

Letter to the Editor

Nitric oxide induces human CLA⁺CD25⁺Foxp3⁺ regulatory T cells with skin-homing potential*To the Editor:*

Ultraviolet (UV) phototherapy is widely used to treat inflammatory skin diseases. Side effects include skin burning and photoaging as well as an increase in the risk of skin cancer. Identification of the molecular mechanisms involved in disease improvement may provide alternative treatments without side effects. Although vitamin D is known to contribute to phototherapy-induced immunoregulation, this still occurs independently of vitamin D, suggesting that alternative key mechanisms are involved.¹

Skin contains large stores of nitrogen oxide (NO) species that are released upon UV irradiation by photochemical reduction, leading to increased nitrites in blood.² Increased levels of urinary NO products correlate with clinical improvement in children with atopic dermatitis (AD).³ Also, NO synthase induces the release of NO from arginine following UV irradiation of the skin. Topical application of the NO synthase inhibitor NG-monomethyl-L-arginine acetate reduces UV-induced immunosuppression in humans, supporting a key role for NO.⁴ NO exerts both proinflammatory and anti-inflammatory effects, with clear differences between humans and mice.⁵ Moreover, the role of NO in T-cell differentiation remains controversial and poorly understood.^{6,7}

We hypothesized that NO may play a therapeutic role in UVB phototherapy for AD. Treatment of human CD4⁺ T cells with the stable NO-donor NOC-18 (100 μ M, a physiological concentration during acute inflammation⁷) did not affect CD25 expression. Only a slight decrease in cell viability was observed (<10%) (see Fig E1, A, in this article's Online Repository at www.jacionline.org). However, NOC-18 almost doubled the percentage of CD25⁺Foxp3⁺CD127⁻ regulatory T (Treg) cells and the Foxp3 expression level (Fig 1, A; see Fig E1, B). Expression of the skin-homing marker cutaneous lymphocyte-associated antigen (CLA) was higher on Treg cells (CD25⁺Foxp3⁺CD127⁻) than on effector T (Teff) cells (CD25⁺Foxp3⁻CD127⁺), and NOC-18 further increased CLA expression on Treg cells (Fig 1, B; see Fig E1, C). CD4⁺ T cells needed to be exposed to NOC-18 for at least 24 hours to induce CLA⁺ Treg cells (Fig E1, D). To investigate whether NO promoted Treg cells through the canonical sGC-cGMP signaling pathway, CD4⁺ T cells were activated with NOC-18 in the presence of the specific competitive inhibitor of sGC activation, 1-H-oxodiazolo-[1,2,4]-[4,3-a]quinoxalin-1-one (ODQ). Addition of ODQ reduced the NO-mediated increase in CD25⁺Foxp3⁺CD127⁻ T cells in a dose-dependent manner (Fig 1, C). No effect on cell viability was observed (not shown). Thus, NO promoted a skin-homing Treg cell phenotype via an sGC-GMP-dependent pathway. We next assessed whether NO could induce *de novo* Foxp3⁺ Treg cells from CD4⁺ T cells by depleting CD25hi cells from total CD4⁺ T cells (Fig E1, E) before addition of NOC-18. This significantly increased the frequency of CD25⁺Foxp3⁺CD127⁻ cells and increased their CLA expression (Fig 1, D). Therefore, NO induced CD25⁺Foxp3⁺CD127⁻ Treg cells *de novo* from CD4⁺CD25⁻ T cells.

The suppressive function of NO-CD4 Treg cells was demonstrated by activating CD4⁺ T cells with NOC-18, which were then cocultured with autologous responder cells prelabeled with ef670. NO-CD4 Treg cells significantly inhibited proliferation and CD25 induction in a dose-dependent manner (Fig 1, E; see Fig E2, A and B, in this article's Online Repository at www.jacionline.org). There was no inhibition of proliferation when control cells activated in the absence of NOC-18 were added to the responder cells (not shown). Moreover, NOC-18 inhibited proliferation of both Treg cells and Teff cells (Fig E1, F), suggesting that NO did not differentially affect the proliferation of a particular subset. To further confirm that CD25⁺Foxp3⁺CD127⁻ cells within the NO-CD4 cells were responsible for suppression, CD25hi cells (NO-Treg cells) were isolated from NOC-18-treated CD4⁺ T cells. These cells expressed high levels of Foxp3 (Fig E2, C). NO-Treg cells showed higher efficiency in inhibiting autologous T-cell proliferation (Fig 1, F; see Fig E2, D), indicating that NO induced functional highly suppressive Foxp3⁺ Treg cells *de novo* from CD4⁺CD25⁻ cells.

The suppressive function of NO-CD4 cells required cell-cell contact as shown using transwells in which responders and Treg cells were separately cocultured (Fig 1, E; see Fig E3, A, in this article's Online Repository at www.jacionline.org). In confirmation, proliferation was not affected when responder cells were activated with increasing doses of supernatants from either NOC-18-treated or control-treated T cells (Fig E3, B). The inhibitory function of NO-Treg cells was partially reversed in the presence of antibodies blocking PD-1/PD-L1 (Fig 1, F), but was unaltered by a CTLA-4 antibody (Fig E3, C). These data suggest that NO-induced Treg cells use PD-1/PD-L1 to exert their immunosuppressive function.

Following UVB irradiation of healthy volunteers, the release of NO and the NO derivative nitrite was assessed. After determination of the minimal erythema dose (MED) (see this article's Methods section in the Online Repository at www.jacionline.org), we irradiated an area of the forearm skin with 2 $\times$ MED. One day later, skin microdialysis was performed at the erythematous irradiated sites as previously described.² Cutaneous blood flow can act as a biomarker for NO. Laser Doppler flow measurements decreased blood flow on dermal microdialysis of the NO synthase inhibitor NG-Nitro-L-arginine methyl ester (L-NAME), but not on dialysis of the inactive enantiomer NG-Nitro-D-arginine methyl ester (D-NAME) (Fig 2, A). In parallel, nitrite in the dialysates was also reduced following L-NAME dialysis (Fig 2, B). These data demonstrate that UVB phototherapy enhanced NOS-derived NO production in the skin and cutaneous circulation and suggest that NO contributed to the immunosuppression induced by phototherapy.

Finally, we investigated circulating Treg cell and Teff cell populations in patients with AD, before and after phototherapy, and determined the improvement in clinical symptoms using SCORAD (SCORing Atopic Dermatitis) assessment (see Table E1 in this article's Online Repository at www.jacionline.org). We observed a significant correlation between AD improvement and the ratio of circulating Treg:Teff cells (Fig 2, C). Whether the accumulation of circulating Treg cells reflects a lack of migration to the skin or an increased pool of Treg cells, even after migration of some CLA⁺ Treg cells, remains to be further investigated.

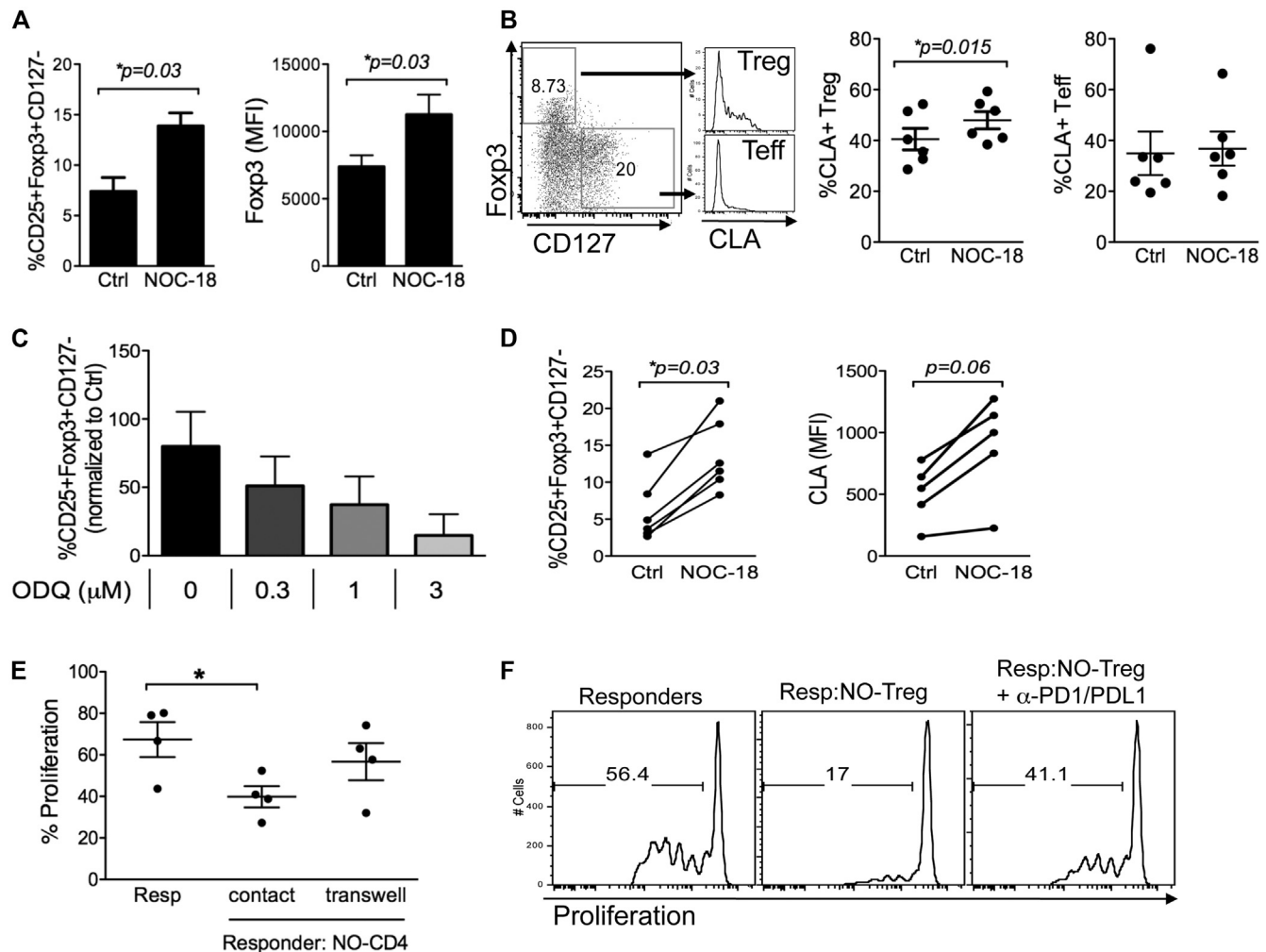


FIG 1. NO induced *de novo* human CLA⁺ Treg cells. CD4⁺ T cells were activated with anti-CD3/CD28 in the presence of NOC-18 (100 μM) for 5 days. **A**, Expression of CD25⁺Foxp3⁺CD127⁻ cells and Foxp3 (n = 6). **B**, CLA expression on Treg cells and Teff cells. **C**, Percentage of NOC-18–induced Treg cells in the presence of various concentrations of the sGC inhibitor ODQ, normalized to control for each donor (n = 3). **D**, Percentage of CD25⁺Foxp3⁺CD127⁻ cells and CLA expression on CD25⁺Foxp3⁺CD127⁻ cells after addition of NOC-18 to purified CD4⁺ T cells (Resp) or physically separated from Resp cells using transwells. **E**, NO-induced Treg cells (NO-CD4) were cocultured with prelabeled autologous CD4⁺ T cells (Resp) or physically separated from Resp cells using transwells. **F**, Proliferation of Resp cells cocultured with NO-Treg cells with antibodies blocking PD-1 and its ligand PD-L1. Representative of 2 donors. P values were calculated using the Wilcoxon paired *t* test or using the Friedman test followed by Dunns posttest for multiple comparisons. Mean ± SEM are represented. Ctrl, Control.

Altogether, our data indicate that NO is a key immunomodulator induced by phototherapy likely to participate in the immunosuppression observed in UV-irradiated patients. Our results demonstrate that NO promoted differentiation of highly suppressive human CD25⁺Foxp3⁺CD127⁻ Treg cells. Most murine studies suggest that NO decreases the suppressive activity of Foxp3⁺ Treg cells,⁶ and that NO induces a specific subset of murine Foxp3⁺ Treg cells, mediating suppression through the release of IL-10, independently of the sGC-cGMP cascade.⁷ In contrast, we found that NOC-18–treated human CD4⁺ T cells did not increase IL-10 production (not shown), used the sGC-cGMP pathway, and required cell-cell contact involving at least the PD-1/PD-L1 pathway to exert their immunosuppressive function. These contrasting findings may relate to intrinsic mouse/human differences.⁵ Human NO-induced Treg cells also expressed increased CLA, suggesting a greater ability to migrate to skin.

Healthy human skin contains functional Treg cells,⁸ and reports on the presence of Treg cells in lesional skin of patients with AD are not consistent.⁹ Our data suggest that NO released during phototherapy may restore/enhance suppressive function and Treg-cell migration to the skin to dampen localized inflammation.

These findings give novel insights for designing therapies targeting this pathway to avoid the deleterious side effects of phototherapy. In addition, NO can induce highly suppressive Treg cells *de novo* from CD4⁺CD25⁻ T cells. This strategy may provide a convenient means to generate high numbers of autologous Treg cells for human therapies aimed at counteracting the exacerbated immune responses observed in autoimmune and atopic diseases, or transplantation.

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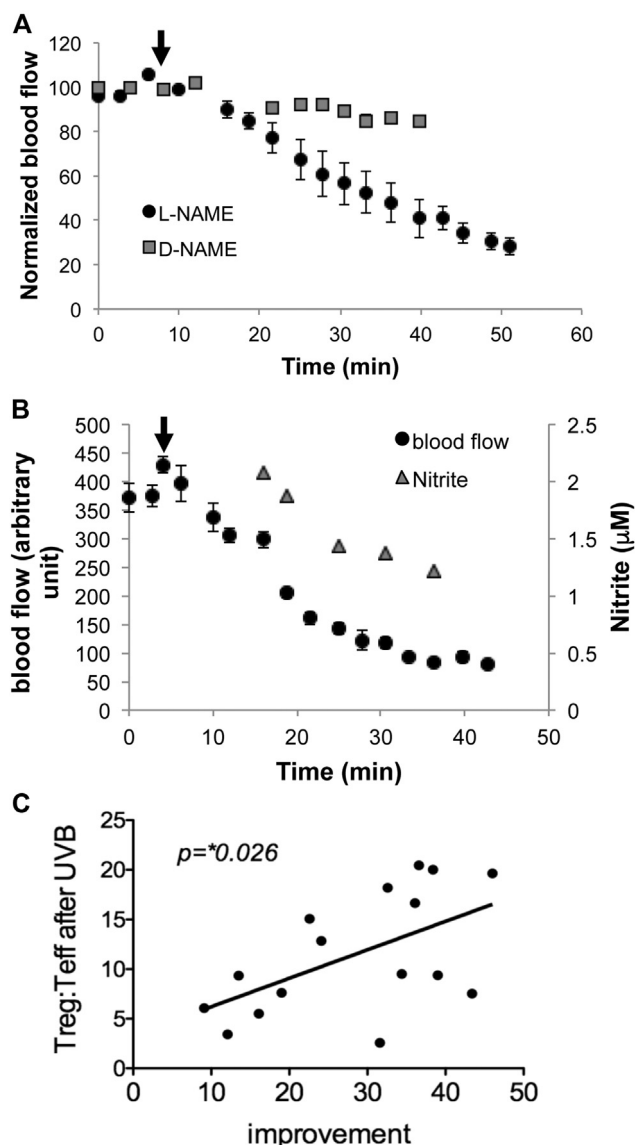


FIG 2. UVB induced NO production and Treg cells *in vivo*. **A**, Twenty-four hours after irradiation of the forearm of healthy volunteers ($n = 5$), cutaneous microdialysis was performed in the presence of L-NAME ($n = 5$, mean $\pm$ SEM), an NO synthase inhibitor, or its inactive enantiomer D-NAME ($n = 3$). Blood flow results are normalized against initial blood flow. **B**, Levels of dialysate nitrite concentration (as a measure of NO, triangles) were plotted against blood flow (arbitrary units) as a measure of erythema (black circles). Figure is representative of 2 subjects. Nitrite was at the lower level of detection of the apparatus and could not be combined for analysis. **C**, A cohort of patients with AD was treated by phototherapy (Table E1). The improvement in disease for each donor was assessed by SCORAD (SCORing Atopic Dermatitis) before and after phototherapy, and correlated with the Treg:Teff cells ratio.

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METHODS

Cell purification and activation

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom [UK]), from the venous blood of the healthy volunteers, obtained after informed consent (Research Ethics Committee reference no. 08/S1103/38). CD4⁺ T cells were negatively isolated using magnetic beads (Stem Cells, Manchester, UK; purification, >95%), and cultured in RPMI 1640 with 10% FCS at 0.5×10^6 per well in a 48-well plate precoated with anti-CD3 (OKT3, 5 µg/mL) or anti-CD3/α-CD28 (CD28.2, 5 µg/mL, ebiosciences, Hatfield, UK). In some experiments, CD25hi cells were isolated from total CD4⁺ T cells by positive selection with EasySep Human CD4⁺CD25high T-Cell Isolation Kit (Stem Cells), according to manufacturer's instructions. In other experiments, CD4⁺CD25[−] T cells were isolated by negative selection using the EasySep Human CD4⁺CD25high T-Cell Isolation Kit (Stem Cells), according to manufacturer's instructions. The stable NO-donating compound 3,3-Bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC-18) (Sigma, Gillingham, UK) was added at the beginning of the culture (100 µM).^{E1}

Flow cytometry

The following antibodies were used for fluorescence-activated cell sorting staining (all from Biolegend, London, UK, except where stated): Viability dye eFluor 780 (ebioscience), anti-CD3 AF700, anti-CD4 FITC (BD Biosciences, Oxford, UK), anti-CD4 PerCP, anti-CD25 AF488, anti-CD25 APC (BD Pharmingen, Oxford, UK), anti-CLA Pacific Blue, anti-CD127 PE (ebioscience), and anti-Foxp3 PE or anti-Foxp3 AF647 (BD Biosciences). For intracellular staining, Foxp3 staining buffer set (BD Biosciences) was used, following the manufacturer's instructions. Staining was analyzed using the Fortessa (BD) flow cytometer. To calculate the relative expression, the mean fluorescence intensity (MFI) obtained with the isotype control was subtracted from the MFI obtained with the specific antibodies.

Proliferation assay

CD4⁺ T cells were stained with the proliferation dye ef670 (ebioscience) according to the manufacturer's instructions. Cells were then activated by plating 5×10^4 in a precoated 96-well plate as described above. After 5 days, proliferation was analyzed using the Fortessa flow cytometer, and data were analyzed using FlowJo software.

Suppression assays

CD4⁺ T cells prestained with the proliferation dye ef670 (5×10^4 cells/well used as responders) were cocultured in 96-well U-bottom plates with different numbers of NO-treated CD4⁺CD25⁺ T cells (as suppressor cells) and activated by anti-CD3/CD28 (5 µg/mL) in 200 µL RPMI. The proliferation of the responder cells was analyzed using the Fortessa flow cytometer.

Transwell assays

CD4⁺ T cells prestained with the proliferation dye ef670 (10×10^5 cells/well used as responders) were cultured in the presence of precoated anti-CD3/CD28 (5 µg/mL) in 800 µL RPMI. Different numbers of NO-treated CD4⁺ T cells as suppressor cells were seeded in the transwell insert, which separates suppressor cells from responder cells. The proliferation of responder cells was analyzed using the Fortessa flow cytometer.

Statistics

Data were analyzed using Graphpad Prism software. Flow cytometry data were analyzed using the Wilcoxon test, when assessing paired samples, or a Friedman test followed by Dunns posttest for multiple comparisons. All data were expressed as mean $\pm$ SEM. All *P* values were 2-tailed, with a 95% CI.

Determination of MED

Consent for the study was obtained from the Lothian Regional Ethics Committee (2002/4/37). Five 2-cm diameter spots on the inner side of the forearm of 5 healthy human volunteers (3 females, 2 males, age 20-23 years) were irradiated with graded doses of broadband UVB from a xenon-arc UV photolamp (Xe/HgXe Arc lamp, Oriel Instruments, Stratford, Conn) with irradiances from 28 to 112 mJ/cm². The lowest dose of UV to produce a clearly defined erythral response after 24 hours was taken as the MED. Volunteers were then irradiated with twice their personal MED for the microdialysis experiment.

Skin microdialysis

Two sites on the inner forearm were selected for microdialysis. At each site, 2 MEDs were administered to a 2-cm diameter area. Twenty-four hours later, the irradiated area was anesthetized with topical lidocaine/prilocain cream (EMLA 5%, AstraZeneca, Luton, UK). After 1 hour a 23-gauge needle (BD Microlance 0.6 mm $\times$ 2.5 mm) was inserted along the dermoepidermal junction for 20 mm length under sterile conditions. The needle was flushed briefly with saline to remove blood from the internal lumen and a microdialysis catheter introduced (prepared locally as described previously^{E2}). The needle was removed, leaving a 20 mm length of the dialysis fiber in place under the epidermis. This was flushed with saline and residual lidocaine cream removed from the skin. Sixty minutes resting time was left between catheter insertion and commencement of microdialysis to allow the cutaneous circulation to recover from the needle insertion and lidocaine application. All experiments were carried out in an ambient temperature of 24°C to 26°C. PBS with 5 mM N^G-Nitro-L-arginine methyl ester (L-NAME) (Sigma-Aldrich Company Ltd, Gillingham, UK) was dialysed through 1 catheter in each subject at 2 µL/min using a micro infusion pump (PHD 2000 infusion pump, Harvard Apparatus, Holliston, Mass) and 5 mM N^G-Nitro-D-arginine methyl ester (D-NAME) (Sigma-Aldrich Company Ltd) dialysed to the second catheter. Nonfunctioning catheters in 2 volunteers meant that D-NAME data were available from only 3 individuals.

Blood flow measurement by laser Doppler flowmetry

Cutaneous blood flow, measured as red blood cell flux, was measured by laser Doppler perfusion (Moor Instruments Ltd, Axminster, UK) with probes placed on skin overlying the microdialysis site recording at 3-minute intervals. Because insertion of the microdialysis catheter and infusion of fluid can cause a slight increase in blood flow, normal saline was infused before commencement of microdialysis experiments and blood flow recorded until 3 consecutive recordings were similar.

Determination of NO concentration in dialysates

NO has a half-life of a few seconds, but its oxidation product, nitrite, is stable for 1 or 2 hours in aqueous solution. Dialysate samples from 3 L-NAME microdialysis samples were immediately frozen at -70°C for later analysis. Nitrite was measured using the chemiluminescence-based tri-iodide reduction.^{E3} Briefly, nitrogen gas (Saffire, BOC, Broxburn, UK) was bubbled through a solution of 1 mol potassium iodide (Sigma Chemicals, Gillingham, UK) and glacial acetic acid at a constant pressure of 5 bar in a glass reservoir. Through a rubber cap on the top of the reservoir, 10 µL of dialysate was introduced into the solution using a 10-µL syringe (SGE Ltd, Milton Keynes, UK). The effluent nitrogen/NO mixture was drawn into a chemiluminescence analyzer (42C NO-NO₂-NOX Analyzer, Thermo Environmental Instruments Inc) connected to an analogue-digital converter (MacLab/4e AD Instruments with Chart v3.2 for Macintosh). For each batch of dialysates, a set of nitrite standards was analyzed for calibration of the equipment. Sodium nitrite (AnalR, BDH Chemicals Ltd, VWR, Lutterworth, UK) was prepared in solution and ascending concentrations from 0.625 to 50 µM NaNO₂ were analyzed in 10 µL triplicates. Nitrite levels in the dialysate were at the lower levels of detection of the chemiluminescence meter and could not be combined for a mean. A representative result has been shown.

Patients with AD

The study was approved by the South East Scotland regional ethics committee REC 02 (REC 13/SS/0151). Patients were recruited from the Department of Dermatology at the University of Edinburgh and University Hospital Birmingham. The exclusion criteria for the recruitment were treatment with systemic corticosteroids or other immune response–modifying systemic drugs such as azathioprine or methotrexate in 4 weeks before the study starts; taking aspirin or other nonsteroidal anti-inflammatory drugs in the week before each blood sampling; inability to give informed consent; pregnancy; skin type 1 (always burns, never tans); and involved in current research or have recently been involved in any research before recruitment. The filaggrin genotype was analyzed by the Wellcome Trust Clinical Research Facility (Edinburgh) and donors with mutation were excluded. In total, 10

males and 6 females aged between 23 and 56 years were analyzed (Table E1). Blood samples were collected before and after 8 weeks of phototherapy.

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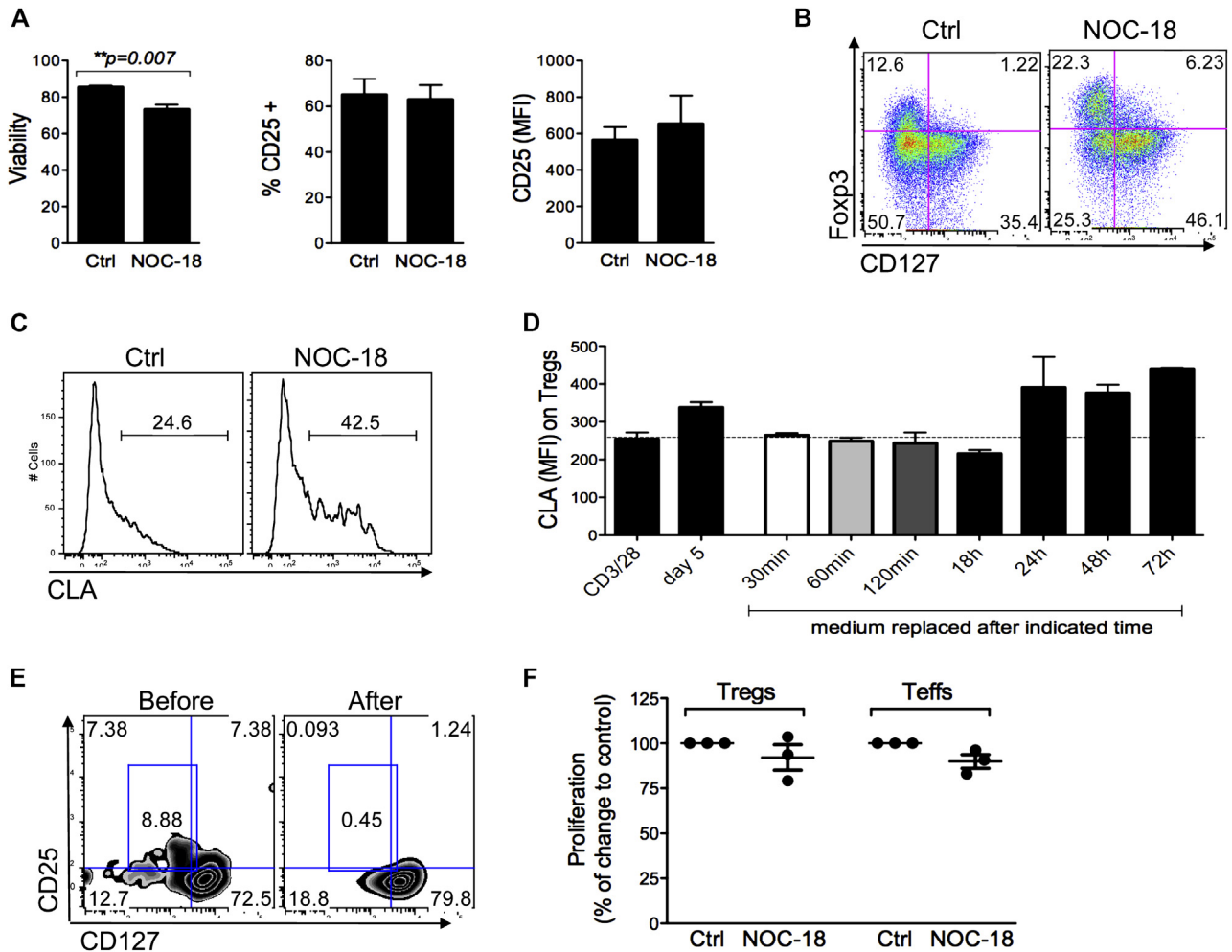


FIG E1. NO promoted CLA⁺ human Treg cells. Purified peripheral CD4⁺ T cells were activated with plate-bound anti-CD3/CD28 in the presence of the NO-donating compound NOC-18 (100 μ M) for 5 days before analysis by flow cytometry. **A**, T-cell viability and CD25 expression (% and MFI) (mean $\pm$ SEM, $n = 7$). **B**, Representative plots showing expression of CD25⁺Foxp3⁺CD127⁻ cells after addition of NOC-18. **C**, Representative plots showing expression of CLA on CD25⁺Foxp3⁺CD127⁻ cells after addition of NOC-18. **D**, CD4⁺ T cells were activated in the presence of NOC-18 for various amounts of time before replacing the culture medium without NOC-18, and CLA expression on Treg cells measured after 5 days (mean $\pm$ SEM, $n = 2$). **E**, Representative FACS plot showing CD25 versus CD127 before (left panel) and after (right panel) CD25⁺ depletion. **F**, CD4⁺ T cells were stained with the proliferation dye ef670 before being activated with plate-bound anti-CD3/CD28 in the presence of 100 μ M NOC-18 for 5 days. Proliferation of Treg cells and Teff cells was then analyzed by flow cytometry and normalized to control ($n = 3$). Ctrl, Control.

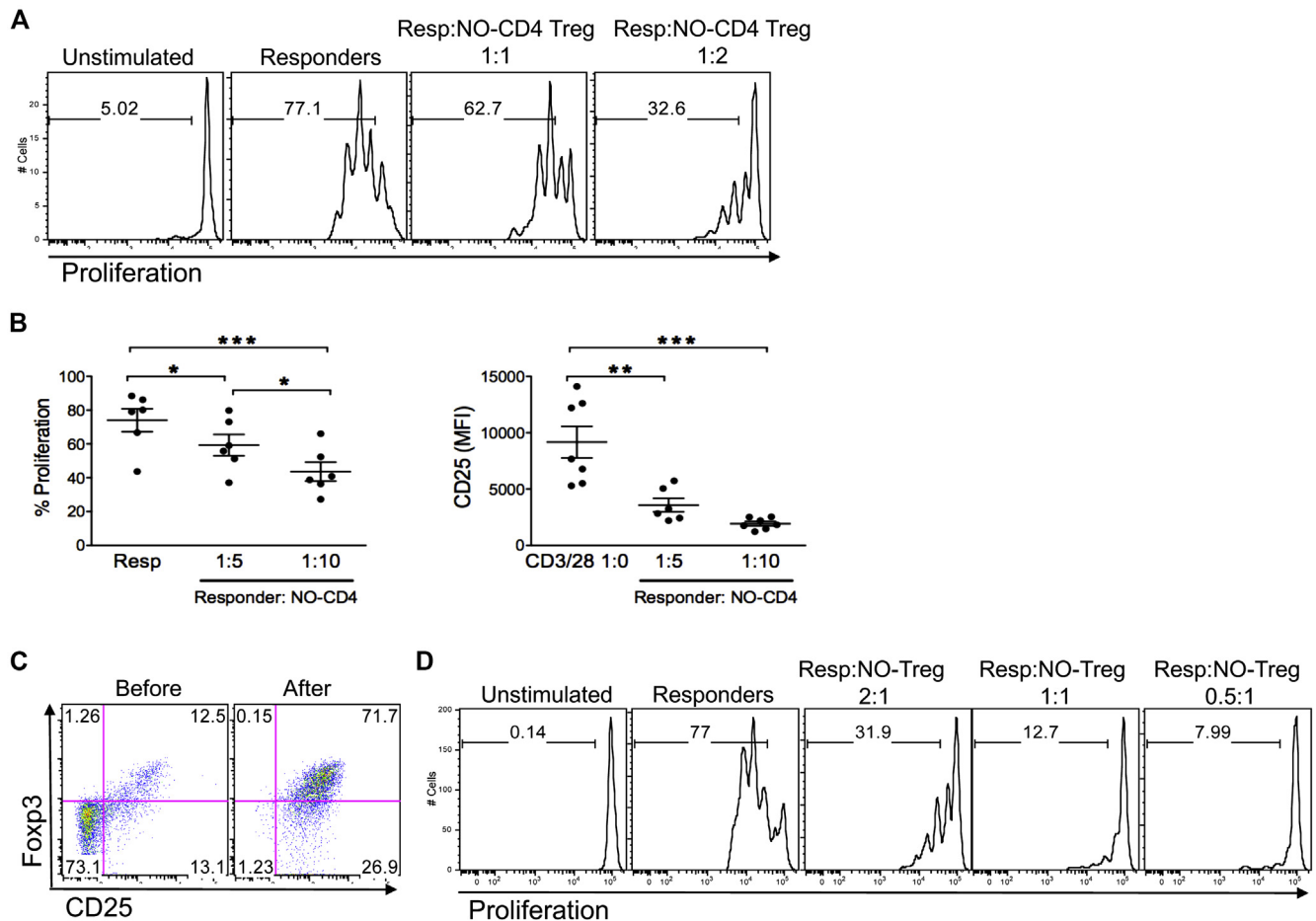


FIG E2. NO-induced CD25⁺Foxp3⁺CD127⁻ Treg cells inhibited the proliferation of autologous CD4⁺ T cells in a dose-dependent manner. CD4⁺ T cells were activated with plate-bound anti-CD3/CD28 in the presence of 100 μ M NOC-18 for 5 days (NO-CD4 Treg cells). Autologous CD4⁺ T cells were used as responder cells (Resp) and cocultured with NO-CD4 Treg cells at different ratios for 4 days. Twenty percent of cells had a CD25⁺Foxp3⁺CD127⁻ Treg-cell phenotype after addition of 100 μ M NOC-18 for 5 days. Therefore, to achieve a responder cells:Foxp3⁺ cells ratio at 1:1 and 1:2, responder cells were cultured with NO-CD4 at 1:5 and 1:10 ratios. **A**, Representative FACS plot showing proliferation of responder cells cultured with NO-CD4 T cells. **B**, Mean $\pm$ SEM data for proliferation and CD25 expression for 6 donors. *P* values were calculated by the Friedman test followed by Dunns posttest. **C**, CD25^{hi} cells were isolated from NO-CD4 Treg cells (NO-Treg cells). Representative FACS plot showing CD25 versus Foxp3 before and after isolation. **D**, NO-Treg cells were cocultured with autologous responder CD4⁺ T cells (Resp) at different ratios for 4 days and proliferation of autologous CD4⁺ T cells was analyzed by flow cytometry. Representative of 3 donors.

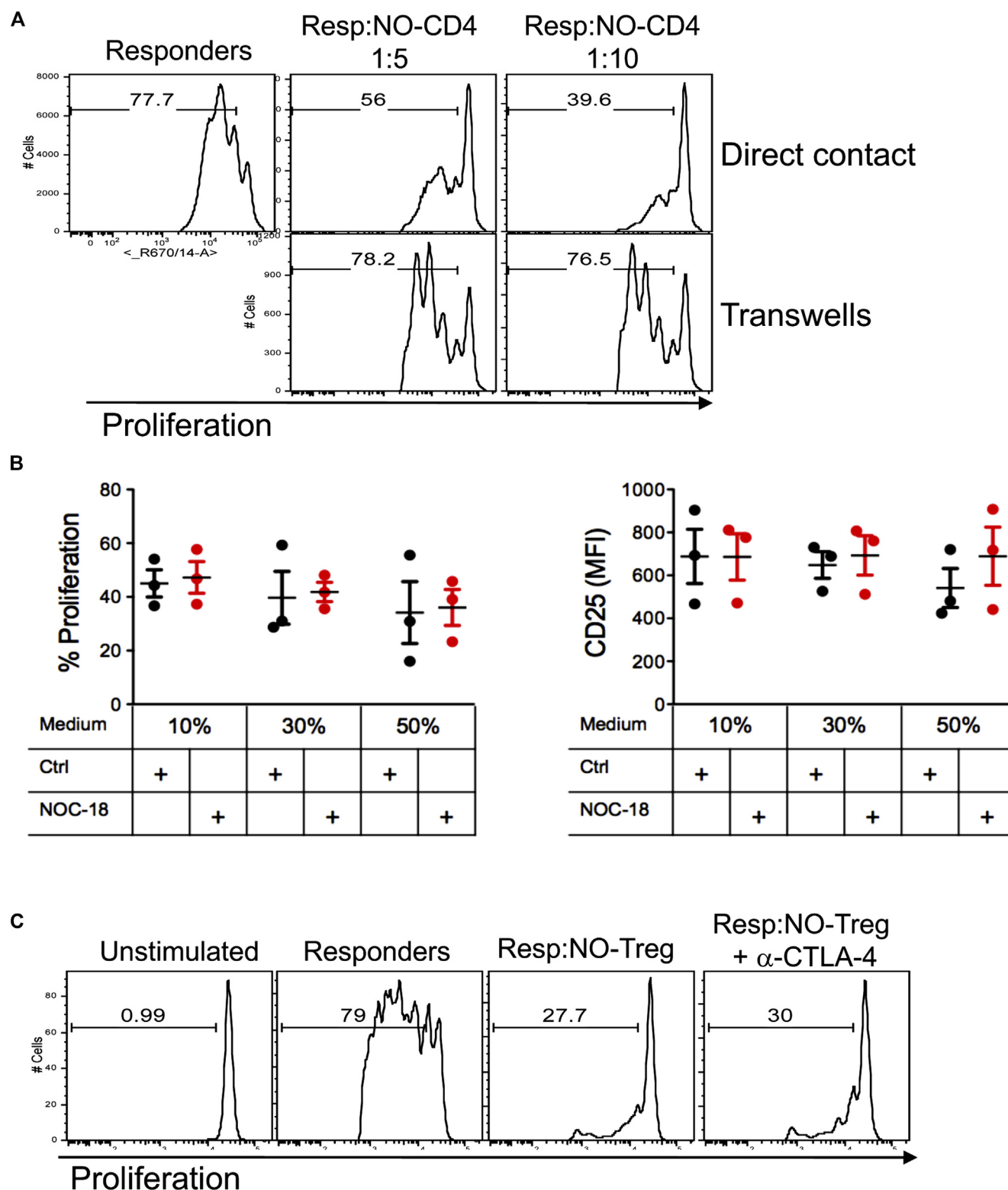


FIG E3. NO-induced Foxp3⁺ Treg cells required cell-cell contact for their suppressive function. **A**, CD4⁺ T cells were activated with anti-CD3/CD28 in the presence of 100 μ M NOC-18 for 5 days (NO-CD4), and used as suppressor cells in coculture with ef670-labeled autologous CD4⁺ T cells or physically separated from autologous CD4⁺ T cells using transwells. Representative FACS plots showing proliferation of autologous CD4⁺ T cells. **B**, Culture supernatants from CD4 T cells cultured with or without 100 μ M NOC-18 for 5 days were mixed with fresh RPMI at different ratios and transferred to ef670-labeled autologous CD4 T cells. Proliferation was analyzed by flow cytometry ($n = 3$, mean $\pm$ SEM). **C**, CD25hi cells (NO-Treg cells) were isolated from total CD4⁺ T cells activated with 100 μ M NOC-18 for 5 days. NO-Treg cells were cocultured with autologous CD4⁺ T cells in the presence of antibodies blocking CTLA-4. Representative of 2 donors. Ctrl, Control.

TABLE E1. Patients' characteristics used in this study

Patient no.	Age (y)	Sex	SCORAD before treatment	SCORAD at 8 wk	Improvement	Total UVB dose (J/cm ²)
UVAD1	39	M	46.1	14.5	31.6	8.8
UVAD2	48	M	50.5	16.1	34.4	10.4
UVAD3	40	M	27.5	15.4	12.1	15.9
UVAD4	43	M	48.7	16.1	32.6	6.7
UVAD5	30	M	26	9.9	16.1	8.8
UVAD6	51	F	28.6	19.5	9.1	7.7
UVAD7	32	F	32.6	13.6	19	14.5
UVAD8	36	F	28.3	5.7	22.6	19.6
UVAD9	54	M	38.3	24.8	13.5	8.5
UVAD10	50	M	73	27	46	11.5
UVAD11	47	F	60.8	24.7	36.1	5.1
UVAD12	23	F	55.9	17.5	38.4	8.9
UVAD13	48	M	57.9	18.9	39	7.6
UVAD14	56	M	40.2	16.1	24.1	5.2
UVAD15	48	M	55.7	12.3	43.4	7.2
UVAD16	43	F	51.5	14.9	36.6	11

F, Female; M, male.