



Glucocorticoid-induced eosinopenia in humans can be linked to early transcriptional events

To the Editor,

Glucocorticoids (GC) are the mainstay of therapy for a variety of eosinophil-associated disorders, including hypereosinophilic syndromes (HES),¹ although responses are not universal and GC are associated with significant toxicity.² Prior to the first therapeutic use of GC, it was observed that the administration of exogenous adrenocorticotrophic hormone or hydrocortisone led to a rapid, profound, and transient eosinopenia in humans.³ Although this is likely an important first step in the clinical response to GC, several factors have hampered progress in understanding the underlying mechanisms. These include spontaneous apoptosis in culture, changes in surface protein expression with techniques used to manipulate and isolate eosinophils in vitro, and difficulties with sample collection for ex vivo studies due to the low number of eosinophils in the peripheral blood of most healthy individuals and their disappearance from the circulation following administration of GC. While important work has been performed on specific candidate genes and proteins, an unbiased view of the global transcriptional response to GC in human eosinophils and its relationship to the kinetics of GC-induced eosinopenia has not yet been generated.

To address this, we performed RNA sequencing (RNA-seq) of peripheral blood eosinophils following a single dose of oral prednisone (1 mg/kg) in three subjects with hypereosinophilia of unknown significance (untreated asymptomatic eosinophilia $\geq 1500/\mu\text{L}$ of >5 years' duration without evidence of end-organ manifestations)⁴ in whom the baseline eosinophil counts were sufficiently high to allow serial sampling of eosinophils after GC administration. The kinetics of GC-induced eosinopenia were consistent across the three subjects, with the initial decline occurring between 60 and 120 minutes after GC administration (Figure 1A). Consequently, RNA-seq was performed on early time points, up to and including 120 minutes after the dose. The number of differentially expressed (DE) genes ($\text{FDR} \leq 0.05$) increased over time and the genes classified as DE were internally consistent: 96.2% of the genes that were DE at 30 minutes were also DE at 60 minutes, and 91.4% of the genes that were DE at 60 minutes were also DE at 120 minutes. A total of 414 unique transcripts were DE following GC administration at one or more time points.

A gene set enrichment analysis revealed that the predominant response involves genes related to apoptosis, immune cell surface receptor signaling, cytokine and growth factor receptor signaling, and metabolism (Figure 1B). Tables of the individual genes and

pathways with evidence of a response to GC in human eosinophils are available as supplementary materials with the Gene Expression Omnibus (GEO) upload for the RNA-seq data (GSE111789). Apoptosis-related genes were the most highly enriched among the early GC-responsive genes. Significant changes were observed in the expression of six genes encoding proteins in the intrinsic pathway of apoptosis (*BCL2L11*, *RNF144B*, *STK17B*, *MCL1*, *PAK1*, and *PRKCE*) and five genes encoding other proapoptotic proteins (*BTG1*, *PRUNE2*, *PTEN*, *RHOB*, and *TXNIP*) (Figure 1C). With the exception of *MCL1*, *PRKCE*, and *RHOB*, the direction of change in gene expression would be predicted to favor apoptosis. Although statistical significance was reached at different time points by individual genes, the direction of change in gene expression was consistent over time for all differentially expressed genes, and the initial change in expression for most genes was already evident within 30 minutes of GC administration. Despite the observed changes, circulating eosinophils purified 120 minutes after in vivo GC administration to healthy volunteers and a patient with HES not receiving treatment showed no evidence of apoptosis as assessed by Annexin-V staining (Figure 1D-E), a finding consistent with prior published in vitro data.⁵

The rapidity of GC-induced eosinopenia is most consistent with eosinophil trafficking out of the circulation. The available data suggest that tissue levels of eosinophil chemoattractants do not increase in response to GC⁶; therefore, a direct effect of GC on eosinophil surface proteins is the most plausible mechanism for GC-induced eosinopenia. As the median half-life of cell surface receptors has been estimated to be approximately 18 hours,⁷ a drop in transcript abundance is unlikely to lead to a significant change in protein expression at the surface within the first 2 hours of GC administration, whereas an increase in transcript abundance might. Of the 414 genes differentially expressed in eosinophils in the first 2 hours after prednisone administration, only 12 are known to be involved in leukocyte migration: *CXCR4*, *RIPOR2*, *PREX1*, *CCR1*, *PTGER4*, *SWAP70*, *IL16*, *PIK3CG*, *NLRP12*, *CD244*, *S1PR1*, and *CSF1* (Figure 2A). Four of these genes (the chemokine receptors *CXCR4* and *CCR1*, the prostaglandin receptor *PTGER4*, and the cytokine *CSF1*) were significantly DE prior to the initial decline in circulating eosinophils, but of these only *CXCR4* showed significantly increased expression within 60 minutes of GC administration. The early response of *CXCR4* to GC at the RNA and protein levels was further investigated by exposing eosinophils from healthy volunteers to GC in vitro. A consistent rise in *CXCR4* transcript abundance was observed between 60 and 120 minutes (Figure 2B), coinciding with the timing of eosinophil egress from the peripheral circulation (Figure 1A). An increase in *CXCR4* surface expression was also observed

Abbreviations: 7-AAD, 7-amino-actinomycin D; DE, differentially expressed; FDR, false discovery rate; GC, glucocorticoid; HES, hypereosinophilic syndrome; HEUS, hypereosinophilia of unknown significance; MFI, median fluorescence intensity; RNA, ribonucleic acid; RNA-seq, RNA sequencing; SEM, standard error of the mean.

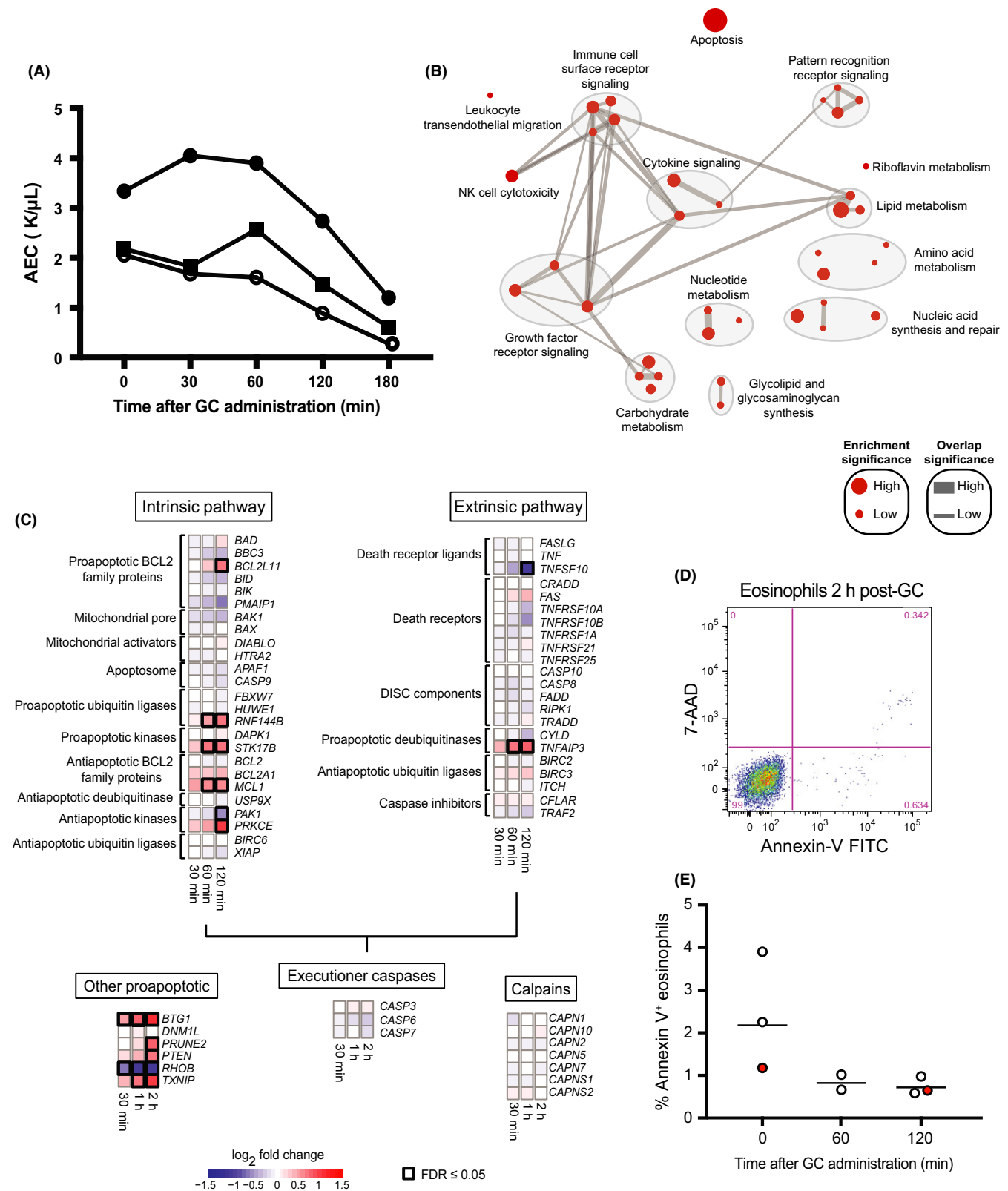


Figure 1. Kinetic and transcriptional response of human circulating eosinophils to a single dose of oral prednisone. A, Eosinophil kinetics after oral prednisone. B, Molecular pathways most affected by in vivo GC administration. Node size is proportional to the significance of pathway enrichment. Edge width is proportional to the number of shared genes between pathways. C, Transcriptional response of apoptosis genes to in vivo GC administration. FDR, false discovery rate. D, Representative plot of viability (7-AAD) and early apoptosis (Annexin-V) of blood eosinophils 2 h after oral prednisone. E, Eosinophil apoptosis before and after oral prednisone. Normal donors are shown as white circles and a subject with HES is shown as a red circle. The horizontal line denotes the geometric mean

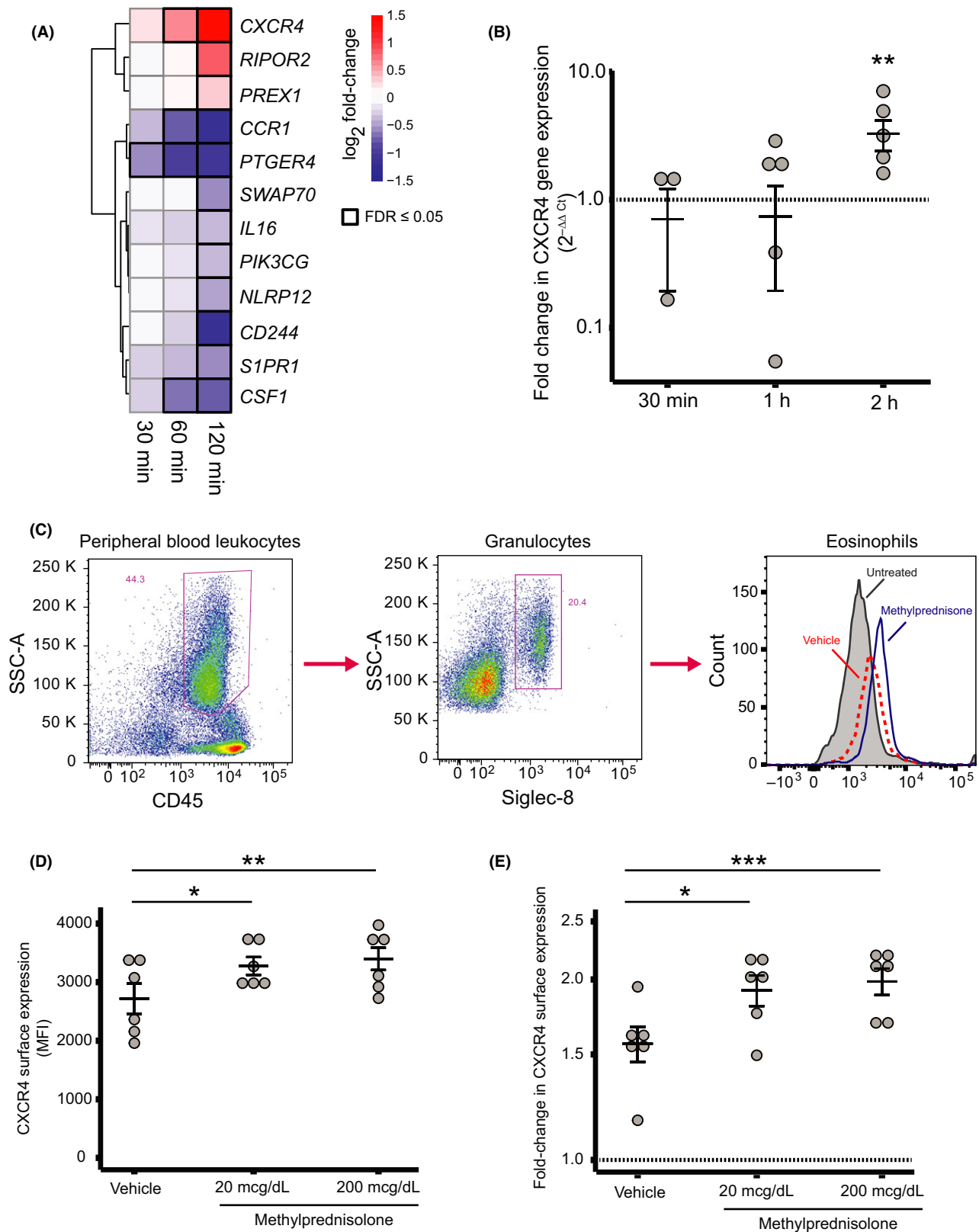


Figure 2. Changes in CXCR4 expression following GC exposure in vivo and in vitro. A, Transcriptional response of leukocyte trafficking genes after GC. B, Fold change in CXCR4 transcript abundance after in vitro exposure of eosinophils to dexamethasone 5 $\mu\text{mol/L}$ or vehicle. Error bars: geometric mean and standard error. C, Flow cytometry gating strategy for eosinophils and representative histogram of CXCR4 signal intensity in untreated eosinophils and eosinophils from the same subject after in vitro exposure to methylprednisolone or vehicle. D, MFI and (E) fold change of CXCR4 expression in human eosinophils after 2 hours of in vitro exposure to methylprednisolone or vehicle. Error bars: mean $\pm$ SEM. * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$

120 minutes after in vitro GC exposure (Figure 2C-E). Preferential upregulation of CXCR4 in eosinophils by GC has been reported previously at 4 hours, with demonstrated effects on eosinophil chemotaxis.⁸ Moreover, the CXCR4 ligand CXCL12 is expressed in human spleen, bone marrow, and lymph nodes, sites where eosinophils have been suspected to migrate after GC exposure.^{9,10}

Taken together, our results are most consistent with a model in which in vivo exposure to pharmacologic levels of GC induces transcriptional changes that prime human eosinophils for apoptosis, with apoptosis occurring after migration out of the circulation, which in turn is mediated by GC-induced upregulation of the gene encoding the chemokine receptor CXCR4.

A complete description of methods is in the Data S1.

FUNDING INFORMATION

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

DATA ACCESSIBILITY

The RNA-seq data generated as a part of this study has been uploaded to the Gene Expression Omnibus (GEO) and will be publicly available upon publication of the article, under GEO Series Identifier GSE111789.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Materials

Khoury et al., Glucocorticoid-induced eosinopenia in humans can be linked to early transcriptional events. This supplement has been provided by the authors to give readers additional information about their work and to enhance the interpretation and reproducibility of the data.

Human subjects

Human subject protocols were approved by the Institutional Review Boards of the National Institute of Allergy and Infectious Diseases or the Department of Laboratory Medicine at the National Institutes of Health. Subjects with hypereosinophilia of unknown significance (HE_{US}) (1) or hypereosinophilic syndrome (HES) were recruited under NIH protocols with ClinicalTrials.gov identifiers NCT00001406 and NCT01524536. Healthy volunteers were recruited under NIH protocols NCT00090662, NCT00001846, and NCT02798523. All subjects provided written informed consent.

RNA-seq on human eosinophils before and after glucocorticoid (GC) administration

Oral prednisone (1 mg/kg) was administered to 3 unrelated subjects with HE_{US}. Whole blood was collected before and 30, 60, 120, and 180 minutes after prednisone administration. Complete blood counts were performed at each time point. At the 0, 30, 60, and 120-minute time points, the blood was also used for negative-selection immunomagnetic purification of eosinophils (2) with MACSxpress Eosinophil Isolation and Erythrocyte Depletion kits (Miltenyi Biotec, Cat. Nos. 130-104-446 and 130-098-196). For all experiments, eosinophil purity > 98% was confirmed by counting a minimum of 300 cells on cytopsin preparations stained with Shandon Kwik-Diff (Thermo Fisher Scientific, Cat. No. 9990700). Isolated eosinophils were placed in 500 µL of TRIzol reagent (Thermo Fisher Scientific, Cat. No. 15596018) and stored at -80° C. Total RNA was isolated by extraction with TRIzol Reagent (Thermo Fisher Scientific, Cat. No. 15596018), followed by column-based purification with the RNA Clean & Concentrator-5 kit (Zymo Research; Cat. No. R1016). RNA quantity was measured on a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Cat. No. Q32866), with RNA BR quantitation assays (Thermo Fisher Scientific, Cat. No. Q10211). RNA quality was assessed by microfluidic electrophoresis on an Agilent 2100 Bioanalyzer system (Agilent, Cat. No. G2939A), with RNA 6000 Nano chips (Agilent, Cat. No. 5067-1511). Sequencing libraries were prepared with the TruSeq Stranded Total RNA with Ribo-Zero Gold kit (Illumina, Cat. No. RS-122-2303). Indexed libraries were normalized and pooled (11-plex), and the cBot system (Illumina Cat. No. SY-301-2002) was used for cluster generation with a TruSeq PE Cluster Kit v3-cBot-HS (Illumina, Cat. No. PE-401-3001). Paired-end sequencing (2 x 94 bp) was performed on an Illumina HiSeq 2000 sequencer (Illumina Cat. No. SY-401-1001), with the TruSeq SBS v3-HS kit (Illumina, Cat. No. FC-401-3001).

Illumina base call (.bcl) files were converted to FASTQ format with bcl2fastq v.2.17.1.14 (Illumina, Inc.). Adapter sequences were trimmed with Cutadapt v.1.10 (3) in Python v.2.7.9. The adapter-trimmed FASTQ files were aligned to the reference human genome assembly (GRCh38) with Bowtie v. 2.2.5 (4) and TopHat v.2.0.14 (5,6). The transcript annotation (GTF) file was obtained from GENCODE (release 23) (7). Only fragments in which both paired-end reads were successfully aligned were kept. The binary alignment files (.bam) were then used for generation of a matrix of read counts with the featureCounts program of the package Subread v.1.5.1 (8). Paired-end exonic fragments were grouped at the level of genes, based on the GENCODE 23 annotation file. Normalization and differential expression analysis with RNA sequencing data were performed with the DESeq2 (9) package in R v.3.3.1 (10). To effectively account for the specifics of RNA-seq data, such as discreteness and dependence of the variability on the mean, DESeq2 uses a generalized linear model, where the read count in gene i from sample j is assumed to follow a negative binomial distribution with mean $s_{ij}q_{ij}$, and variance as a function of the mean and a dispersion parameter. The normalizing factor s_{ij} accounts for differences in sequencing depth between samples. The quantity q_{ij} depends on the gene and the biological condition of the sample through the loglinear model

$$\log_2 q_{ij} = \sum_r \beta_{ir} X_{jr}$$

To identify differentially expressed transcripts after treatment with the GC in this study, $X_{j0}=0, X_{j1}=1$ for the post-treatment samples and $X_{j0}=1, X_{j1}=0$ for the pre-treatment samples. The log-fold change in gene i is reflected by $\beta_{i1} - \beta_{i0}$. To handle the lower power associated with small numbers of samples, an empirical Bayesian procedure is implemented to stabilize the log-fold change estimates. The Wald test is then applied to the log-fold change in each gene, followed by multiple-testing adjustment with the method of Benjamini and Hochberg (11). To identify the molecular pathways that were most affected transcriptionally by the GC stimulus in human eosinophils, a gene-set enrichment analysis was performed at each time point. Gene set definitions were downloaded from the Broad Institute's Molecular Signatures Database (MSigDB) (12), v. 5.2. Three groups of gene sets were included: H: Hallmark gene sets. C2/CP: Canonical pathways, which includes gene sets from KEGG, Biocarta, Pathway Interaction Database, Reactome, Signaling Gateway, Signal Transduction KE, SuperArray, and SigmaAldrich. C5/BP group: Gene Ontology Biological Processes. A gene set enrichment test was then performed, as implemented in function *geneSetTest* of the R package limma (13). This test first ranks all genes by a differential expression statistic, in this case log2 fold-change after prednisone treatment. For each of the MSigDB gene sets, it then tests whether the genes in the gene set tend to be located near the top of the ranked list of differentially expressed genes. This was performed without regard to the direction of change in expression. The ranks of the differentially expressed genes were

used to calculate the enrichment p-values by a Wilcoxon test. Multiple-testing correction was performed separately for each category of gene sets with the method of Benjamini and Hochberg (11).

Detection of CXCR4 cell-surface expression by flow cytometry

Peripheral blood from 6 healthy volunteers was collected in Vacutainer EDTA tubes. Erythrocytes were lysed with ACK lysis buffer (Quality Biological). Peripheral blood leukocytes (PBLs) were incubated in 48-well flat-bottom plates (Corning) at a density of $4 \times 10^6/\text{ml}$ (1 million cells/well) in culture medium, which consisted of Gibco RPMI (Thermo Fisher Scientific, Cat. No. 11835-030) with 10 mM HEPES (Thermo Fisher Scientific, Cat. No. 15630-080). After resting for 2 hours at 37°C with 5% CO₂, a fraction of the cells was sampled for cell surface expression (untreated samples). The remaining PBLs were incubated for an additional 2 hours with media alone, media with vehicle (0.1% ethanol), media with methylprednisolone at 20 µg/dL, or media with methylprednisolone at 200 µg/dL (Sigma-Aldrich, Cat. No. M0639). Before and after *in vitro* exposure of PBLs to methylprednisolone, the cells were stained with the following antibodies: CD45-PerCP, clone 2D1 (BD Biosciences, Cat. No. 347464), Siglec-8-PE, clone 7C9 (Biolegend, Cat. No. 347104), and CXCR4-APC, clone 12G5 (BD Biosciences, Cat. No. 555976). Data acquisition was performed on LSRII or LSR Fortessa flow cytometers (BD Biosciences) and analyzed with FlowJo v.10. After exclusion of cell debris and doublets, eosinophils were gated as CD45⁺SSCA^{Hi} cells expressing surface Siglec-8. Median fluorescence intensity (MFI) of CXCR4 was compared between methylprednisolone-treated and vehicle-treated eosinophils by a paired t-test. Because CXCR4 surface expression increases after *in vitro* incubation even in the absence of dexamethasone, fold-change (treated over untreated) in MFI was also compared across conditions by a paired t-test using log-transformed values.

Detection of cell viability and apoptosis by flow cytometry

Cell viability and apoptosis after *in vivo* administration of a single dose of a glucocorticoid were measured in three unrelated subjects: two healthy volunteers who received intravenous methylprednisolone 250 mg and one subject with HES who received oral prednisone 1 mg/kg. Peripheral blood was collected from each subject before, 1 hour (for the healthy volunteers only), and 2 hours after administration. At each time point, granulocytes were separated from mononuclear cells by sedimentation over Ficoll-Hypaque (GE Life Sciences, Cat. No. 17-1440-03). Erythrocytes were lysed by hypotonic shock with ice-cold double deionized water and then neutralized with phosphate-buffered saline (PBS). Eosinophils were purified by magnetic bead selection with the Eosinophil Isolation Kit (Miltenyi Biotec, Cat. No. 130-092-010). Freshly purified eosinophils were washed and stained with Annexin V-FITC (BD Biosciences, Cat. No. 556419) and 7-AAD (BD Biosciences, Cat. No. 559925). As a positive control, purified eosinophils obtained prior to GC administration were

incubated for 2 hours in culture medium consisting of RPMI with 10mM HEPES at 37°C with 5% CO₂ prior to staining. Data acquisition was performed on LSRII or LSR Fortessa flow cytometers (BD Biosciences).

Detection of CXCR4 transcript abundance by qPCR

Peripheral blood from 5 healthy volunteers was collected in Vacutainer EDTA tubes. Eosinophils were purified with the MACSxpress Eosinophil Isolation and Erythrocyte Depletion kits (Miltenyi Biotec, Cat. Nos. 130-104-446 and 130-098-196). Purified eosinophils were incubated in 12-well plates at a density of 5×10^6 cells per well in 1mL of culture medium consisting of RPMI with 10mM HEPES. After resting for 4 hours at 37°C with 5% CO₂, water-soluble dexamethasone at 0.5 μ M (Sigma-Aldrich, Cat. No. D2915) or medium was added, and the cells were incubated for an additional 30, 60 or 120 minutes. At each time point, dexamethasone- and vehicle-treated eosinophils were harvested and resuspended in 1mL TRIzol reagent (Thermo Fisher Scientific, Cat. No. 15596018) and stored at -80°C. RNA was isolated by extraction with TRIzol reagent (Thermo Fisher Scientific, Cat. No. 15596018), followed by column-based purification with the RNA Clean & Concentrator-5 kit (Zymo Research; Cat. No. R1016). cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat. No. 4368813). Quantitative real-time PCR was performed in duplicate using the QuantStudio6 Flex System (Applied Biosystems, Cat. No. 278861532) with the TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Cat No. 4352042) and target primers for *CXCR4* (TaqMan Gene Expression Assays; Thermo Fisher Scientific, Cat. No. Hs00237052_m1 *CXCR4*) and the internal control 18S rRNA (Applied Biosystems, Cat. No. 4318839). The Δ Ct values (Δ Ct = Ct of the target gene – Ct of internal control) were calculated for each subject, in each condition (dexamethasone or vehicle), at each time point. Because Δ Ct values are in a logarithmic scale, these were used for statistical inference. We performed paired t-tests (dexamethasone-treated versus vehicle-treated) at each time point. For ease of visualization, the data are displayed as fold-changes in gene expression (dexamethasone-treated over vehicle-treated). For this, we employed the $2^{-\Delta\Delta Ct}$ method (14), where the first Δ is the Δ Ct in dexamethasone-treated cells and the second is the Δ Ct in vehicle-treated cells. Because statistical inference was performed in the logarithmic scale, the error bars correspond to the geometric mean and its standard error. The standard error of the geometric mean was calculated by the Delta method, as:

$$\sqrt{\left(\ln 2 \times 2^{\frac{\Delta\Delta Ct}{2}}\right)^2 \times \frac{var(\log_2 FC)}{n}}$$

Where FC = fold-change, $\overline{\log_2 FC}$ = the sample mean of fold-changes in $\log_2$ scale, and n = number of biological replicates.

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