Peripheral Blood and Granuloma CD4⁺CD28⁻ T Cells Are a Major Source of Interferon- γ and Tumor Necrosis Factor- α in Wegener's Granulomatosis

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To elucidate whether the fraction of CD28⁻ T cells within the CD4⁺ T-cell population is a major source of Th1-like and proinflammatory cytokine production driving Wegener's granulomatosis (WG) granuloma formation, we analyzed the phenotype and functional characteristics of peripheral blood CD4⁺CD28⁻ T cells and of T cells in granulomatous lesions of 12 patients with active WG. Surface markers and intracytoplasmic cytokine and perforin expression were assessed by flow cytometry. Cytokine secretion was measured by enzyme-linked immunosorbent assay. Immunohistological studies demonstrated interferon- γ and tumor necrosis factor- α cytokine positivity attributable to CD4⁺CD28⁻ T cells in granulomatous lesions. Peripheral blood CD4⁺CD28⁻ T cells expressed CD57, also found on natural killer cells, and intracytoplasmic perforin. They were generally CD25 (interleukin-2 receptor)-negative. CD18 (adhesion molecule β_2 -integrin) was strongly up-regulated on CD4⁺CD28⁻ T cells, whereas only a minority of CD4⁺CD28⁺ T cells expressed CD18. CD4⁺CD28⁻ T cells appeared as a major source of interferon- γ and tumor necrosis factor- α . In contrast, CD4⁺CD28⁺ T cells were able to produce and secrete a wider variety of cytokines including interleukin-2. One-quarter of CD4⁺CD28⁺ T cells expressed the activation marker CD25, but they lacked perforin. Thus, CD4⁺CD28⁻ T cells appeared more differentiated than CD4⁺CD28⁺ T cells. They displayed Th1-like cytokine production and features suggestive of the capability of CD4⁺ T-cell-mediated cytotoxicity. CD4⁺CD28⁻ T cells may be recruited into granulomatous lesions from the blood via CD18 interaction, and may subsequently promote

monocyte accumulation and granuloma formation through their cytokine secretion in WG. (Am J Pathol 2002, 160:1717-1724)

Wegener's granulomatosis (WG) is an inflammatory disease of unknown origin characterized by disseminated necrotizing granulomas and a systemic vasculitis affecting predominantly small vessels. Frequent relapses and disease, as well as therapy-related mortality, still determine the prognosis of WG.¹ WG granulomas consist of CD4⁺ T cells, monocyte-derived tissue macrophages, giant cells, and neutrophils surrounding a necrotic area. Sometimes macrophages are lined up in a palisading manner. Less organized lesions are frequently seen. Activated CD4⁺ T cells from granulomatous lesions of the respiratory tract and from peripheral blood produce and release interferon (IFN)- γ indicating a predominance of a Th1-like response in WG.²⁻⁴ Moreover, CD4⁺CD26⁺ (CD26 = optional Th1 marker) T cells as well as IFN- γ -positive cells are present in granulomatous lesions of the upper respiratory tract in WG.⁵ In addition, clinical results support the concept that CD4⁺ T cells play a critical role in WG. Patients refractory to conventional immunosuppressive treatment have been successfully treated with monoclonal antibodies directed against T-cell surface antigens CD52 and/or CD4 resulting in partial T-cell depletion.^{6,7}

Activated CD4⁺ T cells promote the transformation of nonspecific microabscesses to granulomatous inflammation in animal models of *Listeria monocytogenes*-induced granulomas. In these animal models granuloma formation takes place in the presence of a dominating Th1 cytokine response. Local concentrations of tumor necrosis factor (TNF)- α , IFN- γ , and interleukin (IL)-2 induce intraparenchymal monocyte accumulation.⁸ Induction of necrotic centers is mediated by antigen-activated T cells.⁹

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Whereas CD8⁺ T cells play a relatively modest role during the first phase of granuloma formation, it has been demonstrated in mouse models of tuberculosis, that they play a more important role in controlling infection at later stages.¹⁰

Recently, a marked expansion of CD4⁺ T cells and CD8⁺ T cells lacking CD28 expression has been observed in WG.^{11–13} The frequency of CD28[−] T cells correlates with the cumulative number of involved organs, indicating that patients with a higher fraction of CD28[−] T cells are prone to more severe disease involvement throughout time.¹² Moreover, CD28[−] T cells are significantly enriched in granulomatous lesions of nasal biopsy specimens and in bronchoalveolar lavage fluid of WG patients with active disease.¹³ Lack of CD28 expression on peripheral blood CD4⁺ T cells is an infrequent finding in healthy patients. The CD28[−] subset of peripheral blood T cells has been reported to express surface markers of activation¹¹ and to secrete IFN- γ .¹⁴ CD28[−] T cells also express CD57, a surface molecule of natural killer cells and mature subsets of T and B cells.^{11,12,14,15}

Based on these findings, we hypothesized that the fraction of CD28[−] T cells within the CD4⁺ T-cell population (CD4⁺CD28[−] T cells) is a major source of Th1-like cytokine production and, thus, may represent the driving force for subsequent monocyte accumulation and granuloma formation in WG. We analyzed phenotypic and functional characteristics of the CD28⁺ and CD28[−] fractions of the peripheral blood CD4⁺ T-cell population. Immunohistological stainings of tissue specimen were performed to ascribe cytokine secretion to CD4⁺CD28[−] T cells within the granuloma.

Materials and Methods

Study Population

Peripheral blood mononuclear cells from 12 patients with biopsy-proven WG were analyzed. All patients met the criteria of the American College of Rheumatology¹⁶ and the Chapel Hill Consensus Conference definition for WG.¹⁷ The disease extension and vasculitis activity were described by the Disease Extension Index and Birmingham Vasculitis Activity Score as outlined elsewhere.^{18–21} In brief, the Disease Extension Index is the equivalent of organ involvement in WG,^{18,19} whereas the Birmingham Vasculitis Activity Score considers clinical features and laboratory data to give a measure of vasculitis activity.^{20,21}

Analysis of Lymphocytes Subsets

Staining of cells was performed using APC-conjugated anti-human CD4; Cy-Chrome anti-human CD3; phycoerythrin (PE)-conjugated CD28; PE-conjugated HLA-DR; mouse anti-human TNF- α ; rat anti-human IL-13, IL-10, IL-8; rat IgG2a; rat IgG1; as well as fluorescein isothiocyanate-conjugated CD28, CD30, CD18, CD57, CD45RA, CD95, perforin, rat anti-human IFN- γ , IL-4, IL-2; and rat anti-human IL-2, IL-5, IgG1, and IgG2, obtained

from Becton Dickinson and Pharmingen (Heidelberg, Germany). Fluorescein isothiocyanate-conjugated anti-CD25 was purchased from Immunotech (Marseilles, France) and fluorescein isothiocyanate-conjugated anti-Fas-Ligand antibody from Holzelt Diagnostika (Kologne, Germany). Bcl-2 expression was determined by intracytoplasmic staining after permeabilization according to the instructions of the manufacturer. Determination of perforin expression was restricted to 3 of the 12 patients.

Cell Separation

Peripheral blood mononuclear cells were sorted into CD4⁺CD28⁺ and CD4⁺CD28[−] T cells. CD4⁺CD28[−] T cells were isolated from five selected patients with a CD4⁺CD28[−] fraction >10%. CD4⁺ T cells were purified from peripheral blood mononuclear cells by negative selection using magnetic bead separation (MACS CD4⁺ T Cell Isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany). CD28⁺ cells were stained by PE-conjugated anti-CD28 antibody (Becton Dickinson, Heidelberg, Germany) and depleted by anti-PE MicroBeads (Miltenyi Biotec). CD28[−] and CD28⁺ fractions of CD4⁺ T cells were cultured in RPMI 1640 supplemented with 5% fetal calf serum, 5% human serum, 5 mmol/L L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin (all from Sigma, Munich, Germany) at 37°C in a humidified atmosphere in a 5% CO₂ incubator. The cells were stimulated with 100-ng/ml anti-human CD3 for 24 hours. Cells were subjected to enzyme-linked immunosorbent assay (ELISA) thereafter.

Surface Marker and Intracytoplasmic Cytokine Staining

Cell surface and intracellular stainings were performed using whole blood. Heparinized peripheral blood (150 μ l) was diluted with 850 μ l of RPMI 1640 supplemented with 10% fetal calf serum, 5 mmol/L L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin (all from Sigma, Munich, Germany) and was stimulated with phorbol myristate acetate (PMA) (10 ng/ml) and ionomycin (1 μ g/ml) at 37°C in a humidified atmosphere in a 5% CO₂ incubator. Monensin (2 μ mol/L) was added to inhibit cytokine secretion. At 5 hours 100 μ l of 20 mmol/L ethylenediaminetetraacetic acid (final concentration, 2 mmol/L) was added. Cells were washed twice (250 $\times$ g, 5 minutes, 4°C) in washing buffer [phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ (Gibco BRL, Karlsruhe, Germany), 0.1% bovine serum albumin, 0.1% sodium azide (Sigma, Munich, Germany), pH 7.4]. The thoroughly resuspended cells were fixed and lysed in 2 ml of diluted FITC Lysing Solution (Becton Dickinson, Heidelberg, Germany) for 10 minutes at room temperature. Cells were washed twice in washing buffer. The pellet was resuspended in 100 μ l of permeabilization buffer [PBS without Ca²⁺ and Mg²⁺, 0.1% bovine serum albumin, 0.1% sodium azide, 0.1% saponin, pH 7.4 (Sigma, Munich, Germany)] for 10 minutes at room temperature. After washing with buffer the cells were stained in 100 μ l of staining

buffer containing previously determined optimal concentrations (0.25 to 1.0 $\mu\text{g}/100 \mu\text{l}$) of fluorochrome-conjugated monoclonal antibodies for cell-surface antigens and anti-cytokine antibodies or appropriate negative (isotype) controls. Incubation was performed at 4°C for 30 minutes in the dark. After staining, the cells were washed and fixed with 500 μl of PBS containing 1.5% paraformaldehyde. Cells were analyzed thereafter.

Flow Cytometry

Four-color 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 CD3 and CD4 staining. Data of 1000 lymphocytes were collected. Positively and negatively stained populations were calculated by quadrant dot-plot analysis determined by isotype controls.

Detection of Cytokines by ELISA

After stimulation with 100 ng/ml of anti-CD3 for 24 hours cytokine secretion was determined from the supernatant of CD28⁺ and CD28⁻ fractions of CD4⁺ T cells using Quantikine human IFN- γ and IL-10 and Quantikine HS human IL-4 ELISA kits (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions.

Immunohistology

Nasal biopsies of three WG patients obtained for diagnostic purposes were submerged in 0.9% NaCl, snap-frozen in liquid nitrogen, and stored until use at -80°C. Six- μm serial cryostat sections were fixed in acetone for 30 minutes, followed by fixation in chloroform for 30 minutes. Incubation with the primary antibody (TNF-E, GZ-4, and CD4;⁵ CD28.1; DAKO, Hamburg, Germany) was performed for 30 minutes and immunostaining was undertaken according to the alkaline phosphatase anti-alkaline phosphatase method with New Fuchsin development.^{5,22} Finally, slides were counterstained with hematoxylin and mounted. Immunostainings were controlled by implementing the secondary reagents alone to confirm specificity or enzyme development by itself to rule out endogenous enzymatic activities. The stainings were evaluated by KH-U and AM.

Statistical Analysis

The SPSS statistical software package (SPSS GmbH, München, Germany) was used for analysis. Data are presented as means $\pm$ SE of mean (SEM). To test for normal distribution the Kolmogorov-Smirnov test was used. For comparison the Mann-Whitney *U* test was used. Correlation was examined by computing Spearman's correlation coefficient. A *P* value of <0.05 was considered to be statistically significant.

Results

Patient Characteristics

Twelve patients with WG were studied. The male:female ratio was 1:1. The patients age was 53.4 ± 3.0 years (mean $\pm$ SEM). Erythrocyte sedimentation rate (ESR) was 44.4 ± 6.3 mm/hour, CRP was 3.9 ± 1.4 mg/dl, leukocytes $7271 \pm 438/\mu\text{l}$, and creatinine 1.4 ± 0.3 mg/dl. The fraction of CD28⁻ T cells within the CD4⁺ T-cell population (CD4⁺CD28⁻) was $14.4 \pm 5.4\%$. The fraction of CD28⁻ T cells within the CD8⁺ T-cell population (CD8⁺CD28⁻) was $40.8 \pm 6.1\%$. In accordance with previous findings,¹² CD28⁻ T cells within the CD4⁺ and CD8⁺ T-cell populations were significantly expanded in WG compared with age- and sex-matched normal controls (*P* < 0.01). Patients had active disease at the time of analysis with a Disease Extension Index of 2.4 ± 0.3 , a Birmingham Vasculitis Activity Score-1 (indicating new or worse disease activity) of 8.0 ± 1.8 and a Birmingham Vasculitis Activity Score-2 (persisting or grumbling disease activity) of 9.7 ± 1.8 . Thus, immunosuppressive treatment (three patients with oral cyclophosphamide, nine patients with either methotrexate, azathioprine, or leflunomide in addition to corticosteroids) was insufficient at the time of analysis and was subsequently intensified.

Phenotype of CD4⁺CD28⁻ T-Cell Subset in WG

Figure 1 shows representative stainings of surface and intracellular markers of the CD28⁻ and CD28⁺ fractions of the (gated) CD3⁺CD4⁺ T-cell population. Using the surface markers CD18, CD25, CD30, CD45RA, CD57, CD95, and the intracellular markers Bcl-2 and perforin, phenotypic distinctions between the fraction of CD28⁺ and the fraction of CD28⁻ T cells within the CD4⁺ T-cell population became apparent. Whereas approximately one-quarter of CD4⁺CD28⁺ T cells expressed CD25 (α -chain of IL-2R), virtually none of the CD4⁺CD28⁻ T cells expressed CD25. In contrast, the majority of CD4⁺CD28⁻ T cells were CD57-positive. Nearly all CD4⁺CD28⁺ T cells were CD57⁻. We found a strong negative correlation between CD28 and CD57 cell-surface expression on the CD4⁺ T cell population (*r* = 0.9317, *P* < 0.001). Perforin was only expressed by CD4⁺CD28⁻ T cells, but not by the CD4⁺CD28⁺ T-cell subset. CD18 (β_2 -integrin) was strongly up-regulated on CD4⁺CD28⁻ T cells, whereas only a minority of CD4⁺CD28⁺ T cells expressed CD18. The majority of CD4⁺CD28⁻ T cells were CD45RA⁻, whereas CD4⁺CD28⁺ T cells appeared predominantly CD45RA⁺-positive. The majority of both, the CD28⁻ and the CD28⁺ fractions of the CD4⁺ T-cell population, were CD30⁻. CD4⁺CD28⁻ T cells were predominantly CD95 (Fas)-positive, as well as their CD28⁺ counterparts. Neither CD4⁺CD28⁻ nor CD4⁺CD28⁺ T cells displayed sufficient Bcl-2 (B cell lymphoma leukemia 2 protein) expression.

To address the question whether CD4⁺CD28⁻ T cells completely lack CD28 expression or have low surface

Figure 1. Representative quadrant dot-plot analysis of peripheral blood T cells ($CD3^+CD4^+$). The fraction of $CD28^+$ and the fraction of $CD28^-$ T cells within the $CD4^-$ T-cell population displayed different surface and intracytoplasmic markers as assessed by flow cytometry: $CD4^+CD28^-$ T cells were mainly $CD57^+CD18^+$ and $CD45RA^+CD25^-$, whereas $CD4^+CD28^+$ T cells were mainly $CD57^-CD18^-$ and $CD45RA^+$ and to some extent $CD25^+$. Neither the fraction of $CD28^-$ nor the fraction of $CD28^+$ T cells within the $CD4^+$ population expressed surface $CD30$ or intracytoplasmic $Bcl-2$.

$CD28$ expression ($CD28^{low}$), we compared the mean fluorescence with negative isotype controls. The $CD28^-$ T-cell fraction of the $CD4^+$ T-cell population and isotype controls displayed similar mean fluorescence suggesting that these cells were completely $CD28$ -deficient (data not shown).

Intracytoplasmic Cytokine Expression of $CD4^+CD28^-$ and $CD4^+CD28^+$ T Cells

Staining for intracytoplasmic $IFN-\gamma$, $IL-2$, $TNF-\alpha$, $IL-5$, $IL-8$, and $IL-13$ demonstrated distinct differences in the capability to produce (not necessarily secrete) and/or store cytokines between the fraction of $CD28^+$ and the fraction of $CD28^-$ T cells within the $CD4^-$ T-cell population. The majority of $CD4^+CD28^-$ T cells expressed $IFN-\gamma$, but not $IL-2$. In contrast, fewer $CD4^+CD28^+$ T cells were $IFN-\gamma$ -positive, but approximately one-third displayed $IL-2$ expression. Approximately half of the $CD4^+CD28^-$ T cells were $TNF-\alpha$ -positive, whereas few $CD4^+CD28^+$ T cells expressed $TNF-\alpha$. Only a few cells of the $CD28^-$ and $CD28^+$ fractions of $CD4^+$ T cells were $IL-5$ -, $IL-8$ -, or $IL-13$ -positive (Figure 2). Taken together, $CD4^+CD28^-$ T cells appeared as a major source of $IFN-\gamma$ and $TNF-\alpha$ production, whereas $CD4^+CD28^+$ T cells were able to produce a wider variety of cytokines, including $IL-2$ (Figure 3).

Cytokine Secretion of $CD4^+CD28^-$ and $CD4^+CD28^+$ T Cells

To assess the capability of $CD4^+CD28^-$ and $CD4^+CD28^+$ T cells to secrete cytokines and to compare secretion with production (evident by intracytoplasmic

cytokine expression in the fluorescence-activated cell sorting analysis), we determined cytokine secretion of $CD4^+$ T cells using ELISA. Peripheral blood mononuclear cells were sorted into $CD4^+CD28^+$ and $CD4^+CD28^-$ T cells. After stimulation with anti- $CD3$ for 24 hours cytokine secretion was measured in the supernatant of $CD28^+$ and $CD28^-$ fractions of the $CD4^+$ T-cell population. $IFN-\gamma$ secretion was six to seven times higher in $CD4^+CD28^-$ T cells compared with $CD4^+CD28^+$ T cells. $IL-10$ secretion was also higher in $CD4^+CD28^-$ T cells compared with $CD4^+CD28^+$ T cells. No significant difference was observed in the overall low level of $IL-4$ secretion (Figure 4).

Staining Results in WG Tissues

The $CD28^-$ fraction of the peripheral blood $CD4^+$ T-cell population appeared as a major source of $IFN-\gamma$ and $TNF-\alpha$. To determine whether a $CD4^+CD28^-$ subset is also present in granulomatous lesions and, thus, may promote granuloma formation through its cytokine secretion, we performed immunohistological studies on nasal biopsy specimen of three patients with inflammatory involvement of the upper and lower respiratory tract. Immunohistochemistry with anti- $CD4$ on serial cryostat sections demonstrated the presence of $CD4^+$ T cells, whereas the number of $CD28^+$ T cells was very low or not detectable at all (Figure 5, B and C). These findings confirm data from a previous study,¹³ in which we found abundant $CD3^+$ T cells lacking $CD28$ in granulomatous lesions. All three cases displayed distinct and specific $TNF-\alpha^+$ lymphocytes (5 to 20 $TNF-\alpha^+$ T cells/high-power field, $\times 400$; Figure 5D). The number of $IFN-\gamma^+$ cells within granulomatous lesions appeared higher compared with $TNF-\alpha^+$ cells (Figure 5E). $CD4$ staining $CD28^-$ T cells

Figure 2. Representative quadrant dot-plot analysis of peripheral blood T cells (CD3⁺CD4⁺). The fraction of CD28⁺ and the fraction of CD28⁻ T cells of the CD4⁺ T-cell population displayed differences in the expression of intracytoplasmic cytokines as assessed by flow cytometry. CD4⁺CD28⁻ T cells expressed IFN- γ , but not IL-2. In contrast, CD4⁺CD28⁺ T cells displayed less often IFN- γ , but approximately one-third of this subset was IL-2-positive. TNF- α expression was more frequent in the CD4⁺CD28⁻ T-cell subset than in CD4⁺CD28⁺ T cells. Only a few cells of the CD28⁻ and CD28⁺ fractions of CD4⁺ T cells were IL-5-, IL-8-, or IL-13-positive.

matched with the sections displaying IFN- γ and TNF- α positivity. Thus, CD4⁺CD28⁻ T cells appeared as a source of IFN- γ and TNF- α production in granulomatous lesions in WG.

Discussion

Animal and *in vitro* models of granuloma induction secondary to infectious agents have demonstrated the important role of activated Th1-cytokine-producing CD4⁺ T cells in promoting the transformation of nonspecific microabscesses to granulomas.⁸⁻¹⁰ Because animal mod-

els of vasculitis do not reproduce granulomatous lesions typical of WG,²³ indirect evidence of a role of CD4⁺CD28⁻ T cells in granuloma formation had to be sought by phenotypical and functional characterization of peripheral blood CD4⁺ T cells and comparison with an immunohistological analysis of T cells located in granulomatous lesions. In accordance with a previous study¹³ we found abundant T cells lacking CD28 in the granulomatous lesions. In the present study, CD4⁺CD28⁻ T cells appeared as a major source of IFN- γ and TNF- α in peripheral blood as well as in granulomatous lesions (Figure 6).

We found marked differences in the expression of surface markers and cytokine production between CD4⁺CD28⁻ and CD4⁺CD28⁺ T cells in WG. The majority of peripheral blood CD4⁺CD28⁻ T cells was CD57-positive, whereas CD4⁺CD28⁺ cells were mainly CD57⁻. Two groups have previously reported on the expression of CD57 and activation markers on different T-cell subsets in Schlesier and colleagues¹¹ demonstrated CD57 expression on CD8⁺ T cells and Giscombe and colleagues¹⁴ detected CD57 on expanded T-cell populations. Moreover, CD4⁺CD28⁻ T cells expressed intracytoplasmic perforin, whereas CD4⁺CD28⁺ T cells did not. Thus, CD4⁺CD28⁻ T cells may be characterized as a putative cytotoxic CD4⁺ T-cell subset in WG. CD4⁺ T-cell subsets capable of perforin-mediated cytotoxicity have been demonstrated recently. However, their role in immunity and diseases is primarily unknown.^{24,25}

The CD4⁺CD28⁻ T cell subset in WG differs from CD4⁺ T cells found to mediate granuloma formation in models⁸⁻¹⁰ with respect to their IL-2 production. The CD4⁺CD28⁻ T cell subset did not produce IL-2 and did not express CD25. Lack of CD25 expression on CD4⁺CD28⁻ T cells may indicate a relative resistance to

Figure 3. Intracytoplasmic cytokine expression of the fraction of CD28⁻ and CD28⁺ T cells of the CD3⁺CD4⁺ T-cell population as assessed by flow cytometry. Data are presented as mean and SD. CD4⁺CD28⁻ T cells expressed IFN- γ (mean, 88.4% versus 32.1%; $P < 0.001$) and TNF- α (64.9% versus 33.2%, $P < 0.01$) more often than CD4⁺CD28⁺ T cells. The CD4⁺CD28⁺ T-cell subset expressed IL-4 (2.3% versus 0.01%, $P < 0.05$), IL-2 (23.8% versus 0.4%, $P < 0.05$), and IL-13 (5.2% versus 0.5%, $P < 0.01$) in a higher percentage than CD4⁺CD28⁻ T cells. *, $P < 0.05$; **, $P < 0.001$.

Figure 4. Cytokine secretion of CD4⁺ T cells sorted into CD28⁺ and CD28⁻ fractions. Cytokine secretion was determined by ELISA from the supernatant after stimulation with anti-CD3 for 24 hours. CD4⁺CD28⁻ T cells were capable of a higher IFN- γ and IL-10 secretion than CD4⁺CD28⁺ T cells. IL-4 secretion was not significantly different between both CD4⁺ T-cell subsets in this analysis.

apoptosis on withdrawal of the T-cell growth factor IL-2.²⁶ This may in turn favor the expansion of this subset. In contrast, expression of the anti-apoptotic protein Bcl-2 (low) and CD95⁺ (fas) surface expression (high) was not different when CD4⁺CD28⁻ and CD4⁺CD28⁺ T cells were compared. The CD4⁺CD28⁻ T-cell fraction in WG phenotypically resembles CD28⁻ T cells from CD28-knockout mice, which are also characterized by a deficient IL-2 production.²⁷

Our findings also raise the question about whether these CD4⁺CD28⁻ T cells migrate from blood into granulomatous lesions, or whether CD28 is down-regulated on migration of CD4⁺CD28⁺ T cells into inflammatory lesions. Direct evidence of migratory routes and of phenotypic changes during migration of T cells is still missing in WG. CD18 (adhesion molecule β_2 -integrin) was strongly up-regulated on CD4⁺CD28⁻ T cells, whereas only a minority of CD4⁺CD28⁺ T cells expressed CD18.

β_2 integrins may promote recruitment of T cells into granulomatous lesions via interaction with endothelial ICAM-1. Up-regulation of endothelial ICAM-1 is found in active WG.²⁸ Recruitment of activated T cells via ICAM-1/LFA-1 interaction has also been demonstrated *in vitro* in vasculitic patients.²⁹ Based on these findings it seems more likely that CD4⁺CD28⁻ T cells are directly recruited from the blood into granulomatous lesions.

We found CD4⁺CD28⁻ T cells in granulomatous lesions (serial sections; Figure 5, B and C). TNF- α and IFN- γ staining could be ascribed to CD4⁺CD28⁻ T cells (Figure 5, D and E). TNF- α staining and detection of TNF- α mRNA by *in situ* hybridization has been demonstrated in antineutrophil cytoplasmic antibodies (ANCA)-positive glomerulonephritis, ie, a vasculitic manifestation.³⁰ To our knowledge, this is the first report describing TNF- α ⁺ T cells in granulomatous lesions of WG. The number of TNF- α -staining T cells seemed low compared

Figure 5. Nasal tissue biopsy of a WG patient. **A** and **B**: H&E staining showing ulceration and geographic necrosis at the left (**A**, open arrowhead), ill-defined epithelioid cell granulomas at the bottom right (**A**, black arrowhead), and an epithelioid cell granuloma with a multinucleated giant cell at the top (**B**). **C** to **F**: Immunohistochemistry. The same nasal tissue displayed a dense infiltrate of CD4⁺ CD28⁻ T lymphocytes (**C**, CD4; **D**, CD28). Within the CD4⁺ T-lymphocytic infiltrate solitary cells were positive for TNF- α (**E**) and numerous cells stained positive for IFN- γ (**F**). Original magnifications: **A**, $\times 200$; **B-F**, $\times 630$.

Figure 6. Summary of the principle structure of granulomatous lesions in WG- and T cell-related findings. WG granulomas consist of T cells (T), monocyte-derived tissue macrophages (M), giant cells of the foreign body and Langhans type (GC), and neutrophils (N) surrounding a necrotic area (blank). CD3⁺ T cells are mainly CD28⁻.¹³ CD4⁺CD26⁺ (CD26 = optional Th1 marker) T cells produce IFN-γ.⁵ CD4⁺CD28⁻ T cells are IFN-γ⁺ and TNF-α⁺ in granulomatous lesions in WG as demonstrated by this study. Moreover, peripheral blood CD4⁺CD28⁻ T cells were shown to be a major source of IFN-γ and TNF-α. CD4⁺CD28⁻ T cells may promote granuloma formation through their cytokine secretion in WG. (Original art by P Lamprecht, A Mueller, and WL Gross WL).

with IFN-γ⁺ T cells. However, rapid secretion of TNF-α may result in a low number of TNF-α⁺ cells and explain the discrepancy between high-TNF-α mRNA and low-TNF-α gene product level as has been described for nasal polyp tissues by Finotto and colleagues³¹ previously. Thus, our findings demonstrate the presence of a CD4⁺CD28⁻ T-cell subset producing IFN-γ and also TNF-α in granulomatous lesions of WG. Moreover, subtle differences in cytokine production and secretion such as TNF-α may contribute to differences between the less organized granulomatous lesion in WG and well-defined granulomas in infections.³²

The restricted cytokine production of peripheral blood CD4⁺CD28⁻ T cells as compared to CD4⁺CD28⁺ T cells may indicate a higher degree of differentiation of the CD4⁺CD28⁻ T-cell subset as compared to their CD4⁺CD28⁺ counterpart. Recently, HIV-specific CD8⁺ T cells have been found to be predominantly preterminally differentiated, ie, CD45RA⁻. A disturbed antiviral response because of a skewed maturation of cytotoxic CD8⁺ T cells has been proposed as a consequence.³³ The majority of the blood CD4⁺CD28⁻ T cells were CD45RA⁻ suggestive of a memory T-cell phenotype in WG. However, because CD45RA may be re-expressed on terminally differentiated CD4⁺ memory T cells,³⁴ it needs to be clarified, whether or not CD4⁺CD28⁻ T cells are preterminally or terminally differentiated in WG, and what effect this may have on their effector function.

The phenotype of CD4⁺CD28⁻ T cells also raises the question of whether CD4⁺CD28⁻ T cells are derived from antigen-challenged and -activated CD28⁺ precursors.

Lack of CD28 surface expression on CD4⁺ T cells seems not simply a consequence of chronic inflammation. Clonally expanded CD4⁺CD28⁻ T cells have been found in rheumatoid arthritis, but not in psoriatic arthritis, both of which are chronic inflammatory diseases.³⁵ In rheumatoid arthritis, CD4⁺CD28⁻ T cells also express surface CD57 and intracytoplasmic perforin. They produce IFN-γ as well as IL-2^{36,37} and bear co-stimulatory killer cell-activating receptors and CD161, another C-type lectin natural killer receptor.^{38,39} CD4⁺CD161⁺ were found to be expanded in blood as well as in rheumatoid synovitis.³⁹ The nature of the antigen or antigens driving CD4⁺CD28⁻ T-cell expansion and the consequences of this expansion in rheumatoid arthritis are also still a matter of debate. Cytomegalovirus reactivation has been proposed to cause lack of CD28 on T cells in rheumatoid arthritis.⁴⁰ Interestingly, several chronic infections such as HIV or cytomegalovirus infection induce expansion of T-cell subsets lacking CD28.^{41,42} As there were reports that cytomegalovirus reactivation may mimic symptoms of WG,⁴³ we currently analyze the role of cytomegalovirus and other putative antigens responsible for CD28 down-regulation on CD4⁺ T cells.

In summary, our findings suggest that peripheral blood- and granuloma-residing CD4⁺CD28⁻ T cells were a major source of IFN-γ and TNF-α. CD4⁺CD28⁻ may favor granuloma formation through their cytokine production in WG. Furthermore, CD4⁺CD28⁻ T cells displayed features suggestive of CD4⁺ T-cell-mediated cytotoxicity. At present, the nature of the antigen driving the evolution of CD4⁺CD28⁻ T cells in WG remains obscure and to be investigated.

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