various death receptors including Fas, TNFR1, DR3, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), presumably by interaction with caspase-8/FADD in the death-inducing signaling complexes (DISCs).

We have recently demonstrated somatic *APT1* mutations in a subset of non-Hodgkin's lymphomas (NHL) associated with extranodal disease and autoimmune features.[6] Because of the proposed association between ALPS and caspase-10, an obvious extension of our work would be to examine whether somatic caspase-10 mutations might also be involved in the pathogenesis of NHL. We initially established a mutation detection assay based on polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) to analyze a 137-bp genomic region of caspase-10 encompassing nucleotides 1186-1322 (codons 396-441). During the establishment of this assay, we observed an altered DGGE pattern in 1 of 10 normal control DNA samples (Figure 1A). Surprisingly, direct sequence analysis of this sample demonstrated the G1228A substitution in the heterozygous constellation (Figure 1B). Subsequent analysis of DNA samples from 85 healthy Danish blood donors (170 alleles) identified 12 additional heterozygotes, corresponding to allele frequencies of 93.2% (for the G1228 allele) and 6.8% (for the A1228 allele). Our preliminary analysis of 96 NHL cases shows a similar allele distribution in NHL.

Because we did not identify V410I homozygotes among our panel of normal controls, we cannot formally exclude that this variant is associated with severe impairment of Fas-mediated apoptosis *in vivo.* Nevertheless, the predicted homozygosity frequency of approximately 1 in 200 in the Danish population strongly argues against a major contribution to the ALPS phenotype. Further documentation is required to solidify the association between caspase-10 mutations and ALPS type II, and to establish whether the V410I variant is a benign polymorphism or may contribute to the ALPS phenotype together with other yet undefined factors involved in the control of apoptosis and lymphoproliferation.

Kirsten Grønbæk
Tine Dalby
Jesper Zeuthen
Elisabeth Ralfkiaer
Per Guldberg
Department of Tumor Cell Biology
Institute of Cancer Biology
Danish Cancer Society
Copenhagen, Denmark
Department of Pathology
Herlev Hospital
Herlev, Denmark

Acknowledgment

We thank V. Ahrenkiel and B. Rasmussen for technical assistance. The study was supported by grants from The Danish Cancer Society, the E. Willumsen Foundation, and the Danish Cancer Research Foundation.

References

1. Straus SE, Sneller M, Lenardo MJ, Puck JM, Strober W. An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. Ann Intern Med. 1999;130:591-601.
2. Fisher GH, Rosenberg FJ, Straus SE, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell. 1995;81:935-946.
3. Wu J, Wilson J, He J, Xiang L, Schur PH, Mountz JD. Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. J Clin Invest. 1996;98:1107-1113.
4. Dianzani U, Bragardo M, DiFranco D, et al. Deficiency of the Fas apoptosis pathway without Fas gene mutations in pediatric patients with autoimmunity/lymphoproliferation. Blood. 1997;89:2871-2879.
5. Wang J, Zheng L, Lobito A, et al. Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. Cell. 1999;98:47-58.
6. Grønbæk K, thor Straten P, Ralfkiaer E, et al. Somatic Fas mutations in non-Hodgkin's lymphoma: association with extranodal disease and autoimmunity. Blood. 1998;92:3018-3024.

To the editor:

HIV-associated dysfunction of in vitro IL-12 production depends on the nature of the stimulus and on the CD4 T-cell count of the patient

A recent paper by Marshall et al suggests that decreased production of interleukin-12 (IL-12) is crucial in human immunodeficiency virus (HIV)-associated immune deficiency.[1] According to these authors, peripheral blood mononuclear cells (PBMC) from HIV-positive subjects produce lower levels of IL-12 in response to a wide range of stimuli, irrespective of the disease stage. Interferon-gamma (IFN-γ) production was decreased in response to IL-12, and upregulation of the IL-12 receptor β2 chain, critical for signal transmission, was impaired. The latter defect could be reversed by rIL-12 pretreatment. The authors logically conclude: "A primary IL-12 defect may lead to secondary deficiencies in expression of the genes for IL-12Rβ2 and IFN-γ, thus amplifying immune deficiency during HIV infection." These data are in line with the "type-1 to type-2 shift" paradigm of HIV pathogenesis.[2-4] Because addition of IL-12 to PBMC cultures from HIV-positive subjects was shown to partly restore responses to antigen stimulation,[5] rIL-12 seems a good candidate for immunotherapy in acquired immunodeficiency syndrome (AIDS).

Other authors, including our group, observed HIV-associated cytokine imbalances, which are partly inconsistent with a type-1 to -2 shift.[6-8] Increased levels of various cytokines, including IFN-γ and IL-12, were measured in serum of HIV-positive subjects, even in advanced disease.[9-11] PBMC stimulation resulted in increased IFN-γ production in early HIV stages and lower IFN-γ levels in AIDS patients, as compared to controls.[12,13] Marshall's claim about a stimulus- and stage-independent impaired IL-12 production has also been challenged. One group demonstrated that IL-12 p40 production, induced by *Staphylococcus aureus* (SAC), inversely correlated with the patients' CD4 T count, and 2 studies indicated that IL-12 induced by lipopolysaccharide (LPS) and *Candida*, was not consistently decreased in HIV-positive subjects.[14,15] Moreover, in Marshall's study, induction by stimuli other than SAC, resulted in lowered production of IL-12 p40, but not of IL-12 p70 (bioactive), in HIV-positive PBMC cultures. We recently studied the physiological pathway of IL-12 production through interaction between CD40-ligand (CD40L) and CD40. Impaired CD3/TCR-mediated induction of CD40L on CD4 T cells was observed in patients with advanced disease. On the contrary, stimulation of PBMC with CD40L and IFN-γ resulted in higher IL-12 p70 in early stages and only slightly decreased IL-12 production in cultures from AIDS patients. We concluded therefore that the main dysfunction was impaired upregulation of CD40L on T cells and not deficient IL-12 production capacity per se.[8]

In view of the discrepancies between various studies, we decided to compare IL-12 p70 production in blood cells from HIV-negative and HIV-positive patients, using the following conditions: (1) RPMI only, (2) A broadly mitogenic combination of phytohemagglutinin (PHA) and LPS, (3) T-cell receptor Vβ-stimulating *Staphylococcus* enterotoxin B (SEB), and (4) monocyte-stimulating SAC. All stimulations were done in whole blood, because it represents most closely the in vivo milieu. Samples were obtained from 18 HIV-negative lab workers and from 38 HIV-positive subjects, recruited at our outpatient clinic. Patients with active opportunistic diseases were excluded, but all Centers for Disease Control classes were represented: 12 patients had more than 500 CD4 T cells/µL, 16, between 200 and 500 CD4 T cells, and 10 had fewer than 200 CD4 T cells/µL (= AIDS). Most patients with CD4 T-cell counts lower than 500 were treated with a variety of highly active antiretroviral therapies (HAART) and all were monitored for viral load (VL) using the Cobas Amplicor HIV-1 Test Version 1.5 (Roche Diagnostics, Brussels, Belgium). VL was below the detection limit of 400 copies/mL (2.6 log) in 22 patients. The VL median ± 95% confidence interval (CI) in the remaining 16 patients was 4.62 ± 0.69 log (range: 3.25 to >5.9 logs).

One hundred microliters of heparinized blood, diluted 1:10 in RPMI (GIBCO BRL, Paisley, Scotland), were cultured 4-fold in round-bottom polystyrene tubes (Falcon 2054, Becton Dickinson, Erembodegem, Belgium). Optimum concentrations of stimuli were determined in preliminary experiments: PHA (Difco, Detroit, IL) was used at 5 µg/mL plus LPS (Difco) at 25 µg/mL; SEB (Sigma, St Louis, Missouri) at 1 µg/mL and SAC (Pansorbin, Calbiochem, La Jolla, CA) at 0.016 vol percent. After an incubation of 24 hours at 37°C in a 5% CO2 incubator, the supernatants were harvested and stored at −70°C. IL-12 concentrations were measured using the p70 ELISA (R&D Systems, Abingdon, UK).

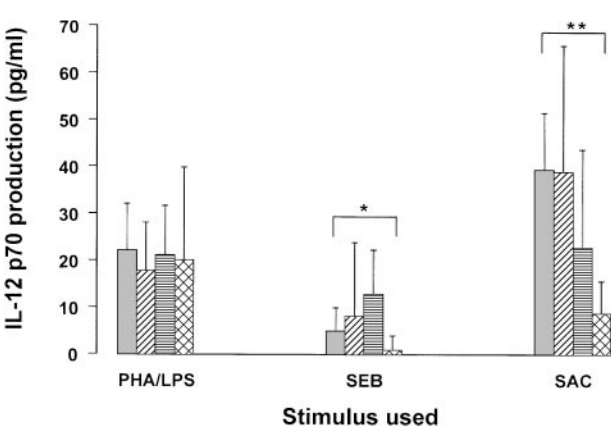

Figure. IL-12 production in whole blood cultures from HIV-negative and HIV-positive subjects. Blood was obtained from 18 HIV-negative controls (▥), 12 HIV-positive subjects with CD4 T-cell counts higher than 500/µL (▨), 16 subjects with CD4 T counts 200-500/µL (▤), and 10 subjects with CD4 T counts lower than 200/µL (▩). The blood was diluted 1/10 in medium and incubated with the indicated stimuli.

IL-12 could not be measured in any supernatant of medium control cultures (not shown). Stimulated IL-12 levels (median and CI) are depicted in the figure. The Mann-Whitney U test was used to calculate the significance of differences between the controls and the patient groups, stratified according CD4 T count. In general, IL-12 production was highly variable, but several tendencies are obvious. In blood cultures of HIV-negative subjects, SAC was the strongest IL-12 inducer, followed by PHA/LPS, whereas SEB was relatively weak. The IL-12 response to LPS/PHA was not statistically different between patients and controls. The response to SAC was unaltered in patients with more than 500 CD4 T cells, slightly (not significant) decreased in patients with intermediate CD4 T counts, and significantly ($p < .01$) reduced in AIDS patients. Interestingly, SEB induced slightly (but n.s.) more IL-12 in the cultures from patients with more than 200 CD4 T cells/µL, whereas in AIDS patients SEB-stimulated IL-12 production was significantly ($p < .05$) decreased. The latter results are similar to those we previously obtained with CD40L in peripheral blood mononuclear cells (PBMC) cultures.[8]

The same data were reanalyzed after stratifying the patients according to VL (fewer than and more than 400 copies, respectively). This analysis showed IL-12 concentrations of 21.1 ± 9.9 and 9.4 ± 10.6 pg/mL ($p = .07$) after PHA/LPS stimulation, 9.3 ± 6.4 and 4.6 ± 5.6 pg/mL (n.s.) after SEB stimulation, and 18 ± 14.5 and 13.3 ± 19.4 pg/ml (n.s.) after SAC. Clearly, there is a tendency of lowered IL-12 production in subjects with a measurable VL, regardless of the stimulus used. Nevertheless, among the latter patients no correlation was found between the IL-12 production and VL (Spearman rank test).

In summary, our present data indicate that the influence of in vivo HIV infection on in vitro IL-12 production is more complex than Marshall et al suggested. Both the nature of the stimulus and the stage of the disease determine the IL-12 p70 output in culture. The IL-12 response to SAC decreased according to CD4 T counts. Other stimuli, including SEB and CD40L, had a double-edged effect: a relative overproduction of IL-12 in non-AIDS patients and a defective IL-12 production in AIDS patients. Unfortunately, the effects of viral load itself and of viral load reduction by highly active antiretroviral therapy (HAART) on IL-12 production remain unclear, even in our present study.

Together with earlier observations, the present data indicate that both immune overactivation and immune deficiency are reflected in IL-12 production during HIV infection. Although IL-12 could be useful to enhance deficient cell-mediated immunity, it also has potential proinflammatory and HIV-enhancing effects. Therefore, more studies are needed to unravel the role of dysregulated IL-12 production in HIV pathogenesis before immunotherapy with IL-12 itself or IL-12 inducers can be considered.

Guido Vanham, Lieve Penne, Katrien Fransen, Luc Kestens
Department of Microbiology
Institute of Tropical Medicine
Antwerp, Belgium

Marc De Brabander
Clinical Research Unit
St Bartholomeus
AZ Jan Palfijn
Merksem, Belgium

This work was supported by a grant from the Janssen Research Foundation.

References

1. Marshall JD, Chehimi J, Gri G, Kostman JR, Montaner LJ, Trinchieri G. The Interleukin-12-mediated pathway of immune events is dysfunctional in human imunodeficiency virus-infected individuals. Blood. 1999;94:1003-1011.
2. Chougnet C, Wynn TA, Clerici M, et al. Molecular analysis of decreased interleukin-12 production in persons infected with human immunodeficiency virus. J Infect Dis. 1996;174:46-53.
3. Meyaard L, Hovenkamp E, Keet IP, et al. Single cell analysis of IL-4 and IFN-gamma production by T cells from HIV-infected individuals: decreased IFN-gamma in the presence of preserved IL-4 production. J Immunol. 1996;157:2712-2718.
4. Klein SA, Dobmeyer JM, Dobmeyer TS, et al. Demonstration of the Th1 to Th2 cytokine shift during the course of HIV-1 infection using cytoplasmic cytokine detection on single cell level by flow cytometry. AIDS. 1997;11:1111-1118.
5. Uherova P, Connick E, MaWhinney S, Schlichtemeier R, Schooley RT, Kuritzkes DR. In vitro effect of interleukin-12 on antigen-specific lymphocyte proliferative responses from persons infected with human immunodeficiency virus type 1. J Infect Dis. 1996;174:483-489.
6. Graziosi C, Pantaleo G, Gantt KR, et al. Lack of evidence for the dichotomy of TH1 and TH2 predominance in HIV-infected individuals [see comments]. Science. 1994;265:248-252.
7. Romagnani S, Maggi E, Del Prete G. An alternative view of the Th1/Th2 switch hypothesis in HIV infection. AIDS Res Hum Retroviruses. 1994;10:iii-ix.
8. Vanham G, Penne L, Devalck J, et al. Decreased CD40 ligand induction in CD4 T cells and dysregulated IL-12 production during HIV infection. Clin Exp Immunol. 1999;117:335-342.
9. Fan J, Bass HZ, Fahey JL. Elevated IFN-gamma and decreased IL-2 gene expression are associated with HIV infection. J Immunol. 1993;151:5031-5040.
10. Navikas V, Link J, Wahren B, Persson C, Link H. Increased levels of interferon-gamma (IFN-gamma), IL-4 and transforming growth factor-beta (TGF-beta) mRNA expressing blood mononuclear cells in human HIV infection. Clin Exp Immunol. 1994;96:59-63.
11. Torre D, Zeroli C, Ferrario G, et al. Serum levels of interleukin-12 in adult and paediatric patients with HIV-1 infection [letter]. AIDS. 1995;9:405-407.
12. Ullum H, Cozzi Lepri A, Bendtzen K, et al. Low production of interferon gamma is related to disease progression in HIV infection: evidence from a cohort of 347 HIV-infected individuals. AIDS Res Hum Retroviruses. 1997;13:1039-1046.
13. Breen EC, Salazar-Gonzalez JF, Shen LP, et al. Circulating CD8 T cells show increased interferon-gamma mRNA expression in HIV infection. Cell Immunol. 1997;178:91-98.
14. Meyaard L, Hovenkamp E, Pakker N, van der Pouw Kraan TC, Miedema F. Interleukin-12 (IL-12) production in whole blood cultures from human immunodeficiency virus-infected individuals studied in relation to IL-10 and prostaglandin E2 production. Blood. 1997;89:570-576.
15. Harrison TS, Levitz SM. Role of IL-12 in peripheral blood mononuclear cell responses to fungi in persons with and without HIV infection. J Immunol. 1996;156:4492-4497.

To the editor:

Autoantibodies neutralizing thrombopoietin in a patient with amegakaryocytic thrombocytopenic purpura

Amegakaryocytic thrombocytopenic purpura (AMTP), first reported by Korn,[1] is a rare disease characterized by severe thrombocytopenia associated with a total absence or a selective decrease in bone marrow megakaryocytes. Previous studies suggest a variety of pathogenetic mechanisms for AMTP, such as the intrinsic defect at the megakaryocyte progenitor cell level and humoral and cellular suppression of megakaryocytic differentiation.[2] Thrombopoietin (TPO), recently isolated, is the hematopoietic factor that potently stimulates megakaryocytopoiesis and platelet production.[3] Since TPO is the principal regulator of platelet production, a decreased availability or function of TPO might lead to a form of AMTP. There was a recent report of a patient with pure red-cell aplasia who had a circulating autoantibody against erythropoietin, the primary regulator of red blood cell production.[4] These findings suggest that a similar mechanism may be responsible for some cases of AMTP, that is, an autoantibody that blocks the action of endogenous TPO on megakaryocytopoiesis. To explore this possibility, we checked for the presence of antibodies against TPO in a patient (a 70-year-old woman) with AMTP who achieved remission following treatment with cyclosporin A (cyA) with some modifications, according to Hill's report.[5]

As shown in Figure 1, the patient's serum before therapy had the highest level of anti-TPO IgG antibodies during the scope of the study. With cyA therapy, the antibody levels declined and did not return to the pretreatment value until the end of the study. Endogenous TPO levels appear to be regulated by c-Mpl, the receptor for TPO, expressed on platelets and megakaryocytes, a mechanism of end-cell regulation. Previous studies showed that serum TPO levels were markedly elevated in patients with AMTP before therapy.[6] In contrast to these findings, Figure 1 shows that in spite of severe thrombocytopenia, the pretreatment level of TPO (0.21 fmol/mL) was lower than baseline values of normal subjects (0.33 to 1.72 fmol/mL) as previously reported.[7] Throughout the study period, circulating platelet counts appeared to increase in response to the elevation in endogenous TPO levels (Figure 1). In an effort to characterize the patient's anti-TPO antibody, we examined the effect of the IgG fractions from the patient's serum on the growth of megakaryocyte colonies from adherent cell-depleted normal human bone marrow mononuclear cells (Figure 2). The IgG fraction before cyA therapy or during therapy (October 14, 1997) completely or partially reduced the number of megakaryocyte colonies stimulated with 0.2 ng/mL of glycosylated recombinant human TPO, respectively. This inhibition of megakaryocyte colony formation was able to be overcome by increasing the concentration of human TPO in the cultures to 10 ng/mL. The IgG fractions,

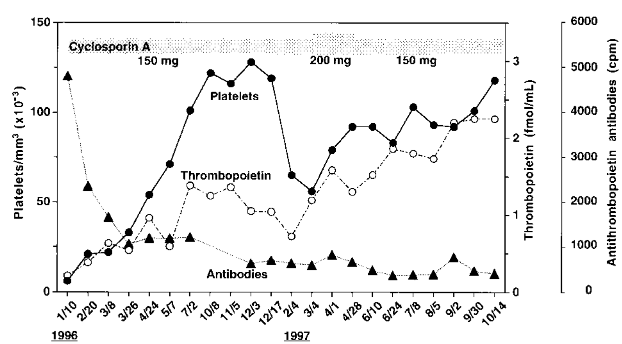

Figure 1. Changes in anti-TPO antibody levels, TPO concentrations, and platelet counts during cyA treatment. Anti-TPO IgG antibodies were measured with a solid-phase radioimmunoassay. Briefly, microtiter plates were incubated with 4 µg/mL of recombinant human TPO at 4°C overnight and washed. Test sera diluted at 1:10 in phosphate-buffered saline were added to the wells and incubated at 37°C for 1 hour. After washing, the wells were incubated with radioiodinated protein A at room temperature for 2 hours. Finally, the radioactivity bound to the wells was measured with a gamma counter. Data from the assay validation using rabbit polyclonal IgG antibodies against human TPO showed that the intra- and interassay coefficients of variation ranged from 6.3% to 10.3% and from 7.9% to 13.4%, respectively. Serum TPO levels were measured as described previously.[7]