The glucocorticoid-induced tumour necrosis factor receptor-related gene (GITR) is expressed on regulatory T-cells (Tregs), which are CD4+CD25+ lymphocytes. Binding of the GITR-ligand (GITRL) leads to downregulation of the regulatory function of Tregs. Patients suffering from a defect in their Tregs exhibit a condition in their skin resembling atopic dermatitis. GITR also exists in a soluble form, and increased levels of this lead to decreased levels of GITRL and thereby increased Treg activity. We have measured the levels of GITR and GITRL in plasma from atopic dermatitis patients and found it not to be increased. Furthermore, plasma levels of GITR and GITRL did not correlate with SCORAD. Both GITR and GITRL correlated with the levels of thymus- and activation-regulated chemokine/CCL17 and cutaneous T-cell-attracting chemokine/CCL27, two chemokines believed to play a major role in the pathogenesis of atopic dermatitis and the migration of Tregs and skin-homing T-cells. Immunohistochemistry showed GITR and GITRL were present in few dermal cells of both patients with atopic dermatitis, and normal healthy volunteers, and often localized in close proximity to each other. Since regulatory T-cells are localized in the vicinity of GITRL-expressing cells in atopic dermatitis skin, the GITR/GITRL interaction may serve to perpetuate the inflammation locally. Key words: atopic dermatitis; regulatory T-cells; GITR; GITRL; FOXP3.

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Our aim was to investigate the plasma levels of GITR and GITRL in patients with AD as well as the expression of GITR in AD skin. Since CCL17 is known to attract Tregs, we wished to examine the correlation between GITR, GITRL and CCL17. Furthermore, since CCL27 and its receptor CCR10 is closely related to the CCR4 positive cells and to the attraction of T-cells into the skin, we also wanted to examine if CCL27 correlates with GITR, GITRL and CCL17.

MATERIALS AND METHODS

All participants gave informed consent and the study was approved by the local ethics committee of Aarhus County.

Blood samples and ELISA

Heparinized blood samples were taken from 78 patients with AD (43 women, mean age 17.6±14.1 years and 35 men, mean age 21.3±15.8 years), after signed consent. The samples were centrifuged and the plasma was stored frozen at –80°C. The patients were scored using the SCORAD scale.

A total of 22 heparinized blood samples were taken from healthy donors (9 women, mean age 37.5±12.8 years and 11 men, mean age 34.5±6.5 years) after signed consent and used as controls.

The plasma was carefully thawed and mixed in a 1:1 solution with 1% bovine serum albumin in phosphate buffered saline (PBS). The samples were analysed using an ELISA (Duoset Elisa Development kit from R&D Systems, Oxon, UK, catalogue numbers DY689, DY694, DDN00, DY376). The ELISA was performed as described by the manufacturer. Briefly, 96-well plates (Nunc-ImmuNoTM Plate MaxiSorpTM Surface NUNC, Roskilde, Denmark) were incubated with the capture antibody (4 µg/ml in PBS) and sealed for 24 h. After incubation the wells were aspirated and washed three times with 0.05% Tween 20 (PBS). The samples were analysed using an ELISA (DuoSet Development kit from R&D Systems, Oxon, UK) consisting of Colour Reagent A and Colour Reagent B (R&D Systems). After washing the wells were aspirated and washed three times with 0.05% Tween 20 and incubated with peroxidase-labelled polymer (conjugated to goat anti-mouse immunoglobulin, DakoCytomation EnVisionTM System). After washing with TBS and TBS 0.05% Tween 20 the samples were stained with substrate-chronom solution with levamizole according to the manufacturer’s instructions and counterstained with Mayer’s haematoxylin and mounted on slides for microscopy.

As control, serial sections incubated with mouse IgG1 10 µg/ml were used, and in all cases were negative. As positive control, frozen sections from tonsillar tissue were used.

Western blotting

Validation of the antibodies used for ELISA and the immunohistochemistry stainings was performed by Western blotting (Fig. 1). Peripheral blood mononuclear cells (PBMC) were separated with Lymphoprep® (Axis-Shield, Oslo, Norway) according to the manufacturer’s instructions from heparinized blood samples from a healthy donor. PBMCs were washed twice with ice cold PBS, lysed with cold lysis buffer and boiled for 5 min. The lysate was centrifuged at 13,000 g for 3 min and the supernatant was collected and assayed for protein concentration as described by Bradford (16). The proteins were separated by Novex 10–20% Tris-glycine gel (Invitrogen, Taastrup, Denmark) and blotted onto nitrocellulose membranes. Membranes were incubated with the appropriate primary antibody and detected with either anti-mouse IgG1-HRP (DakoCytomation) or anti-goat IgG-HRP (DakoCytomation) in a standard enhanced chemiluminescence reaction (Amersham, Buckinghamshire, UK).

Skin biopsies and immunohistochemistry

Skin biopsies were obtained from 10 patients with AD (3 women, mean age 37.7±8.4 years and 7 men, mean age 34.6±7.4 years) selected after the clinical severity of the skin lesions, one biopsy from lesional skin and one biopsy from non-lesional skin, each 4 mm in diameter. Normal skin biopsies were obtained from patients undergoing plastic surgery. The biopsies were snap-frozen in liquid nitrogen, and stored at –80°C until use. The samples were cut in a cryostat and mounted on glass slides for immunostaining. The samples were dehydrated in ice-cold acetone for 10 min, washed in Tris-buffered saline (TBS, DakoCytomation, Glostrup, Denmark, no. S1968) and incubated for 30 min with blocking serum (10% swine serum). The endogenous peroxidase activity was quenched by incubation in 0.03% hydrogen peroxide containing sodium azide for 5 min. The samples were rinsed in distilled water, washed in TBS and TBS 0.05% Tween 20 and incubated with mouse anti-human GITR antibody 10 µg/ml or mouse anti-human GITRL antibody 10 µg/ml (R&D Systems) or goat anti-human FOXP3 antibody 10 µg/ml (Abcam, Cambridge, UK) for 1.5 h. The samples were washed in TBS and TBS 0.05% Tween 20 and incubated with peroxidase-labelled polymer (conjugated to goat anti-mouse immunoglobulin, DakoCytomation EnVisionTM System). After washing with TBS and TBS 0.05% Tween 20 the samples were stained with substrate-chronom solution with levamizole according to the manufacturer’s instructions and counterstained with Mayer’s haematoxylin and mounted on slides for microscopy.

As control, serial sections incubated with mouse IgG1 10 µg/ml were used, and in all cases were negative. As positive control, frozen sections from tonsillar tissue were used.

Statistics

The distribution of GITR and GITRL did not fulfill the demands for normal distribution and is therefore expressed by their median value, with 25% and 75% range in parenthesis.

Correlation of the individual values was performed using non-parametric correlations, Spearman’s rho and Pearson’s correlation.

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RESULTS

Plasma GITR and GITRL levels

Patients with AD had a median (25–75% range) plasma GITR level of 104.0 pg/ml (71.4–143.9 pg/ml), which was not statistically different from that of healthy controls 82.0 pg/ml (24.1–149.0) (Fig. 2A). Their plasma GITRL levels were 996.5 pg/ml (283.6–1859.3) in patients with AD, equal to that of normal healthy controls 873.4 pg/ml (457.0–1730.2) (Fig. 2B).

Because GITR belongs to the TNF superfamily, which is known to regulate the effects of its ligands by soluble receptors, we examined whether individual levels of GITR correlated with GITRL levels. Fig. 3A shows a significant correlation, \( r = 0.63, p < 0.01 \), between the two variables.

Correlations with TARC and CTACK plasma levels

We observed significant correlations of both cytokines with GITR and GITRL. The correlation was most pronounced between TARC and GITR \( r = 0.72; p < 0.01 \) and between TARC and GITRL \( r = 0.51; p < 0.01 \) (Figs 3B–C). The correlations between CTACK and GITR \( r = 0.34 \) and between CTACK and GITRL \( r = 0.38 \) were also significant at the \( p < 0.01 \) level (Figs 3D–E).

Correlation between GITR, GITRL and clinical parameters

Patients with AD were subgrouped according to their SCORAD (mild: <15, moderate: 15–40 and severe: >40): there was no correlation with disease activity and plasma levels of GITR and GITRL. Neither did individual plasma values of GITR and GITRL correlate to the level of IgE, number of eosinophils in the blood, or age.

Localization of GITR and GITRL expressing cells in the skin

Skin samples from patients with AD and from normal controls contained only very few GITR- and GITRL-expressing cells. Both antibodies resulted in a cytoplastic staining pattern of cells distributed throughout the dermis, often localized in lymphoid aggregates, and never observed in the epidermis (Figs 4A–B).

Staining of sequential sections from AD samples showed that most of the GITR- and GITRL-expressing cells were localized in the close proximity to each other.

Double-staining of AD skin sections using a combination of the mouse anti-GITR or mouse anti-GITRL with polyclonal goat anti-FOXP3 antibody, showed that GITR and FOXP3-expressing cells were the same, and GITRL-expressing cells were often localized side by side with FOXP3-expressing cells (Fig. 4C). This observation suggests a close interaction between these two different cell types locally, and indicates that GITR-expressing cells are indeed Tregs.

DISCUSSION

In this study we did not observe an increase in the levels of GITR and GITRL in the plasma from patients with AD; nor did we see any correlations with disease activity measured by SCORAD. Patients with AD had significant correlations between plasma levels of GITR and GITRL, and both GITR and GITRL correlated with TARC and CTACK.
The serum concentration of TARC and CTACK correlates with disease activity (17, 18), and possibly also reflects the effect of treatment (15). In these studies very low to undetectable concentrations of both CTACK and TARC were found (17, 18).

Both TARC and CTACK have been implicated in the pathogenesis of AD (13). TARC and CTACK are chemotactic for Th2 and skin homing lymphocytes expressing the chemokine receptors CCR4 and CCR10, respectively. Both chemokines are highly expressed in AD skin and both receptors are expressed by CLA-positive effector cells. Little is known of the regulation of Treg migration. Tregs have been described to express CCR4 and CCR8 and to migrate towards MDC (CCL22), TARC (CCL17) and I-309(CCL1) (10). CCR10 is co-expressed with CCR4, and of the circulating CCR10+ T-cells approximately 30% are CCR4 positive (11); yet no data for CCR10 expression on Treg is available.

TARC, therefore, has the potential to attract both CLA+ lymphocytes and Tregs. Co-localization of GITR-positive lymphocytes and GITRL-expressing cells indicates that the Tregs in AD skin may lose their immunosuppressive function, thus perpetuating the local inflammatory reaction. It should be taken into account that we have chosen to examine chronic AD, in which a mixed Th1/Th2 cytokine profile can be found, and that the cytokine pattern and T-cell function is different in acute AD, which is dominated by a Th2 cytokine profile. Thus, the Treg function could be different in this situation.

We have recently shown that the NF-κB pathway is crucial for CTACK transcription, and since GITRL is also known to induce NF-κB activation (19), the close correlation between GITRL and CTACK in plasma of patients with AD suggests that the NF-κB pathway is a common pathway in both the GITRL-induced downregulation of Treg activity and CTACK induced inflammation in AD.

GITR belongs to the TNF receptor super-family, where soluble receptors function as inhibitors of their soluble ligands. The regulation of the TNF super-family is, however, complex. It has become clear that members of

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**Fig. 3.** (A) Correlation between soluble glucocorticoid-induced tumour necrosis factor receptor (sGITR) and glucocorticoid-induced tumour necrosis factor receptor ligand (GITRL) in patients with atopic dermatitis (AD) is significant (\( p < 0.01 \), \( r = 0.63 \)). (B) Correlation between thymus and activation-regulated chemokine or CCL17 (TARC) and GITR (\( r = 0.72 \) and \( p < 0.01, n = 70 \)) and (C) between TARC and GITRL in patients with AD (\( r = 0.51 \) and \( p < 0.01, n = 78 \)). Plasma levels of cutaneous T-cell attracting chemokine (CTACK) correlated with plasma levels of (D) sGITR (\( p < 0.01 \) and \( r = 0.34 \), \( n = 78 \)) and (E) GITRL in AD (\( p < 0.01 \) and \( r = 0.38, n = 78 \)).
of the TNF super-family possess the ability to induce bi-directional signalling (16). This means that binding of ligand to receptor is not the only way to induce a signal. Binding of receptor to the membrane bound ligand can also induce a signal through the ligand and thereby lead to activation of cells expressing the ligand.

Evidence that bi-directional signalling is possible for GITR/GITRL is increasing (16). The pro-inflammatory capacity of GITR to stimulate monocytic cells that express membrane-bound GITRL and soluble GITRL ability to inhibit Tregs is a potentially dangerous condition, as this would lead to activation of cells of monocytic linkages and decreased activity of Treg at the same time. Binding of GITR to GITRL in the blood will render both inactive and lead to removal of both these pro-inflammatory peptides. We believe this is the explanation for the close correlation of GITR with GITRL in chronic AD and also for the missing correlation with disease activity.

In summary, the correlation of plasma concentrations between GITRL and GITR and CTACK and TARC suggest that GITRL and GITR function as inflammatory signals in AD, through downregulation of Treg activity or perhaps through a common NF-κB link, since GITRL activates NF-κB, which in turn, among other things, induces expression of CTACK.

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