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# Immunophenotyping of Inclusion Body Myositis Blood T and NK Cells

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## Abstract

### Background and Objectives

To evaluate the therapeutic potential of targeting highly differentiated T cells in patients with inclusion body myositis (IBM) by establishing high-resolution mapping of killer cell lectin-like receptor subfamily G member 1 (KLRG1<sup>+</sup>) within the T and natural killer (NK) cell compartments.

### Methods

Blood was collected from 51 patients with IBM and 19 healthy age-matched donors. Peripheral blood mononuclear cells were interrogated by flow cytometry using a 12-marker antibody panel. The panel allowed the delineation of naive T cells (T<sub>n</sub>), central memory T cells (T<sub>cm</sub>), 4 stages of effector memory differentiation T cells (T<sub>em</sub> 1–4), and effector memory re-expressing CD45RA T cells (T<sub>emRA</sub>), as well as total and subpopulations of NK cells based on the differential expression of CD16 and C56.

### Results

We found that a population of KLRG1<sup>+</sup> T<sub>em</sub> and T<sub>emRA</sub> were expanded in both the CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subpopulations in patients with IBM. KLRG1 expression in CD8<sup>+</sup> T cells increased with T-cell differentiation with the lowest levels of expression in T<sub>n</sub> and highest in highly differentiated T<sub>emRA</sub> and CD56<sup>+</sup>CD8<sup>+</sup> T cells. The frequency of KLRG1<sup>+</sup> total NK cells and subpopulations did not differ between patients with IBM and healthy donors. IBM disease duration correlated with increased CD8<sup>+</sup> T-cell differentiation.

### Discussion

Our findings reveal that the selective expansion of blood KLRG1<sup>+</sup> T cells in patients with IBM is confined to the T<sub>emRA</sub> and T<sub>em</sub> cellular compartments.

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## Glossary

**ATG** = anti-T-lymphocyte globulin; **CPT** = cell preparation tube; **ENMC** = European Neuromuscular Centre; **FACS** = fluorescence-activated cell sorting; **HC** = healthy control; **hPBMC** = human peripheral blood mononuclear cell; **IBM** = inclusion body myositis; **IBMFRS** = Inclusion Body Myositis Functional Rating Scale; **KLRG1** = killer cell lectin-like receptor subfamily G member 1; **NK** = natural killer; **T<sub>cm</sub>** = central memory T cells; **T<sub>conv</sub>** = conventional T cells; **T<sub>eff</sub>** = effector T cells; **T<sub>em</sub>** = effector memory T cells; **T<sub>emRA</sub>** = effector memory re-expressing CD45RA cells; **T<sub>n</sub>** = naive T cells; **T<sub>reg</sub>** = regulatory T cell.

Inclusion body myositis (IBM) is an inflammatory autoimmune disorder of skeletal muscle resulting in progressive limb weakness and loss of function.<sup>1-8</sup> A direct cause of IBM muscle damage is T cell–mediated cytotoxicity of IBM myofibers, which was first described in seminal studies by Engel et al.<sup>9-13</sup> Light and immuno-electron microscopy studies have shown that myofibers are invaded by cytotoxic CD8 T cells with perforin granules oriented towards the muscle membrane and spike-like projections invading myofiber substance.<sup>9-13</sup> A large body of literature has noted the clonality of these T cells,<sup>14</sup> suggesting they are directed toward unknown muscle antigens.

Over the past decade, it has been recognized that IBM muscle-invading T cells are highly differentiated, expressing markers typical of highly differentiated T cells<sup>15-19</sup> such as killer cell lectin-like receptor subfamily G member 1 (KLRG1).<sup>17,19,20</sup> As the highly differentiated T-cell population is known to be relatively resistant to apoptosis and other mechanisms of immune cell control, as well as corticosteroids,<sup>21-24</sup> the treatment-refractory nature of IBM appears to relate to the abundance of this population of cells. However, it is not clear whether T cells expand ubiquitously across all T-cell subsets or if their expansion is confined to a particular subset.

The repertoire of functionally distinct T-cell subsets is largely described by naive, effector, and memory T cells. Naive T cells (T<sub>n</sub>) are most abundant in healthy individuals and are considered immunologically inexperienced (i.e., they have not encountered their cognate antigen). After antigen-induced activation, T-cell differentiation bifurcates into short-lived effector or memory T-cell pathways. Effector T cells (T<sub>eff</sub>) rapidly expand when they encounter their cognate antigen and contract within weeks following their initial activation. T<sub>eff</sub> play a critical role in promoting immunity through their helper or cytotoxic function. A subset of activated T cells differentiates into 2 populations of memory T cells—central memory T cells (T<sub>cm</sub>) and effector memory T cells (T<sub>em</sub>)—which are further subcategorized into 4 stages of differentiation (T<sub>em1</sub>–T<sub>em4</sub>). Chronic activation of T<sub>em</sub> is marked by re-expression of CD45RA, which is normally expressed in T<sub>n</sub>, thus named effector memory re-expressing CD45RA cells (T<sub>emRA</sub>). T<sub>emRA</sub> are resistant to apoptosis, retain high cytotoxic capacity, and secrete proinflammatory cytokines. T<sub>emRA</sub> and memory T cells play an essential role in immunologic memory and autoimmunity. The regulation of these populations in IBM and whether any express KLRG1 has not been defined.

To further understand the phenotype of cytotoxic T and natural killer (NK) lymphocytes and the regulation of T-cell subsets in IBM and their relationship to disease duration, we conducted a cross-sectional single-visit prospective study of patients with IBM and age-matched healthy participants. We collected blood for deep immunophenotyping by flow cytometry.

## Methods

### Standard Protocol Approvals, Registrations, and Patient Consents

Approval by an ethical standards committee on human experimentation for the experiments using human participants in this study was given by the Institutional Review Board at University of California, Irvine (HS 2019-5134). All participants provided written informed consent and Health Insurance Portability and Accountability Act authorization for data collection and to perform the research in this study.

### Patients

We identified all patients diagnosed with IBM seen at the UC Irvine neuromuscular clinic between July 1, 2019, and November 30, 2019. Patients who met the European Neuromuscular Centre (ENMC) 2011 criteria for a diagnosis of clinically defined or probable IBM were enrolled in this cross-sectional study. Baseline demographics including age, age at onset of disease, disease duration, and Inclusion Body Myositis Functional Rating Scale (IBMFRS) score were collected; there were no specific inclusion or exclusion criteria with respect to these demographics. To meet the inclusion criteria for the study, the patients with IBM could not be on immunotherapy at the time of the study and for at least 1 year prior to inclusion into the study (if there was a history of prior immunosuppressant use). Healthy controls (HCs) were also included in this study. The HCs were only enrolled if they were over the age of 40 years and had no history of neurologic or autoimmune disorders, cancer, or use of immunosuppressants (current or remote).

### Isolation of Human Peripheral Blood Mononuclear Cells

Approximately 8 mL of blood was collected into vacutainer cell preparation tubes (CPTs) containing sodium heparin (BD Biosciences) and processed within 24 hours. CPT tubes were centrifuged at room temperature for 30 minutes at

1,800 × g with no brake for the isolation of human peripheral blood mononuclear cells (hPBMCs). Following isolation, hPBMCs were washed, centrifuged, and resuspended in freezing media (50% Dulbecco's Modified Eagle Medium, 40% fetal bovine serum, and 10% dimethyl sulfoxide) at 3.0 × 10<sup>6</sup> cells/mL. Multiple 1-mL aliquots of hPBMCs were partitioned into cryovials and stored in liquid nitrogen for subsequent flow cytometry analysis. Samples were stored in liquid nitrogen between 1 and 5 months (panel 1) and 9 and 13 months (panel 2) prior to flow cytometry analysis (see the Table for a description of flow cytometry panels). Analysis of markers of T-cell subsets, including regulatory T cells, and T-cell differentiation in peripheral blood lymphocytes was done through multiplex flow cytometry.

### Flow Cytometry Analysis

All patient samples were processed simultaneously for flow cytometry analysis. A total of 3.0 × 10<sup>6</sup> cells were thawed in a

37°C water bath, transferred into 15 mL conical tubes, and washed with RPMI only. Viability was assessed by staining cells with Zombie-NIR viability dye (BioLegend) diluted in RPMI at 1:1,000. After a 15-minute incubation on ice while protected from light, cells were washed 3× with RPMI supplemented with 1% bovine serum albumin (fluorescence-activated cell sorting [FACS] buffer). After centrifugation (550× g), cells were resuspended with Fc blocking solution (FACS buffer and human TruStain FcX diluted 1:50; BioLegend), raised to a volume of 50 µL, and placed on ice for 15 minutes prior to staining. For cell surface staining, a 2× staining cocktail was prepared by diluting the antibodies listed in the Table in FACS buffer, and 50 µL of this 2× cocktail was added to the 50 µL of cells in Fc blocking solution to yield a final 1× staining solution. Staining was performed in the dark for 30 minutes on ice. Cells were then washed 3×, resuspend with FACS buffer, and filtered through a 70 µm nylon mesh prior to analysis. For samples involving the intracellular

**Table** Antibodies Used for Flow Cytometry

	Marker	Color	Vendor	Catalog number	Clone	Dilution
<b>Antibodies for panel 1</b>						
1	Viability	Zombie-NIR	Biolegend	423106	—	1:1,000
2	CD3	BUV395	BD Biosciences	564000	SK7	1:200
3	CD4	PerCP-Cy5.5	Biolegend	300530	RPA-T4	1:100
4	CD8	BV605	Biolegend	344742	SK1	1:200
5	Kv1.3	FITC	Alomone	APC-101-F	Polyclonal	1:50
6	KLRG1	PE-Cy7	ThermoFisher	25-9488-41	13F12F2	1:100
7	CD57	Pac Blue	Biolegend	359608	HNK-1	1:200
8	CD28	BV510	BD Biosciences	563075	CD28.2	1:25
9	Helios	PE	Biolegend	137216	22F6	1:25
10	FoxP3	A647	Biolegend	320214	259D	1:50
<b>Antibodies for panel 2</b>						
1	Viability	APC-Cy7	Biolegend	423106	—	1:1,000
2	CD3	BUV395	BD Biosciences	564000	SK7	1:200
3	CD8	BV605	Biolegend	344742	SK1	1:200
4	KLRG1	PE-Cy7	ThermoFisher	25-9488-41	13F12F2	1:100
5	CCR7	APC	Biolegend	353213	G043h7	1:100
6	CD45RA	Pac Blue	Biolegend	304118	HI100	1:200
7	CD28	PE	Biolegend	563075	CD28.2	1:400
8	CD27	PerCP-Cy5.5	Biolegend	302819	O323	1:100
9	CD56	BV650	Biolegend	362531	5.1H11	1:200
10	CD16	BV785	Biolegend	360733	B73.1	1:100
11	CD4	BUV496	BD Biosciences	612937	SK3	1:300
12	CD57	FITC	Biolegend	359603	HNK-1	1:900

staining of transcription factors (panel 1), cells were fixed and permeabilized with the eBioscience Foxp3/Transcription factor staining buffer set (Thermo Fisher) after cell surface staining. Intracellular staining was performed in the dark for 30 minutes on ice. Stained cells were analyzed on a BD LSRFortessa flow cytometer. Flow cytometry data analysis and the design of representative flow plots were performed with FlowJo version 10.7.1.

### Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 8.0. Pearson correlation was used to evaluate linear relationships among T-cell subpopulations and clinical correlates of IBM disease. Comparisons between multiple groups were performed by 2-way analysis of variance, followed by a post hoc Bonferroni test to determine significance of differences between 2 groups. Values of  $p \leq 0.05$  were considered significant. Comparisons between 2 groups were performed using an unpaired 2-tailed Student *t* test or a nonparametric Mann-Whitney test.

### Data Availability

Anonymized data not published within this article will be made available by request from any qualified investigator.

## Results

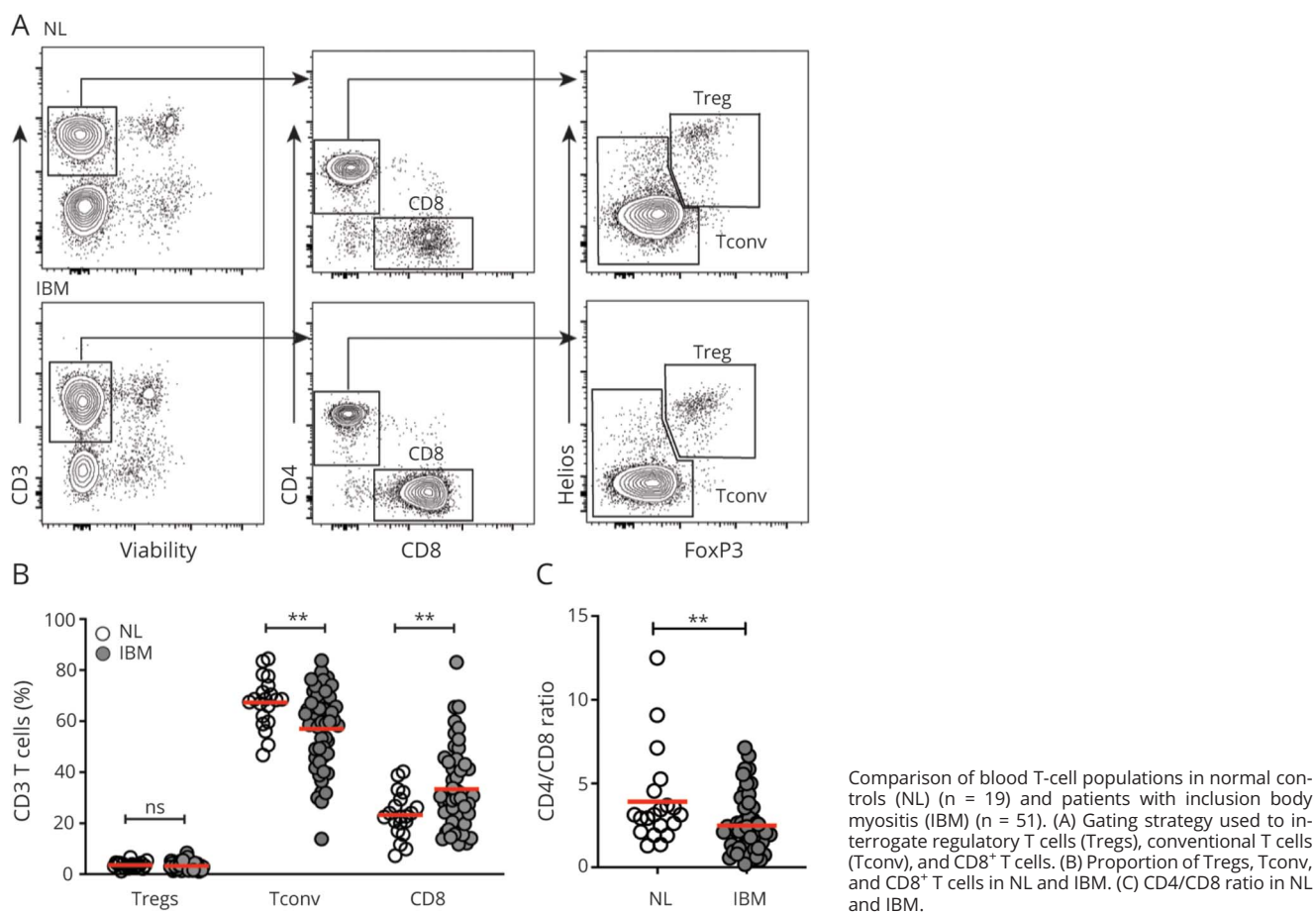
### Patients

Fifty-one patients with IBM ( $65.7 \pm 8.47$  years) who met the ENMC 2011 criteria for a diagnosis of clinically defined or probable IBM were included in this study. The baseline demographics included age 47–84 years, age at onset of disease 42–76 years, disease duration 2–30 years, and IBMFRS 1–40. An additional 19 HCs ranging from 40 to 75 years of age ( $60.6 \pm 8.67$  years) were included. A secondary analysis was performed on a subset of patients to achieve age matching. Four patients with IBM and 6 HCs were not included in the secondary analysis, resulting in a mean age of 64.2 years and 64.1 years for patients with IBM and healthy donors, respectively. The results of the secondary analysis were similar to those presented in the following (eFigures 1–3, [links.lww.com/WNL/B807](https://links.lww.com/WNL/B807)).

### Expansion of CD8<sup>+</sup> T Cells in IBM Blood

We developed a flow cytometry panel (Table) to examine various subsets of blood T cells, including CD4<sup>+</sup> regulatory T cells (Tregs), CD4<sup>+</sup> conventional T cells (Tconv), and CD8<sup>+</sup> T cells in IBM compared with age-matched healthy controls (HCs) (Figure 1A). The flow cytometry analysis

**Figure 1** Skewed Distribution of the Blood T-Cell Population in IBM Towards CD8 Cytotoxic T Cells



showed that there was no difference in the mean proportion of CD4<sup>+</sup>Foxp3<sup>+</sup>Helios<sup>+</sup> Tregs among CD3<sup>+</sup> T cells in HCs and patients with IBM (3.4% vs 3.6%;  $p = 0.62$ ), nor did they differ as a percentage of CD4<sup>+</sup> T cells (5.1% vs 5.6%;  $p = 0.34$ ) (Figure 1B). There was a decrease in the mean proportion of CD4<sup>+</sup> Tconv in IBM (57.1% vs 67.5%;  $p = 0.007$ ) and an increase in mean proportion of CD8<sup>+</sup> T cells among total T cells in IBM (33.4% vs 23.3%;  $p = 0.095$ ) compared to HCs. The net effect was a decrease in IBM of the CD4/CD8 T-cell ratio (2.5 vs 3.9;  $p = 0.01$ ) (Figure 1C).

### IBM CD4 and CD8 T Cells Shift to More Highly Differentiated Populations Marked by KLRG1

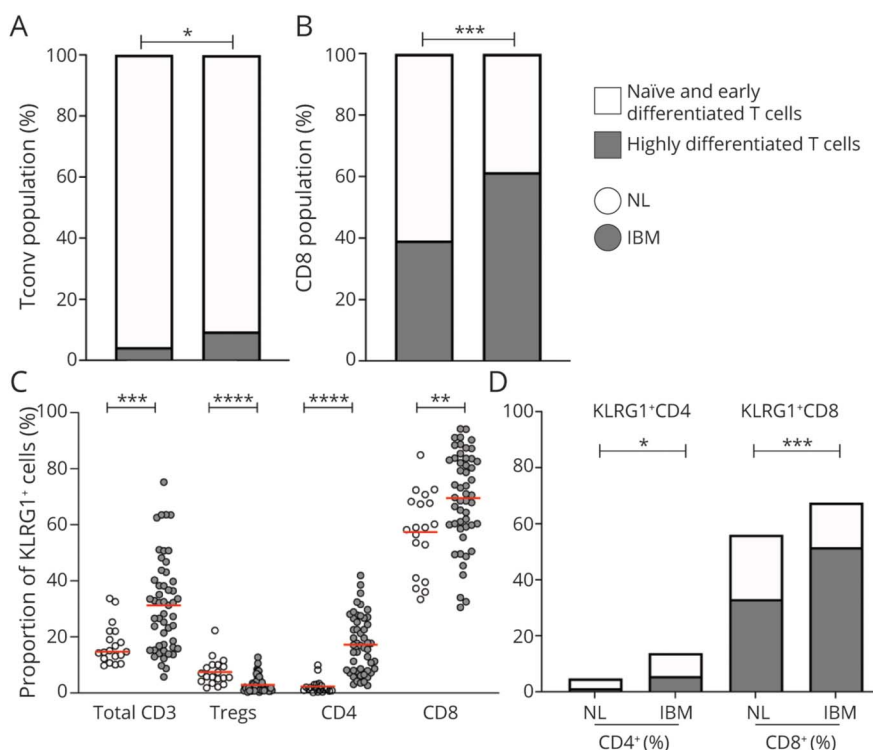
Naive T cells chronically exposed to antigen proceed through a differentiation pathway culminating in highly terminally differentiated T cells.<sup>25</sup> We initially dichotomized each of the CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations according to their cell surface expression of CD28 and CD57. An early differentiated population (collectively T<sub>n</sub>, T<sub>cm</sub>, and early Tem) was defined as CD28<sup>+</sup>CD57<sup>-</sup>. A highly differentiated population (collectively late Tem and TemRA) was composed of CD28<sup>-</sup>CD57<sup>-</sup>, CD28<sup>-</sup>CD57<sup>+</sup>, and CD28<sup>+</sup>CD57<sup>+</sup> T cells. These studies showed that within the CD4<sup>+</sup> Tconv and CD8<sup>+</sup> T-cell populations, there was a shift in IBM towards a highly differentiated T-cell phenotype for the CD4<sup>+</sup> Tconv population (IBM compared to normal highly differentiated CD4<sup>+</sup> Tconv, 9.5% vs 4.4%;  $p = 0.02$ ; Figure 2A) and the CD8 population (IBM compared to normal highly differentiated CD8, 61.4% vs 39.1%;  $p = 0.002$ ; Figure 2B).

We further examined the expression of KLRG1, a T cell surface molecule that marks highly differentiated T cells, in the total CD3<sup>+</sup>, CD4<sup>+</sup> Tconv, and CD8<sup>+</sup> T-cell populations. The proportion of KLRG1<sup>+</sup> T cells was increased in IBM compared to HCs within the total CD3<sup>+</sup> (31.4% vs 17.0%;  $p = 0.0004$ ), CD4<sup>+</sup> Tconv (17.2% vs 2.3%;  $p < 0.0001$ ), and CD8<sup>+</sup> (69.5% vs 57.4%;  $p = 0.005$ ) T-cell populations (Figure 2C). The expansion of the CD4<sup>+</sup> KLRG1<sup>+</sup> population was particularly striking, with nearly complete discrimination of IBM from HCs (96% of patients with IBM vs 0% of HCs having KLRG1<sup>+</sup> of CD4 T cells >3.4%). KLRG1 was less expressed on Tregs in IBM compared with normal Tregs (2.9% vs 7.5%;  $p < 0.0001$ ). The expansion of KLRG1<sup>+</sup> CD4 and CD8 T cells predominantly occurred in the highly differentiated populations (late Tem and TemRA) (Figure 2D).

### Deeper Phenotyping of the Highly Differentiated Tem and TemRA Populations

To further define the relationship between highly differentiated T cells and traditionally defined T-cell subsets, we used a second panel of markers (Table) that in addition included CD45RA, CCR7, and CD27. This panel permitted the interrogation of T<sub>n</sub>, T<sub>cm</sub>, 4 stages of Tem (Tem1–Tem4), and TemRA, using a previously described classification scheme.<sup>26–28</sup> The CD4 T<sub>n</sub> were decreased in IBM (23.4% vs 32.5%;  $p = 0.0000002$ ) vs the CD4<sup>+</sup> Tem2 (10.0% vs 6.6%;  $p = 0.07$ ) and Tem4 populations (4.2% vs 1.8%;  $p = 0.21$ ) (Figure 3A). For the IBM CD8 population, there was a significant increase in the TemRA population (30.4% vs 19.8%;  $p = 0.00002$ ) and

**Figure 2** Highly Differentiated CD4<sup>+</sup> and CD8<sup>+</sup> T Cells are Elevated in IBM



(A, B) Proportion of blood naive and early differentiated T cells (CD57<sup>-</sup>CD28<sup>+</sup>) and heterogeneous highly differentiated T cells (CD57<sup>-</sup>CD28<sup>-</sup>; CD57<sup>+</sup>CD28<sup>-</sup>; CD57<sup>+</sup>CD28<sup>+</sup>) among CD4<sup>+</sup> conventional T cells (Tconv) (A) and CD8<sup>+</sup> T cells (B). (C) Proportion of killer cell lectin-like receptor subfamily G member 1 (KLRG1)<sup>+</sup> cells among total CD3<sup>+</sup> T cells, regulatory T cells (Tregs), CD4<sup>+</sup> Tconv, and CD8<sup>+</sup> T cells. (D) Expansion of the KLRG1<sup>+</sup> T-cell population lies largely within the highly differentiated compartment. IBM = inclusion body myositis.

nonsignificant increase in the Tem4 population (11.2% vs 6.8%;  $p = 0.07$ ) (Figure 3B).

To further refine the expansion of KLRG1<sup>+</sup> populations (Figure 2), we examined how this marker was expressed within the populations defined by the second deeper phenotyping panel. Within the CD4<sup>+</sup> T-cell subsets, there was a small but significant expansion in the KLRG1<sup>+</sup>CD4<sup>+</sup> Tem4 population (2.8% vs 1.0%;  $p = 0.003$ ) (Figure 3C). Within the CD8<sup>+</sup> T-cell subsets, there was a significant expansion in KLRG1<sup>+</sup>CD8<sup>+</sup> TemRA (22.9% vs 15.0%;  $p = 0.000008$ ), nonsignificant expansion in CD8<sup>+</sup> Tem4 (7.2% vs 4.2%;  $p = 0.09$ ), and a concomitant decrease in Tem1 (7.4% vs 12.7%;  $p = 0.003$ ) populations (Figure 3D).

### CD56<sup>+</sup>CD8<sup>+</sup> T Cells are KLRG1<sup>+</sup>

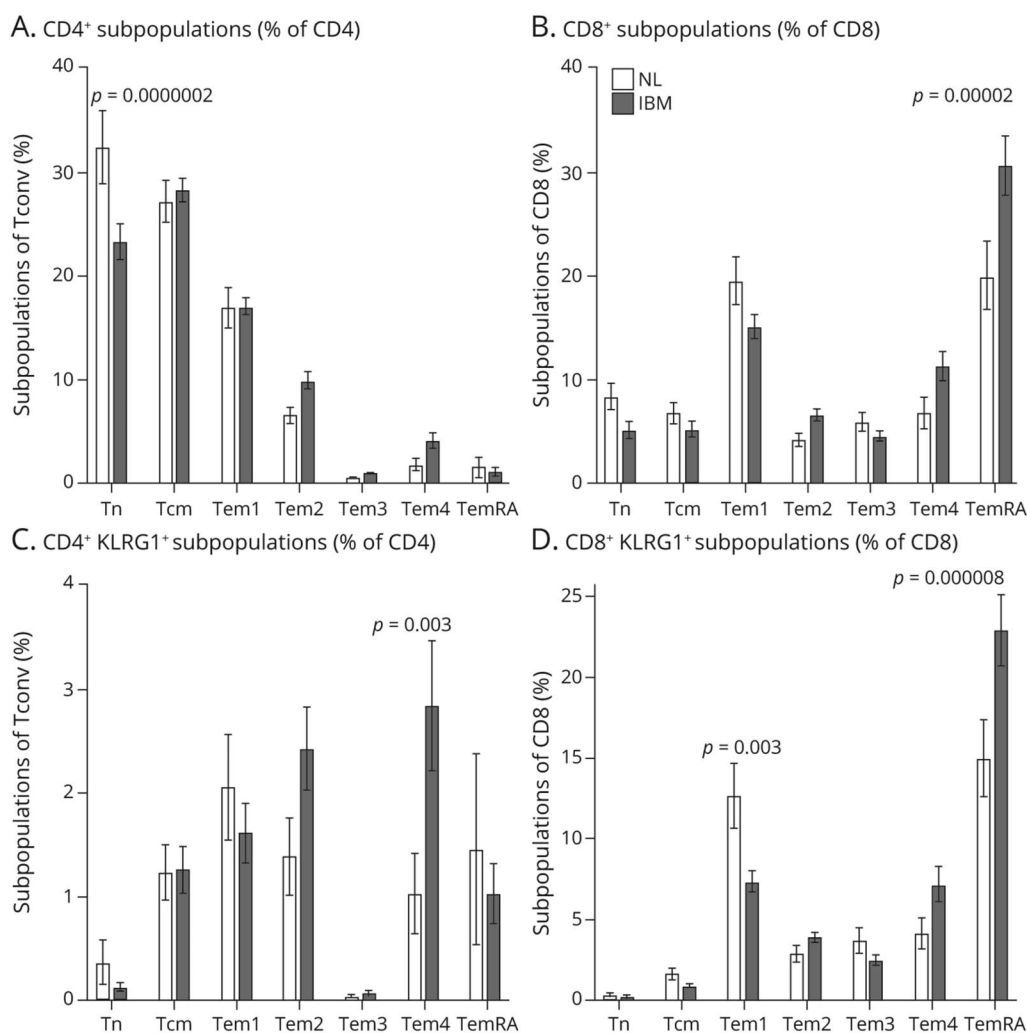
A population of highly cytotoxic T cells that are CD56<sup>+</sup>CD8<sup>+</sup>CD3<sup>+</sup> have been speculated to play a significant role in other autoimmune

diseases.<sup>29,30</sup> The mean proportion of CD8<sup>+</sup>CD3<sup>+</sup> T cells that were CD56<sup>+</sup> in IBM blood was not different than normal (2.65% vs 2.17%,  $p = 0.97$ ), although some patients with IBM had relatively higher levels (greater than 6%, in 14% of patients with IBM vs 0% of HCs) (Figure 4A). KLRG1 was an excellent marker of CD56<sup>+</sup>CD8<sup>+</sup>CD3<sup>+</sup> T cells (93% were KLRG1<sup>+</sup>) (Figure 4B). The mean fluorescence intensity of KLRG1, a measurement related to the density of KLRG1 surface expression per cell, across T-cell populations showed graded increases with T-cell differentiation state and was highest in the CD56<sup>+</sup>CD8<sup>+</sup>CD3<sup>+</sup> T-cell population (Figure 4C).

### NK Cells in IBM

NK cells are innate lymphocytes with potent cytotoxic effector function, but their regulation in IBM is not well defined. Therefore, we measured changes in blood NK cell populations in IBM and HCs. Three populations of NK cells were

**Figure 3** Deeper Phenotyping of CD4 and CD8 Populations Shows Expansions Lie Within the Tem4 and TemRA Populations



(A, B) Distribution of naive T cells (Tn), central memory T cells (Tcm), effector memory T cells (Tem)1–4, and effector memory re-expressing CD45RA cells (TemRA) in bulk CD4<sup>+</sup> (A) and bulk CD8<sup>+</sup> (B) T cells in normal controls (NL) vs patients with inclusion body myositis (IBM). (C, D) Proportion of killer cell lectin-like receptor subfamily G member 1 (KLRG1)<sup>+</sup>CD4<sup>+</sup> (C) and KLRG1<sup>+</sup>CD8<sup>+</sup> (D) T cells among Tn, Tcm, Tem1–Tem4, and TemRA.

distinguished by cell surface expression of CD56 and CD16 within CD3<sup>-</sup> lymphocytes, including the CD16<sup>-</sup>CD56<sup>bright</sup>, CD16<sup>+</sup>CD56<sup>dim</sup>, and CD16<sup>+</sup>CD56<sup>-</sup> populations.<sup>31</sup> There were no overall differences in the frequency of total NK or NK cell subpopulations in IBM (Figure 5A). KLRG1 was expressed by 14% of NK cells (Figure 5B).

### Longer Disease Duration Is Associated With Greater IBM Blood T-Cell Differentiation

We examined the correlation between disease duration (time from symptom onset to blood immune cell profiling), IBMFRS, and T-cell differentiation status. We found a positive linear correlation to the proportion of CD8<sup>+</sup> T cells that were highly differentiated TemRA ( $R = 0.36$ ;  $p = 0.01$ ) and the proportion of CD8<sup>+</sup> TemRA that were KLRG1<sup>+</sup> ( $R = 0.35$ ;  $p = 0.01$ ) (Figure 6, A and B). However, no relationship was found between the IBMFRS and T-cell differentiation status. Furthermore, we found that the proportion of CD8<sup>+</sup> T cells that were TemRA and the proportion of CD8<sup>+</sup> TemRA that were KLRG1<sup>+</sup> were not associated with age, suggesting that correlations between these CD8<sup>+</sup> T-cell subsets and disease duration were not attributed to changes associated with aging (Figure 6, C and D).

## Discussion

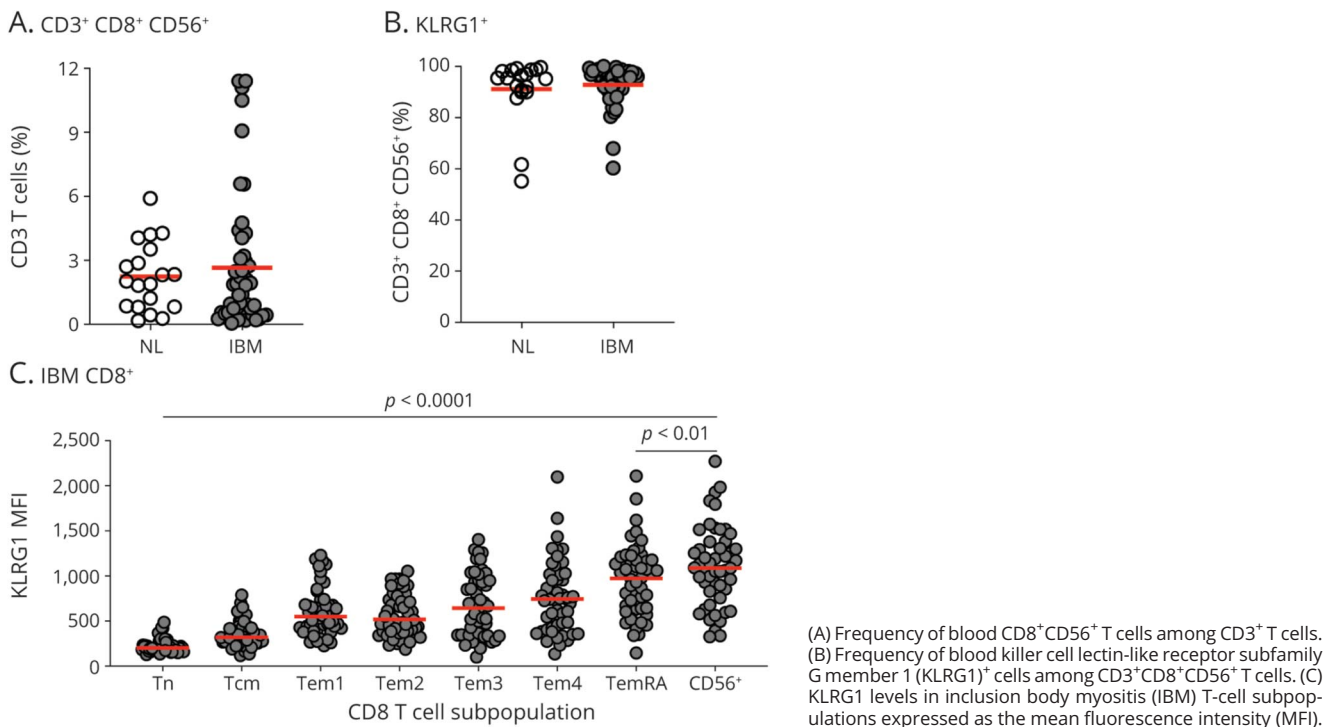
Previous studies of IBM muscle have identified predominant T-cell infiltration also skewed towards a cytotoxic CD8

phenotype (CD8 T cells constituting 85% of muscle endomysial T cells).<sup>9</sup> More recently, studies have focused on understanding the specific phenotype of these muscle-invading T cells and have identified a highly differentiated phenotype marked by expression of intracellular transcripts (e.g., granzymes) and the surface molecule KLRG1, and also noted the presence of this phenotype in blood CD8<sup>+</sup> T cells.<sup>17,19</sup>

We performed deep immunophenotyping of the IBM blood T-cell compartment to resolve at higher resolution the nature of the T-cell expansions and found that CD4<sup>+</sup> and CD8<sup>+</sup> T cells were skewed towards the highly differentiated Tem2, Tem4, and TemRA phases. This skewing suggests that IBM T cells are chronically exposed to undefined antigens. CD8<sup>+</sup> T cells in the Tem and TemRA phases, unlike Tn, are resistant to corticosteroids and apoptosis.<sup>32-35</sup> Indeed, in vivo administration of corticosteroids to healthy volunteers resulted in a decrease in blood CD8<sup>+</sup> Tn but a relative increase in CD8<sup>+</sup> TemRA.<sup>36</sup> Our findings that T cells shift to a highly differentiated state, together with the previously reported terminally differentiated phenotype of muscle T cells,<sup>8,17,19</sup> could explain the relative refractoriness of IBM to corticosteroids.

We also focused on KLRG1, a recently identified biomarker of IBM muscle cytotoxic T cells, for its potential therapeutic relevance. KLRG1 is a potential drug target for IBM<sup>19</sup> and other autoimmune diseases including primary biliary cholangitis.<sup>37</sup> Patients with IBM had greater proportions of KLRG1<sup>+</sup> cells in

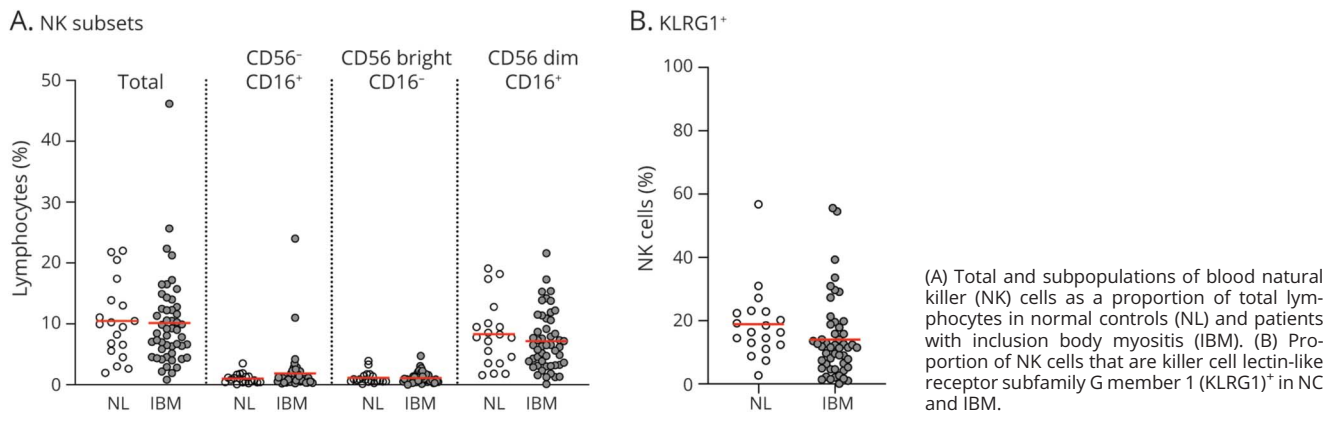
**Figure 4** CD8<sup>+</sup>CD56<sup>+</sup> T Cells in Blood Are KLRG1<sup>+</sup> and Have the Highest KLRG1 Receptor Density of All CD8 T-Cell Populations



(A) Frequency of blood CD8<sup>+</sup>CD56<sup>+</sup> T cells among CD3<sup>+</sup> T cells. (B) Frequency of blood killer cell lectin-like receptor subfamily G member 1 (KLRG1)<sup>+</sup> cells among CD3<sup>+</sup>CD8<sup>+</sup>CD56<sup>+</sup> T cells. (C) KLRG1 levels in inclusion body myositis (IBM) T-cell subpopulations expressed as the mean fluorescence intensity (MFI).



**Figure 5** Blood NK Cell Population Proportions of Lymphocytes in IBM Are Unchanged



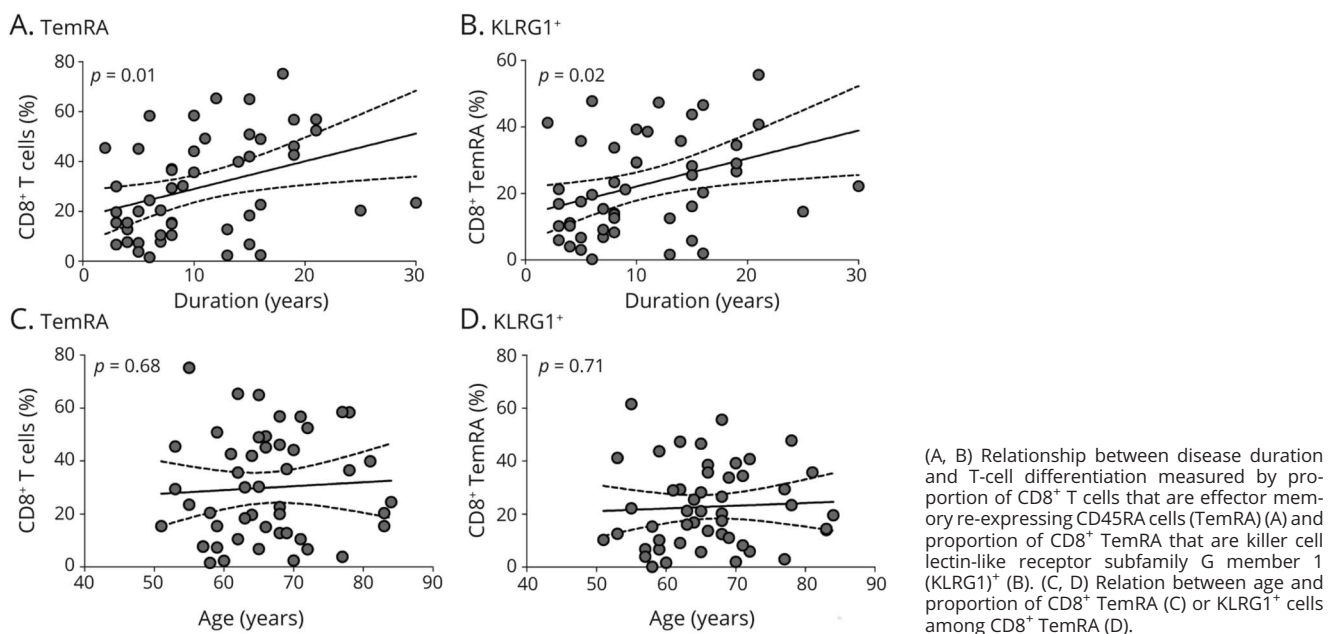
the overall CD3<sup>+</sup> T-cell compartment and both the CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets. Further refinements of the CD4 and CD8 compartments identified most of the expansion coming from the highly differentiated CD4<sup>+</sup> Tem2 and Tem4 and CD8<sup>+</sup> Tem4 and TemRA compartments. The expansion of the KLRG1<sup>+</sup> CD4 conventional population was particularly striking. Especially high proportions of KLRG1<sup>+</sup> CD8<sup>+</sup> T cells (>73%) were present in 41% of patients with IBM, in contrast with 0% of HCs having levels above this threshold.

The identification of minimal KLRG1 expression on IBM patient blood Tregs (3%) suggests that a therapeutic strategy aimed at depleting highly differentiated T cells by targeting KLRG1 would not deplete Tregs. Because Tregs are critical in

suppressing undesired autoimmunity, avoiding Treg depletion is an absolute requirement for an immunotherapeutic approach. Other T-cell depleting strategies have not avoided Treg depletion, and some have provoked autoimmunity (e.g., alemtuzumab, daclizumab),<sup>38-40</sup> which have limited their use or led to withdrawal from the market.

The expansion of a differentiated CD4<sup>+</sup> population in IBM has not been noted previously. This expansion included the CD28<sup>-</sup> Tem4 population, suggesting that these CD4<sup>+</sup> T cells may function as cytotoxic T cells, not helper T cells, as the CD4<sup>+</sup>CD28<sup>-</sup> population has cytotoxic capacity and has been identified in other autoimmune diseases.<sup>41-44</sup> An open-label IBM clinical trial with anti-T-lymphocyte globulin (ATG)<sup>45</sup>

**Figure 6** IBM Disease Duration Is Correlated With Increased CD8<sup>+</sup> T-Cell Differentiation



showing a trend towards efficacy and the preferential depletion of CD28<sup>-</sup>CD4<sup>+</sup> Tem4-like cells by ATG<sup>46</sup> provides strong scientific premise for further studies aimed at defining the functional role of highly differentiated CD4<sup>+</sup> T cells in IBM.

No apparent alterations in NK cell phenotypes were identified. The predominant mature cytotoxic NK population and the CD56<sup>dim</sup> subset in particular showed no increase. These findings continue to suggest a central role for highly differentiated KLRG1<sup>+</sup> T cells in the pathogenesis of IBM and a minimal involvement of NK cells, in agreement with quantitative studies in muscle that have suggested far fewer muscle-invading NK cells than T cells.<sup>9</sup> We also found that CD8<sup>+</sup> T cells that coexpressed the NK cell marker CD56 expressed the highest levels of KLRG1. Prior in vitro studies have shown that activation induces CD56 expression in a subset of T cells that proliferate less, express more inflammatory cytokines, and are capable of human leukocyte antigen-unrestricted cytotoxicity.<sup>47</sup> The presence of CD56<sup>+</sup>CD8<sup>+</sup> T cells that express KLRG1 is noteworthy as it speaks to the “NK cell–like” behavior and increased cytotoxicity of the CD8<sup>+</sup> T cells in IBM.

As a single time point (cross-sectional) study, the current findings do not inform about changes in highly differentiated T cells with increasing disease duration. Furthermore, the longitudinal relationship to disease-related severity and clinical measures of these cell populations was not studied. These measures will be investigated in a recently launched natural history study, INSPIRE-IBM, a longitudinal multicenter study in 150 patients with IBM over a 2-year period with data collection at 5 distinct time points.

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## Disclosure

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Greenberg is an inventor of intellectual property related to myositis diagnostics and therapeutics, owned and managed by Brigham and Women’s Hospital (BWH); is a founder of Abcuro, Inc. Partners HealthCare, the owner of BWH; and has financial interests in Abcuro. The financial interests were reviewed and managed in accordance with the conflict of interest policies of Partners HealthCare. T. Mozaffar and S.A. Villalta report no disclosures relevant to the manuscript. Go to [Neurology.org/N](http://Neurology.org/N) for full disclosures.

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## References

- Amato AA, Barohn RJ. Inclusion body myositis: old and new concepts. *J Neurol Neurosurg Psychiatry*. 2009;80(11):1186-1193.
- Benveniste O, Guiguet M, Freebody J, et al. Long-term observational study of sporadic inclusion body myositis. *Brain*. 2011;134(pt 11):3176-3184.
- Schmidt J, Dalakas MC. Inclusion body myositis: from immunopathology and degenerative mechanisms to treatment perspectives. *Expert Rev Clin Immunol*. 2013;9(11):1125-1133.
- Dimachkie MM, Barohn RJ. Inclusion body myositis. *Neurol Clin*. 2014;32(3):629-646.
- Machado PM, Ahmed M, Brady S, et al. Ongoing developments in sporadic inclusion body myositis. *Curr Rheumatol Rep*. 2014;16(12):477.
- Mastaglia FL, Needham M. Inclusion body myositis: a review of clinical and genetic aspects, diagnostic criteria and therapeutic approaches. *J Clin Neurosci*. 2015;22(1):6-13.
- Needham M, Mastaglia FL. Sporadic inclusion body myositis: a review of recent clinical advances and current approaches to diagnosis and treatment. *Clin Neurophysiol*. 2016;127(3):1764-1773.
- Greenberg SA. Inclusion body myositis: clinical features and pathogenesis. *Nat Rev Rheumatol*. 2019;15(5):257-272.
- Arahata K, Engel AG. Monoclonal antibody analysis of mononuclear cells in myopathies: I: quantitation of subsets according to diagnosis and sites of accumulation and demonstration and counts of muscle fibers invaded by T cells. *Ann Neurol*. 1984;16(2):193-208.
- Arahata K, Engel AG. Monoclonal antibody analysis of mononuclear cells in myopathies: III: immunoelectron microscopy aspects of cell-mediated muscle fiber injury. *Ann Neurol*. 1986;19(2):112-125.
- Arahata K, Engel AG. Monoclonal antibody analysis of mononuclear cells in myopathies: IV: cell-mediated cytotoxicity and muscle fiber necrosis. *Ann Neurol*. 1988;23(2):168-173.
- Hohlfeld R, Engel AG, Goebels N, Behrens L. Cellular immune mechanisms in inflammatory myopathies. *Curr Opin Rheumatol*. 1997;9(6):520-526.
- Dalakas MC, Hohlfeld R. Polymyositis and dermatomyositis. *Lancet*. 2003;362(9388):971-982.
- Salajegheh M, Rakocevic G, Raju R, Shatunov A, Goldfarb LG, Dalakas MC. T cell receptor profiling in muscle and blood lymphocytes in sporadic inclusion body myositis. *Neurology*. 2007;69(17):1672-1679.
- Pandya JM, Fasth AE, Zong M, et al. Expanded T cell receptor Vbeta-restricted T cells from patients with sporadic inclusion body myositis are proinflammatory and cytotoxic CD28null T cells. *Arthritis Rheum*. 2010;62(11):3457-3466.
- Allenbach Y, Chaara W, Rosenzweig M, et al. Th1 response and systemic Treg deficiency in inclusion body myositis. *PLoS One*. 2014;9(3):e88788.
- Greenberg SA, Pinkus JL, Amato AA, Kristensen T, Dorfman DM. Association of inclusion body myositis with T cell large granular lymphocytic leukaemia. *Brain*. 2016;139(pt 5):1348-1360.
- Hohlfeld R, Schulze-Koops H. Cytotoxic T cells go awry in inclusion body myositis. *Brain*. 2016;139(pt 5):1312-1314.
- Greenberg SA, Pinkus JL, Kong SW, Baecher-Allan C, Amato AA, Dorfman DM. Highly differentiated cytotoxic T cells in inclusion body myositis. *Brain*. 2019;142(9):2590-2604.
- Knauss S, Preusse C, Allenbach Y, et al. PD1 pathway in immune-mediated myopathies: pathogenesis of dysfunctional T cells revisited. *Neurol Neuroimmunol Neuroinflamm*. 2019;6(3):e558.
- Schirmer M, Vallejo AN, Weyand CM, Goronzy JJ. Resistance to apoptosis and elevated expression of Bcl-2 in clonally expanded CD4<sup>+</sup>CD28<sup>-</sup> T cells from rheumatoid arthritis patients. *J Immunol*. 1998;161(2):1018-1025.
- Posnett DN, Edinger JW, Manavalan JS, Irwin C, Marodon G. Differentiation of human CD8 T cells: implications for in vivo persistence of CD8<sup>+</sup> CD28<sup>-</sup> cytotoxic effector clones. *Int Immunol*. 1999;11(2):229-241.
- Spaulding C, Guo W, Effros RB. Resistance to apoptosis in human CD8<sup>+</sup> T cells that reach replicative senescence after multiple rounds of antigen-specific proliferation. *Exp Gerontol*. 1999;34(5):633-644.
- Vallejo AN, Schirmer M, Weyand CM, Goronzy JJ. Clonality and longevity of CD4<sup>+</sup>CD28null T cells are associated with defects in apoptotic pathways. *J Immunol*. 2000;165(11):6301-6307.
- Strioga M, Pasukoniene V, Characiejus D. CD8<sup>+</sup> CD28<sup>-</sup> and CD8<sup>+</sup> CD57<sup>+</sup> T cells and their role in health and disease. *Immunology*. 2011;134(1):17-32.
- van Aalderen MC, van den Biggelaar M, Remmerswaal EB, et al. Label-free analysis of CD8(+) T cell subset proteomes supports a progressive differentiation model of human-virus-specific T cells. *Cell Rep*. 2017;19(5):1068-1079.
- Koch S, Larbi A, Derhovanessian E, Ozelcik D, Naumova E, Pawelec G. Multiparameter flow cytometric analysis of CD4 and CD8 T-cell subsets in young and old people. *Immun Ageing*. 2008;5:6.
- Larbi A, Fulop T. From "truly naive" to "exhausted senescent" T cells: when markers predict functionality. *Cytometry A*. 2014;85(1):25-35.
- tHart BA, Kap YS, Morandi E, Laman JD, Gran B. EBV infection and multiple sclerosis: lessons from a marmoset model. *Trends Mol Med*. 2016;22(12):1012-1024.
- Van Acker HH, Capsomidis A, Smits EL, Van Tendeloo VF. CD56 in the immune system: more than a marker for cytotoxicity? *Front Immunol*. 2017;8:892.
- Poli A, Michel T, Theresine M, Andres E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology*. 2009;126(4):458-465.
- Pearl JP, Parris J, Hale DA, et al. Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. *Am J Transpl*. 2005;5(3):465-474.
- Hodge G, Hodge S. Steroid resistant CD8(+)CD28(null) NKT-like pro-inflammatory cytotoxic cells in chronic obstructive pulmonary disease. *Front Immunol*. 2016;7:617.
- Pandya JM, Loell I, Hossain MS, et al. Effects of conventional immunosuppressive treatment on CD244<sup>+</sup> (CD28null) and FOXP3<sup>+</sup> T cells in the inflamed muscle of patients with polymyositis and dermatomyositis. *Arthritis Res Ther*. 2016;18:80.
- Gupta S, Su H, Bi R, Agrawal S, Gollapudi S. Life and death of lymphocytes: a role in immunosenescence. *Immun Ageing*. 2005;2:12.
- Olnes MJ, Kotliarov Y, Biancotto A, et al. Effects of systemically administered hydrocortisone on the human immunome. *Sci Rep*. 2016;6:23002.
- Li Y, Li B, You Z, et al. Cytotoxic KLRG1 expressing lymphocytes invade portal tracts in primary biliary cholangitis. *J Autoimmun*. 2019;103:102293.
- Devonshire V, Phillips R, Wass H, Da Roza G, Senior P. Monitoring and management of autoimmunity in multiple sclerosis patients treated with alemtuzumab: practical recommendations. *J Neurol*. 2018;265(11):2494-2505.
- Willis MD, Harding KE, Pickersgill TP, et al. Alemtuzumab for multiple sclerosis: long term follow-up in a multi-centre cohort. *Mult Scler*. 2016;22(9):1215-1223.
- Williams T, Chataway J. Immune-mediated encephalitis with daclizumab: the final nail. *Mult Scler*. 2019;25(5):753-754.
- Patil VS, Madrigal A, Schmiedel BJ, et al. Precursors of human CD4(+) cytotoxic T lymphocytes identified by single-cell transcriptome analysis. *Sci Immunol*. 2018;3(19):eaan8664.
- Schafflick D, Xu CA, Hartlehnert M, et al. Integrated single cell analysis of blood and cerebrospinal fluid leukocytes in multiple sclerosis. *Nat Commun*. 2020;11(1):247.
- Oja AE, Vieira Braga FA, Remmerswaal EB, et al. The transcription factor hobit identifies human cytotoxic CD4(+) T cells. *Front Immunol*. 2017;8:325.
- Maly K, Schirmer M. The story of CD4<sup>+</sup> CD28<sup>-</sup> T cells revisited: solved or still ongoing? *J Immunol Res*. 2015;2015:348746.
- Lindberg C, Trysberg E, Tarkowski A, Oldfors A. Anti-T-lymphocyte globulin treatment in inclusion body myositis: a randomized pilot study. *Neurology*. 2003;61(2):260-262.
- Duftner C, DeJaco C, Hengster P, et al. Apoptotic effects of antilymphocyte globulins on human pro-inflammatory CD4<sup>+</sup>CD28<sup>-</sup> T-cells. *PLoS One*. 2012;7(3):e33939.
- Kelly-Rogers J, Madrigal-Estebas L, O'Connor T, Doherty DG. Activation-induced expression of CD56 by T cells is associated with a reprogramming of cytolytic activity and cytokine secretion profile in vitro. *Hum Immunol*. 2006;67(11):863-873.