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 (T_{EM}) 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 (T_{EM}) 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 T_{EM} 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 T_{EM} phenotype compared to controls (64.8±4.9% vs. 19.8±2.9, respectively, p<0.001).*

Conclusion. *In CSS, CD8+ T_{EM} 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 (T_{EM}) (9). Circulating CD8+Vβ+ T_{EM} may infiltrate target organs and participate to the inflammatory cascade of both vasculitic and granulomatous lesions.

Our hypothesis is that CD8+ T_{EM} 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 T_{EM} 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, $CD8^+$ αβ 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 $CD4^+CD28^-$ 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 1:10, 2µl (eBioscience, San Diego, CA, USA); CD4-PB 1:20, 1µl, (Life Technologies, NY, USA), CD28-FITC 1:20, 1µl (BD Biosciences, Franklin Lakes, NJ, USA); NKGD2-PE 1:12.5, 1.6µl (eBioscience, San Diego, CA, USA); CD45RA-A647 1:40, 0.5µl (Serotec, 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 $CD8^+V\beta^+$ 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

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

Patient no.	Sex	Age	p-ANCA (anti-MPO UI/ml).	Involved organs	Previous treatment	CD8+ TCRVβ expansion	BVAS	Eosinophils (K/µl)	Ongoing therapy
1	F	54	+ (24)	PNS, lung	CCS, AZA	Vβ1	3	890	PDN 12.5mg, AZA 100mg
2	M	45	+ (42)	PNS, skin, kidney	CCS, CYC	Vβ 18, Vβ 9	6	1280	PDN 17.5mg
3	F	59	-	PNS	CCS, AZA	Vβ5.3	0	720	PDN 5/0mg
4	M	38	+ (63)	PNS, skin, joints	CCS, CYC	Vβ8	6	800	PDN 17.5mg
5	F	54	-	PNS, hearth	CCS, CYC	Vβ4, Vβ13.1 Vβ13.2	0	1100	PDN 25mg
6	M	55	+ (28)	PNS, skin, kidney	CCS, CYC	Vβ1, Vβ16	3	1690	MP 8mg
7	M	57	+ (46)	PNS, lung (DAH), kidney	CCS, CYC, MTX	Vβ 3, Vβ 18	0	250	None
8	M	60	+ (35)	PNS, skin	CCS, AZA, MTX	Vβ23	0	650	MTX 12.5mg/week
9	M	67	+ (32)	PNS, lung (DAH), kidney	CCS, CYC	Vβ13.1, Vβ18	6	620	PDN 10/0mg

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

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 (3µM). After a 5-hour incubation at 37°C, all samples were collected and stained with antibodies against CD8-PerCP 1:20, 1µl (eBioscience, San Diego, CA, USA); CD4-APC 1:40, 0.5µl (BDBiosciences, Franklin Lakes, NJ, USA); CD62L-PE 1:20, 1µl (Immunotech, Marseille, France); CD45RA-A647 1:40, 0.5µl (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 T_{EM} 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 $CD8^+CD28^-$ T_{EM} 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 T_{EM} 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 no. 3, 7, 8), the $CD8^+$ T_{EM} within the $NKG2D^+$ cells were lower than 40% of the total $CD8^+$ population.

Finally. we analysed NKG2D expression in 5/9 CSS patients $CD8^+V\beta^+$ 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* 1.7±2.5%, respectively, p=0.120), neither in the $CD4^+CD28^-$ (1.9±3.4% *versus* 1.0±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 poly-clonal stimulation in both $CD8^+$ and $CD4^+$ population of CSS and healthy controls are reported in Table II. The $CD8^+$ population from CSS upregulates CD107 on its surface in a significantly higher proportion that healthy subjects. (p=0.0031, Fig. 2, panel A).

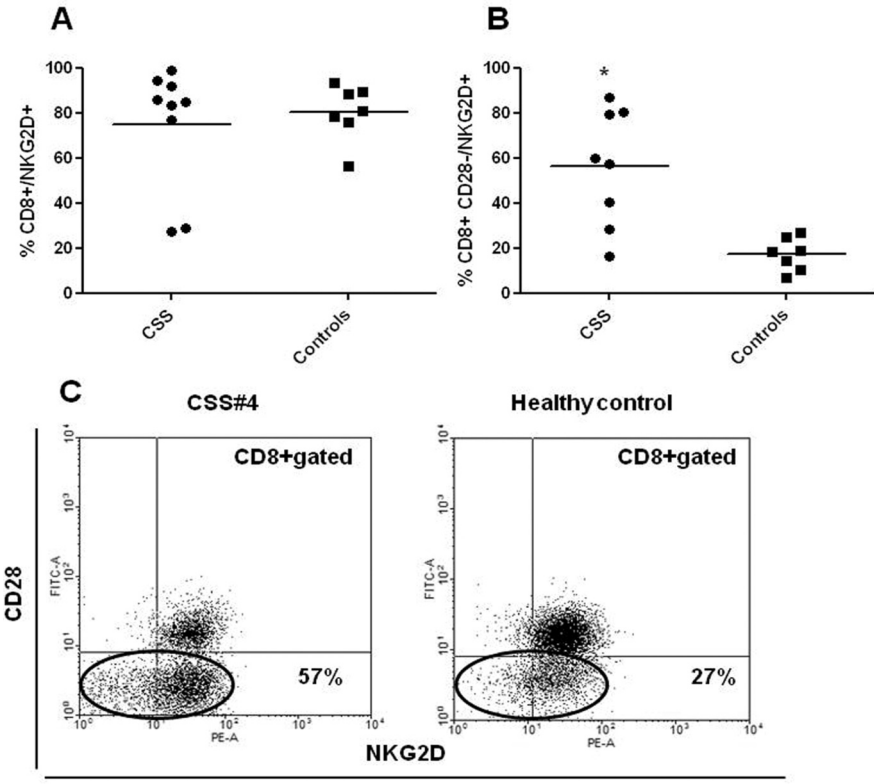

Fig. 1. NKG2D expression in $CD8^+$ (Panel **A**) and $CD8^+CD28^-$ fraction T_{EM} cells (Panel **B**) observed in CSS patients and healthy controls. The mean of $NKG2D^+$ T_{EM} cells is statistically different between CSS and controls (*p=0.002). The dot plots show a representative double staining 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 T_{EM} phenotype (Panel **C**).

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.

CSS no.	NS % $CD8^+CD107^+$	STIM % $CD8^+CD107^+$	NS % $CD4^+CD107^+$	STIM % $CD4^+CD107^+$
1	1.02	11.59	2.58	2.13
2	2.1	18.29	0.84	7.67
3	na	na	na	na
4	0.1	21	0.35	4.32
6	0.84	29.7	0.02	21.60
7	0.56	45.44	1.64	19.8
8	1.5	29.83	1.02	5.52
9	2.85	27.48	2.5	3.81
MEAN	1.28	26.19	1.28	9.26
SD	0.94	10.80	1.01	8.01
Healthy controls				
1	1.1	9.45	1.2	1.65
2	0.6	6.68	0.6	1.49
3	0.94	4.53	0.78	2.09
4	2.64	6.79	2.04	3.27
5	0.19	4.81	0.69	3.23
6	1.64	10	2.36	2.95
7	1.32	10.9	0.49	4.48
8	0.4	12.6	0.3	4.34
MEAN	1.10	8.22	1.06	2.94
SD	0.78	2.94	0.76	1.13

NS: not stimulated; STIM: stimulated; SD: standard deviation; na: not available.

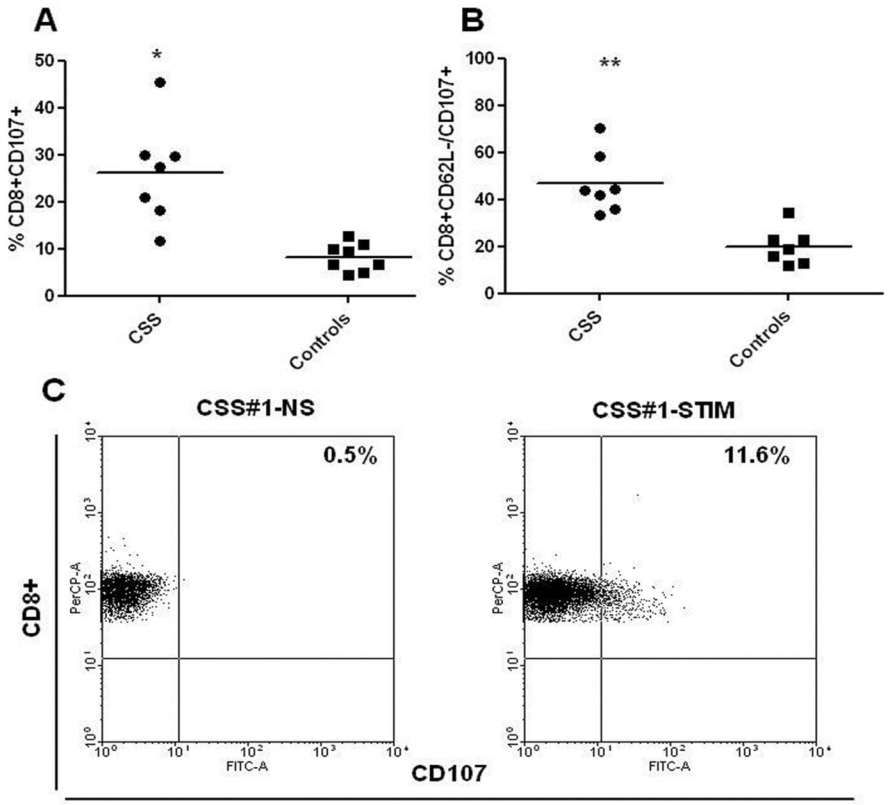

Fig. 2. CD107a cytotoxic assay in $CD8^+$ (Panel **A**) and $CD8^+CD62^-$ fraction T_{EM} cells (Panel **B**) observed in CSS patients and healthy controls. The mean of $CD107^+$ is statistically different between CSS and controls in both whole $CD8^+$ and T_{EM} cells (*p=0.0031 and **p<0.001, respectively). 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.

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 $CD8^+$ T_{EM} ($CD8^+CD62L^-$) 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

T_{EM} are antigen driven cells which may infiltrate target organs and participate to the inflammatory cascade of vasculitic diseases. Actually, the percentage of $CD4^+CD45RO^+CD62L^{low}$ T_{EM} cells in patients with MPO ANCA-associated vasculitis has been found significantly expanded (24) as well as the circulating $CD4^+$ T_{EM} 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 T_{EM} 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 $CD4^+CD28^-$ T_{EM} could favour unbalanced proinflammatory responses in WG. We here described NKG2D recep-

tor expression in both $CD8^+$ T_{EM} and expanded $CD8^+V\beta^+$ cell populations of CSS. These results support our hypothesis that monoclonally- or oligoclonally- expanded $CD8^+$ T_{EM} 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_{EM} 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 $CD8^+V\beta^+$ 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 $CD8^+V\beta^+$ 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 monoclonally/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 granulomatous 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.

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