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Human CD25$^+$ Regulatory T Cells Maintain Immune Tolerance to Nickel in Healthy, Nonallergic Individuals[1]

Andrea Cavani,[2]* Francesca Nasorri,* Chiara Ottaviani,* Silvia Sebastiani,* Ornella De Pità,[†] and Giampiero Girolomoni*[§]

We investigated the capacity of CD25$^+$ T regulatory cells (Treg) to modulate T cell responses to nickel, a common cause of allergic contact dermatitis. CD4$^+$ T cells isolated from the peripheral blood of six healthy, nonallergic individuals showed a limited capacity to proliferate in response to nickel in vitro, but responsiveness was strongly augmented (mean increment ± SD, 240 ± 60%) when cells were depleted of CD25$^+$ Treg. Although CD25$^+$ Treg were anergic to nickel, a small percentage up-regulated membrane CTLA-4 upon nickel exposure. CD25$^+$ Treg strongly and dose-dependently inhibited nickel-specific activation of CD25$^-$ T lymphocytes in coculture experiments in a cytokine-independent, but cell-to-cell contact-dependent, manner. Approximately 30% of circulating CD25$^+$ Treg expressed the cutaneous lymphocyte-associated Ag (CLA), and CLA$^+$CD25$^+$ Treg were more efficient than CLA$^-$CD25$^+$ cells in suppressing nickel responsiveness of CD25$^-$ T cells. The site of a negative patch test in response to nickel showed an infiltrate of CD4$^+$CLA$^+$ cells and CD25$^+$ cells, which accounted for ~20% of the total T cells isolated from the tissue. Skin-derived T cells suppressed nickel-specific responses of peripheral blood CD25$^-$ T cells. In addition, 60 ± 14% of peripheral blood CD25$^+$ Treg expressed the chemokine receptor CCR7 and strongly inhibited naive T cell activation in response to nickel. Finally, CD25$^+$ T cells isolated from peripheral blood of nickel-allergic patients showed a limited or absent capacity to suppress metal-specific CD4$^+$ and CD8$^+$ T cell responses. The results indicates that in healthy individuals CD25$^+$ Treg can control the activation of both naive and effector nickel-specific T cells. *The Journal of Immunology,* 2003, 171: 5760–5768.

T he skin is continuously challenged by environmental Ags that may evoke protective or detrimental immune responses. In the case of small chemicals (haptens) accessing the skin, the development of vigorous T cell responses may be responsible for undesired immunomediated diseases, such as drug reactions and allergic contact dermatitis (ACD)[3] (1). ACD is a worldwide disease whose expression depends upon the engagement of hapten-specific cytotoxic CD8$^+$ T cells (2, 3). CD4$^+$ Th1 cells contribute to the magnitude and the persistence of the inflammatory reaction by releasing IFN-γ, TNF-α, and IL-17 (4, 5). In contrast, IL-10-producing CD4$^+$ cells may have a role in limiting the immune response and thus the tissue damage (6–8).

Although most people come into contact with ubiquitous chemicals with sensitizing potentials, only a few individuals develop ACD. This suggests that under physiologic conditions the skin immune system possesses regulatory mechanisms to avoid immunopathologic reactions to haptens. Indeed, in mouse models the existence of specific tolerogenic mechanisms that prevent immune responses to skin-applied haptens has been clearly documented. Hapten-specific tolerance occurs in animals fed the allergen before cutaneous sensitization. Oral tolerance can be adoptively transferred to

naive animals with spleen or lymph node CD4$^+$ T cells or with a mixture of CD4$^+$ and CD8$^+$ T cells and requires IL-10 (9, 10). Moreover, haptens painted onto UV light-irradiated skin expand IL-10-secreting CD4$^+$ T cells, which express high levels of CTLA-4 and suppress hapten-specific immune responses in vivo (11, 12).

Recently, a subset of CD4$^+$ T regulatory cells (Treg) constitutively expressing the α-chain of the IL-2R (CD25) has been identified in both mice and humans (13–15). The current view is that CD25$^+$ Treg originate from the thymus as a distinct T cell lineage involved in the maintenance of tolerance to self Ags (16, 17). CD25$^+$ Treg constitute 5–10% of peripheral blood CD4$^+$ T cells, express high levels of CTLA-4, and are naturally anergic in vitro to mitogens (18–20). Upon TCR triggering, CD25$^+$ Treg inhibit CD4$^+$ and CD8$^+$ T cell proliferation and IL-2 release in an Ag- and cytokine-independent, but cell-to-cell contact-dependent fashion (21, 22). CD25$^+$ Treg may originate also in peripheral lymphoid organs and regulate immune responses to environmental Ags. Consistent with this hypothesis, CD25$^+$ Treg are expanded following oral feeding with Ags (23, 24). Moreover, CD25$^+$ Treg can regulate immune responses to microorganisms and transplantation Ags (23–29). Interestingly, a conspicuous number of circulating CD25$^+$ T cells coexpresses the skin-homing receptor cutaneous lymphocyte associated Ag (CLA), thus indicating their possible engagement in skin immunity (30, 31).

Here we investigated the capacity of CD25$^+$ T cells to control immune responses to nickel. Our results indicate that CD25$^+$ T cells actively regulate nickel-specific T cell responses in healthy individuals and may have a critical role in maintaining a condition of "silent inflammation" to ubiquitous environmental substances penetrating the skin.

Materials and Methods

Blood and skin samples

Blood samples were obtained from six healthy individuals (aged 25–38 years, four women and two men) with no history of allergies to metals and

negative patch test to 5% nickel sulfate and from six patients (aged 28–36 years, four women and two men) with a documented allergy to metals and moderate to intense positive patch test to nickel. Skin biopsies were obtained at the site of nickel application from three nonallergic subjects and three nickel-allergic patients after informed consent was obtained. The study was approved by the institute ethical committee.

Cell purification

PBMCs were separated by density gradient centrifugation over Lymphoprep (Nycomed-Pharmacia, Oslo, Norway). After extensive washing, PBMCs were left to adhere for 2 h at 37°C in petri dishes (6×10^6 cells/ml, 6 ml/dish) suspended in RPMI 1640 complemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen Italia, San Giuliano Milanese, Italy; complete RPMI) with 5% autologous plasma added. The nonadherent fraction was collected for T cell purification, whereas adherent cells were incubated for 30 min in 0.2% EDTA, detached with a cell scraper, and used as APCs for most of the functional assays. $CD8^+$ T cells ($\geq$95% pure) were positively selected by incubating nonadherent cells with immunomagnetic beads coated with anti-CD8 mAb, followed by DETACHaBEAD CD4/CD8 (Dynal, Oslo, Norway) according to the manufacturer's protocol. $CD4^+$ T cells ($\geq$97% pure) were obtained from the $CD8^+$-depleted fraction after incubation with a cocktail of anti-CD8, -CD11b, -CD16, -CD19, -CD36, and -CD56 mAbs conjugated with magnetic beads (Untouched $CD4^+$ T cell isolation kit; Miltenyi Biotec, Gladbach, Germany). $CD4^+CD25^+$ ($\geq$85% pure) and $CD4^+CD25^-$ ($\geq$93% pure) T cells were separated by positive and negative selection from the $CD4^+$ population using MACS CD25 microbeads (Miltenyi Biotec), respectively. In selected experiments $CD4^+CD25^+$ cells were further purified in CLA^+ ($\geq$90% purity) and CLA^- ($\geq$95% purity) fractions using a biotin-conjugated, anti-CLA mAb (HECA-452, rat IgM; BD PharMingen, San Diego, CA), followed by anti-biotin magnetic beads (Miltenyi Biotec). $CD4^+CD25^-CD45RA^+$ ($\geq$95% pure) and $CD4^+CD25^-CD45RO^+$ ($\geq$95% pure) T cells were isolated by negative immunomagnetic separation by incubating total $CD4^+CD25^-$ cells with anti-CD45RO (UCHL1, IgG2a) or anti-CD45RA (HI100, IgG2b) mAbs (both from BD PharMingen) for 20 min at 4°C, followed by anti-mouse IgG conjugated to immunomagnetic beads (Dynal).

T cell isolation from skin biopsies

Four-millimeter punch skin biopsies were performed 36 h after application of 5% nickel sulfate in petrolatum on three nonallergic subjects and three nickel-allergic patients. Skin samples were minced with a scalpel and placed in culture in complete RPMI with 5% autologous plasma and 60 U/ml of IL-2. After 9 days T cells emigrated from tissue samples were collected and left for 2 days in 96-well microplates in complete RPMI without IL-2 before phenotypic characterization and functional assays.

Preparation of monocyte-derived dendritic cells

Adherent PBMCs were depleted of $CD2^+$, $CD19^+$, and $CD56^+$ cells by negative selection with mAb-coated immunomagnetic beads (Dynal). The resulting monocyte population ($\geq$90% $CD14^+$ cells) was cultured in complete RPMI supplemented with 10% FBS (HyClone, Logan, UT) in the presence of 100 ng/ml recombinant human GM-CSF (Mielogen; Schering-Plough, Milan, Italy) and 200 U/ml recombinant human IL-4 (R&D Systems, Minneapolis, MN) at 37°C with 5% CO$_2$. Medium was replaced after 3 days, and cells were used as APCs in proliferation assays on days 6–7 of culture. This procedure produced a $\geq$95% pure $CD1a^+$ $CD14^-$ dendritic cell preparation. To induce maturation, LPS from *Escherichia coli* 055:B5 (Sigma-Aldrich, Milan, Italy) was added at 50 μg/ml for the last 24 h of culture.

T cell proliferation assays

PBMCs alone (250,000 cells/well) or purified T cell subsets (160,000 cells/well) plus autologous gamma-irradiated adherent cells (150,000 cells/well) were cultured in flat-bottom, 96-well plates in the presence or the absence of 20 μg/ml of nickel sulfate (Sigma-Aldrich) in complete RPMI plus 5% autologous plasma. To check their suppressive function, conditioning cells (either $CD4^+CD25^+$, $CD4^+ CD25^+CLA^+$, or $CD4^+CD25^+CLA^-$ T cells subsets, or $CD4^+CD25^-$ T cells as a control) were added to the responder T cells. To investigate whether $CD4^+CD25^+$ Treg required physical contact with responder T cells, $CD4^+CD25^-$ T cells (500,000 cells/well) with irradiated autologous APCs (400,000/well) and 20 μg/ml nickel were placed in the lower chamber of Transwell plates (0.4-μm pore size, 24-well format; BD Biosciences, Franklin Lakes, NJ), whereas the conditioning $CD4^+CD25^+$ T cells (250,000 cells/well) were seeded in the upper chamber in the presence of APCs and 20 μg/ml nickel. As a positive control, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were seeded together in the lower chamber. In some experiments 10 μg/ml of anti-TGF-β (clone 9016.2, mouse IgG1) or anti-IL-10 (clone 23758.11, mouse IgG2b) mAbs (R&D Systems) or control IgG were added to the cultures. After 5 days cell cultures were pulsed with 2 μCi/ml of [^{3}H]thymidine (Amersham Pharmacia Biotech, Little Chalfont, U.K.) for 16 h and harvested onto fiber-coated 96-well plates. Radioactivity incorporation was measured in a beta counter. Results are given as the mean counts per minute $\pm$ SD of triplicate cultures.

ELISA for cytokine release

T cell supernatants were collected from cultures after 3 days, filtered, and tested for IL-4, IL-10, and IFN-γ content the using DuoSet ELISA System (R&D Systems) following the manufacturer's instructions.

Antibodies

For immunohistochemistry and FACS analysis, pure mAb anti-human CD4 (SK3, mIgG1), CD8 (SK1, mIgG1), CD25 (2A3, mIgG1), CTLA-4 (BNI3, mIgG2a), CCR7 (2H4, mIgM), and CLA; PE-conjugated anti-CD25 (M-A251, mIgG1); FITC-conjugated anti-CD4 (SK3, mIgG1), anti-CD69 (L78, mIgG1), and anti-HLA-DR (L243, mIgG2a); and mouse and rat isotype controls were obtained from BD PharMingen. Secondary FITC- or PE-conjugated goat anti-mouse IgG were purchased from Southern Biotechnology Associates (Birmingham, AL); secondary, biotin-conjugated, goat anti-rat IgM was purchased from BD PharMingen, and PE-conjugated streptavidin was obtained from DAKO (Carpinteria, CA).

Immunohistochemistry

A patch test to 5% nickel sulfate applied on the back of healthy individuals and nickel-allergic patients was punch-biopsied at 36 h and frozen at −80°C in OCT compound. Five-micron cryostat sections were fixed in 4% paraformaldehyde for 5 min and pretreated for 15 min with PBS containing 0.3% hydrogen peroxide to block the endogenous peroxidase activity, and with protein block serum-free solution (DAKO) to block the nonspecific binding of primary and secondary Abs. Slides were incubated for 1 h at room temperature in a humid atmosphere with 2.5 μg/ml of anti-CD4, 1.2 μg/ml of anti-CD8, 0.5 μg/ml of anti-CD25, or 50 μg/ml of anti-CLA mAbs, followed by biotinylated anti-mouse (DAKO) or biotinylated anti-rat (BD PharMingen) secondary Ab for 10 or 30 min, respectively. Streptavidin-peroxidase (DAKO) was added for 10 min to the samples, and 3-amino-9-ethyl-carbazole (Vector Laboratories, Burlingame, CA) was used as substrate. Every step was followed by extensive washing in PBS. Slides were counterstained with hematoxylin.

Flow cytometric analysis

Purified cell populations were washed with PBS added to 1% human serum and 0.01% NaN$_3$ and were stained with FITC- or PE-conjugated mAbs or primary mAbs, followed by the appropriate secondary FITC- or PE-conjugated Ab. Staining with matched isotype control Ig was included. Cells were analyzed with a FACScan equipped with Cell Quest software (BD Biosciences).

Statistical analysis

Wilcoxon's signed rank test was used to compare differences in T cell proliferation. A value of $p \leq 0.05$ was considered significant.

Results

Peripheral blood $CD4^+$ T cells isolated from healthy donors strongly proliferate in response to nickel when depleted of the $CD4^+CD25^+$ fraction

Nickel is a potential skin sensitizer that continuously stimulates the immune system, given its ubiquitous distribution and the easy permeation through the skin barrier. As a result, most individuals, independently from their allergic status, possess circulating nickel-specific memory T cells that are mostly restricted to the $CD4^+$ compartment (8). Here we investigated the nickel responsiveness of a purified fraction of PBMCs isolated from six healthy, nonallergic individuals. All subjects showed a significant $CD4^+$, but not $CD8^+$, nickel-specific T cell response (Fig. 1), confirming previously published data (8). Interestingly, when $CD4^+$ T lymphocytes were depleted of the $CD25^+$ fraction, resulting in 95% pure
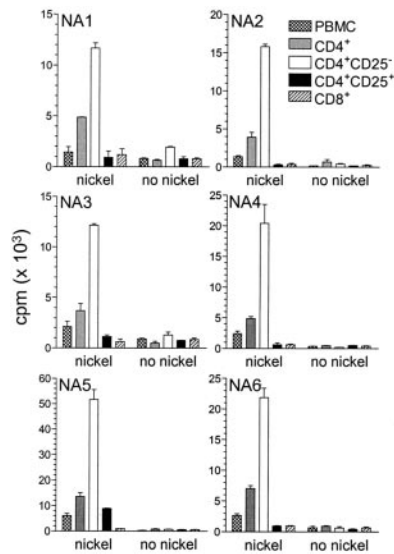
FIGURE 1. Peripheral blood CD4$^+$CD25$^-$, but not CD25$^+$, T cells isolated from healthy individuals strongly proliferate in response to nickel sulfate in vitro. T cell subpopulations were purified from the peripheral blood of six nonallergic individuals (NA1 to NA6) and were assayed for nickel (20 μg/ml) responsiveness in vitro in the presence of adherent cells as APCs for 5 days (3). [H]thymidine was added at 2 μCi/well during the final 16 h of coculture. Data are expressed as the mean counts per minute $\pm$ SD of triplicate cultures.

CD4$^+$CD25$^-$ T cells, a strong increase (mean $\pm$ SD, 240 $\pm$ 60%; range, 144–316%; $p < 0.001$) in nickel-specific responses was observed in all individuals. In contrast, CD4$^+$CD25$^+$ T cells were unresponsive to nickel in five of the six donors examined and responded poorly to the metal in the individual NA5.

CD4$^+$CD25$^+$ T cells from healthy subjects are anergic to nickel, but up-regulate CTLA-4 after nickel exposure

At an optimal nickel concentration (20 μg/ml), CD4$^+$CD25$^+$ T cells failed to respond to the metal presented by adherent cells. To rule out the possibility that the threshold of activation or the APC requirement of CD25$^+$ Treg could differ from those of CD25$^-$ T cells, responsiveness to increasing concentrations of nickel was assayed using adherent monocytes or monocyte-derived dendritic cells as APCs. The results confirmed the incapacity of CD4$^+$CD25$^+$ T cells to respond in vitro to the metal in terms of both proliferation and cytokine release (Fig. 2). CD25$^+$ T cells spontaneously released a small amount of IL-10, independently of the presence of nickel in the culture. However, 72-h culture with monocytes in the presence, but not in the absence, of nickel induced surface expression of CTLA-4 Ag on both CD25$^+$ (mean $\pm$ SD percentage of CTLA-4$^+$ cells in the absence and the presence of nickel, 0.66 $\pm$ 0.23 and 3.56 $\pm$ 1.03%, respectively) and CD25$^-$ (mean $\pm$ SD percentage of CTLA-4$^+$ cells in the absence and the presence of nickel, 0.09 $\pm$ 0.12 and 1.82 $\pm$ 1.14%, respectively) T lymphocytes (Fig. 2*B* and Table I), indicating that they were both triggered by the metal through the TCR (32). This result is in line with previous findings indicating higher CTLA-4 expression on CD25$^+$ compared with CD25$^-$ T cells (20).

Nickel-specific CD4$^+$CD25$^-$ T cell responses are dose-dependently inhibited by CD4$^+$CD25$^+$ T cells in a cytokine-independent, but cell-contact-dependent, manner

To definitively assess the inhibitory function of CD25$^+$ T cells in T cell responses to nickel, we cocultured blood-derived CD4$^+$
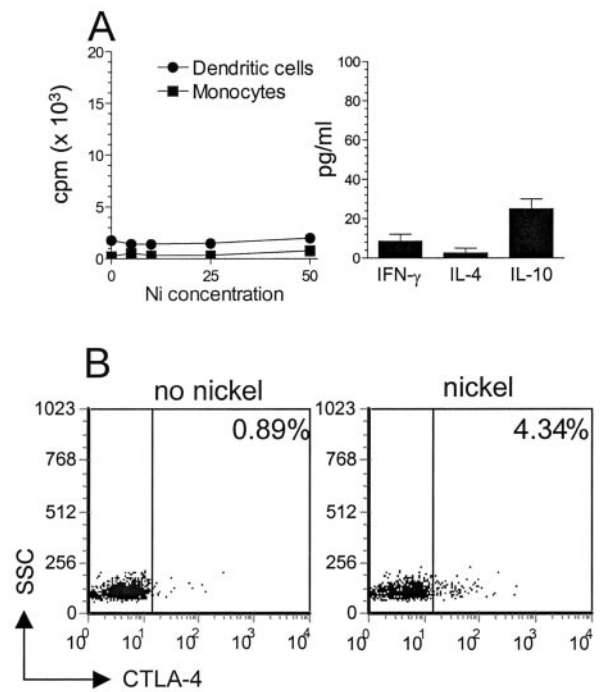

FIGURE 2. CD4$^+$CD25$^+$ T cells from healthy individuals fail to respond to nickel independently from the Ag concentration and the type of APC used in the assay, but up-regulate CTLA-4 upon nickel exposure. *A*, CD25$^+$ T cells were stimulated with nickel and either adherent monocytes (150,000 cells/well) or LPS-matured monocyte-derived dendritic cells (5,000/well) and were then assayed for proliferative response. Culture supernatants of CD25$^+$ T cells stimulated with nickel (20 μg/ml) and adherent monocytes were collected on day 3, and cytokine content was determined by ELISA. *B*, FACS analysis of a representative experiment performed on CD25$^+$ T cells from subject NA2; cells were cocultured for 3 days with adherent monocytes with or without nickel and stained with FITC-conjugated anti-CTLA-4 mAb.

CD25$^-$ T cells with autologous CD25$^+$ lymphocytes in the presence of APCs. At a CD25$^-$/CD25$^+$ ratio of 2/1, the proliferative response of CD25$^-$ cells was strongly reduced (mean $\pm$ SD, 74 $\pm$ 12%; range, 54–85%; $p < 0.001$) by the presence of CD25$^+$ T cells in all six healthy individuals investigated (Fig. 3). In contrast, the addition of an equal number of CD25$^-$ T lymphocytes did not change or even augment T cell proliferation. Titration of the number of CD25$^+$ cells added to the cocultures demonstrated that the magnitude of the inhibition, in terms of cell proliferation and IFN-γ release, depended on the CD25$^-$/CD25$^+$ ratio, with maximal inhibition at 1/1 (Fig. 4). Neither anti-IL-10 nor anti-TGF-β

Table I. *Nickel-induced CTLA-4 expression by CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells from non allergic individuals[a]*

Donor	CD4$^+$CD25$^+$		CD4$^+$CD25$^-$	
	No nickel	Nickel	No nickel	Nickel
NA1	0.71	2.72	<0.1	1.70
NA2	0.89	4.36	<0.1	0.75
NA3	0.54	5.37	0.12	3.92
NA4	0.45	2.94	0.19	1.53
NA5	0.45	3.22	0.34	2.14
NA6	1.03	2.91	<0.1	0.91

[a] Purified CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ T cells were incubated for 72 h with or without nickel in the presence of APCs and assayed for membrane expression of CTLA-4 by flow cytometry. Data are expressed as the percentage of CTLA-4$^+$ cells subtracted from cells stained with isotype-matched Ig.
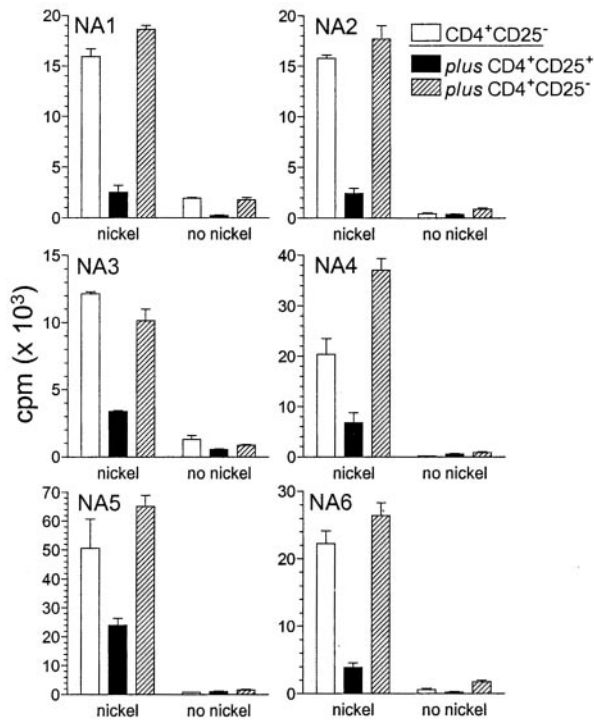
FIGURE 3. CD4$^+$CD25$^+$ T cells isolated from healthy nonallergic donors suppress specific CD4$^+$CD25$^-$ T cell responses to nickel. CD4$^+$CD25$^-$ T cells (160,000 cells/well) and conditioning CD25$^+$ or CD25$^-$ T cells (both at 80,000 cells/well) were cocultured for 5 days with APCs and nickel. Data are expressed as the mean counts per minute ± SD of triplicate cultures.

blocking Abs could revert the suppression induced by CD25$^+$ T cells (Fig. 5A), in line with the observation that cell-to-cell contact, but not soluble factors, mediates the inhibitory activity of CD25$^+$ T cells (22). Indeed, CD25$^+$ T cells failed to suppress nickel-specific CD25$^-$ T cell responses in Transwell plate experiments (Fig. 5B).

A relevant percentage of CD4$^+$CD25$^+$ T lymphocytes expresses the CLA and suppresses nickel-specific T cell responses more efficiently than CD4$^+$CD25$^+$ CLA$^-$ T cells

CLA is a sialylated membrane glycoprotein expressed by 15–20% of circulating T lymphocytes that allows preferential homing to the skin. To evaluate the capacity of CD4$^+$CD25$^+$ T cells to be en-
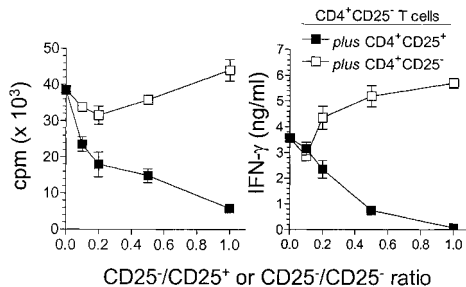

FIGURE 4. CD4$^+$CD25$^+$ T cells dose-dependently inhibit nickel-specific CD4$^+$CD25$^-$ T cell responses. CD4$^+$CD25$^-$ T cells (80,000 cells/well) were cocultured with increasing numbers of CD25$^+$ or CD25$^-$ T lymphocytes and assayed for proliferation (A; mean counts per minute ± SD of triplicate cultures) and IFN-γ release (B) measured by ELISA of supernatants collected on day 3 of coculture (mean ± SD, nanograms per milliliter).
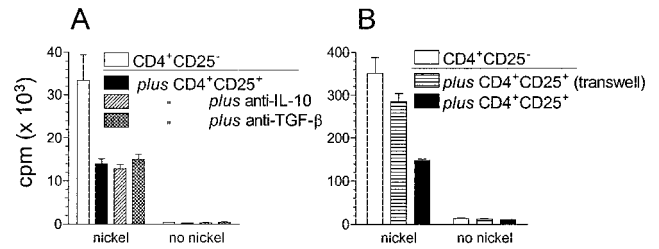

FIGURE 5. Suppressive function of CD4$^+$CD25$^+$ T cells is independent from TGF-β or IL-10 release and requires cell-to-cell contact with CD4$^+$CD25$^-$ T cells. A, CD4$^+$CD25$^-$ and CD25$^+$ T cells were seeded at a ratio of 2/1 with APCs and 20 μg/ml nickel for 5 days in the presence or the absence of anti-TGF-β and anti-IL-10 mAbs (10 μg/ml). B, Transwell experiments were performed in 24-well microplates by seeding CD25$^-$ (500,000 cells/well) in the lower chamber and CD25$^+$ (250,000 cells) lymphocytes in the upper chamber, separated by a 0.4-μm pore size filter. APCs (400,000 cells) and nickel (20 μg/ml) were added to both chambers. As a control, CD25$^-$ and CD25$^+$ cells were cocultured together in the lower chamber. Data are expressed as the mean counts per minute ± SD of triplicate cultures.

gaged in cutaneous immune responses, the expression of CLA was analyzed on circulating CD25$^+$ T cells isolated from healthy individuals. As shown in Fig. 6A, peripheral blood CD25$^+$ T cells failed to coexpress the CD69 activation marker and variably expressed HLA-DR. A high percentage of CD25$^+$ T cells (20–30%) expressed CLA. Similar results were obtained when CD25$^+$ T cells from nickel-allergic individuals were analyzed (not shown). The majority of T cells involved in immune responses to skin-applied haptens expressed CLA (33). Thus, we wondered whether CD4$^+$CD25$^+$CLA$^+$ and CD4$^+$CD25$^+$CLA$^-$ T cells differed in
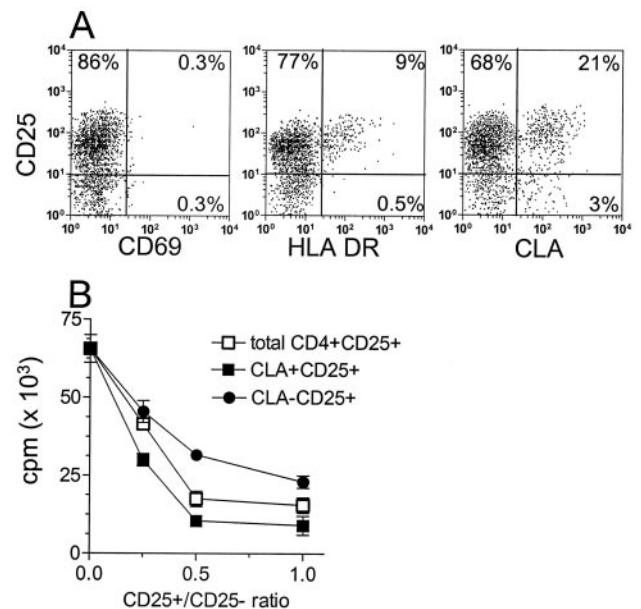

FIGURE 6. CLA$^+$CD4$^+$CD25$^+$ T cells exert a stronger suppressive activity on nickel-specific responses compared with the CLA$^-$ fraction. A, CD25$^+$ cells were purified by immunomagnetic separation from peripheral blood CD4$^+$ T cells and processed for double-staining FACS analysis as described in *Materials and Methods*. The figure shows the expression of CD69, HLA-DR, and CLA on CD25$^+$ T cells from a representative healthy individual. B, Total CD25$^+$ T cells or fractioned CLA$^+$ and CLA$^-$ CD25$^+$ T cells were cocultured with CD25$^-$ T cells at a 1/2 ratio with APCs and nickel (20 μg/ml) for 5 days. Data are expressed as the mean counts per minute ± SD of triplicate cultures.
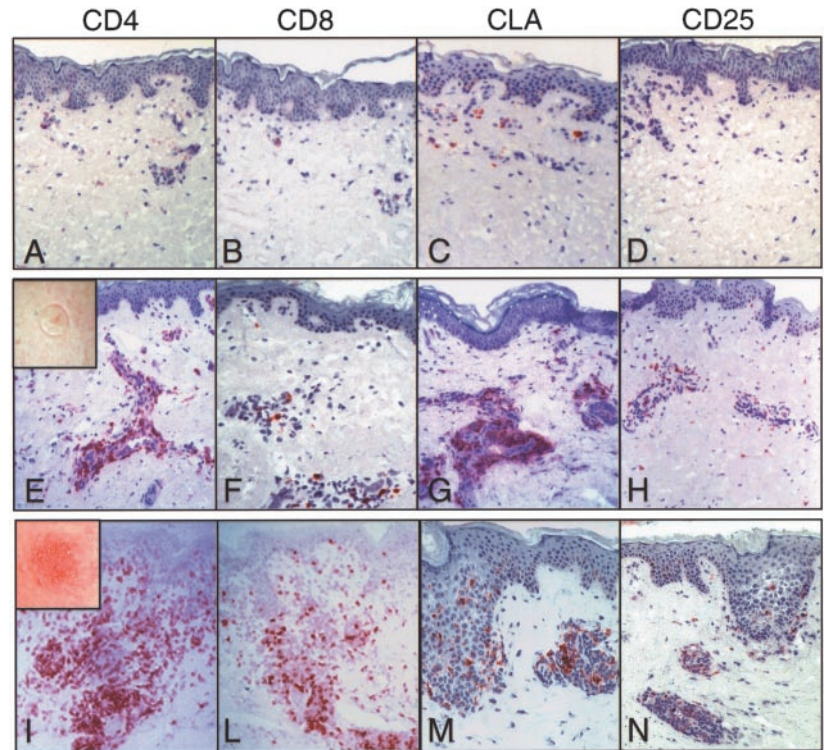
FIGURE 7. Negative patch tests to nickel sulfate applied on the skin of nonallergic individuals display a CD4$^+$, CD8$^+$, and CLA$^+$ infiltrate along with CD25$^+$ cells. Normal skin (*A–D*) or the site of patch test to nickel (*E–H*) applied on the back of healthy nonallergic subjects (*n* = 3) or nickel-allergic patients (*I–N*; *n* = 3) was punch biopsied at 36 h and processed for immunohistochemistry. The insets in *E* and *I* show a negative and a positive patch test reaction, respectively, where biopsy was performed.

their capacity to inhibit nickel-specific T cell responses. The results indicated that purified CD25$^+$CLA$^+$ T cells were indeed more potent than CD25$^+$CLA$^-$ cells to suppress nickel-specific CD4$^+$CD25$^-$ T cell proliferation (83 vs 51% inhibition, respectively; *p* < 0.002; Fig. 6*B*).

The skin site of hapten application in healthy individuals is infiltrated by CD4$^+$, CD8$^+$, CLA$^+$, and CD25$^+$ T cells

Application of 5% nickel sulfate under occlusion on the skin of nickel-allergic individuals induces a strong inflammatory reaction, which develops in 24–72 h and mimics natural occurring ACD. In contrast, in nonallergic individuals, nickel application to the skin is not followed by a clinically evident inflammatory response. Since nickel-reactive T cells can be isolated from the peripheral blood of nonallergic individuals, we hypothesized that in healthy donors, CD25$^+$ Treg may have a role in preventing nickel-specific T cell activation at the site of hapten penetration. Biopsies at the site of a negative patch test to nickel (Fig. 7*E*, inset) were taken from three nonallergic donors at a relatively early time point (36 h) and were processed for immunohistochemistry. Infiltration of CLA$^+$ (Fig. 7*G*) and CD4$^+$ (Fig. 7*E*) T cells and a lower number of CD8$^+$ T cells (Fig. 7*F*) were detected along with the presence of a significant number of CD25$^+$ cells (Fig. 7*H*). In contrast, normal skin (Fig. 7*D*) contained rare CLA$^+$, CD4$^+$, or CD8$^+$ cells and no CD25$^+$ cells. This finding demonstrates that despite the absence of clinically visible inflammation, T lymphocytes, including CD25$^+$ cells, are recruited at the site of hapten application in healthy individuals.

T lymphocytes isolated from negative patch tests to nickel are enriched in CD4$^+$CD25$^+$ T cells and strongly inhibit nickel-specific responses of blood-derived CD4$^+$CD25$^-$ T cells

The latter observation neither excluded that CD25$^+$ T cells could represent recently activated CD25$^-$ lymphocytes nor indicated their regulatory function. Thus, to functionally assess T cells infiltrating a negative patch test to nickel, punch biopsies were taken

36 h after nickel application, mechanically fragmented, and cultured as described in *Materials and Methods*. After 9 days, T cells emigrated from the tissue samples were collected and starved (without IL-2) for 48 h before cytofluorometric analysis and functional assays. The number of T cells obtained from a 4-mm skin biopsy was low compared with that in nickel-positive skin reactions from nickel-allergic subjects (9 × 10^5 ± 2 × 10^5 cells vs 8 × 10^6 ± 1.5 × 10^6 cells, respectively; *n* = 3). In contrast to the cells infiltrating positive skin reactions, T cells emigrated from negative skin tests were unable to proliferate or release cytokines in response to the metal (Fig. 8, *A* and *B*). CD25$^+$ cells accounted for ~30% of the total number of T cells isolated from the biopsy on day 6 (not shown), which was reduced to 20% after a 2-day period of starvation (Fig. 8*C*). At a later time point (4 days), the percentage of CD25$^+$ T cells did not change significantly (not shown). Importantly, T cells derived from a negative patch tests, but not from a positive patch test, strongly inhibited nickel-specific responses of autologous CD4$^+$CD25$^-$ T cells isolated from peripheral blood (*p* < 0.001; Fig. 8*D*). These results demonstrate that T cells recruited at the site of nickel challenge in healthy donors contain a fraction of CD25$^+$ Treg and block the activation of nickel-reactive T lymphocytes.

CD4$^+$CD25$^+$ T cells express the CCR7 chemokine receptor and inhibit naive T cell responses to nickel

Although the suppressive activity of CD25$^+$ Treg on nickel T cell responses prevailed in the CLA$^+$ fraction, a significant regulatory function was also provided by CD25$^+$CLA$^-$ T cells. These Treg are unlikely to be attracted to the skin following hapten exposure, but may prevent nickel-specific T cell priming in secondary lymphoid organs. Indeed, cytofluorometric analysis of PBMCs revealed that 60 ± 14% (*n* = 4) of CD4$^+$CD25$^+$ T cells expressed the chemokine receptor CCR7, the ligand for CCL21, which strongly influences lymphocyte positioning into secondary lymphoid organs (34) (Fig. 9*A*). To assess the capacity of CD25$^+$ Treg to regulate nickel-specific T cell priming in vitro, CD4$^+$CD25$^-$ T
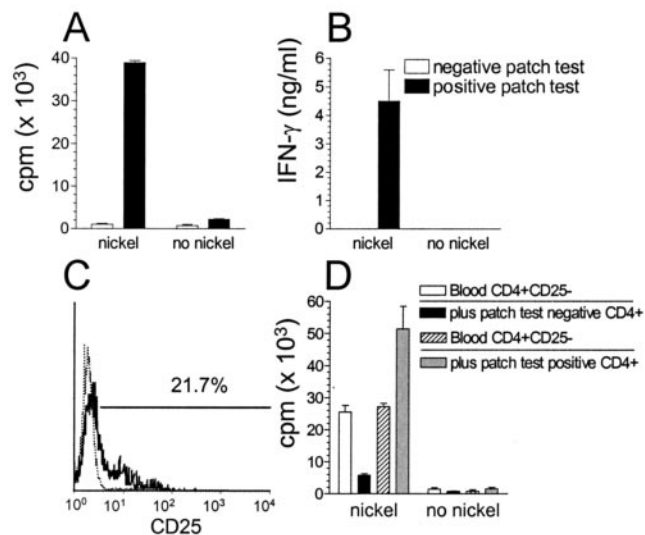
FIGURE 8. T cells isolated from a negative patch test to nickel applied on the skin of healthy donors fail to proliferate in vitro in response to the metal and strongly inhibit nickel-specific T cell responses of autologous peripheral blood CD4$^+$CD25$^-$ T cells. Skin biopsies were minced with a scalpel and placed in culture in complete RPMI with 5% autologous plasma plus 60 U/ml recombinant human IL-2. On day 9 T cells emigrated from tissue samples were collected and were left for 2 days in 96-well microplates in complete RPMI without IL-2 before phenotypic characterization and functional assays. Nickel-specific proliferation (*A*) and IFN-γ release (*B*) of T cells emigrated from the skin (80,000/well) in a negative patch test compared with those obtained from a positive patch test of a nickel-allergic patient are shown. *C*, FACS analysis after staining with anti-CD25 mAb of total T cells isolated from the negative skin reaction to nickel. *D*, Inhibition of nickel-specific autologous CD4$^+$CD25$^-$ (80,000 cells/well) T cell responses by peripheral blood CD25$^+$ T cells (80,000 cell/well) or lymphocytes (80,000 cell/well) isolated from the negative patch test or from a positive patch test from a nickel-allergic patient. Data are representative of experiments performed with T cells isolated from three healthy nonallergic individuals and three nickel-allergic patients. Results represent the mean counts per minute ± SD of duplicate cultures.

FIGURE 9. CD25$^+$ T cells express the lymph node-homing receptor CCR7 and inhibit both memory and naive T cell responses to nickel. *A*, Purified CD4$^+$ T cells were double-stained and examined by FACS analysis for CD25 and CCR7 coexpression. *B*, CD4$^+$ T cells from peripheral blood of four nonallergic individual (NA2 to NA5) were purified with immunomagnetic beads as described in *Materials and Methods* to obtain pure CD45RO$^+$RA$^-$ and CD45RO$^-$RA$^+$ T cell populations. Responder cells were cocultured with CD4$^+$CD25$^+$ T cells at a 2/1 ratio in the presence of APCs and nickel (20 μg/ml) for 5 days, and proliferation was determined.

cells from nonallergic subjects were separated into naive CD45RO$^-$RA$^+$ and memory CD45RO$^+$RA$^-$ T lymphocytes and assayed for their nickel responsiveness in the presence of CD25$^+$ Treg. Both memory and naive T cells from peripheral blood of healthy allergic individuals responded to nickel, with a higher proliferation of CD45RO$^+$RA$^-$ T cells and a lower response of naive CD45RO$^-$RA$^+$ T lymphocytes (Fig. 9*B*). CD4$^+$CD25$^+$ T cells inhibited to a comparable degree both T cell subsets ($p < 0.002$), indicating that CD25$^+$ Treg could have a role not only in blocking hapten-specific T cell activation in peripheral tissues, but also in preventing the expansion of specific memory-effector T cells at any contact with the hapten.

CD4$^+$CD25$^+$ T cells from peripheral blood of allergic patients proliferate in response to nickel in vitro, display a limited capacity to inhibit CD4$^+$CD25$^-$ T cell proliferation, and fail to inhibit nickel-specific CD8$^+$ T cells responses

Our findings strongly suggested an important contribution of CD4$^+$CD25$^+$ T cells in the modulation of cutaneous immunity to nickel. In line with this hypothesis, we asked whether CD4$^+$CD25$^+$ T cells from nickel-allergic patients and healthy individuals differed in the capacity to suppress nickel-specific responses. The percentage of CD4$^+$CD25$^+$ T cells obtained from the peripheral blood of nickel-allergic patients did not differ significantly from that of nonallergic donors (not shown). However,
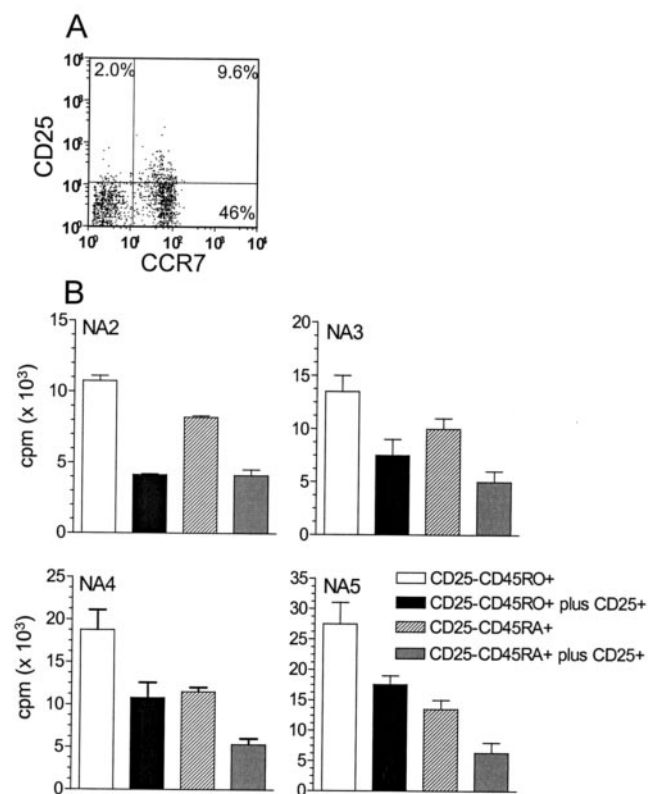
in contrast to CD25$^+$ T cells obtained from peripheral blood of nonallergic individuals, CD25$^+$ T cells from nickel-allergic donors strongly proliferated in response to the metal (Fig. 10). The regulatory function of CD4$^+$CD25$^+$ T cells from nickel-allergic patients was absent or much fainter compared with that of CD25$^+$ Treg from nonallergic subjects. In particular, CD4$^+$CD25$^-$ cells cocultured with CD25$^+$ T cells at a 2/1 ratio showed 50 and 25% decreased responses to nickel in patients A4 and A6 ($p < 0.05$), respectively, and nonsignificant effects in the remaining patients (Fig. 11*A*). Finally, CD25$^+$ T cells from all allergic patients failed to inhibit nickel-specific CD8$^+$ T cell responses in coculture experiments (Fig. 11*B*). Interestingly, when CD25$^+$ T cells from nickel-allergic and nonallergic individuals were compared in their capacity to suppress allogeneic T cell responses, no significant differences were observed, indicating that the overall CD25$^+$ Treg activity in nickel-allergic individuals was not compromised (data not shown).

Discussion

We have shown here that in healthy, nonallergic individuals, CD4$^+$CD25$^+$ T cells dose-dependently regulate primary and secondary nickel-specific T cell responses and are recruited in the skin after nickel application. The maximal inhibitory effect of CD25$^+$ Treg on nickel responsiveness of CD4$^+$CD25$^-$ T cells was achieved at 1/1 and 1/2 ratios, in line with previous reports that
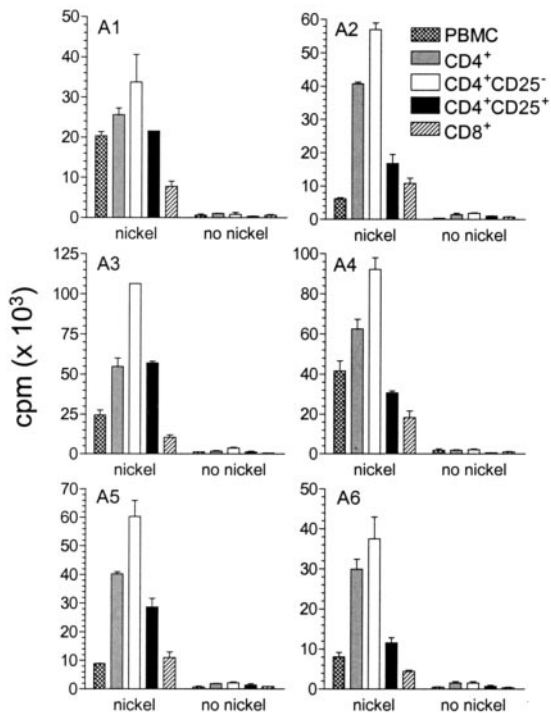
FIGURE 10. Peripheral blood CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ isolated from nickel-allergic donors proliferate in response to nickel sulfate in vitro. T cell subpopulations were purified from the peripheral blood of six patients (A1 to A6) and assayed for nickel (20 μg/ml) responsiveness in vitro in the presence of adherent cells as APCs for 5 days. [^{3}H]thymidine was added at 2 μCi/well during the final 16 h of coculture. Data are expressed as the mean counts per minute $\pm$ SD of triplicate cultures.

FIGURE 11. CD4$^+$CD25$^+$ T cells from nickel-allergic patients show a limited suppressive activity on CD4$^+$CD25$^-$ T cells and fail to suppress CD8$^+$ T cell responses to nickel. CD4$^+$CD25$^-$ T cells (*A*) or CD8$^+$ T lymphocytes (*B*) were cultured with conditioning CD25$^+$ or control CD25$^-$ cells at a 2/1 ratio in the presence of APCs and nickel for 5 days. Data are expressed as the mean counts per minute $\pm$ SD of triplicate cultures.

studied the effects of Treg on allospecific or mitogen-induced T cell activation (18). CD25$^+$ Treg also exert a suppressive effect at physiologic numbers, as suggested by the strong increase in the nickel-specific CD4$^+$ T cell proliferation of peripheral blood T cells upon removal of the CD25$^+$ T cell fraction. In addition, coculture experiments indicated that 40% inhibition occurred at a CD25$^-$/CD25$^+$ ratio of 1/8. CD4$^+$CD25$^+$ cells from nonallergic individuals failed to respond to nickel in terms of both proliferation and cytokine release, according to the idea that the anergic status of Treg is correlated with their suppressive function (35). Direct evidence of a CD25$^+$ Treg antigenic repertoire is missing, mostly because of their unresponsiveness in vitro. However, when stimulated with nickel, a small fraction (~3%) of CD25$^+$ cells up-regulates membrane expression of CTLA-4, which is induced by TCR triggering (32), suggesting that a minority of CD25$^+$ Treg could specifically recognize the metal. CTLA-4 is a negative regulator of T cell activation by competing with CD28 for binding to the B7 family members expressed on APCs. CTLA-4 expression is higher on CD25$^+$ T cells compared with the CD25$^-$ counterpart, and it can be involved in the suppressive activity of Treg (20, 36, 37). CLA expression identifies a fraction of T cells with a propensity to recirculate in the skin. CLA is acquired by T cells after Ag encounter in skin-draining lymph nodes and is modulated by multiple factors, including the type of APC and the cytokine environment (38). Interestingly, CD25$^+$CLA$^+$ and CD25$^+$CLA$^-$ T cells differed in their suppressive activity, with CD25$^+$CLA$^+$ Treg much more efficient in regulating nickel-specific T cell responses.

In humans, CD25$^+$ T cell positioning in peripheral tissues during inflammation and tumor progression has been clearly demonstrated (39, 40). To validate the hypothesis of a direct role of CD25$^+$ Treg in cutaneous hypersensitivity to nickel, we investi-
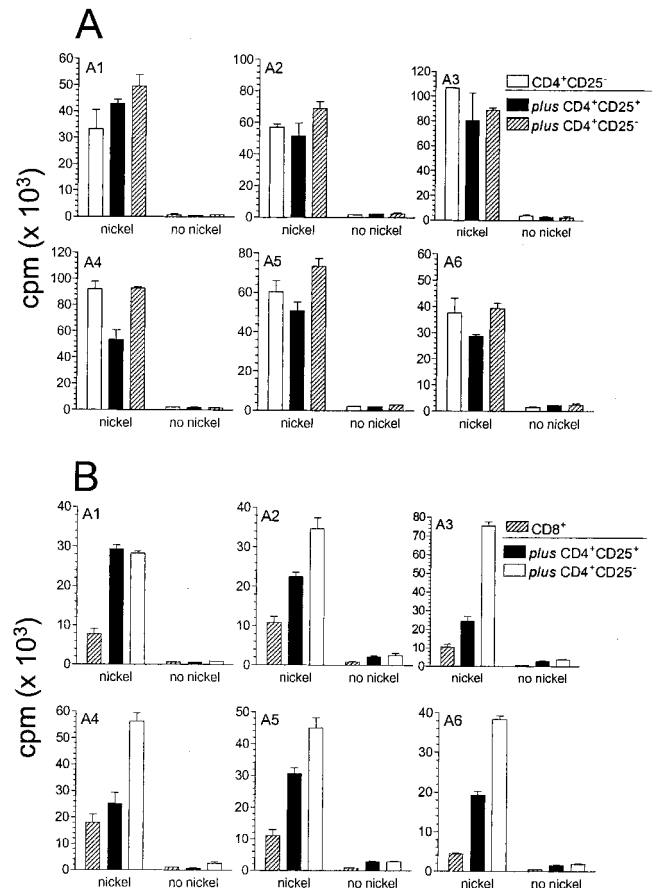
gated the characteristics of T cells infiltrating the site of a patch test to nickel applied on the skin of nonallergic individuals. In contrast to nickel-allergic patients, nonallergic subjects do not develop cutaneous inflammation in response to nickel applied at a concentration of 5% in petrolatum under occlusion for 48–72 h despite the presence of nickel-reactive CD4$^+$ T cells in their peripheral blood. Although such apparent contradiction may be explained in different ways, our data demonstrate that nickel-challenged skin of nonallergic individuals showed a significant infiltrate of mononuclear cells, including CD4$^+$, CD8$^+$, CLA$^+$, and some CD25$^+$ cells. These CD25$^+$ cells comprise ~20% of T cells isolated from nickel-challenged skin, they are anergic upon nickel restimulation in vitro, and importantly, they strongly suppressed nickel-specific activation of blood-derived CD4$^+$CD25$^-$ lymphocytes. In comparison, T cells isolated from nickel-allergic donors strongly proliferate and secrete IFN-γ when stimulated with nickel in vitro. These results indicate that in both allergic and nonallergic individuals, hapten application is followed by a rapid recruitment of T cells. However, in the latter, Treg recruitment appears to prevent the activation of effector T cell populations and the development of the inflammatory reaction. In contrast to Treg isolated from nonallergic subjects, CD25$^+$ T cells isolated from the peripheral blood of nickel-allergic individuals display a limited capacity to regulate nickel-specific CD4$^+$CD25$^-$ T cell responses and completely fail to suppress CD8$^+$ T cell responsiveness to the

metal. Interestingly, the reduced regulatory activity of CD25$^+$ T cells isolated from nickel-allergic individuals appears restricted to nickel-specific responses, since they suppress allo-specific T cell responses with the same efficiency as Treg isolated from healthy donors (data not shown). Thus, subjects that develop ACD in response to nickel may have a deficient function of their CD25$^+$ Treg and a high number of metal-reactive CD8$^+$ T cells. These two findings might be correlated, since it has been shown that CD25$^+$ Treg are strongly involved in regulation of the memory/effector CD8$^+$ T cell population (41). We also found that >50% of CD25$^+$ Treg expressed the chemokine receptor, CCR7, which is involved in T cell homing to peripheral lymph nodes (34), and that CD25$^+$ Treg strongly inhibit the in vitro activation of naive T cells to nickel. In this scenario, we hypothesize that CD25$^+$ Treg modulate the outcome of immune responses toward skin-applied sensitizers with two mechanisms: a central mechanism mediated by CD25$^+$CCR7$^+$ Treg, which may limit the emergence of memory-effector hapten-specific T cells, CD8$^+$ T lymphocytes in particular, and a peripheral mechanism mediated by CLA$^+$CD25$^+$ Treg, which can migrate in response to specific chemotactic stimuli in the skin and down-regulate activation of effector T cells at the site of hapten penetration, thus impeding the clinical manifestations of the allergic reaction.

Further investigations are required to establish the relative contributions of CD25$^+$ Treg and other regulatory T cell subsets in the control of ACD reactions. In particular, we have previously characterized a subset of nickel-specific CD4$^+$ T lymphocytes with regulatory activity that down-regulates the immune responses to the metal through the release of a high amount of IL-10. In contrast to CD25$^+$ Treg, IL-10-releasing CD4$^+$ T cells, also named Tr1 cells, do not require cell-to-cell contact for their suppressive function and act on APCs by blocking their IL-12 release and Ag-presenting functions (7). Tr1 recruitment at the site of nickel application is mostly promoted by the local release of CCL1, released by keratinocytes and infiltrating leukocytes late during ACD reaction (42). This finding suggests that Tr1 cells may be involved in the termination of the immune response to avoid excessive tissue damage.

In aggregate, our data strongly support the role of adaptive CD25$^+$ Treg in the control of cutaneous immune responses to contact sensitizers and suggest that defective development of specific Treg may be critically involved in the expression of ACD in response to haptens. Insights into the origin and differentiation of adaptive CD25$^+$ Treg could represent a critical step for therapeutic or preventive approaches of hapten-induced skin allergies.

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