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Reciprocal modulation of circulating CD4+CD25+bright T cells induced by extracorporeal photochemotherapy in Cutaneous T-cell Lymphoma and Chronic Graft-Versus-Host-Disease patients

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Abstract. The mechanisms of action of extracorporeal photochemotherapy (ECP) in cutaneous T-cell lymphoma (CTCL) are poorly understood. Recently, ECP has been shown to induce an increase in regulatory T cell (Treg) expression and functional activities in Graft-versus-host-disease (GvHD), whereas no data are available in CTCL patients. The aim of this study is to evaluate whether ECP is able to modulate the expression levels of the circulating CD4+CD25+bright subset in CTCL patients and whether these modifications are related to the disease course. The patient population included 43 CTCL and 15 chronic GvHD patients treated by ECP at our institutions since 1992. The expression of the circulating CD4+CD25+bright subset was analysed at baseline and sequentially during treatment by flow-cytometry. Fifty healthy donors were used as controls. The baseline circulating CD4+CD25+bright percentage values in CTCL (median: 4.3%) were similar to those of healthy donors, whereas GvHD showed significantly lower values (median: 1.5%; $p<0.001$). During treatment, CTCL patients were characterised by an early decrease (from 4.3% to 2.4% median after 6 months). The CD4+CD25+bright decrease was associated to the disease course, as it occurred in 91.3% of responding but in only 25% of PD patients ($p=0.0001$). On the other hand, a significant increase of CD4+CD25+bright cells was observed in GvHD. ECP induces a reciprocal modulation of the circulating CD4+CD25+bright cells in CTCL and GvHD, with a downregulation in CTCL potentially associated with the response mechanisms.

Key words: extracorporeal photochemotherapy, cutaneous T-cell lymphoma, graft-versus-host-disease, T regulatory cells, flow-cytometry

Extracorporeal photochemotherapy (ECP) is a therapeutic procedure in which leukapheresed peripheral blood mononuclear cells are exposed to ultraviolet A (UVA) light in the presence of the photosensitizer DNA-intercalating agent 8-methoxypsoralen (8-MOP) (1). It represents a frontline treatment for erythrodermic cutaneous T-cell lymphoma (CTCL) patients (2), with a 63% mean response rate (range 43%-100%) (3-4). In recent years, ECP has been shown to induce significant clinical improvement also in

refractory/resistant graft-versus-host-disease (GvHD) after allogeneic bone marrow transplantation (5) and has been successfully used in the prevention of solid organ 344 transplant rejection (6).

In spite of this increasing clinical evidence, the mechanisms of action are still poorly understood. The activity of ECP in CTCL patients has been suggested to be dependent on the induction of a vaccine-like clonotypic immune-mediated response against T-cellclones (7). An apparently opposite mechanism takes place in GvHD, in which the ECP clinical activity is mediated by the down-regulation of the activity of T-cell clones and autoallogeneic immune responses (8). In both CTCL and GVHD, however, the first step is represented by the induction of lymphocyte apoptosis, followed by the phagocytosis of apoptotic lymphocytes by antigen-presenting cells (APCs) or by DCs (9).

Recently, increasing evidence supports the hypothesis that ECP induces an up-regulation of T lymphocyte cell subsets with immunosuppressive roperties (regulatory T cells, Treg) in GvHD patients. Treg cells play a central role in immune homeostasis by regulating the maintenance of self-tolerance and are therefore essential for the control of autoimmunity and immune responses o transplanted allografts (10-11). Distinct Treg opulations have been identified, among which the most extensively studied is the CD4+CD25+bright subset. Circulating CD4+CD25+bright cells represent a small subset in healthy individuals accounting for 2% to less than 5% of peripheral blood mononuclear cells. CD4+CD25+bright can be antigen-induced, but also naturally occurring cells acting in an antigen non-specific manner through contact-dependent T cell-to-T cell interactions. Naturally-occurring CD4+CD25+bright cells are characterised by the constitutive expression of specific markers, such as the transcription factor forkhead box P3 (FoxP3) (12), the glucocorticoid-induced tumour-necrosis factor receptor-related protein (GITR) and the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), whereas antigen-induced are mainly identified by the expression of immunosuppressive cytokines (IL-10) and/or transforming growth factor-beta (TGF-β) (10-11).

Several in vitro and in vivo studies ascertained the induction of Treg subsets and functions in GvHD patients. Indeed, the infusion of syngeneic ECPtreated apoptotic cells results in increasing Treg levels in murine models, through the development of tolerogenic DCs (13-14). Moreover, the transfer of ECP-treated cells reverses experimental GvHD by increasing donor Treg cells (15). An increase of circulating CD4+CD25+bright functional Treg has been recently shown in clinical series of 10 (16) and 14 (17) GvHD patients undergoing ECP; a stabilization or even an increase in the CD4+CD25+ Treg cells has been also found in small series of ECP-treated solid transplant recipients who did not experience a graft immune rejection (18-19).

Data on CD4+ CD25+bright Treg cells in CTCL are limited and controversial. The first issue concerns the possibility that neoplastic cells display a regulatory T phenotype. In vitro studies by Berger et al. (20) showed that CTCL cells express a Treg CD25+/CTLA4+FoxP3+ phenotype after interaction with DCs loaded with apoptotic T cells. A regulatory T-cell phenotype was also demonstrated in vivo in large transformed T cells from 4/5 mycosis fungoides patients (21). Capriotti et al. (22) found using real-time quantitative PCR that at least one subset of SS patients (less than 30%) express FoxP3 on leukemic cells, whereas other studies (23-25) failed to demonstrate these results showing that CTCL cells from both skin and peripheral blood do not express FoxP3. A second issue deals with the expression of Treg subsets within the reactive inflammatory compartment. Klemke et al. (23) found a very low percentage of Treg cells in both the skin and peripheral blood of the 8 SS patients included in the study. Tiemessen et al. (24) partially confirmed these results, failing to demonstrate significant differences in Treg levels between 10 CTCL patients and controls, even if CTCL patients showed a lower suppressive activity, which inversely correlated with the extent of peripheral blood involvement. More recently, increasing number of FoxP3+ reactive infiltrating cells were shown in early or infiltrated mycosis fungoide plaques with a marked reduction in tumours or large cell lymphoma transformation (25). No data are reported in literature as to circulating CD4+CD25+bright subsets in CTCL patients undergoing ECP.

In this paper, the circulating levels of the CD4+CD25+bright cells have been sequentially analysed during ECP and related to the clinical response in a group of 43 CTCL and 15 GvHD patients treated at our institutions since 1992. Our purpose is to evaluate the ECP-induced modulation of the CD4+CD25+bright cells in CTCL patients and whether these modifications are related to the disease course.

MATERIALS AND METHODS

Patient population

Forty-three CTCL (17 females and 26 males; median age: 55, range: 25-85 years) and 15 chronic GvHD (7 females and 8 males; median age: 48, range: 24-59 years) patients were treated with ECP at our Department from 1992 until 2008 and were included in this study after informed consent. CTCL patients included 28 Sézary syndrome (SS) and 15 Mycosis fungoides (MF), diagnosed according to the standard EORTC classification (26). MF patients were staged according to the recently ISCL/EORTC revised TNM classification (27). The majority of MF patients showed an erythrodermic clinical picture (T4, n=8); the remaining had patch- (T2a, n=3), plaque- (T2b, n=3) and nodular-disease (T3, n=1). Lymph node involvement was found in 3 MF patients.

Peripheral blood involvement was graded according to the criteria of the International Society for Cutaneous Lymphoma, as follows: absent (less than 5% circulating Sézary cells, B0); minimal (Sézary cell count of less than 1,000 mm-3 or less than 20% atypical T cells on peripheral smears (B1); leukemic (B2) (28). The B2 score was defined by at least one of the following criteria: 1) absolute circulating Sézary cell count of ≥1,000 mm-3; 2) CD4/CD8 ratio of ≥10 caused by an increase in circulating T-cells; 3) increased lymphocyte counts with evidence of a T-cell clone in the peripheral blood by polymerase chain reaction (PCR); 4) circulating CD4+CD7- value of ≥40%; 5) aberrant expression of T-cell markers; and 6) chromosomally abnormal T-cell clone. In addition, CD4+CD26- percentage values were used for diagnosis (threshold value: more than 30%) and monitoring of peripheral blood involvement (29). The peripheral blood involvement was scored as B0 in 8, B1 in 4 and B2 in 3 MF patients. All the SS patients showed a B2 leukemic score. Chronic GvHD patients eligible for ECP were in complete remission of primary disease and had GvHD refractory or resistant to steroids plus at least two different lines of immuno-suppression. Chronic GvHD was defined according to the classic clinical manifestations and date of onset (after day 100 from transplantation). One patient received allogeneic transplant for metastatic kidney cancer; all the others had haematological malignancies (2 acute lymphoblastic leukaemia,2 acute myeloblastic leukaemia, 3 non-Hodgkin lymphoma and 8 multiple myeloma). All the GvHD patients carried out ECP together with immunosuppressive drugs.

Treatment plan and clinical response evaluation

Extracorporeal photochemotherapy (ECP) treatment was administered by trained nursing staff under medical supervision, using the UVAR II system from 1992 to 1999 and thereafter the UVAR XTS device (Therakos, Johnson and Johnson, King of Prussia, PA, USA), according to standard procedures. Each procedure consisted of 5 to 6 cycles of collection and separation of buffy coat leukocytes (2%-5% of total circulating leukocytes), followed by exposure of the pooled leukocytes to UVA and 8-MOP, and then reinfusion to the patient. From 1992 to 1996, the photoactive drug 8-methoxypsoralen (8- MOP) was given orally at a dose of 0.6 mg/kg one hour before ECP; since 1997, the liquid form of 8-MOP has been added directly to the buffy coat, resulting in reliable and sufficient drug levels in the cell suspension during the irradiation period.

CTCL patients underwent ECP on 2 consecutive days once a month (1-4); GvHD patients were treated on 2 consecutive days every week for the first month, then every 2 weeks for 2-3 months, thereafter monthly (5-6). The patients were required to have received ECP for at least 2 months for GvHD and for at least 6 months for CTCL. Response assessment in CTCL patients was based on the measurement of clinically apparent disease in the skin, lymph nodes, and peripheral blood, according to the pre-treatment staging, and defined according to standard criteria as complete response (CR), partial response (PR), stable disease (SD) or progression (PD).

CR was defined as the disappearance of all evidence of clinical disease for at least 4 weeks, PR as a 50% tumor regression or greater and at least a 50% reduction in the SC counts (in the patients with pre-treatment peripheral blood involvement), for a minimum of 4 weeks, without the appearance of new lesions. A patient was defined as “responder” only if a response was achieved in all the pre-treatment sites of disease involvement. The cutaneous response was based on the evaluation of the Severity Weighted Assessment Tool (SWAT) score, obtained as the product of the percentage total body surface area involvement of each lesion type (patch, plaque, and tumor or ulceration), multiplied by a weighting factor (1 for patches, 2 for plaques, 3 for tumors/ulcers) (30). Lymph node response was assessed by physical

examination of palpable nodes and, if necessary, biopsy. The response in the blood was determined by comparing both the percentage and absolute number of circulating atypical lymphoid cells as determined by morphology and flow cytometry before and after treatment.
Response criteria for GvHD patients were defined as complete response (CR) in the presence of a complete resolution of all GvHD manifestations, partial response (PR) in the presence of an improvement in skin involvement on at least 50% of the body surface area.

Peripheral blood lymphocyte (PBL) flow cytometry

Peripheral blood lymphocytes (PBL) were analysed according to their immunofluorescence reactivity, using a FACSCalibur cytometer (Becton-Dickinson, S. José, CA, USA). Three- and four-colour immunofluorescence analyses were performed simultaneously using FITC-, PE-, PerCP- and APC-conjugated antibodies. At least 10,000 lymphocytes were collected for each antibody combination. Lymphocyte purity was verified by the usual FSC/SSC parameters by means of a CD45 gating analysis. Isotype-matched negative controls conjugated to each fluorochrome were used to set the location of the cursor for each blood sample. A wide panel of monoclonal antibodies directed against T-cell antigens, as well as activation and proliferation markers, was tested routinely, including CD4 (Leu-3, clone SK3), CD26 (clone L272), purchased from BD Biosciences, San Jose, CA, USA, and CD25 (clone CD25-3G10). As reported in the literature (31), the CD4+CD25+ population can be divided in two different levels of expression: cells that expresses a low level of CD25 (defined CD4+CD25+low) and cells with a higher level of CD25 (defined CD4+CD25+bright) that appear as a tail in respect to the major population, containing both CD4+CD25+low and the CD4+CD25- cells, in the presence of a fluorescence CD25 intensity equal or more than 100. Clonal TCR rearrangement was identified using a panel of 28 MoAbs directed against the variable regions of the β-chain (Serotec Ltd., Oxford, UK; Immunotech Coulter Company, Marseille, France; Endogen, Cambridge, MA, USA; Pharmingen, S. Diego, CA, USA). For each patient, samples were collected at baseline (the day before the beginning of the first ECP course), after 3 and 6 months of treatment, and thereafter every 6 months. The samples collected at baseline and after 3 and 6 months of treatment were available in all patients; thereafter, a progressive reduction in the number of samples available was observed due to treatment discontinuation for progression or other reason, ongoing treatment or end of follow-up period. All the samples were collected the day before the beginning of the ECP cycle. A cohort of 50 healthy donors (HD) age- and sex-matched were used as controls.

Statistical analysis

The results of lymphocyte subsets are given as median, 25th percentile and 75th percentile, minimum and maximum values. Non-parametric tests were applied to analyze the differences in sample distribution. The Mann-Whitney U test was used to evaluate the differences between two groups of patients at a set interval. The Wilcoxon signed-rank test for paired samples was applied to evaluate differences in repeated measures at set intervals. The statistical analysis was applied only to baseline, 3rd and 6th month determinations in which all the patients had their samples available (43 CTCL and 15 GVHD). Fisher's exact probability test was used for cross-table comparisons.

RESULTS

CD4+CD25+bright baseline values

No significant difference could be found at baseline in the circulating CD4+CD25+bright percentage values between CTCL (median: 4.3%; 25%-75% percentile: 2.1-6.1%) and healthy donors (HD)(median: 3.0%; 2.5-3.6%). GvHD patients showed significantly lower values (median: 1.5%; 1.2-1.7%) than both CTCL and HD ($p<0.001$) (Fig. 1). No differences were found between MF and SS patients or according to the circulating Sézary cell (SC) counts.
The TCR-vβ families were evaluated on the CD4+CD25+bright population in 19 patients in whom a specific restriction of the TCR β-chain variable region was detectable by flow-cytometry. CD4+CD25+bright cells did not show a monoclonal restriction since only 2.9% to 4.1% of this subset expressed the TCR-vβ which characterized the clonal population (Fig. 2A, B).

CD4+CD25+bright kinetics during ECP treatment

An opposite trend was observed between CTCL and GvHD patients during ECP. CTCL patients showed an early significant decrease in the median percentage values after 3 months from the beginning of ECP, in respect to baseline levels (from 4.3% to 2.5%; p=0.0001). The reduction in the CD4+CD25+bright values was maintained after 6 months (median: 2.4%; p=0.0026 in respect to baseline), 12 and 18 months of treatment (Fig. 3A). At two years of treatment, median values appeared to slightly increase, even if they were still lower in respect to baseline levels. All the determinations performed afterwards did not disclose significant modifications: the 4 patients in whom the CD4+CD25+bright values were available after more than 2 years of treatment maintained lower than baseline values. No differences in the CD4+CD25+bright trends could be demonstrated between MF and SS. A constant increase in the median percentage values of the CD4+CD25+bright subpopulation was observed from baseline to the 3rd (median: 2%; p=0.0015) and 6th month determination (median: 2.4%; p= 0.0003 in respect to baseline) in GvHD patients (Fig. 3B). The increase continued for all the treatment period; the median percentage values after 18 months of treatment were nearly double than the baseline levels (from 1.5% up to 2.6%).

CD4+CD25+bright modulations and disease course

A clinical response was achieved in 23/43 CTCL patients (53.5%), with 5 CR (11.6%). A SD was obtained in 12 patients, whereas 8 progressed. Overall, CD4+CD25+bright cells decreased during ECP in 33 CTCL patients, with a maximum reduction in respect to baseline levels ranging from 10% to 86.2% (median: 56.6%); on the other hand, CD4+CD25+bright cells increased in 10 patients (Fig. 4). The decrease of the CD4+CD25+bright levels was significantly associated with the achievement of clinical response: it was obtained in 91.3% responders (5/5 CR + 16/18 PR) and in 12/20 (60%) non-responders (Fisher test. P= 0.018). The difference was more pronounced if we consider only PD patients who showed a decrease in only 2/8 cases (25%) (Fisher test vs responders: p=0.0001). Patients who obtained a long-standing SD during ECP showed a CD4+CD25+bright level behavior similar to that of responders, with 10/12 decreases. As to GvHD, 7 patients (46.7%) achieved a clinical improvement. All the GvHD patients underwent a significant increase in CD4+CD25+bright without differences between responders and nonresponders.

DISCUSSION

In this study spanning over a 15-year period, the expression levels of the circulating CD4+CD25+bright subpopulation were sequentially analyzed during ECP treatment in 42 CTCL and 15 GvHD patients. Two main findings are shown for the first time: 1) ECP induces a predominant down-regulation of circulating CD4+CD25+bright cell subsets in CTCL patients; 2) the reduction of CD4+CD25+bright cells is potentially associated with the disease outcome in CTCL. These results therefore highlight the capability of ECP to induce a reciprocal modulation of this subpopulation in CTCL in respect to GvHD, even if such comparison needs to be taken with caution, given the profound differences in aetiology, immunology and clinical outcome which characterize the two diseases. CD4+CD25+bright cells were higher at baseline but decreased during ECP in CTCL patients, whereas they were lower at baseline but increased during ECP in GvHD patients. Indeed, the reduction in CTCL was evident from the 3rd month, with a median value decrease from 4.3% at baseline to 2.4% obtained at the 6-month control. Thereafter, CD4+CD25+bright remained roughly unchanged, even if the value distribution of this subset implies that further studies are needed to confirm these results. The reduction in the circulating CD4+CD25+bright levels is associated with the clinical disease course, as it occurs in 91.3% (21/23) of responding but in only 25% (2/8) PD patients. It is interesting to note that all the 5 CR patients developed a marked decrease of CD4+CD25+bright levels. Moreover, 10/12 patients with a long standing SD status during ECP behave like responders, showing a decrease in the CD4+CD25+bright levels. Indeed, the maintenance of a cutaneous and haematological stabilisation with a good quality of life, no infections and tolerable itching in an aggressive CTCL form such as SS, is to be considered a sign of ECP clinical activity. In contrast, CD4+CD25+bright levels constantly increased in GvHD during ECP; the up-regulation of CD4+CD25+bright cells was not associated to the disease outcome as happens in CTCL. The analysis of the percentage modifications during ECP from baseline levels well illustrates this progressive opposite behaviour which broadens during ECP between CTCL and GvHD. Rubegni et al. (17) already reported an increase in the CD4+CD25+ circulating subset in 14

patients with chronic GvHD after 12 months of ECP treatment. Biagi et al. (16) obtained similar results in 10 GvHD patients; moreover, these authors showed that CD4+CD25+ cells expressed high levels of Foxp3 and CD62L and were functionally able to exert inhibitory activities. Recent experimental evidence strongly supports these data, showing that GvHD can be considered an imbalance of effector T cells and that the transfer of ECP-treated cells reverses GvHD by increasing Treg and reducing effector T lymphocytes (15). The expression and role played by Treg in CTCL patients is far less clearly defined due to the presence of few and controversial findings without data in ECP-treated patients. As a whole, SS seems to be characterized by a peculiar reduction in Treg expression and functions in both skin and peripheral blood, in respect to solid tumours which share a Treg increase correlated with an unfavourable prognosis (11). Our findings of similar baseline CD4+CD25+bright levels between CTCL patients and healthy donors, confirm previous data which reported similar (18) or even lower (17) Treg levels in 10 and 8 SS patients, respectively, with respect to controls. CD4+CD25+bright cells were not found in our study to belong to the clonal population, in agreement with previous studies (23-25). Even if Berger et al. (20) showed that the conversion of CTCL cells to Treg cells is induced by the exposure to immature DCs loaded with apoptotic CTCL cells, a condition which represents an in vitro model of ECP, the same authors reported a Treg in vivo phenotype only in 2/50 CTCL cases. The main limit of our study is that the CD25bright expression, which was the first and most extensively used phenotypic marker for CD4+ Treg, does no exclusively identify this T-cell population as it can be found at various degrees also on activated T cells and APC (10-11). Indeed the long time span of this study, with patient enrolment beginning in 1992, implies that the analysis of the transcription factor forkhead box P3 (FoxP3), the most exclusive intracellular marker for the identification of Treg, is available only in a minority of patients. FoxP3 represents a crucial transcription factor for the functionality of CD4+CD25+bright cells, as it has been shown that the inhibitory properties lies in the FoxP3+ subset (10- 12). Functional in vitro studies should therefore also be carried out to ascertain the effective suppressive activity of the CD4+CD25+bright subset in SS patients. A FoxP3 cytoplasmic expression was found by flow-cytometry in a percentage of CD4+CD25+bright cells ranging from 36% up to 69% (data not shown from 7 SS patients included in this study). These preliminary results suggest that SS patients are characterized by a lower and more variable FoxP3 expression than normal subjects, in whom nearly all the CD4+CD25+bright cells show an uniformly positive staining (32), and are in keeping with Klemke et al. (23) and Tiemessen et al. (24), who reported a lower FoxP3 expression and suppressive activity of Treg cells in CTCL with respect to healthy subjects. A variable FoxP3 pattern by SS patients has also been found by Walsh et al. (33) who showed an increased expression in association with HTLV-I infection.

The biological significance of the present data, assuming CD4+CD25+bright cells as a surrogate marker for CD4+Treg, is intriguing. The functional down-regulation of circulating Treg subsets in CTCL, if confirmed by further phenotypic and functional studies, could induce a less effective suppression of the anti-tumour immune responses and thus contribute to the development of the immunemediated response against pathogenic T-cell clones. The favourable prognostic role played by the Treg down-regulation in ECP-treated patients appears to be in contrast with the increased accumulation of FoxP3+Treg cells in indolent patch/plaque with respect to nodular CTCL lesions (25) and the more aggressive SS disease course associated with the lack of Treg activity (23). However, the prognostic role played by Treg expression is still a matter of controversy also in literature: in fact, recent evidence provides the view that FoxP3 expression could be related to a worse prognosis (22). Some more insights into this controversial issue could be given by the analysis of the re-circulation of Treg cells between peripheral blood and skin. Indeed, results on patients with rheumatoid arthritis demonstrated that the percentage of CD4+CD25+ Treg was significantly increased in the synovial fluid with respect to the peripheral blood (34), suggesting therefore that the recruitment to the inflammatory sites may be responsible for the Treg reduction in the peripheral blood. It could therefore be useful in future studies to evaluate Treg expression and the modifications induced by ECP also in cutaneous lesions. In conclusion, the analysis of the circulating CD4+CD25+bright subset may represent, from a clinical point of view, an additional tool in CTCL management during ECP for the identification of responsive patients, whereas, from a biological perspective, it offers a new intriguing scenario for a better understanding of ECP-induced immunemodulation.

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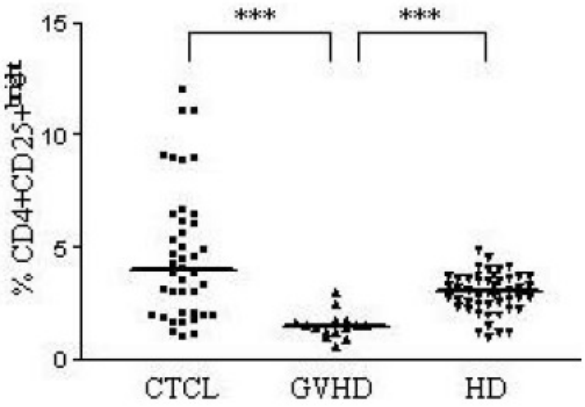

Fig. 1. Percentage values of the Pbl CD4+CD25+bright subset as determined at baseline before the beginning of ECP in CTCL, GvHD and HD. The horizontal bars represent median values. ***: p<0.001.

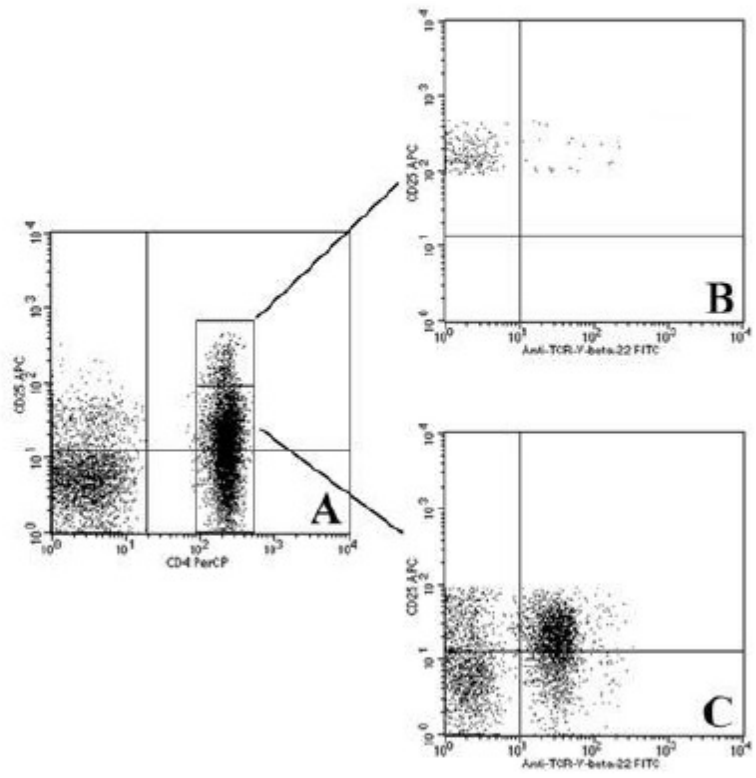

Fig. 2. A representative dot plot from an SS patient, showing that the large majority of CD4+ circulating cells are CD25 negative or CD25+low (A, lower rectangle); only a small subset of CD4+CD25+bright can be identified in the peripheral blood (A, upper rectangle). CD4+ cells were gated on the basis of CD25 expression. In B, CD25+bright gated cells do not express the TCR-vβ of the clonal population, whereas gated CD25negative and CD25+low cells show a specific restriction of the TCR β- chain variable region (Fig. 2C).

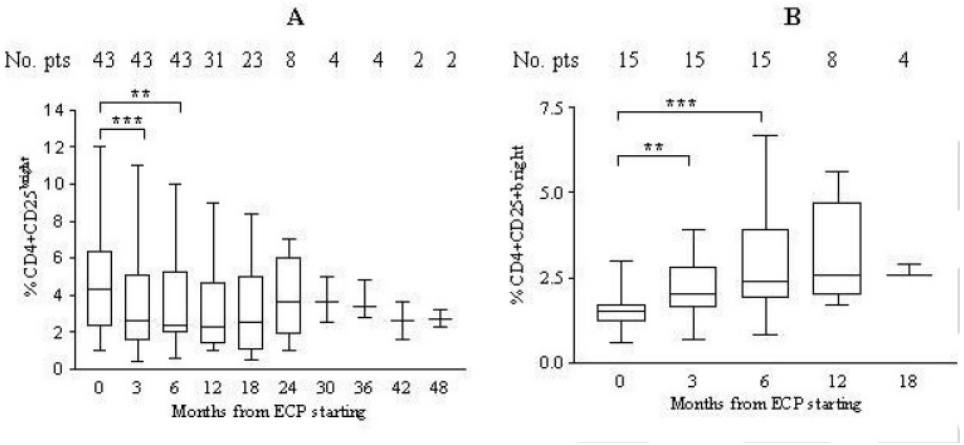

Fig. 3. Kinetics of the CD4+CD25+bright percentage values in CTCL (A) and GvHD (B) patients during ECP. Data are represented as a a box and whisker plot: the ends of the box are the 25th (lower end) and 75th percentile (upper end), the median is marked by an horizontal line inside the box and the whiskers are the two lines outside the box that extend to the highest and lowest observations. Numbers above each graphic represent number of patients tested at each time interval. **: p<0.01; ***: p<0.001.

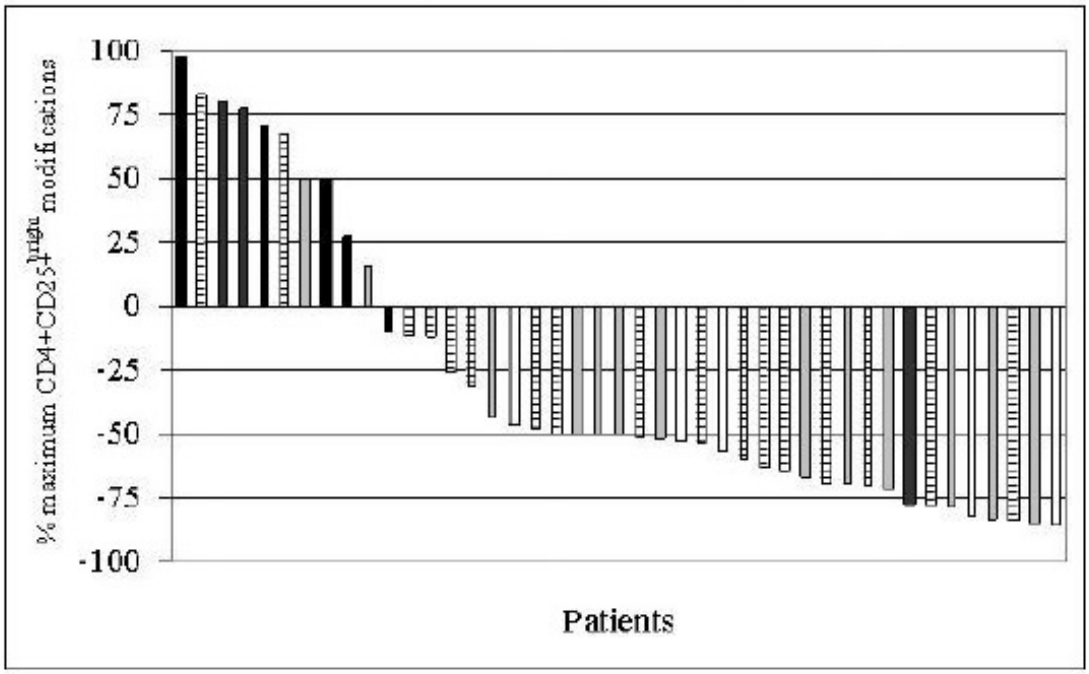

Fig. 4. Highest percentage modifications of the CD4+CD25+bright levels during ECP treatment with respect to baseline values in CTCL patients. The columns above the x axis represent patients who showed an increase of the CD4+CD25+bright subset during ECP; the columns below the x axis represent patients who obtained a CD4+CD25+bright decrease during ECP. The value of each column indicates the highest percentage modification with respect to baseline values obtained at whichever time during ECP. Column colour represents the response obtained by ECP: white=CR; white with horizontal lines=PR; grey=SD; black=PD.