Expression of NKG2D and CD107 in CD8+ effector memory lymphocytes in Churg-Strauss syndrome

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ABSTRACT

Objectives. Churg-Strauss syndrome (CSS) is a necrotising vasculitis of small vessels in which oligoclonally expanded TCR Vβ CD8+ effector memory T cells populations (TEM) may be involved in vasculitic damage. The aim of this study was to assess the functional role of CD8+ T cells in CSS patients by flow cytometry analysis of membrane expression of cytotoxic markers NKG2D and CD107a.

Methods. Immunostaining of peripheral T cells and effector memory lymphocytes (TEM) from CSS patients and controls was performed by gating CD28 and CD45RA in the CD8+NKG2D+ and CD4+NKG2D+ populations. CD107a expression was evaluated in both whole CD8+ and CD4+ and the TEM cells by gating CD62 and CD45RA following polyclonal stimulation.

Results. NKG2D expression was shifted toward the CD8+CD28- fraction of T cells in CSS patients compared to healthy controls (56.1±25.8% versus 17.2±7.3%, respectively, p=0.002). CD8+Vβ expanded T cells showed a significantly increased expression of NKG2D compared to the whole CD8+ T cell population (91.4±1.9% versus 79.7±3.8%, respectively, p=0.015). Moreover the CD8+ population from CSS upregulates CD107a on its surface upon polyclonal stimulation in a significantly higher proportion than healthy subjects (26.2±10.8% versus 8.2±2.9%, p=0.0031) and the majority CD8+ CD107+ cells from CSS patients showed a TEM phenotype compared to controls (64.8±4.9% vs. 19.8±2.9, respectively, p<0.001).

Conclusion. In CSS, CD8+ TEM lymphocytes show markers of cytotoxic activity, which suggests a role for these cells in vasculitic damage.

Introduction

Churg-Strauss syndrome (CSS) is a disease characterised by vasculitis of small and medium arteries, which commonly involves the lung, skin and peripheral nerves, with prominent peripheral blood eosinophilia (1, 2). A pathogenic role for Th2 lymphocytes was postulated to explain the first two phases of this disease, which are the allergic phase, characterised by rhinitis and asthma, and the phase of eosinophilic infiltration of tissues (3, 4). Affected tissue of CSS patients highly express CCL17/thymus and activation-related chemokine (TARC), which recruits CD4+CD45RO+ T cells producing Th2 cytokines (5). CSS is usually considered a Th2 mediated disease, but Th1 and Th17 lymphocytes might also play a role (6, 7). CD8+ and CD4+ T cells are commonly found in biopsies from patients with active disease, including samples from both vasculitic and granulomatous lesions, suggesting T cell-mediated damage of blood vessels (8). Recently, we have described an expanded CD8+ cell population in the peripheral blood of CSS patients, which is characterised by specific Vβ families and monoclonal or oligoclonal T cell receptors (TCR), and which showed an effector memory phenotype (TEM) (9). Circulating CD8+Vβ+ TEM may infiltrate target organs and participate to the inflammatory cascade of both vasculitic and granulomatous lesions. Our hypothesis is that CD8+ TEM lymphocytes, which are monoclonally or oligoclonally expanded, may be involved in vasculitic damage acting by a mechanism of NK cell-like cytolysis in target organs, whereas CD4+ cells with a Th2 profile drive the activation of eosinophils, which secrete a variety of harmful mediators.

In this study, the expression of CD107a and NKG2D by CD8+ and CD4+ T cells, including TEM lymphocytes, was analysed in peripheral blood samples of CSS patients. CD107a is known as lysosomal-associated membrane pro-
tein 1 (LAMP1) and is considered to be a sensitive marker of degranulation for CD8+ cytotoxic T lymphocytes (CTLs) and NK cells (10, 11). In contrast to NK cells, CD8+ T cells do not constitutively express perforin, granzymes or CD107a; rather, these markers are expressed by CD8+ T cells only following their activation and clonal expansion (12).

NKG2D is an activating immunoreceptor that was first recognised on NK cells, but subsequently found on γδ T cells, CD8+ T cells and macrophages (13, 14). NKG2D is important not only for T cell- and NK cell-mediated immunity against viruses and tumours, but it has been shown also to play a role in autoimmune diseases, allogeneic transplantation, and xenotransplantation (15). The expression of ligands that are able to engage NKG2D results in target cell killing, and some studies implicate NKG2D in human and murine autoimmune disease pathology (16). Recently, NKG2D receptor overexpression has been found in oligoclonally-expanded CD4+CD8- T EM cells in Wegener’s granulomatosis. These cells have been shown to produce TNF-α, a cytokine that is important for the development of granulomas (17).

We thus reasoned that both CD107a and NKG2D receptors, whenever overexpressed in CD8+ T cells subsets, might suggest a mechanism of NK cell-like cytolysis in target organs of patients with CSS.

### Materials and methods

**Subjects**

Nine patients with confirmed CSS, who were diagnosed according to the American College of Rheumatology classification (ACR) (18), were included in the study. All patients had asthma, hypereosinophilia, rhinosinusitis and clinical manifestations that were consistent with systemic vasculitis (2). No patient had laboratory evidence of CMV and EBV reactivation. Patients clinical data are summarised in Table I. Samples from seven patients were recruited from the Allergy and Clinical Immunology, University of Torino, Italy, and the remaining two patients were recruited from Saint Vincent de Paul Hospital, Paris, France. All subjects gave their informed consent to participate in the study, which was approved by the local ethics committee. At the time of the study, no patient showed active vasculitis, while persistent disease was detected in 5 cases according to the Birmingham Vasculitis Activity Score (BVAS3) item list (19). The remaining patients were considered to be in remission, with the exception of asthma or neurologic and renal sequela, for at least six months prior to the study. Six healthy subjects, matched for age, were studied as controls.

**NKG2D immunostaining**

T cell immunostaining of whole peripheral blood from patients and controls was performed by four-colour flow cytometry (Facs Calibur, BD Biosciences, San José, CA, USA) and analysed by CellQuest (BD Biosciences, San José, CA, USA) software using the following panel of monoclonal antibodies (MoAb): CD8–A700 1:20, 1μl (BD Biosciences, Franklin Lakes, NJ, USA); NKG2D-PE 1:12.5, 1.6μl (eBioscience, San Diego, CA, USA); CD45RA-A647 1:40, 0.5μl (Sero tec, Oxford, UK). By gating CD28 and CD45RA in the CD8+NKG2D+ and CD4+NKG2D+ population, CD28- cells, either CD45RA- or CD45RA+, were considered effector memory lymphocytes (T EM) (20–22). Flow cytometry analysis was then performed in 5/9 patients using the specific Vβ families (FITC-conjugated) (Beckman Coulter, Miami, Florida, USA) of expanded T cell populations for each patient (9) (Table I) and the CD8, CD4 and NKG2D MoAb in order to quantify the NKG2D expression in the expanded CD8/Vβ families. Naïve and memory subsets and the expanded Vβ families were compared to normal controls (n=6). Each antibody was checked against an appropriate isotype-control antibody.

### CD107a assay

Peripheral blood mononuclear cells (PBMC) were separated from whole blood samples of patients by density

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**Table I. Clinical characteristic of patients.** BVAS, eosinophil counts and therapy are reported at the moment of blood sample analysis.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age</th>
<th>p-ANCA (anti-MPO U/ml)</th>
<th>Involved organs</th>
<th>Previous treatment</th>
<th>CD8+ TCRVβ expansion</th>
<th>BVAS</th>
<th>Eosinophils (K/μl)</th>
<th>Ongoing therapy</th>
</tr>
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<tr>
<td>1</td>
<td>F</td>
<td>54</td>
<td>+ (24)</td>
<td>PNS, lung</td>
<td>CCS, AZA</td>
<td>Vβ1</td>
<td>3</td>
<td>890</td>
<td>PDN 12.5mg, AZA 100mg</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>45</td>
<td>+ (42)</td>
<td>PNS, skin, kidney</td>
<td>CCS, CYC</td>
<td>Vβ18, Vβ9</td>
<td>6</td>
<td>1280</td>
<td>PDN 17.5mg</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>59</td>
<td>-</td>
<td>PNS</td>
<td>CCS, AZA</td>
<td>Vβ5.3</td>
<td>0</td>
<td>720</td>
<td>PDN 50mg</td>
</tr>
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<td>4</td>
<td>M</td>
<td>38</td>
<td>+ (63)</td>
<td>PNS, skin, joints</td>
<td>CCS, CYC</td>
<td>Vβ8</td>
<td>6</td>
<td>800</td>
<td>PDN 17.5mg</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>54</td>
<td>-</td>
<td>PNS, heart</td>
<td>CCS, CYC</td>
<td>Vβ4, Vβ13.1, Vβ13.2</td>
<td>0</td>
<td>1100</td>
<td>PDN 25mg</td>
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<tr>
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<td>55</td>
<td>+ (28)</td>
<td>PNS, skin, kidney</td>
<td>CCS, CYC</td>
<td>Vβ1, Vβ16</td>
<td>3</td>
<td>1690</td>
<td>MP 8mg</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>57</td>
<td>+ (46)</td>
<td>PNS, lung (DAH), kidney</td>
<td>CCS, CYC, MTX</td>
<td>Vβ3, Vβ18</td>
<td>0</td>
<td>250</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>60</td>
<td>+ (35)</td>
<td>PNS, skin</td>
<td>CCS, AZA, MTX</td>
<td>Vβ23</td>
<td>0</td>
<td>650</td>
<td>MTX 12.5mg/week</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>67</td>
<td>+ (32)</td>
<td>PNS, lung (DAH), kidney</td>
<td>CCS, CYC</td>
<td>Vβ13.1, Vβ18</td>
<td>6</td>
<td>620</td>
<td>PDN 100mg</td>
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</table>

p-ANCA: perinuclear-staining antineutrophil cytoplasmic antibodies, reported as positive indirect immunofluorescence (IFI) titer; BVAS: Birmingham Vasculitis Activity Score; PNS: peripheral nervous system; DAH: diffuse alveolar haemorrhage; CCS: corticosteroids; PDN: prednisone; MP: methylprednisolone; CYC: cyclophosphamide; AZA: azathioprine; MTX: methotrexate.

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Cytotoxic CD8+ lymphocytes in Churg-Strauss syndrome / M. Boita et al.
gradient centrifugation (Lymphoprep, Nycomed, Roskilde, Denmark). Approximately 300,000 cells/well were plated into a 96-well plate in 150 μl of RPMI containing 10% human serum and prepared in three different conditions: stained against CD107a MoAb FITC-conjugated (5 μl/well, BD Bioscience, San Diego, CA, USA) with or without the addition of anti-CD3 MoAb (for polyclonal stimulation) to obtain a final concentration of 100 ng/ml and unstained. Cells were then centrifuged at 1600 rpm for 1 minute and left for 1 hour at 37°C, followed by the addition of monensin (3 μM). After a 5-hour incubation at 37°C, all samples were collected and stained with antibodies against CD8-PerCP 1:20, 1 μl (eBioscience, San Diego, CA, USA); CD4-APC 1:40, 0.5 μl (BD Biosciences, Franklin Lakes, NJ, USA); CD62L-PE 1:20, 1 μl (Immunotech, Marseille, France); CD45RA-A647 1:40, 0.5 μl (Serotec, Oxford, UK). Cells were then sorted by flow cytometry (Facs Calibur, BD Biosciences, San José, CA, USA) and analysed using CellQuest software (BD Biosciences, San José, CA, USA). CD107a expression was evaluated in both whole CD8+ and CD4+ and the Tem cells by gating CD62 and CD45RA in the CD8+CD107+ and CD4+CD107+ population. The difference in CD107a expression between unstimulated (NS) and stimulated (STIM) conditions was evaluated for each patient and control.

Statistical analysis
Data were analysed with SPSS software (version 13.0 for Windows, Chicago, IL, USA). Normal distributions of variables were assessed according to Kolmogorov-Smirnov’s test of normality. In cases of non-normal distributions, the variables were analysed on a logarithmic scale. Comparisons between continuous variables were estimated with the unpaired Student’s t-tests or the Mann-Whitney U-test, depending on the distribution of the variables. All values are reported as the mean ± standard deviation (SD).

Results
NKG2D immunostaining
NKG2D expression was evaluated in CD4+ and CD8+ cells of all 9 patients and compared to age matched healthy subject. CD8+ T lymphocyte populations within both patients and controls had a high proportion of NKG2D expression (mean 74.76%±27.1% and 80.4%±12.4% in patients and controls respectively, p=0.621) (Fig. 1, panel A). These data confirm that NKG2D is constitutively expressed on CD8+ T cells in humans. (23). However, in CSS (n=8) the phenotype of CD8+NKG2D+ T cells was significantly shifted toward the expanded CD8+CD28 Tem cell fraction as compared to healthy controls. (56.1±25.8% versus 17.2±7.3%, respectively, p=0.002) (Fig. 1, panel B). Figure 1 (panel C) shows Tem cells in the CD8+NKG2D+ subpopulation as observed in one case of CSS compared to one control. It is of notice that in 3/9 CSS patients, who were in complete remission at the time of the study (CSS n. 3, 7, 8), the CD8 Tem within the NKG2D+ cells were lower than 40% of the total CD8+ population. Finally, we analysed NKG2D expression in 5/9 CSS patients CD8+Vβ expanded T cell populations. A significantly increased expression of NKG2D compared to the total CD8+ T cell population was observed (91.4±1.9% versus 79.7±3.8%, respectively, p=0.015). Mean NKG2D expression amongst CD4+ cells was not significantly different between patients and controls (13.1±18% versus 17.2±25.5%, respectively, p=0.120), neither in the CD4+CD28+ (19.3±3.4% versus 10.1±1.9%, respectively p=0.592) nor in the CD4+CD28+ (6.4±12.3% versus 0.8±1.1%, respectively p=0.248) fraction of T cells.

CD107a assay
Results of CD107a expression upon polyclonal stimulation in both CD8+ and CD4+ population of CSS and healthy controls are reported in Table II. The CD8+ population from CSS up-regulates CD107 on its surface in a significantly higher proportion that healthy subjects. (p=0.0031, Fig. 2, panel A).

Fig. 1. NKG2D expression in CD8+ (Panel A) and CD8+CD28 fraction Tem cells (Panel B) observed in CSS patients and healthy controls. The mean of NKG2D+ Tem cells is statistically different between CSS and controls (p=0.002). The dot plots show a representative double staining for NKG2D/CD28 on gated CD8+ T-cells in one patient (CSS n. 4) and one control. In CSS patient CD8+/NKG2D+ cells are shifted toward a Tem phenotype (Panel C).
CD107a upregulation in CD8+ cells following polyclonal stimulation as observed in one patient is shown in Figure 2, panel C.

On the contrary, mean CD107 expression was not different after stimulation of CD4+ cells in patients and controls. However, a subgroup of patients (CSS no. 2, 6, 7) showed an upregulation similar to their CD8+ counterpart (Table II). Higher proportions of CD8+ TEM (CD8+CD62L-) from CSS patients have been found to express CD107a following polyclonal stimulation (anti-CD3) compared to normal subjects (64.8±4.9% vs. 19.8±2.9, respectively, p<0.001, Fig. 2, panel B).

### Discussion

TEM are antigen driven cells which may infiltrate target organs and participate to the inflammatory cascade of vasculitic diseases. Actually, the percentage of CD4+CD45RO+CD62Llow T EM cells in patients with MPO ANCA-associated vasculitis has been found significantly expanded (24) as well as the circulating CD4+ TEM with no or little CD28 expression in Wegener granulomatosis (25). One or more Vβ families were found numerically expanded by flow cytometry among CD8+ T cells population of CSS patients and these expanded T cells were mostly represented by CD8+ lymphocytes with effector memory phenotype (9).

In this study, we found that the expression of NKG2D receptor and CD107a on circulating CD8+ T cells of CSS is significantly shifted toward an effector memory phenotype, compared to healthy controls.

NKG2D is an activating immunoreceptor that was first recognised on NK cells (13) and subsequently found on γδ T cells, CD8+ αβ T cells and macrophages (14), and has been recently found on CD4+CD28- oligoclonally-expanded TEM cells from patients with Wegener’s granulomatosis (17). These cells have been shown to produce TNF-α and to participate in granuloma formation as the expression of the activating NKG2D receptor on CD4+CD28- TEM could favour unbalanced proinflammatory responses in WG. We here described NKG2D recep-

### Table II

Mean values of CD107a expression in CD8+ and CD4+ cells upon polyclonal stimulation by anti-CD3 (STIM) and without stimulation (NS) in both T cells from CSS patients and healthy controls.

<table>
<thead>
<tr>
<th>CSS no.</th>
<th>NS % CD8+CD107+</th>
<th>STIM % CD8+CD107+</th>
<th>NS % CD4+CD107+</th>
<th>STIM % CD4+CD107+</th>
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<td>2.13</td>
</tr>
<tr>
<td>2</td>
<td>2.13</td>
<td>18.29</td>
<td>0.84</td>
<td>7.67</td>
</tr>
<tr>
<td>3</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>21.04</td>
<td>0.35</td>
<td>4.32</td>
</tr>
<tr>
<td>5</td>
<td>0.84</td>
<td>29.7</td>
<td>0.02</td>
<td>21.60</td>
</tr>
<tr>
<td>6</td>
<td>0.56</td>
<td>45.44</td>
<td>1.64</td>
<td>19.8</td>
</tr>
<tr>
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<td>29.83</td>
<td>1.02</td>
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<tr>
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<td>26.19</td>
<td>1.28</td>
<td>9.26</td>
</tr>
<tr>
<td>MEAN</td>
<td>0.94</td>
<td>10.80</td>
<td>1.01</td>
<td>8.01</td>
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Healthy controls

<table>
<thead>
<tr>
<th>CSS no.</th>
<th>NS % CD8+CD107+</th>
<th>STIM % CD8+CD107+</th>
<th>NS % CD4+CD107+</th>
<th>STIM % CD4+CD107+</th>
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<td>9.45</td>
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<td>6.68</td>
<td>0.6</td>
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<td>3</td>
<td>0.94</td>
<td>4.53</td>
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<td>2.94</td>
<td>0.76</td>
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NS: not stimulated; STIM: stimulated; SD: standard deviation; na: not available.

**Fig. 2.** CD107a cytotoxic assay in CD8+ (Panel A) and CD8+CD62L fraction TEM cells (Panel B) observed in CSS patients and healthy controls. The mean of CD107+ is statistically different between CSS and controls in both whole CD8+ and TEM cells (p<0.0031 and p<0.001, respectively). CD107a cytotoxic assay in CD8+ cells upon polyclonal stimulation in one CSS patients is shown in panel C. NS: basal state without stimulation; STIM: Polyclonal stimulation with anti-CD3. Analysis was performed on whole gated CD8+ population.
tor expression in both CD8+ T Emb and expanded CD8+Vβ8+ cell populations of CSS. These results support our hypothesis that mononclonally- or oligoclonally- expanded CD8+ T Emb cells may be involved in vasculitic damage. CD8+ populations from CSS upregulate CD107a on its surface following polyclonal stimulation and the majority of CD8+CD107+ cells from CSS patients showed a T Emb phenotype. CD107a, a sensitive marker of degranulation (10, 11), indicates activated antigen-specific CD8+ T cells that are ready to degranulate and promote cytolysis of target tissues (26).

NKG2D receptor expression in expanded CD8+Vβ8+ cell populations, as well as overexpression of CD107a, observed in CSS patients, suggests that these cells may cause cytolysis in target organs through an NK cell-like mechanism. Expanded CD8+Vβ8+ cells would infiltrate target organs, where they would then orchestrate granuloma formation and contribute to vascular injury. According to this pathogenic model, the vascular injury would be a secondary phenomenon, rather than the primary autoimmune target. In patients with CSS, the higher levels of NKG2D and CD107a overexpression was found in patients with signs of persistent disease. During remission, circulating subsets of CD8+ lymphocytes with an effector memory phenotype preserve the immunophenotypic characteristics of cytotoxic cells, even if at lower levels (patients no. 3, 7, 8). This fact can be due to the long lasting immunosuppressive treatment. Based on our results, we hypothesise that mononclonally/oligoclonally expanded populations of effector CD8+ lymphocytes are involved in vasculitic damage. Whether it is an exogenous antigenic trigger (virus, bacterial, drug) or a self-antigen, the force that drives the expanded populations of CD8+ TCRVβ in patients with CSS is not presently known. We suggest that the initial trigger may stimulate CD8+ cells to expand clonally and polarise toward a proinflammatory and cytotoxic phenotype, which in turn drives the development of vasculitis.

Our data confirm that, in CSS, the CD8+ T cell population contains terminally differentiated cells with an effector function that are able to infiltrate target organs and participate in the inflammatory cascade of vascular and granulomatosus lesions, which are commonly instigated by Th1 and more recently by Th17 cells (6, 7).

Whether these cells play a major role in vasculitic damage should be confirmed by tissue biopsies of involved organs.

References