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Premature Cell Senescence and T Cell Receptor–Independent Activation of CD8+ T Cells in Juvenile Idiopathic Arthritis

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Objective. **CD8+ T cells lacking CD28 were originally reported to be a characteristic feature of juvenile idiopathic arthritis (JIA), but the relevance of these unusual cells to this disease remains to be elucidated. Because of recent evidence that loss of CD28 cells is typical of terminally differentiated lymphocytes, the aim of this study was to examine functional subsets of CD8+ T cells in patients with JIA.**

Methods. **Blood and/or waste synovial fluid samples were collected from children with a definite diagnosis of JIA (n = 98). Deidentified peripheral blood (n = 33) and cord blood (n = 13) samples from healthy donors were also collected. CD8+ and CD4+ T cells were screened for novel receptors, and where indicated, bioassays were performed to determine the functional relevance of the identified receptor.**

Results. **JIA patients had a naive T cell compartment with shortened telomeres, and their entire T cell pool had reduced proliferative capacity. They had an overabundance of CD31+CD28^{null}CD8+ T cells, which was a significant feature of oligoarticular JIA (n = 62) as compared to polyarticular JIA (n = 36). CD31+ CD28^{null}CD8+ T cells had limited mitotic capacity and expressed high levels of the senescence antigens histone γH2AX and/or p16. Ligation of CD31, which was independent of the T cell receptor (TCR), sufficiently induced tyrosine phosphorylation, vesicle exocytosis, and production of interferon-γ and interleukin-10.**

Conclusion. **These data provide the first evidence of cell senescence, as represented by CD31+CD28^{null} CD8+ T cells, in the pathophysiology of JIA. Activation of these unusual cells in a TCR-independent manner suggests that they are maladaptive and could be potential targets for immunotherapy.**

Juvenile idiopathic arthritis (JIA) is the most prevalent rheumatic disease of childhood, occurring before the age of 16 years (1). Oligoclonal T cells comprise a large proportion of joint infiltrates; some clones go into the circulation and persist for years (2). Clonotypes reactive to endogenous proteins or to pathogens have been reported, but clones that are diagnostic/prognostic of JIA or those common to all patients are unknown. Neither the driving force of T cell oligoclonality nor the identity of pathogenic clonotypes is known. Nevertheless, T cells play an important role in the pathophysiology of JIA because they are important sources of inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) that are the targets of biologic therapies (3). Some CD4+ T cells also have suppressive activity, the dysregulation of which is thought to contribute to the disease process of JIA (4). Hence, there is impetus to examine subsets of T cells and how they relate to JIA disease (1). Identification of culprit T cells could pave the way to cell-targeted therapies as an alternative to current broad-spectrum anticytokine biologic agents and synthetic pharmaceuticals.

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In the late 1990s, Wedderburn et al (5) reported that children with JIA have an overrepresentation of CD8+ T cells lacking CD28, the major costimulatory receptor required to sustain conventional T cell receptor (TCR)–driven activation. This observation has become increasingly significant, as more-recent studies demonstrated that $CD28^{null}$CD8+ T cells are biologic indicators of normal chronologic aging of the immune system (6). Furthermore, young adults with chronic inflammatory diseases, such as rheumatoid arthritis (RA), also carry disproportionately large numbers of similar $CD28^{null}$CD4+ T cells for their age, and the size of this CD4 subset has been correlated with the severity of RA (7).

Such differential representation of $CD28^{null}$ T cells in the CD8 and CD4 compartments in JIA and adult RA, respectively, is consistent with clinical and genetic evidence that these two diseases are separate entities, albeit with some documented similarity in the joint pathology (8). JIA does not become RA when the affected child enters adulthood. An exception, however, are patients diagnosed in adolescence (mostly girls) with rheumatoid factor (RF)–positive polyarthritis, which could be an early presentation of RA (9). Therefore, investigating the biology of $CD28^{null}$CD8+ T cells in patients with JIA will improve our understanding of the immunologic pathways unique to this childhood disease.

In healthy adults, $CD28^{null}$CD8+ T cells have shortened telomeres as compared to those on CD28+ cells (10), indicating a long replicative history consistent with somatic cells that are already in an advanced stage(s) of senescence. Such cells are nevertheless functionally active, and some of their effector functions have been attributed to other costimulatory receptors that replace the defunct CD28 (11). Since human $CD28^{null}$ T cells have a memory phenotype and classic memory T cell responses are less dependent on CD28 costimulation (12), we evaluated a provocative hypothesis that $CD28^{null}$CD8+ T cells in children with JIA represent a prematurely senescent subset with maladaptive function mediated by novel receptors in a TCR-independent manner. Plausibility of this suggestion could be deduced from animal models of lymphopenic states demonstrating low levels of TCR-independent cytokine-driven cellular activation in peripheral T cell homeostasis (13). We therefore screened for receptors other than traditional costimulators that are expressed on $CD28^{null}$CD8+ T cells in JIA.

PATIENTS AND METHODS

Study subjects. Research on human subjects was conducted in accordance with the Declaration of Helsinki, using protocols approved by Institutional Review Boards of the University of Pittsburgh and the Mayo Clinic. Children with JIA, with the consent of their legal guardians, were recruited from the rheumatology clinics of the University of Pittsburgh Medical Center (UPMC) Children's Hospital of Pittsburgh, and Mayo Clinic. Diagnosis and determination of oligoarticular and polyarticular JIA were based on the International League of Associations for Rheumatology revised clinical criteria for JIA (14). Following signed informed consent/assent, blood samples were obtained by routine venipuncture, and the patients' clinical data at the time of consent were abstracted from the medical record. In patients who underwent arthrocentesis as part of routine care prescribed by their rheumatologists, the waste synovial fluid (SF) was collected. In 8 patients, paired samples of SF and blood were obtained on the same day. Waste, deidentified peripheral blood samples from healthy children, and deidentified cord blood samples from uncomplicated births were also obtained from Laboratory Services of UPMC Children's Hospital, and UPMC Magee Women's Hospital Research Institute, respectively.

The clinical characteristics of the study subjects are summarized in Table 1. The JIA patients were of various ages at disease onset, had various disease durations, and were taking multiple medications. The age and sex distribution were comparable between the healthy subjects and the JIA patients. While not all of the patients had serologic data on record, half of the patients who had been tested were antinuclear antibody positive, and 2 of the patients with polyarticular JIA were RF positive. Some of the patients had uveitis, as indicated by the use of topical ocular steroids.

Flow cytometry. The phenotypes of T cells in peripheral blood mononuclear cells (PBMCs), SF, and cell cultures were determined by multicolor flow cytometry protocols established previously (6,15). Fluorochrome-conjugated antibodies (from BD, eBioscience, and BioLegend) to the classic T cell markers TCR$\alpha\beta$, CD3, CD4, CD8, and CD28, the activation antigens CD25 and CD69, and the adhesion molecule CD31 were used to identify T cell populations. To identify senescent cells, permeabilized cell preparations were stained with antibodies to p16 and/or histone γH2AX, 2 classic markers of senescence in somatic cells (16,17).

Telomeres were measured using the flow cytometry and fluorescence in situ hybridization technique (18). Analysis focused on CD45RA+CD4+ T cells, since CD45RA, as a naive cell marker, is reliable for CD4+ T cells, but not for CD8+ T cells (19). Quantum TM24 beads (Bangs Laboratories) with a quantitative range of fluorochrome molecules were used for cytometer calibration and to establish a standard curve for the assay according to the manufacturer's specifications. Telomere length was expressed as the mean equivalent soluble fluorescence (MESF), which was calculated from the following linear regression equation: MESF = (fluorescence units of the sample – fluorescence units of the blank)/slope.

All cytometry experiments included antibody isotype controls, cells stained individually with each indicated marker, and multicolor beads (Bangs Laboratories) for signal calibration and for offline calculation of compensation matrices.

Table 1. Characteristics of the study cohort*

	Healthy subjects,	Oligoarticular JIA patients		Polyarticular JIA patients	
	PBMCs	PBMCs	SF	PBMCs	SF
No. of samples	33	20	51	21	17
No. male/female	13/20	7/13	16/35	7/14	5/12
Mean age, years					
Boys					
At consent for study	6.1	9.4	10.4	9.4	10.4
At disease onset	–	9.4	10.9	12.5	9.3
Girls					
At consent for study	8.1	9.7	12.5	9.7	12.1
At disease onset	–	8.4	10.0	10.9	11.7
Mean disease duration, years	–	4.4	5.1	7.7	6.3
No. of medications taken†					
NSAIDs	–	20	45	21	17
Steroids, oral	–	2	3	10	6
Steroids, IA	–	17	43	13	15
Steroids, ocular (topical)	–	1	7	2	1
Steroids, IV	–	0	2	1	0
Methotrexate	–	8	19	21	12
Biologic agents	–	9	12	15	12
Other medications	–	0	5	1	7
Autoantibodies, no. positive/ no. tested					
ANA+	–	7/16	19/33	7/12	5/7
RF+	–	0/5	0/23	1/14	1/7
HLA–B27+	–	1/8	4/10	0/0	2/5

* Peripheral blood mononuclear cell (PBMC) and synovial fluid (SF) samples were obtained at different time points, except for 8 paired samples of PBMCs and SF from 6 patients with oligoarticular juvenile idiopathic arthritis (JIA) and 2 with polyarticular JIA (see Results). Clinical data from the latter 8 patients were entered only once. IA = intraarticular; IV = intravenous; ANA = antinuclear antibody; RF = rheumatoid factor.

† Total numbers of medications were determined by simple tally at the time of consent for study. Some patients were taking multiple medications. Nonsteroidal antiinflammatory drugs (NSAIDs) consisted of ibuprofen, naproxen, meloxicam, tolmetin, indomethacin, and diclofenac. Biologic agents consisted of etanercept, infliximab, and adalimumab. Other medications consisted of hydroxychloroquine and sulfasalazine.

Cytometry was performed on an LSRII cytometer (BD), and analysis of cell populations was done offline using FlowJo software (Tree Star). Cell populations were determined using previously described electronic gating strategies (6,15).

Cell activation assays. T cell activation assays were performed using primary CD31+CD8+ T cells purified from PBMC or SF samples (negative selection with Rosette Sep; StemCell Technologies). Jurkat and JRT3 cells (ATCC) were used as internal system controls. Activation bioassays with 2 μg/ml of anti-TCRα/β (1P26; BioLegend) or 5 μg/ml of anti-CD3 (OKT3; Centocor Ortho Biotech) followed established procedures (15,20). For stimulations using anti-CD31 (WM59; eBioscience) and recombinant CD38-Ig (R&D Systems), optimal concentrations of 1 μg/ml and 10 μg/ml, respectively, were empirically determined. Stimulating agents were either cross-linked in solution with species-specific anti-Ig or immobilized in tissue culture plates according to published procedures (15,20). All activation assays included Ig isotype and medium-only controls.

At the indicated time periods, cells were harvested and immunostained for CD25, CD69, phospho-tyrosine (4G10; Millipore), and granzyme/CD107a (BD). The latter 2 molecules were determined from permeabilized cells using Cytoperm reagent (BD). For granzyme detection, GolgiPlug/Stop reagent (BD) was added to cultures during the last 6 hours of incubation. Markers of cell activation were measured by multicolor flow cytometry as described above. The culture supernatants were also harvested, and the cytokine content was measured by the Luminex system (6,15).

Proliferation assays on primary T cells in PBMC or SF samples were performed using standard bioassays using 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE-DA) (6). Subsets of dividing or nondividing cells were identified by immunostaining for CD4, CD8, CD28, CD31, and histone γH2AX after 5 days in culture.

For the analysis of in vitro senescence cultures, purified CD28+ T cells from PBMCs were subjected to repeated stimulations with irradiated allogeneic PBMCs and Epstein-Barr virus–transformed lymphoblastoid cells, anti-CD3 (OKT3), and recombinant IL-2 (Proleukin; Chiron), as described previously (21). Cell phenotypes of the cultures were determined by flow cytometry 7 days after each stimulation cycle.

For analysis of T cell population doubling, cultures of T cells derived from PBMC or SF samples were monitored according to the procedure described by Koetz et al (22).

Statistical analysis. Data were analyzed nonparametrically using PASW Statistics version 18 software (SPSS). Data for each sample group were displayed as box-and-whisker median plots. Kruskal-Wallis analysis of variance (ANOVA) was used to determine differences between 3 or more groups, with post hoc comparison between any 2 groups examined by least squares difference or by Tukey's statistic. Curve-fitting ANOVA was carried out for repeated measures over a given time period, as appropriate. P values less than 0.05 were considered significant.

RESULTS

Intrinsic activated state and replicative pressure of T cells in JIA. The physiologic milieu in autoimmune disorders is considered an environment of persistent immune activation. Cytometric survey of cell phenotypes in blood samples from patients with JIA showed larger proportions of CD4+ and CD8+ T cells coexpressing the classic activation antigens CD25 and CD69 (range 5–15%) as compared to healthy controls (Figure 1A). JIA patients also had higher expression of CD25/CD69 (up to 35%) on SF T cells. Associated with this activated state, naive CD45RA+CD4+ T cells from JIA patients had significantly shorter telomeres than did those from either healthy children or cord blood controls (Figure 1B). The proliferative population-doubling capacity of the entire blood T cell compartment was also signifi-

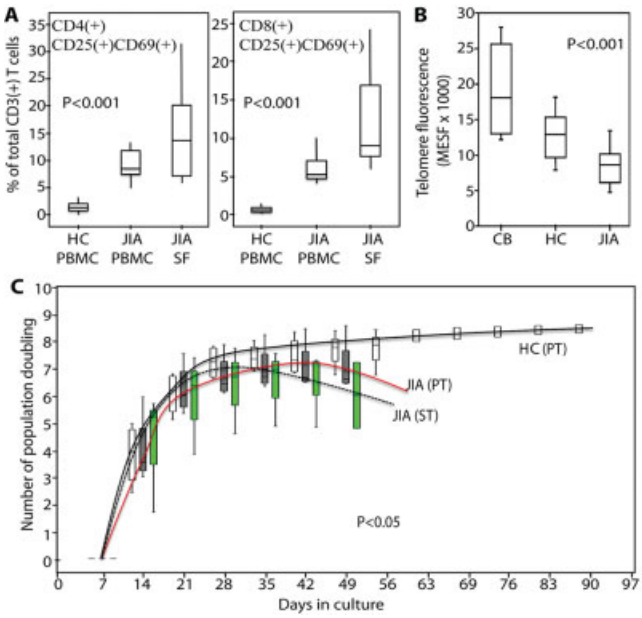

Figure 1. T cells from patients with juvenile idiopathic arthritis (JIA) have an activated phenotype and are under replicative pressure. **A,** Peripheral blood mononuclear cell (PBMC) and synovial fluid (SF) samples from JIA patients and PBMCs from healthy controls (HC) were analyzed by multicolor flow cytometry. Results are expressed as the percentage of CD25+CD69+ T cells in the CD4+ and CD8+ T cell compartments (n = 23 per group). **B,** Cord blood (CB) mononuclear cells, PBMCs from JIA patients, and PBMCs from healthy controls were analyzed by flow cytometry and fluorescence in situ hybridization. Results are expressed as the mean equivalent soluble fluorescence (MESF) units of telomere fluorescence in CD45RA+CD4+ T cells (n = 13 per group). **C,** T cells derived from PBMC (PT) and SF (ST) samples obtained from JIA patients as well as PBMC samples obtained from healthy controls were examined by population-doubling assay, according to the method of Koetz et al (22). Results are expressed as the number of doublings at 7-day intervals (n = 5 per group), and a fitted curve based on the complete data set of cell counts was constructed. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. *P* values were determined by Kruskal-Wallis analysis of variance (ANOVA) (**A** and **B**) or by curve estimation ANOVA (**C**).

cantly reduced among the patients (Figure 1C). Additionally, SF T cells had overall lower proliferative capacity than blood T cells, a property consistent with their more rapid turnover (23). All of these data indicate that T cells are under replicative pressure in JIA.

CD28 deficiency and CD31 expression on CD8+ T cells with limited mitotic capacity in JIA. The phenotypes of nondividing T cells were further explored under conditions of potent stimulation with allogeneic cells and anti-CD3. By standard CFSE-DA assay, >30% of JIA blood T cells remained CFSE-DA^{high}, indicating limited or no capacity for cell division (Figure 2A). In contrast, the vast majority (>98%) of blood T cells from healthy controls underwent mitosis, with a negligible number of CFSE-DA^{high} cells (Figure 2B). The mitotically restricted population was predominantly CD8+ T cells that lacked CD28, but expressed CD31, a molecule known to facilitate tissue invasion by normal granulocytes (24). Virtually all CD31+CD28^{null}CD8+ T cells expressed high levels of CD69, indicating full cellular activation, rather than quiescence, in response to stimulation (Figure 2A). These unusual CD8 T cells also

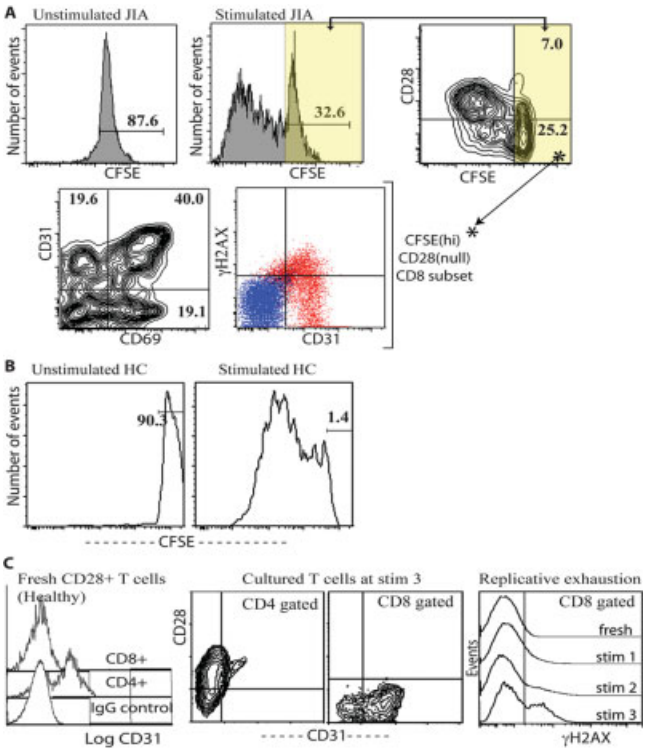

Figure 2. CD8+ T cells with restricted mitotic capacity obtained from patients with juvenile idiopathic arthritis (JIA) lack CD28, but express CD31 and histone γH2AX. **A** and **B,** Peripheral blood mononuclear cells (PBMCs) from JIA patients (**A**) and healthy controls (HC) (**B**) were analyzed by 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE-DA) assay. Results are representative of 7 experiments. CD3+ T cells that remained CFSE-DA^{high} (yellow area in **A**), were further examined, and it was shown that >90% were CD8+ T cells that lacked CD28 but expressed various levels of CD31, CD69, and histone γH2AX, depending on the donor. Histone γH2AX expression in CD4+ T cells was negligible. Numbers in each compartment are the percentage of positive cells. **C,** As an internal system control, phenotypes of purified CD28+ T cells from healthy controls were monitored through several rounds of stimulation (stim 1–3) using an in vitro senescence system (23). Results show the CD28, CD31, and histone γH2AX expression profiles after the third stimulation cycle and are representative of 10 experiments.

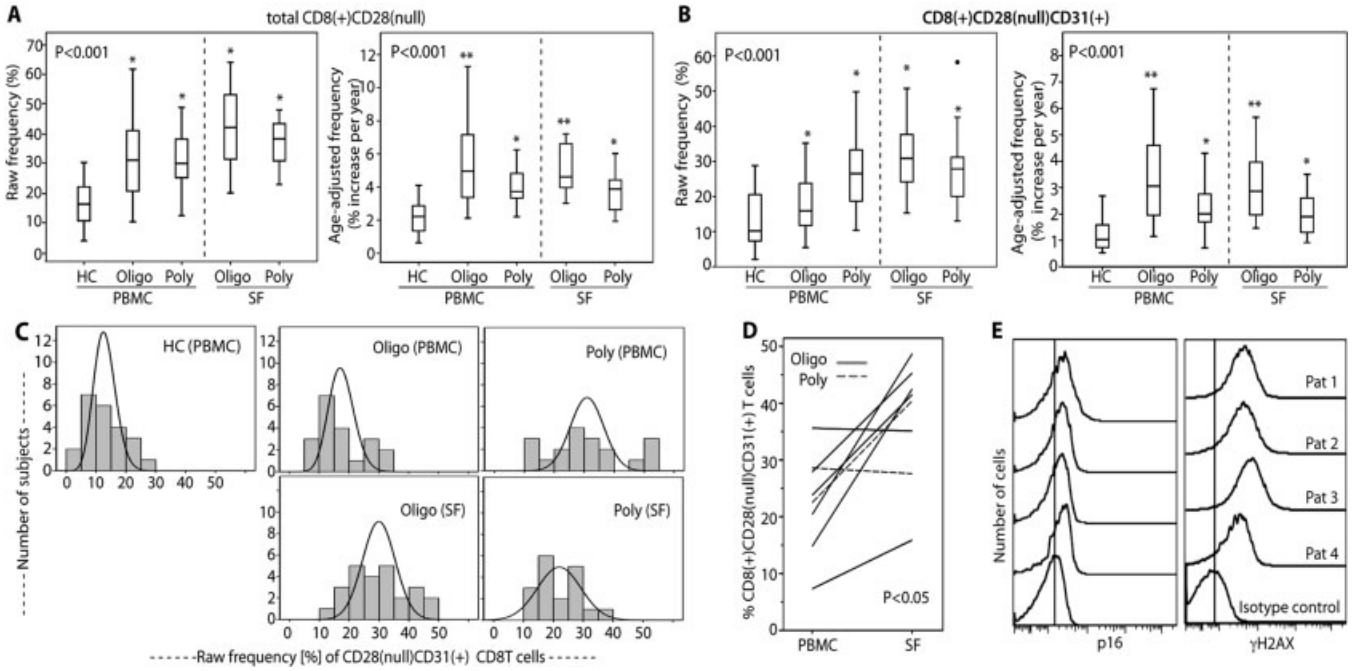

Figure 3. CD31+CD28^{null}CD8+ T cells with features of senescence are overrepresented in patients with juvenile idiopathic arthritis (JIA). Peripheral blood mononuclear cell (PBMC) and synovial fluid (SF) samples from JIA patients with oligoarticular (oligo) or polyarticular (poly) disease and PBMC samples from healthy controls (HC) were analyzed for T cell phenotypes by multicolor flow cytometry. CD28^{null} T cells (**A**) and CD31+CD28^{null} T cells (**B**) are expressed as a percentage of the CD8+ T cell compartment (n = 15–51 samples per group), both the raw frequency (left) and the age-adjusted frequency (right). Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. *P* values shown in **A** and **B** were determined by Kruskal-Wallis analysis of variance. $* = P < 0.05$ versus healthy controls; $** = P < 0.05$ versus healthy controls and polyarticular JIA patients, by Tukey's test. **C–E,** Frequency distribution patterns of CD31+CD28^{null}CD8+ T cells (**C**) (same data set as in **B**), cell frequency data from 8 patients with paired PBMC and SF samples (**D**), and cytograms of gated CD8+ T cells from representative patients (patients 1–4) coexpressing the senescence antigens histone γH2AX and p16 (**E**).

coexpressed histone γH2AX, a senescence antigen known to accompany telomere shortening (16).

As a system control, we also analyzed T cell populations in our previously established in vitro senescence system (21) to track phenotype changes in purified CD28+ T cells from healthy controls (Figure 2C). The parental CD28+ T cell culture had negative staining for histone γH2AX but had constitutive staining for CD31 on CD4+ T cells and negligible staining for CD31 on CD8+ T cells. After 3 rounds of repeated stimulation, many CD4+ T cells in culture retained CD28 but lost CD31. In contrast, most CD8+ T cells lost CD28 but gained CD31. Histone γH2AX was negligible in cultured CD4+ T cells but was expressed by ~30% of cultured CD8+ T cells (Figure 2C).

Collectively, these data showing a loss of CD28 expression, limitation of mitotic activity, and a gain of CD31/histone γH2AX expression among CD8+ T cells are consistent with cellular senescence.

Preponderance of CD31+CD28^{null}CD8+ T cells with features of senescence in JIA. PBMCs and SF samples from JIA patients and PBMCs from healthy controls were examined to determine whether similar T cells were found in vivo. Corroborating the report by Wedderburn et al (5), we found an abundance of CD28^{null}CD8+ T cells in blood samples from JIA patients as compared to healthy controls, with even higher proportions in the patients' SF samples (Figure 3A). Considering age-dependent loss of CD28 (6), the data were normalized for age and showed higher annual increases in CD28^{null}CD8+ T cells in both blood and SF from patients with oligoarticular JIA patients than in those with polyarticular JIA. Inclusion of CD31 expression in the cell population analysis also showed higher annual increases in CD31+CD28^{null}CD8+ T cells in patients with oligoarticular JIA (Figure 3B).

Inspection of the cell frequency distributions showed trends toward higher levels of CD31+CD28^{null}

CD8+ T cells in all patients as compared to the healthy controls (Figure 3C). In 8 patients for whom paired blood and SF samples were available (6 with oligoarticular JIA and 2 with polyarticular JIA) (Figure 3D), the lowest frequency of CD31+CD28^{null}CD8+ T cells corresponded to the high-end frequency for healthy controls. In addition, fresh CD8+ T cells from the JIA patients showed varying levels of histone γH2AX, with cases of coexpression of p16 (Figure 3E). In the CD4+ T compartment however, CD28^{null} cells, with or without CD31 expression, were found sporadically, and their frequency was not significantly different between healthy controls and the 2 JIA groups (see Supplementary Figure 1, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38015/abstract).

The prevalence of CD31+CD28^{null}CD8+ T cells was unrelated to disease duration in either the oligoarticular or the polyarticular JIA group. There was, however, an increasing, but not significant, trend toward association with disease duration when the patients were pooled (Figure 4A). Neither the age at disease onset (Figure 4B) nor the medications taken (Figure 4C) was related to the abundance of these cells regardless of whether the patients were examined separately as 2 subgroups or together as 1 group.

CD31-driven TCR-independent activation of CD8+ T cells. Bioassays of primary CD31+CD28^{null} CD8+ T cells from JIA patients showed that ligation of CD31 was sufficient to elicit protein phosphorylation within 5 minutes and at levels equivalent to those seen with ligation of the CD3–TCR complex (Figure 5A). CD31 ligation with antibody or with recombinant CD38, a known CD31 ligand (25), resulted in the induction of CD25 and CD69 (Figure 5B) as early as 30 minutes and the induction of granzyme and its cytolytic vesicle partner CD107a (Figure 5C) within 6 hours. CD31 ligation also induced high levels of interferon-γ and IL-10 in culture supernatants at concentrations comparable to those elicited by TCR/CD3 ligation (Figure 5D). TCR/CD3 ligation, but not CD31 ligation, led to TNFα production, indicating differential outcomes of CD31 and TCR/CD3 triggering. These results were recapitulated in an internal experimental system control using JRT3 and Jurkat cells (Figure 5E), 2 classic models of T cell activation (26). JRT3, a TCR/CD3-deficient somatic variant Jurkat cell, displayed only a CD31-driven response. Clearly, CD31 is a transducer of a cell-activating signal in a TCR/CD3-independent manner.

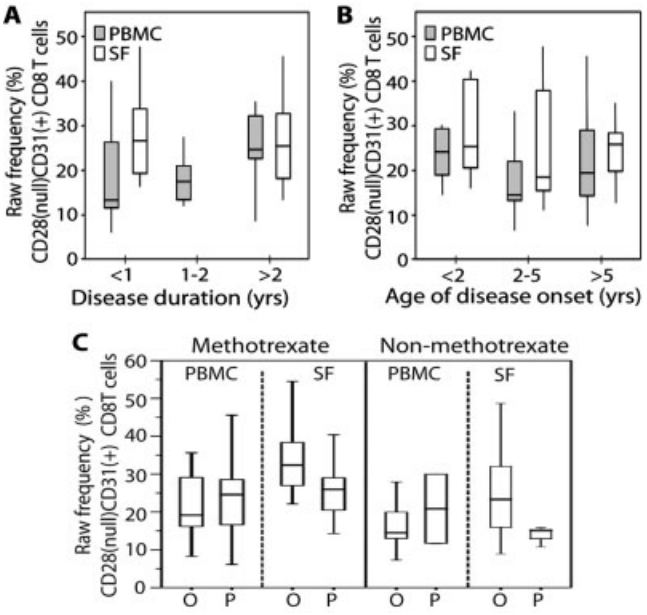

Figure 4. Frequency of CD31+CD28^{null} CD8+ T cells in patients with juvenile idiopathic arthritis (JIA) is unrelated to disease duration, age at disease onset, or medication. Cell frequency data in patients with oligoarticular (O) and polyarticular (P) JIA from Figure 3B (n = 15–51 samples per group) were plotted against **A,** the disease duration, **B,** the age at disease onset, and **C,** the medications taken. As depicted in **A**, there was a trend toward increasing CD31+CD28^{null} CD8+ T cell frequencies in peripheral blood mononuclear cell (PBMC), but not synovial fluid (SF), samples with increasing duration of disease, but this was not significant. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles.

DISCUSSION

The present study shows unequivocally that the loss of CD28 expression on CD8+ T cells is a property of JIA, corroborating the original Wedderburn report (5). Given that CD28 is normally sensitive to downmodulation during conventional TCR-driven cell activation (27), accumulation of CD28^{null}CD8+ T cells among JIA patients is predictable due to the prevailing immune-activated state in this disease. This is consistent with our previous findings about the derivation of CD28^{null} cells in vitro from precursor CD28+ cells cultured in TNFα, an effector cytokine in JIA (3), or those repeatedly stimulated in senescence culture systems (20,21). In vivo, loss of CD28 occurs progressively with age (6,15) and is thought to be a consequence of infection with common and/or persistent pathogens (28).

Here, we report that the overabundance of CD28^{null}CD8+ T cells in JIA is significantly dispropor-

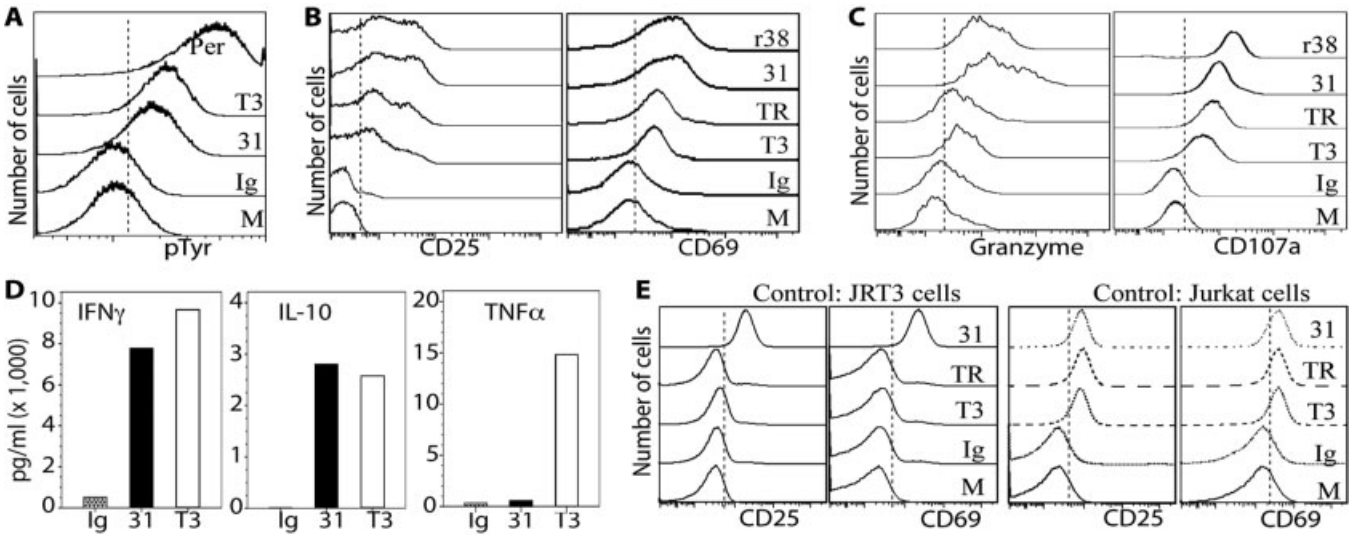

Figure 5. Ligation of CD31, independently of the T cell receptor (TCR), is sufficient to activate T cells. Peripheral blood mononuclear cell (PBMC) and synovial fluid (SF) samples from JIA patients that had been enriched in CD31+CD28^{null}CD8+ T cells were incubated in anti-CD3 (T3), anti-CD31 (31), IgG (Ig), medium (M), recombinant CD38-Ig (r38), or anti–T cell receptor α/β (anti-TCR$\alpha\beta$ [TR]). Shown are the induction of **A,** protein tyrosine phosphorylation (pTyr) within 5 minutes (hydrogen peroxide [Per] positive control), **B,** CD25 and CD69 expression within 30 minutes, **C,** granzyme and CD107a expression within 6 hours, and **D,** interferon-γ (IFNγ), interleukin-10 (IL-10), and tumor necrosis factor α (TNFα) production in culture supernatants after overnight incubation. Similar CD31-driven, TCR-independent activation was noted in **E,** JRT3 and Jurkat cells, which expressed constitutively high levels of CD31, as compared with controls. Results are representative of 5–10 independent experiments.

tionate with age. While we did not test for infection in our cohort since there was no medical indication for it, prevalence of these unusual cells is unlikely to be associated with infection with persistent pathogens, such as cytomegalovirus (CMV) that has been reported to cause perturbations in the T cell repertoire in adults (28). Repertoire studies in children with JIA and their healthy counterparts have shown no significant differences in the levels of CD28 expression or in the overall size of clonal populations, with or without exposure to CMV (29). CMV surveillance in the US also indicates that most Americans are exposed to the virus during very early childhood, with up to 80% already CMV seropositive by the age of 6 years (30). Thus, we suggest that the prevalence of CD28^{null}CD8+ T cells among children with JIA is truly characteristic of the disease state and is reminiscent of the age-disproportionate prevalence of CD28^{null}CD4+ T cells in young adults with RA (7). We also found a very low frequency of CD28^{null}CD4+ T cells in JIA, which is consistent with a previous report (31). This differential representation of CD28^{null} T cells in the CD8 and CD4 compartments in JIA and RA, respectively, is consistent with the consensus clinical opinion that JIA and RA are separate diseases (1).

We have reported that the loss of CD28 expression on T cells is generally irreversible due to transcriptional inactivation (11). CD28 loss is often associated with gains in expression of novel receptors, as we have shown in culture systems, in healthy adults of various ages as well as in adults with RA (15,21,32). Here, we show an unusually high level of expression of CD31 on CD28^{null}CD8+ T cells in patients with JIA, and the frequency of this cell subset was, again, highly disproportionate with age.

CD31 is a molecule present on granulocytes that facilitates their normal transmigration to sites of injury/inflammation (24). Kimmig and colleagues (33) also reported that CD31 is coexpressed with TCR excision circles (TRECs) in naive CD4+ T cells. CD31 then becomes down-regulated following activation of such naive CD4+ T cells and is irreversibly lost upon their subsequent conversion to memory cells. The present work confirms this observation. Our data show abundant constitutive expression of CD31 on fresh blood CD4+ T cells, with a loss after repeated stimulation of the CD4+ T cells. In contrast to the Kimmig report, however, our data show that CD31 is expressed sporadically and at low levels on blood CD8+ T cells but is gained at high levels by these CD8+ T cells after repeated activation.

Although TREC was not measured in this study, both our in vitro and in vivo data clearly show that CD31 expression on CD8+ T cells was predominantly among the $CD28^{null}$ T cells. Since $CD28^{null}$ T cells have a memory/activated phenotype (20), CD31 expression on CD8+ T cells in JIA may be attributed to a post-thymic activation event, which is in stark contrast to its constitutive expression on naive CD4+ T cells.

The age-disproportionate prevalence of $CD31+CD28^{null}CD8+$ T cells in both SF and blood from JIA patients indicates a role of these cells in disease pathophysiology. Our data clearly show higher annualized increases of $CD31+CD28^{null}CD8+$ T cells in patients with oligoarticular JIA as compared to those with polyarticular JIA. This observation corresponds to the overall higher annualized frequency increase of $CD28^{null}$ cells, regardless of CD31 expression, in oligoarticular JIA as well. These are unexpected findings, since oligoarticular JIA is generally considered a less severe disease subtype than polyarticular JIA (1). The prevalence of $CD31+CD28^{null}CD8+$ T cells was not significantly associated with disease duration, age at disease onset, or medications. A longitudinal analysis of a larger cohort of patients is needed to determine whether or not an abundance of $CD31+CD28^{null}CD8+$ T cells reflects overall stability of oligoarticular JIA or perhaps an outcome trajectory toward either a "persistent" or "extended" disease course of oligoarticular JIA (1), or if perhaps they are bioindicators of disease activity, irrespective of JIA subtype.

Whether the prevalence of $CD31+CD28^{null}$ CD8+ T cells is specific to childhood arthritis or is a general manifestation of a noninfectious chronic inflammatory state needs to be examined. Large populations of $CD28^{null}CD8+$ T cells have been found in some inflammatory conditions, such as ankylosing spondylitis (34), but not in others, such as psoriasis, except when there is concurrent CMV disease (29,35). Whereas the relevance of CD31 in these diseases has yet to be examined, an inflammatory state might not fully explain the emergence of these cells in vivo.

$CD31+CD28^{null}CD8+$ T cells represent lymphocytes in advanced stages of senescence. Whether they are derived in vitro or are naturally occurring in vivo, our data show that these cells express high levels of 2 cell senescence and mitotic inhibitory molecules, histone γH2AX and p16 (16,17). They also have limited or no mitotic activity, even under highly stimulatory conditions with allogeneic cells and anti-CD3 ligation. This lack of mitotic activity may not be attributed to anergy, since IL-2, a cytokine required for T cell proliferation and a protector from anergy (36), is a component of the stimulating milieu. Indeed, the culture microenvironment is one that favors activation, as indicated by the expression of CD69 on otherwise nondividing cells, since senescence is not quiescence (17).

It might be noted that Prelog et al (37) have argued that JIA has a "premature aging" phenotype, based on their single observation that T cells from patients have shorter telomeres than do those from healthy children. Controversy remains as to whether telomere shortening alone constitutes immune aging (38). Aging of the organism is also not synonymous with cell senescence (39). Nevertheless, our study reproduced the observation about telomere shortening reported by Prelog, but our study goes beyond this phenotype. Here, we provide evidence for the overall restriction of the proliferative capacity of the entire T cell compartment in JIA, as well as the expression of senescence antigens and the lack of mitotic activity of $CD31+CD28^{null}CD8+$ T cells. To our knowledge, these data constitute the first evidence of premature cellular senescence in JIA.

Despite their feature of senescence, CD31+ $CD28^{null}CD8+$ T cells are functionally active. CD31 serves as a TCR-independent immune receptor, in contrast to the classic costimulatory role of the defunct CD28. CD31 triggering alone sufficiently elicits the effector function of $CD31+CD28^{null}CD8+$ T cells, with equivalent magnitudes of cellular responses achieved by antibody ligation and by CD38, a natural ligand of CD31 (25). CD31 triggering alone induces the expression of granzyme, consistent with the original reports about T cell cytotoxicity as a pathogenic mechanism of JIA (40).

Our data also show that CD31 triggering is a potent inducer of IL-10, but not TNFα, which is more effectively elicited by TCR triggering. Whether such CD31-driven IL-10 production indicates regulatory activity of $CD31+CD28^{null}CD8+$ T cells similar to that ascribed to naturally occurring and inducible regulatory CD4+ T cells (41) needs to be examined further. It may be noted, however, that $CD28^{null}CD8+$ T cells have been reported to have a regulatory/suppressive activity in transplant settings in adults (42). It is not clear what the relevant subset is, but an inhibitory subset of $CD28^{null}CD8+$ T cells expressing CD56, rather than CD31, has been reported in a xenograft model of rheumatoid synovium derived from adults with RA (43). Although the relevance of such an inhibitory subset in vivo has yet to be examined, the latter report is consistent with emerging observations about differences in regulatory activity between CD4+ and CD8+ T cells, with IL-10–producing CD4+ T cells being a distinct

regulatory subset (41). However, CD4+ T cell–derived IL-10 also has well-documented immune stimulatory effects in addition to its regulatory/inhibitory effect especially in humans (41,44). Better understanding about the context in which T cell–derived IL-10 promotes or inhibits immune responses is needed.

While the biologic and/or clinical relevance of CD8+ T cell–derived IL-10 in JIA will have to be examined further, systemic IL-10 has been implicated in disease pathophysiology (45). There is also correlative evidence that improvement of clinical symptoms with steroid therapy is associated with lower plasma IL-10 levels (46). Of interest, therefore, is the source of systemic IL-10 and whether the preponderance of CD31+CD28^{null}CD8+ T cells might be linked to IL-10–regulated pathways of JIA. This is especially true for oligoarticular JIA, where certain IL-10 gene polymorphisms have been reported to distinguish between "persistent" and "extended" courses of oligoarticular disease (47). An important clinical consideration is whether or not CD31-driven IL-10 production reflects regulatory activity of CD31+CD28^{null}CD8+ T cells that influence the outcome of JIA.

In summary, we report here our finding of a preponderance of CD8+ T cells that lack CD28 and express CD31 in children with JIA. Our data show that CD31+CD28^{null}CD8+ T cells represent a cell subset in the advanced stages of senescence. This provides the first evidence of premature T cell senescence in children, which is consistent with our original thesis concerning the role of cell senescence in immune-mediated diseases (48). TCR-independent activation of these JIA T cells, akin to the situation in adult-onset autoimmune and chronic infectious diseases (49,50), is a form of immune dysregulation. Further elucidation of CD31-driven immune pathways and their influence on disease manifestations will provide insights into the improvement of therapy for JIA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. de Vallejo had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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