

# CD28 negative T cells are enriched in granulomatous lesions of the respiratory tract in Wegener's granulomatosis

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## Abstract

**Background**—Lack of CD28 expression on peripheral blood CD4+ and CD8+ T cells has been reported in patients with Wegener's granulomatosis (WG), suggesting a pathogenetic role of CD28- T cells in WG. **Methods**—Ten patients with WG and six with sarcoidosis (disease control) were analysed. Fluorescence activated cell sorter (FACS) analysis was used to detect CD28 expression on T cells from peripheral venous blood and from bronchoalveolar lavage (BAL) fluid. T cells in biopsy specimens from granulomatous lesions of the upper respiratory tract were analysed for CD28 expression by double immunofluorescence staining.

**Results**—A significantly higher fraction of CD28- T cells was found in the CD4+ and CD8+ T cell compartment in BAL fluid (65.6 (5.4)% and 76.3 (4.1)%, respectively) than in blood (13.4 (6.2)% and 42.9 (6.2)%;  $p < 0.001$ ) in patients with WG but not in those with acute sarcoidosis (6.7 (2.2)% and 53.4 (7.3)% in BAL fluid *v* 4.1 (2.5)% and 52.0 (9.4)% in blood). The total number of CD4+/CD28- T cells but not of CD8+/CD28- T cells was also significantly higher in BAL fluid than in blood in patients with WG ( $p < 0.05$ ). Patients with WG had a significantly higher fraction of CD28- T cells in the CD4+ and CD8+ T cell compartment in BAL fluid than patients with acute sarcoidosis (65.6 (5.4)% *v* 6.7 (2.2)%;  $p < 0.001$ ; and 76.3 (4.1)% *v* 53.4 (7.3)%;  $p < 0.05$ ). The total number of CD4+/CD28- and CD8+/CD28- T cells was also significantly higher in patients with WG than in those with sarcoidosis ( $p < 0.01$ ). An abundance of CD28- T cells was found in granulomatous lesions by double immunofluorescence staining in patients with WG.

**Conclusions**—Our data indicate enrichment of CD28- T cells in BAL fluid and suggest recruitment of CD28- T cells into granulomatous lesions in WG. Further analysis of the phenotype and function of T cell subsets in WG is needed to better understand leucocyte homing in WG and to find new therapeutic targets.

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**Keywords:** Wegener's granulomatosis; sarcoidosis; CD28

Several recent reports have supported the concept that T cells play an important role in Wegener's granulomatosis (WG).<sup>1</sup> Moreover, recruitment of specialised functional T cell subsets into areas of inflammation has recently been reported in animal models and some human diseases.<sup>2,3</sup> Granulomatous lesions in WG are made up of CD4+ T cells, monocyte derived tissue macrophages, giant cells, and neutrophils. An increased production and release of interferon (IFN)- $\gamma$  by activated CD4+ T cells from granulomatous lesions of the respiratory tract and peripheral blood characterises a predominantly Th1-like response in WG.<sup>4-6</sup> High numbers of IFN- $\gamma$  staining cells and CD4+/CD26+ (CD26 is an optional Th1 marker) double positive T cells are seen in granulomatous lesions of the upper respiratory tract in patients with WG.<sup>7</sup> Lack of CD28 expression on peripheral blood CD4+ and CD8+ T cells has been reported in WG.<sup>8-10</sup> Expansion of the CD28- T cell fraction correlates with a high cumulative number of involved organs—that is, a high disease extension index (DEI),<sup>8</sup> suggesting a pathogenetic role of the CD28- fraction of CD4+ and CD8+ T cells in WG.

The present study was designed to investigate whether CD4+/CD28- and CD8+/CD28- T cells are enriched in granulomatous lesions of the respiratory tract in WG. Phenotypic characterisation of T cells homing into granulomatous lesions in WG would improve our understanding of the pathogenesis. Subsequent studies could address the functional role of T cells homing into granulomatous lesions in WG. Tissue homing T cell subsets may become targets of better directed, less toxic future treatments. Lack of animal models ideally reflecting WG has prevented in vivo studies on lymphocyte homing in this condition to date.

We analysed CD28 surface expression of T cells from peripheral venous blood, bronchoalveolar lavage (BAL) fluid, and in biopsy specimens from the upper respiratory tract. Lack of CD28 expression on T cells found in BAL fluid and in granulomatous lesions may suggest recruitment of these cells from blood to sites of inflammation in WG. Patients with acute sarcoidosis served as disease controls in order to identify potential differences in the recruitment of this T cell subset between these two granulomatous diseases. As granulomatous diseases may be found in the presence of Th1 as well as Th2 responses, acute sarcoidosis was chosen as the disease control because it

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is a granulomatous disease with a predominantly Th1-like response similar to the cytokine response found in WG.<sup>11</sup> Despite similarities in the cytokine response in WG and acute sarcoidosis, the natural course, response to treatment and outcome suggest that there are fundamental differences between the diseases which may be related to differences in the nature of the cells recruited into the inflammatory lesions.<sup>12</sup>

## Methods

### CHARACTERISTICS OF STUDY POPULATION

Ten patients with WG were studied, all of whom met the criteria of the American College of Rheumatology<sup>13</sup> and the Chapel Hill Consensus Conference definition for WG.<sup>14</sup> The disease extension and vasculitis activity were described by the DEI and Birmingham vasculitis activity score (BVAS) as outlined elsewhere.<sup>15, 16</sup> In brief, the DEI is the equivalent of current organ involvement in WG,<sup>15</sup> whereas the BVAS considers clinical features and laboratory data to give a measure of vasculitis activity.<sup>16</sup> Partial remission of WG was defined as improvement in vasculitis activity, although clinical or serological signs of inflammation were still detectable. Respiratory tract involvement was diagnosed when pulmonary infiltrates were seen on the chest radiograph, an increase in neutrophils and/or lymphocytes was found in the BAL fluid, and transbronchial biopsy specimens and/or biopsy specimens from the nasal mucosa supported the diagnosis.

Six patients with acute sarcoidosis served as disease controls. The term "acute" sarcoidosis refers to the rapid onset of disease. Löfgren's syndrome is one well known form of acute sarcoidosis with a classical triad of active disease manifestations (bilateral hilar lymphadenopathy (BHL), oligoarthritis, and erythema nodosum). Chronic sarcoidosis is the insidious form of the disease in which a Th2 cytokine pattern may be seen.<sup>11</sup> Patients with chronic sarcoidosis were not therefore used as disease controls. The diagnosis of sarcoidosis was based on clinical symptoms such as new or progressive dyspnoea, cough, active uveitis, new radiographic abnormalities on the chest radiograph, an increase in lymphocytes in BAL fluid with an increased CD4/CD8 ratio of lymphocytes and raised levels of serum angiotensin converting enzyme. The diagnosis was supported by transbronchial lung biopsy specimens and biopsy specimens from other affected organs, in line with recently published guidelines.<sup>17</sup>

The fraction of CD28<sup>+</sup> T cells in blood was also determined in 15 healthy controls for comparison. The erythrocyte sedimentation rate (ESR, normal range <10 mm/h), C reactive protein (CRP, <5 mg/l), complete blood count (including leucocytes: 4–10 × 10<sup>3</sup>/mm<sup>3</sup>, thrombocytes 150–300 × 10<sup>3</sup>/mm<sup>3</sup>), and serum creatinine levels (6–12 mg/l) were routinely determined. Normal values for other routinely determined parameters are: CD4/CD8 ratio in blood, 0.6–3.2; BAL fluid leucocyte count, <20 × 10<sup>6</sup>; BAL fluid lymphocyte

count, <15%, <3 × 10<sup>6</sup>; CD4/CD8 ratio in BAL fluid, 1–3. All patients provided written informed consent.

### BRONCHOSCOPY AND BAL CELL PREPARATION

Fibreoptic bronchoscopy was performed as described previously.<sup>18</sup> In brief, local anaesthesia with lidocaine and premedication with atropine and pethidine were given prior to the procedure. BAL was preferentially performed in radiographical abnormal lung segments in the patients with WG and in the middle lobe or the lingula in patients with sarcoidosis. Twelve fractions of 20 ml sterile saline 0.9% were instilled and aspirated in a single segment. The fluid recovery was at least 60% and all materials were placed immediately on ice. The first two fractions were separated and the third to 12th fractions were pooled and further analysed. Aliquots of the material were routinely examined for conventional bacterial pathogens, acid fast bacteria, *Legionella* species, *Chlamydia* species, *Mycoplasma* species, and *Pneumocystis carinii*. Any infected material was omitted from further study.

The lavage material was filtered through surgical gauze and centrifuged at 4°C. The cell pellet was resuspended in RPMI 1640 medium (GIBCO, Eggenstein, Germany) containing 0.01% human serum albumin (Sigma, München, Germany) to a density of 10<sup>6</sup> cells/ml.

### ANTIBODIES, REAGENTS AND FACTS

#### IMMUNOPHENOTYPING

Antibodies for FACS analysis were fluorescein (FITC) conjugated anti-CD4 and FITC conjugated anti-CD8, phycoerythrin (PE) conjugated anti-CD28, and peridin-chlorophyll (PerCP) conjugated anti-CD45 which were obtained from Becton-Dickinson (Heidelberg, Germany). FITC conjugated IgG1 and PE conjugated IgG2a (also from Becton-Dickinson) were used for negative controls. Antibodies for immunohistochemistry used in this study were rabbit anti-CD3 antiserum and APAAP-complex from Dako (Glostrup, Denmark), monoclonal mouse anti-CD28 antibody (Biosource International, Nivelles, Belgium), anti-CD8 antibody (clone Leu2a, BD Biosciences, Heidelberg, Germany), Cy-3 conjugated goat anti-mouse IgG, and FITC conjugated donkey anti-rabbit IgG from Dianova (Hamburg, Germany).

Identical double staining protocols were used for BAL cells and heparinised peripheral venous blood. 1 × 10<sup>6</sup> cells per sample were incubated on ice with previously determined optimal concentrations of FITC or PE labelled antibodies for 30 minutes. After staining the cells were washed and fixed with PBS containing 1.5% paraformaldehyde. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton-Dickinson). Data were acquired with CELL-QUEST software (Becton-Dickinson). Lymphocytes were gated for analysis based on light scattering properties and on CD45 and CD4 or CD8 staining. Data on 1.5 × 10<sup>3</sup> lymphocytes were collected. Positively

and negatively stained populations were calculated by quadrant dot plot analysis determined by isotype controls.

#### IMMUNOHISTOLOGY

Nasal biopsy specimens of patients with WG obtained for diagnostic purposes were submerged in 0.9% NaCl and snap frozen in liquid nitrogen and stored until use at  $-80^{\circ}\text{C}$ . Cryostat frozen sections were fixed in acetone for 30 minutes, followed by fixation in chloroform for 30 minutes. Incubation with the primary antibody was performed for 30 minutes and immunostaining was undertaken according to the APAAP (alkaline phosphatase anti alkaline phosphatase) method with New Fuchsin development. Finally, slides were counterstained with haematoxylin and mounted. Immunostaining was controlled by implementing the secondary reagents alone in order to confirm specificity or enzyme development alone to rule out endogenous enzyme activities. Double immunofluorescence staining was performed after a blocking step with 10% bovine serum albumin in Tris buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) with the primary antibodies for 30 minutes. After washing in TBS the secondary antibodies were added and incubated for 30 minutes. Specimens were mounted in DABCO anti-fading solution (2.5% DABCO (1,4-diazabicyclo(2,2,2)-octane) in 90% glycerol, pH 8.6) and viewed under a fluorescence microscope. Stainings were controlled by using only the secondary antibodies to detect non-specific and background fluorescence. Images were obtained using a Leica TCS SP confocal microscope (Bensheim, Germany). The single fluorescence intensities were adjusted using identically stained tonsillar tissue as a measure. Subsequent images of patient tissues stained under the same procedure were acquired using the same instrument settings as for tonsil tissue.

#### STATISTICAL ANALYSIS

Data are expressed as mean (SE). Data referring to CD4+/CD28- and CD8+/CD28- T cells indicate the fraction of CD28- cells of the respective CD4+ and CD8+ T cell subsets stained for CD28 expression. We also determined total numbers of leucocytes, lymphocytes, CD4+ T cells, CD8+ T cells, CD4/CD8 ratio, and CD4+/CD28- and CD8+/CD28- T cells to test the possibility that the percentage of CD4+/CD28- and CD8+/CD28- T cells in a compartment (blood, BAL fluid) may be different in WG and sarcoidosis, whereas the total number of recruited CD28- T cells may be similar because of different CD4/CD8 ratios in blood or BAL fluid in the two diseases.

The non-parametric Mann-Whitney U test was used to compare CD4+/CD28- and CD8+/CD28- T cell populations in patients with WG and sarcoidosis. *p* values of <0.05 were considered significant. Spearman rank order correlation coefficients were determined to assess associations of clinical and laboratory

parameters. Analysis was performed applying SPSS software package.

## Results

#### STUDY POPULATION

Ten patients with WG were studied. One patient had newly diagnosed WG, seven had suffered a relapse of their disease, and two were in partial remission. Thus, eight patients had active disease and in two patients WG activity was still not under full control. Immunosuppressive therapy was ineffective and was intensified subsequently in the seven patients with relapse. Not surprisingly, we found a DEI of 4.7 (0.4) and BVAS of 15.1 (1.4), indicating substantial disease activity. All patients with WG were c-ANCA positive. Eight patients had upper and lower respiratory tract involvement; in two the respiratory tract involvement was restricted to the lower tract at the time of the study. Six patients with acute sarcoidosis served as disease controls. Five patients presented with classical Löfgren's syndrome (BHL, oligoarthritis, and erythema nodosum) and one had an incomplete Löfgren's syndrome (BHL, oligoarthritis) and heart involvement. Pretreatment consisted of non-steroidal anti-inflammatory drugs in two patients.

There was no statistically significant difference between patients with WG and sarcoidosis with regard to disease duration (3.5 (1.1) *v* 0.8 (0.5) years), ESR (48 (10) *v* 63 (14) mm/h), CRP (20 (8) *v* 51 (16) mg/l), blood leucocyte count (5.9 (0.4) *v* 6.0 (0.5)  $\times 10^3/\text{mm}^3$ ), blood lymphocyte count (0.9 (0.1) *v* 1.1 (0.05)  $\times 10^3/\text{mm}^3$ ), CD4/CD8 ratio in blood (1.3 (0.4) *v* 2.1 (0.3)), BAL fluid leucocyte count (26.6 (3.8) *v* 45.4 (12.9)  $\times 10^6$ ), and BAL fluid lymphocyte count (7.8 (3.1) *v* 16.1 (5.1)  $\times 10^6$ ). Age (63 (4) *v* 44 (6) years; *p*<0.05), thrombocyte count (263 (21) *v* 451 (86)  $\times 10^3/\text{mm}^3$ ; *p*<0.05), creatinine level (15 (3) *v* 8 (1) mg/l; *p*<0.05), and CD4/CD8 ratio in BAL fluid (5.1 (1.7) *v* 18.8 (6.7); *p*<0.05) were significantly different between patients with WG and those with sarcoidosis.

To address the question whether treatment related effects could influence the phenotype of T cells, patients with different treatments were compared. As the number of patients was small, cyclophosphamide was compared with other treatments. No statistically significant differences were seen for the aforementioned laboratory values and the frequencies (percentage and absolute numbers) of CD4+/CD28- and CD8+/CD28- T cells between different treatment modalities (cyclophosphamide *v* methotrexate or azathioprine) in our patients with WG.

#### FREQUENCY OF CD4+/CD28- AND CD8+/CD28- T CELLS IN PERIPHERAL BLOOD AND BAL FLUID IN WG AND SARCOIDOSIS

The fraction of CD4+/CD28- T cells in peripheral blood (13.4 (6.2)%) was significantly different from the fraction of CD4+/CD28- T cells in BAL fluid (65.6 (5.4)%) in patients with WG (*p*<0.001). The fraction of CD8+/CD28- T cells in peripheral blood (42.9 (6.2)%) was also significantly different

Table 1 Mean (SE) data with 95% confidence intervals (CI) of study subjects

Parameter	WG		Sarcoidosis		p Value <sup>f</sup>
	Mean (SE)	95% CI	Mean (SE)	95% CI	
Age (years)	63 (4)	54 to 71	44 (6)	27 to 60	<0.05
Disease duration (years)	3.5 (1.1)	0.9 to 6.1	0.8 (0.5)	0.1 to 2.1	NS
Current DEI	4.7 (0.4)	3.5 to 5.8			
BVAS	15.1 (1.4)	8.8 to 12.2			
ESR (mm/h)	48 (10)	26 to 69	63 (14)	28 to 97	NS
CRP (mg/l)	20 (8)	2 to 39	51 (16)	12 to 89	NS
Leucocyte count ( $\times 10^3/\text{mm}^3$ )	5.9 (0.4)	4.9 to 6.9	6.0 (0.5)	4.4 to 7.5	NS
Thrombocyte count ( $\times 10^3/\text{mm}^3$ )	263 (21)	224 to 300	451 (86)	280 to 622	<0.05
Creatinine (mg/l)	15 (3)	7 to 22	8 (1)	6 to 9	<0.05
CD4+/CD28- blood (%)	13.4 (6.2) <sup>‡</sup>	0.5 to 27.4	4.1 (2.5)	1.8 to 10.2	NS
CD4+/CD28- BAL (%)	65.6 (5.4) <sup>‡</sup>	53.4 to 77.8	6.7 (2.2)	1.4 to 12.2	<0.001
CD8+/CD28- blood (%)	42.9 (6.2) <sup>‡</sup>	28.9 to 56.9	52.0 (9.4)	29.1 to 74.9	NS
CD8+/CD28- BAL (%)	76.3 (4.1) <sup>‡</sup>	67.1 to 85.5	53.4 (7.3)	35.5 to 68.2	<0.05

WG = Wegener's granulomatosis; DEI = disease extension index<sup>15</sup>; BVAS = Birmingham vasculitis activity score<sup>16</sup>; ESR = erythrocyte sedimentation rate; CRP = C reactive protein; BAL = bronchoalveolar lavage; NS = no significant difference.

The fraction of CD28- T cells within the CD4+ and CD8+ T cell populations is indicated by CD4+/CD28- and CD8+/CD28-.<sup>†</sup>p value indicates statistically significant differences between WG and sarcoidosis.

<sup>‡</sup>Frequencies of CD4+/CD28- and CD8+/CD28- T cells from peripheral venous blood and BAL fluid were also significantly different in WG (p<0.001 and p<0.01) but not in acute sarcoidosis.

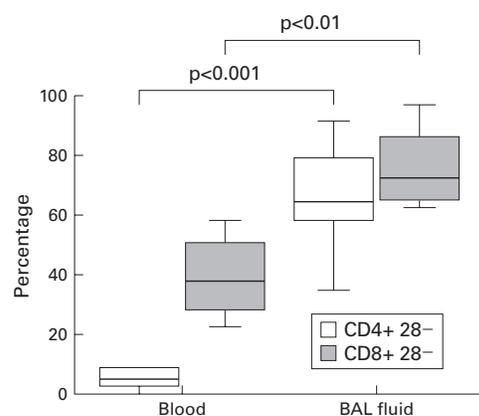


Figure 1 Fraction of CD28- T cells in the CD4+ and CD8+ T cell compartment in bronchoalveolar lavage (BAL) fluid and blood of patients with Wegener's granulomatosis.

from the fraction of CD8+/CD28- T cells in BAL fluid (76.3 (4.1)%) in patients with WG (p<0.01). The total number of CD4+/CD28- T cells in peripheral blood was also significantly different from the number of CD4+/CD28- T cells in BAL fluid (p<0.01), whereas the total number of CD8+/CD28- T cells in peripheral blood was not significantly different from the number of CD8+/CD28- T cells (table 1, fig 1).

There was no statistically significant difference between the fraction of CD4+/CD28- T cells (4.1 (2.5)% v 6.7 (2.2)%) and the fraction of CD8+/CD28- T cells (52.0 (9.4)% v 53.4 (7.3)%) in peripheral blood compared with BAL fluid in patients with sarcoidosis. The total numbers of CD4+/CD28- T cells and CD8+/CD28- T cells in peripheral blood and BAL fluid were also not significantly different in this group of patients.

The fraction of CD4+/CD28- T cells and CD8+/CD28- T cells was 2.1 (1.8)% and 28.5 (6.0)%, respectively, in healthy controls. Thus, CD4+/CD28- T cells and CD8+/CD28- T cells were more prevalent in peripheral blood in patients with WG than in healthy controls (p<0.05). CD8+/CD28- T cells were also more prevalent in peripheral blood in patients

with acute sarcoidosis than in healthy controls (p<0.05), whereas CD4+/CD28- T cells were not.

#### COMPARISON OF FREQUENCIES OF CD4+/CD28- AND CD8+/CD28- T CELLS BETWEEN WG AND SARCOIDOSIS

In peripheral blood the fraction of CD4+/CD28- T cells was 13.4 (6.2)% in patients with WG compared with 4.1 (2.5)% in those with sarcoidosis, while that of CD8+/CD28- T cells was 42.9 (6.2)% in patients with WG and 52.0 (9.4)% in patients with sarcoidosis. There was no statistically significant difference between the percentage of CD4+/CD28- and CD8+/CD28- T cells in the peripheral blood of patients with WG and sarcoidosis. Total numbers of CD4+/CD28- and CD8+/CD28- T cells in the peripheral blood of patients with WG and sarcoidosis were also not different.

The fraction of CD4+/CD28- T cells in BAL fluid was 65.6 (5.4)% in patients with WG and 6.7 (2.2)% in those with sarcoidosis. The fractions of CD4+/CD28- T cells in BAL fluid differed significantly between patients with WG and those with sarcoidosis (p<0.001). The fractions of CD8+/CD28- T cells were also significantly different between patients with WG (76.3 (4.1)%) and those with

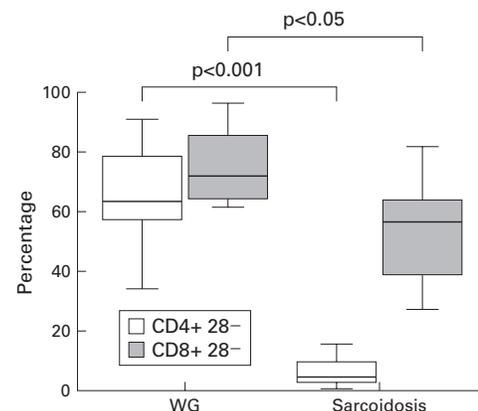
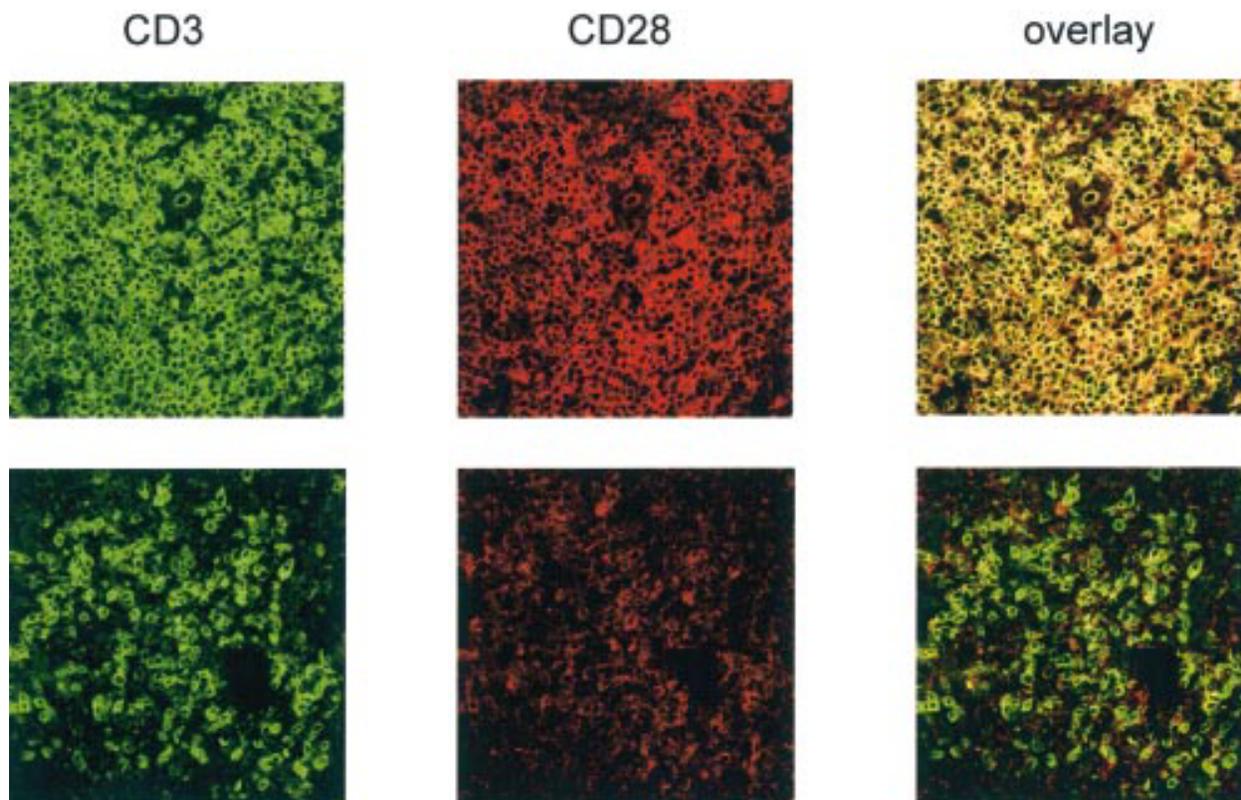


Figure 2 Fraction of CD28- T cells in the CD4+ and CD8+ T cell compartment in bronchoalveolar lavage (BAL) fluid of patients with Wegener's granulomatosis (WG) compared with patients with acute sarcoidosis.



**Figure 3** Tonsillar tissue (top row) from patients without Wegener's granulomatosis (WG) undergoing surgery and tissue of a granulomatous lesion in a patient with WG (bottom row) were stained with anti-CD3, anti-CD28, and both antibodies (overlay). Double immunofluorescence staining (overlay) indicated extensive CD3+/CD28+ double positive staining of T cells in the tonsil, demonstrating efficacy of the staining protocol. In contrast, CD3+ cells did not stain for CD28 and were therefore CD3+/CD28- in the granulomatous lesion in WG. Similar results were obtained from five biopsy specimens from patients with WG.

sarcoidosis (53.4 (7.3)%;  $p < 0.05$ , fig 2). The total numbers of CD4+/CD28- and CD8+/CD28- T cells in the BAL fluid of patients with WG and sarcoidosis also differed significantly ( $p < 0.05$ ).

#### IMMUNOHISTOCHEMISTRY

Biopsy specimens of tissue from granulomatous lesions of patients with WG were stained with anti-CD3, anti-CD28, and both antibodies. Tonsillar tissue from patients without WG undergoing surgery was identically stained. Double immunofluorescence staining (overlay) showed extensive CD3+/CD28+ double positive staining of T cells in the tonsil. Thus, the staining protocol correctly indicated double or single positive cells, and the absence of CD28 on T cells in granulomatous lesions from patients with WG was not due to failure of the staining procedure. Nearly all the CD3+ cells did not stain for CD28 (CD3+/CD28- T cells) in biopsy specimens of the upper respiratory tract in patients with WG (fig 3). Similar results were obtained from five biopsy specimens.

#### CORRELATION OF PARAMETERS

A good correlation was found between ESR and BVAS ( $r = 0.68$ ;  $p < 0.05$ ) and for CRP and BVAS ( $r = 0.83$ ;  $p < 0.01$ ) in patients with WG. There was no correlation between CD4+/CD28- or CD4+/CD28- T cells and CD8+/CD28- T cells from peripheral venous blood or BAL fluid and age or other parameters in WG or sarcoidosis.

#### Discussion

Expansion of the fraction of CD28- T cells has previously been observed in peripheral blood in patients with WG.<sup>8-10</sup> The present study was designed to identify recruitment of CD28- T cells into granulomatous lesions of the respiratory tract and BAL fluid in patients with WG. Patients with acute sarcoidosis served as disease controls because both diseases are characterised by a predominantly Th1-like cytokine profile but have a different outcome. Patients with WG had a significantly higher fraction and total number of CD4+/CD28- and CD8+/CD28- T cells in BAL fluid than those with acute sarcoidosis. The fraction of CD4+/CD28- and CD8+/CD28- T cells was significantly higher in BAL fluid than in blood in patients with WG but not in those with acute sarcoidosis. The total number of CD4+/CD28- T cells, but not CD8+/CD28- T cells, was also significantly higher in BAL fluid than in blood in patients with WG. In addition, CD28- T cells were seen in significant numbers in biopsy specimens from granulomatous lesions of the upper respiratory tract in five patients with WG.

To our knowledge, this is the first study to show enrichment of CD28- T cells in BAL fluid and detection of this T cell subset in granulomatous lesions of WG. Our data are in line with previous reports on leucocyte numbers and the lymphocytic cell profile and resultant CD4/CD8 ratios in BAL fluid in patients with WG and sarcoidosis.<sup>18</sup> Our results

are also in agreement with a recent report on the frequency of CD28<sup>-</sup> T cells in patients with acute sarcoidosis.<sup>19</sup> Phenotypical characterisation of BAL T cells from patients with sarcoidosis in that study demonstrated IFN- $\gamma$  expression and high level expression of CD28. Moreover, in vitro stimulation with the Th1-related cytokine IL-15 induced strong expression of IFN- $\gamma$  and CD28, suggesting a role for macrophage derived cytokines in the activation of T cells in sarcoidosis.<sup>19</sup>

Lack of CD28 expression is an infrequent finding in peripheral blood CD4<sup>+</sup> T cells of healthy humans. Expansion and selective recruitment of CD28<sup>-</sup> T cells to sites of inflammation have recently been observed in distinct infections and some autoimmune diseases.<sup>20–25</sup> CD8<sup>+</sup> and CD4<sup>+</sup> T cells may lose CD28 expression during some chronic infections. Respiratory challenge of mice with *Bordetella pertussis* induces a pulmonary infiltration with CD28<sup>-</sup> T cells.<sup>20</sup> Inflammatory lesions in ulcerative colitis contain aggregates of unusual T cell subsets such as CD4<sup>+</sup>/CD28<sup>-</sup> and  $\gamma\delta$ T cell receptor expressing CD4<sup>-</sup>/CD8<sup>-</sup> T cells.<sup>21</sup> The severity of extra-articular disease manifestations correlates with the expansion of the CD4<sup>+</sup>/CD28<sup>-</sup> IFN- $\gamma$  producing T cell compartment in rheumatoid arthritis.<sup>22</sup> In WG, expansion of the CD28<sup>-</sup> T cell subset correlates with a high cumulative number of involved organs—that is, a high DEI.<sup>8</sup> In rheumatoid arthritis CD4<sup>+</sup>/CD28<sup>-</sup> T cells were found to undergo clonal expansion and lack CD40L, rendering them incapable of providing help for B cell differentiation and immunoglobulin production. Skewing of the immune response towards autoreactive responses may be one of the consequences.<sup>23–24</sup> Despite lacking CD28 molecules, clonal expansion of CD4<sup>+</sup>/CD28<sup>-</sup> T cells has been attributed to the aberrant expression of killer cell activating (KAR) molecules in the absence of inhibitory receptors on CD4<sup>+</sup>/CD28<sup>-</sup> T cells in patients with rheumatoid arthritis. Stimulation of KAR enhances the proliferative response of CD4<sup>+</sup>/CD28<sup>-</sup> T cells to TCR mediated stimulation in rheumatoid arthritis.<sup>25</sup>

Lack of CD28 expression on CD4<sup>+</sup> T cells has also been reported to increase with age.<sup>26</sup> We found no correlation with age in patients with WG or sarcoidosis. In a previous report<sup>8</sup> we also found no correlation of CD28<sup>-</sup> T cells with age in WG, and Schlesier *et al* reported similar findings.<sup>9</sup> Although enlargement of the of CD28<sup>-</sup> T cell compartment with age has been reported, one has to be aware that the “healthy” old age population in many reports was unselected. Search for unreported concomitant diseases may result in a different observation—namely, the absence of an increase in CD4<sup>+</sup>/CD28<sup>-</sup> or CD8<sup>+</sup>/CD28<sup>-</sup> T cells with age (Dr Michael Schirmer, Innsbruck, personal communication).

Clonal expanded CD4<sup>+</sup>/CD28<sup>-</sup> T cells can be detected in the inflamed synovia of patients with rheumatoid arthritis but they do not enrich in the joint.<sup>22</sup> In contrast, our data indicated enrichment of CD4<sup>+</sup>/CD28<sup>-</sup> T cells in BAL fluid compared with blood in patients

with WG. We detected CD28<sup>-</sup> T cells in granulomatous lesions of WG, suggesting recruitment of CD28<sup>-</sup> T cells into the granulomatous lesions from blood. Alternatively, CD28<sup>+</sup> T cells could also be recruited from blood and may lose CD28 expression at the site of inflammation. Since loss of CD28 expression is a feature of highly differentiated T cells arising from CD28<sup>+</sup> T cell precursors—for example, during acute Epstein-Barr (EBV) and human immunodeficiency virus (HIV) infection<sup>27–28</sup>—CD28<sup>-</sup> T cells could be generated in a “longstanding” granuloma such as in WG. This would be less likely in an acute sarcoid granuloma. However, influx of neutrophils forming microabscesses, poorly formed granulomatous lesions, and parenchymal necrosis indicate a higher cellular turnover in WG compared with the well organised epithelioid cell granulomas of acute sarcoidosis.<sup>29</sup> Thus, further analysis of phenotype, clonality, and function of CD28<sup>-</sup> T cells in blood and at sites of inflammation is needed to understand leucocyte homing in WG. Preliminary data from our current analysis of CD4<sup>+</sup>/CD28<sup>-</sup> T cells in WG indicate that CD4<sup>+</sup>/CD28<sup>-</sup> T cells are phenotypically and functionally different from the CD4<sup>+</sup>/CD28<sup>-</sup> T cell subset found in rheumatoid arthritis.<sup>22–25</sup>

In conclusion, this study has shown enrichment of CD28<sup>-</sup> T cells in BAL fluid and granulomatous lesions of the respiratory tract in patients with WG but not in those with acute sarcoidosis. Homing and compartmentalisation of CD28<sup>-</sup> T cells into granulomatous lesions in WG may be one mechanism which finally determines the phenotypically different evolution of the inflammatory lesions in WG and sarcoidosis. Furthermore, specialised T cell subsets preferentially homing into areas of inflammation in WG may become therapeutic targets for selective treatments in the future.

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