Depletion of T regulatory cells through selection of CD127-positive cells results in a population enriched in memory T cells: implications for anti-tumor cell therapy

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ABSTRACT

Background

Donor lymphocyte infusions can induce remissions in patients with relapse after allogeneic hematopoietic stem cell transplantation. Nevertheless, some grafted patients never display any signs of alloreactivity, either following allogeneic hematopoietic stem cell transplantation or after donor lymphocyte infusions. Consequently, they do not develop graft-*versus*-host disease and frequently do not respond to donor lymphocyte infusions. In a recently published clinical trial, we observed that elimination of $CD4^+CD25^+Foxp3^+$ natural regulatory T cells from the donor lymphocyte product could improve alloreactivity and the associated anti-tumor effect in a small proportion of patients with relapsed hematologic malignancies. Here, we aimed to improve the effect of donor lymphocyte infusion by modifying the procedure for depletion of T regulatory cells.

Design and Methods

We directly compared depletion of regulatory T cells from human peripheral blood mononuclear cells achieved by selection of CD127-positive cells or by selection of CD25-negative cells. We tested the manipulated products (i) *in vitro* in mixed lymphocyte reactions and against pathogen-derived recall antigens and (ii) *in vivo* in experimental graft-*versus*-host disease.

Results

In vitro, we found that depletion of regulatory T cells through CD127 positive selection improved both alloreactive and pathogen-specific immune responses. *In vivo*, we observed accelerated donor T-cell division and enhanced graft-*versus*-host disease due to efficient regulatory T-cell depletion accompanied by enrichment in memory T cells.

Conclusions

Our results show that the strategy of CD127 positive selection is an efficient way of eliminating regulatory T cells from donor lymphocyte infusions and improves alloreactivity. This supports the investigation of CD127 positive selection in place of elimination of CD25-positive cells for clinical applications.

Key words: CD127 positive selection, Treg, alloreactivity, donor lymphocyte infusion.

Citation: Touil S, Rosenzwajg M, Landau DA, Le Corvoisier P, Charlotte F, Klatzmann D, Maury S, and Cohen JL. Depletion of T regulatory cells through selection of CD127-positive cells results in a population enriched in memory T cells: implications for anti-tumor cell therapy. Haematologica 2012;97(11):1678-1685. doi:10.3324/haematol.2011.056374

Funding: this work was supported by "APP recherche clinique translationnelle Inserm-DHOS-INCA 2008" and "Agence de la Biomédecine". ST is supported by a grant from the French government.

Acknowledgments: the authors would like to thank Eliane Piaggio for critical reading of the manuscript and Pierric Parent, Yamina Hamel and Maude Guillot-Deloste for technical support.

Manuscript received on October 5, 2011. Revised version arrived on March 26, 2012. Manuscript accepted on May 7, 2012.

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The online version of this article has a Supplementary Appendix.

Introduction

Donor lymphocyte infusions (DLI) can induce a graft-*versus*-tumor (GVT) effect in patients with a relapse of hematologic malignancies after allogeneic hematopoietic stem cell transplantation (HSCT). The anti-tumor effect of donor T cells was initially suggested by the increased risk of leukemia relapse observed in patients who received a T-cell-depleted allogeneic stem cell transplant.[1] During the 1980s, the immunotherapeutic properties of donor T cells were more directly illustrated by the successful application of DLI for the treatment of relapse following allogeneic HSCT, notably for patients with chronic myelogeneous leukemia.[2] Both allogeneic HSCT and DLI are also associated with a risk of graft-*versus*-host disease (GVHD), a major cause of treatment-related morbidity and mortality.[3,4]

Unfortunately, some grafted patients never display any signs of alloreactivity, either following allogeneic HSCT or after DLI, despite incompatibility in human leukocyte antigens (HLA) and minor histocompatibility antigens.[5] They do not develop GVHD and frequently relapse after allogeneic HSCT and even after one or several DLI. This may be due to a poorly immunogenic donor/recipient HLA combination and/or to mechanisms of immunomodulation rendering donor T cells incapable of mediating a marked GVH/GVT effect. Natural immunoregulatory $CD4^+CD25^+Foxp3^+$ T cells (Treg) are a likely candidate for such immunomodulation resulting in an abrogated anti-tumor effect.[6] We and others have previously described that Treg have a key role in the control of alloreactive responses in experimental allogeneic HSCT. Indeed, Treg depletion from the transplant before infusion led to significantly accelerated GVHD in mice.[7-9] In humans, a low content of Treg in the graft or in recipients was associated with GVHD and GVT effects after allogeneic HSCT[10] or after DLI.[11] These studies supported the hypothesis that eliminating Treg from the DLI product could improve alloreactivity and consequently the associated GVT effect in patients with relapsed disease. We recently validated this approach in a clinical trial and observed that Treg depletion from DLI through $CD25^+$ cell depletion could set off clinical GVHD associated with partial or complete remission of the malignant disease in patients previously unresponsive to DLI. However, this treatment was effective in only a small proportion of patients.[12]

We hypothesized that the low proportion of patients responding may be due to the method of Treg depletion relying on CD25 negative selection. Since conventional non-Treg $CD4^+$ T cells also transiently express CD25 after activation, the elimination of $CD25^+$ cells from the DLI may also deplete these transiently-activated effector or memory T cells, potentially prone to mediate alloreactive responses and the GVT effect.[13]

Expression of CD127 (the interleukin-7 receptor α chain) is commonly associated with central and effector memory functions in both CD4 and CD8 peripheral T cells.[14] Interestingly, it has also been observed that most $Foxp3^+$ Treg have weak or absent surface expression of CD127.[15,16] Thus, positive selection of $CD127^+$ cells instead of elimination of $CD25^+$ ones could lead to efficient Treg depletion while maintaining a fraction of $CD127^+FOXP3^-CD25^+$ T cells with putative strong immune responses. Based on this hypothesis, we directly compared these two strategies for DLI Treg depletion using good manufacturing practice-compatible procedures.

Design and Methods

Mice

Ten- to 14-week old NOD/SCID/$\gamma C^{-/-}$ (NSG) mice were bred in our animal facility under specific pathogen-free conditions. Experiments were performed according to European Union guidelines and approved by our institutional review board (CREEA Ile de France n. 3).

Depletion of regulatory T-cell fractions

Cells were obtained from leuko-apheresis samples freshly collected from healthy donors by the *Etablissement Français du Sang*, after written, signed informed consent had been obtained. The study was evaluated and approved by the French National Cancer Institute (INCA) and the French National Institute for Health and Research in Medicine (Inserm) in March 2008.

Peripheral blood mononuclear cells (PBMC) were separated on a Ficoll-Hypaque gradient. Treg were depleted following two strategies: (i) $CD25^+$ cells were depleted from PBMC using CD25 microbeads II (20 μL per 10^7 cells), an LD column and a MidiMACS separator (Miltenyi Biotec, Germany), and (ii) PBMC were first stained with a biotin-coupled anti-CD127 monoclonal antibody (0.5 μL per 10^7 cells), (eBioRDR5, eBioscience, San Diego, CA, USA) and then positively selected using anti-biotin microbeads (4 μL per 10^7 cells), MS columns, and a MiniMACS separator (Miltenyi Biotech). Treg depletion and cell contents were evaluated by flow cytometry.

Antibodies and flow cytometry analysis

The following conjugated monoclonal antibodies were used for cell surface staining: anti-CD3 (UCHT1)-ECD, CD3 (UCHT1)-APC, CD4 (SFCI12T4D11)-PC7, CD4 (SFCI12T4D11)-ECD, CD8 (B9.11)-PE, CD45-PC7 (J33), CD45RA (2H4LDH11LDB9)-FITC (purchased from Beckman Coulter, Villepinte, France), CD25 (M-A251)-PE (BD Biosciences, Le Pont De Claix, France), and CD127 (eBioRDR5)-FITC (eBioscience). Intranuclear FOXP3 labeling was performed after CD3, CD4, CD127 and CD25 membrane staining using an APC anti-human Foxp3 kit (PCH101 clone, eBiosciences) according to the manufacturer's instructions. Rat IGg2a APC was used as an isotypic control (eBiosciences). Cells were acquired and analyzed by flow cytometry using a FC500 Cytometer (Beckman Coulter). The instrument setting parameters (gains, compensations, and threshold) were set with the machine's software (CXP Software; Beckman Coulter) in conjunction with calibration beads (Flow-set beads, Cytocomp kit, and CYTO-TROL Control Cells). The instrument's reproducibility was verified with standardized beads (Flow-check). Data were analyzed with CXP analysis software (Beckman Coulter).

In vitro functional assay

Tests were performed at 37°C in a 5% CO_2 atmosphere in 96-well round-bottomed plates in RPMI 1640 medium supplemented with 10% AB-human-serum, 1% L-glutamine, 1% penicillin and 1% streptomycin (RPMI complete) in a final volume of 200 μL. Responding cells were tested for their capacity to respond to allogeneic PBMC or autologous PBMC pulsed with specific antigens: tuberculin protein: purified protein derivative, tetanus toxin and candidine. For specific antigen sensitization, autologous PBMC were pulsed for 2 h at 1×10^6 cells/mL in RPMI complete with 20 μg/mL of purified protein derivative, 10 μg/mL of tetanus toxin, or 10 μg/mL of candidine. The numbers of responding cells were normalized to the percentage of $CD3^+$ cells determined by flow cytometry in order to stimulate, in each condition, the same number of $CD3^+$ cells (25×10^3) with 25×10^3 irradiated (15 Gy) allogeneic or antigen-pulsed autologous PBMC as the stimulating cells.

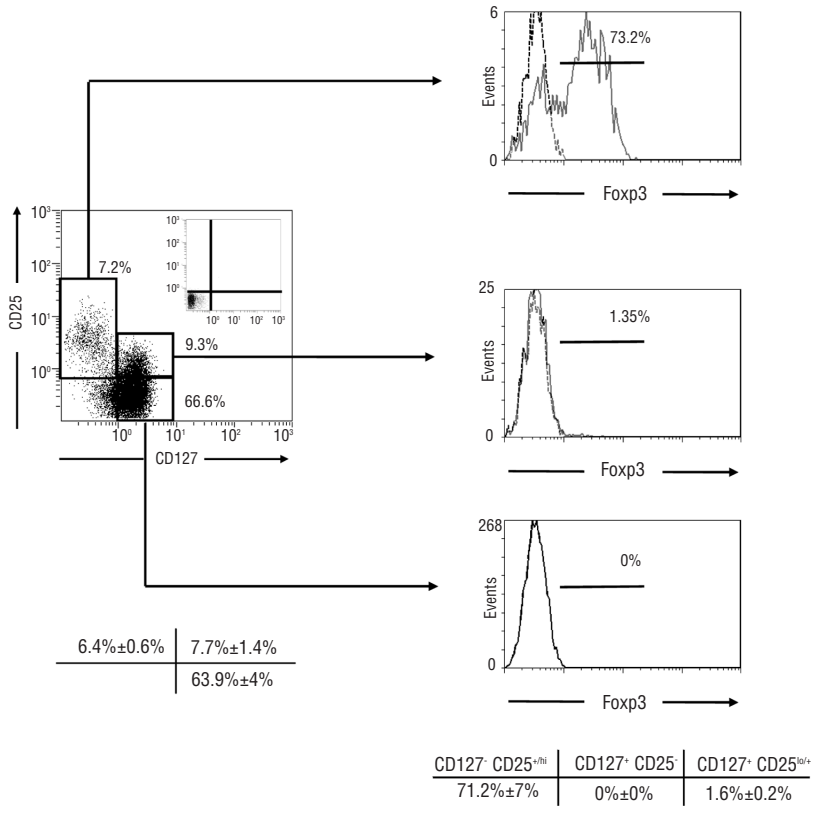

Figure 1. Foxp3 expression in the different subsets of CD3+CD4+ T cells. Freshly isolated PBMC were gated into CD3+CD4+CD25highCD127-, CD3+CD4+CD25lowCD127+ and CD3+CD4+CD25-CD127+ cells and the percentages of cells in each gate is shown together with expression of Foxp3. Isotype controls for CD25 and CD127 staining are shown within the dot blot (top right). The histogram illustrates Foxp3 expression (full line) with isotype control (dashed line) in the corresponding gate. The tables under the figure show mean percentages ± SEM of 14 independent experiments.

Experiments were performed in triplicate for each culture condition. After 5 days, cell proliferation was determined by incorporation of 1 μCi (0.037 MBq) of ^{3}H-thymidine (Amersham, Buckinghamshire, UK) for an additional 16 to 18 h and measured using a β-counter (counter-WALLAC).

In vivo *functional assay*

Treg-depleted cell populations were also tested *in vivo* for their capacity to divide and to induce xenogeneic GVHD in immunodeficient NSG mice. For each experiment, CD25^{-} or CD127^{+} CD3^{+} T cells were compared to the same number of CD3^{+} cells from PBMC of the same donor. Thus, 8×10^{6} CD3^{+} cells were labeled with CFSE and injected intravenously into the animals (into the retro-orbital sinus). At day 7, the mice were sacrificed and their spleens were harvested. Splenocytes were labeled with an anti-human CD45 monoclonal antibody to first detect human cells. The CFSE dilution was then analyzed by flow cytometry, focusing on three different cell populations: CFSE^{high} cells that did not divide, CFSE intermediate (CFSE^{int}) that had undergone one to six divisions and CFSE^{neg} that had undergone more than six cell divisions.

The same protocol was applied, without CFSE staining, for the xenogeneic GVHD. Progression of the disease was monitored daily through several parameters: weight loss, hunching, ruffled fur, skin lesions and diarrhea. Animals were sacrificed when they developed strong clinical signs of GVHD, such as severe weight loss (more than 30%), hunched posture and reduced mobility associated with severe diarrhea.

Statistical analysis

Data are expressed as mean ± standard error of mean. The statistical significance was calculated using the two-tailed paired Student's t-test and the log-rank test for the Kaplan-Meier survival curve. A *P* value of less than 0.05 was considered statistically significant. The degree of statistical significance is indicated as follows: (*) $P<0.05$, (**) $P<0.01$, and (***) $P<0.001$.

Results

CD127^{+} positive selection efficiently depletes T regulatory cells while maintaining a subpopulation of CD25^{+}CD45RA^{-}FOXP3^{-} activated/memory T cells

We measured the expression of CD25, CD127 and FoxP3 on PBMC from healthy volunteer donors. Confirming previously published results,[15,16] we observed that 71.2%±7.0 of CD3^{+}CD4^{+}CD25^{high}CD127^{-} T cells expressed a high level of Foxp3, whereas Foxp3 was virtually absent in the CD25^{low}CD127^{+} cells and in CD25^{-} CD127^{+} cells. Thus, among the CD3^{+}CD4^{+} T cells, three subpopulations could be clearly identified based on CD25 and CD127 expression: (i) CD25^{high}CD127$^{low/neg}$ cells that contain most of the Foxp3^{+} Treg, and (ii) CD25^{-}CD127^{+} cells, and (iii) CD25^{low}CD127^{+} cells that do not include Foxp3^{+} cells (Figure 1). Our goal was to preserve the subpopulation of CD127^{+}CD25^{low} cells (less than 8%) after Treg depletion, which potentially contains conventional T cells with a memory/activated phenotype.

When we compared Treg depletion by CD25 negative *versus* CD127 positive selection on total PBMC, we observed effective Treg depletion with both strategies, as illustrated in *Online Supplementary Figure S1*. CD25-depleted and CD127-positively selected fractions contained 0.5%±0.1 and 0.8%±0.1 Foxp3^{+} Treg among CD3^{+}CD4^{+}, respectively, compared with 5.1%±0.4 in unmanipulated PBMC ($P<0.001$ for both types of Treg depletion, Figure 2A, left panel). Comparing the two strategies, Treg depletion was better with CD25 negative selection than with CD127 positive selection ($P<0.05$).

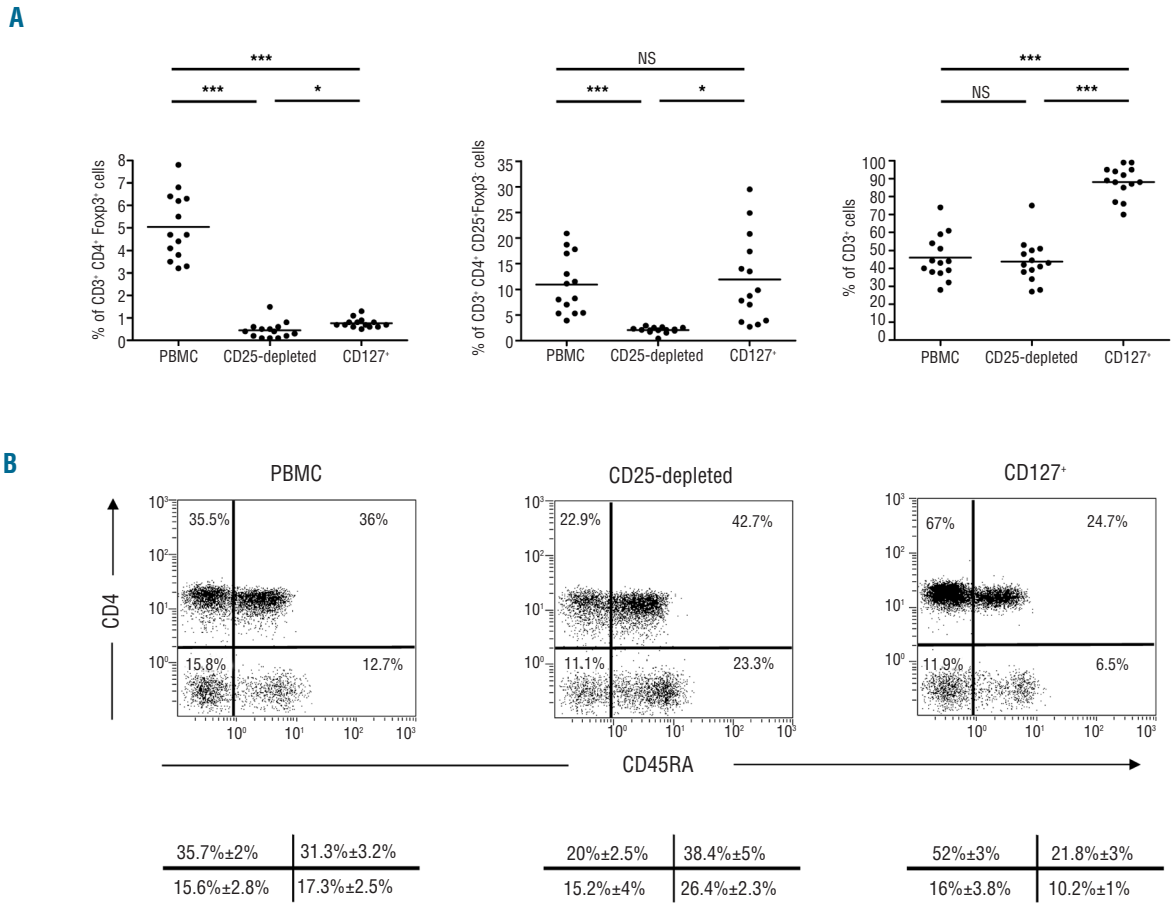

Figure 2. Phenotypic characterization of cells following Treg depletion. (A) Scatter plots show the percentage of Foxp3$^+$ (left) and CD25$^+$ Foxp3$^-$ cells (middle) in CD3$^+$ CD4$^+$ gated non-manipulated PBMC and Treg depleted cells. Right scatter plot shows the percentage of CD3$^+$ T cells in different cell populations. Horizontal bars represent the mean of 14 independent experiments for each group. (B) The naïve/memory CD45RA phenotype is shown in CD3$^+$ gated PBMC and Treg depleted cells. The numbers in each quadrant indicate the percentage of the representative experiment. The tables under the figure show mean percentages ± SEM of five independent experiments.

Importantly, CD127 selection did not alter the content of CD25$^+$Foxp3$^-$ non-Treg cells, as illustrated in *Online Supplementary Figure S1* since we observed similar proportions of CD3$^+$CD4$^+$CD25$^+$Foxp3$^-$ in PBMC and CD127-positively selected cells (10.9%±1.5 and 11.9%±2.3, respectively). In contrast, this population was dramatically reduced (2.1%±0.2) following CD25 Treg depletion (Figure 2A, middle panel). Of note, the CD127 method significantly enriched the product in CD3$^+$ T cells (88.2%±2.3) when compared with PBMC and CD25-depleted cells (43.1%±3.3 and 43.8%±3.2, respectively, Figure 2A, right panel, P<0.001 and *Online Supplementary Figure S2*).

We also studied the naïve/memory phenotype of CD3-gated T cells by analyzing CD45RA expression before and after Treg depletion (Figure 2B). First, neither CD25 negative selection nor CD127 positive selection significantly altered the relative proportions of CD3$^+$CD4$^+$ and CD3$^+$CD4$^-$ cells within each fraction (*data not shown*). Second, when compared to unmanipulated PBMC, we observed that CD127 positive selection led to an enrichment in CD45RA$^-$ memory cells in both the CD3$^+$CD4$^+$ and the CD3$^+$CD4$^-$ T-cell compartments (70.6%±4.0 and 61.6%±8.0 *versus* 53.5%±2.0 and 48.3%±5.1, respectively, P<0.01 for both). An opposite effect was observed after CD25 depletion, when an increase in CD45RA$^+$ naïve cells was observed as compared to unmanipulated PBMC among CD3$^+$CD4$^+$ and CD3$^+$CD4$^-$ cells (65.9%±4.0 and 60.6%±6.0 *versus* 46.5%±2.0 and 51.7%±5.1, respectively, P<0.01 for both). Additionally, we found no impact of either CD25 or CD127 selection on the repartition between CD45RA$^-$CCR7$^+$ central memory and CD45RA$^-$CCR7$^-$ effector memory cells among CD3$^+$CD4$^+$ and CD3$^+$CD4$^-$ cells (*data not shown*).

Thus, CD127 positive selection efficiently eliminates Treg cells while enriching the cell product in a subpopulation of CD4$^+$ and CD8$^+$ T cells with an activated/memory phenotype.

Elimination of T regulatory cells through CD127 positive selection improves both alloreactive and pathogen-specific T-cell responses in vitro

We next tested whether Treg depletion increases allogeneic immune responses *in vitro*. We cultured an adjusted number of 25×10^3 CD3$^+$ cells collected from PBMC or Treg-depleted preparations in the presence of allogeneic irradiated stimulating PBMC obtained from a pool of donors. CD25 depletion had no effect on allogeneic

immune responses. In contrast, we observed a dramatically improved T-cell response for CD127+ selected cells after allogeneic stimulation with a nearly 6-fold greater proliferation index as compared to PBMC and CD25 depleted cells ($P<0.05$ for both). We subsequently tested the capacity of Treg-depleted T cells to proliferate when stimulated *in vitro* in the presence of tetanus toxin, tuberculin and candidine proteins for 6 days. This combination of antigens enables the evaluation of an immune response against three types of pathogens derived from a bacterium, a mycobacterium and a fungus. Here again, compared with PBMC, CD25 depletion had no effect on immune responses. In contrast, the proliferation index of CD127 positively selected cells was significantly higher than that of PBMC after stimulation with candidine and tetanus toxin, whereas the difference did not reach statistical significance after stimulation with tuberculin (Figure 3). Of note, Treg depletion via CD25 or CD127 selection did not induce

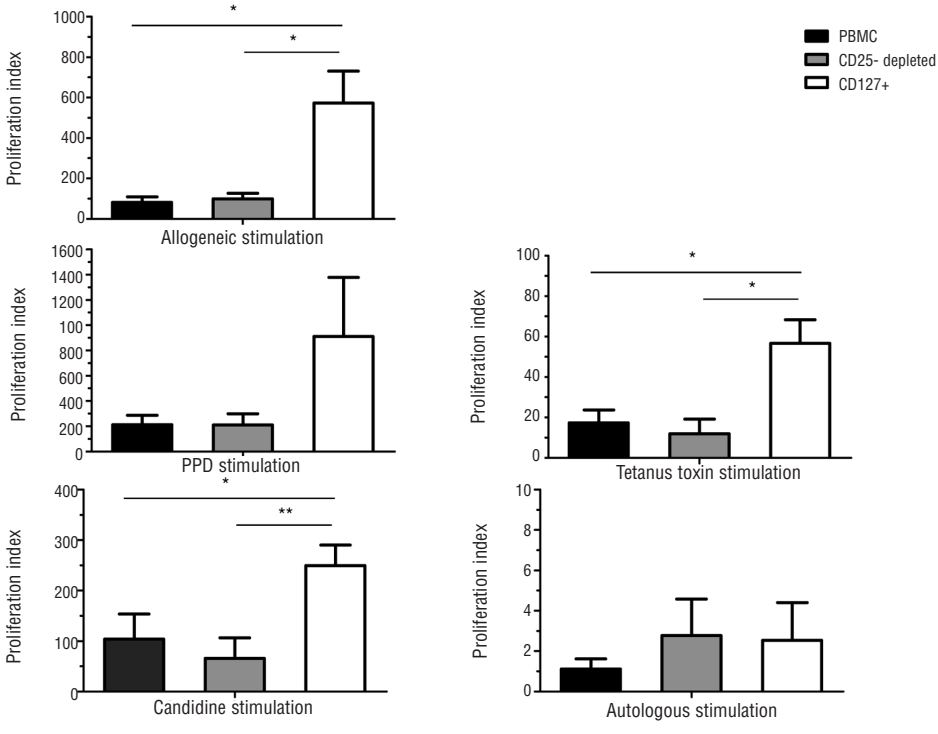

Figure 3. ***In vitro*** **functional analysis of cells following Treg depletion. PBMC and Treg-depleted cell populations were tested for their capacity to respond to allogeneic, pathogen-specific or autologous stimulation after 6 days of culture. Histograms represent the proliferation index calculated as proliferation (cpm) of stimulated cells divided by proliferation of non-stimulated cells. Data represent mean ± SEM of four [allogeneic and purified protein derivative (PPD) stimulation] and five [tetanus toxoid (TT) and candidine] independent experiments.**

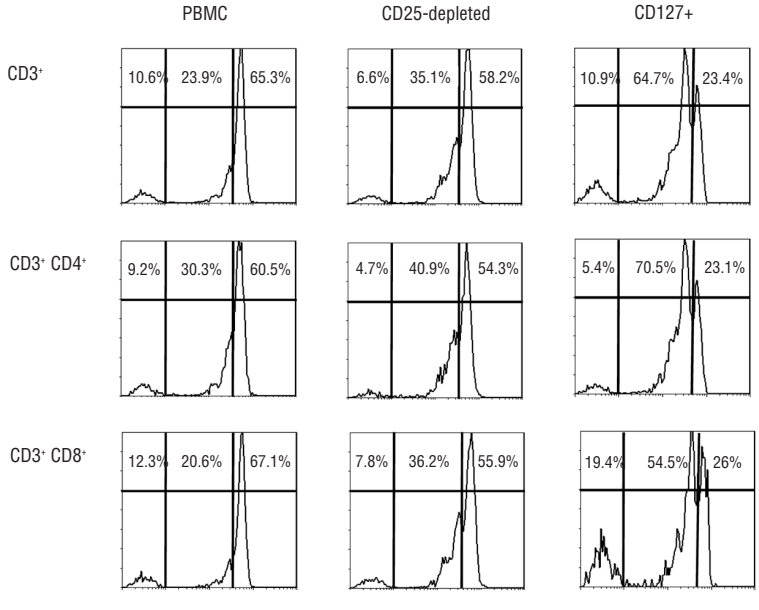

Figure 4. ***In vivo*** **functional analysis of cells following Treg depletion. NOD/SCID/$\gamma c^{-/-}$ mice were grafted with CFSE-stained PBMC or Treg depleted cells. At day 7, division profiles of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ in $CD45^+$ gated collected splenocytes were determined by CFSE dilution. The numbers in quadrants indicate the percentage of $CFSE^{high}$ cells that did not divide, CFSE intermediate ($CFSE^{int}$) that had undergone one to six divisions and $CFSE^{neg}$ that had undergone more than six cell divisions in the representative experiment. The table under the figure shows mean percentages ± SEM of three independent experiments. Each experiment involved two mice per group.**

	PBMC			CD25-depleted			CD127+		
	$CFSE^-$	$CFSE^{low}$	$CFSE^+$	$CFSE^-$	$CFSE^{low}$	$CFSE^+$	$CFSE^-$	$CFSE^{low}$	$CFSE^+$
% of $CD3^+$	8.4 ± 4.1	29.7 ± 7.6	62.3 ± 3.8	6.8 ± 0.1	30.8 ± 5.2	61.8 ± 5	7.1 ± 3.8	69.8 ± 4.7	22.2 ± 0.8
% of $CD3^+$ $CD4^+$	7.0 ± 2.2	39.4 ± 9.1	52.9 ± 9.4	4.5 ± 0.1	34.1 ± 7.7	61.2 ± 7.6	3.5 ± 4.8	74.6 ± 3.2	22 ± 1.4
% of $CD3^+$ $CD8^+$	7.6 ± 3.1	29.4 ± 6.8	62.4 ± 3.7	8 ± 0.2	33 ± 4.9	57.4 ± 4.9	12.8 ± 6.7	62.5 ± 6.5	24.9 ± 0.6

autoreactivity, as attested by proliferation indices after autologous stimulation. Similar experiments were performed with read outs at days 3, 4, 5 and 6 to ensure that the observed differences were not due to differences in the kinetics of the immune response. We consistently observed a superior immune response for the CD127 selected T cells in all the experimental conditions, and independently of the duration of the stimulation (*Online Supplementary Figure S3*). Thus, Treg depletion through CD127 positive selection not only increases allogeneic immune responses but also reinforces the capacity of sorted cells to respond to different recall antigens *in vitro*, in accordance with the enrichment observed in memory-phenotype T cells in the CD127 positively selected fraction.

We further aimed to test whether the preservation of $CD127^{+}CD25^{low}$ cells obtained through the CD127 but not the CD25 selection process was critical for mediating immune responses. By directly comparing functional responses of FACS-sorted $CD25^{low}CD127^{+}$ and $CD25^{-}CD127^{+}$ cells, we observed that the former population led to an increased response, although not statistically significant, to both allogeneic and pathogenic antigens (*Online Supplementary Figure S4*).

Elimination of T regulatory cells through CD127 positive selection induces increased T-cell division in vivo *and exacerbates graft-*versus*-host disease in a model of xenogeneic transplantation*

To validate our approach *in vivo*, we developed a model of xenogeneic GVHD by infusing human T cells into NSG mice. This model not only leads to clinical signs of xenogeneic GVHD but also enables a comparison of the kinetics of T-cell division after adoptive transfer of CFSE-stained cell preparations. Thus, NSG mice were grafted with human PBMC, CD25-depleted or CD127-positively selected cells after *ex vivo* CFSE staining.

First, we analyzed the division of $CD3^{+}$, $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T cells by CFSE dilution. At day 7 after adoptive transfer, the percentage of undivided ($CFSE^{high}$) $CD3^{+}$ cells only decreased in mice grafted with CD127-positively selected cells, as compared to the percentages in mice grafted with PBMC or CD25-depleted cells (22.2%±0.8 *versus* 62.3%±3.8 and 61.8%±5.0, respectively). Consequently, we also observed an increased proportion of $CFSE^{int}$ (1 to 6 divisions) in these mice grafted with $CD127^{+}$ cells compared to in mice grafted with PBMC or CD25-depleted cells (69.8%±4.7 *versus* 29.7%±7.6 and 30.8%±5.2, respectively). Similar observations were made regarding the $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ cell populations, with a more pronounced effect for the latter (Figure 4). We, therefore, witnessed accelerated T-cell division upon xenogeneic stimulation in mice receiving CD127 positively selected cells as compared to the rate in mice grafted with PBMC or CD25-depleted cells. This behavior of donor T cells *in vivo* could also be due to homeostatic proliferation rather than to alloreactivity. However, this is not in accordance with long-term experiments of xenogeneic GVHD. Indeed, to study the *in vivo* relevance of these two methods of cell manipulation further, we examined whether CD127 positively selected cells increase or accelerate xenogeneic GVHD manifestations from day 0 to

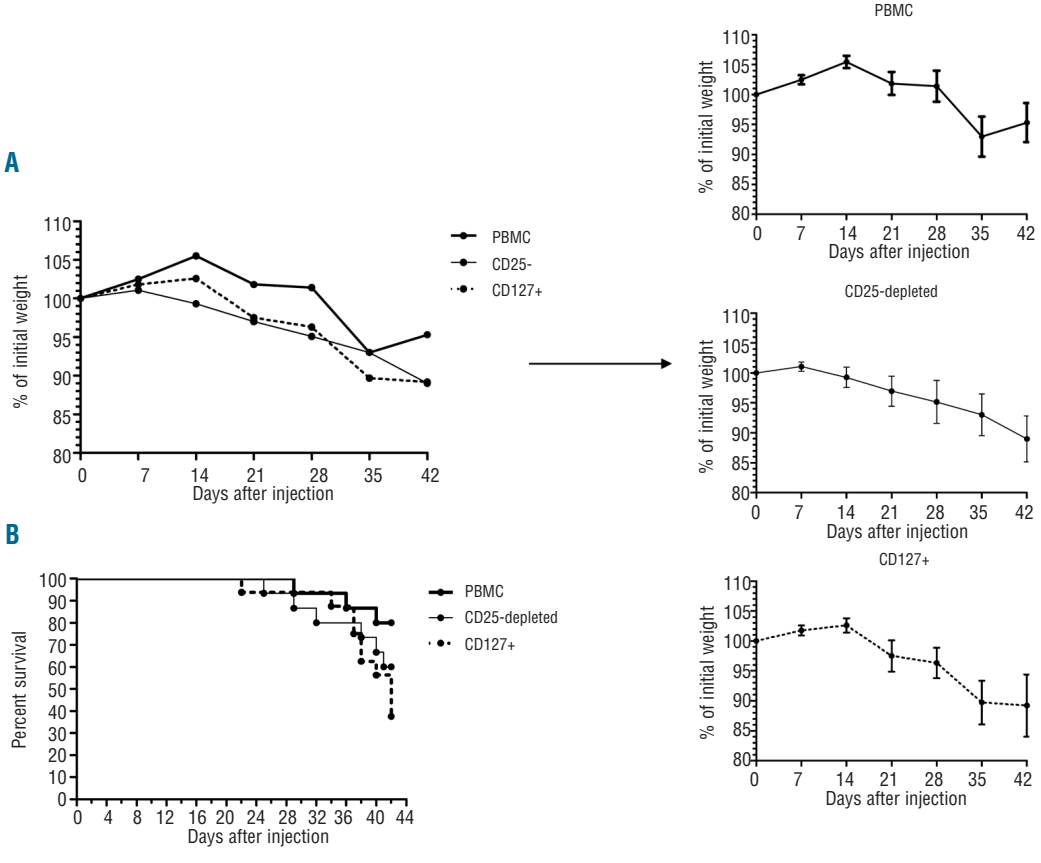

Figure 5. Effect of Treg elimination on GVHD in a model of xenogeneic transplantion. Xenogeneic GVHD was induced by the adoptive transfer of human cells into NOD/SCID/$\gamma C^{-/-}$ mice. Mice received PBMC (thick line), CD25-depleted PBMC (thin line) or CD127-selected PBMC (dashed line). (A) Progression of the disease was determining by monitoring the percentage of weight loss. (B) Kaplan-Meier survival curve for mice grafted with PBMC (n=15), CD25-depleted PBMC (n=15) or CD127-positively selected cells (n=16) pooled from five independent experiments.

week 6 after cell transfer. In these experiments, NSG mice were grafted without any conditioning regimen with 8×10^6 $CD3^+$ T cells from PBMC or from Treg-depleted cell preparations. Infusion of CD25-depleted or CD127 positively selected cells resulted in accentuated clinical GVHD manifested by an accelerated weight loss compared with that of mice grafted with unmanipulated cells (Figure 5A, $P<0.01$ $CD127^+$/PBMC and $P<0.05$ $CD25^-$/PBMC). The Kaplan-Meier survival curves from day of transplantation to day 42 also revealed differences between mice grafted with PBMC and Treg-depleted cells, with the proportion of mice that died being higher in the group grafted with Treg-depleted cells (Figure 5, right panel). Of note, only mice grafted with CD127-positively selected cells showed a statistically significant increase in mortality compared to the group given an unmanipulated graft (63% and 20% mortality at day 42, respectively, $P<0.05$).

Discussion

Treg depletion represents a promising therapeutic approach in the arsenal of anti-tumor strategies. In the field of allogeneic HSCT, we recently provided the proof of principle that Treg elimination prior to DLI could indeed set off GVHD in previously allo-resistant patients and consequently could improve the GVT effect.[12] We hypothesized that eliminating Treg through CD25 negative selection could also remove a fraction of effector T cells with a strong alloreactive and anti-tumor potential. Consequently, we developed a new Treg depletion strategy that preserves the $CD25^+$ non-Treg cell population to improve GVHD and the associated GVT effect. Notably, we tested the use of CD127 to discriminate between activated T cells and Treg, both of which express CD25. When comparing CD25 negative to CD127 positive selection, we observed that both strategies efficiently eliminated Treg, although the result was better with the CD25-based method. However, this advantage was offset by a consistently superior activity in mixed lymphocyte reactions, in pathogen-specific immune responses and in xenogeneic GVHD using immunodeficient mice, an appropriate model that enables evaluation of *in vivo* immunomodulation strategies with human cells before applying them to clinical use.[17,18] Collectively, these results suggest that despite better Treg elimination with the CD25 approach, CD127 positive selection produces a cellular product that is more prone to an immune response, and specifically an alloreactive response.

CD127 positive selection radically modifies the cell content of the DLI compared with unmanipulated PBMC or CD25-depleted PBMC. Whereas T cells represented 40-50% of unmanipulated PBMC, they constituted virtually 90% after CD127 positive selection. In the setting of DLI, both GVHD and the GVT effect are mostly supported by donor T cells. Consequently, elimination of monocytes, NK or B cells from the DLI by a CD127-based strategy is not likely to affect the recipient's outcome negatively. Conversely, it may actually improve the efficacy of DLI, as NK cells, for example, may inhibit activated alloreactive T cells.[19]

It is also assumed that CD127 is an excellent marker of memory T cells linked to the fact that induction and maintenance of CD4 and CD8 memory cells require IL-7.[14] We did, in fact, observe a higher level of CD127 expression on memory T cells than on naïve T cells (*data not shown*). Consequently, our procedure of Treg elimination through CD127 positive selection led to an enrichment of both $CD4^+$ and $CD8^+$ $CD45RA^-$ memory-phenotype T cells. In this respect, it is important to note that several studies have shown in mice that both $CD4^+$ and $CD8^+$ memory cells can support a strong GVT effect, which is compatible with our approach.[20-22] More surprising is our observation of accelerated GVHD after infusion of CD127 positive cells, which is in sharp contradiction with the findings of several previous studies.[21-25] This improved capacity of CD127-positive cell preparations to induce GVHD could be due to: (i) efficient Treg depletion that could modify the equilibrium in the balance between response or absence of immune response, (ii) the remaining proportion of naïve $CD45RA^+$ cells (more than 20%) after CD127-based Treg depletion which could be sufficient to set off GVHD in a context in which Treg are absent, and (iii) the model of xenogeneic GVHD which could differ from classical allogeneic GVHD with regards to its mechanism of initiation and expansion. Importantly, since recipient mice were profoundly lymphopenic in this model, a mechanism of lymphopenic-induced proliferation might be implicated in T-cell activation and the induction of GVHD. Since lymphopenic-induced proliferation depends strongly on the interaction between IL7 and CD127,[26] this might have an impact on the *in vivo* activation of CD127-positive selected cells. However, our *in vitro* experiments also demonstrated an improved alloreactivity in mixed lymphocyte reactions, which supports the first two hypotheses.

Natural Treg form a powerful immunomodulatory mechanism. Accurate manipulation of these cells, specifically in the context of hematopoietic transplantation and alloreactivity, is emerging as a formidable therapeutic avenue. Nevertheless, it is heavily dependent on effective dissection of the T-cell compartment. CD25-based Treg depletion is an important example in this respect. The accompanying depletion of effector T cells may result in a truncated therapeutic effect. It is, therefore, crucial to assess the capacity of Treg-depleted products to respond to immune challenges. Our results show that a CD127 positive selection strategy is a very efficient method for eliminating Treg from DLI and for improving alloreactivity and, consequently, the GVT effect *in vivo*. It may also improve immune responses to pathogens. These results provide strong support for the clinical development of this strategy. Importantly, due to the risk of inducing severe GVHD in this setting, we plan to test this approach primarily in patients who are refractory to alloreactivity, i.e. who have never developed GVHD manifestations after HSCT and previous unmanipulated DLI.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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