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T Cell Infiltrates in the Muscles of Patients with Dermatomyositis and Polymyositis Are Dominated by CD28null T Cells

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Dermatomyositis and polymyositis are disabling rheumatic diseases characterized by an appreciable number of T cells infiltrating muscle tissue. The precise phenotype, function and specificity of these cells remain elusive. In this study, we aimed to characterize T cells in muscle tissue and circulation and to investigate their association to clinical phenotype. Twenty-four patients with dermatomyositis and 42 with polymyositis were screened for frequency of CD4+ CD28null and CD8+ CD28null T cells in peripheral blood by flow cytometry. Presence of these cells in inflamed muscle tissue from 13 of these patients was analyzed by three-color immunofluorescence microscopy. Effector functions, proliferation and Ag specificity were analyzed by flow cytometry after in vitro stimulation. The clinical relevance of CD28null T cells was analyzed by multiple regression analyses including six separate and combined disease variables. We demonstrate that muscle-infiltrating T cells are predominantly CD4+ CD28null and CD8+ CD28null T cells in patients with dermatomyositis and polymyositis. Muscle-infiltrating CD28null T cells were found already at time of diagnosis. Disease activity correlated with the frequency of CD8+ T cells in the inflamed muscles of polymyositis patients. Circulating CD4+ CD28null and CD8+ CD28null T cells were significantly more frequent in human CMV (HCMV) seropositive individuals, responded to HCMV Ag stimulation, and correlated with disease duration. These cells also display a proinflammatory cytokine profile, contain perforin and lack the costimulatory molecule CD28. Our observations imply that CD28null T cells represent clinically important effector cells in dermatomyositis and polymyositis, and that HCMV might play a role in propagating disease in a subset of patients. The Journal of Immunology, 2009, 183: 4792–4799.

Dermatomyositis and polymyositis are autoimmune diseases clinically characterized by proximal muscle weakness. Histopathologically the muscles display cellular infiltrates of T cells, macrophages, and dendritic cells (DC).2 A perivascular distribution of CD4+ T cells, macrophages and occasional B cells is typical for dermatomyositis (1, 2). In contrast, polymyositis is dominated by CD8+ T cells and macrophages surrounding the muscle fibers (1). Electron microscopy studies of inflamed muscle tissue from polymyositis patients suggest that CD8+ T cells are cytotoxic to muscle fibers (3). Indeed, both CD4+ and CD8+ muscle-infiltrating T cells have been shown to be perforin-positive (4). The aetiopathogenic mechanisms are not known but interplay between genes and environment is likely to contribute. Viral infections have been suggested to confer a risk factor for polymyositis and dermatomyositis but so far no convincing data have been reported (5).

Treatment of dermatomyositis and polymyositis is based on high doses of glucocorticoids in combination with other immunosuppressives, however, with a varying response. In some patients the remaining muscle weakness is associated with persisting inflammatory infiltrates in muscle tissue even after several months of immunosuppressive treatment (6). Strikingly, in some patients the inflammatory cells persist even after high doses of glucocorticoids and other immunosuppressive treatment (6–8). In this context the CD28null T cells, suggested to be resistant to apoptosis, are of particular interest (9, 10). These cells are long-lived, proinflammatory and terminally differentiated T cells lacking CD28. CD8+ CD28null T cells emerge from repeated Ag stimulation and have been reported in peripheral blood of individuals with chronic viral infections like EBV, human CMV (HCMV), and HIV (11, 12). A corresponding CD4+ population is less common, but is over-represented in peripheral blood of patients with autoimmune diseases, such as rheumatoid arthritis, Crohn’s disease, or multiple sclerosis in combination with HCMV seropositivity (13–16).

Despite frequent reports on CD28null T cells in peripheral blood in autoimmune disorders, the distribution in affected...
organisms and clinical impact is poorly understood. In this study, including a large cohort of patients with dermatomyositis and polymyositis, we provide evidence indicating that CD4⁺CD28null and CD8⁺CD28null T cells are important effector T cells in the inflamed muscle tissue and that these cells correlate with disease variables.

Materials and Methods

Patients and healthy controls

Sixty-six patients, 47 women and 19 men, with probable or definite polymyositis (n = 40), dermatomyositis (n = 24), or juvenile dermatomyositis (n = 2), with regular visits at the Rheumatology Clinic at the Karolinska University Hospital (Stockholm, Sweden) were enrolled in the study (17, 18). Patients with inclusion body myositis were excluded (19). The median age was 52 years (range 28–74), and for patients with polymyositis 61 years (range 24–79). Median disease duration was 5 years (range 0–30) for all patients together, 6 years duration (range 0–24) for patients with dermatomyositis and 5 years (range 0–30) for patients with polymyositis. At time of blood sampling, 23 patients were untreated, as these were either recently diagnosed (n = 3), in remission, and 38 patients were treated with prednisolone. CD4⁺CD28null and CD8⁺CD28null T cells in muscle tissue. In the cohort, subjects were also analyzed for the presence (+) or absence (−) of IgG and IgM Abs to HCMV.

Table I. Clinical and cellular characteristics of polymyositis and dermatomyositis patient group

<table>
<thead>
<tr>
<th>Disease</th>
<th>Characteristics</th>
<th>Peripheral Blood⁶</th>
<th>Muscle Tissue Section⁷</th>
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<td>Disease duration (y)</td>
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<tr>
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<tr>
<td>PM-9</td>
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⁶ Patients with dermatomyositis (DM) and polymyositis (PM) investigated for CD4⁺CD28null and CD8⁺CD28null T cells in muscle tissue. In the cohort, subjects were also analyzed for the presence (+) or absence (−) of IgG and IgM Abs to HCMV.

⁷ CD4⁺CD28null and CD8⁺CD28null T cells in peripheral blood (PB) indicate the percentage of these subsets in peripheral blood.

⁸ Indicates the number of infiltrates per biopsy investigated for the presence of CD4⁺CD28null and CD8⁺CD28null T cells.

Indicates the number of CD3⁺ T cells and the range in the number of CD3⁺ T cells in the investigated infiltrates.

Aza, azathioprine; CsA, cyclosporine; Mtx, methotrexate; Pred, prednisolone; VAS, visual analogue scale.

Flow cytometry analyses

PBMC were isolated by ficoll separation (Ficoll-Paque Plus; GE Healthcare) and screened for CD4⁺CD28null and CD8⁺CD28null phenotypes by flow cytometry. Abs used were CD3-PE (clone UCHT), CD28-allophycocyanin (clone CD28-2; BD Pharmingen), and CD4-PerCP (SK3; BD Biosciences). Perforin was detected by intracellular stainings with perforin-FITC (6G9) and isotype control (clone 27-35; BD Pharmingen). Cells were acquired by flow cytometry on a FACSCalibur instrument (BD Immunocytometry Systems) and analyzed with CellQuest (BD Biosciences) or FlowJo (Tree Star) software systems. The indicated percentage of CD4⁺CD28null and CD8⁺CD28null T cells was calculated as the frequency of CD28⁺ cells in the gated CD3⁺ CD4⁺ or CD3⁺ CD8⁺ populations, respectively. Separate immunostainings including CD4 and CD8 Abs verified that the presence of double positive T cells did not have any significant impact on the CD4⁺ or CD8⁺ populations.

Pure CD4⁺CD28null, CD4⁺CD28−, CD8⁺CD28null, and CD8⁺CD28− T cell populations for functional analyses were isolated from PBMC by flow cytometry on a MoFlo high-speed cell sorter (DakoCytomation) after immunostaining with the Abs from BD Biosciences (CD3-FITC, SK7, CD28-PE, L293), and CD4-allophycocyanin (SK3) or the Ab from Immunootech CD4-PE Cy5.5 (13B8.2; Beckman-Coulter).

Functional assays

Flow cytometry-sorted T cell populations were cultured at a density of 20,000 cells/well in 96-well plates (Nalge Nunc International) coated with anti-CD3 Abs (OKT-3) at concentrations 0, 0.1, and 2 μg/ml. All cell cultures were performed in RPMI 1640 with 5% heat-inactivated human AB serum from the Karolinska University Hospital blood pool for 72 h, with the last 18 h in the presence of 0.5 mCi [³H]thymidine. Cells were harvested (Harvester 96; Tomtec), and proliferation measured by the levels of incorporated [³H]thymidine in a scintillation counter (Trilux 1450; Microbeta). Additionally, secreted IL-2, IL-4, IL-6, IL-10, TNF, and IFN-γ levels were measured in cell culture supernatants by using the Human Th1/Th2 Cytokine Cytometric Bead Array (CBA) kit (BD Biosciences).

Muscle biopsy tissue and immunostainings

Biopsy specimens were obtained from the vastus lateralis or tibialis anterior muscle by a “semi-open” technique under local anesthesia (21, 22). The samples were immediately frozen in isopentane chilled by liquid nitrogen and stored at −70°C.

For immunohistochemistry, 7-μm thick biopsy sections were placed on chrome gelatin-coated slides (Cell-Line Associates), air-dried and stored at −70°C. Frozen sections were fixed with acetone. Endogenous peroxidase activity was quenched by incubation in 1% H2O2 in PBS before blocking with 5% human sera. The presence of CD28null T cells in muscle tissue is preferably investigated by expression of a positive surrogate marker to allow a direct quantification and to reduce the risk for inclusion of recent activated T cells temporarily down-regulating CD28. Several reports have indicated that CD28null T cells acquire NK cell-related receptors (23, 24). As we found CD24 to be expressed by the CD28null subset, this molecule was used to detect CD28null T cells. The primary Ab (goat anti-human CD24; R&D Systems) and the isotype-matched control Ab (goat IgG; CalTag Laboratories) were diluted in 1% BSA in PBS. The secondary Ab (donkey anti-sheep/goat Ig-biotin; The Binding Site) was diluted in PBS and incubated before adding ABC standard kit (Vector Laboratories). For
development, dexamethasone (Vector Laboratories) was added. Sections were counterstained with hematoxylin.

For immunofluorescence stainings, frozen muscle biopsy sections were fixed with acetone and blocked by 5% pooled human serum in PBS. Sections were incubated overnight with the mix of primary Abs (goat anti-human CD244; R&D Systems, mouse anti-human CD4 clone SK3; BD Biosciences, and rabbit anti-human CD3; DakoCytomation) or isotype controls (goat IgG; Caltag Laboratories, and mouse IgG1 and rabbit IgG; DakoCytomation) diluted in 1% BSA (Sigma-Aldrich), 1% human serum, and 0.02% azide in PBS. In the second step, donkey anti-sheep/goat Ig-biotin (The Binding Site) was added. In the third step, the tissue was incubated with a mix of (avidin-Oregon Green 488; Molecular Probes, and antimouse IgG-Rhodamine Red-X and anti-rabbit IgG-AMCA; Jackson ImmunoResearch Laboratories), diluted in 1% BSA and 0.02% azide in PBS. Slides were mounted with Mowiol (Calbiochem). Double immunostainings with goat anti-human CD4 (R&D Systems) and mouse anti-human CD28 (Santa Cruz Biotechnology) and mouse anti-human CD28 were performed using the same protocol, and visualized by addition of donkey anti-sheep/goat Ig-biotin (The Binding Site) with avidin-Oregon Green 488 (Molecular Probes) or anti-mouse IgG-Rhodamine Red-X (Jackson ImmunoResearch Laboratories), respectively.

Stained tissue sections were examined with a Leica DM RXA2 microscope equipped with a Leica DC digital color video camera 300F connected to a PC computer.

The frequency of different T cell subsets in the muscle sections were quantified manually on coded samples by two complimentary methods (see below), and the mean value from two independent observers was used. Firstly, T cells were identified by expression of CD3. Coexpression of CD244 and CD3 was used to identify CD28null T cells. To exclude CD4dim macrophages/monocytes and NK cells which also might express CD244, CD4, with or without CD244, identified CD8 T cells were considered as T cells. Colocalization of CD3 and CD4 in the three-color stainings was used to identify CD4 T cells. To identify CD8 T cells in the very same section, a lack of CD4 in presence of CD3 was used. Morphologically identified cell structures in combination with membrane colocalized immunostainings of CD3 were considered as T cells. Colocalization of CD3 and CD4, with or without CD244, identified CD4 CD28null and conventional CD4 T cells, respectively. Expression of CD3, in the absence of CD4, with or without CD244 identified CD8 CD28null and conventional CD8 T cells. In three cases where we had additional muscle tissue with inflammatory infiltrates, CD4 T cells and CD8 T1 subsets were doublestained with anti-CD28 and the frequency of CD28negative cells was estimated in infiltrates containing nine CD3 or more cells.

ELISA
Sera from patients included in this study were tested for human HCMV-specific IgG and IgM in an enzymo-gnost anti-HCMV/IgG ELISA and an enzymo-gnost anti-HCMV/IgM ELISA (Dade Behring) according to the manufacturer’s instructions. Sera from all patients seronegative for HCMV were further analyzed for the presence of IgG against HCMV by an ELISA using HCMV clinical isolate obtained from blood samples from a kidney transplant patient, as previously described (26). A control Ag was isolated from uninfected fibroblasts.

HCMV reactive assays
Response to HCMV-derived peptides was investigated by culturing 1 million PBMCs from five anti-HCMV IgG seropositive and four seronegative patients in the presence of 2 μg/ml pp65 (PepMix pp65 HCMVA; JPT) or immunodominantly presented pp65 in the context (PepMix IE-1 HCMVA; JPT). Influenza A Ag (PepMix, membrane protein M1; JPT), medium only, or 200 ng/ml Staphylococcus aureus enterotoxin B (Sigma-Aldrich) were used as controls. In the last 4 h, brefeldin A (BD Biosciences) was added to a final concentration of 10 μM. Production of IFN-γ by CD4+ and CD8+ T subsets was analyzed by flow cytometry (CyaAn; DakoCytomation) after 12 h of stimulation. The Abs used were CD3-Pacific blue (UCHT1), IFN-γ-PEI (DB-1), CD8-PerCP (SK1), CD8-PerCP (SK1), CD14-allophycocyanin Cy5 (MpdP), and CD28-allophycocyanin CD28.2, all from BD Biosciences.

Statistical analyses

Kruskal-Wallis nonparametric ANOVA was used to compare the frequency of CD4 CD28null and CD8 CD28null T cells in peripheral blood from healthy controls, patients with dermatomyositis, and patients with polymyositis. All pair-wise comparisons were adjusted for using Dunn’s test for multiple comparisons. Correlations between the populations of CD4 CD28null and CD8 CD28null T cells, as well as the frequency of muscle-infiltrating CD8+ T cells and disease activity were investigated by two-tailed Spearman correlation test. Comparison of cell frequency of CD4 CD28null and CD8 CD28null T cells in peripheral blood in patients with dermatomyositis or polymyositis. PBMC from patients with dermatomyositis (DM), polymyositis (PM), or healthy controls (HC) were screened for the frequency of CD28null T cells in the CD4+ (a) and CD8+ (b) T cell population. The frequency of the CD4+CD28null and CD8+CD28null T cells correlates in patients with both dermatomyositis (c) and polymyositis (d). Linear correlation was tested by Spearman correlation test.

Results
CD28null T cells are more frequent in peripheral blood of patients with dermatomyositis and polymyositis compared with healthy subjects
To examine the presence and role of CD28null T cells in patients with dermatomyositis and polymyositis we have analyzed a cohort of 66 patients with varying age, disease duration, disease activity (VAS, 0–57), and treatment. The large number of patients and variation in clinical features allowed study of the contribution of CD28null T cells to different phases of these rare diseases.

Patients with both dermatomyositis and polymyositis displayed a significantly higher median frequency of circulating CD4+CD28null T cells, 9% (p < 0.001) and 4.1% (p < 0.05), respectively, compared with the healthy controls (1%) (Fig. 1a). The frequency in patients with dermatomyositis also tended to be higher compared with our previously published cohort of patients with rheumatoid arthritis (4.4%, p = 0.093 by Mann-Whitney U test) (27). The frequency of circulating CD8+CD28null T cells was significantly higher in patients with polymyositis, median 54% (p = 0.016), and the same tendency was seen for dermatomyositis (50%) compared with healthy controls (30%) (Fig. 1b). There was a significant correlation between the sizes of the circulating
CD8 only a minority of the CD28 of CD28null T cell in both CD4 analyzed for the expression of CD244. CD244 was expressed by a majority populations within the CD4 subsets from eight patients, the CD28null T cells were less prone to pro- and CD28null (null) CD4 contain perforin, and express CD244. Flow cytometry-sorted CD28null T cells from patients with dermatomyositis and polymyositis rapidly secrete TNF, in both CD4 and CD8 cells (median 98%, range 95–99%, n = 6; CD4CD28null and CD8CD28null T cells produced primarily TNF (Fig. 2, a and f), and from some patients also IFN-γ, but not IL-2, IL-4, IL-6 or IL-10 (data not shown). The response from the reciprocal CD28+ T cells was consistently lower. Proliferation was measured in the same experimental setup, where both the CD4CD28null and CD8CD28null T cells responded poorly compared with the CD28+ subsets (Fig. 2, b and g), supporting that the CD28null T cells are terminally differen- 

dicated that both CD4CD28null and CD8 CD28null T cells in patients with dermatomyositis or polymyositis possess effector functions, including secretion of proinflammatory cytokines and a potential to kill by cell-mediated cytotoxicity.

Muscle-infiltrating CD4+ and CD8+ T cells are predominantly of the CD28null phenotype

With the ambition to identify a positive surrogate marker for CD28null T cells we found CD244 to be expressed by 89% (range, 82–97%, n = 7) of the circulating CD4+ CD28null and 98% (range, 96–100%, n = 7) of the CD8+ CD28null T cells (Fig. 2, d and i). Only 3% (range 0–4%, n = 7) of the conventional CD4+CD28+ and 18% (range 5–39%, n = 7) of the CD8+CD28+ T cells expressed this molecule. CD3+ T cells, and not NK cells, constituted the major cell population expressing CD244. Strong correlations were observed between the frequency of T cells lacking CD28 in the CD4+ (Fig. 2e) and CD8+ (Fig. 2f) populations and the proportion of CD244+ cells expressing CD3, recorded in patients with significant CD4+CD28null and CD8+CD28null T cell subsets.

In the muscle tissue CD244 was mainly expressed by cells co-expressing CD3, and sometimes also CD4 (Fig. 3), indicating that the majority of the CD244-expressing cells are T cells, and not NK cells. Such CD3+CD4+CD28nullCD244+ and CD3+CD4nullCD28null T cells in both CD4+ (d) and CD8+ (i) populations, whereas only a minority of the CD28+ T cells expressed this molecule. The dot plots show results from flow cytometry analysis of PBMC from one representative of a total of seven patients. Percentages in the dot plots indicate the frequency of CD28null T cells that expresses CD244 and perforin.
CD28nullCD244+ T cells were present in biopsy samples from all investigated patients with polymyositis and dermatomyositis, and were often found in the proximity of non-necrotic muscle fibers, as well as adjacent to other infiltrating cells. The frequency of CD4+CD28null T cells, defined as CD3+CD4+CD28null cells in peripheral blood and CD3+CD4+CD244+ cells in muscle tissue, was substantially higher in the muscle in patients with dermatomyositis and polymyositis. The scale bar indicates 50 μm.

The increased frequency of CD4+CD28null and CD8+CD28null T cells has been described in peripheral blood of subjects with persistent viral infections, in particular HCMV (11, 28). We analyzed the presence of IgG and IgM Abs to HCMV in our cohort. Of the 66 screened patients, 48 (73%) were IgG seropositive and 12 (18%) were IgM seropositive. Circulating CD4+CD28null T cells were almost exclusively found in patients with HCMV seropositivity (p < 0.0001) (Fig. 4e). HCMV seropositive patients also had a higher frequency of CD8+CD28null cells compared with seronegative patients (p = 0.015) (Fig. 4d). Anti-HCMV IgM seropositive patients did not show further increased percentages of CD4+CD28null or CD8+CD28null T cell populations. Taken together, the increased frequency of circulating CD28null T cells in dermatomyositis and polymyositis was strongly associated with previous exposure to HCMV infection.

Circulating CD28null T cells are predominantly found in myositis patients seropositive for HCMV

The increased frequency of CD4+CD28null and CD8+CD28null T cells have been described in peripheral blood of subjects with persistent viral infections, in particular HCMV (11, 28). Therefore, we analyzed the presence of IgG and IgM Abs to HCMV in our cohort. Of the 66 screened patients, 48 (73%) were IgG seropositive and 12 (18%) were IgM seropositive. Circulating CD4+CD28null T cells were almost exclusively found in patients with HCMV seropositivity (p < 0.0001) (Fig. 4e). HCMV seropositive patients also had a higher frequency of CD8+CD28null cells compared with seronegative patients (p = 0.015) (Fig. 4d). Anti-HCMV IgM seropositive patients did not show further increased percentages of CD4+CD28null or CD8+CD28null T cell populations. Taken together, the increased frequency of circulating CD28null T cells in dermatomyositis and polymyositis was strongly associated with previous exposure to HCMV infection.
the inflamed muscles of patients with dermatomyositis and polymyositis. These cells were found at time of diagnosis as well as during later disease phase and correlated with clinical variables associated with the disease. CD28null T cell subsets were also enriched in peripheral blood in which they may constitute up to 50% of CD4+ and 96% of CD8+ T cells. Their presence correlated with HCMV seropositivity, and they responded to stimulation by HCMV Ags.

Accumulation of CD4+CD28null T cells in tissues have previously been described in the vessel walls in various inflammatory cardiovascular disorders and shown to correlate with clinical disease variables (29, 30). Despite these findings and the results in the present study, highly differentiated CD4+ T cells of the CD28null subset are not a general finding in sites of chronic inflammation. We recently demonstrated that only sporadic CD4+CD28null T cells were present in synovial membrane and synovial fluid from patients with rheumatoid arthritis, despite increased frequency in peripheral blood (27).

The co-occurrence of both CD4 and CD8 CD28null T cells, has been suggested to be associated with HCMV-induced IFN-α production by plasmacytoid DC. Interestingly, plasmacytoid DCs and the IFN-α-induced protein MxA were frequently found in muscle tissue from patients with dermatomyositis and polymyositis (31, 32). Therefore, differentiation of T cells into CD28null could occur in the inflamed muscle due to local IFN-α production by resident plasmacytoid DCs. Even though a close link between HCMV and circulating CD28null T cells, these cells are not necessarily all specific for HCMV Ags. Fletcher et al. (28) demonstrated that repeated exposure to Ags from persistent pathogens can induce pathogen-specific CD28null T cells by HCMV-dependent bystander differentiation. In an autoimmune setting, like in dermatomyositis and polymyositis, persistent Ags might be autoantigens, as was recently suggested for patients with multiple sclerosis in whom CD4+CD28null T cells displayed reactivity to myelin basic protein (15).

CD28null T cells were also found in muscle biopsy sections from HCMV seronegative patients, indicating that HCMV infection is not a prerequisite for disease or local differentiation of T cells into the CD28null phenotype. In patients with dermatomyositis and polymyositis, autoantibodies with specificities to Ro-52/60 or myositis-specific histidyl-tRNA synthetase (Jo-1) are frequently found. Sera from IgG seropositive patients potently induced IFN-α by mononuclear cells in the presence of nuclear material from necrotic cells (32, 33). It is therefore plausible that autoantibody-dependent mechanisms could explain part of the IFN-α levels in the muscle, which in turn could drive a local differentiation of T cells into CD28null in the absence of, or concomitant with, HCMV. Although some of our patients from whom we investigated the infiltrates in muscle tissue had anti-Jo-1 or anti-Ro52 Abs, the number of patients is too small to allow any comparison between subgroups of patients according to autoantibody profiles.

The presence of CD28null T cells in muscle tissue, even at the time of diagnosis, indicates a contribution to disease manifestations. This is supported by the correlation between the frequency of CD8+ T cells in the infiltrates in patients with polymyositis and disease activity. Because the majority of CD8+ T cells in the muscle were of the CD28null phenotype, it is likely that CD8+CD28null T cells contribute to these effects. Although only limited numbers of patients with dermatomyositis were assessed for infiltration of T cells in the muscles, there was a tendency of a lower muscle CD4 to CD8 ratio in polymyositis compared with dermatomyositis. These results are especially interesting because the pathogenesis of polymyositis is believed to be mediated by CD8+ T cells (34–36). The frequency of circulating CD28null T cells in dermatomyositis

**FIGURE 5.** Circulating CD4+CD28null and CD8+CD28null T cells in patients with dermatomyositis and polymyositis decrease with disease duration. Results from multivariate regression analyses show that the frequency of CD4+CD28null T cells in peripheral blood decrease with increased disease duration (a). Also the frequency of circulating CD8+CD28null T cells decrease with disease duration (b), but independently increase with increasing age (c). Trend lines indicate the predicted frequency of CD28null T cells (upper lines) and HCMV- (lower lines) for HCMV-positive patients according to the statistical models for the CD4+CD28null and CD8+CD28null T cells subsets. In patients with polymyositis, the frequency of infiltrating CD8+ T cells in the muscle correlated with global disease activity score (VAS) (d).

**CD4+CD28null and CD8+CD28null T cells respond with cytokine secretion to HCMV Ags**

To further investigate the association between CD28null T cells and HCMV infection, we analyzed whether these cells displayed specificity for the HCMV-derived Ags pp65 and IE. As shown in Fig. 4f, both CD4+CD28null and CD8+CD28null T cells from the HCMV-positive patients (n = 5) responded predominantly to the pp65 Ags with IFN-γ production, whereas CD28null T cells from HCMV-negative patients (n = 4) did not (Fig. 4e), and neither did conventional CD28+ T cells from any of these patients. Neither CD4+CD28null nor CD8+CD28null T cell subsets responded to stimulations with influenza peptides, which served as an irrelevant virus Ag.

The frequency of circulating CD4+CD28null and CD8+CD28null T cells decreases with increased disease duration

Next we used multiple regression analyses to investigate how the frequency of circulating CD28null T cells was associated with clinical features. We found that the percentage of circulating CD4+CD28null and CD8+CD28null T cells was lower with longer disease duration (Fig. 5, a and b). However, this effect on CD8+CD28null T cells was partly compensated for by an increment with age (Fig. 5c). The clinical relevance of these cells was further supported by the striking correlation between global disease activity in polymyositis patients and the frequency of CD8+ T cells, predominantly CD28null, in the tissue infiltrates (r = 0.90, p = 0.01) (Fig. 5d).

**Discussion**

In this study we demonstrate that CD4+CD28null and CD8+CD28null T cells are present and dominate the T cell infiltrates of
and polymyositis decreased with disease duration. Possibly, this could be explained by homing to the muscle, supported by an increase of the percentage of CD8+/CD28null T cells in the tissue with longer disease duration. Although we cannot exclude that long-term immunosuppressive treatment also contributed to the reduction of circulating CD28null T cells, on a group level the frequency of CD28null T cells in the circulation and muscle tissue did not differ between untreated patients and patients with different therapies. In the absence of a specific surface marker for the CD28null T cells we used CD244 together with CD3 as surrogate markers for this T cell subset. Naturally there is a limitation in this as CD244 could also be expressed on NK cells. To circumvent this problem we used triple immunofluorescence microscopy including the T cell lineage marker CD3. Hereby, only T cells expressing CD244 were identified as CD28null T cells in muscle tissue. We further substantiated this strategy by performing some costainings with CD28 and CD4 or CD28 and CD8, which gave very similar results. Even in blood, the proportion of CD3+ cells in the CD244+ cell population was found to be highly dependent on the frequency of CD28null T cells. This reassured that this CD3+ CD244+ T cells in muscle tissue represented CD28null T cells.

We and others have previously shown that CD28null T cells from healthy donors and patients with other chronic inflammatory disorders display a distinct proinflammatory profile (13, 37). There are several possible pathogenic mechanisms for how CD28null T cells could harm the muscle fibers in dermatomyositis and polymyositis. In addition to release of cytotoxic granules containing perforin and granzyme B, CD28null T cells are also potent producers of TNF and IFN-γ which alone and synergistically exert cytotoxic effects and interfere with the contractile properties of muscle fibers (38–41). These proinflammatory cytokines are also important in HCMV reactivation, which further could sustain the inflammation (42). Additionally, IFN-γ and TNF may induce up-regulation of MHC class I or II Ags in muscle fibers which is a characteristic finding in patients with polymyositis or dermatomyositis, but not in differentiated fibers from healthy individuals (43). Expression of MHC molecules on muscle fibers could allow T cell recognition, and although this has been regarded unlikely because the costimulatory molecules CD80/86, normally required for functional interaction, are not expressed by inflamed muscle fibers (44, 45), Still, T cells independent of costimulation by MHC-expressing muscle fibers which is a core set measure for disease outcome assessment in adult and juvenile idiopathic inflammatory myopathies. I. Lundberg, C. Morrison, L. Oakley, I. Oakley, et al. 2001. Proposed preliminary core set measures for disease outcome assessment in adult and juvenile idiopathic inflammatory myopathies. J. Clin. Invest. 108: 1185–1194.

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