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Heterogeneity of CD4⁺ and CD8⁺ memory T cells in localized and generalized Wegener's granulomatosisPeter Lamprecht^{1*}, Anika Erdmann^{1*}, Antje Mueller¹, Elena Csernok¹, Eva Reinhold-Keller¹, Konstanze Holl-Ulrich², Alfred C Feller², Hilke Bruehl³ and Wolfgang L Gross¹¹Department of Rheumatology, University of Luebeck, and Rheumaklinik Bad Bramstedt, Ratzeburger Allee 160, 23538 Luebeck, Germany²Institute of Pathology, University of Luebeck, and Rheumaklinik Bad Bramstedt, Ratzeburger Allee 160, 23538 Luebeck, Germany³Medical Policlinic, University of Munich, Pettenkoferstrasse 8a, 80336 Munich, Germany

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Received: 30 May 2002 Revisions received: 27 September 2002 Accepted: 8 October 2002 Published: 24 October 2002

Arthritis Res Ther 2003, **5**:R25-R31 (DOI 10.1186/ar610)© 2003 Lamprecht *et al.*, licensee BioMed Central Ltd (Print ISSN 1478-6354; Online ISSN 1478-6362). This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any non-commercial purpose, provided this notice is preserved along with the article's original URL.**Abstract**

Memory T cells display phenotypic heterogeneity. Surface antigens previously regarded as exclusive markers of naive T cells, such as L-selectin (CD62L), can also be detected on some memory T cells. Moreover, a fraction of CD45RO⁺ (positive for the short human isoform of CD45) memory T cells reverts to the CD45RA⁺ (positive for the long human isoform of CD45) phenotype. We analyzed patients with biopsy-proven localized Wegener's granulomatosis (WG) ($n = 5$), generalized WG ($n = 16$) and age- and sex-matched healthy controls ($n = 13$) to further characterize memory T cells in WG. The cell-surface expression of CD45RO, CD45RA, CD62L, CCR3, CCR5 and CXCR3 was determined on blood-derived T cells by four-color flow cytometric analysis. The fractions of CCR5⁺ and CCR3⁺ cells within the CD4⁺CD45RO⁺ and CD8⁺CD45RO⁺ memory T cell populations were significantly expanded in localized and generalized WG. The mean

percentage of Th1-type CCR5 expression was higher in localized WG. Upregulated CCR5 and CCR3 expression could also be detected on a fraction of CD45RA⁺ T cells. CD62L expression was seen on approximately half of the memory T cell populations expressing chemokine receptors. This study demonstrates for the first time that expression of the inducible inflammatory chemokine receptors CCR5 and CCR3 on CD45RO⁺ memory T cells, as well as on CD45RA⁺ T cells ('revertants'), contributes to phenotypic heterogeneity in an autoimmune disease, namely WG. Upregulated CCR5 and CCR3 expression suggests that the cells belong to the effector memory T cell population. CCR5 and CCR3 expression on CD4⁺ and CD8⁺ memory T cells indicates a potential to respond to chemotactic gradients and might be important in T cell migration contributing to granuloma formation and vasculitis in WG.

Keywords: CD45RA revertant, CD62L, chemokine receptor, effector memory T cell, Wegener's granulomatosis**Introduction**

Wegener's granulomatosis (WG) is an autoimmune disease of unknown etiology characterized by granulomatous and vasculitic lesions. Localized WG, in which granulomatous lesions are restricted to the respiratory tract, may precede 'classical' generalized WG. Observation of a disease course of localized WG without progression for several years has remained a rare finding [1]. The disease usually progresses to generalized WG, in which clinical

manifestations of frank autoimmune vasculitis prevail, for example pulmonary-renal syndrome. An autoantibody, namely antineutrophil cytoplasmic antibody specific for proteinase 3 (PR3-ANCA), is detected in the vast majority of patients with generalized WG [1,2]. Whereas granulomatous lesions express predominantly T helper type 1 (Th1)-type cytokines in localized WG [3], a shift towards stronger Th2-type cytokine expression is found in granulomatous lesions of the respiratory tract in generalized WG

ANCA = antineutrophil cytoplasmic antibody; APC = allophycocyanine; BVAS = Birmingham Vasculitis Activity Score; CD45RA = long human isoform of CD45; CD45RO = short human isoform of CD45; CD62L = L-selectin; CCR3 = CC chemokine receptor 3; CCR5 = CC chemokine receptor 5; CXCR3 = CXC chemokine receptor 3; DEI = Disease Extent Index; FITC = fluorescein isothiocyanate; HC = healthy controls; PBMC = peripheral blood mononuclear cells; PE = phycoerythrin; PerCP = peridinin chlorophyll protein; PR3 = proteinase 3; Th1 = T helper type 1; WG = Wegener's granulomatosis.

[3,4]. In kidney lesions of generalized WG, the expression of both Th1-type and Th2-type cytokine and chemokine receptors has been described. Thus, the local cytokine milieu might depend on the site and extent of disease activity [4,5]. The shift in the cytokine profile in granulomatous lesions of the respiratory tract might be of importance for disease progression [2,3].

The term effector memory T cells has been used to denote memory T cells that display migratory properties, readily produce cytokines and release granular contents [6–8]. These cells show striking tissue selectivity of migration and are preferentially recruited to sites of inflammation [7,8]. The phenotype of memory T cells is heterogeneous. A fraction of CD45RO⁺ (positive for the short human isoform of CD45) memory T cells reverts to the phenotype CD45RA⁺ (positive for the long human isoform of CD45) [9]. Antigen-experienced T cells, for example virus-specific T cells, were found to display considerable heterogeneity with regard to their CD45 isoform expression [10,11]. Surface antigens formerly regarded as exclusive markers of naive T cells, such as L-selectin (CD62L) or the CC chemokine receptor CCR7, were also found to be expressed on a fraction of memory T cells including tissue-specific cells [10,12]. Thus, distinction between naive and memory T cells cannot be based on the analysis of single surface antigens. A combination of surface markers and the function of the T cell have to be considered in distinguishing naive from memory T cells [9–12].

In granulomatous lesions, CD4⁺ and CD8⁺ T cells express the CD45RO isoform consistent with a memory phenotype [13]. However, the intralesional expression of CD45RA isoforms has yet not been analyzed. RANTES ('regulated upon activation in normal T cells, expressed and secreted'; also known as CCL5), a ligand for the CC-chemokine receptors CCR1, CCR3 and CCR5, has recently been demonstrated in pulmonary granulomatous lesions in WG. The chemokine is expressed mainly in macrophages and may promote the recruitment of effector memory T cells into the lesion [13].

In the present study we analyzed CCR5 and CCR3 expression on T cells to determine the surface antigens important for the recruitment of cells, namely the exertion of migratory functions and a prerequisite for the formation of granulomatous lesions and vasculitis in WG. Because chemokine receptors such as the inducible inflammatory chemokine receptors CCR5 and CCR3 – along with selectins and adhesion molecules – have a pivotal role in effector memory T cell trafficking into inflammatory areas [6–8], we proposed that CCR5 or CCR3 is expressed on CD4⁺ and CD8⁺ effector memory T cells. We further proposed that differences in the chemokine receptor expression might contribute to the recruitment of distinct effector memory T cell populations into granulomatous lesions,

resulting in different cytokine patterns within granulomatous lesions in localized WG and generalized WG. This in turn might also influence the outcome of the disease course (localized versus generalized WG). Moreover, the analysis of relevant chemokine receptor expression unravels new targets for a specific therapy directed against chemokine receptors.

Materials and methods

Study population

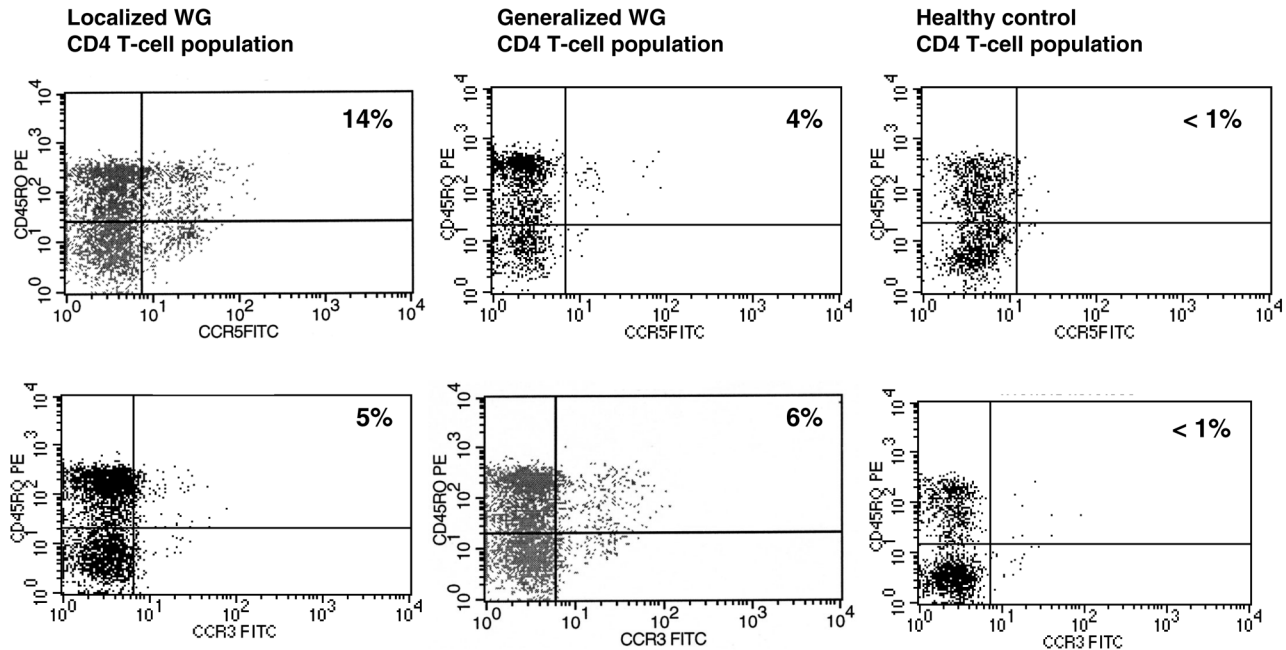
Peripheral blood mononuclear cells (PBMC) from 5 patients with localized WG, 16 patients with generalized WG, and 13 age- and sex-matched healthy controls (HC) were analyzed. All patients met the criteria of the American College of Rheumatology [14] and the Chapel Hill Consensus Conference definition for WG [15]. WG was biopsy-proven in each patient. Biopsies were seen in a German reference center for vasculitis (Department of Pathology, University of Lübeck) by two independent observers (KHU and ACF). All patient charts of patients with localized and generalized WG were critically evaluated by an interdisciplinary group [1].

Subclassification of WG into localized and generalized WG was done in accordance with the definitions given for both disease stages by the European Vasculitis Study Group [16]. PR3-ANCA was detected in all patients with generalized WG. Disease extension and vasculitis activity were documented by using the Disease Extent Index (DEI) and the Birmingham Vasculitis Activity Score (BVAS) as outlined elsewhere [17,18]. In brief, the DEI gives the equivalent of organ involvement attributable to active disease in WG [17], whereas the BVAS considers clinical features and laboratory data to give a measure of vasculitis activity [18]. In its current version BVAS.1 represents a score of new or worse disease activity, namely active disease, whereas BVAS.2 represents a score of disease activity due to persisting disease [18].

There were two groups of patients with generalized WG: nine patients with generalized WG had active disease (BVAS.1 \geq 4, DEI \geq 2), whereas seven patients were in remission (BVAS.1 = 0, BVAS.2 \leq 3), i.e. evidence of partial or complete improvement of vasculitis activity by clinical and serological investigations and by imaging procedures. All patients with localized WG were in remission, with some symptoms still persisting because of the damage caused by preceding active disease. Treatment consisted of corticosteroids (1 patient with localized WG and 14 patients with generalized WG), cyclophosphamide (0/5), methotrexate (1/9), azathioprine (0/2), leflunomide (0/3, in combination with methotrexate) and cotrimoxazole (3/0).

Antibodies and reagents

The following antibodies were used for flow cytometric analysis of cells: CD4-APC (APC = allophycocyanine),

Figure 1

Representative flow-cytometric analysis of cell-surface chemokine receptor expression on CD4⁺ T cells. Peripheral blood mononuclear cells from a patient with localized Wegener's granulomatosis (WG), from a patient with generalized WG and from a healthy control were simultaneously stained with CD4-APC, CD45RO-PE, CD45-PerCP, and either CCR5-FITC or CCR3-FITC. CD4-positive lymphocytes were gated on the basis of light-scattering properties and on CD4 and CD45 staining, then analyzed for expression of CD45RO and CCR5 or CCR3.

CD8-APC, CD45RO-PE (PE = phycoerythrin), CD45RA-FITC (FITC = fluorescein isothiocyanate), CD45-PerCP (PerCP = peridinin chlorophyll protein), CD62L-PerCP (BD, Heidelberg, Germany), CCR5-PE, CCR5-FITC, CCR3-PE, CCR3-FITC, CXC chemokine receptor 3 coupled to FITC (CXCR3-FITC), and RANTES (R&D, Wiesbaden, Germany). Isotype control antibodies were as follows: Rat IgG2a-FITC, mouse IgG2a-FITC, mouse IgG2a-PE, mouse IgG2b-FITC, mouse IgG2b-PE, mouse IgG1-FITC and mouse IgG1-PE (BD, Heidelberg, Germany). To test the specificity of the antibodies, PBMC were incubated for 30 minutes with increasing concentrations of RANTES (1–1000 ng/ml; R&D, Wiesbaden, Germany). After a washing step, staining for CD4, CD45RO and CCR5 was performed. At the highest RANTES concentration, CCR5 staining was almost completely blocked.

Cell-surface marker staining and flow cytometry

PBMC were isolated by Ficoll-Hypaque density-gradient centrifugation. Cells were resuspended in buffer containing 0.1% bovine serum albumin and 0.1% NaN₃ at 10⁶ cells/ml. Nonspecific antibody binding at the Fc receptor was blocked by treating 10⁶ cells with 10 μg human IgG (Chromopure) for 15 minutes at room temperature. Afterwards 10⁵ cells were stained in 100 μl of buffer containing the previously determined optimal con-

centrations (0.25–1.0 μg/100 μl) of fluorochrome-conjugated monoclonal antibodies for cell surface antigens or appropriate negative (isotype) controls. Incubation was performed at 4°C for 30 minutes in the dark. After a washing step, cells were fixed with 300 μl of PBS containing 1.5% paraformaldehyde. Four-color flow cytometric analysis by fluorescence-activated cell sorting was performed with a FACSCalibur™ flow cytometer (BD, Heidelberg, Germany). Data were acquired with CELL-Quest™ software (BD, Heidelberg, Germany). CD4⁺ or CD8⁺ lymphocytes were gated for analysis based on light scattering properties and on CD4 or CD8 and CD45 staining. Data were collected for 10⁴ lymphocytes. Positively and negatively stained populations were calculated by quadrant dot plot analysis determined by isotype controls.

Statistical analysis

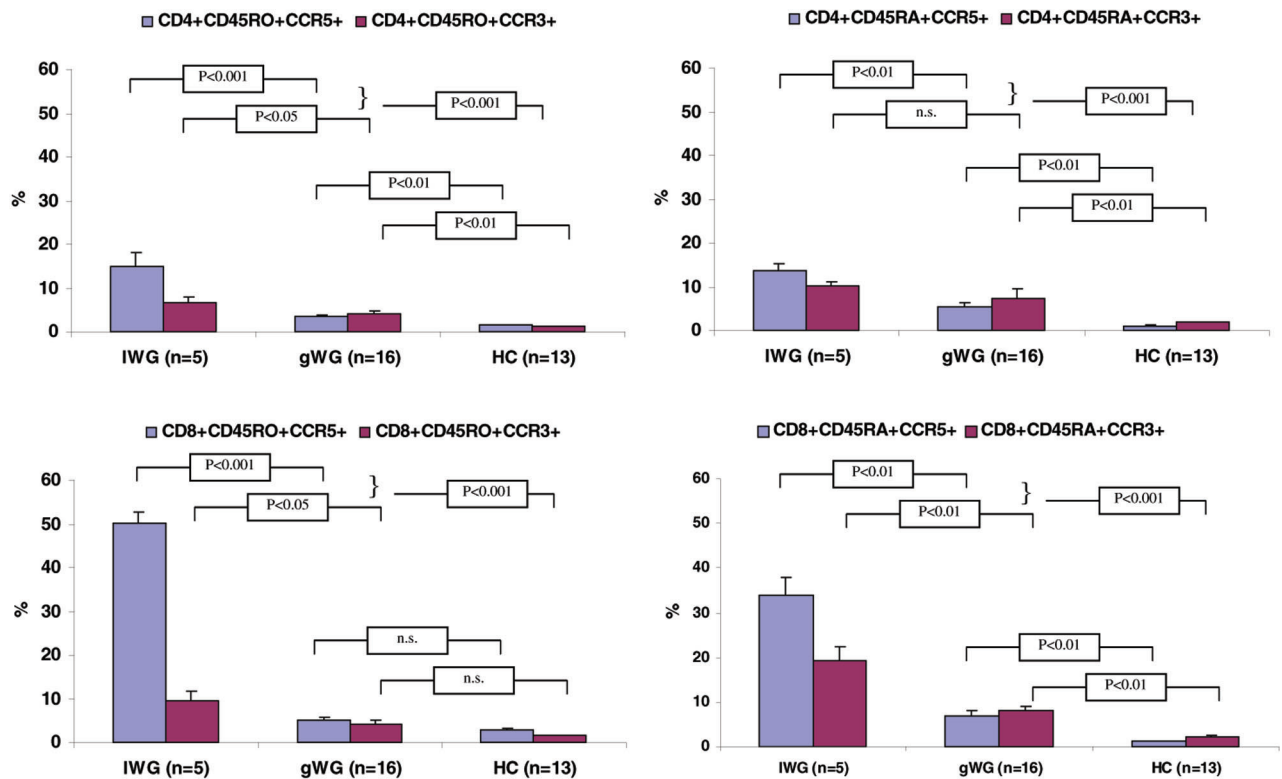
A non-normal distribution was assumed and a nonparametric test (Mann-Whitney test) was performed. $P < 0.05$ was regarded as significant.

Results

CD45RA⁺ and CD45RO⁺ T cells express CCR3, CCR5 and CXCR3

The median CD4⁺/CD8⁺ T cell ratio was 2.5 (range 1.1–4.4) in localized WG, and 1.1 (0.6–2.6) in generalized WG, whereas it was 1.7 (0.9–7.1) in HC. There

Figure 2



Cell-surface expression of the chemokine receptors CCR5 and CCR3 on T cells in localized Wegener's granulomatosis (IWG), generalized WG (gWG) and healthy controls (HC). Bars represent the fractions (given as percentages, means \pm SEM) of either CCR5+ or CCR3+ cells within the CD4+CD45RO+, CD4+CD45RA+, CD8+CD45RO+ and CD8+CD45RA+ T cell populations.

were no differences between the absolute leukocyte and lymphocyte counts in localized WG, generalized WG and HC. HC displayed low-level cell-surface expression of CCR5 and CCR3, which might be in response to some antigenic challenge. CCR5 and CCR3 expression was significantly upregulated on CD4+ and (even more strongly) on CD8+ T cells in both localized and generalized WG compared with HC (Figs 1 and 2). Chemokine receptor expression was not confined to the CD45RO+ population: CD45RA+ T cells also had upregulated CCR5 and CCR3 expression. In localized WG, CCR5 expression was generally significantly higher than CCR3 expression. In generalized WG, CCR3 expression was similar to CCR5 expression. Co-expression of CXCR3 was detected on up to one-quarter of CCR5+ T cells within either the CD45RO+ or the CD45RA+ T cell population.

We found no significant differences in the mean percentages of CCR5 expression and CCR3 expression on the CD4+ and CD8+ memory T cell subsets between active generalized WG and generalized WG in remission. The corticosteroid dose was significantly higher in generalized WG, both active and in remission (10.9 ± 2.0 ,

0–25 mg/day orally; mean \pm SEM, range), compared with localized WG (0.8 ± 0.8 , 0–4 mg/day p.o.; $P < 0.01$).

CD62L expression on chemokine receptor expressing CD45RA+ and CD45RO+ T cell populations

To address the question of whether CD62L expression is confined to naive T cells or is also seen on some memory T cells in WG, we analyzed CD62L expression on T cells expressing chemokine receptors. Approximately half of the CD4+CD45RO+, CD8+CD45RO+, CD4+CD45RA+ and CD8+CD45RA+ T cell populations expressing CCR5 or CCR3 were also expressing CD62L. Thus, some memory T cells might also express CD62L in WG. In contrast, mean CD62L expression was significantly higher on CD4+CD45RA+ and CD8+CD45RA+ T cells not expressing CCR5 or CCR3 (89.2 ± 3.1 , range 80.6–96.4% and 66.0 ± 13.0 , range 21.4–93.0%; $P < 0.05$). Thus, most CD45RA+ T cells not bearing CCR5 or CCR3 have to be regarded as naive T cells expressing CD62L for their homing to secondary lymphoid tissues [5–7].

Discussion

In WG, molecules involved in the recruitment of T cells into granulomatous lesions are E- and P-selectin and

ICAM-1 expressed on the endothelial side, and the β_2 -integrin LFA-1 on T cells [5,19]. In addition to the steps mediated by selectin and adhesion molecules, chemokine receptors support the selective recruitment of differentiated T cells into tissues through interaction with endothelial and tissue-expressed chemokines [6–8]. In the present study we analyzed the expression of the inducible inflammatory chemokine receptors CCR5 and CCR3 on CD4⁺ and CD8⁺ memory T cells, important for their recruitment to inflammatory sites, in other words the exertion of migratory effector functions, in WG. We found the expression of the chemokine receptors CCR5 and CCR3 to be significantly upregulated on peripheral blood CD4⁺ and CD8⁺ T cells in localized and generalized WG. Predominance of CCR5 expression over CCR3 expression was detected in localized WG but not in generalized WG. Moreover, this pattern of chemokine receptor expression was detected similarly on CD4⁺CD45RO⁺, CD4⁺CD45RA⁺, CD8⁺CD45RO⁺ and CD8⁺CD45RA⁺ T cells. CD62L expression was also seen on approximately half of the aforementioned T cell populations expressing chemokine receptors, whereas CD4⁺CD45RA⁺ and CD8⁺CD45RA⁺ T cells not expressing CCR5 or CCR3 displayed a significantly higher expression of CD62L.

Upregulated CCR5 and CCR3 expression on CD4⁺CD45RO⁺ and CD8⁺CD45RO⁺ and also on CD4⁺CD45RA⁺ and CD8⁺CD45RA⁺ T cells indicates activation and the potential to respond to chemotactic gradients in inflammatory areas, which is consistent with an effector memory T cell phenotype [6–8].

Because it remains an important challenge to relate the phenotype of a T cell population to its function, further studies have to address functional aspects such as response *in vitro* to chemotactic gradients, cytokine release and cytotoxic activity of distinct T cell populations. Detection of the inducible inflammatory chemokine receptors CCR5 and CCR3 on CD45RA⁺ T cells suggests that CD45RA⁺ 'revertants' [9,20] might also constitute part of the expanded population of memory T cells bearing chemokine receptors in WG. A lack of CD27 expression has been reported to distinguish effector cells from naive T cells within the CD8⁺CD45RA⁺ T cell population [21]. However, Wills *et al.* [22] demonstrated that cytomegalovirus-specific T cells, that is, antigen-experienced T cells, are found within the fractions of CD27⁺CD28⁻ and CD27⁻CD28⁻ T cells. These cells either express CD45RA or CD45RO. Cytomegalovirus-specific T cells, that is, antigen-experienced T cells, are mainly CD28⁻ and can also express CCR5 [10,23,24]. The fraction of CD28⁻ T cells is also expanded in WG and is correlated with organ involvement [25–28]. These findings support our designation of CD45RA⁺ T cells expressing the inducible inflammatory chemokine receptors CCR5 and CCR3 as T cell 'revertants'.

A fraction of memory T cells also expressed CD62L contributing to the phenotypic heterogeneity of T cells in WG. CD62L expression was significantly higher on CD4⁺CD45RA⁺ and CD8⁺CD45RA⁺ T cells not expressing CCR5 or CCR3, which might represent naive T cells. CD62L mediates the homing of naive T cells through interaction with glycoprotein ligands on high endothelial venules in secondary lymphoid tissues. Memory T cells were previously thought to have lost CD62L expression completely. However, our results are in line with those of two other studies also demonstrating CD62L expression on part of the memory T cell population [12,29]. CD62L expression might in fact be lost only some time after differentiation into tissue-specific memory T cells [12,29]. Thus, CD4⁺CD45RA⁺ and CD8⁺CD45RA⁺ T cells expressing the inducible inflammatory chemokine receptors CCR5 or CCR3 as well as CD62L might constitute part of the CD45RA⁺ 'revertant' memory T cell fraction.

We have previously shown that cytokine production is upregulated in patients with active disease and ineffective therapy [30]. In the present study we analyzed two groups of patients with generalized WG: patients with active disease (failure of immunosuppression) and patients in remission (responding to immunosuppressive therapy). We found no significant differences in mean percentage of CCR5 expression and CCR3 expression on the CD4⁺ and CD8⁺ memory T cell subsets between active generalized WG and generalized WG in remission. Popa *et al.* [31] also found markers of T cell activation such as HLA-DR to be upregulated during remission of generalized WG. The overall level of chemokine receptor expression was higher in patients with localized WG than in those with generalized WG. Higher doses of corticosteroids might have influenced the overall level of chemokine receptor expression in generalized WG but do not explain differences in the expression of CCR5 and CCR3 between localized and generalized WG.

Moreover, the overall level of chemokine receptor expression in generalized WG might have been influenced by a depletion of CCR5⁺ and CCR3⁺ T cells owing to enhanced recruitment into inflammatory sites. Downregulation of the cell-surface expression of the inducible inflammatory chemokine receptors CCR5 and CCR3 during longer phases of disease activity might be another mechanism influencing the overall level of their expression in generalized WG [32]. As stated above, granulomatous lesions of the respiratory tract express predominantly Th1-type cytokines in localized WG [3]; a shift toward stronger Th2-type cytokine expression is found in granulomatous lesions of the respiratory tract in generalized WG [3,4].

CCR5⁺ T cells have been ascribed to the effector memory population producing Th1-type cytokines and CCR3⁺ T cells to the effector memory population producing Th2-

type cytokines [6–8]. In localized WG a higher CCR5 expression on memory T cells might favor the recruitment of Th1-type effector memory T cells into granulomatous lesions of the respiratory tract, whereas in generalized WG the CCR3-mediated recruitment of Th2-type effector memory T cells might have a larger role. In kidney lesions of generalized WG, Th1-type as well as Th2-type cytokine expression and CCR5 and CCR3 expression have been described in generalized WG [4,5]. Changes in the cytokine balance might influence disease activity, as exemplified by a patient with localized WG whose disease activity flared and generalization of WG occurred during an acute hepatitis C virus infection and interferon- α therapy [33].

The present study shows that not only 'classical' chemokine-receptor-bearing CD45RO⁺ T cells, but also CD45RA⁺ 'revertants' within the CD4⁺ and CD8⁺ T cell populations, contribute to the phenotypic heterogeneity of memory T cells in WG. CD62L expression is also found on some memory T cells. Differences in CCR5 and CCR3 expression on effector memory T cells might favor the migration of distinct Th1-type and Th2-type T cell populations into granulomatous lesions and vasculitic areas, resulting in differences in tissue cytokine patterns found in localized WG and generalized WG.

Acknowledgements

This work was supported by grants SFB367/A8 from the German Research Society (Deutsche Forschungsgemeinschaft/DFG) to PL, AM and WL, and grant nos 0.2 and 02.2 from the Verein zur Foerderung der Erforschung und Bekaempfung rheumatischer Erkrankungen Bad Bramstedt e.V. to AM and PL. We thank Linda Carstens for excellent technical assistance. We are grateful to the patients for their cooperation.

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