Phenotype and functional changes of Vγ9/Vδ2 T lymphocytes in Behçet's disease and the effect of infliximab on Vγ9/Vδ2 T cell expansion, activation and cytotoxicity

Antonina Accardo-Palumbo, Anna Rita Giardina, Francesco Ciccia, Angelo Ferrante, Alfonso Principato, Rosalia Impastato, Ennio Giardina and Giovanni Triolo*

Abstract

Introduction: Infliximab is a chimeric monoclonal antibody against tumor necrosis factor alpha (TNF-α) that has been introduced recently for Behçet's disease (BD) patients who were resistant to standard treatment. The aim of this study was to analyse the functional changes of Vγ9/Vδ2 T lymphocytes in both active and inactive disease and the effect of infliximab on Vγ9/Vδ2 T cell expansion, activation and cytotoxicity.

Methods: We investigated 1) cell expansion, 2) expression of TNFRII receptor, 3) perforin and gamma interferon (IFN) content, 4) release of granzyme A (GrA) and 5) phenotype changes, in vitro and in vivo, in Vγ9/Vδ2 T lymphocytes by means of fluorescence-activated cell sorter analysis of lymphocyte cultures from patients with active and inactive BD and healthy subjects.

Results: Cell expansion, expression of TNFRII, perforin and gamma IFN content and release of granzyme A were significantly higher in active patients. In vitro and ex vivo treatment with infliximab resulted in a significant reduction of all parameters together with changes in the phenotype of Vγ9/Vδ2 T cells.

Conclusions: All together these data indicate that infliximab is capable of interfering with Vγ9/Vδ2 T cell function in BD and although cell culture models cannot reliably predict all potential effects of the drug in vivo, our results present the possibility that this drug may find use in a range of immunological disorders, characterized by dysregulated cell-mediated immunity.

Introduction

Behçet's disease (BD) is a multisystemic inflammatory disorder characterized mainly by recurrent oral and genital apthous ulcerations and uveitis. The clinical spectrum of BD is wide, involving skin, blood vessels, joints, nervous system, lungs and intestines. The treatment of BD is based for most patients on the combination of corticosteroids and immunosuppressive agents. Despite the improvement obtained with this strategy, relapses and failure may occur, some patients being also refractory to all treatments. Infliximab a chimeric monoclonal anti-body against TNF-α that has been introduced for patients with Crohn's disease, rheumatoid arthritis, psoriasis, juvenile chronic arthritis and more recently for BD patients who were resistant to standard treatment [1,2].

The immunopathogenesis of BD remains unknown, but it is believed to be T cell-mediated [3-5]. Recently, attention has been focused on the role of γδ T cells and we have demonstrated that Vγ9/Vδ2 circulating lymphocytes are activated in patients with active disease and express increased levels of receptors for TNF-α and IL (interleukin)-12 [6]. Moreover, elevated levels of granzyme A both in the serum and Vγ9/Vδ2 cell supernatants of active BD patients are present, suggesting a role for this kind of lymphocytes in the pathogenesis and in the progression of the disease [7]. In this paper we analysed the
functional changes of Vy9/Vδ2 T lymphocytes in both active and inactive disease and of the effect of infliximab on Vy9/Vδ2 cell expansion, activation and cytotoxicity.

Materials and methods

Patients

Thirteen patients with BD (nine males and four females, mean age 42 ± 24 years), classified according to the International Study Group for Behçet’s Disease [8] were studied. The activity of BD was assessed by collecting clinical symptoms defined according to the BDCAF score [9,10] that includes the presence of several manifestations of the disease, by the uveitis scoring system and by the visual activity measurement [11]. At the time of sampling, disease was active in six patients and inactive in seven. In five of the active patients blood for serum and lymphocyte studies was obtained before and after the anti-TNF-α (Infliximab) therapy. All patients were using colchicine (n = 13), and/or low dose corticosteroids (n = 8). Ten healthy volunteers (age range 21 to 47, mean 30 ± 8 years) were enrolled as controls. None of patients or controls were HIV, CMV, EBV infected. Human studies committee approval and individual informed consent from each patient were obtained.

Cell separation and in vitro expansion by Vy9/Vδ2 T lymphocytes

Peripheral blood mononuclear cells (PBMC) were obtained from each individual by separating heparinized venous blood on Ficoll (Euroclone, Wetherby, Yorkshire, UK). The cells were washed in RPMI-1640 medium (Euroclone), and cultured in 24-well plates (Costar, Cambridge, MA, USA) at a concentration of 5 × 10⁵ cells/ml in RPMI-1640 supplemented with 10% foetal calf serum (Euroclone) 20 mM Hepes (Euroclone), 2 mM L-glutamine (Euroclone) and 100 U/ml penicillin/streptomycin (Sigma, St Louis, MO, USA) at 37°C at 0.5% CO₂. For the expansion of Vy9/Vδ2 T cells, PBMCs were cultured for 10 days in medium alone or in the presence of 0.5 mM DMAPP, Sigma) as previously described [7]. Briefly, 20 μl of supernatants (obtained at the 10th day of Vy9/Vδ2 culture) or diluted (1:100 in PBS) sera were coincubated with 35 μl 1 mM 5,5-dithio-bis-(2nitrobenzoic acid) (Sigma). After incubation at 37°C for 2 hours and 30 minutes respectively, the absorbance at 405 nm was determined. Esterolytic activity of GrA was tested using the synthetic substrate N-α-benzoyloxycarbonyl-L-lysine thiobenzyl ester (z-lys-SBzl; Sigma) as previously described [7].

In vitro effect of Infliximab on Vy9/Vδ2 cultures

In order to examine the effects on Vy9/Vδ2 expansion, TNF-RII expression, perforin and IFN-γ content, and supernatant levels of GrA, Infliximab (Remicade; Centocor Inc., Malvern, PA, USA; Schering Plough SpA, Segrate (Mi), Italy) was added in the medium at a final concentration of 10, 50 (for 3 days) and 100 μg/ml (for 3 and 10 days).

Effect of Infliximab therapy

Five patients with active disease were treated with Infliximab, 5 mg/Kg, by a two hour infusion, at weeks 0, 2, 4 and the patients observed for a further two hours without adverse effects. Sampling, for Vy9/Vδ2 studies, were performed before the start of therapy and at the time of the second infusion.
Statistics
All values are expressed as mean ± SD. We performed analysis of significance in Prism (GraphPad, La Jolla, CA, USA) by the two-tailed t test analysis and by two-way ANOVA.

Results
Functional changes of Vγ9/Vδ2 T lymphocytes
Cumulative results are shown in Table 1. The EF of Vγ9/Vδ2 T lymphocytes from active BD patients was significantly higher (295 ± 44; P < 0.0001) than those obtained from inactive patients (36 ± 8) and from healthy controls (30 ± 8). Vγ9/Vδ2 TNF-R1I was 10,422 ± 1,694 in active patients, 4,087 ± 1,671 in inactive patients and 4,512 ± 1,436 in healthy controls. The intracytoplasmic content of IFN-γ was 40.4 ± 8.2% in Vγ9/Vδ2 standard cultures from active patients, and 18 ± 7% and 11 ± 4% in inactive patients and healthy subjects respectively. Intracytoplasmic perforin content was 27,581 ± 4,611 in active patients and, 5,247 ± 1,230 and 4,980 ± 1,110 in inactive patients and controls respectively.

GrA levels were (818 ± 97 mOD units) significantly higher in the supernatants obtained from cultures of active patients than those obtained from inactive patients (589 ± 21) and controls (550 ± 30). Staining of PBMC cultures with antibodies to Vδ2-TCR, CD45RO and CD27 identified four subsets of Vγ9/Vδ2 cells (Table 2): 1) a subset with a naive CD45RO-CD27+ phenotype, 2) a subset with a memory CD45RO+CD27+ phenotype; 3) a subset with an effector CD45RO+CD27- phenotype and 4) a subset with a terminally differentiated cytotoxic CD45RO-CD27- phenotype. In active patients we observed that the phenotype of Vγ9/Vδ2 T cells is mainly composed of CD45RO+CD27- effector cells (57 ± 13%) and that there is, also, evidence of CD45RO CD27- terminally differentiated cytotoxic cells (20 ± 6%). In contrast, in Vγ9/Vδ2 cultures of inactive patients as well as of normal controls, we found mainly the phenotypes CD45RO+CD27+ (47 ± 9 and 49 ± 9%) and CD45RO-CD27+ (24 ± 7 and 20 ± 8%) that are characteristics of memory and naive cells, respectively.

In vitro studies with Infliximab
The addition of Infliximab to the cultures from active patients determined a significant time and dose dependent inhibition of cell expansion (Figure 1a) and a significant decrease of TNF-R1I expression (Figure 1b). In particular, the mean EF was 295 ± 43.4 in untreated cultures and 50 ± 23 in culture with the highest infliximab concentration (P < 0.001). TNF-R1I MESF was 10,500 ± 1,707 in untreated culture and 4,576 ± 845 (Infliximab 10 μg/ml for 3 days; P < 0.05), 3,466 ± 394 (Infliximab 50 μg/ml for 3 days; P < 0.01) and 833 ± 165 (Infliximab 100 μg/ml for 10 days, P < 0.001) respectively after exposure to infliximab. IFN-γ in untreated cultures was 40.4 ± 8.25%. In the presence of Infliximab we found values of 10.5 ± 3.51% (Infliximab 10 μg/ml for 3 days; P < 0.01), 7.2 ± 1.8% (Infliximab 50 μg/ml for 3 days; P < 0.01) and 5.5 ± 1.7% (Infliximab 100 μg/ml for 10 days; P < 0.01) (Figure 1c).

A significant reduction of cell perforin content (7,040 ± 985 vs 27,573 ± 4,590, P < 0.001) was also observed after infliximab (50 μg/ml for 10 days) (not shown) exposure together with a decrease of granzyme release in the supernatants (Figure 1d); in standard cultures the GrA levels were 818 ± 91.6 mOD, in the presence of Infliximab the levels were 538 ± 72 (10 μg/ml for 3 days; P < 0.05), 518 ± 69 mOD (50 μg/ml for 3 days; P < 0.05) and 240 ± 25 mOD (100 μg/ml for 10 days; P < 0.01). The Vγ9/Vδ2 cells from active patients cultured in presence of Infliximab (50 μg/ml) shown mainly the phenotype of memory (75 ± 15%) and naive (21 ± 3%) cells rather than those of effector (1.76 ± 1.28%) and cytotoxic (0.3 ± 0.21%) cells (Figure 2).

In vivo treatment with Infliximab
Five patients with active disease were infused with 5 mg/Kg of Infliximab, and Vγ9/Vδ2 studies were performed before and after therapy (Table 3). A significant EF differ-

| Table 1: Functional changes of Vγ9/Vδ2 T lymphocytes in BD patients and controls |
|-------------------------------------------------|-----------------|-----------------|-----------------|---------|
| EF of Vγ9/Vδ2 | Active BD | Inactive BD | Controls | P         |
| Vγ9/Vδ2 TNF-R1I | 295 ± 44 | 36 ± 8 | 30 ± 8 | < 0.0001 |
| IFN-γ (%) of Vγ9/Vδ2 | 10,422 ± 1,694 | 4,087 ± 1,671 | 4,512 ± 1,436 | < 0.0001 |
| Perforin (mOD units) | 40.4 ± 8.2 | 18 ± 7 | 11 ± 4 | < 0.001 |
| Granzyme A (mOD units) | 27,581 ± 4,611 | 5,247 ± 1,230 | 4,980 ± 1,110 | < 0.001 |
| BD: Behçet’s disease; EF: Expansion factor. |
ence was found in the Vγ9/Vδ2 cultures from patients before (256 ± 90) and after (80 ± 44; \(P < 0.01\)) Infliximab therapy. Serum GrA levels were 715 ± 174 mOD and 207 ± 72 mOD (\(P < 0.001\)) respectively. After therapy, the cells showed the phenotype of memory (61 ± 11%) and naive (21 ± 3%) cells.

**Discussion**

The immunopathogenesis of BD is unknown. Various micro-organisms such as streptococci and herpes simplex virus have been implicated in the pathogenesis in genetically susceptible HLA-B51+ individuals [12,13]. There is evidence of immunological dysregulation, including neutrophil hyperfunction [14,15], autoimmune manifestations [16] and several phenotypic and functional lymphocyte abnormalities, most of the immunological studies suggesting a central role for T cells in the pathogenesis of this disease [17]. Cytotoxic T cells are considered to play a role in the development of disease. Recent studies [18,19] and our own [6] point also for a role of activated Vγ9/Vδ2 T lymphocytes in the progression and probably in the pathogenesis of the disease.

The treatment of BD comprises mainly systemic corticosteroids for most manifestations of BD. Supplemental therapy with other immunomodulatory agents is often necessary to control serious manifestations such as uveitis and meningoencephalitis and to reduce the incidence of long-term steroid toxicity. Drugs usually used in BD such as glucocorticoids, pentoxifylline and cyclosporine have been demonstrated to modulate peripheral blood gamma delta T lymphocytes [20-22].

The role of TNF-blocking agents on gamma delta T lymphocyte functions has not yet been investigated. In BD, increased serum levels of TNF-α and soluble TNF-RII has been observed during the active stage of disease suggesting a role for TNF-α in the pathogenesis [23-25]. In addition, TNF-α, that has been reported also to be pro-

| CD45RO-CD27+ (%) | Active BD | Inactive BD | Controls | \(P\) |
|------------------|-----------|-------------|----------|-----|
| 7.5 ± 2.25       | 24 ± 7    | 28 ± 8      | < 0.0001 |
| 11 ± 6.44        | 47 ± 9    | 49 ± 9      | < 0.0001 |
| 57 ± 13          | 17.8 ± 9.7| 19.2 ± 6.7  | < 0.001  |
| 20 ± 6           | 8.6 ± 3.8 | 5.5 ± 5.5   | < 0.001  |

BD, Behçet’s disease.
duced by γδ T cells, might stimulate the TNF receptor bearing γδ T cells, in an autocrine or paracrine manner or both, to proliferate [26,27].

In this study we investigated the functional changes of Vγ9/Vδ2 T lymphocytes in both active and inactive Behçet’s disease and the effect of Infliximab on Vγ9/Vδ2 T cell expansion, activation and cytotoxicity. Infliximab is a high affinity monoclonal anti TNF-α antibody that has been introduced for Crohn’s disease and rheumatoid arthritis treatment in patients who are resistant to standard therapy. Our previous studies demonstrated that this drug might block the lineage commitment of naive cells by Infliximab and lead to encourage the possibility that this drug may find widespread use in the treatment of BD.

Abbreviations
BD: Behçet’s disease; EF: expansion factor; FITC: fluorescein isothiocyanate; GrA: granzyme A; MESF: molecular equivalents of soluble Fluorochrome; MoAbs: monoclonal antibodies; PBMCs: peripheral blood mononuclear cells; PE: phycoerythrin; Pf: perforin; z-lys-SbiL: N-a-benzoyloxycarbonyl-L-lysine thiobenzil ester; TCR: T cell receptor; TNF-α: tumor necrosis factor alpha.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AA-P participated in the study design, acquisition of data, analysis and interpretation of data, manuscript preparation. ARG participated in the study design and analysis and interpretation of data, manuscript preparation. FC participated in the study design and interpretation of data. AF, AR, RI and EG participated in the study design, and analysis and interpretation of data. GT participated in the study design, acquisition of data, analysis and interpretation of data, manuscript preparation and carried out overall study supervision.

Acknowledgements
This study was supported in part by a grant from Ministero dell’Università e della Ricerca Scientifica (MIUR) from Italy.

Author Details
Department of Internal Medicine, Division of Rheumatology, University of Palermo, piazza delle Cliniche 2, 90127 Palermo, Italy

Received: 26 February 2010 Revised: 7 May 2010 Accepted: 3 June 2010 Published: 3 June 2010

References
1. Pipitone N, Olivieri I, Cantini F, Triolo G, Salvarani C. New approaches in the treatment of Adamantitides-Behçet’s disease. *Curr Open Rheumatol* 2006, 18:5-9.
2. Giardina A, Ferrante A, Ciccia F, Vadala M, Giardina E, Triolo G. One year study of efficacy and safety of infliximab in the treatment of patients with ocular and neurological Behçet’s disease refractory to standard immunosuppressive drugs. *Rheumatol Int* 2009 in press.
3. Kahan A, Hamzaoui K, Ayed K. Abnormalities of T lymphocyte subsets in Behçet’s disease demonstrated with anti-CD45RA and anti-CD29 monoclonal antibodies. *J Rheumatol* 1992, 19:742-746.
4. Triolo G, Accardo-Palumbo A, Dielli F, Ciccia F, Ferrante A, Giardina E, Licata G. Humoral and cell mediated immune response to cow’s milk proteins in Behçet’s disease. *Ann Rheum Dis* 2002, 61:459-462.
5. Todaro M, Zenilli M, Triolo G, Iovino F, Patiti M, Accardo-Palumbo A, di Gaudio F, Turco MC, Petrella A, de Maria R, Stassi G. NF-kappaB protects Behçet’s disease T cells against CD95-induced apoptosis up-regulating antiapoptotic proteins. *Arthritis Rheum* 2005, 52:2179-2191.
6. Triolo G, Accardo-Palumbo A, Dielli F, Ciccia F, Ferrante A, Giardina E, Sano CD, Licata G. Vγ9Vδ2 T lymphocytes in Italian patients with Behçet’s disease: evidence for expansion, and humour necrosis factor receptor II and interleukin-12 receptor B1 expression in active disease. *Arthritis Res Ther* 2003, 5(R262-R268).

Conclusions
Our observations of disease-specific changes in Vγ9/Vδ2 T cell functions are consistent with the hypothesis that these cells play a role in the pathogenesis of BD. Despite the relatively low number of patients enrolled, data collected in this study point also to a critical role in the regulation of cellular activation and function of Vγ9/Vδ2 T cells by Infliximab and lead to encourage the possibility that this drug may find widespread use in the treatment of BD.

Table 3: Effects of in vivo administration of infliximab on BD patients with active disease

|                      | BD                         | BD                         | P       |
|----------------------|-----------------------------|----------------------------|---------|
|                      | pre-infliximab              | post-infliximab             |         |
| Serum Granzyme mOD   | 715 ± 74                    | 207 ± 72                   | < 0.001 |
| EF of Vγ9/Vδ2        | 256 ± 90                    | 80 ± 44                    | < 0.001 |
| CD45RO-CD27+ (%)     | 4.4 ± 3.6                   | 21 ± 2.6                   | < 0.01  |
| CD45RO-CD27 (%)      | 9.2 ± 7.5                   | 61.4 ± 11.1                | < 0.001 |
| CD45RO+CD27 (%)      | 54 ± 12                     | 7 ± 3                      | < 0.0001|
| CD45RO+CD27 (%)      | 20.6 ± 22                   | 6 ± 6.5                    | < 0.001 |

BD, Behçet’s disease.
7. Accardo-Palumbo A, Ferrante A, Cadelo M, Ciccia F, Parrinello G, Lipari L, Giardina AR, Rilli M, Giardina E, Deli F, Triolo G. The level of soluble Granzyme A is elevated in the plasma and in the Vgamma9/Vdelta2 T cell culture supernatants of patients with active Behçet’s disease. *Clin Exp Rheumatol* 2004, 22:545-549.

8. Criteria for diagnosis of Behçet’s disease. International Study Group for Behçet’s Disease. *Lancet* 1990, 335:1078-1080.

9. Bahcta BB, Brennan P, James TE, Chamberlain A, Noble BA, Silman AJ. Behçet’s disease: evaluation of a new instrument to measure clinical activity. *Rheumatology* 1999, 38:728-735.

10. Lawton G, Bahcta BB, Chamberlain AA, Tennant A: The Behçet’s disease activity index. *Rheumatology* 2004, 43:73-78.

11. Jabs DA, Nussenblatt RB, Rosenbaum JT. Standardization of Uveitis Nomenclature (SUN) Working Group. Standardization of uveitis nomenclature for reporting clinical data. Results of the First International Workshop. *Am J Ophthalmol* 2005, 140:509-516.

12. Havmazouk K, Kahan A, Ayed K, Hamza M. Cytotoxic T cells against herpes simplex virus in Behçet’s disease. *Clin Exp Immunol* 1990, 81:390-395.

13. Hirohata S, Oka H, Mizushima Y. Streptococcal-related antigens stimulate production of IL-6 and interferon-gamma by T cells from patients with Behçet’s disease. *Cell Immunol* 1992, 140:410-419.

14. Takeino M, Kaijone A, Yamashita N, Takiguchi M, Mizushima Y, Kaneoka H, Sakane T. Excessive function of peripheral blood neutrophils from patients with Behçet’s disease and from HLA-B51 transgenic mice. *Arthritis Rheum* 1995, 38:426-433.

15. Accardo-Palumbo A, Triolo G, Carbone MC, Ferrante A, Ciccia F, Giardina E, Triolo G. Polymorphonuclear leukocyte myeloperoxidase levels in patients with Behçet’s disease. *Cell Immunol* 1992, 140:410-419.

16. Triolo G, Accardo-Palumbo A, Triolo G, Carbone MC, Ferrante A, Giardina E. Enhancement of endothelial cell E-selectin expression by sera from patients with active Behçet’s disease: moderate correlation with anti-endothelial cell antibodies and myeloperoxidase levels. *Clin Immunol* 1999, 91:330-337.

17. Sakane T, Kotsani H, Takada S, Tsunematsu T. Functional aberration of T cell subsets in patients with Behçet’s disease. *Arthritis Rheum* 1982, 25:1343-1351.

18. Yamashita N, Kaneoka H, Nanako S, Onieda K, Koizumi H, Kogure M, Inaba G, Sakane T. Role of gammadelta T lymphocytes in the development of Behçet’s disease. *Clin Exp Immunol* 1997, 107:241-247.

19. Freysoldt D, Lau S, Fortune F: γδ T cells in Behçet’s disease (BD) and recurrent aphthous stomatitis (RAS). *Clin Exp Immunol* 1999, 118:451-457.

20. Spinozzi F, Agea E, Bistoni O, Travetti A, Migliorati G, Moraca R, Nicoletti L, Riccardi C, Paoletti FP, Vaccaro R, et al.: T lymphocytes bearing the gamma delta T cell receptor are susceptible to steroid-induced programmed cell death. *Scand J Immunol* 1995, 41:504-508.

21. Lin T, Matsuzaki G, Umesue M, Orito K, Yoshida H, Harada M, Sigraram C, Hiromatsu K, Nomoto K. Development of TCR-gamma delta CD4-CD8+ T lymphocytes in Behçet’s disease (BD) and recurrent aphthous stomatitis (RAS). *Clin Exp Immunol* 1999, 118:451-457.

22. Accardo-Palumbo A, Ferrante A, Ciccia F, Cadelo M, Giardina AR, Impagglato R, Triolo G. Pentoxifylline inhibits Vgamma9/Vdelta2 T lymphocyte activation of patients with active Behçet’s disease in vitro. *Int J Immunopathol Pharmacol* 2007, 20:601-606.

23. Mege JL, Dilten N, Sanguedolce V, Gu L, Bongrand P, Roux H, Ocal L, Inanc M, Capo C. Overproduction of monocyte derived tumor necrosis factor α, interleukin (IL) 6, IL-8 and increased neutrophil superoxide generation in Behçet’ disease: a comparative study with familial Mediterranean fever and healthy subjects. *J Rheumatol* 1993, 20:1544-1549.

24. Saynalp N, Ozcobe OL, Ozdemir O, Nazenderoglu IC, Dundar S, Kirazli S. Cytokines in Behçet’s disease. *J Rheumatol* 1996, 23:321-322.

25. Ueta C, Kawasumi H, Fujiwarw H: Interleukin-12 activates human γδ T cells: synergistic effect of tumor necrosis factor-alpha. *Eur J Immunol* 1996, 26:3065-3073.

26. Bleizoglou AV, Sfikakis PP, Vaiopoulos G, Kapsimali V, Kaklamannis PG. Serum levels of soluble TNF-alpha receptor-II (p75), circulating gammadelta T-cells and Adamantiades-Behçet’s disease activity. *Adv Exp Med Biol* 2003, 528:261-265.

27. Li H, Luo K, Pauza CD. TNF-alpha is a positive regulatory factor for human Vgamma9/Vdelta2 T cells. *J Immunol* 2008, 181:7131-7137.