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Abstract

HIV-1 infection is characterized by a progressive decline in $CD4^+$ T cells leading to a state of profound immunodeficiency. IL-2 therapy has been shown to improve $CD4^+$ counts beyond that observed with antiretroviral therapy. Recent phase III trials revealed that despite a sustained increase in $CD4^+$ counts, IL-2-treated patients did not experience a better clinical outcome (*D. Abrams, Y.Levy, M. Losso et al,* N Engl J Med., 2009, 361(16):1548-59). To explain these disappointing results, we have studied phenotypic, functional, and molecular characteristics of $CD4^+$ T cell populations in IL-2-treated patients. We found that the principal effect of long-term IL-2 therapy was the expansion of two distinct $CD4^+CD25^+$ T cell populations ($CD4^+CD25^{lo}CD127^{lo}FOXP3^+$ and $CD4^+CD25^{hi}CD127^{lo}FOXP3^{hi}$) that shared phenotypic markers of Treg but could be distinguished by the levels of CD25 and FOXP3 expression. IL-2-expanded $CD4^+CD25^+$ T cells suppressed proliferation of effector cells *in vitro* and had gene expression profiles similar to natural regulatory $CD4^+CD25^{hi}FOXP3^+$ T cells (Treg) from healthy donors, an immunosuppressive T cell subset critically important for the maintenance of self-tolerance. We propose that the sustained increase of the peripheral Treg pool in IL-2-treated HIV patients may account for the unexpected clinical observation that patients with the greatest expansion of $CD4^+$ T cells had a higher relative risk of clinical progression to AIDS.

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Introduction

Human immunodeficiency virus (HIV) infection is mainly associated with a progressive decrease in the number of $CD4^+$ T lymphocytes and defective CD4 and CD8 specific T-cell responses against HIV and other pathogens. Quantitative and qualitative CD4 T-cell reconstitution following introduction of antiretroviral therapy (ART) in HIV-infected patients is often incomplete leading to evaluate the impact of immune-based therapy in combination with ART. In the past twenty years, a large set of clinical trials demonstrated that intermittent interleukin (IL)-2 therapy increased the CD4 T-cell pool in HIV-infected patients and induced, in a large majority of patients, significant and sustained increases in CD4 T-cell count beyond that seen in patients treated with antiviral drugs alone. In particular, recombinant IL-2 therapy raised naive and central memory $CD4^+$ cells expressing CD25, the alpha chain of the IL-2 receptor, greater than antiretroviral drugs alone (1-6). This population accounted for up to 70% of the total $CD4^+$ T cell pool of IL-2-treated patients and persisted for a long time.

The clinical impact of IL-2 was evaluated in two long-run large phase III trials (SILCAAT and ESPRIT) involving almost 6000 chronically HIV-1 infected patients. Results recently reported showed that, despite a sustained increase in $CD4^+$ T cell counts, over a median follow up of 7-8 years, IL-2 treated patients did not experience a better clinical outcome (7). These disappointing results have remained unexplained so far.

In mice, IL-2 has been shown to be essential for the maintenance and function of a regulatory T cell subset characterized by the expression of CD25 and of the transcription factor FOXP3 ($CD4^+CD25^{hi}FOXP3^+$ T cells, Treg) (8). Neutralization of IL-2 resulted in a decrease in peripheral Treg and the development of autoimmune pathologies (9). Furthermore, analysis of IL-2 or CD25-deficient mice revealed that IL-2 signalling was essential for peripheral maintenance and function of Treg, but not for their generation in the thymus (10, 11). These data argued for an important role of IL-2 in the homeostasis of Treg in mice, however, it is still debated to which extent IL-2 affects the generation, maintenance and/or function of human Treg. It was shown that IL-2 therapy expanded $CD4^+CD25^{hi}FOXP3^+$ regulatory T cells that displayed suppressive activity *in vitro* in cancer patients (12, 13). On the other hand, IL-2-expanded $CD4^+CD25^+$ T cells in HIV patients were not considered to be Treg (14, 15).

Here, we have determined the long-term effects of IL-2 therapy on $CD4^+$ T cell homeostasis and function in HIV-infected patients. We have focused our analysis on phenotypic, functional, and molecular characteristics of the IL-2-expanded $CD4^+CD25^+$ T cell subset. We found that the main effect of long-term IL-2 therapy was the expansion of two distinct $CD4^+CD25^+$ T cell populations ($CD4^+CD25^{lo}CD127^{lo}FOXP3^+$ and $CD4^+CD25^{hi}CD127^{lo}FOXP3^{hi}$) that shared phenotypic markers of Treg but could be distinguished by the levels of CD25 and FOXP3 expression. IL-2-expanded $CD4^+CD25^+$ T

cells suppressed proliferation of effector cells *in vitro* and therefore shared functional characteristics of Treg from healthy donors. Finally, to assess the molecular characteristics of IL-2-expanded $CD4^+CD25^+$ T cells, we performed a transcriptome analysis of $CD4^+CD25^+$ T cells from HIV patients before and after IL-2 treatment. We found that the gene expression signatures before and after IL-2 treatment cycles are closely related and resemble the gene expression profiles generated from sorted $CD4^+CD25^{hi}$ Treg from healthy donors. Together, our data suggest that IL-2 treatment expands $CD4^+CD25^+FOXP3^+$ T cell populations in HIV patients that share phenotypic, functional, and molecular characteristics with Treg.

Results

IL-2 therapy expands distinct populations of $CD4^+CD25^+$ T cells sharing phenotypic markers of Treg.

To determine the effects of intermittent IL-2 therapy on $CD4^+$ T cell homeostasis we first examined phenotypic characteristics of IL-2-expanded $CD4^+CD25^+$ in a cohort of 31 IL-2-treated patients. $CD4^+$ T-cell counts were 445/µl (95-919) prior to IL-2 treatment and 843/µl (422-2348), at the time of sampling. Patients had received a median (ranges) of 7 (3-18) IL-2 cycles **(Supplementary Table 1)**. IL-2 recipients had in median 29.5% (n=15; ranges: 12.2 - 69.0) and 167/µl $CD4^+CD25^+$ T cells and differed significantly from patients treated with cART alone (n=20) who had 16.6% (9.0 - 34.0) (P= 0.002) and 94 cells/µl (P= 0.012).

Since $CD4^+CD25^+$ T cells in HIV-infected patients may include activated T cells or cells that upregulated CD25 in response to IL-2 treatment, we sought to quantify Treg by analyzing the proportion of $CD4^+CD25^{lo}$ and $CD4^+CD25^{hi}$ T cells expressing low levels of CD127 and the transcription factor FOXP3 (16, 17) **(Fig. 1a).**

The proportion of cells expressing Treg characteristics ($FOXP3^+CD127^{lo}$) among $CD4^+CD25^{hi}$ subset was identical in IL-2 and cART patients (patients 1-6, Table 1) (**Fig. 1a** for representative cases and **Fig. 1b,** see **supplementary Fig. 1** for gating strategy**)**. $CD4^+$ T cells expressing intermediate levels of CD25 ($CD4^+CD25^{lo}$) were previously reported as the main $CD4^+$ T cell population expanded in IL-2-treated patients (6, 15, 18). We found that a higher proportion of $CD4^+CD25^{lo}$ T cells were characterized by a Treg phenotype ($FOXP3^+CD127^{lo}$) in IL-2-treated patients compared to patients treated by cART alone (**Fig. 1a** and **Fig. 1b)**. Furthermore, absolute counts of $CD4^+CD25^{hi}FOXP3^+CD127^{lo}$ and $CD4^+CD25^{lo}FOXP3^+CD127^{lo}$ T cells were significantly increased in IL-2 recipients (n=6, 48.7±11.8 cells/µl and 49 ± 14 cells/µl) as compared with cART-treated patients (n=5, 10.4±2.4 cells/µl and 9.6 ± 2.2 cells/µl; P=0.006 for both comparisons) (**Fig. 1c**). We also noted that the level of FOXP3 expression was lower in the $CD4^+CD25^{lo}CD127^{lo}$ population (MFI FOXP3: 83.8± 11.3) than in the $CD4^+CD25^{hi}CD127^{lo}$ T cell population (MFI FOXP3: 267.7 ± 78.7). These results indicate that IL-2 therapy expands two distinct $CD4^+CD25^+$ T

cell populations that share phenotypic markers of Treg but can be distinguished by the levels of CD25 and FOXP3 expression.

A previous report suggested that the majority of IL-2-expanded $CD25^+$ T cells have phenotypic and functional characteristics that are distinct from Treg (14). More recent reports have established heterogeneity within the human Treg compartment (19-21). In light of these findings, we decided to better characterize IL-2-expanded $CD4^+CD25^+$ T cells in HIV patients. We first compared phenotypic markers of $CD4^+CD25^{lo}$ and $CD4^+CD25^{hi}$ T cells in 15 IL-2-treated patients **(Supplementary Table 1,** patients 7-21**)**. Constitutive expression of intracytoplasmic CTLA-4 (cytotoxic T lymphocyte-associated antigen-4), a negative regulator of T-cell activation, of CD103, the α chain of the αEβ7 integrin and of CD122 (the β chain of the IL-2 receptor), were significantly higher in $CD25^{hi}$ T cells as compared with $CD25^{lo}$ T cells. Also, the expression of the activation markers CD69, CD95, CD40L and HLA-DR was significantly higher on $CD4^+CD25^{hi}$ T cells as compared with $CD4^+CD25^{lo}$ T cells. In contrast, the proportion of $CD4^+$ T cells that express CD54RA and/or CD45RO isoforms was similar in both cell populations. Most of $CD4^+CD25^{hi}$ and $CD4^+CD25^{lo}$ T cells exhibited high levels of CD62L, a characteristics of Treg. Among $CD4^+CD25^{hi}$ T cells, a mean of 25.7% of cells expressed HLA-DR, a marker of mature Treg (19)**.** Compared to $CD25^-$ T cells, a higher proportion of $CD25^{lo}$ T cells expressed CD122 (P= 0.005), CD103 (P=0.046) and high levels of CD62L (P=0.018) **(Fig. 2)**.

$CD4^+CD25^+$ IL-2 expanded T cells exhibit functional characteristics of Treg.

Next, we explored the proliferative potential of IL-2-expanded $CD4^+CD25^+$ T cells. Upon stimulation with immobilized anti-CD3 mAb, the proliferation of $CD4^+CD25^+$ T cells was significantly reduced compared to autologous $CD4^+CD25^-$ T cells (P=0.002). Addition of soluble anti-CD28 mAb restored only partially the proliferative capacity of these cells as described for Treg in healthy individuals and cART-treated patients (22) (**Fig. 3a**). Interestingly, in contrast to $CD4^+CD25^-$ T cells, purified $CD4^+CD25^+$ T cells containing both the $CD4^+CD25^{hi}$ and $CD4^+CD25^{lo}$ T cell populations did not proliferate in response to stimulation with PPD, CMV or HIV-p24 antigens (**Fig. 3b**). These data demonstrate that IL-2 expanded $CD4^+CD25^+$ cells that exhibit functional characteristics of Treg.

Then, we investigated the capacity of $CD4^+CD25^+$ T cells expanded in IL-2-treated patients (n=13) to suppress effector functions of $CD4^+$ T cells. First, we found that depletion of these cells led to a significant increase in CD4 T cell proliferation in response to PPD and HIV-p24 antigens (P< 0.05 for both comparisons) (**Fig. 3c**). Next, addition of purified $CD4^+CD25^+$ T cells (10^4 to 10^5 cells) resulted in approximately 50% and 80% inhibition of $CD4^+CD25^-$ T-cell proliferation to PPD and HIV-p24 antigens at a 1:4 and 1:1 ratio (Treg : Effector), respectively (**Fig. 3d**). It is noteworthy that the levels of inhibition of the $CD4^+CD25^+$ population from IL-2 patients were similar to that observed with $CD25^{hi}$ T cells from cART-

treated patients (22). This suggests that $CD4^+CD25^{hi}$ cells from IL-2 treated patients are highly suppressive or alternatively that expanded $CD4^+CD25^{lo}$ T cells exert also regulatory functions.

$CD4^+CD25^+$ IL-2 expanded T cells exhibit molecular characteristics of Treg

Finally, to assess the molecular characteristics of IL-2-expanded $CD4^+CD25^+$ T cells, we performed a longitudinal analysis of the gene expression profiles of enriched $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from 10 HIV-infected patients (patients 34001 – 59019, **Supplementary Table 1**) before the first (week 0) and eight weeks after the third IL-2 cycle (week 24) using microarrays. We found that 206 genes were differentially expressed ($P<0.001$) between enriched $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells before IL-2 treatment (week 0) **(Supplementary Table 2)**, whereas 60 genes were differentially expressed ($P<0.001$) after three IL-2 cycles at week 24 **(Supplementary Table 3).**

As expected, many of the genes differentially expressed at week 0 (*FOXP3*, *CTLA4*, *IL10*, *FAS, LRRC32* (GARP), *CCR8*, and *IL1R1*) have been associated with phenotype or function of Treg (23-26) **(Fig. 4).** Analysis of the gene expression signature after three IL-2 treatment cycles at week 24 revealed that of the 60 genes differentially expressed at this time point ($P<0.001$), 50 were also differentially expressed at week 0 **(Fig. 4 and Supplementary Table 3).** We noted, however, also several differences between $CD4^+CD25^+$ T cells before IL-2 treatment and $CD4^+CD25^+$ T cells after IL-2 treatment. The chemokine receptor CCR8 (27) was expressed at lower levels after 3 treatment cycles, whereas the dual-specificity phosphatase 6 (DUSP6), a negative regulator of ERK2 activity involved in tuning T cell excitation thresholds (28), was upregulated in $CD4^+CD25^+$ T cells after IL-2 treatment (**Fig. 4**). Then, we have used the 60 genes differentially expressed between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from IL-2-treated HIV patients as a "signature" to analyze sorted $CD4^+CD25^{hi}$ and $CD4^+CD25^-$ T cells from peripheral blood of healthy donors. Clustering according to this gene set could accurately discriminate between $CD4^+CD25^{hi}$ and $CD4^+CD25^-$ T cells **(Fig. 5)**. In contrast, this signature could not discriminate between $CD4^+CD25^+$ T cells from patients before and after IL-2 treatment using hierarchical clustering **(Supplementary Fig. 2)**, suggesting that Treg from HIV patients before and after IL-2 treatment are related and are similar to Treg from healthy donors.

IL-2 expands pre-existing $CD4^+CD25^+$ populations sharing molecular markers of Treg

To visualize quantitative differences in gene expression between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells before and after IL-2 treatment, we plotted the "$\log_2$fold-change" values for differentially expressed genes ($P<0.001$) at week 24 (y-axis) against those at week 0 (x-axis). In this representation, genes that are upregulated in $CD4^+CD25^+$ *versus* $CD4^+CD25^-$ T cells both before (week 0) and after IL-2 treatment (week 24) are located in the upper-right

part of the diagram, whereas genes that are down-regulated are in the lower-left part. This analysis revealed that the majority of the values are located close to the diagonal (Pearson correlation coefficient = 0.922), indicating that fold-changes in gene expression between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells are maintained before and after IL-2 treatment **(Fig. 6)**. Taken together, these data demonstrate that IL-2-induced $CD4^+CD25^+$ T cell populations in HIV patients have molecular characteristics that are closely related to Treg.

Discussion

We report here that administration of IL-2 in HIV-infected patients results in an increase of two distinct populations of $CD4^+CD25^+$ T cells that express low levels of CD127 and various levels of FOXP3. We found also that IL-2 expanded $CD4^+CD25^+$ T cells respond poorly to antigenic stimulation and suppress function of $CD4^+CD25^-$ T cells *in vitro*. For the first time to our knowledge, we provide a molecular characterization of these cells showing that they exhibit gene expression related to Treg. Finally, by comparing gene expression profiles of $CD4^+CD25^+$ T cells before and after IL-2 therapy, we show that expanded cells pre-exist to IL-2 therapy in patients' blood. Globally, these results demonstrate that IL-2 expands $CD4^+CD25^+$ T-cell populations sharing functional and molecular characteristics of Treg.

This observation confirms and extends the previous functional characterization of IL-2-expanded CD4 T cells in HIV-infected patients (14, 15). These studies were mainly focused on expanded naive $CD4^+CD25^{lo}$, the so-called "Cyokine Expanded Naive (CEN)" cells (14). We found that around 70-75% of $CD4^+CD25^{lo}$ and $CD4^+CD25^{hi}$ express CD45RO or both CD45RA/CD45RO, a phenotype of memory T cells. We also noticed some phenotypic differences between expanded $CD4^+CD25^{lo}$ and $CD4^+CD25^{hi}$ cells. The expression of the activation markers CD69, CD95, CD40L and HLA-DR was significantly higher on $CD4^+CD25^{hi}$ T cells as compared with $CD4^+CD25^{lo}$ T cells. We also found that constitutive expression of intracytoplasmic CTLA-4, a negative regulator of T-cell activation recently described as a marker of progression, was significantly higher in $CD25^{hi}$ T cells as compared with $CD25^{lo}$ T cells.

Attempts to better characterize the origin and function of IL-2 expanded $CD4^+CD25^+$ T cells generated several studies in the past years (6, 14, 15, 18, 29). Results reported here are highly reminiscent to recent reports that have established heterogeneity within the Treg compartment. Baecher-Allan et al. reported two distinct Treg populations that can be distinguished based on the expression of MHC class II molecules (19) and Miyara et al. very recently demonstrated the presence of “resting” and “activated” Treg in the periphery (20). Resting Treg ($CD25^+CD45RA^+FOXP3^{lo}$) converted *in vitro* and *in vivo* into activated Treg ($CD25^{hi}CD45RA^-FOXP3^{hi}$), establishing a developmental link between these two populations (20). Of note, the majority of IL-2-expanded $CD4^+CD25^{lo}CD127^{lo}FOXP3^+$ T cells in HIV patients shares phenotypic characteristics with HLA-DR-negative (19) or resting (20) Treg.

However, we also observed an increase in frequency and numbers of $CD4^+CD25^{hi}CD127^{lo}FOXP3^{hi}$ T cells that are reminiscent of HLA-DR-positive (19) or activated (20) Treg. Our data therefore suggest that IL-2 treatment expands the two major Treg populations present in human peripheral blood. This observation is reinforced by the gene profile analysis of IL-2 expanded $CD4^+CD25^+$ T cells showing that these cells express markers of Treg close to those expressed by highly purified Treg from healthy controls. Moreover, this Treg signature allowed us to track the origin of expanded cells in patients treated with IL-2. Taken together, our data indicate that around 30% of circulating $CD4^+$ T cells express phenotypic, functional and molecular markers of Treg in IL-2 treated patients. It is likely that this imbalance between Treg and conventional effector $CD4^+$ T cells may impair the functionality of T cell pool as suggested by results from phase III trials (7).

Studies in mice have shown that *in vivo* neutralization of IL-2 reduces the number of $CD4^+CD25^+FOXP3^+$ Treg resulting in autoimmune manifestations. *In vitro* and *in vivo* studies showed that IL-2 is critical for Treg function and survival (30, 31). Phase II IL-2 studies have shown an inccreased survival of circulating IL-2 expanded $CD4^+CD25^+$ in HIV-1 infected patients (32). Moreover, analysis of T cell cycling using Ki-67 staining showed that these cells exhibit a low rate of turn over even in HIV untreated patients, which is in sharp contrast with the behaviour of conventional T cells (15). Together, these observations remind characteristics of Treg and strengthen our findings that IL-2 administration leads to expansion of Treg. The present study also extends data from previous clinical studies showing an increase of $CD4^+CD25^+FOXP3^+$ T cells in IL-2-treated cancer patients (12, 13, 33) and contributes to better characterize the homeostatic effects of this cytokine on human Treg populations.

Treg play a major role in the maintenance of self-tolerance, the control of immune homeostasis, and are potent suppressors of immune responses. IL-2-induced Treg may not participate to host defences in HIV patients or may even hinder generation of effector responses against pathogens. Our results may therefore provide an explanation for the unexpected clinical observation that patients with the greatest expansion of $CD4^+$ T cells under IL-2 had a higher relative risk of clinical progression (in terms of AIDS progression or deaths) than those with lower $CD4^+$ counts (7). Lessons learnt from the experience of IL-2 therapy over the last 25 years are of interest for the future of immunotherapy of HIV infection. Results indicate that a refinement of CD4 T cell phenotypes is needed since global CD4 T cell count after a cytokine intervention could not be considered as a valid surrogate marker of clinical benefit. In this setting, intermittent IL-7 therapy in HIV and non HIV conditions, also increases CD4 T cells and perturbs significantly T cell homeostasis without any apparent effect on Treg populations, shows promise. Results of large trials with clinical endpoints are eagerly awaited (34).

Materials and methods

A detailed description of procedures is given in the supplementary methods section.

Patients. 31 HIV-infected patients who had received, while on antiretroviral therapy, at least 3 cycles of IL-2 in the frame of ANRS trials or expanded open access to IL-2 were included in the study. Blood samples collected on EDTA tubes were processed within 3 hours. The clinical characteristics of the patients are depicted in Supplementary Table 1. Median (ranges) CD4 T-cell counts were 445/µl (95-919) prior to IL-2 treatment and 843/µl (422-2348), at the time of sampling. All patients but three had plasma HIV-RNA levels below 50 copies/ml. Patients had received a median (ranges) of 7 (3-18) IL-2 cycles. The median time between testing and the last IL-2 cycle was 2 months (ranges: 2-59). Written informed consent was obtained from all the patients, according to human experimentation guidelines from national ethical committees.

Cell Isolation. $CD4^+$ T lymphocytes were purified from peripheral blood as previously described (22, 35-37).

Flow Cytometric Analysis. Phenotyping of cell subsets was performed on fresh PBMC by four-colour flow cytometry using the mAbs listed in the supplementary methods section. Analyses were performed using FACScalibur™ or LSRII cytometer and CellQuest™ software (Becton Dickinson, San Jose, California, USA) on at least 1000 events. Gating was restricted to the population of lymphocytes according to their light scattering properties.

Proliferation and suppression assays. The different subpopulations (unfractionnated $CD4^+$ cells, $CD4^+CD25^-$ cells and $CD4^+CD25^+$ cells) were assessed for their proliferative capacities in response to polyclonal stimulation and to recall antigens and p24 protein, as described previously (22).

Microarray procedures. Gene expression profiling of enriched $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from HIV patients and healthy donors was performed using Applied Biosystems 1700 microarray platform following protocols provided by the supplier as previously described (38). Microarray data were deposited in the MACE database (http://mace.ihes.fr/) with the accession numbers 2560865806 (data from HIV patients) and 2828616462 (healthy donors).

Statistical analyses. Data are expressed as mean ± SEM for percentages and median and ranges for absolute values. Statistical comparisons were performed using two-tailed unpaired non parametric Mann Whitney test for comparisons between groups (IL-2 vs controls) and Wilcoxon rank test for comparisons between CD4 cell subsets. Significance was considered for $P \leq 0.05$.

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Figure legends:

Figure 1 IL-2 therapy expands $CD4^+CD25^{hi}$ and $CD4^+CD25^{lo}$ T cells that share phenotypic characteristics of natural regulatory T cells. (**a**) Proportion of $FOXP3^+CD127^{lo}$ cells among $CD4^+CD25^{hi}$ (left panels) and $CD4^+CD25^{lo}$ T-cells (right panels) in IL-2 recipients (upper panels) and patients treated with retroviral therapy alone (cART, lower panels). Purified $CD3^+$ T cells isolated from PBMC were stained with anti-CD4, anti-CD25, anti-CD127 and anti-FOXP3. Cells were gated according to forward scattering properties and CD25 expression. Co-expression of FOXP3 and CD127 is illustrated for 3 representative IL-2-treated HIV patients (Patient 1, 2, and 3) and 3 representative patients treated with cART alone (C1, C2 and C3). **(b)** Proportion of $FOXP3^+CD127^{lo}$ cells among the $CD4^+CD25^{hi}$ (left panel) and the $CD4^+CD25^{lo}$ population (right panel) in IL-2 recipients (N= 6) and cART-treated controls (N= 5); ** P< 0.01 (Mann Whitney test). **(c)** Absolute numbers of $CD4^+FOXP3^+CD127^{lo}CD25^{hi}$ (conventional Treg cells) and $CD4^+FOXP3^+CD127^{lo}CD25^{lo}$ in peripheral blood of IL-2 recipients (n=6) and cART-treated controls (n=5) ** P< 0.01 (Mann Whitney test).

Figure 2. Phenotype of the $CD4^+CD25^{hi}$, $CD4^+CD25^{lo}$ and $CD4^+CD25^-$ T-cell subsets in IL-2 treated HIV-infected patients (n=15). The membrane or intracellular (CTLA-4) expression of the different molecules was determined in whole blood cells by four-colour direct flow cytometry after successively combining the FSC/SSC, $CD3^+$, $CD4^+$ and CD25 gates. Analysis was performed on $CD25^{hi}$ (black bars), $CD25^{lo}$ (grey bars) and $CD25^-$ (white bars) T cells. Data are presented as mean percent positive cells ± SEM (* P<0.05; ** P< 0.01 using Wilcoxon rank test).

Figure 3. $CD4^+CD25^+$ T cells from IL-2 treated HIV-infected patients are hyporesponsive/anergic to polyclonal and antigen-specific stimulation and suppress antigen-specific proliferation of autologous $CD4^+CD25^-$ T cells. For functional analysis, we selected patients treated in the long term with IL-2 (n= 15; Median (ranges) CD4 T-cell counts: 324/µl prior to IL-2 treatment and 682/µl at the time of sampling; median of IL-2 cycles: 10; Median time between testing and the last IL-2 cycle 8 months) (**a**) $1x10^5$ purified $CD4^+CD25^-$ (white histograms) or $CD4^+CD25^+$ (black histograms) T cells were stimulated for 3 days with 5µg/ml plate-bound anti-CD3 mAb in the presence or absence of soluble anti-CD28 mAb (5µg/ml). (**b**) $1x10^5$ freshly purified $CD4^+CD25^-$ (white histograms) or $CD4^+CD25^+$ (black histograms) T cells were cultured for 5 days with 5µg/ml purified tuberculin (PPD), 5µg/ml p24, 1/50 dilution of the CMV antigen. 0.5µ Ci/well [^{3}H] thymidine was added during the last 16 h of culture. **(c)** Unfractionated $CD4^+$ T cells (grey histograms) or purified $CD4^+CD25^-$ T cells (white histograms) were incubated PPD or HIV-p24 protein. Results are expressed as mean ± SEM [^{3}H] thymidine incorporation from HIV-infected patients treated with IL-2 (N= 13 for polyclonal stimulation; n= 15 for antigen-specific stimulation); * P<0.05; ** P< 0.01. The median (ranges) [^{3}H] thymidine incorporation in control cultures (medium alone) was 462

(140-824), 240 (130-377) and 136 (63-175) for unfractionated $CD4^+$ cells, $CD4^+CD25^-$ cells and $CD4^+CD25^+$ cells, respectively. **(d)** Co-culture of $CD4^+CD25^-$ with $CD4^+CD25^+$ T lymphocytes from IL-2 treated HIV-infected patients, results in a dose-dependent suppression of antigen-specific proliferation. Purified $CD4^+CD25^-$ T cells were stimulated by PPD (circles) or HIV-p24 protein (diamonds) in the presence of increasing numbers of purified $CD4^+CD25^+$ lymphocytes. Results are expressed as mean (± SEM) percentage of inhibition of proliferation (n= 13 for PPD stimulation and n= 9 for p24 stimulation).

Figure 4.

$CD4^+CD25^+$ T cells from HIV patients before and after three IL-2 treatment cycles express Treg marker genes. Gene expression profiles of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from 10 HIV patients were determined using Human Genome Survey Microarrays v2.0 (Applied Biosystems) as described (38). Targets for microarray hybridization were generated by converting RNA from $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells into cDNA followed by a single round of linear amplification in the presence of Digoxigenin-UTP (see Methods). Microarray images were analyzed using Expression Array System Software (Applied Biosystems) and microarray data analysis was performed using open-source R-scripts (ABarray, www.abarray.org). Shown is the box-plot representation (whiskers: Min to Max) of normalized expression levels of the indicated genes in $CD4^+CD25^-$ ($CD25^-$) and $CD4^+CD25^+$ ($CD25^+$) T cells before (WK0) and after (WK24) IL-2 treatment (N = 10, * $P < 0.05$; **$P< 0.01$ using two-tailed Wilcoxon matched pairs test).

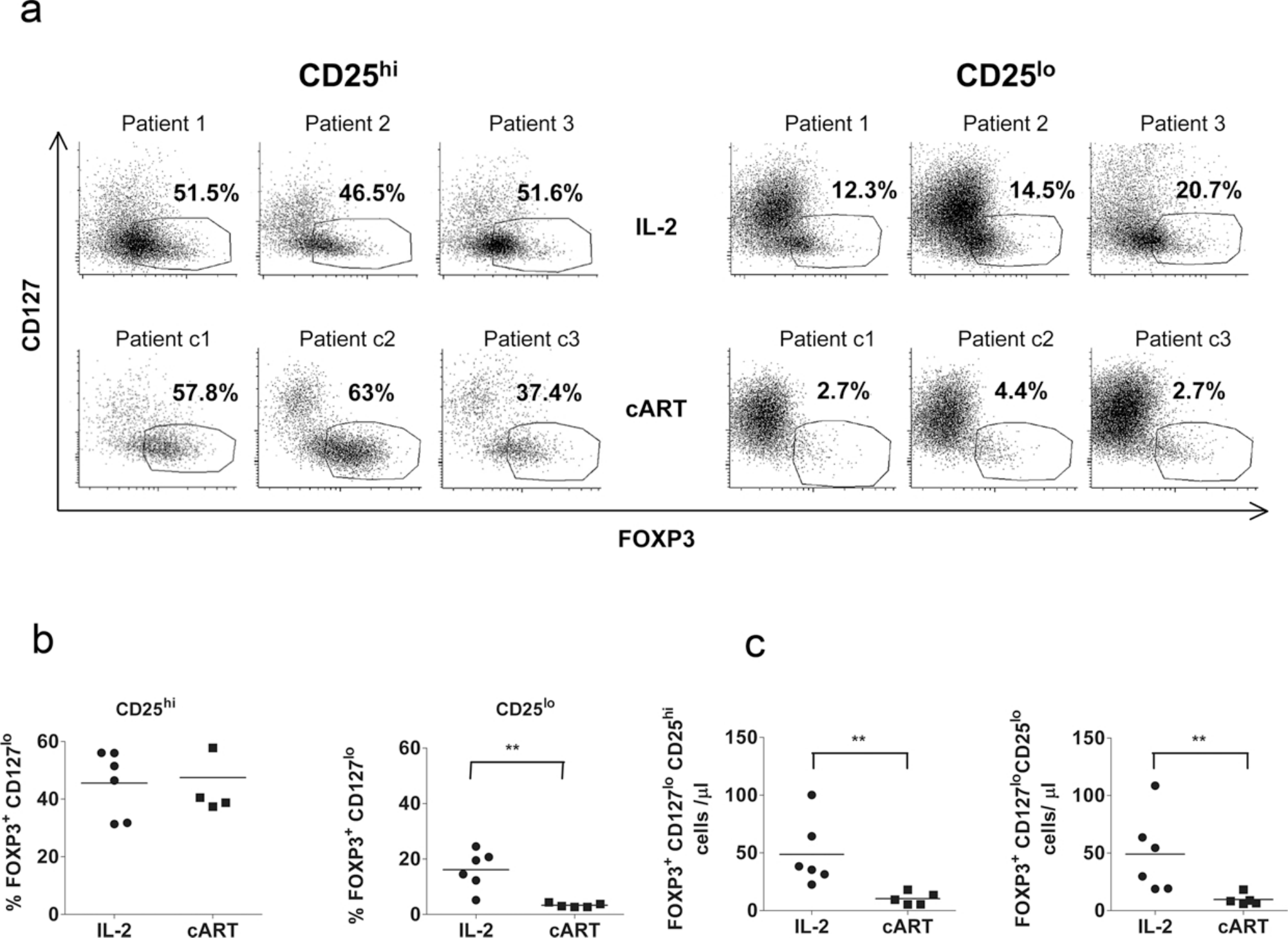
Figure 5.

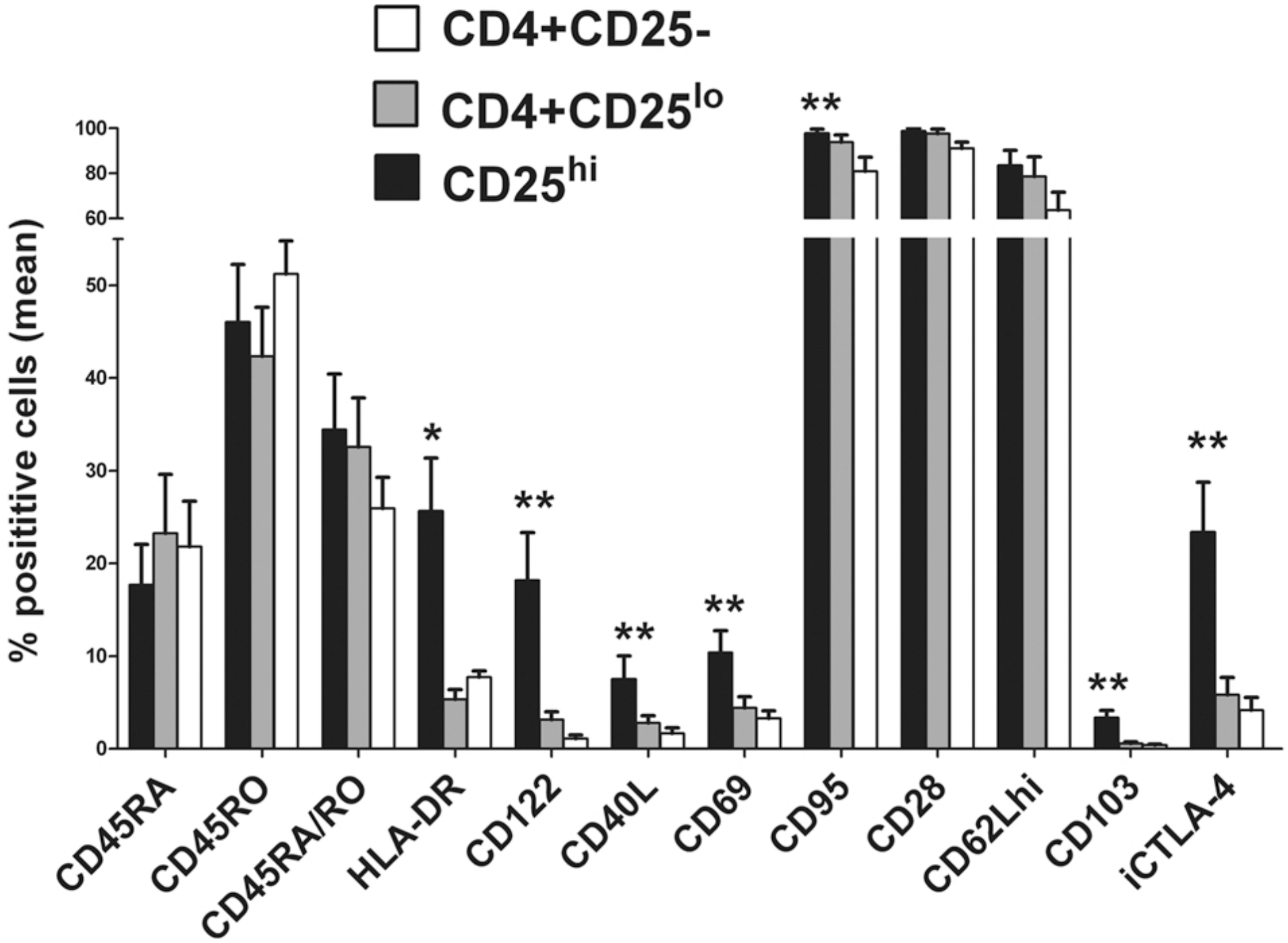
Genes differentially expressed between IL-2-expanded $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells in HIV patients discriminate between sorted Treg and $CD4^+CD25^-$ T cells from healthy donors. Treg and $CD4^+CD25^-$ T cells from peripheral blood of healthy donors were isolated by cell sorting and stimulated for 24h with anti-CD3 and anti-CD28 antibodies. Gene expression profiling using ABI 1700 technology of 6 independent donors was performed as described for samples from HIV patients (see Methods). The 60 genes differentially expressed ($P < 0.001$) between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from HIV patients after IL-2 treatment (Supplementary Table 3) were used to analyze the dataset of healthy donors using a hierarchical clustering tool (Spotfire). This algorithm groups samples (in columns) and genes (in rows) according to their euclidian distances. The dendrogram on top shows that the 60 gene "signature" derived from IL-2-expanded $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from HIV patients groups together samples from $CD4^+CD25^-$ T cells (left side of the diagram) and Treg (right side) from healthy donors.

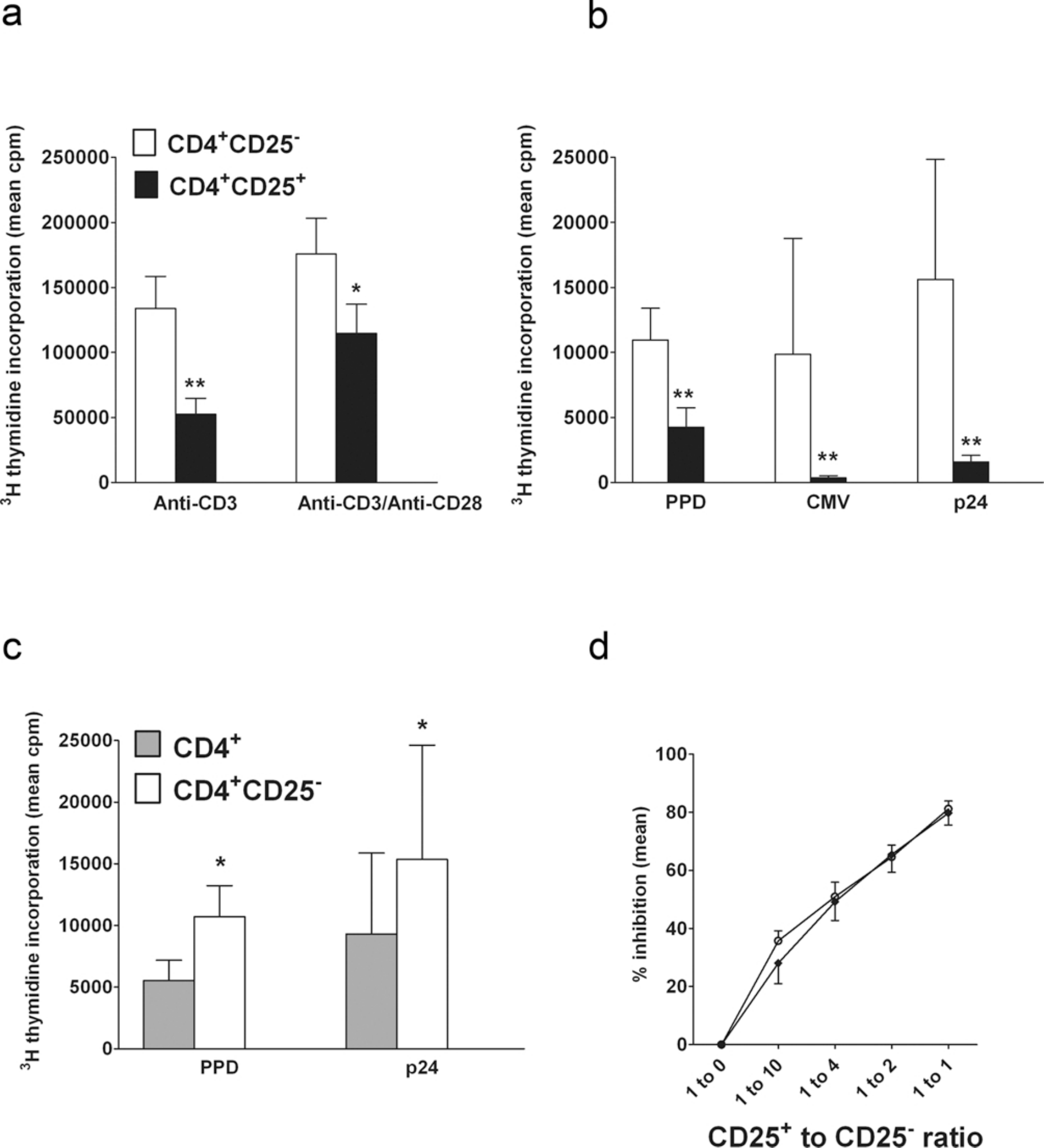
Figure 6.

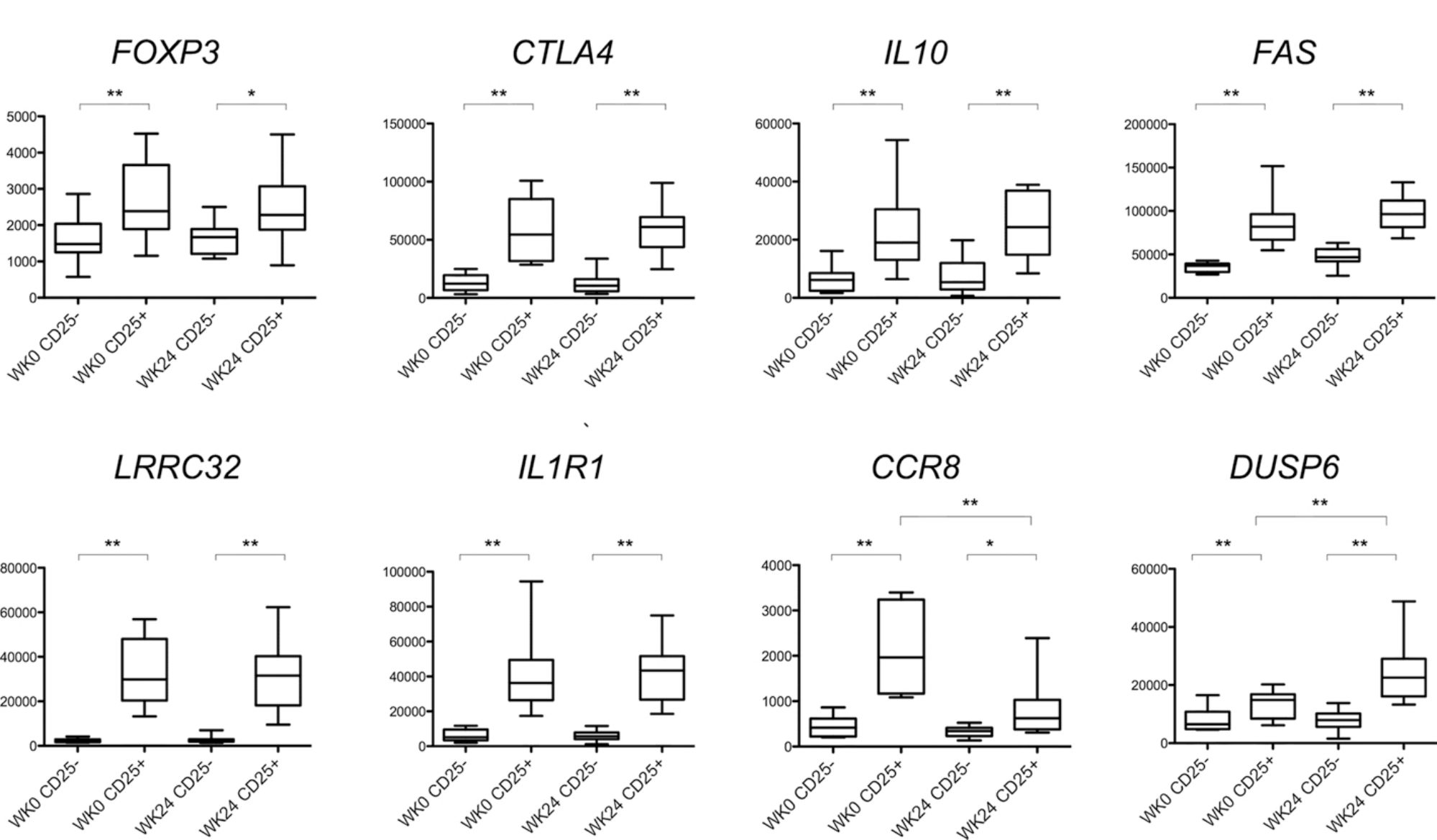
Fold-changes in gene expression between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells are maintained before and after IL-2 treatment. Scatter plot representation of the $\log_2$ fold-change

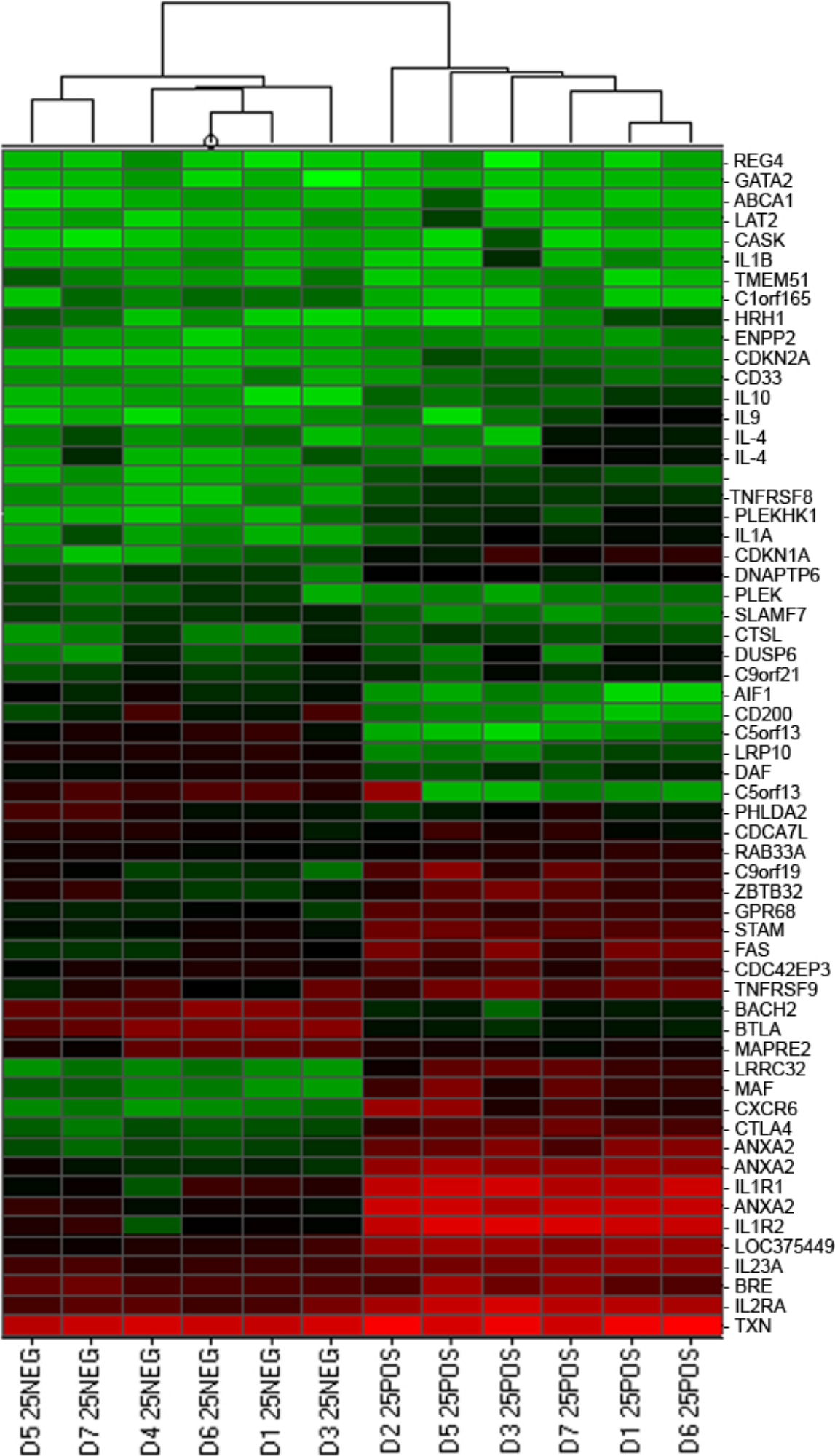
(FC) values of gene expression between $CD4^+CD25^-$ ($CD25^-$) and $CD4^+CD25^+$ ($CD25^+$) T cells before IL-2 treatment (WK0, x-axis) *versus* after IL-2 treatment (WK24, y-axis).


a
CD25hi
CD25lo
Patient 1
Patient 2
Patient 3
51.5%
46.5%
51.6%
12.3%
14.5%
20.7%
IL-2
Patient c1
Patient c2
Patient c3
57.8%
63%
37.4%
2.7%
4.4%
2.7%
cART
CD127
FOXP3
b
CD25hi
% FOXP3+ CD127lo
60
40
20
0
IL-2
cART
CD25lo
**
c
FOXP3+ CD127lo CD25hi cells /µl
150
100
50
FOXP3+ CD127loCD25lo cells/ µl


CD4+CD25-
CD4+CD25lo
CD25hi
% positive cells (mean)
100
80
60
50
40
30
20
10
0
*
**
**
**
**
**
**
**
CD45RA
CD45RO
CD45RA/RO
HLA-DR
CD122
CD40L
CD69
CD95
CD28
CD62Lhi
CD103
iCTLA-4


a
b
c
d
CD4+CD25-
CD4+CD25+
CD4+
3H thymidine incorporation (mean cpm)
250000
200000
150000
100000
50000
0
25000
20000
15000
10000
5000
**
*
Anti-CD3
Anti-CD3/Anti-CD28
PPD
CMV
p24
% inhibition (mean)
100
80
60
40
20
1 to 0
1 to 10
1 to 4
1 to 2
1 to 1
CD25+ to CD25- ratio


FOXP3
CTLA4
IL10
FAS
LRRC32
IL1R1
CCR8
DUSP6
WK0 CD25-
WK0 CD25+
WK24 CD25-
WK24 CD25+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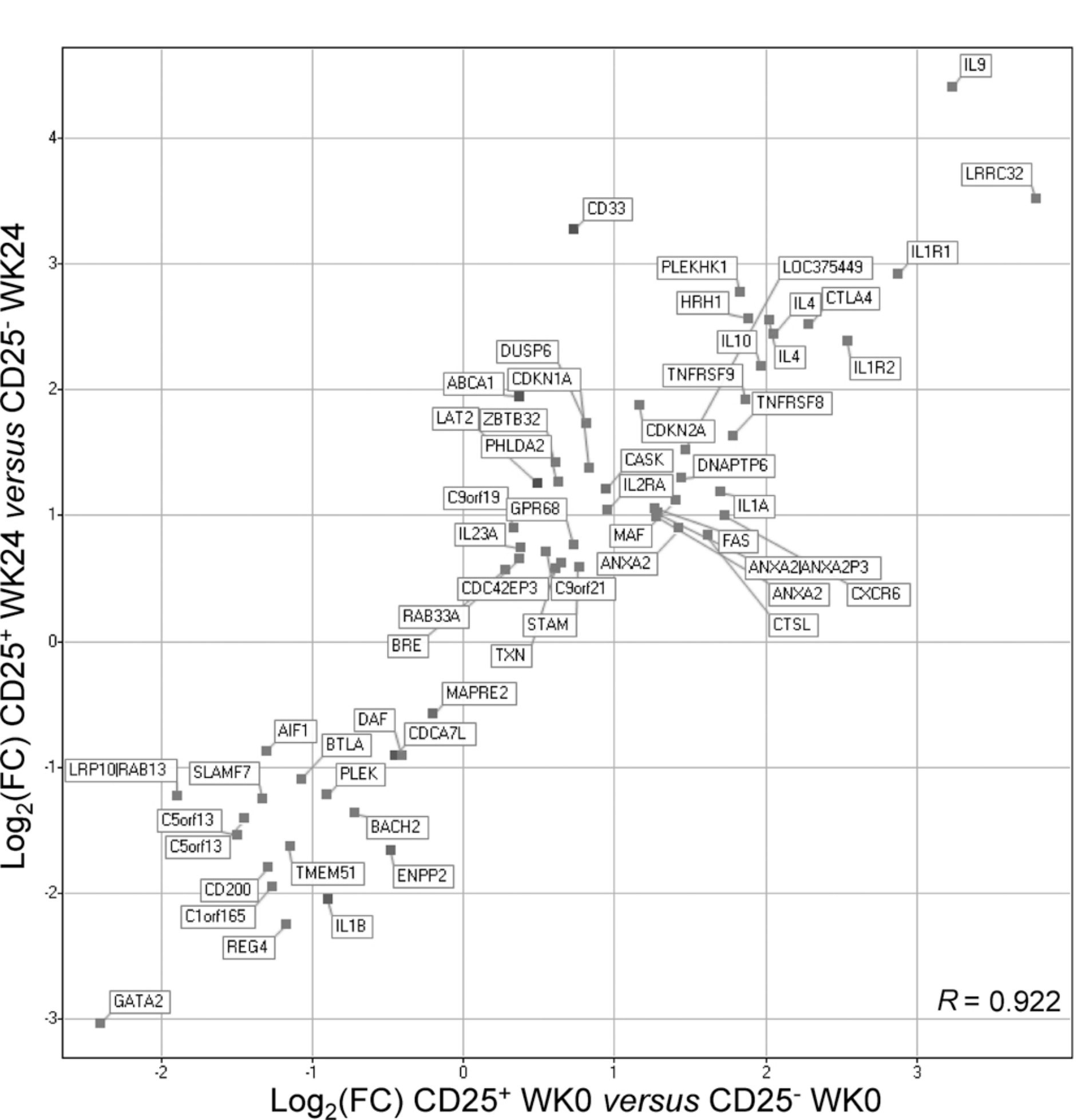
ENPP2
CDKN2A
CD33
IL10
IL9
IL-4
IL-4
TNFRSF8
PLEKHK1
IL1A
CDKN1A
DNAPTP6
PLEK
SLAMF7
CTSL
DUSP6
C9orf21
AIF1
CD200
C5orf13
LRP10
DAF
C5orf13
PHLDA2
CDCA7L
RAB33A
C9orf19
ZBTB32
GPR68
STAM
FAS
CDC42EP3
TNFRSF9
BACH2
BTLA
MAPRE2
LRRC32
MAF
CXCR6
CTLA4
ANXA2
ANXA2
IL1R1
ANXA2
IL1R2
LOC375449
IL23A
BRE
IL2RA
TXN
D5 25NEG
D7 25NEG
D4 25NEG
D6 25NEG
D1 25NEG
D3 25NEG
D2 25POS
D5 25POS
D3 25POS
D7 25POS
D1 25POS
D6 25POS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LRRC32
CD33
IL1R1
PLEKHK1
LOC375449
HRH1
IL4
CTLA4
IL10
IL4
IL1R2
DUSP6
TNFRSF9
ABCA1
CDKN1A
TNFRSF8
LAT2
ZBTB32
CDKN2A
PHLDA2
CASK
DNAPTP6
IL2RA
C9orf19
IL1A
GPR68
IL23A
MAF
FAS
ANXA2
ANXA2|ANXA2P3
CDC42EP3
C9orf21
ANXA2
CXCR6
RAB33A
STAM
CTSL
BRE
TXN
MAPRE2
DAF
AIF1
CDCA7L
BTLA
LRP10|RAB13
SLAMF7
PLEK
C5orf13
BACH2
C5orf13
TMEM51
ENPP2
CD200
C1orf165
IL1B
REG4
GATA2
R = 0.922
4
3
2
1
0
-1
-2
-3
-2
-1
0
1
2
3
Log2(FC) CD25+ WK24 versus CD25- WK24
Log2(FC) CD25+ WK0 versus CD25- WK0