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CD4⁺CD28null T Cells in Autoimmune Disease: Pathogenic Features and Decreased Susceptibility to Immunoregulation

Marielle Thewissen,* Veerle Somers,* Niels Hellings,* Judith Fraussen,* Jan Damoiseaux,† and Piet Stinissen2*

To determine the role of expanded CD4⁺CD28null T cells in multiple sclerosis and rheumatoid arthritis pathology, these cells were phenotypically characterized and their Ag reactivity was studied. FACS analysis confirmed that CD4⁺CD28null T cells are terminally differentiated effector memory cells. In addition, they express phenotypic markers that indicate their capacity to infiltrate into tissues and cause tissue damage. Whereas no reactivity to the candidate autoantigens myelin basic protein and collagen type II was observed within the CD4⁺CD28null T cell subset, CMV reactivity was prominent in four of four HC, four of four rheumatoid arthritis patients, and three of four multiple sclerosis patients. The level of the CMV-induced proliferative response was found to be related to the clonality of the response. Interestingly, our results illustrate that CD4⁺CD28null T cells are not susceptible to the suppressive actions of CD4⁺CD25⁺ regulatory T cells. In conclusion, this study provides several indications for a role of CD4⁺CD28null T cells in autoimmune pathology. CD4⁺CD28null T cells display pathogenic features, fill up immunological space, and are less susceptible to regulatory mechanisms. However, based on their low reactivity to the autoantigens tested in this study, CD4⁺CD28null T cells most likely do not play a direct autoaggressive role in autoimmune disease. The Journal of Immunology, 2007, 179: 6514–6523.

The CD4⁺ T cell displays a variety of helper functions essential for an efficient adaptive immune response. However, a considerable proportion of peripheral CD4⁺ T cells is potentially autoreactive. An important tolerance mechanism preventing the attack of self-tissues encompasses dominant suppression of self-reactive T cells by a specialized subset of CD4⁺CD25⁺ regulatory T (Treg) cells. Both subtypes of CD4⁺ T cells are believed to be involved in the pathogenesis of various autoimmune diseases. A number of studies support a pathogenic role of autoreactive CD4⁺ T cells in the immunopathogenesis of both rheumatoid arthritis (RA) and multiple sclerosis (MS) (reviewed in Refs. 1–3). In addition, Treg cells were shown to be functionally compromised in both patient groups (4–6).

We and others identified an expanded CD4⁺CD28null T cell population within a subgroup of RA and MS patients (7–9). These T cells have undergone clonal expansion in vivo, have lost the expression of CD40L, are highly proinflammatory, and express a variety of killer Ig-like receptors (10–14). An expansion of CD4⁺CD28null T cells was also reported for patients with other autoimmune diseases (13, 15), HIV- and CMV-infected individuals (16), and people suffering from coronary disease (17). Although the role of these cells in disease pathology is not clear, CD4⁺CD28null T cells could be isolated from the disease site, for example, from ruptured unstable coronary plaques (18). In addition, anti-TNF-α treatment in RA patients and statin treatment in patients with unstable angina was associated with a reduction in the frequency of CD4⁺CD28null T lymphocytes (19, 20).

During differentiation in response to chronic stimulation, CD4⁺ T cells gradually lose CD28 from the cell surface, eventually leading to the appearance of CD4⁺CD28null T cells. In chronic inflammatory conditions, CD28 gene expression may additionally be down-regulated by TNF-α (21). To date, the triggers of activation of CD4⁺CD28null T cells have not been fully elucidated. Several studies suggest that CD4⁺CD28null T cells may be autoreactive T cells (8, 22, 23). In contrast, van Leeuwen et al. (16) demonstrated that CD4⁺CD28null T cells could emerge as a consequence of CMV infection. Moreover, detailed characterization of CMV-reactive CD4⁺ T cells showed that these cells were largely CD28null (24).

CMV is a persistent active β-herpesvirus that is present in ~50% of the adult population and 90% of the elderly (25). In healthy, immunocompetent hosts, CMV infection is asymptomatic. However, immunosuppressed individuals may suffer serious, often fatal, consequences as a result of viral re-emergence (26). Maintaining protective immunity against CMV is clearly essential, but may have a significant impact on overall adaptive immune function (25, 27).

In this study, we further characterized the CD4⁺CD28null T cell subset to gain more insight in the role of this aberrant T cell subset in the pathogenesis of RA and MS. It is tempting to speculate that expansion of CD4⁺CD28null T cells in a subset of patients with an autoimmune disease, is caused by chronic stimulation with autoantigens. This study is the first to test reactivity of CD4⁺CD28null T

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3 Abbreviations used in this paper: Treg, regulatory T cell; RA, rheumatoid arthritis; MS, multiple sclerosis; HC, healthy control; SF, synovial fluid; ST, synovial tissue; SFMC, SF mononuclear cell; GrA, granulocyte A; GrB, granulocyte B; MBP, myelin basic protein; bCII, human collagen type II; PF, proliferating fraction; TT, tetanus toxoid; MFI, mean fluorescence intensity.
cells toward a diverse set of foreign and autoantigens in both patients with an autoimmune pathology (RA or MS) and healthy controls (HC). In addition, the cells were analyzed for their tissue-infiltrating and tissue-damaging potential. This study provides new information on the potential role of CD4^+CD28null T cells in autoimmune diseases.

**Materials and Methods**

**Study population and patient material**

To study Ag reactivity, peripheral blood was obtained from four MS patients, four RA patients, and four HC. Patients and controls selected for this study had a CD4^+CD28null T cell population >5%, which was essential to obtain the minimal amount of cells needed for the experiment. Ab reactivity in plasma against CMV was determined at the serology unit of the Virga Jesse Hospital Hasselt with the AxSYM CMV IgM and IgG assay (Abbott Diagnostics) according to the manufacturer’s instructions. MS and RA patients fulfilled the criteria described elsewhere (28, 29). Characteristics of patients and controls are shown in Table I.

In addition, paired blood-synovial fluid (SF) samples were received from six other RA patients. From two of these patients, synovial tissue (ST) was obtained after total knee/hip arthroplasty. Cells were excluded from the ST samples via dissection and sieve homogenization. PBMC and SF mononuclear cells (SFMC) were isolated from the blood samples were obtained after total knee/hip arthroplasty. Cells were excluded from the ST samples via dissection and sieve homogenization. PBMC and SF mononuclear cells (SFMC) were isolated from the blood samples.

**FACS analysis**

Percentages of CD4^+CD28null T cells in paired blood-SF-ST samples were determined by flow cytometric analysis. PBMC-, SFMC-, or the ST-derived cells were stained with FITC-labeled anti-CD4, PE-labeled anti-CD28, and PerCP-labeled anti-CD3 Abs (BD Biosciences). CD4^+CD28null and CD4^+CD28null T cells were identified using PerCP-labeled Ab directed against CD4 and PE-labeled anti-CD28 Ab (both BD Biosciences) or FITC-labeled anti-CD28 Ab (ImmunoTools). Additional phenotypical characterization was performed by surface staining with PE- or FITC-conjugated mAbs specific for CD25, CD45RO, CD62L, TCRαβ (all BD Biosciences), CD11a, CD27, CD44, CD45RA, or CD49d (all ImmunoTools). Intracellular staining was performed with FITC-labeled granulocyte A (GrA), PE labeled granulocyte B (GrB), or FITC-labeled perinonlymphoendothelial cells (ImmuTools). Cells were washed twice and analyzed on a FACs-Calibur flow cytometer (BD Biosciences).

**Isolation and CFSE labeling of sorted T cells**

FITC-labeled anti-CD28 Ab (ImmunoTools), PE-labeled anti-CD28 or anti-CD25 Ab, and PerCP-labeled anti-CD4 Ab (BD Biosciences) were used to stain PBMC. CD4^+CD28null, CD4^+CD28, and CD4^+CD28high T cell populations were sorted using a high-speed FACS Aria cell sorter (BD Biosciences). Isolated CD4^+ T cell subsets were labeled with 1 μM CFSE (Vybrant CFSE cell tracer kit; Molecular Probes/Invitrogen Life Technologies) in PBS (Cambrex Bio) for 7 min at 37°C followed by a 15-min incubation at 37°C in RPMI 1640 medium supplemented with l-glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate, 1% nonessential amino acids (all obtained from Invitrogen Life Technologies), 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen Life Technologies), and 10% heat-inactivated FBS (HyClone). Cells were washed and resuspended in an appropriate volume of culture medium.

**CFSE-based proliferation assay**

Ag specificity of sorted CD4^+CD28null T cells was determined in a CFSE-based proliferation assay. Ag reactivity of CD4^+CD28 T cells was analyzed in parallel to confirm high sensitivity of the assay. CFSE-labeled CD4^+CD28null T cells were cultured in culture medium supplemented with IL-2 (0.5 U/ml) in U-bottom 96-well plates (Nunc). Autologous PBMC were loaded with tetanus toxoid (TT; 20 limit of flocculation/ml; RIVM), myelin basic protein (MBP; 100 μg/ml, purified from white matter of human brain as described (30)), human collagen type II (hCII, 100 μg/ml; Morwell Diagnostics), or CMV (7 μg/ml; BD Biosciences/BD Pharmingen). The CMV peptide pool consisted of 138 peptides of 15-aa residues that completely span the pp65 protein sequence of the AD-169 strain, with 11 aa overlaps (31). Because CMV peptides were dissolved in DMSO, the CMV peptides were dissolved in DMSO, DMSO was included in the assay as a control condition. CFSE-labeled responder cells (2–5 × 10^5) were cultured supplemented with 0.5 × 10^3 Ag-loaded irradiated PBMC. Unloaded PBMC were used in the positive and negative control conditions. As a positive control, anti-CD3 mAb (2 μg/ml, house-made clone 2G3) was added. After 5 days of culture, IL-2 (2 U/ml) was added to the cell cultures. Eight days after set-up, a fraction of the cells was pelletted for subsequent molecular analyses and another fraction of the cells was used for FACS analysis. PE-conjugated anti-CD28 Ab and 7- amino-actinomycin D (BD Biosciences) were used to evaluate the proliferative responses of living CD4^+CD28null T cells. The δ proliferating fraction (ΔPF) was calculated as the percentage of divided cells in a specific condition minus the percentage of proliferated cells in the unstimulated condition (background proliferation).

**Analysis of TCR BV gene usage and CDR3 fragment length of CMV-reactive CD4^+CD28null T cells**

Semiquantitative TCR BV analysis was performed on CMV- or anti-CD3 mAb-stimulated CD4^+CD28null T cells. RNA isolation (Roche Diagnostics) and cDNA synthesis (Promega) were performed according to the manufacturer’s recommendations. The oligonucleotide sequences of the TCR BV-specific primers are shown in Table II. These primer pairs amplify the 23 functional TCR BV genes and are adapted to the current nomenclature (32). PCR amplification was performed with 1 of 23 TCR BV gene-specific primer pairs or a TCR BC gene-specific primer pair. PCR contained 50 ng of cDNA, 1× PCR buffer, 0.2 mM dNTP, 2 pmol TCR BV gene-specific forward primer, 2 pmol BV gene-specific reverse primer, and 1 U of Taq Polymerase in a total volume of 20 μl. The amplification consisted of 35 cycles of 95°C for 20 s, 61°C for 20 s, and 72°C for 40 s. PCR amplicons were subjected to electrophoresis, visualized by staining with ethidium bromide, and DNA band intensities were analyzed with Quantity One software (Bio-Rad). Band intensities of all analyzed TCR BV genes were summed and the relative expression of each TCR BV gene was calculated to this total expression value. The PCR amplicons that were subjected to CDR3 fragment length analysis were reamplified for 25 cycles using a BV gene-specific forward primer (Table II) and a FAM-labeled TCR BC reverse primer (5’-FAM-GT GGC CAG GCA CAC CAG TGT GGC C-3’).

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**Table I. Characteristics of patients and controls**

<table>
<thead>
<tr>
<th>Age (Years)</th>
<th>Gender (M/F)</th>
<th>Disease Duration (Years)</th>
<th>CD4^+CD28null T Cells (%)</th>
<th>Anti-CMV IgG (U/ml)</th>
<th>Medication</th>
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<tbody>
<tr>
<td>HC1</td>
<td>30</td>
<td>M</td>
<td>53</td>
<td>&gt;250</td>
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<tr>
<td>HC2</td>
<td>33</td>
<td>M</td>
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<td>239.8</td>
<td></td>
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<tr>
<td>HC3</td>
<td>30</td>
<td>F</td>
<td>9.4</td>
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</tr>
<tr>
<td>HC4</td>
<td>48</td>
<td>M</td>
<td>11.7</td>
<td>&gt;250</td>
<td></td>
</tr>
<tr>
<td>RA1</td>
<td>76</td>
<td>M</td>
<td>19</td>
<td>6.5</td>
<td>219.9</td>
</tr>
<tr>
<td>RA2</td>
<td>59</td>
<td>F</td>
<td>7</td>
<td>9.6</td>
<td>&gt;250</td>
</tr>
<tr>
<td>RA3</td>
<td>64</td>
<td>F</td>
<td>5</td>
<td>10.5</td>
<td>&gt;250</td>
</tr>
<tr>
<td>RA4</td>
<td>71</td>
<td>F</td>
<td>16</td>
<td>11.8</td>
<td>113.6</td>
</tr>
<tr>
<td>MS1</td>
<td>39</td>
<td>F</td>
<td>10</td>
<td>7.4</td>
<td>&gt;250</td>
</tr>
<tr>
<td>MS2</td>
<td>50</td>
<td>F</td>
<td>2</td>
<td>8.1</td>
<td>NA</td>
</tr>
<tr>
<td>MS3</td>
<td>50</td>
<td>F</td>
<td>2</td>
<td>5.5</td>
<td>Tyroxpine</td>
</tr>
<tr>
<td>MS4</td>
<td>51</td>
<td>M</td>
<td>9</td>
<td>5.7</td>
<td>243.6</td>
</tr>
</tbody>
</table>

* M, Male; F, female; %, percentage; NA, not analyzed; MTX, methotrexate; SSZ, sulfasalazine.
from the cytokine levels measured in the Ag-stimulated conditions. In the shown in Fig. 1

CD4+CD28null T cells express CD45RO and a TCR consisting of an αβ heterodimer (Fig. 1, B and C). In contrast to CD4+CD28+ T cells, CD4+CD28null T cells have lost the expression of another costimulatory molecule CD27. In addition, CD4+CD28null T cells do not express the IL-2R α-chain (CD25) direct ex vivo, whereas CD25 is variably expressed by CD4+CD28+ T cells (Fig. 1, B and C).

To study the expression of cytotoxic markers in CD4+CD28null T cells, PBMC were intracellularly stained for the presence of various cytotoxic molecules. In contrast to their CD28 counterparts, the majority (>75%) of CD4+CD28null T cells have cytoplasmic stores of GrA, GrB, and perforin. Representative dot plots are shown in Fig. 1D.

These results demonstrate that CD4+CD28null T cells are Ag-experienced, terminally differentiated cells, equipped with an extensive cytolytic machinery. CD4+CD28null T cells of HC, RA, and MS patients did not differ for the expression of the studied proteins.

CD4+CD28null T cell infiltration in inflamed tissues

To study whether CD4+CD28null T cells are equipped to infiltrate into tissues, expression of the adhesion molecules CD11a, CD44, CD49d, and CD62L was examined. Representative dot plots for the expression of these markers in CD28+ and CD28null CD4+ T cells are shown in Fig. 2A. Nearly all CD4+CD28+ and CD4+CD28null T cells express CD44 and integrin αI (CD11a; Fig. 2, B and C). However, as indicated by the mean fluorescence intensity (MFI), CD4+CD28null T cells express significantly higher levels of CD11a than their CD28+ counterparts (p < 0.01, Fig. 2, D and E). In contrast to CD4+CD28+ T cells, all CD4+CD28null T cells express integrin αI (CD49d), a molecule that enables interaction with the extracellular matrix (Fig. 2, B and C). As compared with CD28+ T cells, a smaller fraction of CD4+CD28null T cells expresses the lymph node homing receptor CD62L and also the level of expression is decreased in this T cell subset (Fig. 2, B–E). Remarkably, CD62L was expressed by a smaller fraction of CD4+ T cells in MS patients as compared with HC (p < 0.05). Overall, these results clearly show that CD4+CD28null T cells express molecules that allow tissue infiltration. Besides, for CD62L,

### Table II. Sequences of TCR BV gene-specific primer pairs

<table>
<thead>
<tr>
<th>BV</th>
<th>Sequence 5′ → 3′ (Forward)</th>
<th>Sequence 5′ → 3′ (Reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCAAGATGGAACAGGAATGAATTC</td>
<td>GTCCTCCAGGCTGTTGGAGACCA</td>
</tr>
<tr>
<td>2</td>
<td>CCAAAACATCGTGGTACACAGCA</td>
<td>CCAAGGATTGATGAGGAGGAC</td>
</tr>
<tr>
<td>3</td>
<td>CATGGGAATGAAATAAGAAGTCTT</td>
<td>TGGCTGACGGTGTAGTG</td>
</tr>
<tr>
<td>4</td>
<td>GACCTAACAGGCCAAGGAGACCA</td>
<td>AGCCAGCGCTGGTGGAGGAC</td>
</tr>
<tr>
<td>5</td>
<td>TGGAGAACAGGCCAAGGAGACCA</td>
<td>CACCACTGGTGGTGGAGGAC</td>
</tr>
<tr>
<td>6</td>
<td>CTCGAGGTGTTGTTGACATTTCA</td>
<td>ATGTTTCAGATGAGGAGGAC</td>
</tr>
<tr>
<td>7</td>
<td>AAGACAGTCATCGACACAGCACT</td>
<td>TGGGACAGTCGTTCAGACAG</td>
</tr>
<tr>
<td>8</td>
<td>CAGACATCGTGCAGAGAGGAGG</td>
<td>GAGTCCTGGTGTTGGAGGAC</td>
</tr>
<tr>
<td>9</td>
<td>CCCCACCATGAGGCAAGCATCAA</td>
<td>CAGCTCAAGAGAAGCTACATTT</td>
</tr>
<tr>
<td>10</td>
<td>GCCAGATACAGCCAGGAGACCA</td>
<td>AGCTCTCAGAGAGCTACATTT</td>
</tr>
<tr>
<td>11</td>
<td>AGGAGGAACAGGCGACACCTCTG</td>
<td>CACCTGGTGGTGGAGGAC</td>
</tr>
<tr>
<td>12</td>
<td>GTGCCACGCCGACAGGCTATTAA</td>
<td>CAGTCTGACGGTGGAGGAC</td>
</tr>
<tr>
<td>13</td>
<td>CCAAGATACACAGGTGTTCCAGTTT</td>
<td>CACCCCTGGTGGTGGAGGAC</td>
</tr>
<tr>
<td>14</td>
<td>AAGACACCTGTCGACACACAGGGAAG</td>
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<tr>
<td>15</td>
<td>AAGACACCTGTCGACAGAGGAGG</td>
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<tr>
<td>16</td>
<td>GTGTTACTCTTGAAAGTGGACCTT</td>
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<tr>
<td>17</td>
<td>TCACAAACAGGAAAGAAGATTG</td>
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<tr>
<td>18</td>
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<tr>
<td>19</td>
<td>ACCCAAGATACCTCAGTACAGTGGF</td>
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</tr>
<tr>
<td>20</td>
<td>TCGAGATATCTCAGTCAAGAACGCG</td>
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</tr>
<tr>
<td>21</td>
<td>AACCAAGATACCTCAGTACAGTGT</td>
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</tr>
<tr>
<td>22</td>
<td>AAGGAGGAACAGGCGACACCTCTG</td>
<td>CACCCCTGGTGGTGGAGGAC</td>
</tr>
<tr>
<td>23</td>
<td>GACCCTTTGTCAGGCTCTG</td>
<td>GAGGAGGAGCTTCTTAGAACT</td>
</tr>
</tbody>
</table>

PCR mixtures (50 µl) contained 1× PCR buffer, 5 pmol primers, 0.2 mM dNTP, 2 U of Taq polymerase, and 1 µl of PCR product. Fluorescently labeled PCR products were analyzed on the ABI Prism 310 Genetic Analyser (Applied Biosystems).

### Suppression assay

For three HC, the suppressive capacity of CD4+CD25high Treg cells toward CD4+CD28null T cells was measured using a coculture assay. FACs-sorted, CFSE-labeled CD4+CD28null or CD4+CD28+ responder cells (2 × 10³/well) were cultured with 1 × 10⁵ irradiated, autologous PBMC in the presence (1:1 ratio) or absence (1:0 ratio) of FACs-sorted Treg cells. Cells were stimulated with 2 µg/ml anti-CD3 mAb and 0.1 U/ml IL-2. All conditions were performed in triplicate. After 5 days of coculture, proliferation was measured by analyzing the CFSE signal on a FACSCalibur flow cytometer. Proliferation in all conditions was expressed relative to the proliferation in the 1:0 condition.

### Detection of IFN-γ and GrB by ELISA

Cell-free culture supernatants from the suppression assays and the Ag-stimulated conditions were collected. IFN-γ and/or GrB production were measured by ELISA using CytoSets (BioSource International) and a commercially available GrB Ab pair (Mabtech), respectively. Assays were performed according to the manufacturer’s instructions. OD were measured at 450 nm using an ELISA reader (Bio-Rad). Background levels (unstimulated conditions) of IFN-γ and GrB production were subtracted from the cytokine levels measured in the Ag-stimulated conditions. In the coculture experiment, IFN-γ production in the 1:0 ratio (only responder cells) was set at 100%. IFN-γ production in the other conditions was expressed relative to the IFN-γ production in the 1:0 condition.

### Statistical analysis

Statistical analyses were performed using Graphpad Prism version 4.00. To compare the expression of molecules between CD4+CD28null and CD4+CD28+ T cells, data were analyzed by Wilcoxon matched-pairs signed-ranks test. Differences between study groups were calculated by Kruskal-Wallis test. Dunn’s multiple comparison test was used as post-hoc test. A p value <0.05 was considered statistically significant.

### Results

#### Phenotypical characterization of CD4+CD28null T cells

The expression of CD45RO, TCRαβ, CD27, and CD25 on CD4+CD28null T cells of five HC, five RA, and five MS patients was analyzed by flow cytometry. Representative dot plots are shown in Fig. 1A. Like their CD28+ counterparts, the majority of

CD4+ CD28null T cells express CD45RO and a TCR consisting of an αβ heterodimer (Fig. 1, B and C). In contrast to CD4+CD28+ T cells, CD4+CD28null T cells have lost the expression of another costimulatory molecule CD27. In addition, CD4+CD28null T cells do not express the IL-2R α-chain (CD25) direct ex vivo, whereas CD25 is variably expressed by CD4+CD28+ T cells (Fig. 1, B and C).

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no differences in the expression of the studied adhesion molecules were found between the HC (n = 5), RA (n = 5), and MS patients (n = 5) tested.

We then analyzed whether CD4<sup>+</sup>CD28<sup>−</sup> T cells could indeed be detected at (disease relevant) tissue sites. Despite elevated percentages (>4%) of CD4<sup>+</sup>CD28<sup>−</sup> T cells in the blood of three of six RA patients, CD4<sup>+</sup>CD28<sup>−</sup> T cells could not be detected in the SF of any of the studied patients (Fig. 2F). However, CD4<sup>+</sup>CD28<sup>−</sup> T cells were present in both ST samples studied, 3.7 and 11.1%, respectively. These results illustrate that CD4<sup>+</sup>CD28<sup>−</sup> T cells can infiltrate and reside in the ST.

Survival and proliferation of CD4<sup>+</sup>CD28<sup>−</sup> T cells in vitro

To allow analyses of Ag reactivity, the optimal in vitro culture conditions of CD4<sup>+</sup>CD28<sup>−</sup> T cells were determined. Survival and proliferation of CFSE-labeled CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD28<sup>−</sup> T cells were evaluated in the presence of various concentrations (0, 0.1, and 2 U/ml) of IL-2. We showed that CD4<sup>+</sup>CD28<sup>−</sup> T cells do not express the IL-2R complex directly ex vivo (Fig. 1, A and C). Nevertheless, we found that addition of IL-2 to the culture medium was essential for survival and proliferation of the CD4<sup>+</sup>CD28<sup>−</sup> T cells and that the effect was dose dependent (Fig. 3). Upon stimulation, CD4<sup>+</sup>CD28<sup>−</sup> T cells up-regulate CD25 expression (data not shown). However, because of their deficient IL-2 production (10), they depend on external sources of IL-2 for survival and proliferation.

In contrast to CD4<sup>+</sup>CD28<sup>+</sup> T cells, ~20% of the CD4<sup>+</sup>CD28<sup>−</sup> T cells proliferate in the absence of costimulation (condition without feeders). The addition of autologous feeders significantly increased proliferation (PF > 95%) in both cell subsets. Representative dot plots demonstrating the in vitro culture behavior of both CD28<sup>+</sup> and CD28<sup>−</sup> CD4<sup>+</sup> T cells are shown in Fig. 3. Obviously, CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD28<sup>−</sup> T cells behave different in in vitro cultures and addition of low levels of IL-2 to in vitro CD4<sup>+</sup>CD28<sup>−</sup> T cell cultures is essential.

Reactivity of CD4<sup>+</sup>CD28<sup>−</sup> T cells to candidate autoantigens in MS and RA

To determine whether CD4<sup>+</sup>CD28<sup>−</sup> T cells show reactivity to autoantigens, sorted CD4<sup>+</sup>CD28<sup>−</sup> T cells were stimulated with hCII or MBP, two putative autoantigens in RA and MS, respectively. TT was included in the assay as a foreign recall control Ag. Also, reactivity of CD4<sup>+</sup>CD28<sup>−</sup> T cells toward CMV was evaluated because CMV infection has been associated with the expansion of CD4<sup>+</sup>CD28<sup>−</sup> T cells (7, 16). We included DMSO as a control condition, because CMV peptides were dissolved in DMSO. However, in none of the studied subjects was proliferation of CD4<sup>+</sup>CD28<sup>−</sup> T cells induced when DMSO only was added. Example histogram plots illustrating the proliferative responses of both CD4<sup>+</sup>CD28<sup>−</sup> and CD4<sup>+</sup>CD28<sup>−</sup> T cells toward different stimuli are shown in Fig. 4A. In a subset of patients and controls, MBP- and/or hCII-reactive CD4<sup>+</sup>CD28<sup>−</sup> T cells could be detected which illustrates that the sensitivity of this assay is sufficient to detect autoantigen-reactive T cells (data not shown). In present, this assay should be able to detect MBP- or hCII-reactive CD4<sup>+</sup>CD28<sup>−</sup> T cells. Fig. 4B shows the proliferative responses of both patients and controls to the tested Ags. In only one HC (HC1: hCII) and one MS patient (MS1: hCII and MBP), a small proportion of proliferating CD4<sup>+</sup>CD28<sup>−</sup> T cells (ΔPF < 15%) could be detected upon stimulation with candidate autoantigens. TT-specific proliferation was observed in CD4<sup>+</sup>CD28<sup>−</sup> T cells of one HC (HC1, ΔPF = 17%), one RA patient (RA3, ΔPF = 6%), and one MS patient (MS1, ΔPF = 34%). Interestingly, specific proliferation of a fraction of CD4<sup>+</sup>CD28<sup>−</sup> T cells was observed upon CMV stimulation in four of four RA patients, three of four MS patients, and four of four controls (Fig. 4B). In addition, we tested plasma samples for the presence of CMV-specific Abs and found reactivity in all subjects tested (Table I). Despite the presence of anti-CMV Abs, no reactivity of CD4<sup>+</sup>CD28<sup>−</sup> T cells against CMV or any of the other studied Ags was observed in MS4. Fig. 4C shows the fraction of CD4<sup>+</sup>CD28<sup>−</sup> T cells with no
dilution in their CFSE content upon CMV stimulation for every study subject. These cells represent CMV-unresponsive cells. In contrast to RA patients and HC, a substantial fraction (55.1–99.4%) of CD4⁺CD28null T cells of the MS patients appears to be CMV unresponsive. In Fig. 4D, the ΔPF in the CMV-stimulated condition is expressed relative to the ΔPF in the anti-CD3 mAb-stimulated condition. In the RA patients and HC, but not in the MS patients, the level of the CMV response was comparable to the proliferation induced by anti-CD3 mAb stimulation illustrating that almost all responsive cells were CMV reactive.

In addition, IFN-γ and GrB secretion in the culture supernatants was determined by ELISA. In general, these data confirmed the results of the proliferation assay. In the majority of patients and controls, CD4⁺CD28null T cells secreted large amounts (>500 pg/
ml) of IFN-γ and GrB upon CMV stimulation (Table III). Despite the presence of some CD4⁺CD28null proliferating cells, no IFN-γ or GrB release could be detected in MS1 upon stimulation with MBP or hCII. An interesting observation was the low, but detectable, IFN-γ and/or GrB excretion in response to collagen type II stimulation in three of four RA patients and three of four HC. Additionally, TT and MBP induced small amounts of GrB and/or IFN-γ excretion in various study subjects.

Altogether, these data show that CD4⁺CD28null T cells of RA and MS patients are not reactive to the autoantigens tested in this study. CD4⁺CD28null T cells appear to be mainly CMV reactive cells in both RA patients and HC. Of note is that a fraction of the CD4⁺CD28null T cells in MS patients might show reactivity to other Ags than those tested in this study.

TCR BV analysis of the CMV-specific T cell response

To evaluate the diversity of the CMV-specific T cell response, a semiquantitative TCR BV PCR analysis was performed on CMV-stimulated CD4⁺CD28null T cells. In general, a relatively broad set of TCR BV genes was expressed within CMV-reactive CD4⁺CD28null T cells. However, in some individuals (HC2, RA3, MS1, MS2, and MS3) one single predominant TCR BV family, constituting more than one-third of the expressed TCR BV repertoire, could be identified (Fig. 5) and this appeared to be associated with a diminished CMV-induced immune response (Fig. 4D). In all patients and controls, two to five overexpressed BV genes were identified which indicates that several CMV epitopes are recognized. These BV families each represent >10% of the expressed BV genes. To demonstrate the presence of CD4⁺CD28null T cells that are not CMV reactive, TCR BV gene analysis was also performed for anti-CD3-stimulated CD4⁺CD28null T cells for one subject from each study group. In the anti-CD3-stimulated CD4⁺CD28null T cells of the RA patient and HC, the repertoire of expressed TCR BV genes largely corresponded with that of the CMV-stimulated cells (Fig. 5). In contrast, in the MS patient, some TCR BV genes which were expressed in the anti-CD3-stimulated CD4⁺CD28null T cells could not be detected in the CMV-stimulated cells. This could implicate that a fraction of the CD4⁺CD28null T cells is not CMV specific.

In addition, a subset of TCR BV genes overexpressed in the CMV-stimulated condition were subjected to CDR3 fragment length analysis. Results demonstrated that the overexpressed TCR BV families mostly displayed a monoclonal (14 of 22) or oligo-clonal (5 of 22) CDR3 length profile which illustrates that...
CD4+CD28null T cells are clonally expanded cells (results are incorporated in Fig. 5).

The data illustrate that the CMV-induced CD4+CD28null T cell response is polyclonal, but more specifically, the cells involved in the CMV-response are clonally expanded. In addition, a fraction of the CD4+CD28null T cells in MS patients appears to be non-CMV reactive.

Suppressive capacity of CD4+CD25high Treg cells toward CD4+CD28null T cells

CD4+CD25+ Treg cells are an important arm of the immune system that down-regulates potentially harmful effector immune responses. In vitro studies showed that Treg cells are anergic and can suppress proliferation and cytokine production of activated...
CD4^+CD25^- T cells (reviewed by Shevach (33)). To investigate whether potentially pathogenic CD4^+CD28null T cells can be controlled by Treg cells, a CFSE-based suppression assay was performed. According to their CD25 expression, CD4^+CD28null T cells were cultured with varying amounts of Tregs. All conditions were performed in triplicate. Proliferation in the 1:0 ratio (responders alone) was set at 100%. Proliferation of CD4^+CD28^-CD25^- T cells in coculture with varying amounts of Treg cells was calculated relative to proliferation in the 1:0 ratio. A, Suppression of proliferation of CD4^+CD28^-CD25^- T cells. CFSE-labeled CD4^+CD28^-CD25^- responder T cells were cultured with varying amounts of Treg cells and CD4^+CD28null T cells. B, Suppression of cytokine production of CD4^+CD28^-CD25^- T cells. IFN-γ excretion in the culture supernatants was measured by ELISA. Cytokine excretion in the various responder to Treg ratios was expressed relative to the cytokine production in the 1:0 ratio. Bars indicate the mean for three HC; error bars indicate the SEM. C, Suppression of proliferation of CD4^+CD28null T cells. D, Suppression of cytokine production of CD4^+CD28null T cells.

Our results clearly demonstrated that CMV was the driving force behind the differentiation of CD4^+ T cells in RA patients and autoimmune diseases. We previously demonstrated that these cells are expanded in one in three RA patients and one in four MS patients (7). Our results indicate that CD4^+CD28null T cells display several features of pathogenic cells and are less susceptible to regulation by CD4^+CD25^high Treg cells. However, we were unable to demonstrate reactivity to the candidate autoantigens MBP and hCII within the CD4^+CD28null T cell subset of MS and RA patients.

Phenotypic characterization showed that CD4^+CD28null T cells are resting (CD25^-) effector memory cells. The absence of both CD28 and CD27 surface expression and their dependence on exogenous IL-2 indicates that CD4^+CD28null T cells are terminally differentiated cells. However, the profound proliferative and cytokine response observed in our study shows that these cells are not replicatively exhausted as was suggested before (10). A fraction of the CD4^+CD28null T cells can even proliferate in the absence of costimulation showing that costimulatory molecules other than CD28 are involved in complete activation of CD4^+CD28null T cells.

In a CFSE-based proliferation assay, we investigated whether autoantigens like hCII or MBP could be responsible for the expansion of CD4^+CD28null T cells in RA and MS patients. In this study, we found no evidence for autoreactivity of CD4^+CD28null T cells. Ratts et al. (34) did demonstrate that CD8^- T cell differentiation and associated CD28 loss could be caused by chronic stimulation with myelin Ags. But they also showed that the majority of the CD4^- T cell response to myelin Ags was CD28^-CD57^- in both MS patients and HC. Our results are, however, in contrast to the data reported by Markovic-Plese and co-workers (8). Using a different approach, they demonstrated the presence of MBP-reactive cells within the CD4^-CD28null T cell subset. Nevertheless, we believe that the high sensitivity of the assay used in our study together with the strong clonal nature of CD4^+CD28null T cells should have enabled us to detect autoreactive CD4^+CD28null T cells, if present.

Discussion

CD4^+CD28null T cells are chronically activated cells that have lost their classical helper function. We studied various characteristics of these cells to reveal their potential role in the pathogenesis of
HC. The CMV-specific CD4⁺CD28null T cell proliferative response was directed to several CMV epitopes and appeared to be stronger when the number of epitopes recognized was larger. We demonstrated that a large proportion of CD4⁺CD28null T cells in MS patients was not reactive to CMV or any of the other Ags tested in this study. Therefore, the antigenic specificity of these CMV-unresponsive cells remains speculative. In one MS patient, a minor fraction of the CD4⁺CD28null T cells was TT reactive. Reactivity toward other CMV Ags, other self-Ags (myelin oligodendrocyte glycoprotein, phospholipid protein, . . .) or other viruses, like, for example, EBV, could be responsible for our observations. Several studies revealed that EBV infection could be linked with both MS and CD4⁺ T cell differentiation (35, 36).

In general, it has been extremely difficult to demonstrate T cell reactivity against joint derived autoantigens such as hCII in RA patients. One cause may reside in the observed T cell hyporesponsiveness toward recall Ags in RA patients (37, 38). Otherwise, a partial tolerization to hCII may account for the absence of a proliferative response toward hCII in RA patients. Several studies have therefore used cytokine production instead of proliferation as parameter of response (39). Despite the secretion of small amounts of IFN-γ and/or Grb upon stimulation with hCII, it is quite clear that the majority of the highly differentiated CD4⁺CD28null T cells in RA patients are CMV-reactive cells.

Although CD4⁺CD28null T cells do not appear to be autoreactive, their enrichment in 33% of the RA patients and 24% of the MS patients supports their involvement in autoimmune pathogenesis (7). In some individuals, CD28null T cells make up more than half of the CD4⁺ T cell population, in this way diminishing immunological space and survival factors that are otherwise available for functional T cells, including Treg cell subsets. This change may cause a breakdown in tolerance and contribute to autoaggressive immune manifestations. In addition, it cannot be excluded that CMV-reactive CD4⁺CD28null T cells are cross-reactive to self-Ags, other than hCII and MBP which were tested in this study. Indeed, Brok et al. (40) described that the human CMV-UL86 peptide 981-1003 shares a cross-reactive T cell epitope with the encephalitogenic myelin oligodendrocyte glycoprotein peptide 34-56. Nevertheless, the degeneracy in both the TCR and MHC peptide-binding motifs make it very hard to predict cross-reactivity of CMV-reactive CD4⁺CD28null T cells toward a self-peptide (41). Also, it should be noted that this and other studies focused on peripheral CD4⁺CD28null T lymphocytes. It is possible that the disease-relevant CD4⁺CD28null T cells are located within the tissues expressing other TCR specificities.

Based on the surface expression of several adhesion molecules, CD4⁺CD28null T cell posses the capacity to infiltrate into tissues. This finding was confirmed by the detection of these cells in the synovial tissue of RA patients. In addition, we showed that CD4⁺CD28null T cells contain cytoplasmic granules of granzymes and perforin which are depleted upon TCR triggering. In a previous study, Nakajima et al. (18) demonstrated that CD4⁺CD28null T cells from patients with unstable angina can kill endothelial cells through the release of the pore-forming enzyme perforin. It is known that CMV can infect many different types of cells, including epithelial cells, endothelial cells, macrophages, fibroblasts, neuronal cells, smooth muscle cells, and hepatocytes (42). Our preliminary data indicate that CD4⁺CD28null T cells can kill disease relevant cell types, like synovial fibroblasts and oligodendroglial cells, cell types involved in RA and MS pathology, respectively (data not shown). In this way, CD4⁺CD28null T cells may contribute to tissue damage and the release of self-Ags. In addition, activation of CD4⁺CD28null T cells can create a proinflammatory, cytotoxic environment in which autoreactive T cells may be activated in a nonspecific manner (bystander activation).

This study shows that CD4⁺CD28null T cells are only partially susceptible to the regulatory capacities of Treg cells. Fully competent Treg cells can diminish the proinflammatory properties of the CD4⁺CD28null T cells, but cannot prevent their expansion. In mice, it has also been shown that Treg cells may suppress effector cell function but not proliferation (43). Apparently, proliferation and cytokine production are regulated independently. In humans, a target of Treg suppression seems to be transcriptional control of IL-2 in effector cells (44). However, CD4⁺CD28null T cells have been described to have a deficient IL-2 production (10) and may therefore be resistant to the regulatory properties of Treg cells. The failure of Treg cells to suppress CD4⁺CD28null T cell proliferation may contribute to the expansion of this aberrant cell population. The ability of Treg cells to down-regulate immune responses suggests that these cells may have therapeutic applications for the treatment of human autoimmune diseases (45). Results from this study may have implications for the efficacy of Treg therapy in autoimmune diseases as target cells may not be vulnerable to the suppressive capacities of Tregs.

CD4⁺CD28null T cells of RA and MS patients do not differ significantly from CD4⁺CD28null T cells in HC. This may indicate that these cells do not contribute to pathology, and are potentially an epiphenomenon of the disease. However, it may also indicate that the mere presence of these cells is not sufficient to cause pathology but that these cells are involved in the disease in an indirect manner. For instance, it was shown that reactivation of CMV is linked to activation of the immune system (46). In the case of RA, joint trauma may initiate joint inflammation. This inflammatory microenvironment may then result in reactivation of the latent CMV virus, activation of CD4⁺CD28null T cells, and a sustained immune response against the virus and consequently the joint because the immune system cannot eliminate the viral infection. In this way, CD4⁺CD28null T cells could play an indirect role in sustaining the chronic inflammation in the joints.

In conclusion, this study demonstrates that CD4⁺CD28null T cells in autoimmune pathology, although a direct autoaggressive involvement in the pathogenesis is unlikely.

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Disclosures
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References


