Longitudinal Peripheral Blood Lymphocyte Subsets Correlate with Decreased Disease Activity in Juvenile Dermatomyositis

Floranne C. Ernste, Cynthia S. Crowson, Consuelo Lopez de Padilla, Molly S. Hein, and Ann M. Reed

ABSTRACT. ***Objective.*** To determine the clinical characteristics and subsets of peripheral blood lymphocytes (PBL), which correlate with decreased disease activity in patients with juvenile dermatomyositis (JDM).

Methods. Peripheral blood mononuclear cells from 24 patients with JDM were collected at Mayo Clinic Rochester between 2007 and 2011. These were analyzed using fluorescence-activated cell sorting and flow cytometry. Clinical disease activity was determined by visual analog scales (VAS) collected in 2 consecutive visits and correlated with PBL subsets.

Results. The change in CD3+CD69+ T cells correlated with the change in global VAS scores. The change in HLA-DR- CD11c+ myeloid dendritic cells also correlated with the change in extramuscular VAS scores. There were trends toward decreased levels of HLA-DR- CD11c+ cells with decreased muscle and global VAS scores, but these did not reach significance. The change in HLA-DR- CD123+ plasmacytoid dendritic cells negatively correlated with the change in muscle VAS scores. Although not statistically significant, decreased levels of CD3-CD16- CD56+ natural killer (NK) cells and HLA-DR- CD86+ myeloid dendritic cells, and increased levels of CD16+CD56- NK cells, correlated with decreased VAS scores.

Conclusion. Changes in CD3+CD69+ T cells, HLA-DR- CD11c+ myeloid dendritic cells, and HLA-DR- CD123+ plasmacytoid dendritic cells are associated with improved clinical course in JDM and could be used as markers for disease activity, but findings need to be verified in a larger, independent cohort. Lack of significant differences among most of our PBL subsets suggests that lymphocyte phenotyping may be difficult to definitively correlate with disease activity in JDM. (First Release May 15 2013; J Rheumatol 2013:40:1200–11; doi:10.3899/jrheum.121031)

Key Indexing Terms:
PERIPHERAL BLOOD LYMPHOCYTE CELL SUBTYPES T CELLS
DENDRITIC CELLS NATURAL KILLER CELLS JUVENILE DERMATOMYOSITIS

Juvenile dermatomyositis (JDM) is a systemic autoimmune disease affecting the skin and muscle vasculature of children with an incidence of 3.2 per million per year[1,2]. The current model of pathogenesis describes a combination of environmental and genetic factors: immune complex deposition mediates complement activation through the membrane attack complex, upregulation of MHC class I, and inflammation in vessels and muscle[3,4,5,6,7,8,9]. CD4+ T cells, B cells, plasmacytoid dendritic cells (pDC), monocytes, and macrophages are arranged in a perifascicular and perivascular distribution leading to capillary thrombosis and occlusion[8,9,10,11]. Type 1 interferon-α and β also play a role in upregulation of MHC class I, mediation of dendritic cell maturation, and elaboration of proinflammatory cytokines and chemokines[12,13,14].

Perturbations in peripheral blood lymphocyte (PBL) subsets in JDM have been inconsistently reported in active and inactive disease. Fluctuations in T cell subpopulations have been reported in active JDM compared to healthy controls[15,16,17,18,19]. In addition, a higher absolute number and/or proportion of CD19+ B cells have been correlated with increased JDM disease activity compared to healthy controls with a decrement in B cells after remission[15,16,17,18]. In adults with dermatomyositis, the interferon-γ (IFN-γ)/interleukin 4 (IL-4) ratio in CD4+ cells may be a clinical marker of disease activity as levels increase after remission[18].

Natural killer (NK) cells constitute a subset of cells in innate immunity that exert cytolytic activity against

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Accepted for publication March 22, 2013.

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virus-infected, intracellular bacteria, or tumor cells, and produce proinflammatory cytokines and chemokines without antigen specificity or MHC restriction[20,21,22]. The role of NK cells extends beyond cytotoxicity and involves regulating the adaptive immune responses of T cells and dendritic cells[21]. NK cells are divided into NK cell subsets based on the expression of CD56 and CD16. The CD56–CD16+ NK cells constitute the main subset in peripheral blood; they efficiently kill target cells and secrete low levels of IFN-γ. CD3-CD56+CD16- NK cells (< 10% of peripheral blood NK cells) exhibit lower cytolytic activity and produce large amounts of IFN-γ and tumor necrosis factor (TNF) upon stimulation by proinflammatory cytokines and are present in lymphoid tissue[21,22,23]. Little is known about the role of NK cells in the pathogenesis of JDM, although increased numbers of CD56+ NK cells have been found in inflamed muscle tissue in JDM[24]. Patients with untreated JDM have decreased levels of CD3-CD16+, CD54+ (ICAM-1), CD56+ (NK), and CD3+CD8+ T suppressor cells, suggesting that these cells may be actively contributing to disease pathogenesis[25]. The purpose of our study was to determine which PBL subsets correlate with disease activity in JDM, in particular whether NK cells could potentially serve as an indicator of treatment response based on the myositis disease activity assessment visual analog scale (VAS) and Childhood Myositis Assessment Scale (CMAS) scores.

MATERIALS AND METHODS

The study population consisted of 24 patients who fulfilled the Bohan and Peter criteria for JDM and were seen at the Mayo Clinic in Rochester, Minnesota, USA, during the years 2007-2011[26,27]. The study was approved by the pediatric institutional review board committee, and patients or their parents gave informed consent.

Flow cytometric analysis and fluorescence-activated cell sorting (FACS) protocol for sample preparation. Frozen peripheral blood mononuclear cells were obtained from subjects during 2 visits a mean of 9 months apart, and analyzed by flow cytometry. Patients were not instructed to stop corticosteroids or other immunosuppression prior to the blood draws at either of the 2 visits. Venous blood was collected in sodium heparin cell preparation tubes. The manufacturer's protocol was used for processing blood from the patient's arm to the cells. Tubes were spun within 2 h of venipuncture to ensure that the highest quality of PBL was collected. Once the PBL were isolated, they were stored at –140°C. Cells were thawed in 37°C water bath and added to a 15 ml tube with 4 ml warmed RPMI-1640. Cells were spun for 4 min at 1200 rpm and were then resuspended in 500 µl of buffer. The temperature was the one considered most stable for the storage of PBL for assays that require high cell viability for downstream assays. An aliquot of 50 µl of cells was put into 8 wells of a 96-well polystyrene tissue culture plate. Cells were surface-stained with allophycocyanin, FITC, phycoerythrin, or phycoerythrin cyanin 5 (PC5)-conjugated monoclonal antibodies against CD3, CD4, CD8, CD16, CD62L, CD69, CD11c, CD14, CD16, CD19, HLA-DR, CD40, CD80, CD83, or CD86 (BD Pharmingen) and fixed with 2% paraformaldehyde in phosphate buffered saline. The CD3-69 staining was used to further classify T cell subsets and NK cells. Events (range 90–3984, median 825) were acquired immediately using a FACScan flow cytometer (Becton Dickinson). Extra cells were used to make color controls (100 µl per tube). Although each individual sample was not examined for cell viability, a subset of samples and controls was tested for viability using Trypan blue, and both cell groups were similar after freezing with cell viability between 50% and 75%.

Scores on the VAS. Several clinical outcome measures facilitate assessment of clinical response to treatment in patients with JDM. We used the CMAS, a validated core set activity measure used to assess muscle strength, functional capacity, and muscle endurance in patients with JDM[28,29]. The CMAS is endorsed by both the International Myositis Assessment and Clinical Studies Groups (IMACS) and the Pediatric Rheumatology International Trials Organization (PRINTO)[30,31]. The 100-mm VAS was used for disease assessment and included the physician global activity score, the patient/parent global activity score, and the muscle disease activity and extramuscular disease activity scores. Extramuscular disease activity scores included constitutional disease activity, cutaneous disease activity, skeletal disease activity, gastrointestinal disease activity, pulmonary disease activity, and cardiac disease activity. CMAS and VAS scores were obtained at visits 1 and 2 with the differences in the 2 scores correlated with changes in PBL subsets.

Statistics. Descriptive statistics including mean, median, and SD were used to summarize the demographic and clinical features of patients with JDM. Correlation analysis was performed using Spearman's rank order correlation methods and rho. Scatterplots demonstrating each correlation of VAS to change in percentage of PBL phenotype were examined, including linear regression lines to depict trends. A p value < 0.05 was considered statistically significant.

RESULTS

Patient demographics and clinical characteristics. The mean age was 9.5 years, with a minimum of 3 and a maximum of 19 years (Table 1). There were 15 females (63%) and 9 males (37%). Disease duration was a median of 0.2 years, with a minimum of 0 and a maximum of 5 years. Seventy-nine percent of the patients were white. At the initial visit, 42% of the patients were taking prednisone at a mean dose of 56 mg/day, 38% of patients were taking methotrexate (MTX), and 4% were taking mycophenolate mofetil (MMF). Eleven patients (46%) were not taking immunosuppressive treatment. The mean creatine kinase level was 603.6 U/l (SD 1256 U/l). Six of the 24 patients had positive antinuclear antibodies.

A summary of medication changes are as follows (Table 1): the majority of patients began treatment with steroids and MTX at Visit 2. Three patients began MTX in addition to steroids. The rest of the patients either started MMF, MTX, and/or steroids, or remained on the same treatments. Three patients did not receive treatment at either Visit 1 or Visit 2. There were no patients taking rituximab. The mean interval between visits 1 and 2 and the second blood draw was 9 months.

Subsets of PBL. The median of the percentages of PBL subsets at visits 1 and 2 are recorded in Table 2. Relevant subsets were the following: at Visit 1, the T cells (CD3+) were 61.5; at Visit 2, the median of T cells was 62.9. T helper cells (CD3+CD4+) were 39.4 at Visit 1 and 41.6 at Visit 2. The cytotoxic T cells (CD3+CD8+) were 13.6 at Visit 1 and 16.7 at Visit 2. The activated T cells (CD3+CD69+) at Visits 1 and 2 were 0.6 and 0.8, respectively. The B cells (CD3-CD20+) were 8.1 at Visit 1 and 11.0 at Visit 2. At Visit 1, the monocytes (CD3-CD69+)

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Table 1. Clinical characteristics of 24 patients with juvenile dermatomyositis seen at initial visit, grouped by magnitude of change in visual analog scale (VAS) scores.

Characteristic	Large Change in VAS*, n = 10	Minimal Change in VAS, n = 14	All Patients, n = 24	p
Age, mean (SD)	9.9 (5.9)	9.1 (4.8)	9.5 (5.2)	0.86
Female, n (%)	5 (50)	10 (71)	15 (63)	0.29
Race, n (%)				0.53
White	8 (80)	11 (78)	19 (79)	
Unknown	1 (10)	0 (0)	1 (4)	
Other	1 (10)	3 (21)	4 (17)	
MTX, n (%)	3 (30)	6 (43)	9 (38)	
MMF, n (%)	0 (0)	1 (7)	1 (4)	
Rituximab, n (%)	0 (0)	1 (7)	1 (4)	
Cyclosporine, n (%)	0 (0)	1 (7)	1 (4)	
Prednisone, n (%)	4 (40)	6 (43)	10 (42)	
Creatine kinase level, U/l, n = 7, n = 11, mean (SD)	1373.3 (1823.3)	113.7 (89.4)	603.6 (1255.9)	0.13
Global VAS score, mm, mean (SD)	59.8 (12.7)	18.6 (17.6)	35.8 (25.8)	< 0.001
Muscle VAS score, mm, mean (SD)	47.4 (29.3)	7.7 (16.1)	24.3 (29.7)	0.004
Extramuscular VAS score, mm, mean (SD)	41.9 (28.3)	13.6 (12.1)	25.4 (24.5)	0.019
Median CMAS score (min, max), n = 10, n = 12	52 (38, 52)	52 (32, 52)	52 (32, 52)	0.82
Change in global VAS score, mm, mean (SD)	–51.2 (11.3)	–7.1 (19.3)	–25.5 (27.4)	< 0.001
Change in muscle VAS score, mm, mean (SD)	–43.1 (26.7)	–3.6 (16.1)	–20.1 (28.7)	0.006
Change in extramuscular VAS score, mm, n = 9, n = 14, mean (SD)	–37.8 (26.9)	–8.8 (14.9)	–20.1 (24.6)	0.013
Time between 1st and 2nd visit, months, mean (SD)	11.4 (9.9)	7.6 (4.5)	9.2 (7.3)	0.38
Positive antinuclear antibodies, n = 9, n = 12, mean (SD)	3 (33)	3 (25)	6 (29)	0.68
Autoantibody, n = 8, n = 8, mean (SD)				
SS-A 60 kDa	1 (13)	0 (0)	1 (6)	0.30
SS-A 52 kDa	3 (38)	0 (0)	3 (19)	0.05
Smith	1 (17)	1 (14)	2 (15)	0.91
RNP	1 (17)	1 (14)	2 (15)	0.91
Jo-1	1 (17)	0 (0)	1 (8)	0.26
Chromatin	1 (17)	0 (0)	1 (8)	0.26
Medication changes				
None to prednisone and MTX	4	2	6	
Prednisone to prednisone and MTX	1	2	3	
Prednisone and MTX to MTX	1	2	3	
Prednisone and MTX to none	1	0	1	
Prednisone, MTX, and MMF to MTX	0	1	1	
MTX to MTX and HCQ	0	2	2	
Prednisone to prednisone and MMF	1	0	1	
MTX to MTX and prednisone	1	0	1	
Prednisone, MTX, and other to no change	0	1	1	
None to MTX	0	2	2	

* Defined as a decrease of 40 or more mm on a 100 mm scale in global, muscle, or extramuscular VAS between visits 1 and 2. VAS: visual analog scale; CMAS: Childhood Myositis Assessment Scale; MTX: methotrexate; MMF: mycophenolate mofetil; HCQ: hydroxychloroquine.

were 0.7, and at Visit 2, they were 0.5. At Visit 1, the plasmacytoid dendritic cells (CD123+) were 12.5; at Visit 2, they were 15.2.

There appeared to be 3 NK subsets in our patients with JDM (Table 2): CD16+CD56+, CD16–CD56+, and CD16+CD56–. However, the presence of CD16+CD56– NK subset could not be verified because we did not test for the NKp46, NKp30, CD 244, or CD161 markers. At Visit 1, the median percentage of CD16+CD56+ NK cells was 2.5; at Visit 2, it was 7.0. At Visit 1, the median percentage of CD16-CD56+ NK cells was 9.6; at Visit 2, it was 17.0. Finally, at Visit 1, the median percentage of CD16+CD56– was 3.9; at Visit 2, the median percentage was 4.2. There appeared to be a statistically significant increase in the proportion of CD16+CD56+ NK cells at Visit 2 compared to Visit 1 ($p = 0.033$).

Statistically significant results are shown in Figure 1. Figure 1 shows the change in VAS between visits and the change in percentage of PBL subsets in 24 patients. The change in the percentage of CD3+CD69+ T cells was positively correlated to the change in global VAS ($r = 0.43$, $p = 0.037$). There was also a positive correlation between the change in the percentage of HLA-DR- CD11c+ myeloid dendritic cells and change in extramuscular VAS score (r =

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Table 2. Median of percentages of peripheral lymphocyte subsets in 24 patients with juvenile dermatomyositis seen at Visit 1 and Visit 2.

Peripheral Lymphocyte Subsets	Visit 1, median (min, max)	Visit 2, median (min, max)	p*
T cells (CD3+)	61.5 (32.4, 88.5)	62.9 (26.6, 82.4)	0.82
T helper cells (CD3+CD4+)	39.4 (20.7, 62.8)	41.6 (15.6, 62.7)	0.94
Cytotoxic T cells (CD3+CD8+)	13.6 (1.1, 41.8)	16.7 (5.5, 42.7)	0.56
Activated T cells (CD3+CD69+)	0.6 (0.0, 2.2)	0.8 (0.0, 3.3)	0.49
B cells (CD3–CD20+)	8.1 (0.4, 43.2)	11.0 (0.6, 33.2)	0.56
Monocytes (CD3–CD69+)	0.7 (0.1, 6.0)	0.5 (0.1, 2.4)	0.12
pDC (CD123+)	12.5 (0.0, 38.6)	15.2 (2.5, 65.2)	0.17
NK cells (CD16+CD56+)	2.5 (0.0, 25.6)	7.0 (0.0, 24.8)	0.033
NK cells (CD16–CD56+)	9.6 (0.3, 35.9)	17.0 (2.3, 29.4)	0.14
NK cells (CD16+CD56–)	3.9 (0.0, 22.6)	4.2 (0.6, 32.8)	0.98
CD56+	16.0 (1.4, 55.5)	23.6 (2.9, 44.0)	0.026
CD11c+	1.5 (0.0, 16.4)	4.4 (0.0, 9.5)	0.96
CD123+ HLA-DR–	10.9 (0.0, 38.5)	13.4 (2.3, 65.2)	0.10
CD123– HLA-DR+	0.5 (0.0, 17.0)	0.7 (0.0, 10.0)	0.55
CD123+ HLA-DR+	0.5 (0.0, 7.6)	0.4 (0.0, 10.0)	0.66
CD3+CD20–	62.0 (32.4, 88.5)	65.5 (26.6, 82.3)	0.82
CD3+CD20+	0.2 (0.0, 1.4)	0.2 (0.0, 0.5)	0.44
CD3+CD4–	15.2 (3.3, 45.0)	19.8 (10.1, 45.8)	0.53
CD3–CD4+	1.5 (0.1, 24.6)	2.3 (0.0, 13.4)	0.20
CD3–CD8+	2.2 (0, 9.4)	2.7 (0, 8.6)	0.84
CD3+CD8–	40.9 (15.3, 65)	45.4 (18.2, 63.5)	0.79
CD3+CD69–	64.6 (30, 88.7)	62.6 (23.5, 85.1)	0.70
HLA-DR+ CD11c–	16.2 (0, 75.5)	17.3 (5.3, 56.9)	0.89
HLA-DR+ CD11c+	0.7 (0, 16)	1.1 (0, 6.5)	0.45
HLA-DR– CD11c+	0.8 (0, 14.6)	2.7 (0.0, 9.5)	0.46

* P values obtained from paired t-tests. NK: natural killer; pDC: plasmacytoid dendritic cells.

0.43, p = 0.040). The change in the percentage of HLA-DR- CD123+ pDC cells was negatively correlated with the change in muscle VAS (r = –0.44, p = 0.028).

Patients were grouped according to disease activity (active or inactive) at Visit 1. Inactivity was determined by a VAS < 20 mm on either muscle, extramuscular, or global scales. We plotted the change in these PBL subsets: CD3+CD69+ T cells, HLA-DR- CD11c+ myeloid dendritic cells, and HLA-DR- CD123+ pDC. Between visits 1 and 2 they were grouped by disease activity (Figure 2). We found no statistically significant differences in the change in the PBL subsets among active and inactive disease groups (p values for the change in CD3+CD69+ T cells, HLA-DR- CD11c+ myeloid dendritic cells, and HLA-DR- CD123+ pDC were 0.09, 0.21, and 0.13, respectively). Moreover, we plotted the PBL subsets at each visit by disease activity and found no statistically significant differences based on disease activity level (all p > 0.38; Figure 3). Hence the PBL subsets CD3+CD69+ T cells, HLA-DR- CD11c+ myeloid dendritic cells, and HLA-DR- CD123+ pDC cannot be used as predictors of disease activity.

We also looked at NK cell subsets CD16+CD56-, CD16+CD56+, and CD16-CD56+, grouped according to disease activity, active or inactive, between Visit 1 and Visit 2 (Figure 4). There was no statistically significant difference in the NK subsets based on JDM disease activity (p values for CD16+CD56- are 0.42, 0.60, 0.34; for CD16+CD56+ are 0.88, 0.59, 0.06; and for CD16-CD56+ are 0.71, 0.67, 0.49, for global, extramuscular, and muscle VAS, respectively). In addition, we looked at those NK cell subsets grouped by disease activity for both Visit 1 and Visit 2 (Figure 5). There was no statistically significant difference in subsets based on JDM disease activity (p values for CD16+CD56- are 0.43, 0.53, 0.08; for CD16+CD56+ are 0.50, 0.39, 0.66; and for CD16-CD56+ are 0.50, 0.57, 0.79, for global, extramuscular, and muscle VAS, respectively).

Although the results did not reach statistical significance, we noted a few trends. The change in the percentage of HLA-DR- CD11c+ myeloid dendritic cells was positively correlated to the change in muscle VAS scores (p = 0.078) and to the change in global VAS scores (p = 0.084). The change in the percentage of CD16-CD56+ NK cells and of HLA-DR- CD86+ myeloid dendritic cells (mDC) was positively correlated with the change in the extramuscular VAS scores in patients with JDM (p = 0.076, and p = 0.091, respectively). In addition, the change in percentage of CD16+CD56- NK cells was inversely correlated to the change in global VAS (p = 0.094).

Comparison of clinical characteristics of patients with largest change in VAS at Visit 1. The patients were grouped

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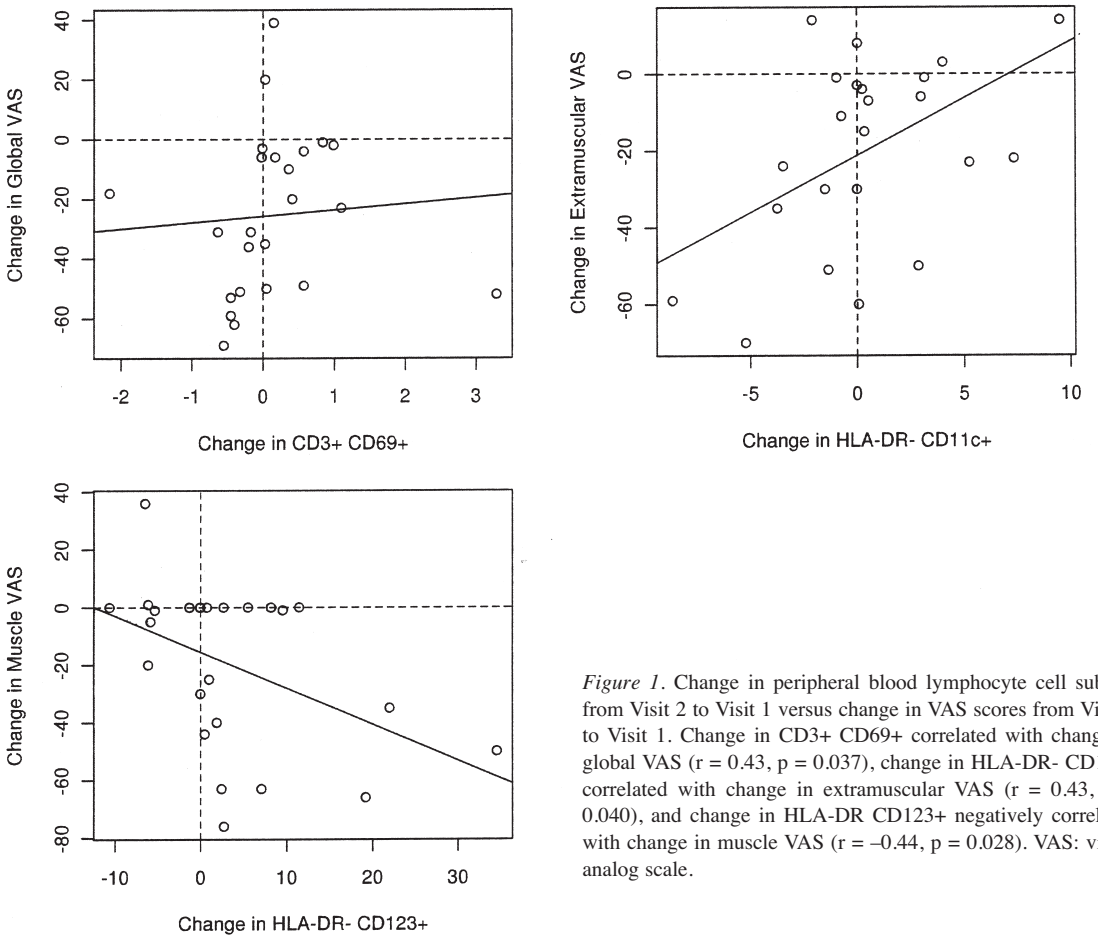

Figure 1. Change in peripheral blood lymphocyte cell subsets from Visit 2 to Visit 1 versus change in VAS scores from Visit 2 to Visit 1. Change in CD3+ CD69+ correlated with change in global VAS (r = 0.43, p = 0.037), change in HLA-DR- CD11c+ correlated with change in extramuscular VAS (r = 0.43, p = 0.040), and change in HLA-DR CD123+ negatively correlated with change in muscle VAS (r = –0.44, p = 0.028). VAS: visual analog scale.

according to the degree of change in VAS scores (Table 1): that is, large change (defined as < 40 mm or more in global, muscle, or extramuscular VAS between visits 1 and 2) and minimal change. Ten patients improved by ≥ 40 mm in VAS scores between visits 1 and 2. These patients had significant findings, with a change in PBL subsets and VAS. In general, there was not a significant difference in mean age or race. Fewer females were among the 10 patients (50% vs 71%). Also, fewer patients were taking MTX (30%), MMF (0%), and prednisone (40%) compared to the 14 patients who had minimal VAS change. The 10 patients who exhibited the largest change in VAS were taking a lower mean dose of prednisone (31 mg) at Visit 1 compared to the 14 patients with minimal change in VAS (73 mg), but prednisone use was not significantly different between the 2 groups. In addition, these 10 patients had a higher mean creatine kinase level (1373 U/l) at Visit 1 compared to the patients with minimal VAS change (114 U/l), suggesting a higher disease activity level or poorly controlled disease. There was no difference in median CMAS scores between the 2 groups. There was not a difference in antinuclear antibodies among the 2 groups. The following autoantibodies were collected: SS-A, SS-B, Smith, RNP, Scl-70, Jo-1, ribosomal P, and chromatin. There were no statistically significant differences in autoantibodies between the 2 groups with the exception of a borderline statistical significance in SS-A 52 kDa antibodies in the group with the higher VAS change (p = 0.05; Table 1). Myositis-specific autoantibodies were not collected in our patients. Finally, medication changes were not significantly different between the 2 groups; in general, most patients began a prednisone and MTX combination by Visit 2.

Comparison of phenotypic expression of peripheral lymphocyte subsets. There were no differences in the change in percentage of B cell or T cell subsets in the 10 patients with the largest change in VAS compared to the group with the minimal change in VAS.

DISCUSSION

This is the first prospective study, to our knowledge, to follow changes in PBL subsets of patients with JDM and to find an association of disease activity with the following: CD3+CD69+ T cells, HLA-DR- CD11c+ mDC, and HLA-DR- CD123+ pDC. Activated T cells, CD3+CD69+, correlated with increased global VAS scores: activated T cells decreased when JDM disease activity decreased. While

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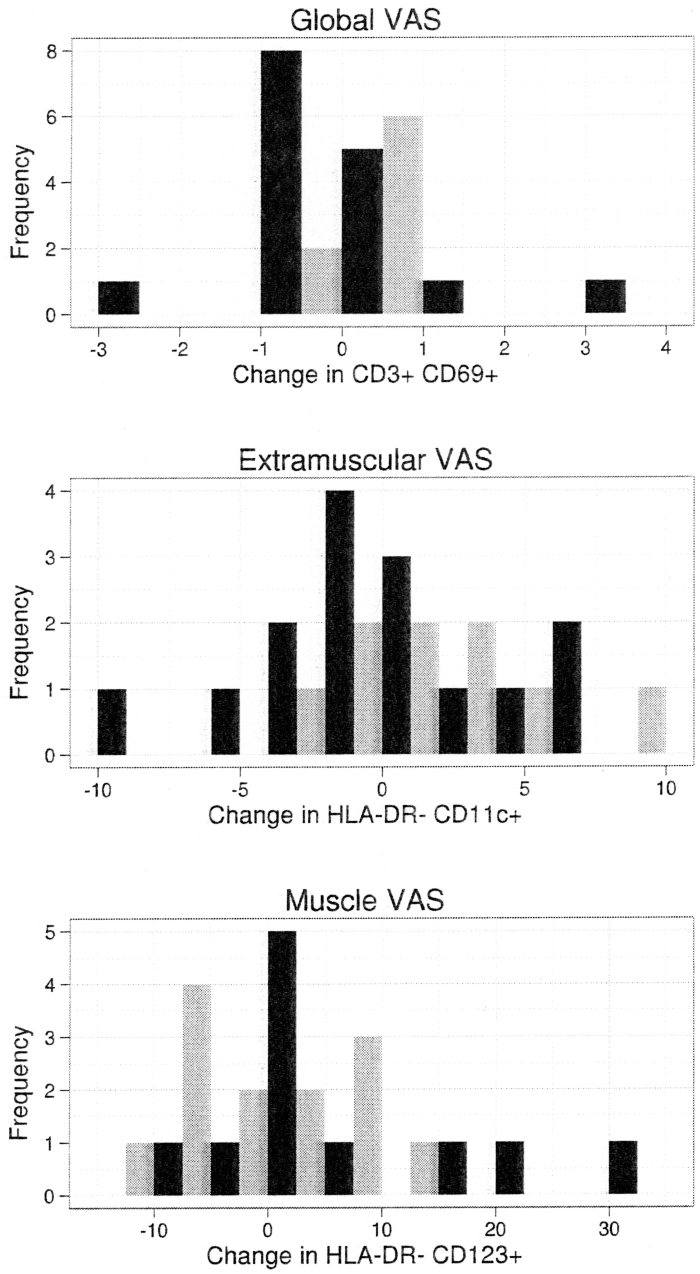

Figure 2. Change in peripheral blood lymphocyte cell subsets, CD3+ CD69+ T cells, HLA-DR- CD11c+ myeloid dendritic cells, and HLA-DR- CD123+ pDC, grouped by disease activity, active (black) or inactive (gray), between visits 1 and 2. There were no statistically significant differences in subsets based on JDM disease activity (p values from top to bottom are 0.092, 0.21, and 0.13). JDM: juvenile dermatomyositis; VAS: visual analog scale.

the role of T cells in JDM has not been fully clarified, we are in agreement with studies reporting a change in T lymphocytes in active JDM and DM[15,16,17,18,19]. Although humorally mediated mechanisms play a role in the pathogenesis of JDM and DM, both B and T cells interact and invade muscle tissue expressing MHC class I and class II antigens[16,17,18,32]. It is possible that as JDM disease activity declines, peripheral T cell populations may decrease[17,25]. Dendritic cells function as antigen-presenting cells to naive T cells, and they inhabit a costimulatory role to CD4+ T and CD8+ T cells[33,34,35,36]. We found a positive correlation with the change in the percentage of HLA-DR- CD11c+ mDC

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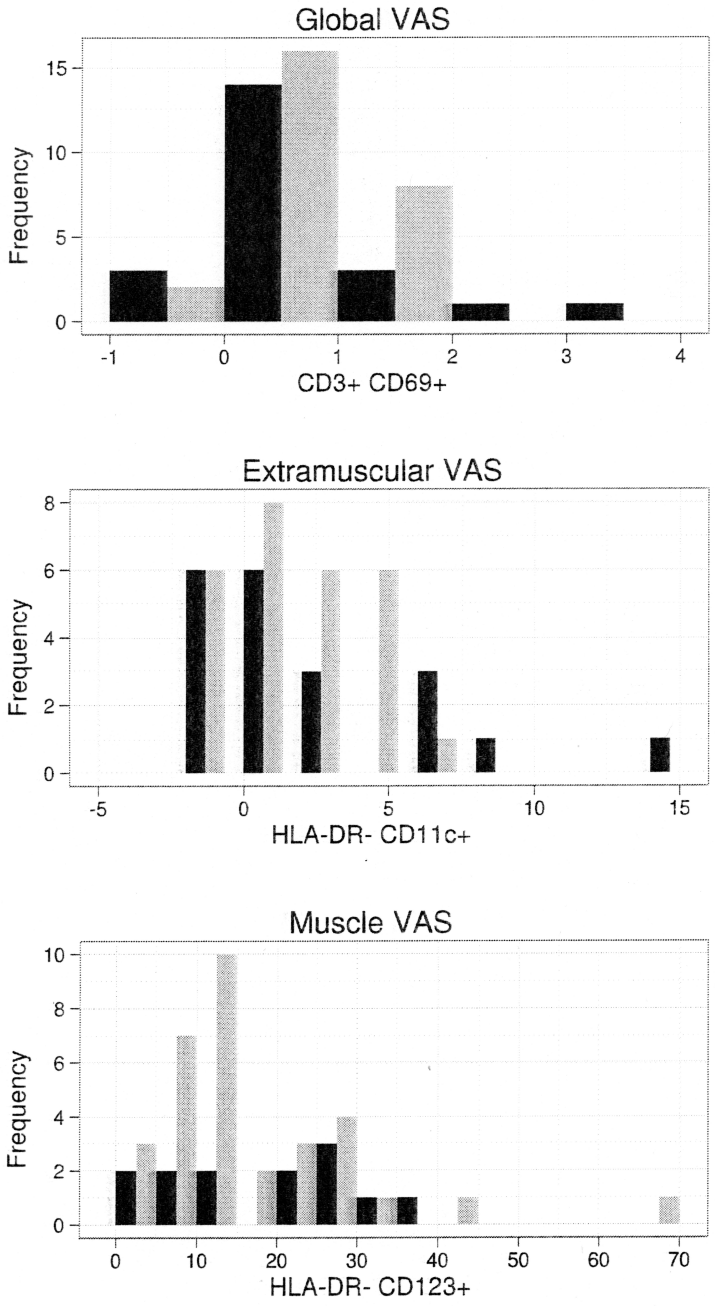

Figure 3. Peripheral blood lymphocyte cell subsets, CD3+ CD69+ T cells, HLA-DR- CD11c+ myeloid dendritic cells, and HLA-DR- CD123+ pDC, grouped by disease activity, active (black) or inactive (gray), for visits 1 and 2. There was no statistically significant difference in subsets based on JDM disease activity (p values from top to bottom are 0.60, 0.39, and 0.73). JDM: juvenile dermatomyositis; VAS: visual analog scale.

with increased extramuscular VAS scores: mDC decreased when JDM disease activity decreased. The significance of the mDC in the pathogenesis of JDM is not clear, but they may be involved in the innate immune response as noted by increased IFN-β production in response to Toll-like receptor triggers[33,34]. Capelletti, *et al* found more mDC in patients with JDM than in patients with DM, which suggests that type I IFN-mediated innate immunity plays a key role in JDM pathogenesis[33]. It is possible that mDC also may trigger an inflammatory infiltrate in muscle fibers in patients with polymyositis and inclusion body myositis[35]. Moreover, although it was not statistically significant, we found that

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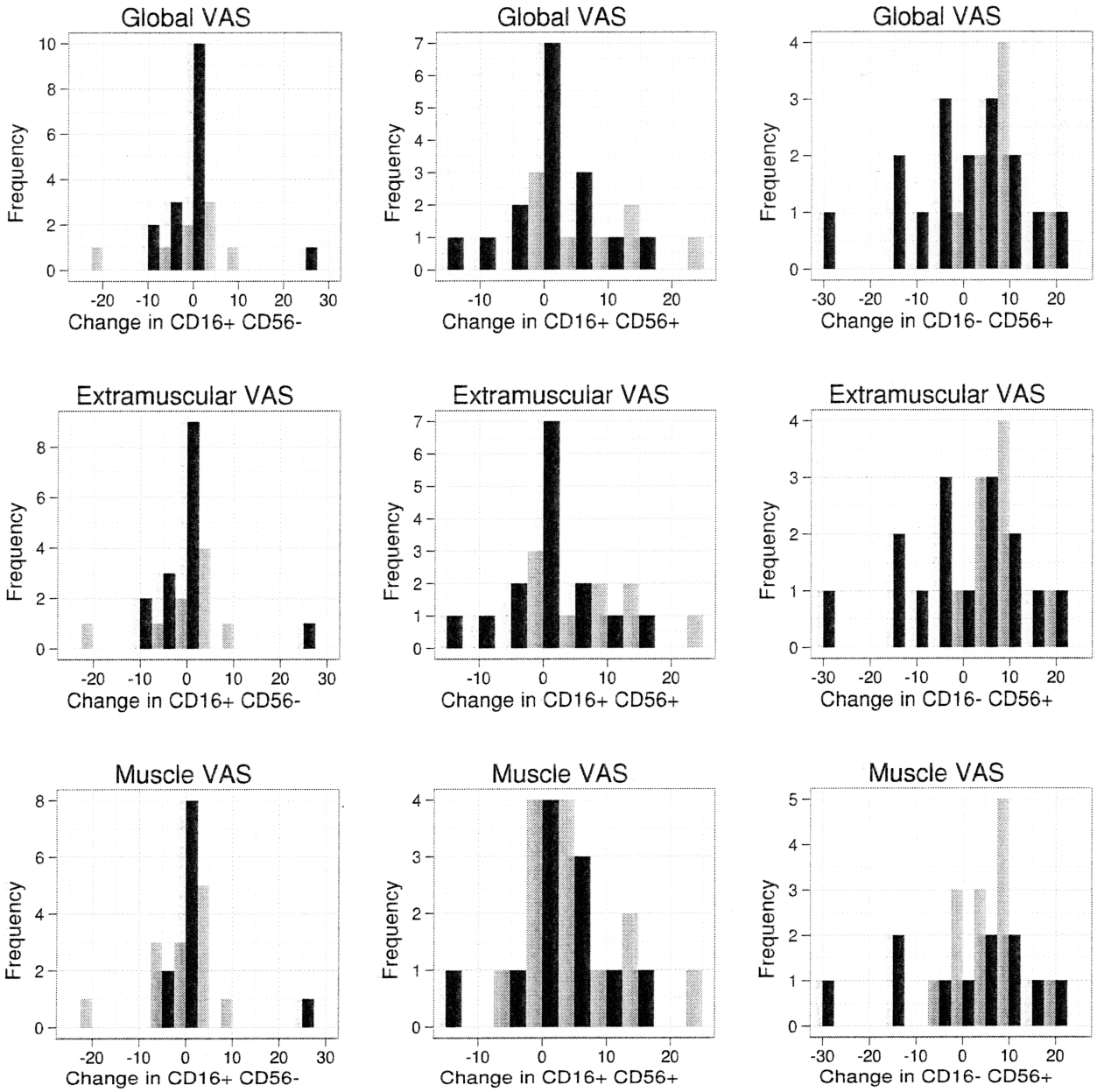

Figure 4. Change in natural killer cells, CD16+ CD56-, CD16+ CD56+, and CD16- CD56+, grouped by disease activity, active (black) or inactive (gray), between visits 1 and 2. There was no statistically significant difference in subsets based on JDM disease activity (p values from top to bottom for CD16+ CD56- are 0.42, 0.60, 0.34; for CD16+ CD56+ are 0.88, 0.59, 0.06; and for CD16- CD56+ are 0.71, 0.67, 0.49). JDM: juvenile dermatomyositis; VAS: visual analog scale.

the changes in the percentage of HLA-DR- CD11c+ mDC were positively correlated to the changes in muscle VAS scores. This seems to be in agreement with its direct correlation to the change in extramuscular VAS scores.

In addition, we found a negative correlation with the change in the percentage of pDC and the change in the muscle VAS scores: as muscle symptoms improved, the pDC increased. These cells have been implicated in JDM and DM pathogenesis because they produce a type I interferon response that may in turn trigger autoimmunity; they may also activate Th2 responses[8,36,37,38,39]. In our patients, we hypothesize that pDC may have been more active in lymphoid and/or muscle tissue during Visit 1, and as the disease course improved, the pDC may have migrated to the peripheral blood.

Although we found an association between JDM disease activity and some PBL subsets, in general, PBL subsets may be poor predictors of disease activity because of a lack of significant differences in PBL subsets grouped by disease activity level. The patients who showed the greatest change

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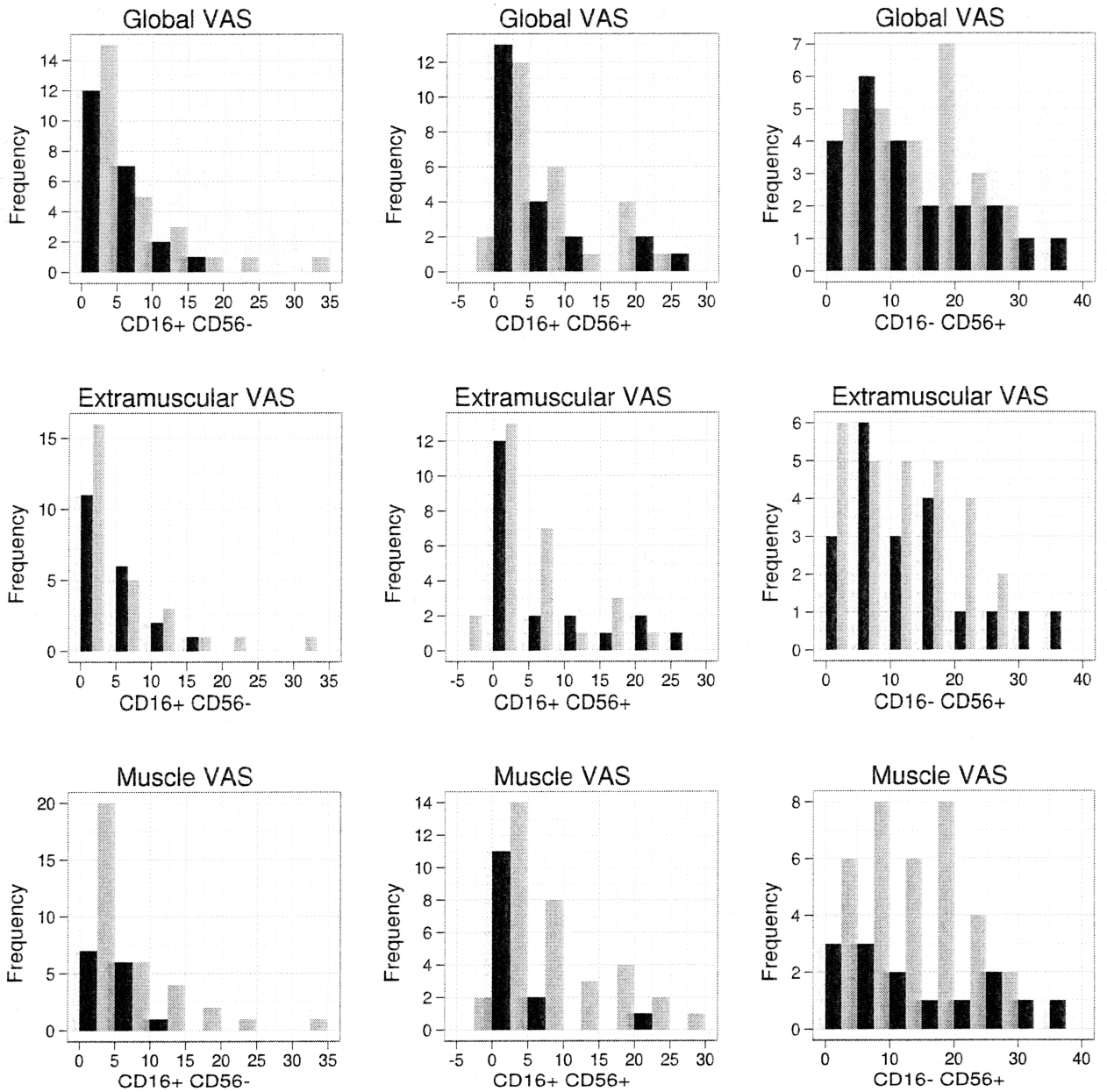

Figure 5. Natural killer cells, CD16+ CD56-, CD16+ CD56+, and CD16- CD56+, grouped by disease activity, active (black) or inactive (gray), for visits 1 and 2. There was no statistically significant difference in subsets based on JDM disease activity (p values from top to bottom for CD16+ CD56- are 0.43, 0.53, 0.08; for CD16+ CD56+ are 0.50, 0.39, 0.66; and for CD16- CD56+ are 0.50, 0.57, 0.79). JDM: juvenile dermatomyositis; VAS: visual analog scale.

in PBL subsets and VAS scores were those with higher disease activity at Visit 1, as noted by increased global, muscle, and extramuscular VAS scores. These patients also were taking fewer immunosuppressive drugs and lower prednisone doses. It is unclear why the patients with higher disease activity were taking fewer drugs and/or lower steroid doses at their initial visit; we can only speculate that they may have been intolerant of higher steroid doses or they may have had inadequate followup during their disease course. Hence, it may be that lymphocyte phenotyping is only clinically relevant in patients with JDM who have poorly controlled or severe disease rather than in those patients with stable or mild to moderate disease, but this is unclear and our findings would need to be verified in a larger, independent cohort.

A major concern of our study was that our patients were taking immunosuppressive therapy such as prednisone, which can alter lymphocyte subsets. Immunosuppressives selectively decrease T cell populations, although some studies have reported no significant differences in the PBL

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subsets in patients with active or inactive JDM and healthy controls with immune suppression, including prednisone use[40,41]. We did not see significant differences in PBL subsets between patients who were taking steroids and those who were not. It may be that peripheral blood T cell subsets decrease as a reflection of increased muscle fiber inflammation. O'Gorman and colleagues, although reporting a lower absolute T cell population in patients with early, active JDM, did find a higher T helper/T suppressor cell ratio in comparison to a healthy control group[15]. We did not find a significant difference in medication adjustments when we compared the proportion of patients who added a medication or increased a dose to patients who had no change in treatment, decreased a dose, or removed a medication, in the PBL subsets when grouped by active or inactive disease.

Of interest, we did not find statistically significant correlations between disease activity and NK lymphocyte subsets. However, we did find 3 NK cell phenotypes: the classic CD3-CD16+CD56+ NK cells, the CD3-CD16-CD56+ NK cell subset, and the CD16+CD56- NK cell subset. The CD3-CD16-CD56+ NK cell is a small subset of PBL (< 2%) in normal individuals; however, subsequent increases in the frequency of CD3-CD16-CD56+ have been described when using IL-2 treatment after bone marrow transplantation and cancer treatment using rIL-2, suggesting that IL-2 might enhance this CD56+ NK cell subset[42,43]. The cytotoxic function of the CD3-CD16-CD56+ NK cell appears to be diminished compared with CD3-CD16+CD56+ NK cells[42]. We speculate that higher percentages of this NK subset at Visit 2 in our study might be the result of activation of NK cells associated with the myositis treatment; however, further studies are needed to clarify the functional role of NK cell subsets. The role of NK cells in autoimmunity is extremely complex and some authors suggest that in addition to the pathogenic functions of NK cells in certain diseases, NK cells may also have a protective effect by inhibiting autoreactive T cell proliferation and activation as well as killing immature DC[21]. Finally, although we were not able to verify this subset with additional NK markers, there appeared to be a CD16+CD56- NK cell subset among our patients with JDM. NK cells are typically defined by expression of CD56; however, there exists an aberrant NK subset, CD16+CD56-, which may be found in patients with human immunodeficiency virus and a high viremic load; this NK subset appears to have poor cytotoxic function *in vitro*, and it has been speculated that the subset impairs the function of the total NK cell population[44]. Further study is needed to clarify the presence and function of this NK subset in patients with JDM.

Although it has been reported that B cells may correlate with DM disease activity[15,16,17,18,19], we did not see changes in the proportion of B cells with disease improvement. While this may be attributed to our small sample size, it has been demonstrated that lymphoid structures in the muscles of patients with JDM may be more relevant when correlating with severe disease activity[36,39].

A strength to our study is the rigorous use of flow cytometric analysis in patients with active JDM who improved prospectively in 2 consecutive visits as determined and correlated with validated instruments of disease activity (CMAS and VAS scores). However, there are several potential areas of concern. First, some patients were at different stages of their disease course, potentially influencing PBL subsets. Moreover, some patients had more disease activity than others, possibly affecting subpopulations at sampling[19]. We acknowledged this limitation by grouping patients according to disease activity level, active or inactive, and found no statistically significant differences in percentages of CD3+CD69+ T cells, HLA-DR- CD11c+ myeloid dendritic cells, and HLA-DR- CD123+ pDC. We also ran a separate analysis adjusting the significant correlations with disease duration and found that the correlations did not change; therefore, the significant relationships described in Figure 1 cannot be explained by disease duration. Also, our patient population was heterogenous and may have included children that could be classified as having overlap syndrome. In particular, there were 4 children who had a positive anti-Sm antibody or positive anti-RNP antibody. Although they had features of JDM clinically, there is a probability that these children may have skewed the PBL subset results. In addition, the viability of each individual sample was not analyzed when thawed; however, a subset of samples was compared to control samples, a procedure that suggested that cell viability was similar. Finally, a significant area of concern was that we did not compare PBL subsets to healthy controls because our focus was on the change in PBL populations related to JDM disease activity.

Declining peripheral levels of activated T cells and mDC may be correlated with reduced disease activity in JDM. Conversely, increasing levels of pDC may be correlated with reduced disease activity in patients with JDM. Our results will need to be verified in a larger, independent cohort of patients. Yet lymphocyte phenotyping may be difficult to use as a means for definitively correlating and/or predicting disease activity because there was a lack of significant differences among most of our PBL subsets in patients with high or low disease activity levels. Further, B cells and NK subsets in our patients did not correlate with JDM disease activity. More research will be needed to clarify the role of activated T cells, B cells, mDC, pDC, and NK subsets in the pathogenesis and disease course of JDM.

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