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## **APOPTOTIC EFFECT OF DEXAMETHASONE AND CYCLOSPORIN A IN MALIGNANT CELLS OF B-CLL PATIENTS**

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B-cell chronic lymphocytic leukemia (B-CLL) is characterized by the accumulation of long-lived CD19+/CD5+ B lymphocytes expressing high levels of Bcl-2. Several studies have demonstrated that glucocorticoids and other cancer chemotherapeutics induce apoptosis in B-CLL. Cyclosporin A, a drug used for the treatment of autoimmune phenomena often occurring in patients with B-CLL, seems to have anti-leukemic effect.

In this study we evaluated the apoptotic effect and related specific mechanisms in leukemic B cells by *in vitro* treatment with cyclosporin A and dexamethasone. Freshly isolated malignant B cells obtained from 23 patients with B-CLL were investigated by flow cytometry for morphological changes and annexin V binding after incubation with cyclosporin A and dexamethasone. Caspases activation was assessed by cleaved caspase substrate determination and monoclonal antibody specific for caspase 3. Furthermore, Bcl-2 expression in fixed malignant B cells has been evaluated.

Flow cytometry analysis of annexin V binding showed that cyclosporin A and dexamethasone induce apoptosis in malignant B cells of B-CLL. After 24 h

of treatment with the two drugs, the cells positive for cleaved caspase substrate were 47.4% vs 27.7% with cyclosporin A and dexamethasone, respectively. In particular, a significant expression of caspase 3 following the treatment with the two drugs has been observed (22.8% and 36.8% with cyclosporin A and dexamethasone, respectively), whereas apoptosis did not occur through Bcl-2 down-regulation. In four patients, following *in vivo* treatment with cyclosporin A or dexamethasone, a decrease in the number of leukocytes has been observed as well as a reduction of the size of lymph nodes and of the spleen, with a clinical improvement.

These data demonstrate that cyclosporin A and dexamethasone induce apoptosis in leukemic B cells of B-CLL. *In vitro* induced apoptosis by the two drugs involves caspase activation but not Bcl-2. In four patients, a correspondence between apoptotic *in vitro* effect and *in vivo* response of the treatment with the two drugs was found. These results may be taken into consideration as a new strategy to treat selected CLL patients.

Keywords: B-cell chronic lymphocytic leukemia, apoptosis, cyclosporin A

## **EXPANSION OF TcR $\alpha\beta$ +, CD4/CD8 DOUBLE NEGATIVE LYMPHOCYTES IN A CASE OF STAPHYLOCOCCAL TOXIC SHOCK SYNDROME**

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A 55-year-old woman presented with toxic shock syndrome (TSS) due to *Staphylococcus haemolyticus*. During the course of the disease a significant leukocytosis ( $20 \times 10^9/l$ ) with lymphocytosis ( $16 \times 10^9/l$ ) appeared and, after flow cytometry evaluation by means of MoAbs, a high number ( $7 \times 10^9/l$ ) of TcR $\alpha\beta$ +CD4/CD8 double negative (DN) T-lymphocytes was observed both in bone marrow and in peripheral blood samples. The immunophenotypic properties of circulating lymphocytes were evaluated during the course of TSS, which was treated successfully. The percentage of DN T-lymphocytes decreased slowly and two weeks after clinical remission a significant number of DN T-lymphocytes ( $1.05 \times 10^9/l$ ) was still observed.

The complete correction of the altered lymphocyte immunophenotype was observed only one month after recovery from TSS, when the WBC differential count was PMN 74%, lymphocytes 23%, monocytes 3% and the immunophenotype was CD3: 75%, CD4: 40%, CD8: 35%, CD5: 76%, CD19: 15%, CD56: 10%. The immunophenotype of circulating and bone marrow lymphocytes was studied also during a phase of an aspecific febrile episode observed two months after recovery, but a subset of CD4/CD8 double negative T-lymphocytes was not found.

Staphylococcal TSS is generally caused by production of a staphylococcal exotoxin named TSST-1, which acts as a superantigen, binds to both MHC class II molecules and specific V $\beta$  regions of the T-cell receptor and leads to the activation of both antigen-presenting cells and T lymphocytes. As a consequence, production of pro-inflammatory cytokines and T cell proliferation occur with: release of IL-1,  $\gamma$ -IFN and TNF by monocytes, powerful proliferative effects on T cells, profound state of T-cell unresponsiveness. However, after an extensive Medline review, this is the first case of TSS characterized by expansion of DN T-lymphocytes.

A very small subset of TcR $\alpha\beta$ +DN T cells can be found in normal bone marrow, liver, thymus, skin. These cells show peculiar immune regulatory properties and can increase in particular autoimmune diseases. Our finding may represent a peculiar effect of lymphocyte stimulation by the staphylococcal exotoxin and suggests that further studies should be car-

ried out in TSS, aimed to achieve a deeper knowledge of the lymphocyte immunophenotype in this peculiar disease.

#### CHEMOKINE RECEPTORS IN CUTANEOUS T-CELL LYMPHOMAS: CORRELATION WITH THE CD4+CD26- SUBPOPULATION

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Cutaneous T-Cell Lymphomas (CTCL) are non-Hodgkin's lymphomas characterized by a proliferation of atypical cerebriform lymphocytes (Sézary cells, SC) infiltrating the epidermis. Circulating SC are always present in the blood of Sézary syndrome (SS) patients, whereas this finding is unusual in mycosis fungoides (MF). In a previous paper we have shown that the lack of CD26 is a specific marker of SC and that the subpopulation CD4+CD26- correctly identifies circulating SC in SS and in B1-MF (non-erythrodermic MF with atypical cells in blood). Chemokines have been recently described as a group of molecules involved in leukocyte trafficking and the chemokine receptor (CR) CCR4 has been associated with skin-homing of T-cells. Furthermore CRs have been associated with TH1 and TH2 lymphocytes.

In this work, the expression of CXCR3, CCR5 and CCR4 in blood samples from 24 CTCL patients (9 SS and 15 MF), 7 erythrodermic inflammatory skin disease (EISD) patients and 10 healthy donors (HD), has been analysed using four colour flow cytometry. In SS patients, the same phenotypical characterization was performed on lymphocyte suspension from skin biopsies. The pattern of cutaneous CRs expression and the CD4+CD26- population have been compared both in the blood and skin.

SS patients were characterized by significantly higher CCR4 ( $64.4 \pm 16\%$ ) and lower CCR5 ( $7.4 \pm 4.4\%$ ) and CXCR3 ( $24.5 \pm 16.3\%$ ) values than the other patients. The CCR4 expression was nearly always confined to the CD4+CD26- subpopulation and therefore correlated with the extension of peripheral blood involvement. On the other hand, the pattern of CR expression of MF patients was similar to that of

HD. CCR4 levels in EISD patients were increased with respect to normal controls, even if to a lesser extent than in SS patients.

When comparing the results obtained from SS patients in the blood and the skin, a similar low expression of both CCR5 and CXCR3 was found. Conversely, the increase in CCR4 expression in the peripheral blood was not observed in the skin, despite the high amounts of CD4+CD26- atypical lymphoid cells. These data are in contrast with Ferenczi's previous report suggesting a high CCR4 expression in CTCL lesions.

The study of chemokine receptors associated to CD4+CD26- subpopulation could help to better define the phenotype of leukemic lymphocytes in Sézary syndrome.

#### **MITOCHONDRIAL ONCOPROTEINS PERFORMED BY FLOW CYTOMETRY ARE A SIGNIFICANT PROGNOSTIC TOOL IN ACUTE MYELOID LEUKEMIA**

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Some mitochondrial oncoproteins (bax, bad o bak) and membrane mitochondrial antigen 7A6 highlight an early event of programmed cell death and therefore they could be a reliable marker of spontaneous apoptosis. The degree of spontaneous apoptosis of leukemic blasts obtained by the ratio between some pro-apoptotic proteins (bax, 7A6) and other anti-apoptotic proteins (bcl-2) may be an interesting tool to predict both chemotherapeutic response and clinical outcome in AML. We now report on a series of 119 patients, 57 females and 62 males, median age 55 years, with *de novo* AML treated with intensive chemotherapy protocols from 1996 to 2000. Bcl-2 and 7A6 proteins were determined in bone marrow or peripheral blood samples by flow cytometry using an anti-bcl-2 124 monoclonal antibody (MoAb) and Apo2.7 MoAb, respectively. Bcl-2 and Apo2.7 were evaluated as mean fluorescence intensities (MFI), calculated as the ratio of bcl-2 or Apo2.7 MoAbs mean/negative controls mean. The results were expressed as an index

(Apo/bcl-2) obtained by dividing Apo2.7 MFI/bcl-2 MFI. The threshold for considering AML cases as apoptotic was set at the Apo/bcl-2 value  $>1.7$  (range 0.02–53.4). Lower Apo/bcl-2 was significantly associated with the immature FAB M0-M1 categories (32/38;  $p = 0.003$ ). Moreover, both  $>20\%$  CD34 and  $>5$  CD117 MFI were significantly correlated to lower Apo/bcl2 (51/67;  $p = 0.001$  and 54/75;  $p = 0.007$ , respectively), confirming that immaturity is associated with lack of apoptosis in AML. Besides the intermediate/good risk cytogenetic classes revealed Apo/bcl-2 levels significantly higher than the poor risk class ( $p = 0.009$ ). With regard to clinical outcome, a significantly higher complete remission (CR) rate was found in patients with higher Apo/bcl2 (86% [30/35] vs. 52% [39/75];  $p = 0.0005$ ). Overall survival (OS) and disease free survival (DFS) were significantly shorter in patients with lower Apo/bcl-2 (10% vs. 38% at 2.5 years;  $p = 0.0005$  and 25% vs. 48% at 2 years;  $p = 0.03$ ). Moreover, lower Apo/bcl-2 was significantly correlated with a shorter time to relapse ( $1.3 \pm 0.6$  months vs.  $3.3 \pm 2.3$  months;  $p = 0.02$ ). Interestingly, Apo/bcl-2 index accurately predicted the clinical response of patients with normal or unknown cytogenetics (59/119). As a matter of fact, within this subset, higher Apo/bcl-2 was significantly associated both with a higher CR rate (94% [15/16] vs 52% [20/38];  $p = 0.003$ ) and a longer OS (42% vs 28% at 2 years;  $p = 0.04$ ). The independent prognostic value of Apo/bcl-2 was confirmed in multivariate analysis with regard to CR ( $p = 0.008$ ), OS ( $p = 0.006$ ) and DFS ( $p = 0.04$ ). In conclusion, mitochondrial proteins represent sensitive indicators of spontaneous apoptotic processes and may be potential targets of novel proapoptotic drugs since spontaneous apoptosis clearly predicts outcome in AML.

#### **P-GLYCOPROTEIN AND BCL-2 LEVELS DETERMINED BY FLOW CYTOMETRY PREDICT OUTCOME IN ACUTE LYMPHOID LEUKEMIAS**

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Multifactorial resistance mediated by P-glycoprotein (P-gp) and apoptosis-related proteins contributes both

to chemotherapy failure and a worse outcome in ALL; however the exact prognostic impact of these resistance mechanisms is still unclear. In order to evaluate the clinical relevance of P-gp and bcl-2, we studied 87 *de novo* ALL patients, 46 males and 41 females, median age 36 years (range 1–73) from 1988 to 2000. Forty out of 73 adult patients were treated with a conventional regimen (protocol 0288 GIMEMA), whereas 33 patients underwent intensive chemotherapy based on high-dose cytarabine and mitoxantrone (HDARA/MTZ), followed by a transplantation procedure. Fourteen paediatric patients were treated according to the AIEOP protocols. After incubation with C219 or JSB-1 or anti-bcl-2 monoclonal antibodies (MoAbs), the expression of P-gp (C219 and JSB1) and bcl-2 was measured by flow cytometry. The results were obtained as mean fluorescence index (MFI), expressed as the ratio of sample mean channel: control mean channel. The thresholds were set at >2 for C219, at  $\geq 5$  for JSB-1, at  $\geq 15$  for bcl-2; 39.1% of the patients were C219 positive, 29.7% JSB-1 positive and 54.6% bcl-2 positive. No significant correlation was observed between P-gp levels (C219 and JSB-1) and WBC count, B or T immunophenotype, CD34 or myeloid antigen expression. On the other hand, significant associations were observed between higher bcl-2 protein levels and age younger than 45 years ( $p = 0.0011$ ), WBC count lower than  $50 \times 10^9/l$  ( $p = 0.039$ ), CD34 expression ( $p = 0.018$ ), normal karyotype ( $p = 0.018$ ). With regard to complete remission rate (CR), no significant difference was found between P-gp positive and P-gp negative patients. Moreover, a longer overall survival (OS) and disease free survival (DFS) was observed in P-gp negative patients ( $p = 0.013$  and  $p = 0.015$  for C219 and  $p = 0.008$  and  $p = 0.0002$  for JSB-1). Furthermore, bcl-2 positive patients showed a higher CR rate (91.5% vs. 69.2%;  $p = 0.008$ ), a longer OS ( $p = 0.002$ ) and DFS ( $p = 0.013$ ). No significant correlation was found between P-gp and bcl-2 levels and relapse rate. Besides, JSB maintained its prognostic significance with regard to OS ( $p = 0.03$  and  $p = 0.03$ ) and DFS ( $p = 0.016$  and  $p = 0.005$ ) within the patients groups treated with 0288 and HDARA/MTZ protocols, whereas only bcl-2 showed a strong prognostic impact on the OS ( $p = 0.002$ ) and DFS ( $p = 0.004$ ) within the paediatric subset. In multivariate analysis JSB-1 was found to be an independent prognostic factors with regard to OS ( $p = 0.04$ ) and DFS ( $p = 0.0009$ ) together with cytogenetics and age. The favourable prognostic impact of higher bcl-2 levels demonstrates that high apop-

otic levels are significantly associated to a worse outcome in ALL. In conclusion, P-gp determination may be added to the other well-known biological factors in order to better stratify the risk groups in ALL.

#### **FLOW CYTOMETRIC DETECTION OF FETAL HAEMOGLOBIN: COMPARISON BETWEEN INDIRECT AND DIRECT IMMUNOFLUORESCENCE**

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The detection of circulating fetal cells in maternal blood is usually performed using the slide based microscopical counting method of acid elution also called Kleinhauer–Betke test: this method show strong limitation in precision and sensitivity. The technology of flow cytometry, capable of both high sensitivity of detection and rapid cell-by-cell analysis, seems ideally suited for such an application.

Aim of this study was the comparison between direct and indirect fluorescence labelling methods using monoclonal antibody directed against hemoglobin F.

Cord blood serial dilution (0%, 1%, 2%, 3%, 5%, 10%, 25%, 50% and 100%) were prepared adding cord blood to venous blood samples obtained from healthy woman with haemoglobin F level of 0.1%. Samples were divided in two aliquotes, which were stained respectively using HbF kit (Bioatlantic, Nantes, France) for indirect immunofluorescence and monoclonal antibody anti HbF (Valter Occhiena, Turin, Italy) fluorescein isothiocyanate labelled for direct immunofluorescence. Precision was performed on replicates obtained from samples with HbF levels of 0.8% (first level) and 13.4% (second level). 30 samples selected from routine haemoglobin screening, performed by Variant (BioRad, Segrate, Italy) were analysed by flow cytometry for results comparison. See Table 1.

#### **IMPAIRED EFFECTOR CD8<sup>+</sup> T CELL DIFFERENTIATION IN ACUTE HEPATITIS C VIRUS INFECTION**

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Table 1

Method	Intra-run CV (SD)	Between run CV (SD)	Correlation with variant
Direct immunofluorescence	10.14% (0.141)	14.71% (0.174)	$r = 0.98$ $y = 0.96x + 0.046$
Indirect immunofluorescence	15.61% (0.168)	16.14% (0.196)	$r = 0.88$ $y = 1.96x + 0.983$

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Hepatitis C virus (HCV) induces chronic infection, frequently leading to cirrhosis and hepatocellular carcinoma, in a large number of infected individuals, despite the presence of virus-specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs), known to be crucial for protection against intracellular pathogens. The recent availability of class I HLA tetramer/peptide complex allowed the *ex vivo* detection of multi-specific CTLs in both the peripheral blood and liver from chronically HCV-infected individuals, although at lower frequencies than in persons resolving acute HCV infection. Acute HCV infection is rarely symptomatic, so it is difficult to determine whether the kinetics, strength, and quality of the early immune responses have a role in dictating recovery versus chronic infection. These findings support both the pivotal role of effector CTLs in controlling HCV and the critical need of CD4 help in inducing and maintaining effective CTL responses. In order to address the issue of a possible immune dysregulation mediated by HCV, we studied the kinetics, phenotype and function of virus-specific CTLs from patients with acute HCV infection using tetramers expressing different viral epitopes, in combination with memory/effector markers, and functional assays.

*HCV-specific CTLs are deficient in the ex vivo effector functions:* Peripheral virus-specific CD8<sup>+</sup>/tetramer<sup>+</sup> cells were significantly expanded and expressed low early CD69 and high late HLA class II activation markers, during acute HCV infection. Their frequencies were directly related to both serum HCV-RNA

and alanine transaminase (ALT) levels. In the functional studies, only a minority of tetramer<sup>+</sup> cells performed effector function *ex vivo*, as detected by interferon (IFN)- $\gamma$  production in response with viral peptides, at all time points tested during the follow-up. Tetramer<sup>+</sup> cells were unable to produce either interleukin (IL)-10 or IL-4 in response to either peptides or phorbol-myristate-acetate (PMA) and ionomycin. Furthermore, the contraction kinetics of the HCV-specific effector CTL responses studied were similar in both patients undergoing stable recovery, and those undergoing chronic infection. In addition, PBMCs derived from the same patients and examined directly for *ex vivo* cytotoxic activity on HCV peptide-pulsed target cells, acted as very poor antigen-specific killers. Virtually all tetramer<sup>+</sup> cells down-regulated T cell receptors in response to antigen, ruling out the possibility that the low performance of effector functions was caused by a defect in TCR engagement or down-regulation upon antigen-contact. This result suggests that the effector CTL function is selectively impaired in acute HCV infection.

*HCV-specific CTLs express a semi-effector cell phenotype:* we next asked whether HCV-specific CTLs expressed memory or effector phenotype. Although only a minority of tetramer<sup>+</sup> cells displayed prompt effector function *ex vivo*, as detected by production of IFN- $\gamma$  in response to a few hrs of contact with viral peptides, the majority of them expressed low levels or had lost the chemokine receptor CCR7, at all time points tested during follow-up. The totality of tetramer<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> cells were confined in the CCR7<sup>-</sup> subset, further corroborating the relationship of this phenotype with effector cells. Therefore, we defined HCV-specific CTLs as semi-effector cells, because they are frozen at a particular differentiation step, in which they have a partial effector cell phenotype (CCR7<sup>low</sup>/IFN- $\gamma$ <sup>low</sup>/killing<sup>low</sup>), which is insufficient to perform terminal effector functions.

*HCV-specific CTLs efficiently proliferate in response to antigen:* fresh CD8<sup>+</sup> T cells in PBMCs derived from all the patients proliferated considerably, follow-

ing 7 days of antigen stimulation in culture. Indeed, tetramer<sup>+</sup> cells increased from <1–10% of the uncultured fresh CD8<sup>+</sup> T cells to >70% after the antigen-culture. Moreover, the majority of CD8<sup>+</sup>/tetramer<sup>+</sup> cells acquired the capacity both to synthesize IFN- $\gamma$  in response to peptide and to perform antigen-specific cytotoxic function, after 1 week co-cultivation with viral antigen, at all time points tested for all patients studied. By contrast, no IL-10 or IL-4 production was evident in response to antigenic stimulus.

*CCR7<sup>+</sup>, but not CCR7<sup>-</sup> cells proliferate in response to antigen:* fresh CCR7<sup>+</sup> and CCR7<sup>-</sup> cells were purified by sorting from PBMCs of HCV-infected individuals, and cultured with antigen for 1 week. Interestingly, only the CD8<sup>+</sup>/CCR7<sup>+</sup> cells were capable of extensive proliferation, since more than 50% became tetramers<sup>+</sup>. The same cells displayed a strong and prompt effector function, since the majority of them rapidly produced IFN- $\gamma$  in a secondary recall response. By contrast, the totality of CCR7<sup>-</sup> cells died, despite their being viable at the start of culture and subjected to the same procedures of antigen-stimulation. This data demonstrates that CTLs, although considerably expanded, are functionally inadequate to fight HCV in a multitude of acutely-infected people, and thus a state of long-lasting liver immunopathology is established.

#### **CORRELATION BETWEEN FLOW CYTOMETRIC IMMUNOPHENOTYPING ANALYSIS AND LYMPH NODE HISTOLOGY IN NON-HODGKIN B-LYMPHOMAS (NHL-B) IN SAMPLES OBTAINED FROM DIFFERENT ANATOMIC SITES**

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Flow cytometry has evolved from a promising new technology to an indispensable tool in the diagnosis of hematologic malignancies and non-Hodgkin lymphoma (NHL) and immunophenotyping has become a necessary complement to morphology and molecular analysis in the diagnosis and monitoring of these malignancies. Of interest a major advance in this field may be achieved by the definition of the patterns of

B and T cell differentiation in bone marrow, peripheral blood and lymph node using multiparameter flow cytometry, in which triple antibody combinations are used together with sensitive data acquisition methods ('live gate'), to obtain a detailed information on the different cell subset involved in the pathogenesis of non-Hodgkin lymphomas. The aim of this study was to determine whether immunophenotypic abnormalities could be defined between B NHL and normal B-cell subpopulations to determine the variability in the patterns of antigen expression of lymphoid antigens using the following triple antibody combinations:  $\kappa/\lambda/CD19$ , IgG/IgD/CD19, IgA/IgM/CD19, FMC7/CD23/CD19, CD5/CD10/CD19, CD79b/CD25/CD19, CD20/CD38/CD19, CD38/CD56/CD19, CD20/CD103/CD19, CD22/CD23/CD19, CD5/CD23/CD19, CD79b/CD5/CD19. We studied a total of 30 specimens (lymph node n. 10, bone marrow n. 10, peripheral blood n. 10) from 10 patients with B NHL in leukemic phase (CLL/SLL-B n. 3 pts, DLBCL n. 4 pts, FL n. 2 pts, MCL n. 1 pts). In addition it has been evaluated the difference of the results between immunohistochemical and multiparameter flow cytometry analysis. In all patients we could detect different patterns of expression between B NHL and normal B-cell subpopulations. Some patients showed patterns expression of antigens stable that remained unchanged over time and was consistent from one specimen to another. Other patients showed more pattern expression of antigens variability from several specimens. These data suggest that several factors may act in the stability or in the variability of multi-antigens expression: (1) the biology and function of the different antigens; (2) the mechanisms of disease dissemination.

#### **DETECTION OF THE MINIMAL RESIDUAL DISEASE IN MULTIPLE MYELOMA BY HIGH RESOLUTION FLOW CYTOMETRY**

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Multiparametric immunophenotyping has proved to be a sensitive and rapid technique to identify and to

quantify bone marrow plasma cells (BMPCs) using specific monoclonal antibodies. In the present study we have used multiparametric immunophenotyping for monitoring the myeloma disease during its clinical phases and during the chemotherapy followed by autologous peripheral blood stem cell (APBSC) transplantation. We have applied high resolution flow cytometry to identify plasma cells in: (1) 62 patients with Multiple Myeloma (MM) at different phases of the disease; (2) 26 patients treated with cycles of Pulse VAD + D-CEP (vincristine, adriamycin, dexamethazone + dexamethazone, cyclophosphamide, etoposide, cisplatin + G-CSF) before the APBSC transplantation; (3) 16 patients treated with cycles of VAD + HD-EDX (vincristine, adriamycin, dexamethazone + high dose-endoxan + G-CSF); (4) 12 APBSC harvests before and after cryopreservation.

At first we compared the specificity of four different modalities of cytometric analysis used to identify better the BMPCs. The BMPCs were identified by their expression of CD138, by the strong reactivity for CD38, by the combination of these two parameters (CD 138/CD38 bright) and by the cell selection performed on the basis of the expression of CD45 (CD45 low/CD138+/CD38+). BMPCs immunological assessment was at the end compared with the results of morphological examination. For the analysis of CD38+ cells a two step acquisition procedure was used: in the first step we acquired 50000 events for each tube; in the second step the acquisition was carried out through a "live-gate" drawn on SSC/CD38 bright fraction. These assays were performed in patients during different stages of the disease: at the onset (15 pts), after chemotherapy (14 pts), in stable phase before the APBSC transplantation (12 pts), after the transplantation (22 pts) and at the moment of relapse (13 pts). Among these four methods the most accurate in identifying and quantifying the BMPCs in MM was the use of the single antibody CD138 or the combination CD138+/CD38+. CD138 had a stronger specificity besides the mean percentage of its expression correlated better with the morphological data. Higher variability was seen in stable MM in phase of plateau pre end post APBSC transplantation where the positivity for CD138 did not always paralleled the morphological data.

This study has not evidenced considerable statistically significant differences of specificity among these 4 methods however CD38 bright, in association with CD56 and CD19 antibodies, was the most useful for phenotyping myelomatous BMPCs (CD38+/CD19-/  
CD56+)

and normal BMPCs (CD38+/CD19+/CD56-).

The phenotypic study of CD38 bright populations was therefore the elective method used for the detection of the minimal residual disease and for comparing the disease eradication efficacy of 2 different treatment strategies: treatment 1 included 2 cycles of Pulse VAD + 2 cycles of D-CEP/G-CSF and treatment 2 included 4 cycles with VAD + HD-EDX + G-CSF.

We compared the number of CD38 bright BMPCs, the number of normal BMPCs CD38+/CD19+/CD56, of myelomatous BMPCs (CD38+/CD19-/CD56+) and mixed - phenotype BMPCs (CD38+/CD19+/CD56+) at the onset, during the first phase of debulking, after the second debulking and mobilization of peripheral blood stem cells, and after the PBSCs transplantation.

Our data suggested that both the chemotherapeutic strategies had the same efficacy in eradicating the disease in fact the number of BMPCs CD38 bright in the different analysed phases were comparable, showing the same decrease from the onset to the transplantation. The phenotype of CD38 BMPCs evidenced that the first treatment (Pulse VAD + D-CEP) was more efficient to destroy the myelomatous plasma cells at the beginning of debulking but it was more aggressive than the second because it did not permit the expansion of normal BMPCs and mixed phenotype BMPCs. Instead VAD + EDX-HD was more efficient in eradicating the myelomatous plasma cells during the second debulking and before the transplantation besides allowed an expansion of normal plasma cells. The myelomatous compartment CD38+/CD56+/CD19- was always present, even if in a small percentage, also after the transplantation.

This last data was confirmed also in the study of peripheral blood autologous stem cells harvests. The analysis of the minimal residual disease in the APBSCs collections using the expression of CD138+/CD38+ and CD38 bright revealed that in the harvests there was a reduced plasma cell contamination and the phenotype was normal. There was always present a minimal number of myelomatous plasma cells and there were no differences between the cell collections before and after cryopreservation.

In summary, this study confirm the importance of the multiparameter flow cytometry immunophenotyping as a rapid, easily standardized and sensitive method for evaluating the plasma cell compartment in patients with MM and it may also offer valuable prognostic information.

### RESVERATROL AND VALPROATE INDUCE DIFFERENTIATION OF AML BLASTS THROUGH NF-KB INHIBITION

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The effects of ATRA treatment in APL demonstrate that differentiative therapy is the more promising approach to cure acute leukemias. In t(15;17) positive leukemia the molecular therapy is well defined, in the other cases of AML a common target, which affect the differentiation of myeloid precursors, needs to be found.

Some experiments attribute to HDAC the role of a general mechanism involved in the block of hemopoietic differentiation, which leads to leukemogenesis. HDAC inhibitors appear to restore terminal differentiation of leukemic blasts. A promising drug of this family is valproate.

An other possible target is NF-kB which is a transcriptional factor constitutively activated in acute myeloid leukemia. Recently the NF-kB inhibitor resveratrol has been described as an inhibitor of proliferation, an inducer of apoptosis and a differentiation agents of leukemic blasts.

In this work we have compared the differentiative effects of resveratrol and valproate in acute myeloid leukemia blasts.

Bone marrow samples of AML patients have been cultured for 4 days with 90  $\mu$ M of Resveratrol or 5  $\mu$ M of valproate. At the end of the incubation, the immunophenotype of blasts has been performed.

The two inhibitors have similar effects on the blast population. First, resveratrol and valproate reduce the number of leukemic blasts, due to an induction of apoptosis, as described in other published works. Second, resveratrol and valproate generate a new cluster of cells with increased expression of markers of differentiation (CD11b and CD16). Both of the drugs do not interfere with the number and the state of activation of lymphocyte, indicating a specific and not toxic activity.

The similar properties of resveratrol and valproate suggest that these two drugs could act on a similar molecular target.

At this scope we have investigated the inhibitory activity on NF-kB. Nuclear extracts have been obtained from bone marrow samples after an 8 hours

of treatment with resveratrol or valproate. DNA binding activity of NF-kB has been evaluated with an ELISA method. Both resveratrol and valproate reduce the NF-kB transcriptional activity. Finally, the acute myeloid leukemic cell line HL-60 has been treated for 4 hours with resveratrol or valproate and stimulated with the NF-kB-inducer TNF (100 ng/ml). Nuclear extracts have been obtained and assayed for NF-kB binding activity. Also in these conditions, both of the drugs inhibit NF-kB transcriptional activity.

We conclude that the similar differentiative properties of resveratrol and valproate could be mediated by the inhibition of NF-kB.

### MULTIPARAMETRIC FLOW CYTOMETRY IMMUNOPHENOTYPING OF BONE MARROW PLASMA CELLS IN MULTIPLE MYELOMA AND MGUS

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Plasma cell (PC) multiparametric immunophenotyping can differentiate between neoplastic cells and their normal counterparts: normal plasmacells are consistently CD19+/CD56-, while myeloma plasma cells are predominantly CD19-/CD56+. Using a flow cytometric technique based on simultaneous triple labeling with CD38/CD56/CD19 and a two-step acquisition procedure, it is possible to enumerate and characterize plasma cells when they represents a few as 0.01% of total bone marrow (BM) cellularity. In the first step 50000 events corresponding to the total of nucleated BM cells were acquired; in the second step only those events included in a "live-gate" drawn on SSC/CD38 bright fraction (where PC are located) were acquired and studied for the relative expression of CD19 and CD56. We have applied this assay to evaluate the BM plasma cells of 24 patients (pts) with monoclonal gammopathy of undetermined significance (MGUS) and of 59 pts with multiple myeloma (MM) submitted to high-dose chemotherapy followed by autologous peripheral blood stem cell transplant (APBSCT). The results of flow cytometric analysis of the relative percentage of malignant vs normal PC within the total num-

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ber of BM PC, obtained at different times during this protocol, were as follows: (1) at diagnosis (18 pts) the mean percentage of CD19<sup>-</sup>/CD56<sup>+</sup> PC was 67±48%, whereas CD19<sup>+</sup>/CD56<sup>-</sup> PC were always <2±0.4%; (2) at the end of induction therapy (22 pts) the relative number of malignant PC decreased to 48±42%, and normal PC were 10±20%; (3) after mobilization (5 pts) malignant PC were 45±55% and normal PC increased to 60±45%; (4) during the plateau phase post-APBSCT (10 pts) malignant PC remained 40±35% and normal PC decreased to 15±33%; (5) at the progression of MM after APBSCT (8 pts) malignant PC increased to 85±20% and normal PC decreased to 5±7%. In all MGUS cases studied the two distinct PC subpopulations could be identified; overall the CD19<sup>-</sup>/CD56<sup>+</sup> and the CD19<sup>+</sup>/CD56<sup>-</sup> PC represented 40±32% and 20±25% of total BM PC, respectively. We conclude that: (1) high-dose chemotherapy followed by APBSCT induces a change in the relative distribution of malignant and normal BM PC with a significant increase of normal PC and a consistent persistence of the malignant clone; (2) following APBSCT, the immunophenotypic profile of BM PC of MM pts becomes similar to that observed in MGUS pts. The last finding deserves further investigation, since it may correlate with phenotypic immunologic reconstitution after APBSCT.

#### MULTIPARAMETER FLOW CYTOMETRY IMMUNOPHENOTYPING OF BONE MARROW PROGENITOR CELLS IN MYELODYSPLASTIC SYNDROMES

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The myelodysplastic syndromes (MDS) are clonal stem cell disorders characterized by pancytopenia, dysplastic hematopoiesis and a propensity to leukemic transformation. Although diagnostic criteria are well established, a significant number of patients have blood and bone marrow findings that make diagnosis and classification difficult. Flow cytometric immunophenotyping is an accurate and highly sensitive method for

quantitative and qualitative evaluation of hematopoietic cells in the different maturative compartments, and several groups have used flow cytometry in the study of MDS. A major advance in this field was achieved by the immunophenotypic definition of the normal and dysplastic patterns of myeloid and erythroid cell differentiation in bone marrow using multiparameter flow cytometry, in which triple antibody combinations were used together with sensitive data acquisition methods ('live gate'), allowing the discrimination between the healthy and neoplastic counterparts. Therefore flow cytometry can be used diagnostically to exclude other causes of cytopenias, document the immunophenotypic manifestations of myeloid and erythroid dysplasia, provide blasts enumeration. We therefore studied by multiparameter flow cytometry the bone marrow precursor cells of 25 patients with MDS (RA n. 8, RARS n. 3, RAEB n. 8, RAEB-T n. 5, CMML n. 1) and of 5 normal donors using the following three colour panel of monoclonal antibodies: CD34/CD117/CD45, CD34/CD33/CD38. Using these triple combinations we were able to define the CD45/SSC blasts cells, CD34<sup>+</sup> progenitor cells, the proportion of CD34<sup>+</sup>/CD117<sup>+</sup>, CD34<sup>+</sup>/CD33<sup>+</sup> and CD34<sup>immature</sup> (CD34<sup>+</sup> cells being CD38 negative or weak positive) progenitor cell subpopulations and the myeloid/lymphoid ratio (ratio of CD34<sup>+</sup>/CD33<sup>+</sup> to CD34<sup>+</sup>/CD33<sup>-</sup> cells). A strict correlation between morphologic blast cells and CD34<sup>+</sup> progenitor cells ( $r = 0.83$ ) or CD45/SSC blast cells ( $r = 0.89$ ) was found. In addition we have demonstrated a strict relationship between the proportion of the different progenitor cell subpopulations and the MDS category defined according to the FAB classification and the IPSS scoring system, particularly in the high risk subtypes. These data underline the clinical relevance of blast cell count and CD34<sup>+</sup> progenitor cells subsetting in MDS.

#### DIFFERENT SENSITIVITY TO APOPTOSIS IN HIV-CHRONICALLY INFECTED CELLS OF MONOCYTIC OR LYMPHOCYTIC ORIGIN

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Programmed cell death/apoptosis of CD4+ T lymphocytes is a main cause of the immunodeficiency during the infection with the human immunodeficiency virus (HIV), but it is still unclear how this process precisely occurs. In particular, it is not known which is the role of the sensitivity to apoptosis of different cell types. Thus, we have analyzed the tendency to undergo apoptosis of chronically HIV-infected CD4+ human cell lines of different origin, along with their capacity to regulate plasma membrane CD95 expression.

We have studied two cell lines: ACH-2 and U1, derived from the T cell line A301 and from the promonocytic line U937, respectively, and have induced apoptosis via several stimuli that trigger different pathways. Flow cytometry has been used to evaluate early and late apoptosis (analyzing the appearance of the typical hypodiploid peak of propidium iodide fluorescence, or considering the binding of FITC-labelled annexin-V to the membrane of apoptotic cells); mitochondrial membrane potential was studied by using the potential-sensitive probe JC-1, a lipophilic carbocyanine that exists in a monomeric form and is able to accumulate into mitochondria. In the presence of a high membrane potential, JC-1 can reversibly form aggregates that, after excitation, emit in the orange/red channel (FL-2). Monomers emit in the green (FL1) channel. The collapse in membrane potential provokes the decrease in the number of JC-1 aggregates (revealed by a decrease in FL2) and a consequent increase of monomers (increase in FL1). Finally, we have evaluated the plasma membrane expression of CD95 by an anti-CD95 (DX2 clone, mouse IgG1). Differences in the plasma membrane expression of CD95 were calculated considering the median fluorescence intensity of the populations stained with fluorescent anti-CD95 mAb, from which the background (i.e., the autofluorescence) was subtracted. The analysis of all samples was performed using a FACScan cytometer (Becton Dickinson, San José, CA) equipped with an argon ion laser tuned at 488 nm. In all cases, a minimum of 10,000 cells per sample were acquired in list mode and analyzed with WinMDI 2.8 software.

Using staurosporine, tumour necrosis factor (TNF)-alpha plus cycloheximide, and gamma-radiations to induce cell death, we observed that ACH-2 were more sensitive to apoptosis than A301, while U1 were less sensitive than U937. Both infected cell types had a lower sensitivity to CD95-induced apoptosis. The analysis of changes in mitochondrial membrane potential corroborates these observations. Plasma membrane CD95 was similarly regulated in all cell types, which however presented a different capacity to produce soluble CD95 molecules (measured by ELISA).

Our *in vitro* results likely mimic the situation that typically occurs *in vivo*, and could explain, at least in part, one crucial point of the pathogenesis of HIV-infection. The virus infects either CD4+ T lymphocytes or monocytes/macrophages, but only the former cell type consistently decreases its number. It can be hypothesized that one of the reasons by which monocytes survive and represent a reservoir of the virus is the capacity of HIV to decrease the sensitivity to apoptosis in this type of cells.

#### **CYTOKINE PROFILE EXPRESSION IN HEALTHY INDIVIDUALS: A INTERLABORATORY FLOW CYTOMETRY STUDY**

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T-lymphocyte and antigen presenting cell activation is a milestone in the generation of the immune response. A specific and differentiated cytokine production (IL-2, IL10, IL12, INF- $\gamma$ , TNF- $\alpha$ ) and their secretion by activated cells drives the immune response to type 1 (cellular) or type 2 (humoral) response.

The flow cytometry technique for the detection of intracellular cytokine production represents the fastest

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Table 2

	IL-2	IL-10	IL-12	IFN- $\gamma$	TNF- $\alpha$
CD4+	4.78 $\pm$ 2.19 2.59–6.97	1.12 $\pm$ 1.11 0.01–2.23	0.54 $\pm$ 0.63 0.0–1.17	3.38 $\pm$ 2.25 1.13–5.63	5.43 $\pm$ 1.38 4.05–6.81
CD8+	2.10 $\pm$ 1.76 0.34–3.86	0.69 $\pm$ 0.63 0.06–1.32	0.86 $\pm$ 0.63 0.23–1.49	4.91 $\pm$ 5.66 0.0–10.57	4.96 $\pm$ 1.98 2.98–6.94
CD14+	3.18 $\pm$ 3.05 0.13–6.23	2.77 $\pm$ 2.60 0.17–5.37	3.38 $\pm$ 3.17 0.21–6.55	3.46 $\pm$ 4.86 0.0–8.3	26.86 $\pm$ 15.59 11.27–42.45

and effective way to detect contemporaneously cytokine production and cell subset. Nevertheless, the type and duration of the stimulus, the technique and reagents used for fixation and permeabilization procedure, the choice of fluorochrome conjugation, origin and specificity of the antibodies contribute, together with the biologic individual variables (age, sex, drugs assumption, etc.) to make the technique difficult to be standardized. To diminish the impact of all these variables on cytokine detection and to evaluate the range of cytokine producing cells in healthy subjects, four flow cytometry laboratories started a common protocol. The first objective was to set up the most reproducible and handling protocol. For this purpose, different culture conditions (type and time of stimulus, cell secretion blocking agent, whole blood or Ficoll–Hypaque fractioned mononuclear cells) were tested and a slightly modification of the protocol proposed by Waldrop et coll (S.L. Waldrop et al., 1997) was chosen (mononuclear cell from Ficoll–Hypaque gradient, SEB + anti CD28 overnight stimulus, Brefeldin A as secretion blocking agent, fixation with paraformaldehyde, permeabilization and surface and intracellular staining in one step using phosphate buffered saline containing 0.5% bovine albumin and 0.05% saponin). The study was conducted on 91 volunteers (52 male, 39 female) of age ranging between 20–70 years old. The cytokine tested were IL-2, IL-10, IL-12, IFN- $\gamma$  and TNF- $\alpha$ , on CD4, CD8 and CD14 cell subsets. Mean  $\pm$  standard deviation (SD) and expression range for the various cytokines on the three cell subsets analyzed are reported in Table 2.

The data obtained by any single laboratory does not differ significantly (Student's *t* test) from each other neither for mean values nor for ranges. The relative high SD observed for almost all the cytokines and all the cell subsets is mainly due to the different individual response rate to the stimulus. To confirm this hypothesis four samples were Ficoll–Hypaque fractioned by a single centre and then distributed to all laboratories which completed the stimulation, staining and analysis individually obtaining fully matching results.

In conclusion, we demonstrate that the standardization of procedures and reagents allows a better reproducibility and feasibility (also on a diagnostic ground) of a highly variable technique. Moreover, we also obtain reliable reference values for cytokine production by main T cell subsets and monocyte.

#### CYTOFLUORIMETRIC ANALYSIS OF LYMPHOCYTIC SUBSETS DURING TREATMENT WITH IL-2 E 5FU IN CONTINUOUS VENOUS INFUSION FOR METASTATIC KIDNEY CARCINOMA: CORRELATIONS WITH CLINICAL RESPONSE

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*Purpose:* The aim of the employment of immunomodulating drugs in the therapy of kidney cancer is based on the evidence of the role of immune system in the control of the tumor growth. The alpha interferon and the interleukin-2 may influence the immunoendocrine network and the immune response against some neoplasia like renal carcinoma. IL-2 can induce an increment of the activity of T and NK lymphocyte. The aim of this study is to demonstrate that such increment may correspond to a better response to the therapy with longer disease free survival (DFS) and overall survival (OS).

*Patients and methods:* We have treated 11 patients with metastatic renal carcinoma from November 1999 to February 2002 with the protocol of the Urologic and Oncologic Group of Piemonte region. This protocol is based on the employment of IL-2, at the dosage of 3 MU/m<sup>2</sup> for six day a week for four weeks, plus 5FU

at the dosage of 200 mg/m<sup>2</sup> every day in continuous venous infusion for four weeks.

We have evaluated the immunological effects of this therapy assessing every week the lymphocyte subsets count with a cytofluorimetric approach using the following panel of monoclonal antibodies: CD3/CD4/CD45, CD3/CD8/CD45, CD3/CD16+56/CD45, CD3/CD19/CD45. We calculated the value of lymphocyte subsets in term of absolute and percent count with a mean follow-up of 26 weeks.

T-test was performed to compare the mean and the absolute value of T and NK lymphocyte subsets obtained during the weeks of observation. The linear regression test was performed to establish the correlation between these value and OS and PFS.

With such therapy protocol we obtained the following results: overall response (OR) 63% (PR = 36%, SD = 27%); median overall survival (OS) 16 months and median disease free survival (PFS) 13 months. The evaluation of the lymphocyte subsets at the 12th week showed an increment of the absolute value of the CD3 lymphocytes and the NK respectively of 27% and 91% in comparison with the value of the first week. There were some differences in these progressive increments between the responder and non-responder groups. The absolute and the percent value of CD3, CD19 and NK, at the first and 12th week, are shown in the table.

	CD3%	CD3 a	CD19 a	NK %	NK a
Week. 1 Resp.	69	1756	187	19	521
Non-resp.	78	1636	186	14	246
Week. 12 Resp.	62	2334	207	30	1268
Non-resp.	83	1575	101	12	308

In the responder's group there was a progressive increase of the CD3 absolute value ( $p = 0.05$ ) and of the percent and absolute count of the NK subset ( $p = 0.04$  and  $p = 0.02$ ). A further analysis was performed to correlate the mean absolute value of the T and the NK lymphocytes and their maximum value with the OS and the PFS. The linear regression test showed a significant correlations between PFS and OS with the mean and the maximum value of the NK ( $r = 0.56$  and  $r = 0.53$ ,  $r = 0.50$  and  $r = 0.64$ ) and a positive trend with the same value of the T lymphocytes.

**Conclusion:** There is a significant increment of the absolute value of T and NK in the responders group; there is a good correlation between the level of the NK and T lymphocyte subsets with PFS and OS.

Further study are needed to confirm these results in order to utilize the analysis of lymphocyte subsets as a parameter of prognostic importance.

## DYSREGULATION OF PROGRAMMED CELL DEATH ACTIVATION IN MULTIPLE SCLEROSIS PATIENTS

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Multiple sclerosis (MS) is a chronic neurologic disease characterized by multifocal inflammation and damage involving the myelin sheath. An immunopathologic mechanism, mainly mediated by the activation of cell mediated immunity (CMI), has repeatedly been linked to the destruction of the myelin sheath. It is postulated that because of molecular mimicry with cross-reacting and yet undefined epitopes, these CTL would be activated, tolerance broken, and disease initiated or reactivated. Apoptosis of autoreactive T lymphocytes is hypothesized to be another major mechanism of tolerance, and in particular, programmed cell death (PCD) of antigen-specific immune cells in MS could potentially modulate immune-mediated destruction of the myelin sheath.

In this study, expression of apoptotic markers was evaluated in peripheral cells of multiple sclerosis (MS) patients with acute (AMS) ( $N = 14$ ) or stable (SMS) ( $N = 15$ ) disease and 6 AMS patients undergoing interferon beta (IFN $\beta$ ) therapy were evaluated as well. Thus we performed flow cytometric analysis of AAD-permeability and we evaluated the percentages of Fas (CD95) and Bcl-2 expressing CD4+, CD8+ after PMA + ionomycin or CD4+, CD8+ and CD14+ after Myelin Basic Protein (MBP) and anti-CD28 incubation. Results showed PMA ionomycin activated either CD4+ or CD8+ FAS+ and 7-AAD+ (apoptotic cells) lymphocytes were increased in IFN $\beta$  treated MS patients compared to SMS and AMS patients, while MBP stimulated either CD4+ or CD8+ or CD14+ FAS+ and 7-AAD+ cells were increased in SMS compared to AMS and IFN $\beta$  treated MS patients. Conversely CD4+, CD8+ and CD14+ cells percentage Bcl-2 expressing was decreased in SMS in confront to IFN $\beta$  MS patients. These observations lead to hypothesize to be a Fas-mediated PCD of polyclonal T lympho-

cytes, in treated IFN $\beta$  MS patients, to dampen immune activation, while in SMS a PCD of antigen-specific immune cells could potentially modulate immune-mediated destruction of the myelin sheath.

#### USE OF COMBINED CYTOPATHOLOGICAL AND FLOW CYTOMETRY ANALYSES IN FINE NEEDLE ASPIRATION BIOPSIES

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Flow cytometry (FC) is generally accepted as a fundamental tool in the diagnosis of hematologic malignancies. Here we reviewed our two-year experience in flow cytometric evaluation of fine needle aspirates (FNA) of lymph nodes or other tissues submitted to our institution between March 2001 and February 2003 for cytologic analysis. Patients were selected on the basis of a clinical or morphological suspicion of a haematological malignancy. FC was performed by multiparameter analysis using three- or four-colour staining methods. A total of 122 cases was evaluated by cytomorphological (CY) and immunocytochemical analysis: an hemopoietic malignancy was identified in 55 cases, a reactive process in 43 cases and a non-hemopoietic lesion in 24 cases. The hemopoietic malignancies were classified as non-Hodgkin B-cell lymphomas (53 cases) and Hodgkin lymphomas (2 cases); 20 of these cases also had a histological confirmation of the lymphoma diagnosis.

FC was performed on a total of 109 cases, being 13 cases could not be adequate due to low cellularity or presence of nonviable cells. Immunophenotypic analysis by FC revealed a lymphomatous proliferation in 49/55 CY positive cases and in one case considered negative by CY analysis. FC failed to detect a B-cell clonal process in the two cases of Hodgkin lymphomas and in four CY+ cases because of insufficient samples. The high number of inadequate samples for FC (11%) is presumably due to the sample collection modality performed by a second needle sticking. The correlation between FC and CY analysis was 97% (106/109 total cases evaluated by FC). In one case FC was able to identify a clonally restricted population, while CY gave a non-conclusive result.

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		FNA cytology		Total
		lymphoma +	lymphoma -	
Flow	lymphoma +	49	1	50
cyto-	lymphoma -	2	57	59
metry	inadequate	4	9	13
	total	55	67	122

The evaluation of FNA by multiparameter FC allows the easy identification of small clonal B-cell populations, hidden among reactive cells, increases diagnostic accuracy through the evaluation of objective parameters and improves the diagnostic accuracy of cytomorphological classification of lymphomas. We conclude that FC in close coordination with cytomorphology is an excellent method for the evaluation of both primary and recurrent hematopoietic conditions, could replace immunocytochemical assessment of lymphocytic markers on smears or cell blocks and could replace the more invasive surgical biopsy in selected cases.

#### FLOW CYTOMETRY IN THE BONE MARROW EVALUATION OF MATURE B-CELL MALIGNANT NEOPLASMS

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Flow cytometry (FC) analysis of bone marrow aspirates is often performed in hemolymphoid disorders both at diagnosis and during disease monitoring but its role has not yet been defined during the staging of B non-Hodgkin's Lymphoma (B-NHL) and B cell lymphoproliferative diseases (B-LPD). The goal of this study is to give an objective evaluation of how FC may help in the detection of bone marrow involvement by the different types of B-cell malignant neoplasms.

210 samples (54 staging and 156 re-staging bone marrow biopsies and bone marrow aspirates) obtained from 185 consecutive patients, were analysed retrospectively. The results of the morphologic examination and the flow cytometry analysis were reviewed independently, and their ability to detect bone marrow involvement was compared. As shown in the table below, FC and morphology agreed in 176 cases

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(83.8%): i.e. both showed 77 positive cases and 99 negative ones. Discrepant results were obtained in 30 cases (14.2%) where morphologic examination showed 25 (11.9%) positive cases whilst FC showed no evidence of disease. Whereas FC detected involvement in 5 cases (2.4%) in the presence of a histologically negative bone marrow biopsy. All morphologically undetermined bone marrow cases (4) were negative by FC.

	Biopsy morpho-logy (BM)+	BM–	BM undeter-mined	Total
FC+	77 (36.7%)	5 (2.4%)	0	82 (39.0%)
FC–	25 (11.9%)	99 (47.1%)	4 (1.9%)	128 (60.9%)
Total	102 (48.6%)	104 (49.5%)	4 (1.9%)	210 (100%)

+: positive for lymphoma involvement; –: negative for lymphoma involvement; undetermined: non-diagnostic for lymphoma.

Discordant cases in which FC fails to detect a fraction of involved bone marrows, consist of a heterogeneous group of lymphomas in which the marrow was characterized essentially by a paratrabeular or nodular infiltration pattern. The 5 BM–/FC+ cases observed, belonging to different NHL subtypes in which monoclonal B cells represented an average of 2.5% of the total cellularity (without signs of haemodilution) suggesting that the presence of neoplastic cells is due to lymphoma bone marrow infiltration and not to peripheral blood contamination. This FC result gives increases the morphologic sensitivity by 2.5%.

On the basis of these data we conclude that neither morphologic examination nor FC alone is adequate for the detection of all cases of B-lymphoid neoplasm bone marrow involvement. FC failed to detect bone marrow involvement in those NHL cases having focal paratrabeular infiltration, but proved to be more sensitive than did histology in detecting small clonal B-cells in NHL which had neoplastic infiltrates of less than 5%. The clinical relevance of minimal disease detected by FC alone needs further evaluation as staging of lymphomas is, at present, based only on morphological data.

#### IMMUNOPHENOTYPIC PROFILE OF DIFFUSE LARGE B-CELL LYMPHOMA IDENTIFIES TWO CATEGORIES OF TUMOR DISPLAYING DIFFERENT HISTOGENESIS

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Diffuse large B cell lymphomas (DLBCL) represent up to 40% of adult lymphoma in western countries. The WHO classification identifies a unique category of DLBCL in which are grouped morphological entities previously described as centroblastic lymphoma, immunoblastic lymphoma and large anaplastic B-cell lymphoma (Kiel classification). Recently, using the micro-array technique, DLBCL have been divided into two groups on the basis of their gene expression: germinal centre B-like lymphoma and activated B-like lymphoma (Alizadeh et al., *Nature* (2000)). The two groups display a significant different survival and a strong correlation with the International Prognostic Index (IPI).

We analyzed, by multicolor flow cytometry, a group of 26 DLBCL using a panel of monoclonal antibodies containing some of the markers analyzed for their gene expression in the cited paper (CD10, CD21, CD27, CD38, CD44, BCL-2) and other markers recognizing differentiation (FMC7, CD24, CD9, CD72, CD74), activation (CD23, CD25, CD80), homing (CD43, CD11c, CD31, CD183, CCR6), apoptosis-related (CD95) and proliferation (MIB-1) antigens. The same antibody panel was also used to analyze B cells from 4 normal tonsils. Large B-cells from neoplastic and normal samples were identified and gated on the basis of their expression for CD19 and high forward scatter properties. The clonality of each neoplastic sample was detected by means of the monotypic expression of kappa or lambda light chain (ratio >90:1) on CD19+ large cells (B-LC). B-LC from all normal tonsil samples showed a phenotype strongly indicating their origin from germinal centre (CD10+, FMC7+, CD21+, CD23+/-, CD27+, CD43-, CD9+, CD38+, CD44+/-, CD72+, CD80+, CD95+, BCL-2-).

Comparing immunophenotypic profile of DLBCL with those of normal B-LC, two groups of tumors were recognized, the first, comprising 15 cases, showing a profile substantially identical to that of the normal B-LC (CD10+, FMC7+/-, CD21+, CD23+/-, CD27+, CD43-, CD9+, CD38+, CD44+/-, CD72+, CD80+, CD95+, BCL-2+) and then defined, as before, germinal centre B-like lymphoma. A second group, comprising 11 cases, showed an almost completely different pattern (CD10-, FMC7-, CD21-, CD23-, CD27+/-, CD43+/-, CD9-, CD38+, CD44+/-, CD72+, CD80+, CD95+, BCL-2+) and

Table 3

Pts	CD 34 +			CD34+\ CD133+			CD34+\ HLA-DR			CD34+\ CD38+		
	PB	IS	ES	PB	IS	ES	PB	IS	ES	PB	IS	ES
1	0.26%	0.23%	0.14%	16%	nv	nv	92%	nv	nv	55%	nv	nv
2	0.86%	1.54%	1.01%	49%	62%	77%	98%	97%	96%	14%	9	6%
3	0.88%	3.53%	3.12%	40%	31%	34%	73%	96%	90%	14%	50	58%
4	5.62%	3.85%	4.86%	48%	30%	32%	77%	88%	89%	40%	47	51%
5	0.30%	1.52%	0.83%	52%	6%	nv	89%	84%	80%	35%	57	nv
6	0.02%	0.10%	0.12%	nv	nv	nv	nv	nv	nv	nv	nv	nv

PB: peripheral blood IS: internal spleen ES: external spleen.

was defined non-germinal centre B-like lymphoma. Moreover, comparing the scattering distribution of each marker of the whole serie of DLBCL to that of the B-LC, a clear “markers clustering” was arising between the germinal centre B-like and the B-LC.

These preliminary results seem to indicate that also on a immunophenotypic basis is possible to distinguish tumors which originate from germinal centre cells from others that display a different histogenesis. Further studies are needed to verify if the two groups differ also for aggressiveness and survival.

#### EXPRESSION OF STEM CELL MARKERS IN THE SPLEEN OF PATIENTS AFFECTED BY MYELOFIBROSIS

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Myelofibrosis with myeloid metaplasia (MMM) is a chronic Philadelphia-negative (Ph<sup>-</sup>) myeloproliferative disorder in which a somatic mutation leads a multipotent hematopoietic progenitor cell to acquire a clonal proliferative advantage. This primary event, common to all chronic myeloproliferative disorders (CMPDs), is specific for MMM in that it produces an abnormal population of hematopoietic cells that inappropriately releases fibrogenic and angiogenic cytokines and/or growth factors in the bone marrow, precociously in-

vades the blood stream and colonizes extramedullary sites.

The origin and the molecular mechanisms responsible for the clonal amplification of the hematopoietic progenitor compartment are presently unknown. In the last years, a new biological characteristic has been described, namely a constitutive release of a high number of CD34+ cells from bone marrow to peripheral blood.

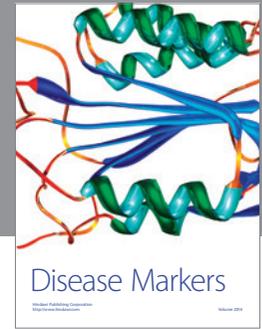
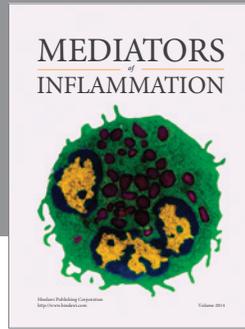
We studied a group of five patients underwent to splenectomy for therapeutical purposes. We analyzed by flow cytometry a sample of the peripheral blood of the patients and two samples of the spleen, from the internal and from the external side respectively reduced to single cells solutions, for the following antigens: CD 34, CD 133, CD 38, CD 45 and Anti HLA-DR.

A FacsCalibur flow cytometer and monoclonal antibodies by BD Biosciences, San Jose, California, USA were utilized except for the CD 133 produced by Miltenyi Biotec, Bergisch Gladbach, Germany and the CD 38 produced by Caltag, Burlingame, California, USA.

In Table 3 are shown the results of the analyses performed on all the samples. A sample obtained from an healthy man submitted to splenectomy for an critical trauma after a road accident, was used as control.

In two samples was not possible to study all the antigens because of the very low number of the CD 34 positive cells.

In conclusion we demonstrated that flow cytometry analysis of stem cell derived from spleen is feasible, with reproducible results, despites the poor number of positive events in many cases. Even if we consider a little group of patients for our study, this first data suggest a good likeness between the samples from peripheral bloods and from the spleen of the same case.



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