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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 may 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.
and healthy subjects gave informed consent. The same two and 100 is maximum disease activity) according to the suggestions by the time point as peripheral blood. The median age for the 41 healthy controls was 52 years (range 28 – 82).

Biopsy specimens were available from four dermatomyositis and nine polymyositis patients before blood sampling was considered as ongoing treatment. Muscle biopsy specimens were obtained from the vastus lateralis or tibialis anterior (y) duration (VAS) Treatment HCMV IgG HCMV IgM Frequency of CD4+CD28null (%) Frequency of CD8+CD28null (%) No. of infiltrates CD4:CD8 ratio Frequency of CD4+CD28null (%) Frequency of CD8+CD28null (%)

| Patient | Age (y) | Disease duration (y) | Disease activity (VAS) | Treatment | DM-1 43 6 30 Aza, Pred + − 22 57 2 9–10 5.3 41 75 16 24 1 50 4.6 4 9 10 |
|---------|---------|----------------------|------------------------|-----------| PM-1 73 0.25 30 None − + 11 85 3 15–36 1.7 86 70 4 73 2 21–40 1.9 57 97 21 91 3 34 1.1 65 67 2 |
| PM-2 61 0.4 50 Mtx, Pred − − 3 88 2 44–53 3.0 45 96 4 57 2 30–60 1.2 47 98 3 49 5 11–96 2.1 59 67 1 |
| PM-3 52 6 30 Mtx, Pred + − 4 49 5 11–96 2.1 59 79 3 54 4 20–40 1.3 49 96 3 57 2 60–120 1.9 57 67 1 |
| PM-4 49 9 37 CsA + − 3 84 1 44 1.2 87 88 1 49 5 30–60 1.1 59 67 3 49 5 11–96 2.1 59 67 1 |
| PM-5 61 0.5 40 None − − 3 84 1 44 1.2 87 88 1 49 5 30–60 1.1 59 67 3 49 5 11–96 2.1 59 67 1 |
| PM-6 70 1.25 45 Pred + + 17 73 2 154–178 1.1 63 76 1 49 5 30–60 1.1 59 67 3 49 5 11–96 2.1 59 67 1 |
| PM-7 57 0.7 50 Mtx − − 2 67 1 53 1.1 65 67 1 49 5 30–60 1.1 59 67 3 49 5 11–96 2.1 59 67 1 |
| PM-8 36 0 60 None + − 6 5 1 11 0.8 54 43 1 49 5 30–60 1.1 59 67 3 49 5 11–96 2.1 59 67 1 |
| PM-9 48 0 20 None − − 0.5 30 3 21–29 1.7 72 69 1 49 5 30–60 1.1 59 67 3 49 5 11–96 2.1 59 67 1 |

1 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.

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

3 CD4+CD28null and CD8+CD28null indicate the average percentage of the indicated T cell subsets in all infiltrates analyzed.

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

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

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 at diagnosis was 55 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 or in relapse, and 38 patients were treated with prednisone in combination with methotrexate (n = 16), azathioprine (n = 10), cyclosporine A (n = 4), azathioprine and anakinra (n = 1), or as monotherapy (n = 7). These patients were treated with i.v. Ig, and two patients were treated with mycophenolate mofetil. Immunoregulatory treatment the last 3 mo before blood sampling was considered as ongoing treatment. Muscle biopsy specimens were available from four dermatomyositis and nine polymyositis patients. Ongoing medication at time of biopsy is shown in Table I. When available, muscle biopsy samples were selected from the same time point as peripheral blood. The median age for the 41 healthy controls was 52 years (range 28–82).

Clinical assessment included the physician’s overall assessment of disease activity on a visual analog scale (0–100, where 0 is no disease activity and 100 is maximum disease activity) according to the suggestions by the International Myositis Assessment and Clinical Studies (20). The same two physicians evaluated all patients. This study was approved by the local Ethics Committee of the Karolinska University Hospital and all patients and healthy subjects gave informed consent.

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 CD28null cells in the gated CD3+ CD4+ or CD3+ CD8+ popula-

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 Immunotech CD4-PE Cy5.1 (15B8.2; 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 pooled human AB serum from the Karolinska University Hospital blood bank for 72 h, with the last 18 h in the presence of 0.5 mCi [3H]Httymidine. Cells were harvested (Harvester 96; Tomtec), and proliferation measured by the levels of incorporated [3H]Httymidine 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 immunohistochecmistry, 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 serum. 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 recently activated T cells temporally 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

The Journal of Immunology

4793
development, diaminobenzidine (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 Ig; 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 anti-mouse 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 Moviol (Calbiochem). Double immunostainings with goat anti-human CD4 (R&D Systems) and mouse anti-human CD28 (clone 204.12; Abcam), or goat anti-human CD8 (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 CD4 was used to identify CD28null T cells. To exclude CD4dim macrophages/monocytes and NK cells which also might express CD244 (25), the combination 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 CD4, 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+ and CD8+ T cells subsets were double-stained with anti-CD28 and the frequency of CD28-negative cells was estimated in infiltrates containing nine CD3+ T cells or more.

ELISA
Sera from patients included in this study were tested for human HCMV-specific IgG and IgM in an enzymo-n anti-HCMV/IgG ELISA and an enzymo-n 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 Ags prepared from 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 reactio ams
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 irrelevant (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) as controls. In the last 4 h, brefeldin A (BD Biosciences) was added to a final concentration of 10 μg/ml. Production of IFN-γ by CD4+ and CD8+ T subsets was analyzed by flow cytometry (CyAn; DakoCytomation) after 12 h of stimulation. The Abs used were CD3-Pacific blue (UCHT1), IFN-γ-FITC (DB-1), CD25-PerCP (SK7), CD8-PerCP (SK1), CD14-allophycocyanin Cy7 (Mop9), 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 by 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.

FIGURE 1. CD4+CD28null and CD8+CD28null T cells are increased 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
CD4⁺ CD28null and CD8⁺ CD28null T cells in patients with dermatomyositis and polymyositis (Fig. 1, c and d). The high frequency of both CD4⁺ CD28null and CD8⁺ CD28null T cells in patients with dermatomyositis and polymyositis implies common factors to be involved in the generation of CD28null T cells of both subsets. The size of each CD28null T cell population was generally stable over a follow-up period of up to 2 years (data not shown).

Circulating CD28null T cells in dermatomyositis and polymyositis rapidly secrete proinflammatory cytokines and contain perforin

The functional characteristics of CD4⁺ CD28null and CD8⁺ CD28null T cells from patients with dermatomyositis and polymyositis were analyzed with regard to cytokine secretion and proliferative capacity after in vitro stimulation. After 4 h of culture with plate-bound anti-CD3 Abs, CD4⁺ CD28null and CD8⁺ CD28null 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 CD4⁺ CD28null and CD8⁺ CD28null T cells responded poorly compared with the CD28⁺ subsets (Fig. 2, b and g), supporting that the CD28null T cells are terminally differentiated (10).

The cytotoxic potential of CD28null T cells was analyzed by their intracellular storage of perforin. Perforin-containing T cells could be found in both the CD8⁺ and CD4⁺ T cell subsets. Almost all perforin-expressing CD4⁺ T cells were within the CD28null subset (median 98%, range 65–99%, n = 6; CD4⁺ CD28null 2%, range 0–3%, n = 7) (Fig. 2c). Also in the CD8⁺ population, the CD28null subset was clearly the dominating perforin-containing T cells (median 98%, range 95–99%, n = 7) compared with CD8⁺ CD28⁺ subset (median 5%, range 1–28%, n = 7) (Fig. 2h).

These data indicate that both CD4⁺ CD28null 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. 2e). 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 coexpressing 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⁺ CD28null/CD244⁺ and CD3⁺ CD4⁻ respectively. The frequency of CD28null T cells of the CD4⁺ (e) and CD8⁺ (j) subsets in peripheral blood correlate with the proportion of CD244⁺ cells comprised of CD3⁺ T cells. Pearson correlation test was used to analyze these correlations in 11 patients from whom PBMC were stained for CD244 and CD28 and analyzed by flow cytometry.
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+CD28− cells in peripheral blood and CD3+CD4+CD244+ cells in muscle tissue, was substantially higher in the muscle in comparison with the circulation (median 57% and 6%, respectively, p < 0.0001) (Fig. 4a). A negative correlation was found between the frequency of CD4+CD28null T cells and 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 = 0.0015) (Fig. 4d). Anti-HCMV IgM seropositive patients did not show further increased percentages of CD4+CD28null T cells compared with seronegative patients (p = 0.015) (Fig. 4d). Anti-HCMV IgM seropositive patients also had a higher frequency of CD8+CD28null T cells compared with HCMV seronegative patients (p = 0.0001) (Fig. 4e).

**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 and CD8+CD28null T cells from the anti-HCMV-positive patients (f) responded to either or both of the HCMV Ags, whereas no such response was seen for T cells from HCMV seronegative patients (e). Data from two representative patients of nine are shown.

indicate that CD4+CD28null and CD8+CD28null T cells are the dominating T cell subsets in muscle tissue in a majority of patients with dermatomyositis and polymyositis and that these cells could be present before clinical debut, during, and after conventional immunosuppressive treatment.
FIGURE 5. Circulating CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> T cells in patients with dermatomyositis and polymyositis decrease with disease duration. Results from multivariate regression analyses show that the frequency of CD4<sup>+</sup>CD28<sup>+</sup> T cells in peripheral blood decrease with increased disease duration (a). Also the frequency of circulating CD8<sup>+</sup>CD28<sup>+</sup> T cells decrease with disease duration (b), but independently with increasing age (c). Trend lines indicate the predicted frequency of CD28<sup>+</sup> T cells (upper lines) and HCMV<sup>+</sup> (lower lines) for HCMV-positive patients according to the statistical models for the CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> T cells subsets. In patients with polymyositis, the frequency of infiltrating CD8<sup>+</sup> T cells in the muscle correlated with global disease activity score (VAS) (d).

**CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> T cells respond with cytokine secretion to HCMV Ags**

To further investigate the association between CD28<sup>+</sup> 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<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> T cells from the HCMV-positive patients (<i>n</i> = 5) responded predominantly to the pp65 Ags with IFN-γ production, whereas CD28<sup>+</sup> T cells from HCMV-negative patients (<i>n</i> = 4) did not (Fig. 4e), and neither did conventional CD28<sup>+</sup> T cells from any of these patients. Neither CD4<sup>+</sup>CD28<sup>+</sup> nor CD8<sup>+</sup>CD28<sup>+</sup> T cell subsets responded to stimulations with influenza peptides, which served as an irrelevant virus Ag.

The frequency of circulating CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> T cells decreases with increased disease duration

Next we used multiple regression analyses to investigate how the frequency of circulating CD28<sup>+</sup> T cells was associated with clinical features. We found that the percentage of circulating CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> T cells was lower with longer disease duration (Fig. 5, a and b). However, this effect on CD8<sup>+</sup>CD28<sup>+</sup> 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<sup>+</sup> T cells, predominantly CD28<sup>+</sup>, in the tissue infiltrates (<i>r</i> = 0.90, <i>p</i> = 0.01) (Fig. 5d).

**Discussion**

In this study we demonstrate that CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> T cells are present and dominate the T cell infiltrates of 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. CD28<sup>+</sup> T cell subsets were also enriched in peripheral blood in which they may constitute up to 50% of CD4<sup>+</sup> and 96% of CD8<sup>+</sup> T cells. Their presence correlated with HCMV seropositivity, and they responded to stimulation by HCMV Ags.

Accumulation of CD4<sup>+</sup>CD28<sup>+</sup> 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<sup>+</sup> T cells of the CD28<sup>+</sup> subset are not a general finding in sites of chronic inflammation. We recently demonstrated that only sporadic CD4<sup>+</sup>CD28<sup>+</sup> 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 CD28<sup>+</sup> 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 CD28<sup>+</sup> 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 CD28<sup>+</sup> 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 CD28<sup>+</sup> 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<sup>+</sup>CD28<sup>+</sup> T cells displayed reactivity to myelin basic protein (15).

CD28<sup>+</sup> 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 CD28<sup>+</sup> phenotype. In patients with dermatomyositis and polymyositis, autoantibodies with specificities to Ro-52/60 or myositis-specific histidyl-IRNA synthetase (Jo-1) are frequently found. Sera from IgG seropositive patients potently induced IFN-α by mononuclear cells in the presence of natural 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 CD28<sup>+</sup> 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 CD28<sup>+</sup> 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<sup>+</sup> T cells in the infiltrates in patients with polymyositis and disease activity. Because the majority of CD8<sup>+</sup> T cells in the muscle were of the CD28<sup>+</sup> phenotype, it is likely that CD8<sup>+</sup>CD28<sup>+</sup> 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<sup>+</sup> T cells (34–36). The frequency of circulating CD28<sup>+</sup> T cells in dermatomyositis
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 co-stainings 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 the 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 myotoxic 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–muscle cell interaction, which has been regarded unlikely because the costimulatory molecules CD80/86, such as CD28null T cells, could be activated following costimulation-independent T cells in multiple sclerosis. Ann. Neurol. 24: 193–208.


