Progressive divergent shifts in natural and induced T-regulatory cells signify the transition from undifferentiated to definitive connective tissue disease

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Abstract

The objectives of the study is to determine clinical signs and distribution of peripheral T-cell subsets, B cells and T regulatory cells in patients with undifferentiated connective tissue disease (UCTD) and during the development toward well-established connective tissue diseases (CTD). The methods include 46 patients with UCTD were followed and investigated for differentiation into defined CTDs for 2 years. Cell subsets were determined on the basis of cell surface markers, intracellular cytokine production by flow cytometry and serum cytokine levels by ELISA. The results are as follows: 45.6% of UCTD patients developed into a defined CTD. The number and percentage of activated T cells, memory T cells and NKT cells were increased in patients compared with controls. In addition, in patients with UCTD, the percentage of CD4+/IFNγ+ T1,1 was significantly higher compared with controls and further increased in patients that developed CTDs. The percentage and absolute number of CD4+/CD25+/Foxp3+ regulatory T cells (Tregs) were diminished in UCTD patients compared with healthy controls, while the number of CD4+/IL-10+ Tregs increased. The conclusions are: Overproduction of IFNγ and the decrease of natural (Foxp3+) Tregs seem to be characteristic features of UCTD patients. The increased IL-10 production of CD4+ T cells might be a compensatory suppressive mechanism; however, it is probably not able to balance the overproduction of IFNγ and the observed decrease of Foxp3+ Tregs. The shift toward T1,1 with increased IFNγ production in patients with UCTD combined with the degree of immunoregulatory disturbances characterized by the progressive divergent shifts in natural and induced T-regulatory cell populations signify the transition from undifferentiated to definitive CTD.

Introduction

The term undifferentiated connective tissue disease (UCTD) was first used in the 1980s to identify a patient group who were recognized as being in the early stages of a connective tissue disease (CTD) but did not yet meet the standard criteria for a well-defined CTD, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), mixed connective tissue disease (MCTD), Sjögren’s syndrome (SS), systemic sclerosis (SSc) or polymyositis/dermatomyositis (PM/DM) (1–5). The prevalence of UCTD varies from 20 to 52%. Approximately 30% of patients with UCTD show differentiation into well-established CTDs and 50–60% of these UCTD patients remain undifferentiated, while in 10–20% of patients the symptoms subside and never evolve into a well-defined CTD.

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The pathogenesis of UCTD, like many rheumatic diseases, is not well understood. Indeed, there have been no rigorous attempts to define the basic pathomechanisms of the disease. However, the clinical symptoms and the presence of the auto-antibodies in patients with UCTD suggest that many of the same immunological mechanisms that play a role in different well-defined CTDs may also be involved in UCTD. Yet, studies on patients with UCTD have not been performed regarding immunoregulatory abnormalities and how these features contribute to the progression to CTD.

Physiologically, both central and peripheral tolerances are crucial to prevent the activation of self-reactive lymphocytes (6). In addition to central tolerance, self-reactive T cells may also be subjected to peripheral deletion, or anergy, which is characterized as a state of functional inertness toward self-antigens (7, 8). Despite the wide array of tolerance mechanisms, some self-reactive T cells do escape tolerance induction and continue to exist in the periphery. In autoimmune diseases, one or several tolerance mechanisms fail due to the interplay of various environmental factors and specific genes.

The differentiation of CD4+ T cells into two functional subsets, T₄₁ and T₄₂, is regulated by different types of cytokines (9). Antigen-presenting cells produce IL-12 and IFNγ, which perpetuate pro-inflammatory reactions that lead to cell and tissue damage. Contrary, IL-4 promotes the T₄₂ polarization that promotes activation of the humoral immune response (10). Several characteristic cell populations exist, which have the capability to suppress autoimmune reactions once they have developed. One important group of such immunoregulatory cells is denoted as regulatory T cells (Tregs).

Tregs derive either from the thymus [CD4⁺CD25⁺Foxp₃ natural Tregs (nTregs)] or in the peripheral blood [induced Tregs (iTregs)] (11). Interestingly, some of these iTregs include counter-regulatory IFNγ-producing T₄₁ and IL-4-producing T₄₂, IL-10-producing Tr1 cells and transforming growth factor-β-secreting T₄₃ (11). If these populations are either diminished or otherwise functionally inactivated, spontaneous development of various autoimmune diseases may occur. In support of this view, a series of reports describe the selective decrease in the number of Tregs or, alternatively, a diminished function of Tregs in patients with autoimmune diseases such as SLE, RA or MCTD (12–14). This shows clearly that Tregs are important in controlling the immune response.

The aim of the present study was to assess whether abnormalities in the distribution of various immune-competent T-cell populations exist in patients with UCTD with a special emphasis on Tregs and, furthermore, whether certain immune phenotypes in these patients predict the development of subsequent defined CTDs.

**Methods**

A total of 46 consecutive patients (45 females and 1 male) who had been diagnosed with UCTD between 2003 and 2005 were enrolled in this study. The mean age was 40.5 ± 13.1 years, and the mean disease duration was 1.83 ± 0.91 years (range: 0.5–2.5 years). Patients were involved based on the following criteria: symptoms and signs suggestive of a CTD not fitting the accepted classification criteria for any of the defined CTDs and the presence of at least one non-organ-specific auto-antibody.

No patients received corticosteroid or any kind of immunosuppressive therapy. Twenty-one healthy individuals served as controls. Patients with UCTD were followed up every 4 months. Laboratory evaluations were performed at each visit. The routine blood tests were carried out, including erythrocyte sedimentation rate (ESR), complete blood count, renal and liver function tests, creatine phosphokinase determinations and urine analysis.

The diagnoses of specific well-established CTDs, such as SLE, MCTD, SSc, SS, PM/DM, RA and systemic vasculitis, were determined by the recognized corresponding classification criteria (15–22).

**Analysis of lymphocyte sub-populations and activated T cells**

In order to determine lymphocyte sub-populations (T₄₁, T cytotoxic, NKTs, B and NK cells) and activated T cells, the following mAbs against T-cell surface markers were used: CD3, CD4, CD8, CD19 and CD56 (BD Biosciences, San Diego, CA, USA and Immunotech, Marseille, France). The expression of T lymphocyte activation markers such as HLA-DR and CD69 was also determined on CD3⁺ cells (BD Biosciences). Samples were processed according to the Coulter Q-PREP Protocol and System (Beckman Coulter Inc., Miami, FL, USA). Briefly, cells from 100 μl of whole blood were stained with 5–10 μl of mAb. After 30 min of incubation, the RBCs were hemolysed and the leukocytes were washed in PBS supplemented with BSA (10 g l⁻¹) and sodium azide (2 mg l⁻¹). The cells were subsequently fixed by using 800 μl of 1% PFA. Mouse IgG1 antibodies were used as isotype control throughout the experiments. Measurements were performed and events were collected on Coulter EPICS XL-M flow cytometer (Beckman Coulter Inc.). Lymphocytes, monocytes and granulocytes were separated based upon their size and granularity pattern, forward and side scatter scattergram. Lymphocyte sub-populations were quantified as their percentage in the entire population.

**Evaluation of intracellular cytokines**

Evaluation of intracellular cytokines of CD4⁺ T cells was performed as described previously (23). Briefly, 1 ml whole blood was diluted 1:2 in RPMI-1640 supplemented with 80 mg l⁻¹ gentamycin and 2 nM glutamin. Cells were stimulated using 25 ng ml⁻¹ phorbol-myristate-acetate (Sigma Aldrich Corp., St Louis, MO, USA) and 1 ng ml⁻¹ ionomycin (Sigma Aldrich Corp.) for 4 h at 37°C and 5% CO₂. Transport of de novo synthesized cytokines from the Golgi apparatus was inhibited by 10 μg ml⁻¹ brefeldin-A (Sigma Aldrich Corp.). Unstimulated cells served as controls. Following stimulation, cells were stained for CD4 and CD8 antigens for 30 min at room temperature. Cell staining was followed by RBC lysis using FACS Lysing Solution (Becton Dickinson, Mountain View, CA, USA) for 10 min at room temperature in dark. Leukocytes were then centrifuged (500 × g, 10 min) and cell membranes permeabilized using FACS Permeabilizing Solution (BD Biosciences) for 10 min at room temperature. Samples were washed in PBS and were
further incubated for 30 min in the dark with specific mAbs: FITC-labeled anti-human IFNγ, PE-conjugated anti-human IL-4 (BD Biosciences) and PE-conjugated anti-human IL-10 (Caltag Laboratories, Burlingame, CA, USA) for 30 min at room temperature in dark. The cells were fixed using 1% PFA. The samples were evaluated by a Coulter EPICS XL-4 flow cytometer (Beckman Coulter Inc.). Lymphocytes, granulocytes and monocytes were gated and separated based on their morphological properties.

**Determination of CD4+CD25+highFoxp3+ Tregs**

Intracellular Foxp3 staining was carried out on the freshly isolated PBMCs by an Intracellular Staining Kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Lymphocytes were gated on the basis of their forward and side scatter properties. Forty thousand gated events (lymphocytes) were collected in each sample on a FACSCalibur equipment (Becton Dickinson, Heidelberg, Germany). Data were analyzed using CellQuest software (Becton Dickinson). CD4+CD25+high suppressor T cells were also positive for Foxp3. The mean fluorescence intensity of Foxp3 was significantly higher in CD4+CD25+high suppressor T cells compared with the CD4+CD25low or CD4+CD25- cells (P < 0.01). The following reagents were used: Ficoll and CD4–FITC mAb (Sigma Aldrich, Steinheim, Germany), CD25–PC5 (Immunotech), Foxp3–PE (clone: PCH101) and Intracellular Staining Kit (eBioscience).

**Determination of serum soluble cytokines**

Serum IL-4, IL-10 and IFNγ were measured by BD OptEIA ELISA Sets (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions.

**Immunoserological assessment**

The detection of anti-nuclear antibodies (ANAs) on HEp2 cell line was carried out by indirect immunofluorescence.

Serum concentrations of auto-antibodies were analyzed by ELISA [anti-SSA, anti-SSB, anti-Jo1, anti-Scl70, anti-dsDNA and anti-cardiolipin (aCL)] (Cogent Diagnostic, Edinburgh, UK) and U1RNP antibodies (Pharmacia and Upjohn, Freiburg, Germany) according to the manufacturer's instructions. Absorbance values were measured by a Labsystems Multiscan MS ELISA reader at 492 nm. The concentrations of control and patient samples were determined using a standard curve obtained from the optical densities of standards with known concentration. By using this method, serum was considered negative at <5 U ml⁻¹.

Serological studies included rheumatoid factor (RF) test, complement factors (CH50, C3 and C4), gamma globulins, C reactive proteins (CRPs) and serum and urine protein electrophoresis.

Anti-cyclic citrullinated peptide (CCP2) antibody reactivity was tested using commercially available ELISA (EliA™ CCP Assay, Phadia GmbH, Freiburg Germany) automatic analyzer according to the manufacturer’s recommendations. Values of 25.0 U ml⁻¹ or greater were considered to be positive.

Diagnostic procedures for SLE, SSs, MCTD, RA, SS and vasculitis included X-ray, CT and MRI imaging, lung function tests, electromyography and electroneurography, Shirmer’s test, Rose-Bengal staining, sialometry, parotis ultrasound and salivary gland biopsy. All patients with UCTD were investigated by 99mTc-DTPA esophageal transit scintigraphy. The intra-esophageal transport of the radiopharmaceutical was followed, images were captured by gamma camera and a series of 128 × 128 images were stored and evaluated as earlier described.

**Statistical analysis**

Data are presented as mean value ± standard deviation. After testing for normality, data were compared with Student’s t-test (paired and unpaired) or Mann–Whitney U test and correlated with Spearman’s rank correlation. Categorical variables were compared using chi-square test. Individual relative risk and 95% confidence intervals were calculated using separate logistic regression for variables found to be significant or approaching significance in the former analysis. P values less than 0.05 were considered statistically significant.

**Results**

**Progression into established CTDs from UCTD**

The patient population at the first visit was denoted as UCTD1, while UCTD2 refers to those 21 patients who remained in the UCTD stage after the 2-year follow-up period. During the mean 2-year follow-up period, 21 (45.6%) of the 46 UCTD patients progressed into a definitive CTD. Of these patients, 2 (9.5%) developed SLE, 5 (23.8%) RA, 5 (23.8%) SS, 5 (23.8%) MCTD and 4 (19%) in SSc.

**Clinical data and immunoserological investigations**

Clinical and immunoserological data obtained from the 46 patients are summarized in Table 1. At the first visit, the frequencies of the most important symptoms were as follows: Polyarthritis in 36 (78.2%), Raynaud's phenomenon in 29 (63%), keratoconjunctivitis in 18 (39.1%), xerostomia in 14 (30.4%), serositis in 9 (19.5%), skin symptoms in 18 (39%), central nervous system involvement in 7 (15.2%), Raynaud's phenomenon in 5 (10.8%), scieractomy in 8 (17.3%) and esophageal dysmotility was found in 15 (32.6%) of patients.

Furthermore, immunoserological investigations and a wide spectrum of auto-antibodies were investigated. ANA titers were positive in 33 (71.7%), anti-native DNA in 4 (8.6%), Waaler–Rose in 15 (39.1%), anti-beta2GPI IgG in 4 (8.6%), anti-beta2GPI IgM in 2 (6.5%), aCL IgG in 3 (6.5%), aCL IgA in 7 (15.2%), aCL IgM in 9 (19.5%), anti-Sm in 2 (4.3%), anti-U1RNP and anti-CCP in 5 (10.8%), anti-SSA in 10 (20.7%), anti-SSB in 7 (15.2%), elevated CRP level in 24 (52.1%) and elevated ESR in 29 (63%).

**Predictive value of clinical and serological data of subsequent CTD development**

Polyarthritis, xerophtalmia, xerostomia and esophageal dysmotility showed correlation with the progression to CTDs (Table 1). Statistically significant correlation with subsequent development of CTD was determined between the presence of ANA (P = 0.0194), aCL IgA (P = 0.03), anti-U1RNP,...
In order to determine whether immunoregulatory abnormalities exist in connection with UCTD and, furthermore, if such abnormalities might play a role in the progression to definitive CTD, we determined first the percentages of CD3+CD8+ and CD3+/IL-4+ T lymphocytes and CD19+ B lymphocytes in the described patient groups and in healthy controls. No significant differences in these T-cell subsets and CD19+B lymphocytes were discovered between the two UCTD patient groups, patients that developed CTD and healthy controls (Fig. 1A).

Interestingly, the percentages of both CD3+/HLA-DR+ and CD3+/CD69+ activated T-cell subsets were elevated in UCTD1 and UCTD2 patients compared with healthy controls (Fig. 1B), suggestive of an active immune reaction. Moreover, those patients that further progressed into definitive CTD exhibited further elevated CD3+/HLA-DR+ and CD3+/CD69+ expression compared with UCTD1, UCTD2 patients and controls (Fig. 1B). Therefore, a progressive increase in activated T cells correlating with severity of disease could be defined, underscoring the progressive immune component toward the development of CTD.

In contrast, the CD4+/IFNγ+ naive T-cell sub-population was decreased in all patient groups when compared with controls. The representation of CD4+/RO+ memory T cells was similarly increased in all patient groups compared with healthy controls. Moreover, two cell populations with immunoregulatory functions [NK cells (CD3−/CD56+) and NKTs (CD3+/CD56+)] were investigated in our patient cohorts.

The distribution of NK cells was increased in patients with UCTD/CTD compared with controls (control–UCTD1: \( P = 0.001 \); control–UCTD2: \( P = 0.01 \); control–CTD: \( P = 0.01 \) (Fig. 1C). Furthermore, the NKT population was significantly increased in patients compared with controls, and interestingly, the observed increase was more evident in the fully developed CTD patients than in the undifferentiated state (\( P < 0.001 \); when patients and controls were compared) (Fig. 1B). Furthermore, the comparison of NKTs between UCTD and CTD revealed a significant increase (\( P = 0.047 \)) (Fig. 1C). Taken together, these results are underscoring the activated immune phenotype of UCTD/CTD.

**Table 1.** Clinical symptoms, auto-antibody profile of patients with UCTD and predictive factors signifying the development of definitive CTD of patients with UCTD

<table>
<thead>
<tr>
<th>Symptoms of patients with UCTD</th>
<th>UCTD patients n = 46</th>
<th>Differentiation into CTD patients</th>
<th>OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyarthritis</td>
<td>36 (78.2%)</td>
<td>0.0003****</td>
<td>29.1 (1.584–535.76)</td>
</tr>
<tr>
<td>Raynaud’s phenomenon</td>
<td>21 (45.6%)</td>
<td>0.001****</td>
<td>15.429 (0.3.434–69.312)</td>
</tr>
<tr>
<td>Xerophthalmia</td>
<td>18 (39.1%)</td>
<td>0.02**</td>
<td>4.222 (1.196–14.900)</td>
</tr>
<tr>
<td>Xerostomia</td>
<td>14 (30.4%)</td>
<td>0.117</td>
<td>4.773 (1.212–18.787)</td>
</tr>
<tr>
<td>Pleuritis/pericarditis</td>
<td>9 (19.5%)</td>
<td>0.0269</td>
<td>2.933 (0.6327–13.598)</td>
</tr>
<tr>
<td>Skin symptoms (erythema, photosensitivity and telangiectasy)</td>
<td>18 (39%)</td>
<td>0.07</td>
<td>3.46 (0.969–12.402)</td>
</tr>
<tr>
<td>Sclerodactyly</td>
<td>8 (17.3%)</td>
<td>0.117</td>
<td>4.8 (0.817–25.892)</td>
</tr>
<tr>
<td>Dysphagia</td>
<td>8 (17.3%)</td>
<td>0.117</td>
<td>4.6 (0.817–25.892)</td>
</tr>
<tr>
<td>Esophageal hypomotility</td>
<td>15 (32.6%)</td>
<td>0.0001****</td>
<td>18.688 (4.349–101.54)</td>
</tr>
</tbody>
</table>

Auto-antibodies

<table>
<thead>
<tr>
<th>Auto-antibodies</th>
<th>patients</th>
<th>Differentiation into CTD patients</th>
<th>OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA positivity</td>
<td>33 (71.7%)</td>
<td>0.0194***</td>
<td>7.464 (1.423–39.166)</td>
</tr>
<tr>
<td>anti-DNA</td>
<td>4 (8.6%)</td>
<td>0.31</td>
<td>0.13 (0.383–41.7725)</td>
</tr>
<tr>
<td>anti-DNA</td>
<td>3 (6.5%)</td>
<td>0.58</td>
<td>2.526 (0.2125–30.028)</td>
</tr>
<tr>
<td>anti-DNA</td>
<td>7 (15.2%)</td>
<td>0.03*</td>
<td>9.6 (1.049–87.83)</td>
</tr>
<tr>
<td>anti-DNA</td>
<td>9 (19.5%)</td>
<td>0.711</td>
<td>1.6 (0.378–7.116)</td>
</tr>
<tr>
<td>anti-DNA</td>
<td>2 (4.3%)</td>
<td>1.0</td>
<td>1.2 (0.07–20.445)</td>
</tr>
<tr>
<td>anti-DNA</td>
<td>8 (17.3%)</td>
<td>0.0161***</td>
<td>12.0 (1.334–107.98)</td>
</tr>
<tr>
<td>anti-DNA</td>
<td>5 (10.8%)</td>
<td>0.0148***</td>
<td>17.0 (0.879–328.48)</td>
</tr>
<tr>
<td>anti-DNA</td>
<td>10 (20.7%)</td>
<td>0.02**</td>
<td>7.077 (0.1.302–38.453)</td>
</tr>
<tr>
<td>anti-DNA</td>
<td>7 (15.2%)</td>
<td>0.036</td>
<td>9.6 (1.049–87.832)</td>
</tr>
<tr>
<td>anti-DNA</td>
<td>15 (32.6%)</td>
<td>0.01</td>
<td>5.775 (1.467–22.732)</td>
</tr>
<tr>
<td>anti-DNA</td>
<td>24 (52.17%)</td>
<td>0.0037****</td>
<td>6.8 (1.835–25.195)</td>
</tr>
</tbody>
</table>

* Denotes significance: \(*P < 0.05, **P < 0.02, ***P < 0.01, ****P < 0.001\).
CTD stages (control–UCTD1: \( P < 0.0001 \); control–UCTD2: \( P < 0.0001 \); control–CTD: \( P < 0.0001 \) and UCTD2–CTD: \( P < 0.0001 \)). In summary, serum cytokine levels of IFN\(_c\)/IL-4 and Th1/Th2 CD4\(^+\) T-cell levels were corresponding and pointed to a shift in T-cell polarization toward Th1. This shift could be due to the disruption of immunological tolerance or the intrinsic defect of T cells and eventually both processes could lead to Th1 dominance.

Circulating Tregs in UCTD patients

Treg populations (nTregs, CD4+CD25\(^{high}\)Foxp3\(^{+}\) and iTregs, such as CD4+IL-10+ Tr1 cells) have come to be appreciated as potent and important mediators of immune regulation. Specifically, Tregs have been shown to play important roles in immunological tolerance and when diminished or rendered dysfunctional may result in several autoimmune features (24, 25). It was therefore of interest to determine the frequency of these Treg populations in order to investigate the possible role on UCTD and progression toward CTD. The percentage of (CD4+CD25\(^{high}\)Foxp3\(^{+}\)) nTregs was significantly lower in peripheral blood of patients with UCTD and with CTD compared with healthy controls (UCTD1: \( P = 0.0044 \); UCTD2: \( P = 0.0014 \); CTD: \( P < 0.0001 \)) (Fig. 3A). The comparison of nTregs between UCTD1 and 2 with CTD also showed a significant decrease in CTDs (UCTD1–CTD: \( P < 0.0001 \); UCTD2–CTD: \( P < 0.0001 \)). Furthermore, the absolute number of nTregs was similarly significantly decreased in UCTD1 (\( P = 0.04 \)) and UCTD2 (\( P = 0.0048 \)) patients compared with controls and further diminished in patients with CTD (\( P = 0.01 \)) (Fig. 3B). Taken together, a progressive decrease in nTreg from healthy controls to UCTD and CTD was observed, suggesting a role for nTregs in the pathogenesis of UCTD and CTD.

Interestingly, an opposite trend was discovered for the IL-10-producing CD4+ Tr1 Tregs. An increase in percentage and absolute cell numbers in patients with UCTD and CTD was observed when compared with controls (Fig. 3A and B). The percentage of Tr1 cells was significantly higher in peripheral blood of patients with UCTD and with CTD compared with healthy controls (UCTD1: \( P = 0.0032 \); UCTD2: \( P < 0.0001 \); CTD: \( P < 0.0001 \)) (Fig. 3A). The comparison of Tr1 cells between CTD and UCTD1 (\( P = 0.00054 \)) and UCTD2 (\( P = 0.0054 \)) further showed a significant increase of Tr1 cells in CTD patients. Moreover, the absolute number of Tr1 cells was similarly significantly increased in UCTD1 and UCTD2 patients and further elevated in patients with CTD (Fig. 3B). When UCTD1 and UCTD2 patients were compared with each other, we found a significant decrease of Foxp3+ Treg absolute numbers (\( P = 0.046 \)) (Fig. 3B). When UCTD patients were compared with fully developed CTD,
an additional increase in IL-10+ Tr1 cells was discovered. These data emphasize the involvement of Treg populations in UCTD and in progression to CTDs.

Finally, we evaluated the distribution of Treg subsets in those patients that progressed into a definitive CTD with respect to the specific disease outcome. Interestingly, we found that from the well-established CTDs, SLE and MCTD patients in our CTD cohort exhibited the most pronounced divergent nTreg/Tr1 shift, although it was also apparent in the rest of the diseases (Fig. 3C).

Discussion

In the last 10 years, several papers have clarified the clinical symptoms characteristic for UCTD. The aim of the present study was to characterize the immunoregulatory malfunctions of patients with UCTD and to pinpoint those abnormalities, which can predict the development of subsequent definitive CTDs.

Concerning the progression of UCTD to definitive CTDs, the follow-up studies on patients with UCTD showed that about half of the patients did not develop further from the UCTD stage, approximately 10–20% of the patients went into remission, while another third progressed toward a definitive CTD (26, 27). In a previous study, we followed up 665 patients with UCTD during a 5-year period (26). The most frequent clinical manifestations of UCTD were arthritis/arthralgia, rashes, usually on the face, Raynaud’s phenomenon, xerostomia/xerophthalmia, fever and sometimes recurrent pleuritis/pericarditis, while the patients’ sera contained at least one auto-antibody to cytoplasmatic or nuclear cell components. In our current study, during the mean 2-year follow-up period, 45.6% of the UCTD patients progressed into a definitive CTD. Of these patients, 23.8% evolved into RA, 23.8% into SS, 23.8% into MCTD, while 19% developed SSc and 9.5% SLE. These findings were in line with our previous study on patients with UCTD (26).

The presence of anti-dsDNA antibodies, anti-phospholipid antibodies and the clinical manifestations such as serositis and photosensitivity rash were predictive for progression to SLE. Xerostomia or xerophthalmia as well as anti-SSA or anti-SSB antibodies were strongly associated with progression to SS. Raynaud’s phenomenon, sclerodactyly and nucleolar ANA were correlated with the evolution to SSc. Furthermore, polyarthritis and swollen hands and fingers along with anti-U1RNP auto-antibodies had a strong predictive value for the subsequent development to MCTD, while polyarthritis and morning stiffness with elevated levels of RF were a compelling predictor of subsequent RA development. Using the purified and recombinant human nuclear antigens, dsDNA, U1RNP, RNP/Sm, SSA, SSB, Scl-70, Jo1, centromer, PM/Scl and new antigens, such as CCP, helps to distinguish the auto-antibody specificities in the initial stage of various systemic autoimmune diseases. Recent findings support the idea that anti-CCP antibodies may serve as a powerful serological marker for early diagnosis of RA and prognostic
prediction of joint destruction (28). In this study, five patients with UCTD were positive for anti-CCP antibodies. Each patient with anti-CCP antibodies progressed into RA during the 2-year follow-up period. The presence of RF in our patients' sera was also predictive of differentiation into CTD; however, RF was found not only in patients with RA but also in patients with other CTDs as well. At the first visit in the UCTD stage, 15 patients were RF positive, while 2 years after the initial RF-positive patients, 5 patients remained UCTD, 3 patients developed into RA, 4 patients progressed into MCTD and 3 patients developed SS. Previous studies concluded that the sensitivity of RF is 60–80% for RA, while the specificity is low, since RF is also detected widely and frequently in many other conditions, such as infectious diseases (29).

The pathogenesis of UCTD has not been extensively investigated previously. Indeed, there have been no rigorous attempts to define the basic pathogenic events of this condition. It is presumed that many of the same immunological mechanisms that play a role in definite CTDs may be involved at a lower and less severe level. Since UCTD patients have similar symptoms as patients with well-established CTDs and the patients develop auto-antibodies to nuclear or cytoplasmic components as well, it is likely that these patients have immunoregulatory abnormalities.

In our study, no differences in the number of T cells (CD3+), T-cell subsets (CD4+ and CD8+) and B cells between UCTD patients and healthy controls were detected, whereas the frequencies of naive and memory T cells differed in patients with UCTD compared with controls. An increase in CD4+ memory and a decrease of naive CD4+ T cells were detected in patients with UCTD, clearly pointing to an ongoing active immune reaction.

NKTs are a heterogeneous T-cell population that co-express TCR and NK cell-related surface markers, including NKR-P1A (CD161), CD56, CD57 and CD122 (NK.1.1) (30, 31). NKTs rapidly express a wide array of Th1 (IFN-γ) and TNF-α and Th2 (IL-4) cytokines, which can significantly modulate Th1 and Th2 differentiation (32). Interestingly, in our UCTD patients, the NKT number was elevated compared with healthy controls. NK cells are important members of innate immunity, which can directly kill the target T cells, as well as mediating antibody-dependent cytotoxicity via membrane receptors binding to the Fc receptor (FcγRIII, CD16) of IgG antibodies (33). We show here that the level of NK cells is increased in patients with UCTD. Taken together, an increase in the Th1 numbers and the overproduction of IFN-γ possibly by Th1-polarized T cells and NK cell and NKT populations in the circulation of patients with UCTD could explain the elevated pro-inflammatory cytokine levels. This may

![Fig. 3.](https://academic.oup.com/intimm/article-abstract/20/8/971/665350)
contribute to sustained cell and tissue damage and progression into different types of CTDs.

In our patients at the point of the diagnosis of UCTD, the relative and absolute number of nTregs was found to be decreased compared with controls; moreover, in the 21 patients who developed definitive CTDs, the number of nTregs was further decreased. Statistically, we found a significant decrease of Foxp3+ Treg absolute numbers when UCTD1 (CTD non-developing) and UCTD2 (CTD developing) patients were compared. Based on these findings, we presume that by evaluating Foxp3+ Treg numbers in UCTD patients, in those, having lower numbers are likely to be more prone to develop subsequently toward a definitive systemic autoimmune disease. Interestingly, in line with our findings, in the active stage of MCTD, the frequency of CD4+CD25^high Tregs was found to be further decreased compared with inactive stages of disease (12). Furthermore, in support of our data, decreased levels of nTregs have been associated with different autoimmune disorders and reduced levels of CD4+CD25^high suppressor cells in the peripheral blood of patients with SLE have been described (14). Additionally, reduced number of nTregs during active SLE in human pediatric patients has been demonstrated (34). In accordance with these findings, when the proportions of nTregs in our CTD patient cohort were analyzed, a clear trend was detected showing the lowest levels of this cell population in patients with SLE and MCTD. Although it is important to mention that based on the low number of developed definite CTD patients in this aspect, the drawn conclusions show only tendencies and outline the developmental propensities of UCTDs to CTDs. Besides, in another study, the number of CD4+CD25^high cells was reduced in patients with myasthenia gravis but elevated in the inflamed synovium in patients with RA (35). Our study showed that RA patients did not exhibit severe reduction of nTregs, although an increase could not be determined. This slight discrepancy could be due to the low number of RA patients within CTD patients and could also be explained by disease heterogeneity and the fact that the two patient cohorts studied were of different origins. However, it is an interesting point that RA patients in our cohort showed the least significant reduction of nTregs of the individual CTD patient groups.

Another Treg population shown to secrete the regulatory cytokine IL-10 are designated as Tr1 Tregs, which have been implicated in various autoimmune diseases (36). IL-10 is a multifunctional cytokine that can suppress IFNγ production by Th1 and regulate growth and/or differentiation of B cells, NK cells, cytotoxic T cell and Th1 (37, 38). Interestingly, the percentage and absolute number of Tr1 cells in our patient cohort showed an opposite trend compared with the findings for nTregs. A significant increase in Tr1 regulatory cells was determined when UCTD patients were compared with healthy controls, and a further increase in patients progressing into definitive CTDs were established. Interestingly, patients that developed SLE and MCTD exhibited the most pronounced low nTreg/high iTreg (Tr1) feature.

In summary, these findings give insight into the progressive decrease in levels of nTregs, while IL-10-producing Tr1 levels are similarly progressively elevated in UCTD and CTD. Studies have shown that IFNγ- or IL-10-producing CD4+ T cells specific for the major RBC auto-antigen, RhD, were both found in the peripheral blood of patients suffering from autoimmune hemolytic anemia (39), indicating that, although present, Tr1 cells do not have the ability to fully inhibit the progression of autoimmune disease. We therefore suggest that the IL-10 overproduction by Tr1 cells may be a compensatory mechanism; however, this may not be sufficient to down-modulate the effects of the observed IFNγ overproduction, and subsequently, this may lead to tissue damage and development of definitive CTDs. The observed change in the T1/T2 rate toward T1 with increased IFNγ production in patients with UCTD, combined with the degree of immunoregulatory disturbances, might determine whether the disease process remains in the UCTD stage or progress into CTD.

In this study, we have assessed the peripheral blood T-cell sub-populations, the characteristic cytokine production and investigated the basal regulatory disorders in patients with UCTD and followed the progression into definitive CTDs. We determined that CTDs are characterized by elevated levels of Tr1 and reduced nTreg populations, which suggests that in the development of these diseases a disproportional distribution of these regulatory type T cells contribute to the pathogenesis. We believe that these findings can contribute to subsequent diagnostics and hopefully therapeutic innovations in the future management of these autoimmune diseases.

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Abbreviations
aCL anti-cardiolipin
ANA anti-nuclear antibody
CCP cyclic citrullinated peptide
CRP C reactive protein
CTD connective tissue diseases
ESR erythrocyte sedimentation rate
iTreg induced Treg
MCTD mixed connective tissue disease
NKT NK T cell
nTreg natural Treg
PM/DM polymyositis/dermatomyositis
RA rheumatoid arthritis
RF rheumatoid factor
SLE systemic lupus erythematosus
SS Sjögren’s syndrome
SSc systemic sclerosis
Treg regulatory T cell
UCTD undifferentiated connective tissue disease
U1RNP U1 ribonucleoprotein

References


