Glucocorticoids and the cytokines IL-12, IL-15, and IL-18 present in the tumor microenvironment induce PD-1 expression on human natural killer cells

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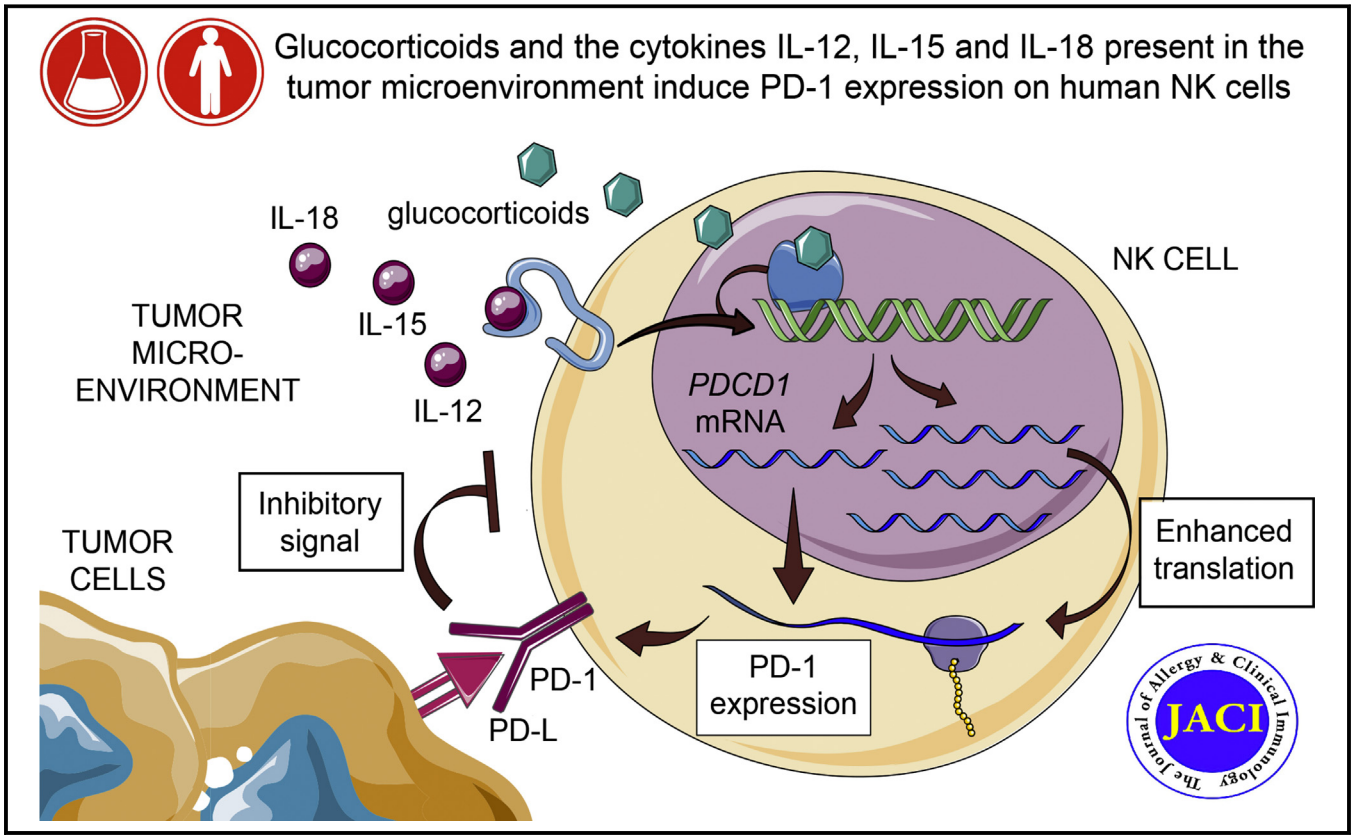
GRAPHICAL ABSTRACT



Background: Programmed cell death protein 1 (PD-1)–immune checkpoint blockade has provided significant clinical efficacy across various types of cancer by unleashing both T and natural killer (NK) cell–mediated antitumor responses. However, resistance to immunotherapy occurs for many patients, rendering the identification of the mechanisms that control PD-1

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expression extremely important to increase the response to the therapy.

Objective: We sought to identify the stimuli and the molecular mechanisms that induce the *de novo* PD-1 expression on human NK cells in the tumor setting.

Methods: NK cells freshly isolated from peripheral blood of healthy donors were stimulated with different combinations of molecules, and PD-1 expression was studied at the mRNA and protein levels. Moreover, *ex vivo* analysis of tumor microenvironment and NK cell phenotype was performed.

Results: Glucocorticoids are indispensable for PD-1 induction on human NK cells, in cooperation with a combination of cytokines that are abundant at the tumor site. Mechanistically, glucocorticoids together with IL-12, IL-15, and IL-18 not only upregulate *PDCD1* transcription, but also activate a previously unrecognized transcriptional program leading to enhanced mRNA translation and resulting in an increased PD-1 amount in NK cells.

Conclusions: These results provide evidence of a novel immune suppressive mechanism of glucocorticoids involving the transcriptional and translational control of an important immune checkpoint. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: *NK cells, glucocorticoids, PD-1, immune checkpoint, translational control, immunotherapy, cancer immunology*

Abbreviations used
Dex: Dexamethasone
GC: Glucocorticoid
GO: Gene Ontology
GR: Glucocorticoid receptor
HD: Healthy donor
NK: Natural killer
PB: Peripheral blood
PD-1: Programmed-cell death protein 1
PD-Ls: PD-1 ligands
PE: Pleural effusion
TLR: Toll-like receptor

Glucocorticoids (GCs) are steroid hormones synthesized and secreted in the circulation on activation of the hypothalamic/pituitary adrenal axis in response to stress and inflammation. Their immune-suppressive effects have been exploited through the development of synthetic GCs, which now represent the standard therapy in many inflammatory disorders.[1] Regarding cancer, recent studies indicate that GC administration should be carefully evaluated in patients with tumors, because GCs may exert a protumoral activity enhancing tumor progression,[2] inhibiting effector T-cell responses,[3] and even compromising therapy-induced antitumor immunity.[4]

While the therapeutic effects of GCs have been known for a very long time, major progress in discovering the underlying molecular mechanisms has only been made recently.[5] Nearly every cell type of the organism is sensitive to GCs, because the glucocorticoid receptor (GR) is ubiquitously expressed and mediates pleiotropic genomic effects that depend on the cellular target, the tissue, and the microenvironment.[6] Recently, in animal models, a novel immune-suppressive mechanism of action of GCs has been identified, acting on innate lymphocytes and involving the induction of immune checkpoint inhibitory receptors.[7,8] In particular, it was shown that endogenous GCs released on infection with murine cytomegalovirus induce programmed-cell death protein 1 (PD-1) expression on natural killer (NK) cells. This study demonstrated that specific signals from the tissue microenvironment cooperate with the signal transduced by the GR to induce the expression of the inhibitory receptor PD-1 at the transcriptional level in NK cells.[7]

PD-1 is an inhibitory receptor playing an important beneficial role in maintaining peripheral tolerance and T-cell homeostasis. However, on interaction with PD-1 ligands (PD-Ls: PD-L1 and PD-L2) that may be expressed on tumor cells, PD-1 inhibits T-cell function, contributing to immune escape mechanisms frequently occurring in patients with cancer. Recently, immunotherapy with monoclonal antibodies that target the PD-1/PD-L1 axis has successfully improved cancer treatment and has been approved in a variety of cancers, including several solid tumors.[9-12] The strategy of PD-1 blockade has mainly focused on enhancing T-cell responses, but there has been an increasing interest recently in harnessing the NK cell compartment for therapeutic interventions, which is particularly relevant for the treatment of T cell–resistant tumors (mostly HLA-cl-I-deficient).[13-18] While PD-1 is expressed by T-cell subsets, it is virtually absent on NK cells in peripheral blood (PB) of healthy donors (HDs), with the exception of a fraction of cytomegalovirus-seropositive individuals.[19] On the other hand, PD-1$^+$ NK cells have been identified in the PB and in the ascitic fluid of patients with ovarian carcinoma, and in the pleural effusions (PEs) of patients with primary and metastatic lung cancer .[19,20] Notably, these PD-1$^+$ NK cells were much more abundant at the tumor site than in the PB of the same patient, suggesting that the tumor microenvironment may deliver signals capable of inducing the *de novo* expression of PD-1. Of note, recent studies revealed that human NK cells display an intracytoplasmic pool of PD-1 mRNA and PD-1 protein localized in the Golgi;[21] however, the stimuli required for its expression on the membrane are unknown.

In the present study, we demonstrate that PD-1 expression on human NK cell surface requires the presence of GCs in combination with the inflammatory cytokines IL-12, IL-15, and IL-18. This induction involves not only the transcription of PD-1–encoding gene (*PDCD1*), but also the activation of a transcriptional program leading to enhanced mRNA translation. Remarkably, *in vitro* PD-1 expression is not associated to the acquisition of an exhausted phenotype, but to the expansion of the less mature CD56^{bright} cells, which represent the most abundant NK cell subset at the tumor sites.

METHODS

Human blood and tissue samples

HDs' PBMCs and plasma were obtained from buffy coats collected in the early morning from volunteer blood donors admitted to the blood transfusion service of Istituto di Ricovero e Cura a Carattere Scientifico Bambino Gesù

Pediatric Hospital (Rome, Italy) after obtaining informed consent, following approval of the hospital's Ethics Board (ID AIRC5x1000 #21147).

Blood samples and PEs following thoracentesis were obtained from patients with primary or metastatic tumor of different origin (as described in Table E1 in this article's Online Repository at www.jacionline.org) who were admitted to Villa Scassi Hospital (Genoa, Italy), following approval of Azienda Sanitaria Locale 3 Ethics Board (ID 33533184).

The study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki.

Cell isolation and stimulation

Human MCs from PB and PE were isolated by Lympholyte-H Cell Separation Medium (Cedarlane Laboratories, Burlington, Ontario, Canada) density gradient centrifugation. NK cells were isolated from HDs' PBMCs by negative selection using the RosetteSep Human NK Cell Enrichment Cocktail (Stemcell Technologies, Vancouver, British Columbia, Canada). T cells and monocytes were isolated from HDs' PBMCs by positive magnetic selection on incubation with anti-CD3-Fluorescein isothiocyanate (FITC) antibody (Ab) (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD14-FITC Ab (Miltenyi), respectively, followed by anti-FITC MicroBeads (Miltenyi). NK cells were stimulated *in vitro* in medium (10% heat-inactivated FBS [Gibco, Thermo Fisher Scientific, Waltham, Mass]-supplemented RPMI 1640) with the addition of dexamethasone (Dex), or cortisol, or the same volume of vehicle (dimethyl sulfoxide; all from Sigma Aldrich [St Louis, Mo]). The following cytokines were used: IL-2 (200 U/mL; Novartis, Basel, Switzerland); IL-12 (10 ng/mL; Miltenyi); IL-15 (25 ng/mL; Miltenyi); IL-18 (100 ng/mL; R&D Systems, Bio-Techne, Minneapolis, Minn); TNF-α (20 ng/mL; R&D Systems); TGF-β (10 ng/mL; PeproTech, Rocky Hill, NJ). The following Toll-like receptor (TLR) ligands were used: ODN2006 (1 mmol/L); Pam3CsK4 (5 μg/mL); Poly(I:C) (1 μg/mL); R848 (6 μmol/L), all from InvivoGen (San Diego, Calif).

NK cells previously cultured *in vitro* for 6 days were washed and restimulated with IL-12 and IL-18, or with phorbol 12-myristate 13-acetate (25 ng/mL; Sigma Aldrich) and ionomycin (1 μg/mL; Sigma Aldrich), or with K562 target cells at an effector/target ratio of 1:1, or incubated with medium alone for 4 hours at 37°C. To evaluate the functional activity of PD-1, NK cells were incubated with P815 cells (an $Fc\gamma R^+$ mastocytoma murine cell line) in the presence of anti-NKp46 (BAB281 clone, IgG1), anti-NKp30 (AZ20 clone, IgG1) and anti-NKp44 (Z231 clone, IgG1) mAbs in combination or not with anti-PD-1 mAb (PD-1.3.1.3 clone, IgG2b [kind gift of Dr Daniel Olive]). The effector/target ratio was 1:1 and the cell density was the same for all conditions: 2×10^5 NK cells were stimulated with 2×10^5 target cells in 200 μL medium, with 1 μg antibody / 2×10^5 P815 cells. Golgi Plug (1:500; BD, Franklin Lakes, NJ), Golgi Stop (1:750; BD), and anti-CD107a-Allophycocyanine (APC) (BD) (for evaluation of NK cell degranulation) were added to the medium during stimulation.

Cytofluorimetric analysis and cell sorting

For cytofluorimetric analysis, cells were stained with Zombie NIR Fixable Viability Kit (BioLegend, San Diego, Calif) and surface antibodies in PBS 5% FCS for 20 minutes at 4°C. For intracellular staining, cells were fixed, permeabilized, and stained with the FoxP3 Staining Buffer Set (Miltenyi). The following antibodies were used: anti-human CD69 FITC, anti-human PD-1 PE, and anti-human PDL2 PE-Vio615 (Miltenyi); anti-human CD16 BV510, anti-human DNAM APC, anti-human TIGIT PE-DAZZLE, anti-human MICA/B PE-Cy7, and anti-human Nectin2 PE (BioLegend); anti-human CD16 FITC, anti-human CD19 PECF594, anti-human CD56 BV650, anti-human NKG2D BV605, anti-human TIM3 BV421, anti-human PDL1 PECF594, and anti-human PVR AlexaFluor647 (BD Biosciences, San Jose, Calif); anti-human CD56 PE-Cy7, anti-human CD158e1/e2 APC, anti-human CD158a/h APC, and anti-human NKG2A PE-Cy7 (Beckman Coulter, Brea, Calif); anti-human perforin FITC (Ancell Corporation, Bayport, Minn); anti-human IFN-γ eF450 (eBiosciences, Thermo Fisher Scientific); anti-human GR Alexa488 and rabbit IgG XP Alexa 488 (Cell Signaling Technology, Danvers, Mass); anti-human ULBP2/5/6 PE, anti-human ULBP3, and anti-human B7-H6 APC (R&D Systems). For PD-1 staining after NK cell stimulation with P815 targets, surface antigens and intracellular stainings were preceded by incubation with unconjugated anti-PD-1 IgG2b mAb (kind gift of Dr Daniel Olive) followed by goat anti-mouse IgG2b PE (SouthernBiotech, Birmingham, Ala). Apoptosis was detected with the AnnexinV FITC Apoptosis Kit (Millipore Sigma, Burlington, Mass) according to the manufacturer's protocol. Proliferation was measured by labeling freshly isolated NK cells before culture with carboxyfluorescein succinimidyl ester (CFSE) (Sigma Aldrich) according to the manufacturer's instructions. Stained samples were analyzed with a Cytoflex S or LX (Beckman Coulter). A MoFlo Astrios cell sorter (Beckman Coulter) was used to sort $CD56^{bright}$ NK cells from total NK cells enriched from HDs' PB using the RosetteSep Human NK Cell Enrichment Cocktail (Stemcell Technologies). Approximately 7×10^5 $CD56^{bright}$ NK cells were sorted from each HD.

Cytofluorimetric data were analyzed with Flow Jo (version 10.6.0; BD Biosciences).

Cell lines and cytotoxicity assays

K562 (a human erythroleukemic cell line) were grown in suspension in 10% FBS-supplemented RPMI 1640, and A549 (a human lung adenocarcinoma cell line) were grown in adhesion in 10% FBS (Gibco)-supplemented Dulbecco modified Eagle medium. For NK cell cytotoxicity assay against K562, target cells were labeled with Green Cell Tracker (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions and 10^4 cells were mixed at varying ratios with NK cells. After 4 hours, cells were stained with 5 μg/mL propidium iodide (Sigma Aldrich) and analyzed by flow cytometry. Specific lysis was calculated as the percentage of propidium $iodide^+$ cells among total Green $Tracker^+$ cells, minus the background level (as determined by target cells incubated without effectors). For NK cell cytotoxicity assay against A549, target cells were labeled with 10 μmol/L Calcein-AM (Sigma Aldrich) and 10^4 cells/well were seeded in a flat-bottom 96-well plate in triplicate. After 1 hour, NK cells were added at varying ratios and after 4 hours of coculture, fluorescence was measured in 100 μL of supernatant using a BioTek Synergy H1 reader (BioTek Instruments, Agilent, Winooski, Vt) (excitation: 485 nm, emission: 530 nm).[22] Specific lysis was calculated according to the formula [(test release − spontaneous release) / (maximum release − spontaneous release)] × 100. Spontaneous release represents florescence release from target cells in medium alone, and maximum release is the florescence release from target cells lysed in medium plus 2.5% Triton X-100 (Sigma Aldrich).

Lytic units (the number of effector cells required to lyse a specified percentage of target cells) were calculated taking the reference lysis level at 30% and expressed as the number of lytic units contained in 10^6 effector cells.[23]

RNA isolation, library construction, sequencing, and analysis

Total RNA was prepared from freshly isolated NK cells, T cells, monocytes, cultured NK cells, or sorted and cultured $CD56^{bright}$ NK cells with an RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). For RNA-sequencing of sorted $CD56^{bright}$ NK cells, 3 biological replicates were generated for each condition (Dex and control). The DNA libraries were generated with the TruSeq stranded mRNA prep kit (Illumina, San Diego, Calif), and RNA-sequencing was performed with a NextSeq 500 (Illumina; paired-end reads 2×75 with 30 mol/L reads per sample). The fastq files were assessed with the fastqc program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and cutadapt (https://journal.embnet.org/index.php/embnetjournal/article/view/200) was used to remove the adapter sequence (if present) and the very short reads (reads length <20). The samples were mapped on reference *Homo sapiens* genome (HG38) using the bioinformatics tool STAR (version 2.7.0f),[24] and the number of reads mapped to each gene was determined with featureCounts.[25] Normalization and differential analysis were performed with DESeq2.[26] Differentially expressed genes between Dex and control samples were then identified from the normalized gene-level counts using the following cutoff values: fold-change of 1.5 and adjusted *P*

value of .05. GOrilla was utilized to identify Gene Ontology (GO) terms (biological processes) enriched in the differentially expressed genes. The significantly enriched GO terms identified were then summarized using REVIGO (Ruđer Bošković Institute, Zagreb, Croatia) in a number of functional categories, each represented by a cluster representative. Genomix4Life (Salerno, Italy) processed the RNA samples and performed differential expression bioinformatics analyses.

Reverse transcription and real-time PCR

RNA was reverse transcribed with the SuperScript IV First-Strand Synthesis System (Invitrogen). For real-time PCR, ready-to-use primer and probe sets predeveloped by Applied Biosystems (Thermo Fisher Scientific) (TaqMan Gene Expression Assays: Hs01550088_m1 for *PDCD1* and Hs00266705_g1 for *GAPDH*) and a QuantStudio 6 Flex instrument (Applied Biosystems) were used. The relative abundance of *PDCD1* mRNA was calculated by normalization to *GAPDH* by ΔΔCt method.

Western blot

Whole cell lysates were prepared from cells using radioimmunoprecipitation assay buffer (10 mmol/L Tris-Cl (pH 8.0), 1 mmol/L EDTA, 0.5 mmol/L ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mmol/L NaCl) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Protein quantification and normalization was done using the Quick Start Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, Calif). Six micrograms of total protein were resolved by SDS-PAGE and transferred to Amersham Protran Western blotting membranes (Sigma Aldrich). Membranes were probed with primary antibodies (anti-human PD-1 from Cell Signaling and anti-human GAPDH from Sigma Aldrich) diluted in 5% milk in Tris-buffered saline with Tween, and species-specific horseradish peroxidase conjugated antibodies (SouthernBiotech). After anti-PD-1 blot the membrane was stripped using ReBlot Plus Stripping solution (Millipore) and reblotted with anti-GAPDH antibody. Chemiluminescent image acquisition was performed using Uvitec Imaging System (Cleaver Scientific, Warwickshire, UK).

Cortisol and cytokines concentration determination

Blood and PE samples were centrifuged at 800*g* for 10 minutes to obtain plasma and PE supernatant, respectively. Cortisol concentration was measured using Cortisol ELISA Kit (Enzo Life Sciences, Farmingdale, NY) and cytokines concentrations were measured with the LEGENDplex Human Cytokine Panel 2 (BioLegend) according to the manufacturers' instructions.

Statistical analysis

Statistical analysis was performed with Prism 6 (GraphPad Software, San Diego, Calif). Normality was tested with the Shapiro-Wilk test. Unpaired 2-tailed Student *t*-tests or paired Student *t*-tests (when data from the same donor were compared) were used to compare 2 groups. One-way ANOVA was used for multigroup comparisons. Differences were considered significant for $P < .05$.

Data and code availability

The data that support the findings of this study are available from the corresponding author upon request. The RNA sequencing data have been deposited in the GEO repository under accession code GSE149113.

RESULTS

GCs in combination with IL-12, IL-15, and IL-18 induce PD-1 expression on NK cells

To identify which signals may induce PD-1 expression on NK cells and whether GCs could be involved, we screened *in vitro* several putative stimuli together with the synthetic GC Dex. It was previously shown that splenic mouse NK cells express high amounts of surface PD-1 on stimulation with IL-18 and IL-15 in combination with the mouse endogenous GC corticosterone for 2 days, while addition of IL-12 to this mix could abrogate this effect.[7] Starting from these evidences, we cultured NK cells isolated from the PB of HDs with different combinations of the cytokines IL-12, IL-18, and IL-15, with the addition of Dex or vehicle alone (dimethyl sulfoxide, control) for 2 (not shown) and 6 days (Fig 1, *A* and *B*). Conversely to murine NK cells, we found that the combination of IL-15, IL-18, and GCs was not able to induce PD-1 expression on human NK cells, while the addition of IL-12 resulted in PD-1 induction (Fig 1, *A*). A very high PD-1 protein surface expression was induced only when NK cells were incubated with all 3 cytokines together and, importantly, this induction required the addition of Dex. Moreover, PD-1 expression was not observed after 2 days of stimulation but after 6 days, suggesting that the molecular mechanisms controlling PD-1 expression are dependent on different species-specific cellular processes. We had similar results when we replaced IL-15 with IL-2 and stimulated NK cells with IL-12, IL-18, and IL-2 for 6 days in combination with Dex (see Fig E1, *A* and *B,* in this article's Online Repository at www.jacionline.org), suggesting that PD-1 induction requires NK cell proliferation. Analogously, by replacing Dex with the endogenous GC cortisol, we could reproduce the same result (Fig E1, *C*), even though the percentage of PD-1^+ NK cells was lower, reflecting the lower potency of the endogenous hormone.

We then tested the ability to induce PD-1 on NK cells of other factors (TLR-ligands and cytokines) that could be part of the microenvironment in infectious or tumor conditions. We stimulated NK cells for short (from 2 hours to overnight) or long (2-6 days) time periods and verified that NK cells were activated by measuring CD69 expression (Fig E1, *D-F*). Neither TLR-ligands (in synergy with IL-12 for shorter time intervals or IL-15 for longer culture periods) (Fig E1, *D* and *E*), nor the cytokines TNF-α and TGF-β (alone for shorter culture intervals or in synergy with IL-15 for longer time intervals) (Fig E1, *F*) were able to induce PD-1 expression on NK cells, alone or in combination with Dex.

By analyzing the kinetics of PD-1 expression by the combined use of IL-12, IL-15, and IL-18, we observed that, only in combination with Dex, PD-1 induction on NK cells started at 4 days and peaked at 6 days (Fig 1, *C*). Therefore, we performed the next experiments to study GC-dependent induction of PD-1 on NK cells by comparing NK cells stimulated under these experimental conditions (IL-12, IL-15, and IL-18 for 6 days) in the presence of Dex or vehicle (control).

Because NK, T, and B cells express comparable amounts of GR (Fig 1, *D*), we verified whether the combinations of IL-12, IL-15, IL-18, and Dex could upregulate PD-1 on other lymphocytes. We stimulated total PBMCs for 6 days, and an increase of PD-1 surface expression was observed only on NK cells (Fig 1, *E*), implying that this regulatory mechanism is cell type–specific. Notably, Dex was not able to further increase PD-1 expression on $CD3^+$ cells because PD-1 induction on T cells requires T-cell receptor engagement, regardless of GC addition, as already reported.[27,28]

All together, these results demonstrate that a sustained activation by IL-12 and IL-18 in the presence of a proliferation stimulus (IL-15 or IL-2) and GCs represents the combination of signals required to induce PD-1 expression on human NK cells.

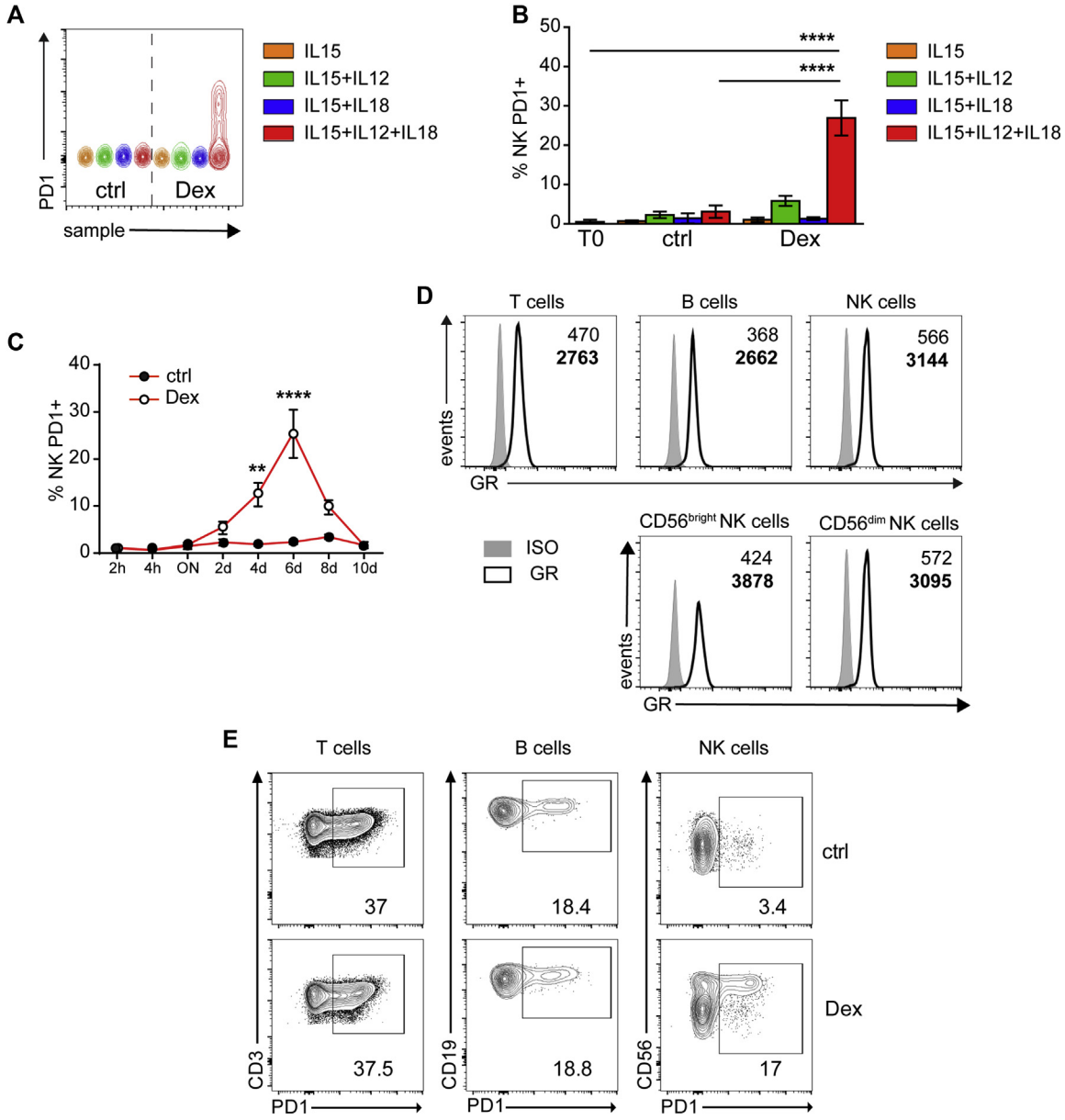

FIG 1. PD-1 expression is induced on NK cells by GCs in combination with IL-12, IL-15, and IL-18. **A,** Cytofluorimetric analysis of PD-1 expression and percentage of PD-1$^+$ NK cells freshly isolated from HDs' PBMCs and stimulated with the combinations of the indicated cytokines in the presence of Dex (500 nmol/L) or vehicle alone (*ctrl*) for 6 days. In **A**, the representative cytofluorimetric dot plot of concatenated samples from 1 experiment is shown. In **B**, data are shown as mean ± SD of 3 experiments. **C,** Percentage of PD-1$^+$NK cells stimulated with IL-15 + IL-12 + IL-18 in the presence of Dex (500 nmol/L) or ctrl for the indicated time points. Results are shown as mean ± SEM of 4 experiments. **D,** GR expression in lymphocytes from HD's PBMCs. Among viable cells, T cells were gated as CD3$^+$CD56$^-$, B cells were gated as CD19$^+$, and NK cells were gated as CD3$^-$CD56$^+$. MFI for isotype control (*ISO*) and GR are shown in each plot. One representative HD is shown. **E,** Percentage of PD-1$^+$ T, B, and NK cells after stimulation of total HDs' PBMCs with IL-15 + IL-12 + IL-18 in the presence of Dex (500 nmol/L) or ctrl for 6 days. One representative experiment is shown (n = 3). $^{**}P < .01$, $^{****}P < .0001$; 1-way ANOVA.

GCs preferentially induce PD-1 expression on CD56^{bright} NK cells

Because PD-1 upregulation on T cells is a marker for cell exhaustion, we verified whether GC-induced PD-1 expression on NK cells was associated with a loss of responsiveness and a phenotype characteristic of terminal differentiation.

Firstly, we compared the effector functions of NK cells treated or not with Dex in addition to IL-12, IL-15, and IL-18 (Dex NK cells vs control NK cells) for 6 days. We found that control NK cells displayed a higher accumulation of intracellular IFN-γ than did Dex-treated NK cells in the absence of restimulation (Fig 2, *A*). This finding confirms previous data obtained on murine NK cells showing that GCs inhibit IL-12 plus IL-18–induced IFN-γ

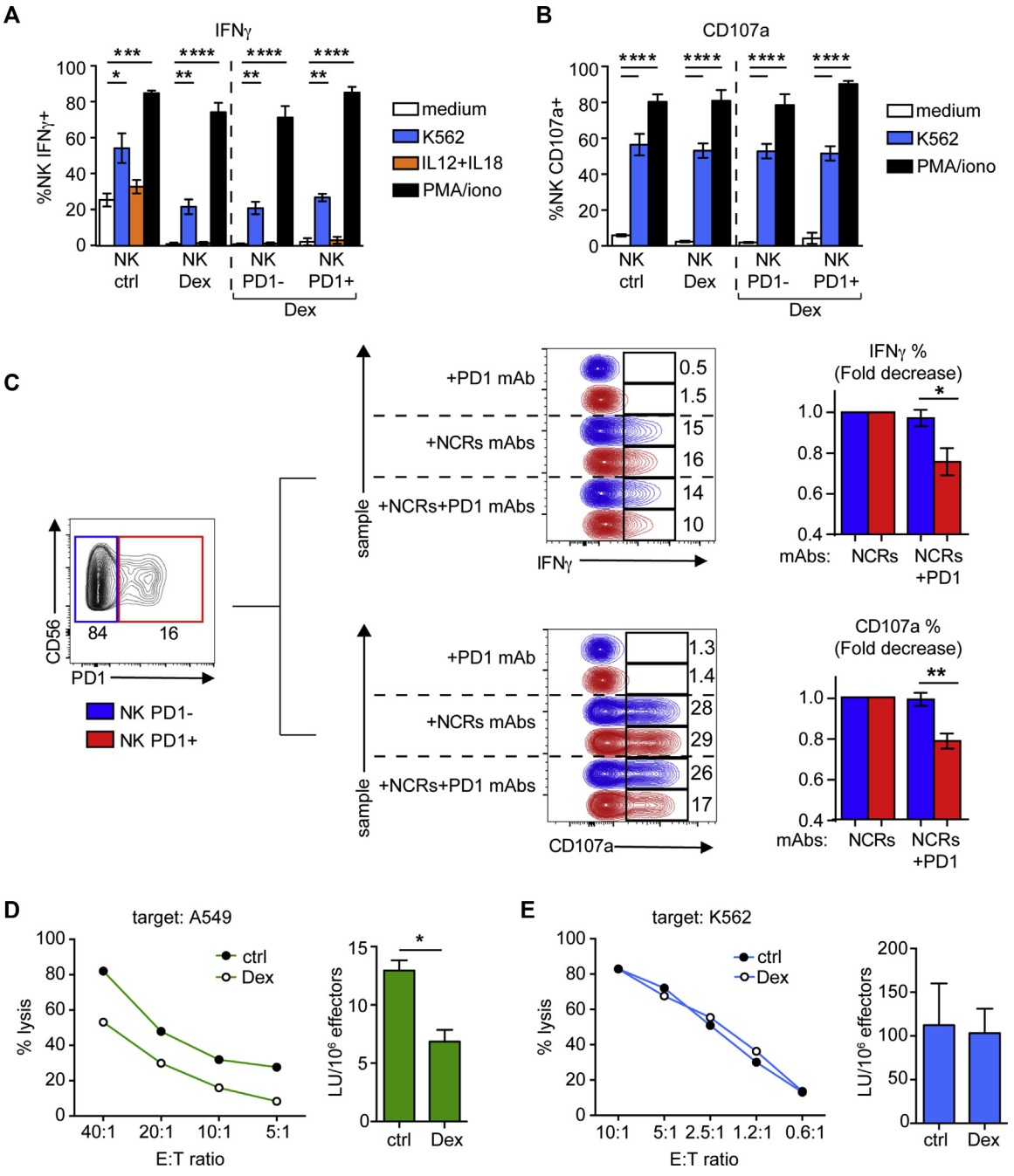

FIG 2. PD-1 expression is not associated to NK cell exhaustion. IFN-γ production **(A)** and CD107a surface expression **(B)** by NK cells previously stimulated with IL-15 + IL-12 + IL-18 and Dex (500 nmol/L) or vehicle (ctrl) for 6 days and then cocultured with K562 target cells, or cultured with IL-12 + IL-18, or phorbol 12-myristate 13-acetate/ionomycin (*PMA/iono*), or medium alone for 4 hours. Data are shown as mean ± SEM of 3 experiments. **C,** IFN-γ production and CD107a surface expression by PD-1$^-$ and PD-1$^+$ NK cells previously stimulated with IL-15 + IL-12 + IL-18 and Dex (500 nmol/L) for 6 days and then incubated with the FcRγ$^+$P815 target cells in the presence of a mix of anti-natural cytotoxicity receptor (NCR) mAbs ± anti-PD-1 mAb for 4 hours. Data are presented as cytofluorimetric dot plots of concatenated samples from 1 representative experiment and as histogram fold decrease, calculated as ratio between percentage of positive cells in NCRs+PD-1 stimulation and percentage of positive cells in NCRs stimulation. Data are shown as means ± SEM of 3 IFN-γ and 5 CD107a experiments. Cytotoxicity against A549 **(D)** and K562 **(E)** targets by NK cells previously stimulated as in **A.** Data are shown as percentage of specific target cell lysis at different effector/target (E/T) ratio of a representative donor *(left panels)* and expressed as mean lytic units (LU) contained in 10^6 effectors cells + SEM of 4 independent experiments *(right panels)*. **P* < .05, ***P* < .01, *** *P* < .005, **** *P* < .0001; 1-Way ANOVA **(A** and **B),** unpaired Student *t*-test **(C-E).**

production by NK cells.[29] When control NK cells were restimulated, intracellular IFN-γ further increased on coculture with K562 target cells, but not in response to reincubation with IL-12 and IL-18 (Fig 2, *A*). Similar results were obtained when Dex NK cells were restimulated in the absence of GCs: even if the absolute frequency of IFN-γ^+ cells was lower compared with that of control cells, the increases induced by K562 stimulation were similar (around 20%) (Fig 2, *A*). In addition, the exocytosis of $CD107a^+$ lytic granules was not impaired by previous Dex incubation (Fig 2, *B*). Moreover, among Dex-treated NK cells, no differences were found between PD-1^- and PD-1^+ cells (Fig 2, *A* and *B*). All together these data demonstrate that, in NK cells, Dex-induced PD-1 expression was not associated to cell exhaustion.

We then stimulated Dex-treated NK cells with $Fc\gamma R^+$ P815 target cells in the presence of anti–natural cytotoxicity receptor antibodies to achieve cell triggering via reverse antibody-dependent cell-mediated cytotoxicity, and anti-PD-1 antibodies were added to this experimental setting. These experiments demonstrate that PD-1 is functional in NK cells because it could inhibit both IFN-γ production and degranulation induced by the engagement of the activating receptors (Fig 2, *C*).

Finally, we assessed NK cell cytotoxicity toward the lung adenocarcinoma cell line A549 expressing PD-L1 and PD-L2, and we used K562 cells as control targets not expressing PD-Ls (see Fig E2 in this article's Online Repository at www.jacionline.org). An impaired cytotoxic activity by Dex-treated NK cells was observed only against A549 cell line (Fig 1, *D* and *E*), demonstrating that the expression of PD-1 by a fraction of NK cells leads to a significant defect in the killing of PD-Ls^+ targets.

We then analyzed whether the extent of PD-1 induction was associated to the stage of maturation of NK cells. We found that IL-12, IL-15, and IL-18 NK cell stimulation for 6 days induced an expansion of $CD16^-$ NK cells, regardless of Dex addition (Fig 3, *A*). Moreover, by analyzing separately the frequency of $CD16^-$ and $CD16^+$ PD-1^+ NK cells, we found that PD-1 was mainly expressed on the $CD16^-$ subset (Fig 3, *B*), which proliferated more and showed lower apoptosis in comparison with $CD16^+$ NK cells, independently of Dex (see Fig E3 in this article's Online Repository at www.jacionline.org). Importantly, PD-1^+ and PD-1^- NK cells showed a comparable rate of proliferation (Fig E3, *A*). Finally, by sorting $CD56^{bright}$ and $CD56^{dim}$ NK cells from HDs' PBMC (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org) and by stimulating these 2 subsets separately, we could directly demonstrate that PD-1 was mainly induced by GCs on $CD56^{bright}$ NK cells (Fig 3, *C*). Of note, these $CD56^{bright}$ NK cells downregulated CD56 expression on stimulation but did not express CD16 (Fig 3, *C*). We further compared the expression of other NK surface receptors (NKG2A, TIM3, TIGIT, KIRs, NKG2D, and DNAM) and of perforin on stimulated $CD56^{bright}$ NK cells. The *de novo* induction of PD-1 by Dex was accompanied by a further increase of NKG2A expression, but it was not associated to other phenotypic changes (Fig E4, *B*). Notably, PD-1 was expressed by $NKG2A^{high}$ $CD56^{bright}$ NK cells (Fig E4, *C*).

Altogether, these data demonstrate that on persistent cytokine stimulation, mature $CD56^{dim}$ $CD16^+$ NK cells become activated and undergo apoptosis, while $CD56^{bright}$ NK cells expand and, in response to GC, upregulate PD-1 expression. Thus, PD-1 expression does not identify exhausted NK cells but rather functions as a "brake" to limit excessive NK cell activation.

GR signaling activates a transcriptional program responsible for the enhanced mRNA translation

To identify the transcriptional program responsible for the GC-dependent induction of PD-1 expression on NK cells, we performed RNA-sequencing on $CD56^{bright}$ NK cells that had been sorted from PBMCs of HDs and stimulated with IL-12, IL-15, and IL-18 in combination with Dex or vehicle (control) for 6 days. By comparing gene expression between Dex and control conditions, we identified a series of genes repressed or induced by GR. As shown in Table E2 in this article's Online Repository (available at www.jacionline.org), we found that many genes related to the immune response were downregulated by GCs, including those encoding for a variety of surface receptors (IL-23R, S1PR5, TLR4, IL-2RB, IL-12RB2, CXCR6). GO enrichment analysis was performed on the set of genes downregulated by GCs, and clusters of close GO terms were joined in "superclusters" of related terms (Fig 4, *A*). GO analysis showed that GCs repressed a transcriptional program responsible for responsivity to activating signals, and to the synthesis and secretion of cytokines (Fig 4, *A*), in line with the well-known effects of GCs as immune suppressors. We performed the same analysis on the set of genes upregulated by GCs. We found that the only genes related to the immune response upregulated by GCs were *IL1R2* and *CXCR4* (Table E2). GO analysis revealed that GCs, instead, induced a transcriptional program that promoted mRNA metabolism, translation, and protein targeting to plasma membrane (Fig 4, *B*).

We then performed quantitative PCR experiments to directly quantify *PDCD1* expression in unstimulated and stimulated NK cells. We also measured *PDCD1* expression in freshly isolated T cells and monocytes as positive and negative controls, respectively. While *PDCD1* transcript was undetectable in monocytes, it was measurable in unstimulated NK cells, confirming that it is expressed in steady-state conditions (Fig 5, *A*). IL-12–, IL-15–, IL-18–, and Dex-stimulation induced an increase of *PDCD1* transcript in NK cells after 4 days to a level that did not further increase at 6 days, and which was around 10-fold lower than that in T cells (Fig 5, *A*). Similar results were obtained using either *GAPDH* or *ACTB* as housekeeping gene (data not shown). Analyzing by Western blot PD-1 expression, we found that the total PD-1 amount was increased on the combined stimulation of IL-12, IL-15, IL-18, and Dex already at 4 days and then further at 6 days, to a level almost similar to that of freshly isolated T cells (Fig 5, *B*).

Collectively, these data suggest that GCs initially induce PD-1 transcription in NK cells, and then further increase its surface expression by promoting a transcriptional program resulting in enhanced mRNA translation and protein targeting to plasma membrane.

PD-1 expression on NK cells in the tumor microenvironment is associated with high GCs and cytokine levels

To verify whether the tumor microenvironment may display conditions similar to those that, *in vitro*, induced PD-1 expression on NK cells, we analyzed a pathological condition in which PD-1 expression by NK cells was reported. It was previously demonstrated that NK cells present in the malignant PEs from primary (mesothelioma) or metastatic (adenocarcinoma and carcinoma)

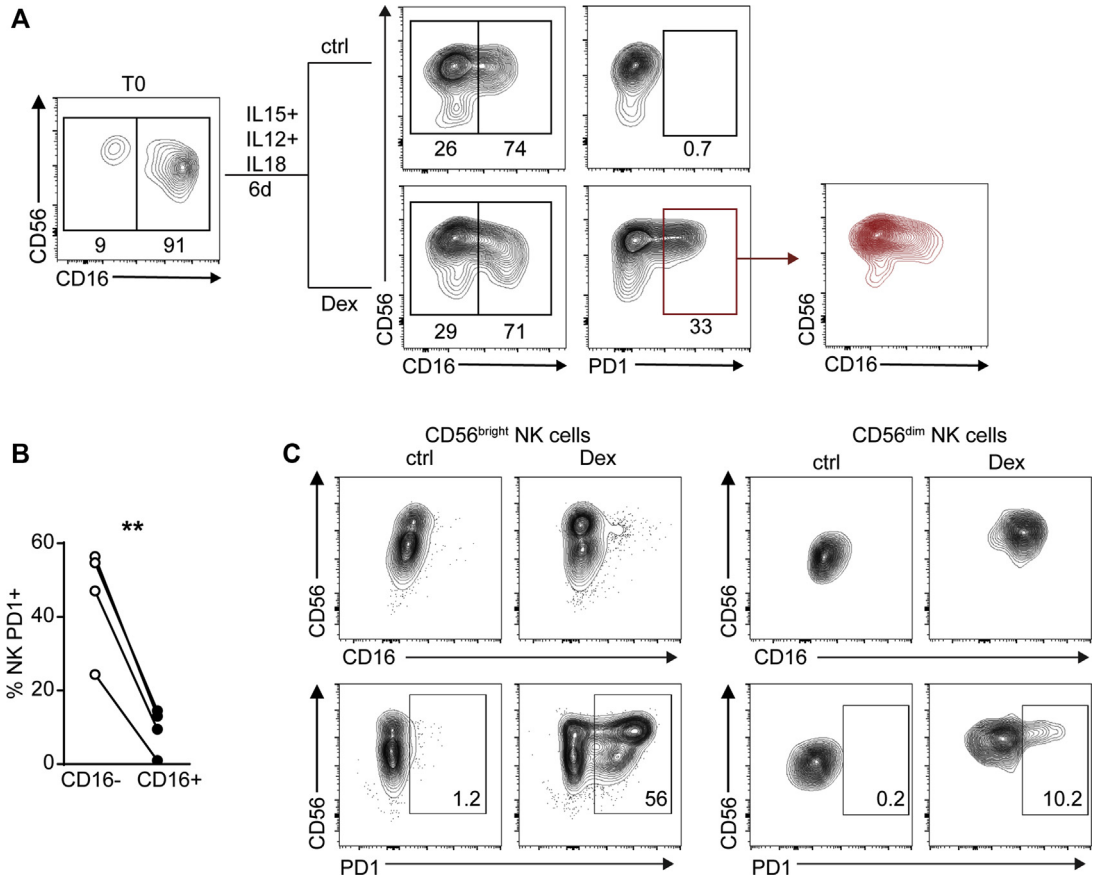

FIG 3. PD-1 expression is preferentially induced on CD56^{bright} NK cells by GCs. **A,** Cytofluorimetric analysis of NK cells freshly isolated from HDs' PBMCs (T0) and after 6 days of stimulation with IL-15 + IL-12 + IL-18 and Dex (500 nmol/L) or ctrl, showing the frequencies of CD16$^+$, CD16$^-$, and PD-1$^+$ NK cells. **B,** Percentage of PD-1$^+$ cells among CD16$^-$ and CD16$^+$ NK cells stimulated as in **A.** Each line represents a single HD. **C,** Cytofluorimetric analysis of CD56^{bright} and CD56^{dim} NK cells sorted at T0 and stimulated as in a, showing the frequency of PD-1$^+$ cells. $^{**}P < .01$; paired Student *t*-test **(B)**.

tumors express PD-1 at higher frequencies than do NK cells from autologous PB (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org).[20] PE NK cells were not anergic, as they could release cytokines and efficiently kill tumor targets including autologous tumor cells.[30,31] However, PD-1 engagement resulted in sharp inhibition of cytokine production.[20]

We measured the concentration of the endogenous GC cortisol in the plasma and PE of patients with mesothelioma and metastatic tumors (see Table E1). Our data clearly indicate that GC concentrations in both the PE and the plasma were much higher as compared to those in the plasma of HDs (Fig 6, *A*). It is conceivable that this may be consequent to the inflammatory state of these patients, suggesting that GR signaling may contribute to shape the antitumor immune responses. Moreover, we found that the phenotypic characteristics of PE NK cells were similar to those of NK cells treated *in vitro* with Dex in addition to IL-12, IL-15, and IL-18 (Fig 6, *B*). Indeed, PD-1 expression was mostly confined to NK cells with a CD56^{bright} phenotype, whose frequency was higher in PE than in PB,[30] and PD-1 was coexpressed with NKG2A (Fig 6, *B*). We then analyzed the cytokine microenvironment in the PE and PB of the same patient, confirming relevant differences (Fig 6, *C*; Fig E5, *B*; and Table E1). Indeed, IL-15, IL-18, and IL-12 were present at the tumor site, and IL-12p40 and IL-15 concentrations were higher as compared to the plasma (Fig 6, *C*). It is conceivable that the actual concentrations of GCs and cytokines acting on NK cells in a solid tumor microenvironment may be even higher than those detected in fluid PE, where soluble components may be substantially diluted.[32]

Collectively, these data revealed that the release of endogenous GCs is increased in the plasma of cancer patients in comparison to HDs, and this increase is associated with high concentrations of this hormone at the tumor site. The higher proportion of PD-1$^+$ NK cells in PE as compared to in PB is consistent with the concept that GCs alone are not sufficient for the induction of this inhibitory checkpoint, and that the cytokines present in the tumor microenvironment (including IL-12, IL-15, and IL-18) are fundamental for inducing PD-1 expression on NK cells.

These results strongly suggest that the mechanism we have identified *in vitro* may occur also at the tumor site in cancer patients.

DISCUSSION

PD-1 immune checkpoint blockade, alone or in combination with other antibodies/drugs, represents a highly successful therapeutic tool to unleash cytotoxic lymphocytes, including NK cells, and attack tumor cells in

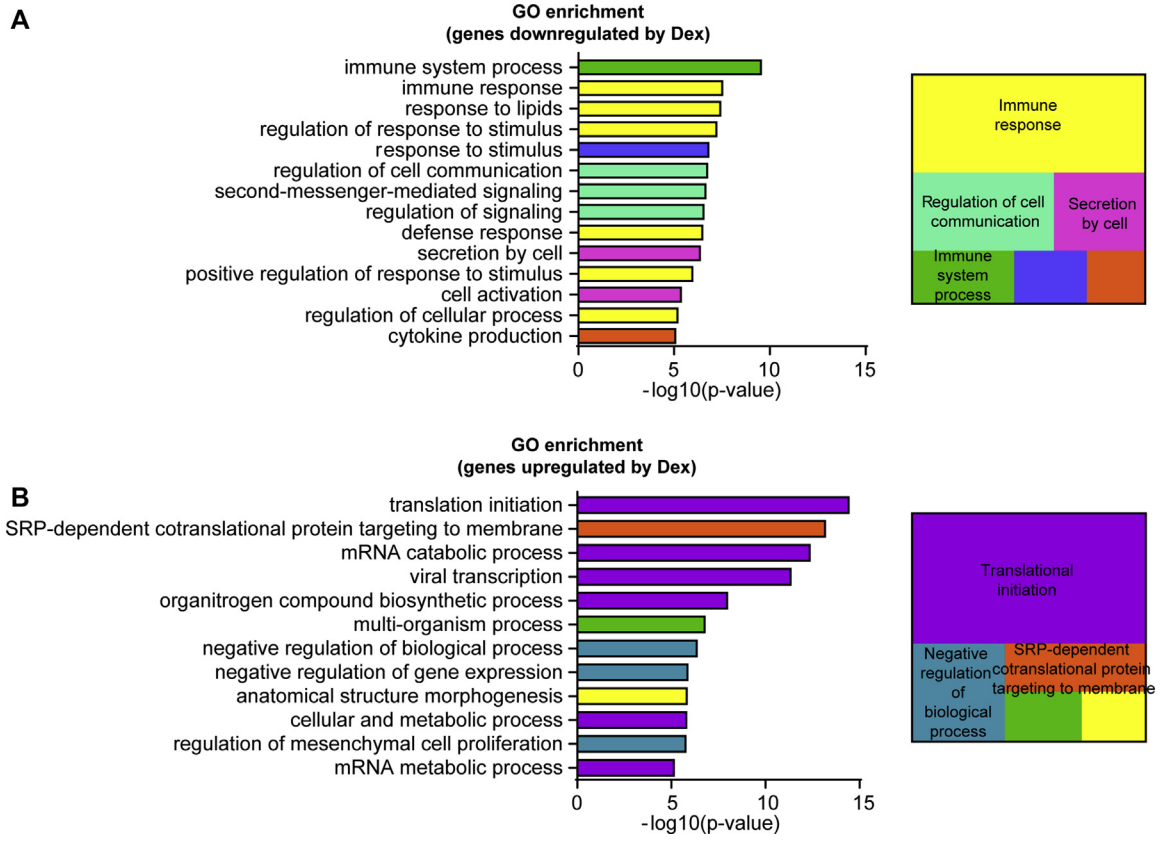

FIG 4. GCs induce a transcriptional program responsible for enhanced translation in CD56^{bright} NK cells. GO enrichment analysis performed with GOrilla on the set of differentially expressed genes between IL-15 + IL-12 + IL-18–stimulated CD56^{bright} NK cells in the presence of Dex (500 nmol/L) or ctrl identified by RNA-sequencing (n = 3 samples per group). Clusters of close GO terms joined with REVIGO in "superclusters" of related terms are visualized in different colors, with the size of rectangles adjusted to reflect their *P* value. The name of these superclusters is the name of the single representative GO term that has a more significant *P* value compared with the others of its family. *SRP*, Signal-recognition particle.

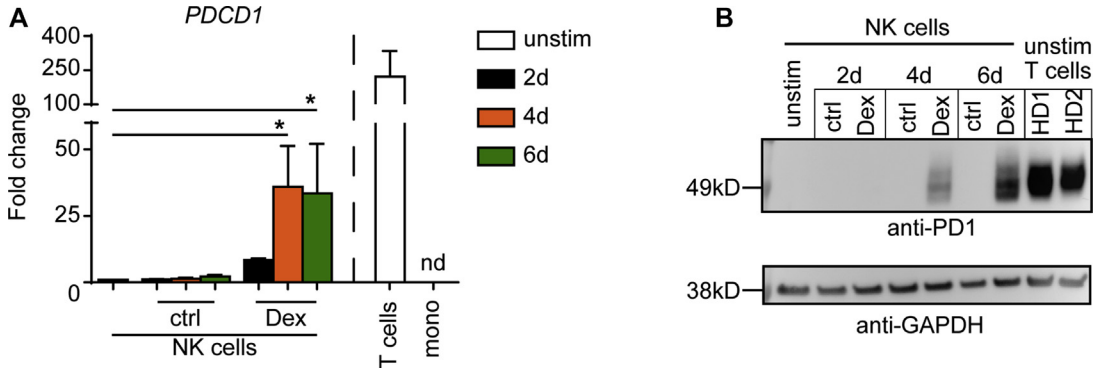

FIG 5. GCs increase PD-1 expression by enhancing *PDCD1* transcription and mRNA translation. **A,** *PDCD1* expression in freshly isolated NK cells, T cells, monocytes (*mono*), and in NK cells stimulated for the indicated time points with IL-12 + IL-15 + IL-18 in the presence of Dex (500 nmol/L) or ctrl, represented as fold change compared with *PDCD1* expression in freshly isolated NK cells. Data from 3 HDs are shown as means + SEM. **B,** PD-1 and GAPDH protein expression in total cell lysates of the same NK samples analyzed in **A** and in freshly isolated T cells from 2 HDs. Data from 1 representative experiment out of 3 performed are shown. **P* < .05; 1-way ANOVA. *nd*, Not detected; *unstim*, unstimulated.

different types of advanced cancers.[33,34] However, resistance to immunotherapy occurs for many patients, rendering the identification of the molecular mechanisms that control PD-1 and PD-L1 expression extremely important to allow the development of strategies for increasing the efficacy of the PD-1/PD-L1 axis blockade.

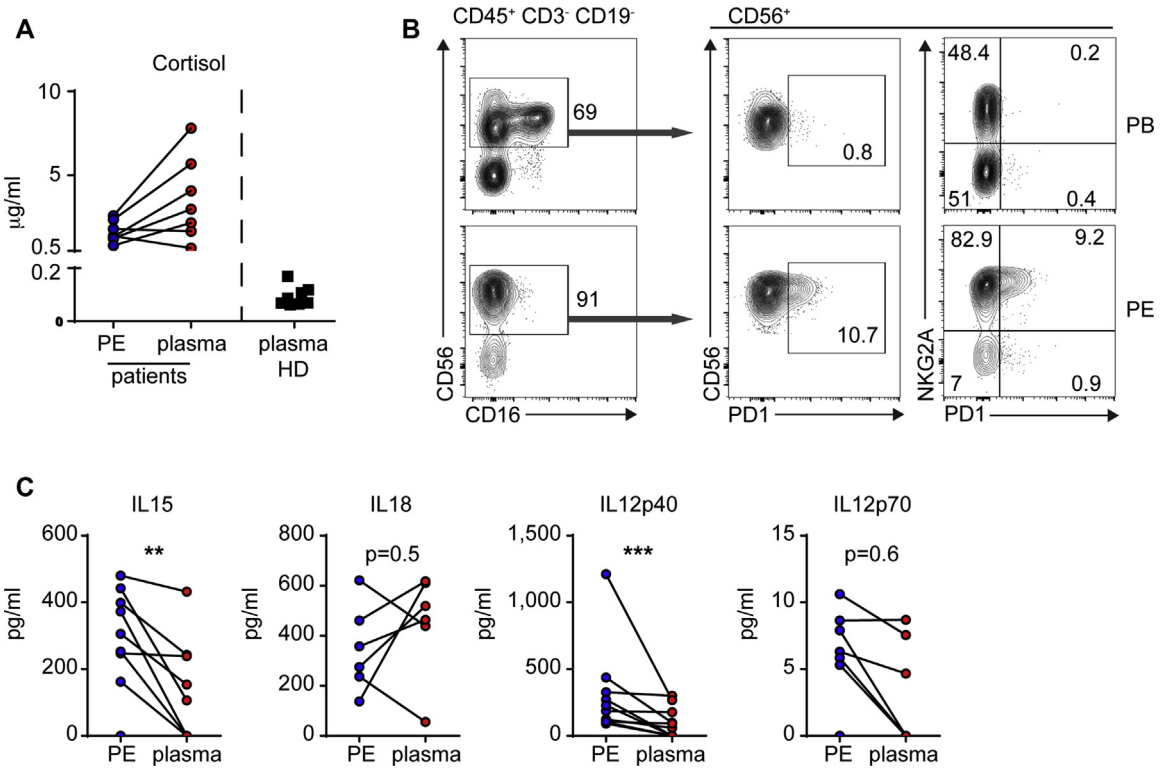

FIG 6. GCs, IL-12, IL-15, and IL-18 concentrations are elevated in the tumor microenvironment. **A,** Cortisol concentrations in the PEs and plasma of patients (n = 7) and in the plasma of HDs (n = 8). **B,** Cytofluorimetric analysis of NK cells gated as $CD45^+CD3^-CD19^-CD56^+$ among viable MCs isolated from PE and autologous PB of a representative patient, among 8 PE and 7 PBMC samples analyzed. **C,** Concentration of IL-15 (n = 10), IL-18 (n = 6), IL-12p40 (n = 10), and IL-12p70 (n = 10) in the PE and plasma of patients. Each line in **A** and **C** represents a single patient. $^{**}P < .01$, $^{***}P < .005$ paired Student *t*-test.

Here we identified a regulatory pathway induced by GCs in combination with cytokines responsible for the *de novo* expression of high levels of PD-1 on the membrane of $CD56^{bright}$ NK cells, which are the most abundant subset of tumor-infiltrating NK cells.[35] *In vitro* experiments demonstrated the specific requirement of GCs in combination with IL-12, IL-15, and IL-18 for PD-1 expression. Notably, this particular combination induced PD-1 expression specifically on NK cells but not in the other PB lymphocyte populations that we have analyzed ($CD3^+CD56^-$ T cells and $CD19^+$ B cells). Remarkably, the regulatory mechanism we identified is not only cell type–specific, but also species-specific because human NK cells show a different cytokine requirement than do murine NK cells. Indeed, different from what was previously shown in the mouse setting, IL-15, IL-18, and GCs signaling are not sufficient for PD-1 induction on human NK cells. Notably, IL-12 plays an indispensable synergistic role for the effect described in the present study. In contrast, IL-12 completely inhibited PD-1 induction on murine NK cells. Moreover, while 2 days of stimulation were sufficient for PD-1 expression in the mouse setting, human NK cells require a longer stimulation period. Altogether, the comparison of the data collected on NK cells from the 2 species demonstrates a strict requirement for GCs for the expression of the inhibitory checkpoint PD-1 on these innate lymphocytes. Moreover, this comparison suggests that the molecular mechanisms involved in NK cells from the 2 species are not the same, highlighting that it may be inappropriate to translate mouse data to human physiology and disease.

It has previously been shown that GR induces PD-1 expression in splenic murine NK cells at the transcriptional level.[7] However, the comparison of PD-1 transcript, total protein, and surface protein kinetics of expression, together with the RNA-sequencing data, strongly suggests a model in which GCs induce PD-1 expression in human NK cells not only by increasing *PDCD1* transcription, but also by enhancing mRNA translation. Indeed, the gene expression comparison between NK cells treated or not with GCs allowed the identification of the wide genomic effects induced by GR signaling. GCs shift the immune response transcriptional program induced by cytokines toward reactions and pathways involving RNA and enhance translation and protein translocation to plasma membrane. Therefore, our present study identifies a previously unappreciated genomic effect of GCs that not only inhibits the response of effector lymphocytes to immune stimulation, but also enhances protein translation leading to the expression of a critical immune checkpoint in the plasma membrane.

In addition to gene expression regulation at the transcriptional and posttranscriptional levels, the translational control of gene expression is increasingly considered as a critical effector mechanism in cancer biology.[36,37] For example, the oncogene transcription factor MYC was shown to exert selective effects on the translation of specific mRNAs that exceed the increase in their transcription. Analogously to what we found for GR, MYC was shown to control the global mRNA translation efficiency by inducing changes in the expression of ribosomal proteins, key translation factors, or cotranscriptional changes in

mRNA capping.[38] Interestingly, the posttranscriptional control of inhibitory checkpoint molecules, including PD-L1, by specific oncogenic pathways has recently emerged as a highly tunable mechanism for controlling the abundance of effector proteins that guide the immune evasion of cancer cells. In particular, in hepatocellular carcinoma, the cooperation of MYC and RTK/RAS signaling was shown to drive an increased tumor growth and metastatic spread by inducing PD-L1 expression selectively at the translational level.[39] Moreover, it has been reported that the eukaryotic translation initiation complex eIF4F regulates the translation of the mRNA encoding STAT1, which is responsible for the IFN-γ–induced PD-L1 expression in melanoma cells.[40]

Notably, the enhancement of translational initiation induced by GCs is exclusively associated to PD-1 induction, while the expression of other inhibitory checkpoint receptors is not affected. A remarkable exception is NKG2A, which is constitutively expressed on $CD56^{bright}$ NK cells. Remarkably, NK cells isolated from PE of patients with primary and metastatic cancers show substantial similarities with *in vitro* GC-treated NK cells. Thus, they both display PD-1 expression, an enrichment in the $CD56^{bright}$ $CD16^{-}$ subset and, importantly, PD-1 coexpression with high NKG2A levels. Further GC-induced NKG2A upregulation detected *in vitro* is in line not only with our findings on PE NK cells, but also with previous data on tumor-infiltrating $CD8^{+}$ T cells showing that PD-1 is often coexpressed with NKG2A.[13] *In vitro* GC-treated NK cells and PE NK cells show also functional similarities, as they are both responsive to stimulation, are not exhausted, and are inhibited by PD-1 engagement.[20,30] Importantly, *ex vivo* analysis of the composition of the tumor microenvironment confirmed that PD-1 induction on tumor-associated NK cells correlates with high levels of both GCs and cytokines. High endogenous cortisol levels were found both in the plasma and PE of these patients and are equivalent to the GC concentrations used in our *in vitro* experiments. Instead, the concentrations of cytokines detected in PE are lower compared with the doses used *in vitro*. However, in solid tumor environment, NK cells are in close proximity to vascular structures, stromal cells, and other cell types that release these relevant cytokines. Therefore, it is likely that the actual concentrations of cytokines acting on NK cells are higher at the tumor site compared with what we measured in exudates, which, because of their fluid nature, lack both cellular interactions and gradients present in solid microenvironments. These data support the notion that the critical cytokines (IL-12, IL-15, IL-18) we detected at the tumor site cooperate with GCs for the induction of PD-1 expression in NK cells, although we cannot exclude the contribution of additional factors *in vivo*. Notably, some of the cancer patients analyzed received corticosteroid treatment, which could further synergize with the endogenous cortisol to induce PD-1 expression in tumor-associated NK cells. In this context, in solid tumors, corticosteroids are often used to treat side effects of malignancies, such as edema, or to reduce the toxicity of chemo- and radiotherapy and to improve patients' quality of life.[41] Importantly, patients treated with immune checkpoint blockade who experience immune-related adverse events (eg, autoimmune reactions) are often treated with corticosteroids as immunosuppressive drugs. Our findings are in line with recent evidences suggesting that caution is needed when administering corticosteroids to patients with cancer. In particular, it was reported that both stress-dependent GC surge and the exogenous supply of GCs are sufficient to subvert chemotherapy-induced tumor-growth inhibition, thus reducing its efficacy.[4] Moreover, it has been shown that corticosteroids decrease high-affinity memory T-cell responses, thus substantially inhibiting the antitumor efficacy of immune checkpoint blockade,[3] and that GCs enhance PD-1 expression induced by T-cell receptor engagement on T lymphocytes.[27,28] Altogether, these findings clearly support the notion that corticosteroids can interfere with chemotherapy and counteract the antitumor immune responses elicited by immune checkpoint blockade.

In conclusion, our data show that the GR, in combination with a specific set of cytokines that may be present in the tumor microenvironment, contributes to the immune suppression of NK cells by inducing the expression of the inhibitory checkpoint PD-1 on $CD56^{bright}$ subset. Thus, GCs can "brake" this NK cell subset, largely predominant at the tumor site, which is characterized by proliferative capacity and cytolytic activity and is potentially efficient in antitumor responses.[42] These findings pave the way for further retrospective or prospective studies in patients with cancer to define whether administration of corticosteroids should be limited, whenever possible. Moreover, the finding that key immune-checkpoint proteins such as PD-1 and PD-L1 can be induced at the translational level on NK cells and tumor cells, respectively, suggests that the use of translation inhibitors might reveal a novel important approach for the therapy of cancer.

Clinical implications: PD-1 expression on NK cells is induced by GCs. Therefore, corticosteroid therapy may be counterproductive in combination with PD-1 immune checkpoint blockade in patients with cancer.

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