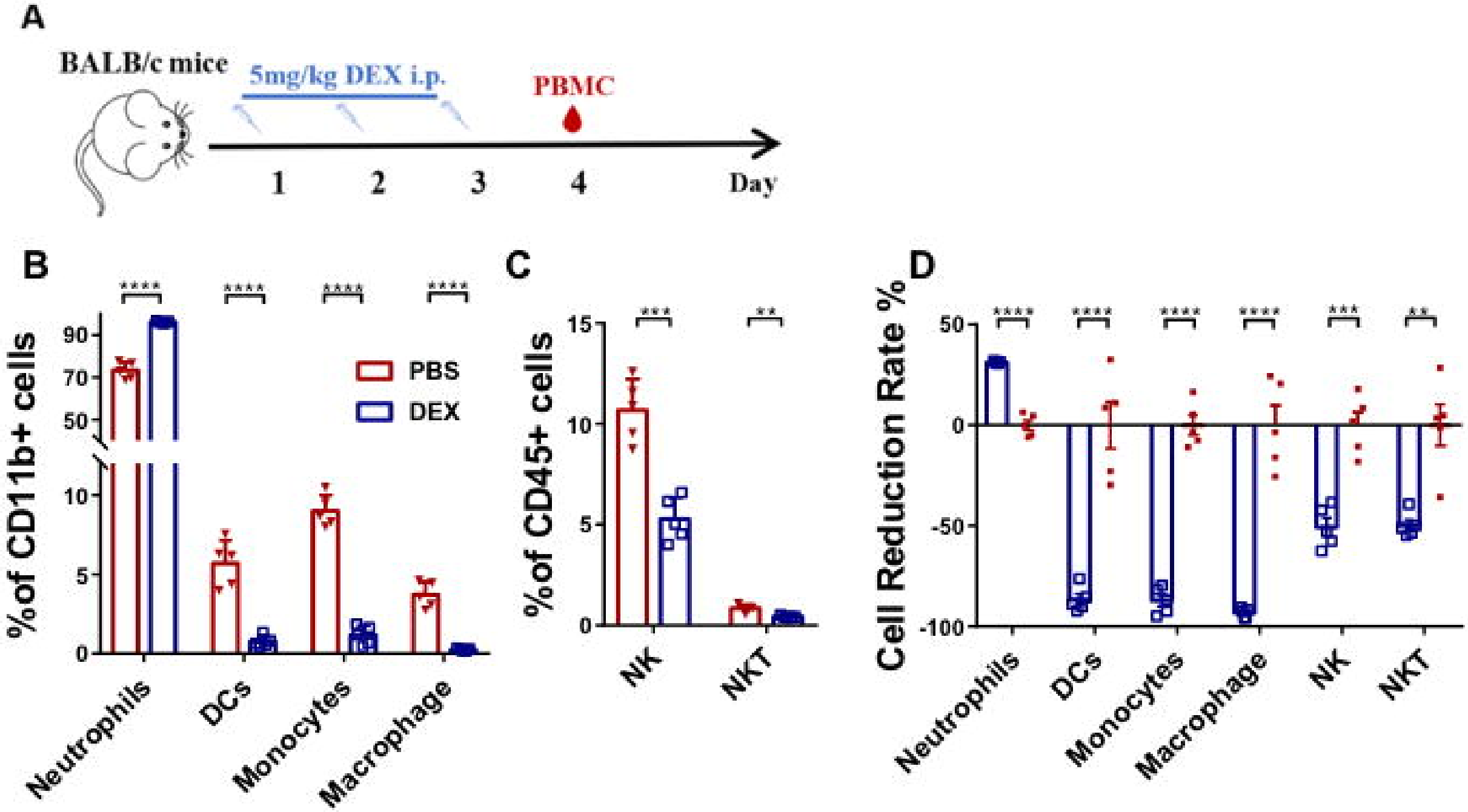
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Figure 2

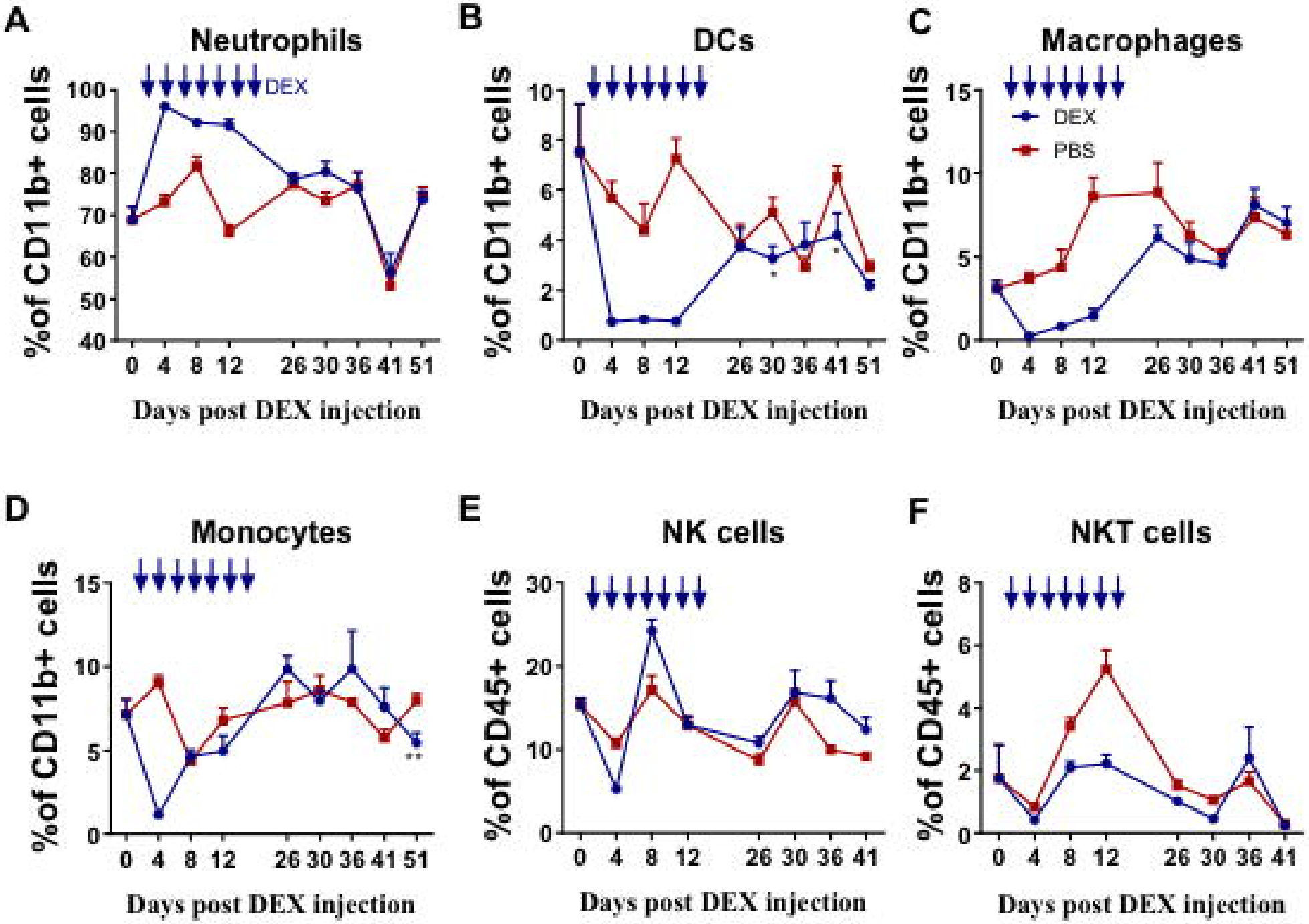
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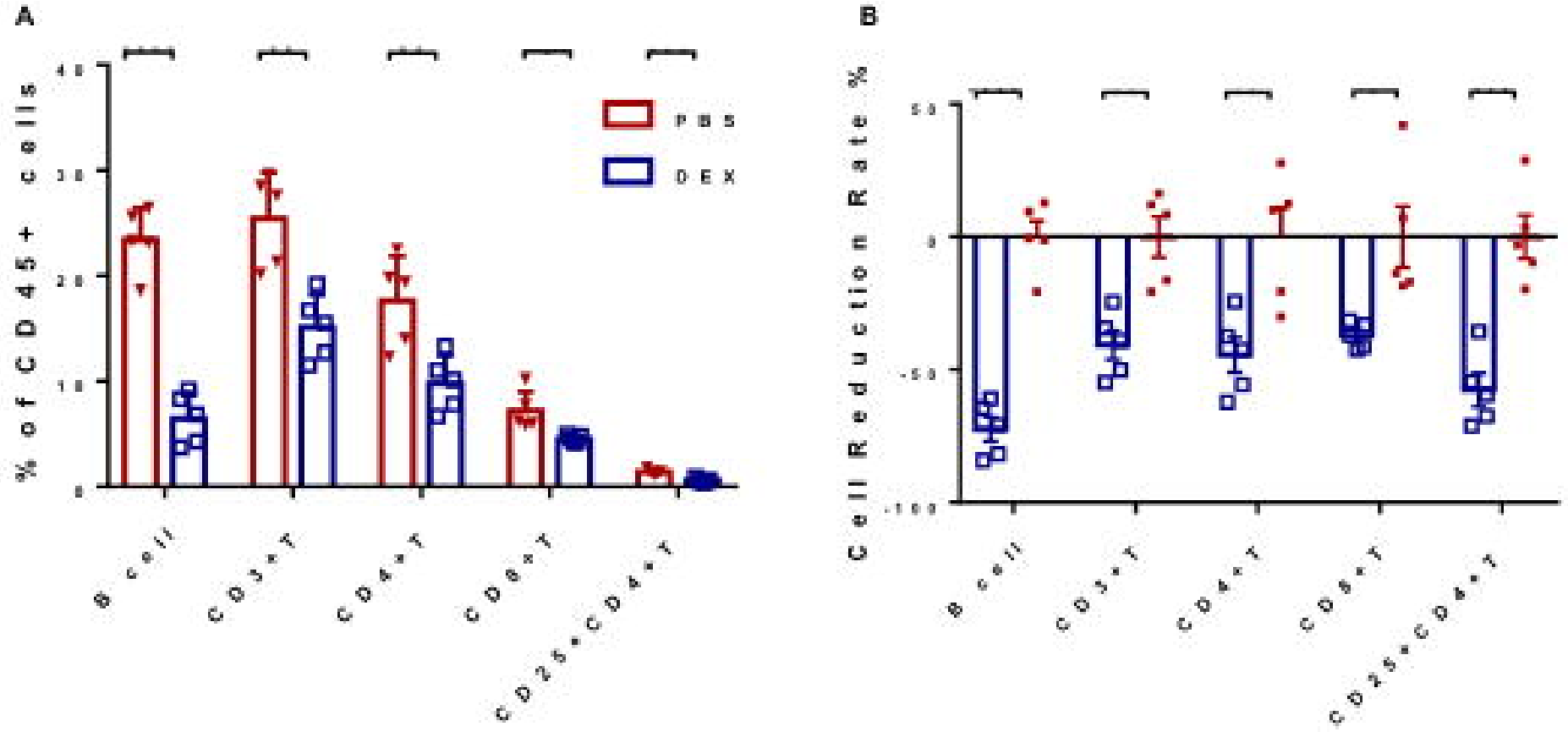
Figure 3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% of CD45+ cells
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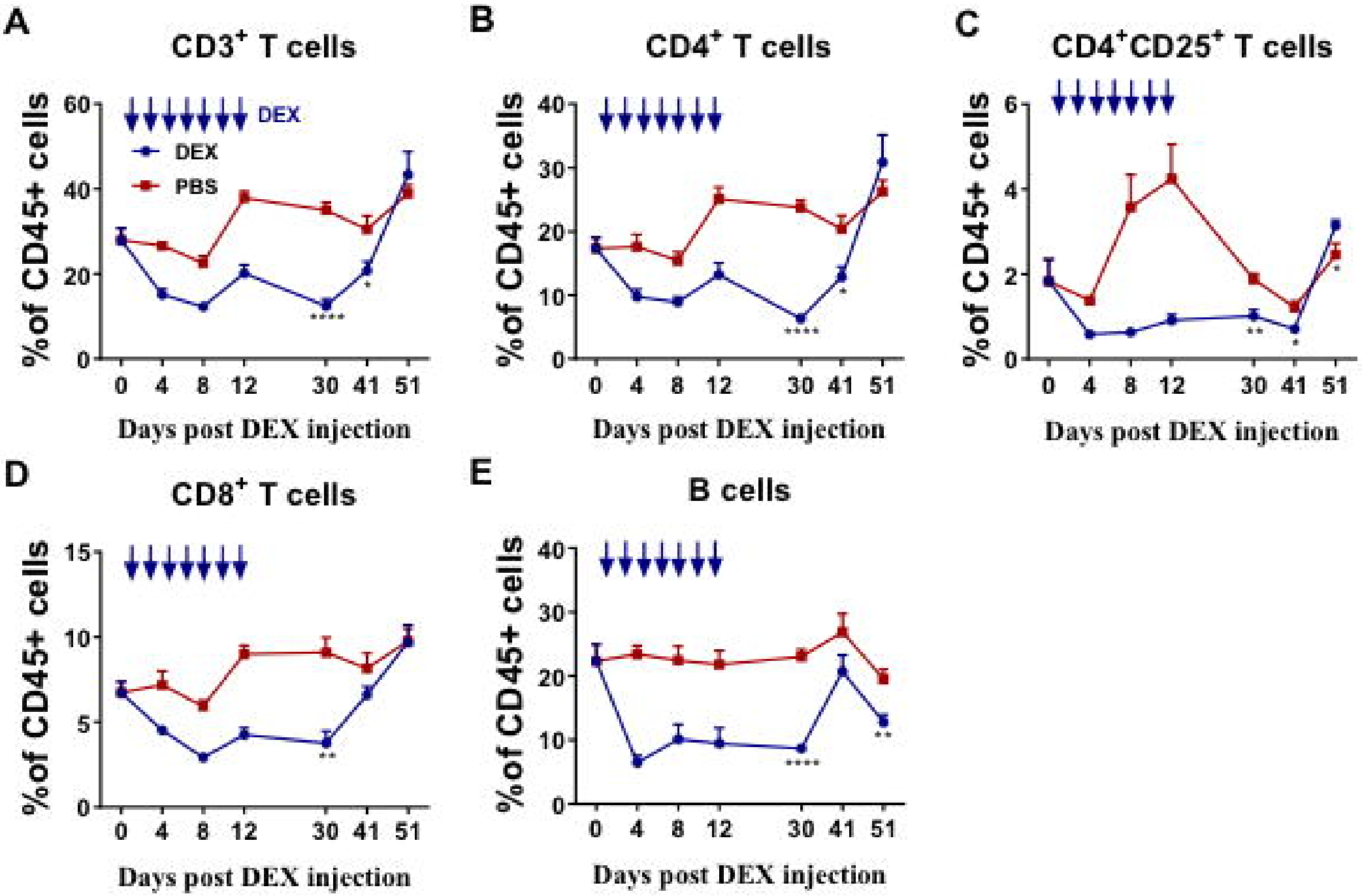
Figure 4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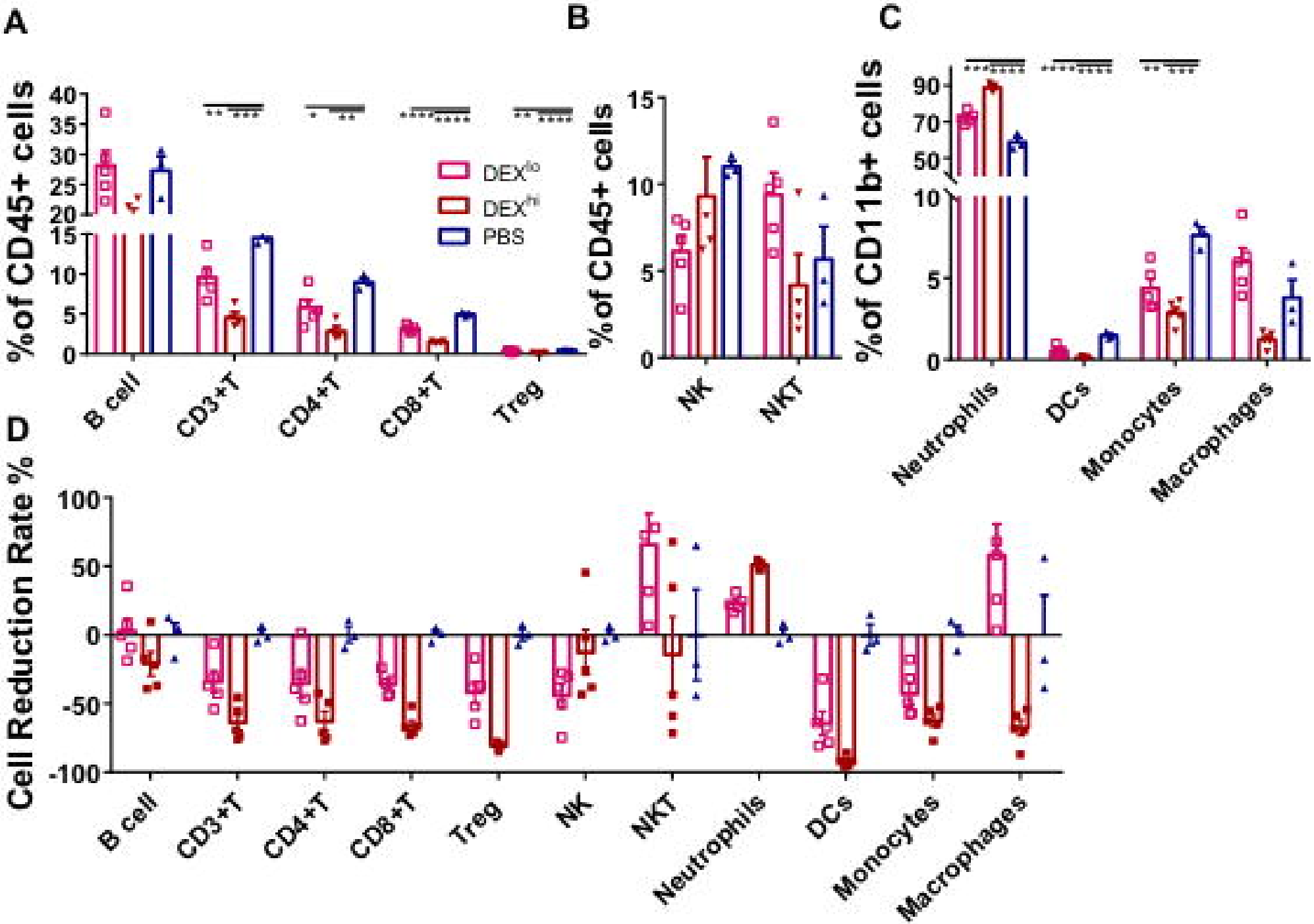
Days post DEX injection

Figure 5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CD4+T
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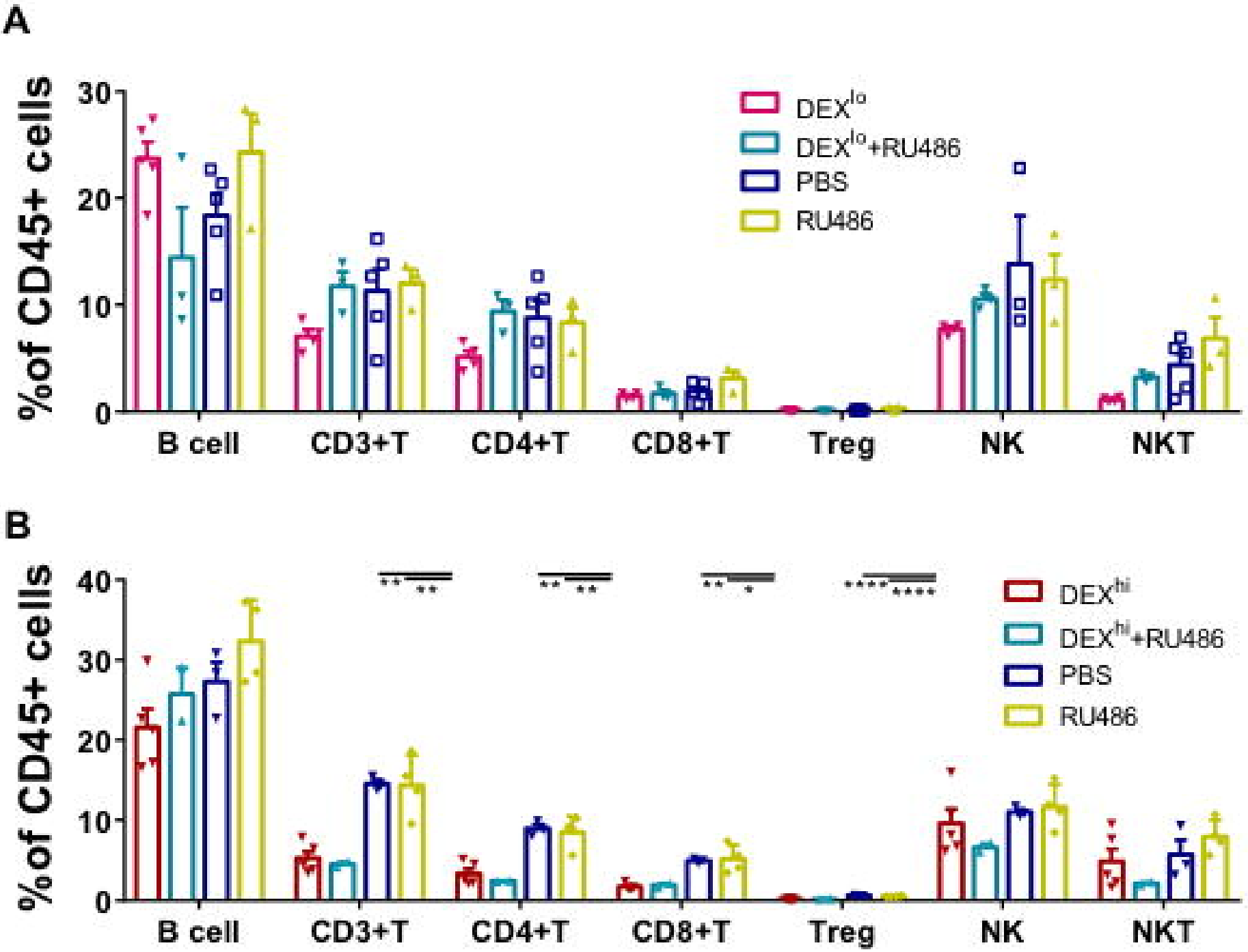
Treg
NK
NKT
Neutrophils
DCs
Monocytes
Macrophages

Figure 6


A
%of CD45+ cells
30
20
10
0
DEXlo
DEXlo+RU486
PBS
RU486
B cell
CD3+T
CD4+T
CD8+T
Treg
NK
NKT
B
%of CD45+ cells
40
30
20
10
0
**
**
**
**
**
*
****
****
DEXhi
DEXhi+RU486
PBS
RU486
B cell
CD3+T
CD4+T
CD8+T
Treg
NK
NKT