

Regulation of nickel-induced T-cell responsiveness by CD4⁺CD25⁺ cells in contact allergic patients and healthy individuals

H. MOED¹, B. M. E. VON BLOMBERG², D. P. BRUYNZEEL¹, R. J. SCHEPER², S. GIBBS¹ AND T. RUSTEMEYER¹

¹Departments of Dermatology and ²Pathology, VU university medical center Amsterdam, the Netherlands

In this study, we investigated the capacity of CD4⁺CD25⁺ regulatory T cells to suppress nickel-specific effector T cells, both in nickel-allergic patients and healthy controls. CD4⁺ cells isolated from allergic patients showed an increased proliferative response to nickel, whereas CD4⁺ cells from negative controls did not respond to allergen. When CD4⁺CD25⁺ cells were depleted, nickel-specific responsiveness was strongly increased both in allergic and in non-allergic individuals, with the most pronounced effect in allergic patients. These regulatory T cells were anergic to nickel but inhibited nickel-specific CD4⁺CD25[−] effector T cells in coculture experiments. CD4⁺CD25⁺ cells from nickel-allergic patients showed only a limited capacity to suppress effector T-cell responsiveness, because an increased nickel reactivity could still be detected in these cocultures. None of the isolated CD4⁺CD25⁺ cells, either isolated from healthy controls or allergic patients, produced IL-10 in response to nickel. Overall, these results support the view that CD4⁺CD25⁺ cells can control the activation of nickel-specific effector T cells in non-allergic individuals, whereas this regulatory capacity is impaired in allergic patients. To investigate the presence of allergen-specific regulatory T cells in truly naïve, non-sensitized individuals, T-cell reactivity should also be studied with non-environmental contact allergens, such as para-phenylenediamine.

Key words: regulatory T cells; allergic contact dermatitis; nickel; CD4⁺CD25⁺. © Blackwell Munksgaard, 2005.

Accepted for publication 26 April 2005

A controlled balance between initiation and downregulation of the immune response is important in maintaining immune homeostasis. When this balance is disturbed by the dysfunction of professional suppressor/regulatory T cells, autoimmune diseases or allergic diseases can develop as a consequence of an exaggerated immune response against harmless non-self antigens.

Regulatory mechanisms, which can diminish excessive immune reactivity, are now thought to play an important role in termination of disease. Specialized regulatory T cells have been described that modulate the immune response through two principal pathways: the release of anti-inflammatory cytokines, or a cell-to-cell contact mechanism. IL-10-producing regulatory T cells (or Tr₁) (1–3) have been identified *in vitro* and isolated from various tissues including the skin (4, 5). These Tr₁ cells block specific T-cell responses in an IL-10-dependent manner by targeting professional antigen-presenting cells

(APC); IL-10 release impairs the maturation of DCs as well as their IL-12 release and thus inhibits the activation of effector T cells (4, 6). The major role of these Tr₁ cells in the skin may be to regulate the magnitude of T-cell reactivity and to induce termination of the local inflammatory response.

Besides these IL-10-producing regulatory T cells a second type of T_{reg} inhibits T-cell responses in a cell-to-cell contact-dependent, cytokine-independent manner. These cells are described as circulating CD4⁺ cells expressing the IL-2 receptor α -chain (CD25) (7–9). They are anergic to stimulation and can suppress proliferation and cytokine production of CD25[−] responder T cells (10). CD4⁺CD25⁺ T_{reg} cells represent about 5–10% of circulating CD4 cells and express high levels of the T-cell inhibitory receptor CTLA-4 and often show overexpression of the *foxp3* gene, associated with expression of CD25 and IL-10 release (11, 12). These cells have been isolated from several peripheral tissues and

from skin explants, indicating their role in peripheral immune responses (13, 14). Moreover, some circulating $CD25^+$ cells express the skin-homing marker CLA, which indicates an involvement of these cells in the regulation of skin inflammation (15).

Cavani et al. (14) investigated the capacity of $CD4^+CD25^+$ cells to modulate T-cell responses to nickel. Their results suggested that in healthy individuals T_{reg} cells can control the activation of both naïve and effector nickel-specific T cells. In this study, we obtained evidence supporting this view. We hypothesized that in non-allergic individuals exposure to ubiquitous nickel might induce circulating nickel-specific effector T cells, which are suppressed by $CD4^+CD25^+$ cells. In allergic patients, with increased exposure, allergen-specific effector T cells might dominate and therefore allergic reactivity to nickel sulphate may develop in these patients. In order to test this hypothesis, we measured nickel-induced proliferative responses of whole $CD4^+$ T cells, $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells, and combinations of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells. Moreover, IL-10 production in these different T-cell populations was measured to assess the putative relevance of this immunosuppressive cytokine in regulation of ACD. Whereas we observed no allergen-induced proliferation or IL-10 production by $CD4^+CD25^+$ T cells, depletion of this subset resulted in an increased reaction to nickel sulphate in both allergic patients and healthy donors. In accordance therefore with the results of Cavani et al. (14), we conclude that $CD4^+CD25^+$ play an important role in actively suppressing the nickel specific T-cell function.

Materials and Methods

Isolation of T-cell subsets

Peripheral blood mononuclear cells (PBMC) of three nickel-allergic patients (with a documented allergy to nickel and a moderate-to-intense positive patch test to nickel) and three healthy individuals (with no history of metal allergy and a negative patch test) were isolated from heparinized blood by density centrifugation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and cryopreserved in liquid nitrogen until further testing. The isolated PBMCs were thawed and $CD4^+$ T cells were labelled using a $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, Gladbach, Germany). $CD4$ -positive cells were negatively selected with a negative selection column yielding a purity of approximately 90%. Next, $CD4^+$ T cells were incubated with anti- $CD25$ antibody (BD Pharmingen, San Diego, CA, USA),

followed by incubation with goat anti-Mouse Microbeads (Miltenyi Biotec). The negative $CD4^+CD25^-$ fraction was separated from the positive $CD4^+CD25^+$ fraction by making use of a positive selection column following the standard protocol described by the manufacturer (MACS, Miltenyi Biotec). Separated subsets were measured by flow cytometry to check for the purity of the subsets. Therefore, cells were stained with $CD4$ -FITC (CLB, Amsterdam, the Netherlands) and $CD25$ -PE (BD Pharmingen).

T-cell stimulation assay

Purified T-cell subsets were resuspended in complete medium [Iscove's Modified Dulbecco's Medium (Biowhittaker, Verviers, Belgium) supplemented with 50 IU/ml penicillin-G, 50 µg/ml streptomycin, 50 µM DTT (Merck, Darmstadt, Germany) and 10% HPS (Biowhittaker)] and cultured in a round bottom 96-well plate in addition to 50 µM nickel sulphate hexahydrate (Merck) for 6 days at 37°C in a 5% CO_2 -containing humidified atmosphere. To determine background proliferation, we cultured cells in complete medium only. Cells were cultured in triplicate at a concentration of 2×10^5 cells per well in a volume of 200 µl. For optimal nickel specific stimulation of the selected T-cell subsets, APC are needed. Therefore, autologous PBMCs were irradiated in a Cobalt-60 source (3000 rad) and added during culture at a concentration of 5×10^4 cells/well. For suppression assays, 0.67×10^5 $CD4^+CD25^+$ cells were cultured in combination with 1.33×10^5 $CD4^+CD25^-$ cells, resulting in a suppressor: responder ratio of 1:2.

After 6 days, T-cell proliferation was measured by ^3H thymidine incorporation as follows: cells were pulsed with 0.5 µCi ^3H thymidine (Amersham, Pharmacia, UK) per well during the last 5 h of culture. Radioactivity was measured by a liquid scintillation counter (Wallac, Turku, Finland).

Cytokine measurement

After 6 days of culture, supernatants were partly removed and stored at -30°C for cytokine measurement. Production of IL-10 was measured by IL-10 PeliPair reagent set (CLB; lower detection level 10 pg/ml) following the manufacturer's instructions.

Statistical analysis

For statistical analysis the paired *T*-test was used. *P*-values <0.1 were considered significantly different.

Results

In order to determine the suppressive capacity of regulatory T cells in healthy individuals and in patients with allergic contact dermatitis, we examined the proliferative capacity of $CD25^+$ and $CD25^-$ T-cell subsets in response to allergen. T-cell subsets ($CD4^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$) isolated from PBMC of non-allergic individuals and nickel-allergic patients were stimulated with nickel and incubated during 6 days. After stimulation, proliferative capacity was determined by measurement of ^3H incorporation. $CD4^+$ cells from allergic patients showed an increased reactivity to nickel (Fig. 1A, $P = 0.02$). In contrast, nickel-stimulated $CD4^+$ cells from healthy controls showed no difference from unstimulated cells (Fig. 1B, $P = 0.35$). Depletion of $CD4^+CD25^+$ cells from total PBMC resulted in an increased nickel-induced T-cell response. Interestingly, this increased reactivity was observed both in allergic patients ($P = 0.09$) and in healthy controls ($P = 0.04$). However, the proliferative increase was found to be highest in the allergic patient group. Purified $CD4^+CD25^+$ cells of both groups demonstrated no increased proliferation in response to nickel.

We next addressed the question whether these non-proliferative regulatory cells were capable of suppressing $CD4^+$ effector T cells. Thereto, $CD4^+CD25^+$ cells were added to $CD4^+CD25^-$ cells in a ratio of 1:2. From Fig. 1, it becomes clear that effector cells can indeed be suppressed by $CD4^+CD25^+$ cells (compare $CD4^+CD25^-$ to $CD4^+CD25^-$ plus $CD4^+CD25^+$). Proliferative responses of the $CD4^+CD25^-$ plus $CD4^+CD25^+$ group are similar to responses of the total $CD4^+$ population.

Because regulatory T cells suppress nickel-specific effector T cells, we next examined the production of IL-10 by these cells. None of the isolated $CD25^+$ cells, either isolated from healthy controls or allergic patients, were capable of producing IL-10 in response to nickel (data not shown). This suggests that $CD25^+$ cells regulate their suppressive function by other mechanisms than IL-10 production.

Taken together, these results indicate that $CD4^+CD25^+$ play an important role in actively suppressing nickel-specific proliferation. These cells by themselves do not proliferate, whereas depletion of regulatory cells leads to an increased reaction to nickel in both allergic patients and healthy donors. Suppression by $CD4^+CD25^+$ cells does not seem to be mediated by IL-10 production.

Discussion

In this report, we provide evidence supporting the view that $CD4^+CD25^+$ T_{reg} cells regulate nickel-

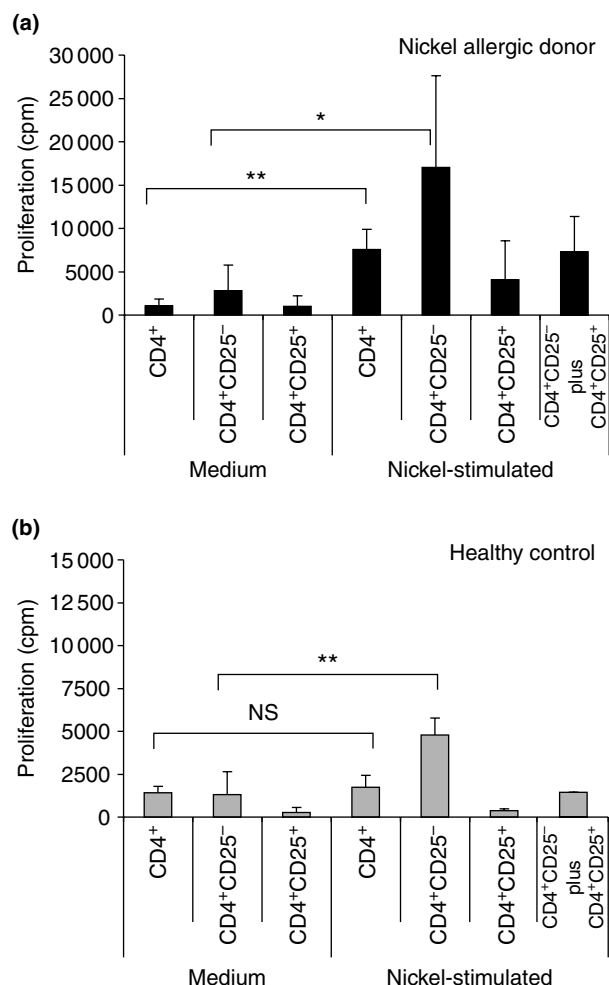


Fig. 1. Proliferative capacity of $CD4^+CD25^-$ effector and $CD4^+CD25^+$ regulatory T cells in response to nickel. Mean values ($\pm$ SD) obtained from 3 different allergic individuals (a) or healthy controls (b) are shown. * $P < 0.1$; ** $P < 0.05$.

specific T-cell responses not only in allergy but also in healthy, non-allergic individuals. $CD4^+CD25^+$ cells have an inhibitory effect on nickel responsiveness of $CD4^+CD25^-$ cells. In addition, these $CD4^+CD25^+$ cells do not respond to allergen in terms of proliferation and IL-10 cytokine production. This is in line with earlier publications that T_{regs} are anergic themselves and have an intrinsic suppressive capacity (10, 14).

In nickel allergic patients, proliferative responsiveness of $CD4^+CD25^-$ effector cells could still be increased when $CD25^+$ regulatory cells were depleted. When $CD4^+CD25^+$ regulatory cells were added to $CD4^+CD25^-$ effector cells, nickel-specific response could be suppressed. However, this response was not completely suppressed, as observed in healthy individuals, suggesting that $CD25^+$ cells isolated from the blood of allergic patients display a limited capacity to regulate nickel specific $CD4^+CD25^-$ T-cell responses.

The relevance of IL-10 production by CD4⁺CD25⁺ regulatory T cells is controversial. Some articles suggest that these cells mediate their suppressive effects in a cell-contact dependent, antigen-independent manner, without the requirement of cytokines such as IL-10 (7, 8). Others show that these CD4⁺CD25⁺ cells are capable of producing high amounts of IL-10 when stimulated in the appropriate fashion (16–18). Also Rustemeyer et al. (19) demonstrated that nickel-specific IL-10 production is important as an immuno-regulatory cytokine in actively tolerized individuals. Notwithstanding, data obtained in this study indicate that CD25⁺ cells can exert their suppressive function by other mechanisms than IL-10 production. These mechanisms may include absorption of IL-2. Because T_{reg} cells contain the IL-2 receptor α -chain, they may be capable of catching IL-2, thus interfering with T-cell proliferation.

In summary, our data support the role of CD4⁺CD25⁺ cells in controlling cutaneous responses to nickel. Presence of well-functioning regulatory T cells in non-allergic individuals, who only were exposed to low doses of environmental nickel, may explain why they do not develop an eczematous reaction after skin contact with nickel. Whether allergen-specific regulatory T cells can be detected in naïve individuals against non-environmental allergens, should be determined in future experiments. When regulatory mechanisms fail (in the case of allergic patients) nickel-specific effector T cells are not suppressed properly, finally resulting in an allergic contact dermatitis reaction. Insight in the mechanisms of ACD regulation is of great importance, because this should help in the development of therapeutical or preventive approaches of allergen-induced skin reactivity.

References

- Sebastiani S, Allavena P, Albanesi C et al. Chemokine receptor expression and function in CD4⁺ T lymphocytes with regulatory activity. *J Immunol* 2001; 166: 996–1002.
- Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries J E, Roncarolo M G. A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997; 389: 737–742.
- Lecart S, Boulay V, Raison-Peyron N et al. Phenotypic characterization of human CD4⁺ regulatory T cells obtained from cutaneous dinitrochlorobenzene-induced delayed type hypersensitivity reactions. *J Invest Dermatol* 2001; 117: 318–325.
- Cavani A, Nasorri F, Prezzi C, Sebastiani S, Albanesi C, Girolomoni G. Human CD4⁺ T lymphocytes with remarkable regulatory functions on dendritic cells and nickel-specific Th1 immune responses. *J Invest Dermatol* 2000; 114: 295–302.
- Roncarolo M G, Bacchetta R, Bordignon C, Narula S, Levings M K. Type-1 regulatory T cells. *Immunol Rev* 2001; 182: 68–79.
- Corinti S, Albanesi C, la Sala A, Pastore S, Girolomoni G. Regulatory activity of autocrine IL-10 on dendritic cell functions. *J Immunol* 2001; 166: 4312–4318.
- Shevach E M. Certified professionals: CD4(+)CD25(+) suppressor T cells. *J Exp Med* 2001; 193: F41–F46.
- Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk A H. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; 193: 1285–1294.
- Annunziato F, Cosmi L, Liotta F et al. Phenotype, localization, and mechanism of suppression of CD4(+)CD25(+) human thymocytes. *J Exp Med* 2002; 196: 379–387.
- Taams L S, Smith J, Rustin M H, Salmon M, Poulter L W, Akbar A N. Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol* 2001; 31: 1122–1131.
- Ramsdell F. Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity* 2003; 19: 165–168.
- Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; 299: 1057–1061.
- Cao D, Malmstrom V, Baecher-Allan C, Hafler D, Klareskog L, Trollmo C. Isolation and functional characterization of regulatory CD25brightCD4⁺ T cells from the target organ of patients with rheumatoid arthritis. *Eur J Immunol*, 215–223 2003.
- Cavani A, Nasorri F, Ottaviani C, Sebastiani S, De Pita O, Girolomoni G. Human CD25⁺ regulatory T cells maintain immune tolerance to nickel in healthy, nonallergic individuals. *J Immunol* 2003; 171: 5760–5768.
- Colantonio L, Iellem A, Sinigaglia F, Dambrosio D. Skin-homing CLA⁺ T cells and regulatory CD25⁺ T cells represent major subsets of human peripheral blood memory T cells migrating in response to CCL-1/I-309. *Eur J Immunol* 2002; 32: 3506–3514.
- Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 2001; 194: 629–644.
- Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med* 2001; 193: 1303–1310.
- Levings M K, Sangregorio R, Roncarolo M G. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 2001; 193: 1295–1302.
- Rustemeyer T, Blomberg B M E, Hoogstraten I M W, Bruynzeel D P, Scheper R J. Analysis of effector and regulatory immune reactivity to nickel. *Clin Exp Immunol* 2004; 34: 1458–1466.

Address:

T. Rustemeyer
Department of Dermatology
VU university medical center
PO Box 7057
1007 MB Amsterdam
The Netherlands
Tel: +31 20 4440145
Fax: +31 20 4440148
e-mail: t.rustemeyer@vumc.nl