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Glucocorticosteroids Modify Langerhans Cells To Produce TGF- β and Expand Regulatory T Cells

Georg Stary,* Irene Klein,* Wolfgang Bauer,* Frieder Koszik,* Bärbel Reininger,* Sabine Kohlhofer,* Kristina Gruber,* Hans Skvara,* Thomas Jung,[†] and Georg Stingl*

Although glucocorticosteroids (GCSs) have been used for many decades in transplantation and (auto)inflammatory diseases, the exact mechanisms responsible for their immunosuppressive properties are not fully understood. The purpose of this study was to characterize the effects of oral GCSs on the cutaneous immune response. We analyzed, by immunofluorescence staining and quantitative RT-PCR, residual skin biopsy material from a clinical study in which we had used oral GCS as positive control for determining the effects of candidate anti-inflammatory compounds on epicutaneous patch tests of Ni-allergic patients. Expectedly, oral GCS treatment led to a reduction of clinical symptoms and infiltrating leukocytes. Notably, we observed increased numbers of dermal FOXP3⁺CD25⁺ T cells and epidermal Langerhans cells (LCs) that were associated with upregulated mRNA expression of TGF- β in lesions of GCS-treated Ni-allergic patients. To investigate this phenomenon further, we exposed purified LCs to GCS. They exhibited, in contrast to GCS-nonexposed LCs, 1) a more immature phenotype, 2) higher intracellular amounts of TGF- β , and 3) increased receptor activator for NF- κ B expression, conditions that reportedly favor the expansion of regulatory T cells (Tregs). Indeed, we observed an enhancement of functionally suppressive FOXP3⁺ T cells when CD3⁺ cells were incubated with GCS-pretreated LCs. The expansion of Tregs was inhibited by TGF- β blockage alone, and their suppressive activity was neutralized by a combination of anti-TGF- β and anti-IL-10 Abs. Our data show that systemically applied GCSs endow LCs with Treg-promoting properties and thus shed new light on the mechanisms of GCS-mediated immunosuppression. *The Journal of Immunology*, 2011, 186: 000–000.

The ideal immunosuppressive drug should not only be very effective but also exhibit a high degree of specificity for the exaggerated immune/inflammatory response and thus be well tolerated and not accompanied by injurious effects on other cells and tissues. Glucocorticosteroids (GCSs) are highly effective immunosuppressive drugs but, unfortunately, do not target the cause of disease and are therefore associated with considerable adverse events (1). Characterization and segregation of both the desired and the undesired effects of GCSs on immune cells should be a promising strategy for the development of more specific anti-inflammatory therapies (2). GCSs are small lipophilic compounds that mediate their biological effects by binding to an intracellular receptor that translocates to the nucleus and targets specific DNA sequences resulting in the blockage of several inflammatory pathways (3, 4) and in the induction of apoptosis (5). These events affect different cellular subsets and lead to the inhibition of T cells (6, 7) and the modulation of dendritic cell (DC) function (8).

Depending on the pathogen, the microenvironment, and the profile of costimulatory molecules, DCs drive the development of

either proinflammatory T cell subsets (e.g., Th1, Th2, or Th17) (9–12) or regulatory T lymphocytes (13–15). This is also true for epidermal Langerhans cells (LCs) (16–18). In mice, the interaction of the receptor activator for NF- κ B (RANK) with its ligand (RANKL), originally described as regulators of bone remodeling and T cell/DC communication (19), is apparently crucial for the induction of regulatory T cells (Tregs) by LCs (17, 18). In man, the role of this receptor–ligand interaction in governing the quality of LC-induced immune responses has not yet been investigated.

Certain immunosuppressive drugs and anti-inflammatory compounds, such as 1 α ,25-dihydroxyvitamin D₃ (20) or rapamycin (21), endow DCs with tolerogenic properties. In the case of GCS, it has been reported that dexamethasone (Dex)-treated DCs remain in an immature state and cause T cell responses of only low magnitude (22–24). T cell priming by Dex-exposed DCs leads to a decrease of IFN- γ -producing and an increase of IL-10-producing T cells, causing a prolonged survival of skin and heart allografts in mice (25, 26). This T cell hyporesponsiveness can be explained by either a survival advantage (27) or the active induction of Tregs. Increased mRNA levels of FOXP3, a marker for Tregs, are found in lymph node cells of mice treated with Dex-DCs (26), and GCS amplifies the IL-2-dependent expansion and function of Tregs in an autoimmune encephalomyelitis mouse model (28). Human Dex-treated DCs expressing the glucocorticoid-induced leucine zipper are capable of inducing Tregs (29). Phenotypic studies of markers expressed by Tregs speak for an increase of such cells within the peripheral blood of patients with autoimmune or allergic diseases upon oral glucocorticoid treatment compared with that of the pretreatment situation (30–32). These data indicate that GCSs actively induce regulatory functions of the immune system and do not simply suppress inflammatory pathways in both leukocytes and nonleukocytes.

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Abbreviations used in this paper: CHS, contact hypersensitivity; DC, dendritic cell; Dex, dexamethasone; EPT, epicutaneous patch test; GCR, glucocorticoid receptor; GCS, glucocorticosteroid; LC, Langerhans cell; mDC, myeloid dendritic cell; NS, nonlesional skin; pDC, plasmacytoid dendritic cell; RANK, receptor activator for NF- κ B; Treg, regulatory T cell; TRITC, tetramethylrhodamine isothiocyanate.

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We conducted a study to investigate the variability and reproducibility of epicutaneous patch test (EPT) reactions in Ni-allergic patients. Oral GCSs were used to modify the cutaneous immune response and to analyze cellular and molecular mechanisms in the skin that are associated with its immunosuppressive properties. Our data indicate that GCSs are able to actively induce a T regulatory response in human skin as one mechanism to control inflammation.

Materials and Methods

Patients and study design

Twenty-four Ni-sensitive healthy individuals (20 women, 4 men; mean age, 36.3 ± 7.5 y; range, 23–49 y), recruited from the allergy outpatient clinic of the Department of Dermatology, Medical University of Vienna (Vienna, Austria) and with a history of a positive Ni EPT reaction within the last 6 wk and no other clinically significant abnormalities, were enrolled into this double-blind, placebo-controlled study. All subjects underwent two periods of EPT challenges by cutaneous application of Ni allergen and vehicle control to the back for 48 h using standard patch testing methodology. The two EPT periods were separated for 30 d. The subjects received oral pretreatment only prior to and during the second EPT period (Fig. 1A). Seventy-two hours after the first patch test application, lesional and non-lesional skin samples were collected. Thereafter, subjects were randomly assigned to the prednisone group (40 mg per day orally) or placebo group and treated for 10 d. Their compliance was monitored by visits at the study site every other day to pick up their medication. A second EPT was conducted at the end of the treatment period (Fig. 1A), and one lesional skin biopsy was taken. EPT reactions were assessed 48 h and 72 h after patch test application by physician grading (0, no dermatitis; 0.5, erythema not covering the test area; 1, erythema, infiltration, possibly papules; 2, erythema, infiltration, papules, vesicles; 3, intense erythema, infiltration, coalescing vesicles, IR-irritative reaction) and by subject grading of itch on a visual analogue scale (0 [no itch] to 10 [most severe]).

All subjects gave their written informed consent after approval by the ethics committee (EK 122/2006) of the Medical University of Vienna (Vienna, Austria) and the health authorities (Bundesministerium für Gesundheit). The study was conducted according to the guidelines of the Declaration of Helsinki.

Immunofluorescence staining and flow cytometry

Single and multicolor immunofluorescence staining was performed as previously described (33). To determine the quantity of Tregs and the phenotype of cord blood-derived LCs, we performed FACS staining for FOXP3, CD25, CD4, and CD3 as well as CD1a, HLA-DR, CD80, CD83, CD86, and CD40, respectively, as described (33). The mAbs used in this study and their sources are shown in Table I.

Cell preparations from cord blood and peripheral blood

CD34⁺ hematopoietic progenitor cells were separated from cord blood mononuclear cells by positive immunoselection (Direct CD34⁺ Progenitor Cell Isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany) as described (34). A total of 50 nM Dex was added to the culture medium for the last 3 d. On day 10, cells were harvested, and LCs were purified by CD1a labeling and MACS.

Peripheral blood samples from healthy individuals were purchased as buffy coats from the Vienna Red Cross Center (Vienna, Austria). Myeloid and plasmacytoid DCs and T cells were isolated as described previously (33). The purity of isolated DC populations was 93–99%, as determined by flow cytometric analysis (FACSscan; BD Biosciences, San Jose, CA). DCs were divided and incubated with or without 50 nM Dex overnight at 37°C.

To assess the potency of different DC subsets to induce Tregs, T cells were incubated for 12 h with DCs at 37°C in a ratio of T cells/DCs of 5:1 and then either analyzed by FACS staining or isolated for functional assays. To evaluate key factors operative in the expansion of Tregs, we added neutralizing anti-RANK, anti-TGF- β , anti-IL-10 Abs, or an isotype control (all 5 μ g/ml) to the 12-h coculture of LCs and T cells in one set of experiments (Fig. 6A).

Normal human epidermal keratinocytes from infant foreskin were purchased from Provitro (Berlin, Germany). The cells were cultured as recommended in the instructor's manual and used after two to four passages. When 70–80% confluence was reached, they were exposed to 50 nM Dex. After 24 h, cells were harvested and further subjected to phenotypic

analysis after determination of their viability by 7-aminoactinomycin D (Sigma-Aldrich, Vienna, Austria) uptake by flow cytometry.

Evaluation of the inhibitory activity of Tregs

After the 12-h coculture of allogeneic T cells with cord or peripheral blood-derived DCs, we quantified Tregs by FACS or determined their inhibitory properties by proliferation assays. Therefore, we either separated them by positive immunoselection with the CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation Kit II (Miltenyi Biotec) (Fig. 5B) or depleted DCs by anti-IgG beads after immunolabeling (Fig. 6A, 6C). Proliferation of responder T cells was measured by [³H]thymidine uptake after their stimulation with plate-bound anti-CD3 (5 μ g/ml) and soluble anti-CD28 (3 μ g/ml). Irradiated Tregs or total T cells were added to the responder T cells after the 12-h coculture of T cells with DCs in different dilutions. To assess relevant cytokines and molecules responsible for the enhancement and/or function of Tregs, we added neutralizing anti-TGF- β , anti-IL-10, or anti-RANK Abs (all 5 μ g/ml) either to the T cell-LC coculture (Fig. 6A) or to the anti-CD3/anti-CD28-induced proliferation assay (Fig. 6C). For control purposes, irradiated CD4⁺CD25⁺FOXP3⁺ non-regulatory T cells as filler cells were added to responder T cells in a concentration matched to that of Tregs.

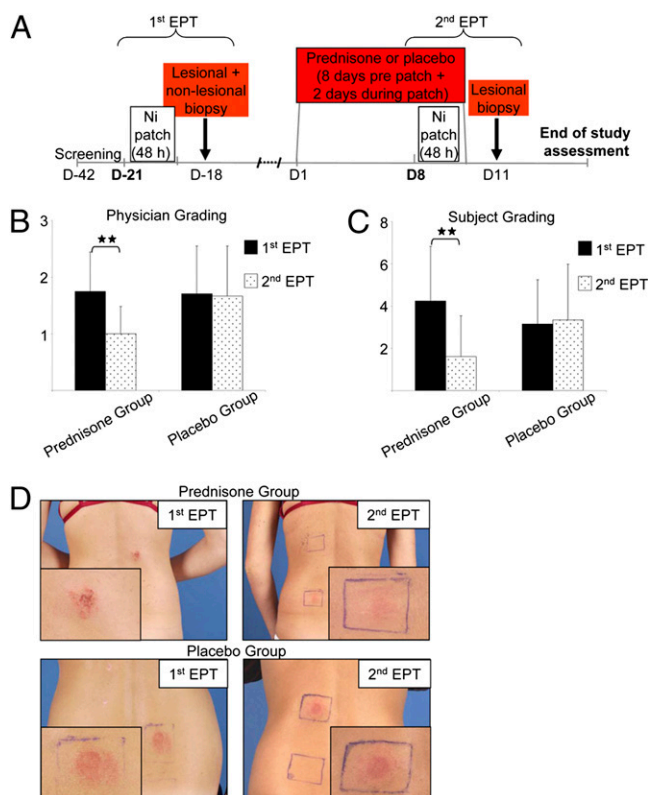


FIGURE 1. Reduction of EPT reactions by oral GCS treatment. **A**, Schematic illustration of the study protocol: 24 Ni-sensitive, healthy individuals were enrolled into the study and randomly assigned to the prednisone group or placebo group. The 10-d treatment period was started on day 1 (D1). Two EPT challenges were performed in each patient: the first prior (on day -21 [D-21]) and the second at the end of the treatment period (on day 8 [D8]). Seventy-two hours after patch test application, a lesional skin biopsy was taken at each of the time points (on day -18 [D-18] and day 11 [D11]). In addition, one nonlesional skin sample was collected after the first EPT reaction. **B**, Skin reactions were graded by physicians (0 to 3). **C**, Subjects scored itch on a visual analogue scale (0 to 10) 72 h after patch test application. **D**, Representative clinical pictures illustrate the reduction of skin inflammation upon prednisone treatment and unchanged skin reactions in a placebo-receiving subject on day -18 (D-18) and day 11 (D11). Note that a vehicle control was applied additionally to the Ni-patch test at each time point. ** $p < 0.01$.

Table I. List of Abs used in this study

Ab Specificity	Clone (Source of Ab)	Isotype
BDCA-2 (pur.)	124B3.13 (Dendritics)	mIgG1
CD1a (FITC)	HI149 (BD Biosciences)	mIgG1
CD1c (pur.)	M241 (Ancell)	mIgG1
CD3 (FITC)	SK-7 (BD Biosciences)	mIgG1
CD4 (pur.)	SK-3 (BD Biosciences)	mIgG1
CD8 (pur.)	C8/144B (DakoCytomation)	mIgG1
CD14 (pur.)	MΦP9 (BD Biosciences)	mIgG2b
CD15	10.1 (DakoCytomation)	mIgG1
CD25 (pur.)	2A3 (BD Biosciences)	mIgG1
CD40 (FITC)	5C3 (BD Pharmingen)	mIgG1
CD45 (pur.)	2D1 (BD Biosciences)	mIgG1
CD56 (biot.)	B158 (BD Biosciences)	mIgG1
CD80 (FITC)	MAB104 (Immunotech/Beckman Coulter)	mIgG1
CD83 (FITC)	HB15a (Immunotech/Beckman Coulter)	mIgG1
CD86 (FITC)	BU63 (DakoCytomation)	mIgG1
CD123 (biot.)	9F5 (BD Biosciences)	mIgG1
CD207 (pur.)	DCGM4 (Beckman Coulter)	mIgG1
FOXP3 (biot.)	236A/E7 (eBioscience)	mIgG1
Glucocorticoid receptor (pur.)	41/Glucocorticoid receptor (BD Biosciences)	mIgG1
HLA-DR (FITC, allophycocyanin)	L243 (BD Biosciences)	mIgG2a
Ki-67 (pur.)	Ki-67 (DakoCytomation)	mIgG1
Neutr. IL-10 (pur.)	JES3-9D7 (BioLegend)	rat IgG1
Pancytokeratin (FITC)	C-11 (Abcam)	mIgG1
RANK (pur.)	MAB683 (R&D Systems)	mIgG1
Neutr. RANK (pur.)	Polyclonal (LIFSPAN Biosciences)	Rabbit Ig
RANKL (pur.)	12A668 (Alexis)	mIgG1
TGF-β (pur.)	Polyclonal (Santa Cruz Biotechnology)	Rabbit Ig
Neutr. TGF-β (pur.)	Polyclonal (R&D Systems)	Goat Ig

biot., biotinylated; m, mouse; neutr., neutralizing; pur., purified.

Quantitative RT-PCR

For quantification of TGF-β in the skin samples, sections of frozen biopsies ($n = 3 \times 24$) were thawed on ice. RNA isolation, its transcription to cDNA, and further quantification and analysis were performed as described previously (35).

Statistical analysis

The levels of significance in the grading of skin reactions, distribution of leukocytes, and the TGF-β expression in skin samples between different time points within the prednisone and placebo groups were verified by the two-sided paired Student *t* test. Differences in the grading of skin

Table II. Leukocyte subsets occurring in nonlesional skin and EPT lesions prior to and after oral GCS treatment

Cell Type	Ag(s)	Nonlesional Skin		First EPT		Second EPT	
		Prednisone	Placebo	Prednisone	Placebo	Prednisone	Placebo
Epidermis (Positive Cells/mm)							
Pan-leukocytes	CD45 ⁺	16.5 ± 4.2	17.2 ± 5.3	32.3 ± 5.1	29.5 ± 7.1	20.8 ± 4.8	27.9 ± 6.5
T cells	CD3 ⁺	0.3 ± 0.5	0.4 ± 0.5	5.8 ± 3.2	4.9 ± 4.5	4.0 ± 3.5	4.5 ± 3.0
Helper T cells	CD4 ⁺ /CD3 ⁺	0	0.1 ± 0.2	0.6 ± 1	1.5 ± 1.6	0.3 ± 0.4	1.5 ± 1.2
Cytotoxic T cells	CD8 ⁺ /CD3 ⁺	0.2 ± 0.3	0.1 ± 0.2	2.3 ± 3.1	2.8 ± 0.9	0.4 ± 0.9	2.0 ± 1.4
Tregs	CD25 ⁺ /FOXP3 ⁺	0	0	0	0	0	0
NK cells	CD56 ⁺ /CD3 ⁺	0	0	0	0	0	0
LCs	CD207 ⁺ /CD1a ⁺	11.6 ± 2.9	12.3 ± 3.3	8.5 ± 9.3	9.3 ± 2.1	14.7 ± 3.0	8.5 ± 2.1
Dermal DCs	CD1c ⁺ /HLA-DR ⁺	1.0 ± 0.6	0.8 ± 0.7	5.3 ± 3.6	4.1 ± 3.8	2.1 ± 1.2	5.1 ± 4.7
Inflammatory DCs	CD207 ⁺ /CD1a ⁺	0.6 ± 0.9	0.8 ± 0.2	13.1 ± 5.5	12.3 ± 3.5	5.8 ± 6.1	12.8 ± 3.3
pDCs	BDCA-2 ⁺ /CD123 ⁺	0	0	3.2 ± 2.8	2.5 ± 2.2	0.2 ± 0.4	3.2 ± 2.6
Neutrophils	CD15 ⁺ /HLA-DR ⁺	0	0	2.1 ± 1.9	1.8 ± 2.3	0.5 ± 0.6	2.0 ± 1.9
Mononuclear phagocytes	CD14 ⁺ /HLA-DR ⁺	0	0	0.1 ± 0.3	0.3 ± 0.6	0.1 ± 0.1	0.1 ± 0.3
Dermis (Positive Cells/mm ²)							
Pan-leukocytes	CD45 ⁺	121.4 ± 13.5	130.1 ± 15.3	592.1 ± 54.1	583.2 ± 41.8	345.8 ± 31.7	552.6 ± 46.4
T cells	CD3 ⁺	44.8 ± 16.6	39.8 ± 17.2	336.8 ± 114.9	359.9 ± 209.3	252.9 ± 120.8	335.8 ± 137.1
Helper T cells	CD4 ⁺ /CD3 ⁺	7.3 ± 4.2	6.1 ± 2.6	82.5 ± 19.8	81.1 ± 26.0	50.1 ± 16.1	83.8 ± 18.8
Cytotoxic T cells	CD8 ⁺ /CD3 ⁺	15.5 ± 15.3	15.3 ± 11.8	188.0 ± 80.4	168.2 ± 75.0	107.4 ± 70.2	163.9 ± 71.5
Tregs	CD25 ⁺ /FOXP3 ⁺	3.4 ± 3.8	1.5 ± 1.8	18.1 ± 10.1	21.2 ± 21.6	40.6 ± 23.2	19.9 ± 21.8
NK cells	CD56 ⁺ /CD3 ⁺	7.3 ± 6.9	6.5 ± 12.1	39.1 ± 16.3	33.1 ± 23.5	25.9 ± 9.6	33.3 ± 19.3
LCs	CD207 ⁺ /CD1a ⁺	0.5 ± 0.5	0.9 ± 1.0	2.6 ± 1.2	1.9 ± 1.5	1.2 ± 0.7	2.3 ± 1.9
Dermal DCs	CD1c ⁺ /HLA-DR ⁺	46.3 ± 12.0	63.1 ± 20.4	128.2 ± 45.1	122.6 ± 25.9	45.3 ± 29.0	116.7 ± 21.3
Inflammatory DCs	CD207 ⁺ /CD1a ⁺	10.7 ± 0.7	15.1 ± 8.8	18.4 ± 2.2	22.2 ± 10.1	12.1 ± 1.0	21.2 ± 10.0
pDCs	BDCA-2 ⁺ /CD123 ⁺	0.5 ± 0.7	0.4 ± 0.6	65.0 ± 32.1	56.4 ± 20.0	11.3 ± 6.5	53.4 ± 17.8
Neutrophils	CD15 ⁺ /HLA-DR ⁺	0.3 ± 0.4	0.2 ± 0.5	35.6 ± 15.3	33.1 ± 13.8	19.4 ± 10.1	34.7 ± 16.5
Mononuclear phagocytes	CD14 ⁺ /HLA-DR ⁺	9.5 ± 6.7	8.7 ± 6.2	41.9 ± 27.5	44.8 ± 30.2	29.5 ± 19.5	43.1 ± 17.2

reactions, cellular numbers, and the TGF- β expression in skin samples at identical time points between subjects of the prednisone cohort and those of the placebo group as well as FACS data and functional results were assessed by the two-sided unpaired Student *t* test. A *p* value <0.05 was considered as statistically significant.

Results

Reduction of Ni EPT reactions upon prednisone treatment

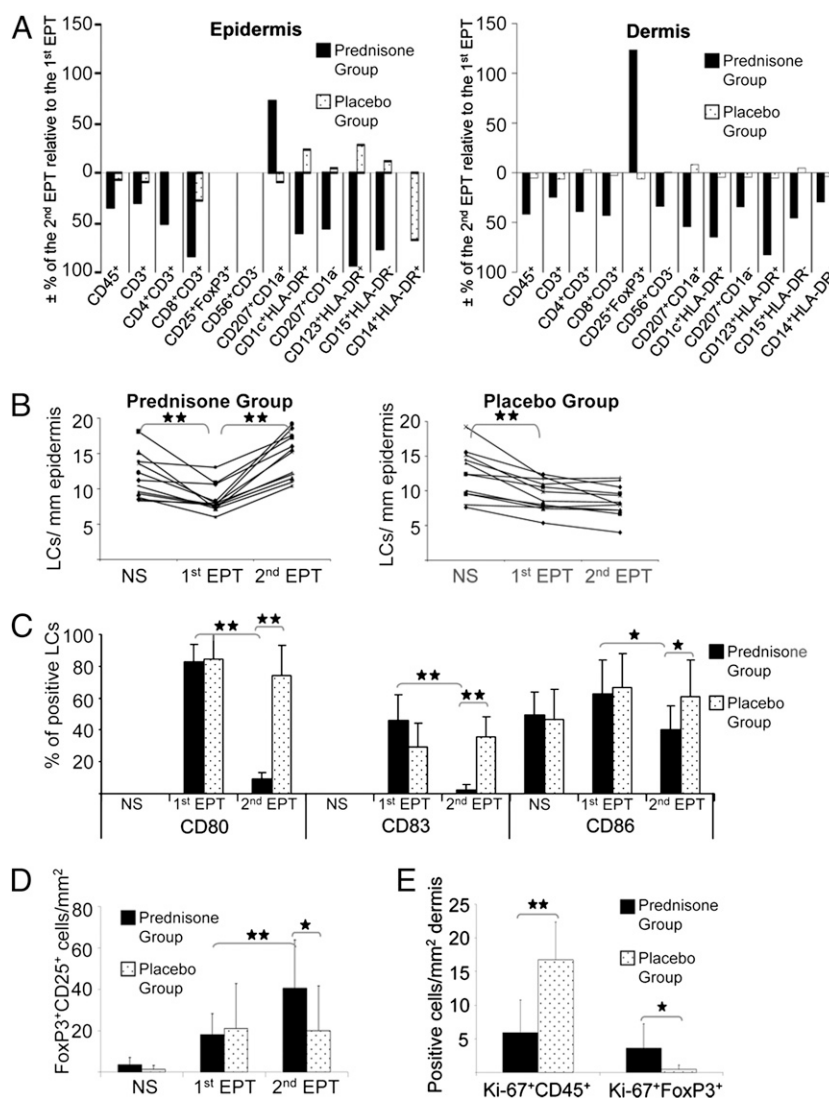
Twenty-four Ni-sensitive but otherwise healthy individuals were enrolled into this study and equally randomized to a prednisone group or placebo group. The first EPT reaction was triggered prior to, the second at the end of, a 10-d treatment period with a 4-wk interval between both EPT challenges (Fig. 1A). Lesional skin biopsies were taken 72 h after the two patch test applications, and a nonlesional skin sample was collected only after the first EPT procedure. The first EPT reaction displayed similar features in the prednisone and the placebo groups according to both the physician and subject grading (Fig. 1B, 1C). After the 10-d treatment period with prednisone, a significant decline of the skin reactions and subjective symptoms was recorded (Fig. 1B–D). Importantly, EPT reactions similar to the first ones were found in the placebo group, arguing against the possibility that the first EPT procedure influences the outcome of the second EPT challenge. These results illustrate the reduction of experimentally induced skin inflammation by oral GCS (pre)treatment and form the basis for further

exploration of the immunological mechanisms underlying this event.

Number and distribution of leukocytes in EPT reaction prior to oral GCS treatment

To unravel the effect of oral GCS treatment on the composition of the inflammatory infiltrate, we aimed at giving a detailed view of the different cell types orchestrating the eczematous reaction in the effector phase of EPTs and their modification by oral GCS treatment. The mAbs used in this study are given in Table I, and the results are summarized in Table II. Immunofluorescence staining of frozen skin sections revealed an increase of the epidermal and dermal leukocytic infiltrate (CD45⁺ cells) in EPT reactions prior to prednisone treatment compared with that of nonlesional skin (NS). The majority of these leukocytes represent T lymphocytes or cells of the DC/macrophage family. Table II shows that approximately two thirds of the T cells belong to the CD8⁺ subset and one third to the CD4⁺ subset in EPT reactions. T cells with a “regulatory” phenotype (FOXP3⁺CD25⁺) constitute ~5% of total T cells. Notably, all FOXP3⁺ cells are CD3⁺. CD56⁺CD3[−] NK cells and neutrophils also account for a substantial part of the inflammatory infiltrate (Table II). In nonlesional skin, we found abundant CD1a⁺CD207⁺ LCs in the epidermal compartment and moderate numbers of CD1c⁺HLA-DR⁺ DCs in the dermal compartment (Table II). Immune-mediated inflammation (i.e., EPT) leads to a slight

FIGURE 2. Increase of epidermal LCs and dermal Tregs in EPT lesions after GCS treatment. *A*, Immunofluorescence stainings of frozen section were performed for the indicated markers. Absolute cell numbers are presented in Table II. The alterations of cell populations of skin reactions after prednisone or placebo treatment are displayed in relation to cellular subsets of the first EPT lesions within the epidermis or dermis. *B*, Quantitative analysis of LCs by double labeling with anti-CD207 (Langerin, tetramethylrhodamine isothiocyanate [TRITC]) and anti-CD1a (FITC) mAbs. Individual results for each patient of the prednisone group (*n* = 12) and placebo group (*n* = 12) are shown. *C*, The maturation status of CD207⁺ LCs was assessed by double staining with anti-CD80, anti-CD83, or anti-CD86 mAbs. Mean percentages of double-positives of total CD207⁺ cells $\pm$ SD are displayed. *D*, The occurrence of FOXP3⁺CD25⁺ cells was analyzed by immunofluorescence double labeling with indicated markers of all patients enrolled. The data are given as absolute numbers of double-positive cells per square-millimeter dermis $\pm$ SD. *E*, Immunofluorescence double staining of the second EPT lesions of the prednisone group (*n* = 12) and placebo group (*n* = 12) with the proliferation marker Ki-67 (TRITC) and either anti-CD45 (FITC) or anti-FOXP3 (FITC) revealed a decreased leukocyte proliferation that is paralleled by increased levels of Ki-67/FOXP3 double-positive cells. Data are given as mean of the absolute numbers of double-positive cells $\pm$ SD. **p* < 0.05; ***p* < 0.01.



decrease of epidermal LCs (Table II, Fig. 2B) and increased levels of inflammatory epidermal CD1a⁺CD207⁺, epidermal and dermal CD1c⁺ DCs, BDCA-2⁺HLA-DR⁺ plasmacytoid DCs, CD14⁺ HLA-DR⁺ mononuclear phagocytes, and neutrophils (Table II).

Increase of epidermal LCs and dermal Tregs upon oral GCS treatment

To illustrate the impact of prednisone or placebo treatment on the composition of the inflammatory infiltrate, Fig. 2A highlights, in addition to absolute cell numbers displayed in Table II, the changes of leukocyte subsets in the second EPT in relation to the first EPT reaction. Consistent with the decline of clinical symptoms, we found a reduction of the leukocytic infiltrate (CD45⁺ cells) upon oral GCS treatment (Table II, Fig. 2A) caused by a decrease of the major cellular subsets of the lymphocytic lineage (both CD4⁺ and CD8⁺ T cells), of the DC/macrophage family (inflammatory epidermal CD1a⁺, epidermal and dermal CD1c⁺ DCs,

and BDCA-2⁺HLA-DR⁺ plasmacytoid DCs), and of neutrophils (Table II, Fig. 2A).

Importantly, LCs were the only epidermal leukocyte population that was increased upon oral GCS therapy compared with populations in skin lesions of placebo recipients (Table II, Fig. 2A, 2B). Most LCs in EPT reactions exhibited a less mature phenotype after GCS treatment, as evidenced by a decrease of CD80⁺, CD83⁺, and CD86⁺ LCs (Fig. 2C).

Notably, we found higher numbers of CD25⁺FOXP3⁺ Tregs in the dermis of EPT lesions upon prednisone treatment as opposed to the placebo group or EPT reactions prior to GCS treatment (Table II, Fig. 2A, 2D). Although formal proof is lacking, this phenomenon appears to be mainly due to a GCS-induced local proliferation of these cells. The reason for this assumption is our observation of decreased numbers of total Ki-67⁺CD45⁺ leukocytes yet increased levels of Ki-67⁺FOXP3⁺ cells after GCS treatment compared with those after placebo (Fig. 2E). Of note, epidermal LCs did not stain positive for Ki-67 (data not shown).

TGF- β and RANK/RANKL are upregulated in EPT lesions upon prednisone treatment

In a search for factors responsible for the GCS-induced expansion of LCs and Tregs, we determined the tissue levels of TGF- β by quantitative RT-PCR. We found a remarkable upregulation of TGF- β mRNA only in skin lesions after oral prednisone treatment compared with EPT reactions prior to prednisone treatment and

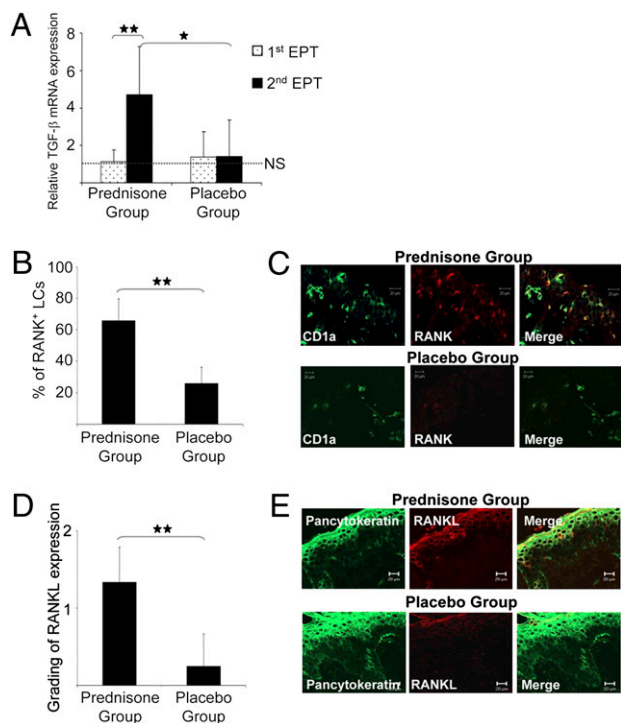


FIGURE 3. Upregulation of TGF- β , RANK, and RANKL in EPT lesions upon oral GCS treatment. **A**, Quantitative RT-PCR was performed from frozen sections of lesional and nonlesional skin samples and analyzed for TGF- β mRNA expression. Data are normalized to β_2 -microglobulin (β_2m) of each specimen and represent the mean values of fold changes relative to healthy skin $\pm$ SD. **B**, Quantitative analysis of immunofluorescence double staining of the second EPT lesions of the prednisone group ($n = 12$) and placebo group ($n = 12$) with anti-RANK (TRITC) and anti-CD1a (FITC) mAbs. Data are given as mean of the percentage of double-positive cells to total CD1a⁺ epidermal cells $\pm$ SD. **C**, Representative images of immunofluorescence staining of EPT lesions with anti-RANK (TRITC) and anti-CD1a (FITC) mAbs after prednisone or placebo treatment. **D**, Quantitative analysis of immunofluorescence double staining of EPT lesions after prednisone ($n = 12$) and placebo ($n = 12$) treatment with anti-RANKL (TRITC) and anti-pancytokeratin (FITC) mAbs. Data are given as mean of RANKL expression on keratinocytes (0, absent; 1, mild, on single cells; 2, moderate, <50% of keratinocytes positive; 3, intense, >50% of keratinocytes positive) $\pm$ SD. **E**, Representative images of immunofluorescence staining of EPT lesions with anti-RANKL (TRITC) and anti-pancytokeratin (FITC) after prednisone or placebo treatment. **C** and **E**, Original magnification $\times 400$. Images were recorded with a Zeiss Axiovert 200M microscope (Zeiss, Vienna, Austria). * $p < 0.05$; ** $p < 0.01$.

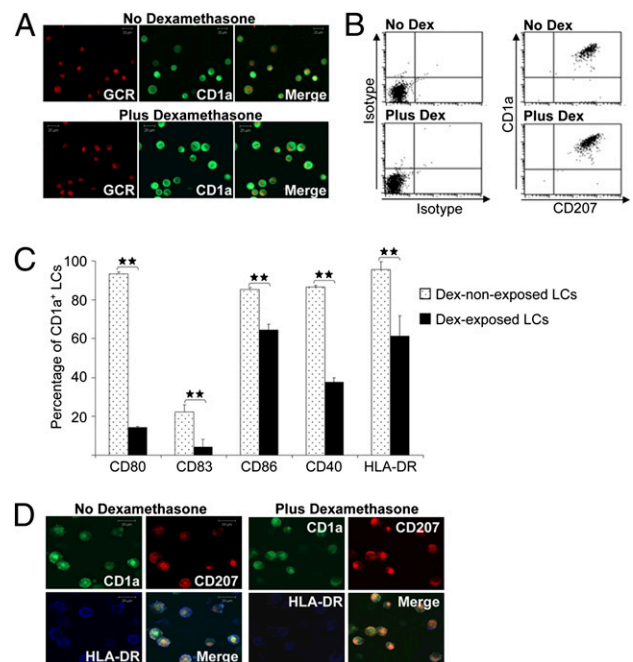


FIGURE 4. Immature phenotype of GCS-exposed LCs. **A**, Cord blood-derived LCs that had been generated in the presence or absence of 50 nM Dex for the last 3 d of culture were stained on adhesion slides for the glucocorticoid receptor (GCR; TRITC) and CD1a (FITC). **B**, Representative dot plots of cord blood-derived LCs show their viability and purity after purification by anti-CD1a immunomagnetic sorting. **C**, Analysis of the maturation status of GCS-exposed or GCS-nonexposed cord blood-derived LCs by flow cytometry. Data are given as mean percentages of double-positive cells for CD1a and the indicated markers $\pm$ SD of two independent experiments. **D**, Characterization of cord blood-derived LCs on adhesion slides as CD1a (FITC)/CD207 (TRITC)/HLA-DR (allophycocyanin) triple-positive cells. Please note the downregulation of HLA-DR on LCs upon exposure to GCS. **A** and **C**, Original magnification $\times 400$. Images were recorded with a Zeiss Axiovert 200M microscope. ** $p < 0.01$.

those of the placebo group with similar TGF- β levels to nonlesional skin (Fig. 3A).

As the interaction of RANK-expressing LCs with RANKL⁺ keratinocytes is apparently crucial for the induction of Tregs in a mouse model of UV-induced immunosuppression (18), we performed RANK and RANKL immunofluorescence stainings of EPT lesions. Epidermal RANK and RANKL staining on keratinocytes and LCs was weak in normal skin (data not shown). However, we found that the fraction of epidermal RANK-expressing LCs (Fig. 3B, 3C) as well as that of RANKL⁺ keratinocytes (Fig. 3D, 3E) was elevated upon prednisone treatment compared with those of EPT lesions of the placebo group. These findings are compatible with the assumption that TGF- β (36) and, similar to the situation in UV-treated animals, RANK/RANKL interactions (18) keep LCs in an immature state, thus favoring the generation/expansion of Tregs.

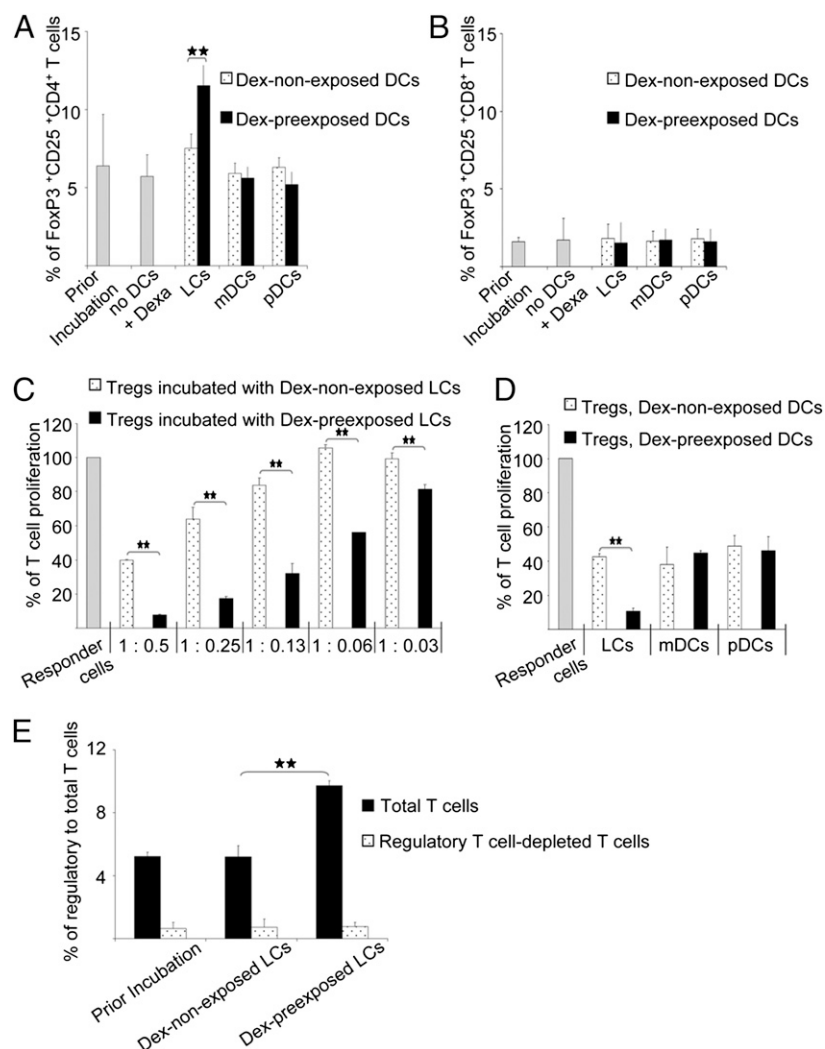
GCS-exposed LCs exhibit an immature phenotype and induce Tregs

To test this hypothesis, we decided to examine the effects of GCS on LC-lymphocyte/Treg interactions under defined in vitro conditions (37). For this purpose, we generated LCs from cord blood-derived CD34⁺ cells for 10 d with or without the addition of 50 nM Dex to the medium during the last 3 d of incubation. All LCs expressed the intracellular glucocorticoid receptor at the end of

the culture period (Fig. 4A). Viability, recovery, and purity of CD1a⁺ cells that coexpressed CD207 after the 10-d culture prior to the incubation with T cells were consistent in the Dex-exposed and Dex-nonexposed cell fractions (Fig. 4B). In contrast to Dex-nonexposed LCs, Dex-treated LCs displayed weaker anti-CD80, anti-CD83, anti-CD86, and anti-CD40 staining on their surfaces (Fig. 4C), consistent with the immature phenotype of LCs seen in skin lesions upon oral GCS treatment. HLA-DR was also downregulated on Dex-exposed LCs (Fig. 4C, 4D).

To elucidate whether Dex-modulated LCs have the capacity to enhance Tregs, we incubated purified total T cells with allogeneic Dex-preexposed and Dex-nonexposed LCs and, for control purpose, myeloid and plasmacytoid DCs isolated from peripheral blood as well as cord blood-derived myeloid non-LC DCs. Prior to this 12-h incubation, Dex-exposed cells were subjected to extensive washes. Analysis of the quantity of regulatory FOXP3⁺CD25⁺CD4⁺ T cells by FACS revealed a significant increase of FOXP3⁺CD25⁺CD4⁺ T cells upon a 12-h incubation period of T cells with Dex-preexposed LCs but not with other Dex-preexposed or Dex-nonexposed DC subsets (Fig. 5A). No significant differences in the absolute T cell counts or the apoptosis rate of CD3⁺ cells, as determined by trypan blue staining, were observed upon incubation with different DC subsets in the presence or absence of Dex (data not shown). It was recently shown that FOXP3⁺CD8⁺ T cells are enhanced during allergen immunotherapy (38). We therefore

FIGURE 5. Expansion of Tregs by GCS-exposed LCs. *A* and *B*, CD25⁺FOXP3⁺ T cells were analyzed by flow cytometry prior to and after 12-h incubation with indicated DC subsets. Data represent the mean percentage of (A) CD25⁺FOXP3⁺CD4⁺ T cells and (B) CD25⁺FOXP3⁺CD8⁺ T cells to total T cells $\pm$ SD of two independent experiments. *C* and *D*, The suppressive capacity of isolated Tregs after incubation with (C) LCs alone or (D) LCs and control-DC subsets was assessed by an anti-CD3/anti-CD28-induced proliferation assay. *C*, Irradiated Tregs were added in indicated dilutions to 50,000 responder T cells (responder T cells/Tregs). *D*, A total of 25,000 irradiated Tregs was added to 50,000 responder T cells. Data for *C* and *D* are given as mean percentage of T cell proliferation in the presence of Tregs relative to T cell proliferation in the absence of Tregs ($= 100\%$) $\pm$ SD of two independent experiments. *E*, Analysis of CD25⁺FOXP3⁺ T cells by flow cytometry prior to and after incubation of Dex-exposed and Dex-nonexposed LCs with either total T cells or T cells that had been depleted of Tregs prior to incubation. Data represent the mean percentage of CD25⁺FOXP3⁺ T cells to total T cells $\pm$ SD of two independent experiments. ** $p < 0.01$.



analyzed whether Dex-preexposed LCs can promote, in addition to CD4⁺ Tregs, regulatory cells with a CD8 phenotype. However, we found low percentages of FOXP3⁺CD25⁺CD8⁺ T cells prior to incubation that were neither affected by Dex-preexposed or Dex-nonexposed LCs nor by control-DC subsets (Fig. 5B).

To determine the regulatory capacity of FOXP3⁺CD25⁺CD4⁺ T cells after their encounter with Dex-exposed or Dex-negative LCs, we subjected them to MACS, which yielded a purity of >95% FOXP3-expressing T cells. We added them in rising concentrations to CD3/CD28-stimulated responder T cells and found a suppressive activity in both groups. However, the suppressive capacity of Tregs that had been incubated with Dex-preexposed LCs far exceeded that of the control group and resulted in an almost complete inhibition of responder T cell proliferation at the highest concentration (Fig. 5C). For control purpose, we substituted LCs by myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs), which did not influence the suppressive activity of Tregs (Fig. 5D).

To investigate whether Dex-exposed LCs push preexisting Tregs into proliferation or, alternatively, make naive T cells differentiate into Tregs, we depleted Tregs from the total T cell population with the Regulatory T Cell Isolation Kit II (Miltenyi Biotec) and then incubated these FOXP3[−] T cells with Dex-preexposed or Dex-nonexposed LCs. In this setting, we did not detect significant amounts of FOXP3⁺ cells, whereas they were increased in the

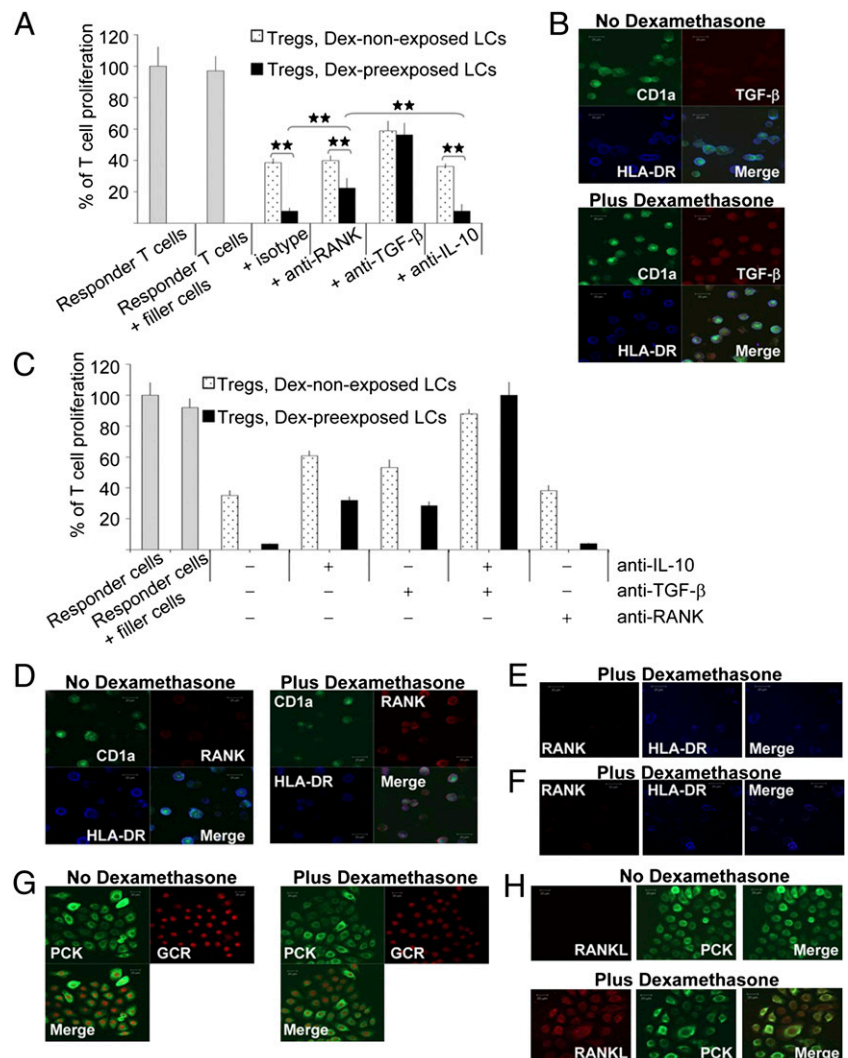
unfractionated T cell population upon incubation with Dex-exposed LCs (Fig. 5E).

These data illustrate the potency and, in comparison with other DCs, specificity of Dex-preexposed LCs to promote the proliferation and functional activity of Tregs.

TGF-β as key cytokine is involved in the expansion of Tregs by GCS-exposed LCs

To define the factors involved in the enhancement of LC-promoted Tregs, we added neutralizing anti-RANK, anti-TGF-β, and anti-IL-10 Abs to the coculture of LCs and T cells (Fig. 6A). The anti-CD3/anti-CD28-driven proliferation of responder T cells in the presence of Tregs that had been incubated with LCs and an isotype control for the neutralizing Abs revealed, as noted earlier, a difference in the inhibitory capacity between Tregs that had been incubated with Dex-preexposed LCs and Dex-nonexposed LCs (Fig. 6A). This difference was not influenced by anti-IL-10 and slightly altered by RANK inhibition (Fig. 6A). However, when TGF-β had been blocked during the T cell–LC coculture, we found Tregs of both groups to be equally suppressive, indicating that TGF-β is a key cytokine involved in the promotion of proliferation of Tregs by Dex-preexposed LCs. The concept that Dex directly affects LCs to enhance Tregs is further strengthened by anti-TGF-β staining of Dex-exposed and Dex-nonexposed LCs fixed on adhesion slides, showing that intracellular TGF-β is

FIGURE 6. TGF-β and RANK/RANKL contribute to the interaction of LCs with Tregs. **A**, Addition of neutralizing anti-RANK, anti-TGF-β, and anti-IL-10 mAbs to the 12-h incubation of T cells and LCs prior to the anti-CD3/anti-CD28 proliferation assay. A total of 25,000 irradiated T cells separated from LCs after their coculture was admixed with 50,000 responder T cells. CD4⁺CD25[−]FOXP3[−] T cells served as filler cells in the same dilutions as those of irradiated Tregs. Data are given as mean percentage of T cell proliferation in the presence of Tregs relative to T cell proliferation in the absence of Tregs (= 100%) ± SD of two independent experiments. **B**, Cord blood-derived LCs were fixed on adhesion slides and stained for TGF-β (TRITC), CD1a (FITC), and HLA-DR (allophycocyanin). **C**, Neutralizing Abs to TGF-β and IL-10 were added directly to the proliferation assay described for **A**. **D**, Cord blood-derived LCs were fixed on adhesion slides and stained for RANK (TRITC), CD1a (FITC), and HLA-DR (allophycocyanin). **E** and **F**, Peripheral blood-derived mDCs and pDCs were fixed on adhesion slides and stained for RANK (TRITC) and HLA-DR (allophycocyanin). **G** and **H**, Primary human keratinocytes were exposed to Dex for 24 h and stained for pancytokeratin (PCK; FITC) and (**G**) the glucocorticoid receptor (GCR; TRITC) or (**H**) RANKL (TRITC). **B**, **D**–**H**, Original magnification ×400. Images were recorded with a Zeiss Axiovert 200M microscope. ***p* < 0.01.



induced by Dex in LCs (Fig. 6B) but not in mDCs or pDCs (data not shown). These experiments evidence a gain of function of LCs by GCS and strongly corroborate our *in vivo* data of upregulated levels of TGF- β in skin lesions upon oral GCS treatment.

We next investigated the role of the immunoinhibitory candidate cytokines and molecules in the suppression of responder T cells by Tregs by adding neutralizing Abs against these moieties directly to the proliferation assays (Fig. 6C). Whereas a slight increase of T cell proliferation was noticed upon either IL-10 or TGF- β blockage alone, inhibition of both cytokines completely reversed the suppressive capacity of Tregs of both groups (Fig. 6C). RANK blockage had no effect in this setting (Fig. 6C). These data strongly suggest that both IL-10 and TGF- β are synergistically needed for optimal function of Tregs.

As RANK and RANKL were upregulated in skin lesions upon oral GCS treatment and inhibition of RANK on LCs decreased the suppressive capacity of Tregs that had been incubated with Dex-exposed LCs (Fig. 6A), we investigated whether Dex directly induces RANK on LCs and/or RANKL in keratinocytes. Indeed, upon incubation with Dex, LCs (Fig. 6D), but not mDCs (Fig. 6E) or pDCs (Fig. 6F), acquired expression of RANK, and RANKL was upregulated in keratinocytes that were all glucocorticoid receptor-positive (Fig. 6G, 6H), reflecting the situation in EPT skin lesions upon oral GCS treatment. Of note, LCs stained negative for RANKL, and less than 5% of keratinocytes expressed RANK.

Discussion

LCs, a subset of tissue DCs located in the epidermis, are sentinels of the skin surface and are responsible for promoting an immune response against invading pathogens (39). The paradigm of LCs as stimulators of a cutaneous immune response was based on both *in vitro* (40, 41) and *in vivo* studies, for example, mouse models for contact hypersensitivity (CHS), where elimination of LCs by UV light inhibits the development of CHS (42). More recent data demonstrate that LCs that have emigrated to the draining lymph nodes can directly present Ags secreted from keratinocytes (43) and are sufficient for the development of cutaneous graft-versus-host disease (44). The concept of LCs as initiator of skin immune responses was further corroborated by decreased CHS responses after inducible depletion of LCs in a diphtheria toxin-based mouse model (45). Although these observations indicate that LCs promote and even prime T cell responses, opposite conclusions have been drawn in other settings. After removal of epidermal LCs by GCS, enhanced CHS responses were observed (46), and LCs were found not to be needed for the induction of a skin-/mucosa-derived T cell response to HSV or *Leishmania* infection (47–49). In murine LC knockout models, LCs were dispensable for the development of CHS reactions (50) or even exhibited downregulatory properties (51). Induction of Tregs, as observed in different murine models of CHS (16–18) and in patients with LC histiocytosis (52), is one possibility for the tolerogenic potential of LCs. In this study, we show for the first time to our knowledge that treatment of contact-allergic patients with oral GCS endows LCs with Treg-enhancing properties.

Immunosuppression of GCS is thought to be triggered by downregulation of inflammatory cytokines (5) and inhibition of DC functions (8). This assumption was corroborated by observations where topical and systemic GCSs deplete murine LCs (53), directly downregulate proinflammatory molecules on their surfaces (54), and thereby inhibit murine CHS reactions (55). Depletion of murine LCs was attributed to apoptosis of LCs induced by GCS (56, 16), which was confirmed in patients with atopic dermatitis upon topical GCS treatment (57). Consequently, prevention of

CHS in mice by topical GCS was found to be independent of Tregs (16). Our data of increased levels of LCs with Treg-promoting capacity upon oral GCS treatment compared with that of lesions prior to GCS therapy are seemingly different. One possible explanation for this discrepancy could be a dosage phenomenon (58), as higher concentrations of GCS within the epidermis upon topical treatment could cause apoptosis, whereas lower amounts of GCS in the epidermis after oral treatment would inhibit the maturation of LCs but not affect their viability. *In vitro* studies support this theory, as GCSs do not induce apoptosis in LCs *in vitro* (59), but keep them in an immature stage (60).

Increased numbers of LCs upon GCS treatment could be due to different reasons. Although a higher rate of LC proliferation was reported in skin lesions of atopic dermatitis patients compared with that of normal skin (61), we could hardly see any Ki-67⁺ LCs. As LC homeostasis is controlled by autocrine/paracrine TGF- β (62), which prevents maturation of LCs even under inflammatory stimuli (36) and the RANKL system (63), synergistic effects of higher levels of TGF- β in and RANK on LCs as well as RANKL on keratinocytes (Figs. 3B–E and 6D, 6E) could therefore be responsible for retaining LCs within the epidermis.

The interaction of LCs and Tregs could take place within the skin or the draining lymph nodes. Elegant studies in mice have shown that inhibition of contact sensitization takes place in the lymph node, whereas suppression of the elicitation reaction occurs only when Tregs are present within the skin (64). Our data let us speculate that, upon entry in the skin, Ni-sensitive T cells (including Tregs) receive stimuli from different (dendritic) APCs (65) and that, upon oral GCS treatment, LCs induce an overproportionate expansion of Ni-specific Tregs. Our finding of proliferating FOXP3⁺ cells upon oral GCS treatment supports this idea. As GCS, when added to T cells without additional DCs, did not influence FOXP3 expression, we argue that GCSs alone are not capable of inducing Tregs. The question as to where the putative interaction between LCs and T cells occurs cannot be answered on the basis of our data. Perhaps LCs on their way into (66) or out of (67) the epidermis do encounter and communicate with Tregs. Our findings of elevated levels of TGF- β within the skin and the enhancement of Tregs make us speculate that GCS-induced TGF- β in LCs not only affects their maturation status (62, 36) but also mediates proliferation of Tregs within the dermis. Direct antiproliferative effects of GCSs on T cells have been known for a long time (68). Although our data favor the participation of Tregs in GCS-induced immunosuppression, direct effects of GCSs on responder T cells might also contribute to decreased skin inflammation *in vivo*.

Our results imply that GCSs actively modulate the cutaneous immune system by induction of TGF- β and RANK in and/or on LCs and RANKL in keratinocytes. We therefore propose the following concept: GCSs modify the epidermal milieu by targeting LCs and keratinocytes, resulting in retention of immature, RANK-expressing, and TGF- β -producing LCs. These modulated LCs interact with Tregs, which then mediate *in situ* the suppression of the cutaneous inflammatory response.

Disclosures

T.J. is a Novartis employee, and G.S. has previously received consultancy fees from Novartis.

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