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Alemtuzumab as Antirejection Therapy: T Cell Repopulation and Cytokine Responsiveness

Anne P. Bouvy, MD,[1] Mariska Klepper, BSc,[1] Michiel G.H. Betjes, MD, PhD,[1] Willem Weimar, MD, PhD,[1] Dennis A. Hesselink, MD, PhD,[1] and Carla C. Baan, PhD[1]

Background. Alemtuzumab induction therapy in kidney transplant patients results in T cell depletion followed by slow immune reconstitution of memory T cells with reduced immune functions. The kinetics and functional characteristics of T cell reconstitution when alemtuzumab is given during immune activation, ie, as antirejection therapy, are unknown. **Methods.** Patients (n = 12) with glucocorticoid-resistant or severe vascular kidney transplant rejection were treated with alemtuzumab. Flow cytometric analysis was performed on whole blood to measure cell division by the marker Ki-67, and cytokine responsiveness by IL-2–mediated and IL-7–mediated phosphorylation of signal transducer and activator of transcription 5 of T cells before and during the first year after rejection therapy. **Results.** At 1 year after alemtuzumab antirejection therapy, the total T cell population recovered to baseline level. Repopulation of CD4+ and CD8+ T cells was associated with increased percentages of Ki-67+ proliferating T cells ($P < 0.05$). In addition, both populations showed a phenotypic shift toward relatively more memory T cells ($P < 0.01$). At the functional level, IL-7 reactivity of CD4+ memory T cells was diminished, reflected by a decreased capacity to phosphorylate signal transducer and activator of transcription 5 during the first 6 months after alemtuzumab treatment ($P < 0.05$), whereas reactivity to IL-2 was preserved. CD8+ T cells were affected in terms of both IL-2 and IL-7 responses (both $P < 0.05$). After reconstitution, relatively more regulatory T cells were present, and a relatively high proportion of Ki-67+ T cells was observed. **Conclusions.** Preliminary data from this small series suggest that alemtuzumab antirejection therapy induces homeostatic proliferation of memory and regulatory T cells with diminished responsiveness to the homeostatic cytokine IL-7. IL-2 responsiveness was affected in repopulated CD8+ T cells.

(*Transplantation Direct* 2016;2: e83; doi: 10.1097/TXD.0000000000000595. Published online 25 May 2016.)

T cell depleting antibody therapy is the treatment of choice for severe or glucocorticoid-resistant kidney transplant rejection.[1] The most commonly used T cell depleting agent is rabbit antithymocyte globulin (rATG), but in recent years, the use of alemtuzumab to treat rejection has gained popularity.[2-6]

Alemtuzumab (Campath-1H) is a humanized monoclonal antibody directed against the cell surface antigen CD52, which is expressed not only by T cells but also by B cells, NK cells, monocytes, macrophages, and dendritic cells. Ligation of alemtuzumab with CD52 induces apoptosis and lysis of immune cells through antibody- and complement-dependent cytotoxicity, which leads to profound and long-lasting lymphocyte depletion. Studies in kidney transplant patients given alemtuzumab as induction therapy have shown that low T cell numbers persisted for more than 1 year and that CD8+ T cells reach baseline levels earlier than CD4+ T cells.[7]

After T cell depletion therapy, T cell repopulation results from 2 processes: (i) thymopoiesis, the formation of new, naive T cells called recent thymic emigrants and (ii) homeostatic proliferation, the expansion of residual naive but mainly memory T cells. Naive recent thymic emigrant can be identified by the expression of CD31, which is lost on antigen binding and proliferation of the naive cell.[8,9] Homeostatic proliferation of both naive and memory cells is the result of antigen binding to the T cell receptor and/or binding of the signal transducer and activator of transcription (STAT5) activating cytokines IL-7 and IL-15 to their cytokine receptor.[9-13] As thymopoiesis decreases with age, homeostatic proliferation is the main contributor to T cell reconstitution in T cell–depleted adults. Furthermore, memory cells are relatively

Received 4 February 2016. Revision requested 24 March 2016.

Accepted 10 April 2016.

[1] *Department of Internal Medicine, Nephrology and Transplantation, Erasmus MC, University Medical Center, Rotterdam, the Netherlands.*

The authors declare no funding or conflicts of interest.

A.P.B. participated in research design, performance of the research, data analysis and writing of the article. M.K. participated in performance of the research and data analysis. M.G.H.B. participated in writing of the article. W.W. participated in research design and writing of the article. D.A.H. participated in research design and writing of the article. C.C.B. participated in research design and writing of the article.

Correspondence: Anne P. Bouvy, MD, Section Transplantation and Nephrology, Department of Internal Medicine, Erasmus Medical Center, Room Nc-523, PO Box 2040, 3000 CA Rotterdam, the Netherlands. (a.bouvy@erasmusmc.nl).

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantationdirect.com).

ISSN: 2373-8731/16/0000-00

DOI: 10.1097/TXD.0000000000000595

resistant to depletion and proliferating naive cells can also adapt a memory phenotype, resulting in a T cell pool which mainly comprises memory T cells after T cell depletion therapy.[6,14-17] In addition to higher numbers of memory cells, higher percentages of regulatory T (Treg) cells have also been found after T cell depletion therapy.[18-20] Homeostatic proliferation, in an activated immune environment, that is, high level IL-2 might play a role in the induction of Treg cells.[19,21]

Memory T cells can rapidly and vigorously respond to donor antigen, a response difficult to inhibit by immunosuppressive drugs. Therefore, memory cells are thought to endanger transplant survival.[22,23] However, several studies reported that patients treated with T cell depletion therapy can be treated with reduced doses of maintenance immunosuppression, suggesting reduced immune functions of the repopulated T cells.[24-28] In vitro, this impaired T cell function is reflected by hampered T cell responses to donor, third-party and recall antigens.[7,16,20,29] Furthermore, after T cell depletion, T cells showed diminished homeostatic proliferation despite incomplete T cell reconstitution, and the phosphorylation capacity of STAT5 of recovered cells in response to IL-2 and IL-7 is affected.[9,30] These recovered T cells also have increased expression of coinhibitory molecules.[30] Impaired STAT signaling as well as increased expression of coinhibitory molecules are features of T cell exhaustion, a phenomenon induced by persistent antigen exposure resulting in dysfunctional T cells that is thought to contribute to donor hyporesponsiveness after kidney transplantation.[30,31]

During rejection, the immune system is highly activated, resulting in high concentrations of cytokines and antigen presentation, processes capable of influencing T cell reconstitution and function of the T cell pool by affecting the formation of Treg and the induction of T cell exhaustion. We therefore speculated that after alemtuzumab antirejection therapy, T cell reconstitution and T cell functions are altered and impaired due to the activated environment. In the present study, we monitored the kinetics of T cell repopulation and their cytokine responsiveness in kidney transplant patients before and after T cell depletion therapy during rejection.

MATERIALS AND METHODS

Patients

Blood samples were collected from 12 consecutive kidney transplant patients (Table 1) with biopsy-proven acute rejection (graded according to the Banff criteria[32]) which was resistant to glucocorticoid treatment (ie, 1000 mg of methylprednisolone intravenously for 3 consecutive days) or very severe. All patients were treated with alemtuzumab (Campath-1H; Sanofi, Paris, France) at a dose of 30 mg subcutaneously on 2 consecutive days. Blood samples were drawn before alemtuzumab treatment was initiated (after glucocorticoid treatment), and again at week 1 and months 1, 3, 6, and 12 after alemtuzumab therapy. The maintenance immunosuppressive regimen after alemtuzumab treatment was left to the discretion of the attending physician but typically consisted of a calcineurin inhibitor and mycophenolate mofetil, with or without prednisolone (Table 2). After alemtuzumab treatment, patients received *Pneumocystis jirovecii* pneumonia and cytomegalovirus (CMV) prophylaxis when T cell numbers were below 200 cells/μL blood. Cytomegalovirus-PCR status was determined before transplantation, and subsequently if there was a clinical suspicion of CMV infection. The medical ethic committee of the Erasmus MC approved the study (MEC-2010-022).

Flow Cytometry

Absolute counts of CD4+ and CD8+ T cells were determined in freshly drawn ethylenediamine tetraacetic acid blood using BD MultiTest 6-color reagent and BD TruCount tubes (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. In brief, ethylenediamine tetraacetic acid blood (50 μL) was added to 20 μL MultiTest reagent in a BD TruCount tube containing a known number of fluorescent

TABLE 1.

Patient characteristics

Patient	Sex	Age at time of KT, y	Primary kidney disease	Preemptive	Transplant no.	Donor type	HLA mismatch A/B/DR	CMV D/R	CMV infection (<1 y after treatment)
1	Male	19	Alport syndrome	Yes	1	Living-related	0/1/1	neg/neg	No
2	Male	39	Focal segmental glomerulosclerosis	Yes	2	Living-unrelated	1/2/1	pos/neg	No
3	Female	72	Polycystic kidney disease	Yes	1	Living-unrelated	1/2/1	neg/pos	Yes
4	Male	38	Focal segmental glomerulosclerosis	No	2	Living-unrelated	1/2/1	pos/pos	Yes
5	Male	48	Polycystic kidney disease	No	2	Non-heart beating postmortal	1/2/0	pos/neg	No
6	Male	59	Polycystic kidney disease	Yes	1	Living-unrelated	2/2/0	neg/pos	No
7	Male	51	Reflux nephropathy/chronic pyelonephritis	No	1	Living-related	1/1/1	pos/neg	No
8	Male	62	Diabetic nephropathy	Yes	1	Living-unrelated	1/2/0	pos/neg	No
9	Female	40	Hypertensive nephropathy	No	3	Heart-beating postmortal	1/1/2	pos/pos	Yes
10	Male	14	Reflux nephropathy/chronic pyelonephritis	No	1	Heart-beating postmortal	1/2/0	neg/neg	No
11	Male	32	Hypertensive nephropathy	No	1	Heart-beating postmortal	1/2/2	neg/pos	Yes
12	Female	37	Reflux nephropathy/chronic pyelonephritis	Yes	1	Living-related	1/1/1	pos/neg	No

KT, kidney transplantation; HLA, mismatch at DR, 0, 1 or 2 mismatches on DR-locus; CMV D/R, cytomegalovirus donor-recipient combination; CMV infection within 1 year after alemtuzumab treatment; defined by a positive CMV polymerase chain reaction test.

TABLE 2.

Rejection therapy

Patient	Time of rejection episode, d	Methyl-prednisolone	IVIg	Immunosuppressive therapy at time of rejection						Time of graft loss, d
				Tac	CsA	MMF	Aza	Pred	ERL	
1	260	Yes	Yes	x	—	x	—	x	—	—
2	745	Yes	No	x	—	—	x	x	—	—
3	68	Yes	No	x	—	x	—	x	—	—
4	33	Yes	Yes	x	—	x	—	x	—	—
5	10	No	No	x	—	x	—	x	—	239
6	55	Yes	No	x	—	x	—	x	—	—
7	253	Yes	No	—	—	x	—	—	x	—
8	156	Yes	Yes	—	x	x	—	—	—	—
9	2	Yes	No	x	—	x	—	x	—	—
10	1740	Yes	Yes	x	—	x	—	—	—	189
11	514	Yes	Yes	x	—	x	—	x	—	250
12	104	Yes	No	x	—	x	—	x	—	—

Methylprednisolon; 1000 mg methylprednisolone for 3 days, IVIg, intravenous immunoglobulin; Tac, tacrolimus; CsA, cyclosporine A; MMF, mycophenolate mofetil (Cellcept); Aza, azathioprine; Pred, prednisolone; ERL, everolimus; time of graft loss, days after alemtuzumab therapy.

beads and incubated for 15 minutes in the dark at room temperature. After lysis with BD FACSLysing solution, samples were measured on a FACSCantoII flow cytometer and analyzed by FACSDiva software (BD Bioscience).

CD4+ and CD8+ T cell subsets were studied; naive (chemokine receptor (CCR)7+ CD45RO–), central memory (CM; CCR7+CD45RO+), effector memory (EM; CCR7–CD45RO+), terminally differentiated effector memory (EMRA; CCR7–CD45RO–) and Treg cell (CD4+CD25+CD127–) T cells. Phosphorylation (P) of STAT5 was determined by phosphospecific flow cytometry. In brief, heparinized blood, 200 μL, was stained using; CD3-AmCyan, CD8-fluorescein isothiocyanate (FITC), CCR7-phycoerythrin (PE)-Cy7, or with CD25-PE-Cy7 and CD127-FITC (all BD Bioscience). Samples were stimulated with 100 ng/mL IL-7 (PeproTech, London, UK), IL-2 (Proleukin 2000 IU/mL; Novartis Pharma GmbH, Nürnberg, Germany) or medium (unstimulated sample) for 30 minutes at 37°C. Next, cells were lysed and fixed using Lyse/Fix Buffer. For intracellular staining, cells were permeabilized with cold 70% methanol for 30 minutes at –20°C, washed in staining buffer with 0.5% bovine serum albumin (BSA), and stained with CD4-peridinin chlorophyll protein (PerCP) (BD Bioscience), CD45RO-Pacific Blue (Biolegend), and pSTAT5-PE (BD Bioscience). Staining with PerCP and Pacific Blue–conjugated antibodies was performed after permeabilization with 70% methanol because this affects their staining. Thymopoiesis and homeostatic proliferation were determined in the previously described naive and memory CD4+ and CD8+ T cells and in Treg cells which were here due to the staining protocol, characterized as CD4+CD127–FoxP3+ T cells (Treg cell). Heparinized blood (100 μL) was stained for 30 minutes with CD3-AmCyan (BD Bioscience), CD4-PacBlue (BD Bioscience), CD8-allophycocyanin (APC)-Cy7 (BD Bioscience), CD45RO-APC (BD Bioscience), CCR7-PE-Cy7 (BD Bioscience), and CD31-PE (Biolegend). Regulatory T cell samples were stained with CD3-AmCyan, CD4-APC-Cy7 (BD Bioscience), CD127-PE-Cy7 (BD Bioscience), CD45RO-PerCP-Cy5 (BD Bioscience), and CD31-PE. Hereafter, red blood cells were lysed using RBC lysis Buffer (BD Bioscience), washed with staining buffer with 0.5% BSA, and then incubated for 60 minutes at 4°C with fixation and permeabilization solution (BD Bioscience). After washing the cells with staining buffer with 0.5% BSA, cells were intracellular stained for 60 minutes in the dark using Ki-67-FITC (BD Bioscience) and FoxP3-APC (eBioscience).

Cells were measured and analyzed as described above. A minimum of 100 events per T cell subset was required for analysis. The effect of IL-2 and IL-7 on STAT5 phosphorylation was calculated by the percentage pSTAT5-positive cells after stimulation minus the unstimulated sample.

Statistical Analysis

Statistical analyses were performed using Graphpad Prism version 5.01. For comparisons between time points, the Wilcoxon signed rank test was used. Data are presented as median plus interquartile range. *P* values 0.05 or less were considered statistically significant.

RESULTS

Clinical Data

A total of 12 kidney transplant recipients received alemtuzumab for glucocorticoid-resistant or severe biopsy-proven acute rejection, which occurred in 5 of 7 patients within the first 90 days after transplantation and in 7 of 12 patients after this period. After antirejection therapy, kidney function as assessed by serum creatinine levels recovered or stabilized in 9 of the 12 patients, whereas 3 patients lost their graft.

The clinical characteristics of the patients are shown in Tables 1 and 2. Patient and rejection characteristics and type of immunosuppression were not associated with immunological outcomes.

T Cell Compartment and Immune Reconstitution

Alemtuzumab therapy resulted in depletion of both CD4+ and CD8+ T cells, which was followed by T cell repopulation (Figures 1A and B). T cell repopulation was more marked in CD8+ than in CD4+ T cells. Six months after alemtuzumab treatment, there were clear signs of CD8+ T cell recovery in the peripheral blood ($P < 0.05$), whereas the numbers of CD4+ T cells were barely measurable. For both populations, repopulation after alemtuzumab treatment was accompanied

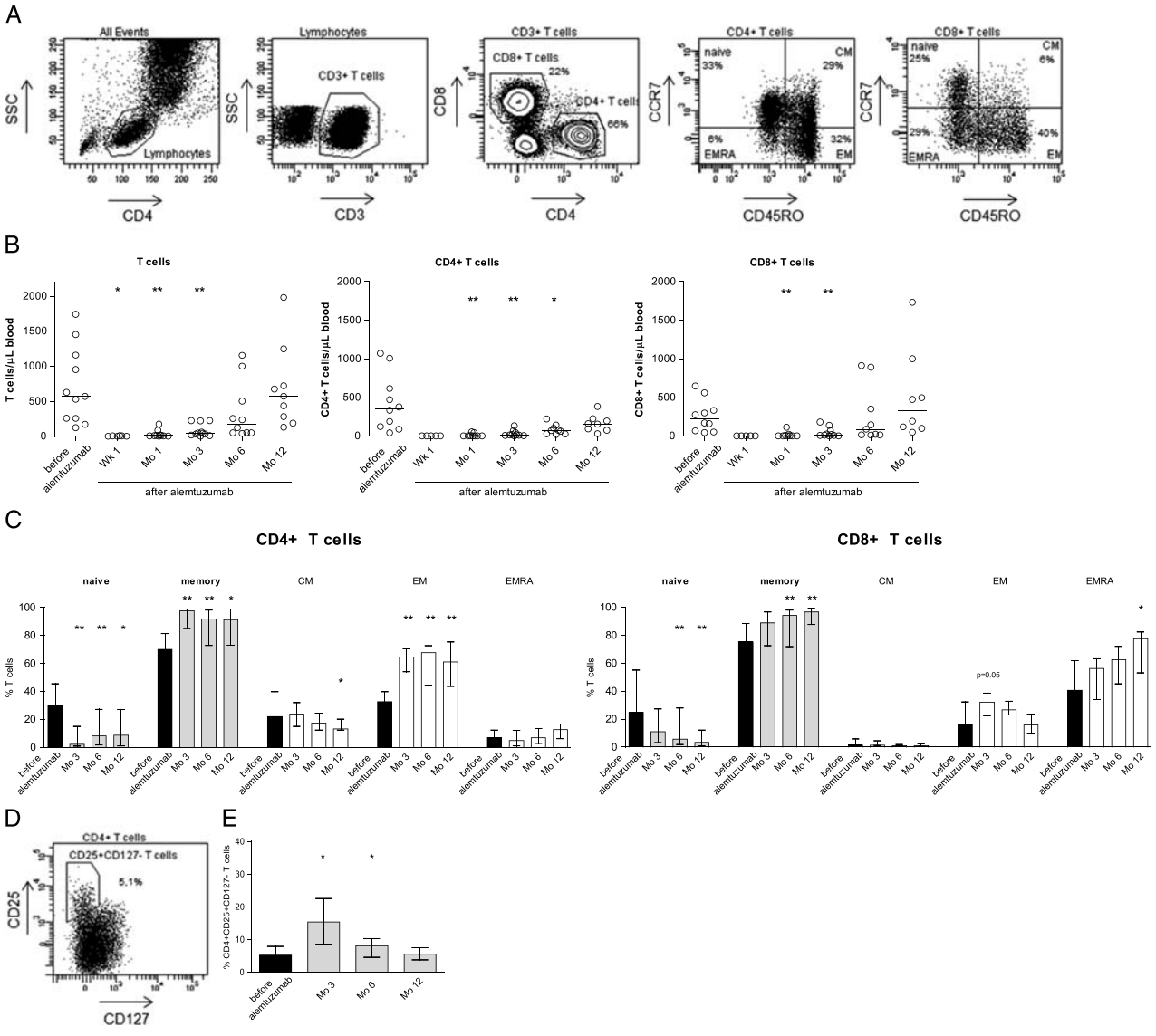

FIGURE 1. T cell repopulation after alemtuzumab therapy. A, Typical example of the gating strategy; CD4+ and CD8+ naive (CCR7+CD45RO–), central memory (CCR7+CD45RO+), effector memory (CCR7–CD45RO+) and EMRA (CCR7–CD45RO–) T cells. B, Absolute number of the total (left), CD4+ (middle) and CD8+ (right) T cell population before and at months 1, 3, 6, and 12 after alemtuzumab treatment. C, Percentage CD4+ (left) and CD8+ (right) naive, total memory, central memory, effector memory and EMRA T cells before and at months 1, 3, 6, 12 after alemtuzumab therapy. D, Typical example of the gating strategy of CD4+CD25+CD127– regulatory T cells. E, The percentage of CD4+CD25+CD127– Treg cells before and after alemtuzumab therapy. Data are shown as median ± IQR, *$P < 0.05$, ** $P < 0.01$; significant differences comparing pre-transplant values to post transplant values the Wilcoxon Signed-Rank test. Due to low cell numbers not all samples could be analysed reliable and are therefore not depicted. IQR, interquartile range.

by a shift toward more memory T cells (Figure 1C, $P < 0.05$). In the CD4+ T cell pool, the increase in memory T cells was the result of an increased frequency of EM T cells, whereas the repopulated CD8+ T cells largely consisted of EMRA T cells. In addition to the naive and memory subsets, the presence of Treg cells after alemtuzumab antirejection therapy was analyzed (Figure 1D). As shown in Figure 1E, an increased percentage of these Treg cells was observed at months 3 and 6 after therapy, which recovered to baseline levels at 12 months ($P < 0.05$).

Thymopoiesis and Homeostatic Proliferation of T Cells

In a limited number of samples (due to low naive T cell counts after treatment) we analyzed, CD31 expression and observed no changes in the percentage of CD31+ T cells, suggesting that thymopoiesis and peripheral repopulation contributed equally to T cell repopulation after alemtuzumab therapy (Figure 2B).

Homeostatic proliferation was measured by the expression of the proliferation marker Ki-67, a nuclear antigen selectively expressed in dividing cells (Figure 2C).[33] At 3 months, the percentages of Ki-67+ CD4+ and of CD8+ T cells were higher than before alemtuzumab treatment, demonstrating high rates of cell division (Figures 2D and E, $P < 0.05$). For both CD4+ and CD8+ T cells, the highest percentages of proliferating cells were measured in memory cells, with no differences in the median percentage Ki-67+ cells between the various memory subsets (month 3: CD4+ CM median, 22.6%; CD4+ EM median, 20.9%; CD8+ EM median, 11.7%; CD8+ EMRA median, 7.9%).

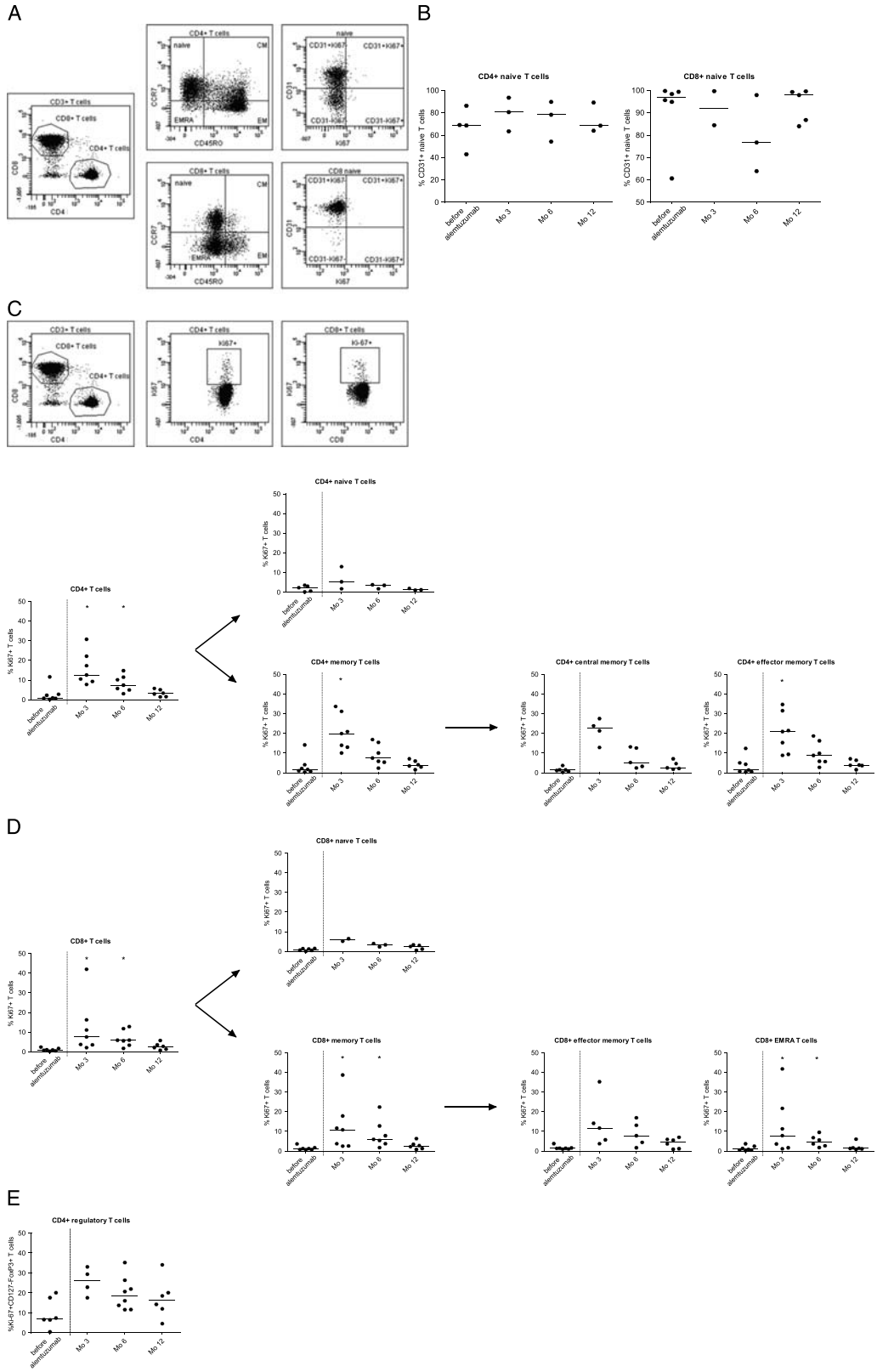

FIGURE 2. Thymopoiesis and homeostatic proliferation. A, Typical example of the gating strategy: expression of CD31 within CD4+ and CD8+ naive (CCR7+CD45RO–) T cells. B, percentage of RTE; naive T cells expressing CD31, before and after alemtuzumab therapy, before and after alemtuzumab therapy. C, Typical example of the gating strategy of CD4+ and CD8+ Ki-67+ T cells. D, the percentage Ki-67 expressing T cells within the total CD4+, CD4+ naïve, CD4+ total memory and CD4+ central memory and effector memory T cells before and after alemtuzumab therapy. E, the percentage Ki-67 expressing T cells within the total CD8+, CD8+ naive, CD8+ total memory, and CD8+ effector memory and EMRA T cells before and after alemtuzumab therapy. F, The percentage of Ki-67+ Treg cell. Because of low number of cells, we could not determine the percentage RTE and Ki-67+ cells for all patients. Data are shown as median, $*P < 0.05$; significant differences comparing pretransplant values to posttransplant values using the Wilcoxon Signed-Rank test. RTE, recent thymic emigrants.

Increased proliferation continued until 6 months after therapy only for CD8+ memory T cells (Figure 2E).

At 3 months after alemtuzumab therapy, high percentages of Ki67+ Treg cells were also measured (Figure 2F). Only 1 patient received everolimus as an immunosuppressant, in whom no high percentage of Treg cells was found.

Cytokine Responsiveness of Repopulated T Cells

Cytokine responsiveness of T cells was measured by pSTAT5 (for gating strategy and STAT5 expression; see Figure 3A). First, pSTAT5 was measured ex vivo in freshly drawn blood samples. Very low percentages of pSTAT5+ T cells were observed during rejection, that is, before antirejection treatment was given (Figure 3B). Three months after T cell depletion treatment, increased percentages of pSTAT5+ in CD4+ and CD8+ T cells were found, indicating T cell activation by members of the common-γ chain cytokine family (ie, IL-2, IL-7, and IL-15).

Second, to assess whether the repopulated T cells had functional potential, their responsive capacities to IL-2 and IL-7 were determined. At months 3 and 6 after alemtuzumab therapy, CD4+ memory T cells showed decreased responsiveness to IL-7 (Figure 3D, $P < 0.05$), which returned to baseline at month 12. Decreased responsiveness was also observed in CD8+ T cells, albeit with different kinetics: the responses decreased with time and minimal responsiveness was present at month 12 (Figure 3E). The kinetics of the different memory subsets followed the same pattern (**Figure S1, SDC,** http://links.lww.com/TXD/A26).

With regard to IL-2 response, CD4+ memory T cells showed a trend toward decreased percentages of pSTAT5 in response to IL-2 (**Figure 3F and Figure S1, SDC,** http://links.lww.com/TXD/A26). Memory CD8+ T cells were affected in functionality because 12 months after antirejection therapy, their STAT5 phosphorylation capacity was still diminished (**Figure 3G and Figure S1, SDC,** http://links.lww.com/TXD/A26).

The pSTAT5 capacity in the Treg cell population in response to IL-7 and IL-2 was also determined (Figures 3I and H). No difference was found in the expression of pSTAT5 by Treg cells before or after transplantation. After antirejection therapy, Treg cells showed poor responsiveness to IL-7 which partly recovered at month 12 (Figure 3I). As shown in Figure 3I, IL-2 responses of Treg cells were unaffected.

DISCUSSION

Results from this preliminary study suggest that use of alemtuzumab to treat early or late acute rejection after kidney transplantation results in a profound depletion of both CD4+ and CD8+ T cells from the circulation, followed by slow reconstitution, which for CD4+ T cells is incomplete at 1 year after treatment. Furthermore, both CD4+ and CD8+ T cells appear to be repopulated by means of homeostatic proliferation rather than thymopoiesis. The reconstituted T cell pool consisted predominantly of CD4+ effector memory and CD8+ effector memory and EMRA T cells which showed a decreased capacity for IL-7– and IL-2–dependent STAT5 phosphorylation. Finally, after reconstitution relatively more Treg cells were present, which again seemed to result from homeostatic proliferation. These repopulated Treg cells responded poorly to IL-7, whereas their response to IL-2 was largely unaffected.

The suggestion that reconstitution after alemtuzumab results from homeostatic proliferation, as defined here by the proliferation marker Ki-67, is in line with our previous findings regarding T cell reconstitution after rATG induction therapy.[9,19] In the current study, a higher frequency of Ki-67+ memory T cells was also measured. Firm conclusions about the kinetics and function of naive T cells from the current study are hard to draw because their numbers were extremely low after treatment. From the limited data collected, it appeared that thymopoiesis before and after T cell depletion therapy was comparable, but we cannot rule out the possibility that increased thymopoiesis also contributed to a certain degree. In contrast to studies of T cell reconstitution after alemtuzumab induction therapy, the patients in this study were pretreated with high-dose corticosteroids. Corticosteroids exert pleiotropic immunosuppressive effects by preventing protein synthesis, which ultimately results in decreased chemokine and cytokine synthesis and affected lymphocyte activation, migration, and recruitment. Lymphopenia after corticosteroid therapy is induced by lysation of cells but mainly by redistribution of T cells toward the bone marrow, spleen, thoracic duct, and lymphocytes.[34-36] Because of these effects, corticosteroids could have modified T cell numbers before alemtuzumab treatment and the reconstitution process. In our study, we have not monitored T cell numbers and characteristics before and after corticosteroid therapy and can therefore not rule out the possibility that steroid influenced the repopulation of T cells. However, the data presented here do not suggest different kinetics and dynamics of T cell repopulation when compared with repopulation after T cell induction therapy.[9]

The higher proportion of memory T cells which was observed during immune reconstitution can be explained by 2 mechanisms. First, memory T cells are relatively resistant to depletion and are thus the proliferating subtype during immune reconstitution. Second, proliferating naive cells develop a memory phenotype due to proliferation in a lymphopenic environment.[9-11,17,22,37] A higher proliferation capacity of a certain memory subset within CD4+ of CD8+ T cells was excluded in this study, because at 3 months, the percentages of Ki67+ CD4+ EM and CM were comparable, as were the percentages of CD8+ EM and EMRA T cells. Although the percentage of proliferating CD8+ T cells was lower than that of CD4+ cells, at 12 months, the absolute number of CD8+ cells exceeded the number of CD4+ T cells. A possible explanation is a relative resistance of CD8 T cells to depletion.

It is of interest to understand whether the T cells that escape depletion by alemtuzumab are alloreactive cells, especially in patients suffering from graft rejection. Published reports have shown that T cell depletion therapy in kidney transplant patients resulted in impaired T cell function of the repopulated cells, for example, decreased T cell reactivity to donor, third party and recall antigen, impaired cytokine responsiveness and low numbers of interferon-γ–producing cells using enzyme-linked immunospot assays.[7,16,20,29,30]

The increase in ex vivo STAT5 phosphorylation reflects the response of T cells to cytokine stimulation observed in vivo. This may have resulted from any pSTAT5 activating cytokine (eg, IL-21, IL-15, thymic stromal lymphopoietin), including

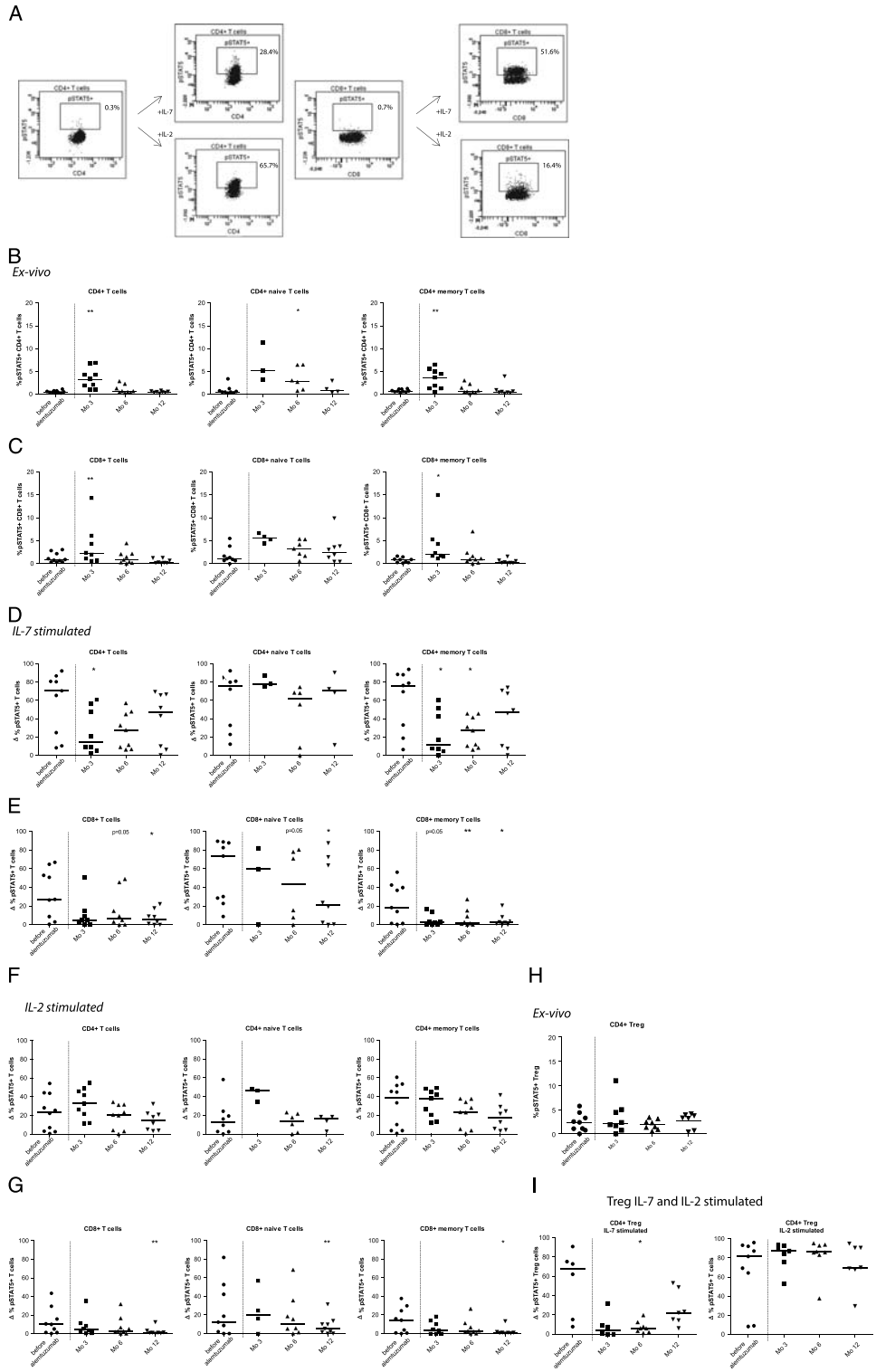

FIGURE 3. IL-7 and IL-2 induced STAT5 phosphorylation capacity; IL-7/IL-2 stimulated percentage pSTAT5+ cells minus unstimulated percentage pSTAT5. A, Typical example of the pSTAT5 gating, unstimulated and after stimulation. B, Percentage of pSTAT5+ total, naïve and memory CD4+ cells before and after alemtuzumab therapy in unstimulated blood (ex vivo). C, Percentage of pSTAT5+ total, naïve and memory CD8+ cells before and after alemtuzumab therapy in unstimulated blood (ex vivo). D, STAT5 phosphorylation capacity in response to IL-7 of total, naive, total memory CD4+ T cells; before and at 3, 6, and 12 months after alemtuzumab therapy. E, STAT5 phosphorylation capacity in response to IL-7 of total, naive, total memory CD8+ T cells; before and at 3, 6, and 12 months after alemtuzumab therapy. F, STAT5 phosphorylation capacity in response to IL-2 of total, naive, total memory CD4+ T cells; before and at 3, 6, and 12 months after alemtuzumab therapy. G, STAT5 phosphorylation capacity in response to IL-2 of total, naive, total memory CD8+ T cells; before and at 3, 6, and 12 months after alemtuzumab therapy. H, Percentage of pSTAT5+ CD4+ Treg cells before and after alemtuzumab therapy in unstimulated blood (ex vivo). I, IL-7 and IL-2 induced STAT5 phosphorylation capacity in Treg cells. Left: The percentage pSTAT5+ Treg cell after IL-7 stimulation. Right: The percentage pSTAT5+ Treg cell after IL-2 stimulation. Data are shown as median, * $P < 0.05$, ** $P < 0.01$, significant differences comparing pretransplant values to posttransplant values using the Wilcoxon Signed-Rank test.

the cytokines IL-2 and IL-7 studied in vitro. It is well known that also other proteins might activate STAT5, such as growth hormone and erythropoietin.

After in vitro stimulation, the repopulated T cells exhibited reduced pSTAT5 capacity in response to IL-7 and, to a lesser extent, to IL-2. These findings indicate that the repopulating cells after antirejection depletion therapy are also functionally hampered in cytokine-driven T cell responses. This can be explained by several mechanisms. First, high basal levels of pSTAT5 are reported to alter STAT signaling. These pSTAT5 levels indicate chronic immune activation that might be the first incentive for impaired STAT signaling.[30,38] Second, alemtuzumab treatment resulted in increased percentages of functionally active Treg cell controlling T cell responses.[19,39-41] A third mechanism could be the predominant expansion of terminally differentiated, functionally impaired immunosenescent CD28–CD8+ T cells.[15] These functionally impaired T cells compete for "immune space" with CD4+ T cells, suppressing their proliferation and may therefore also explain their incomplete repopulation. Additionally, we observed increased percentages of terminally differentiated CD8+ EMRA cells. Fourth, T cell exhaustion, the loss of T cell effector functions induced by persistent donor antigen stimulation, may explain our findings.[31] As well as the loss of proliferative capacity, cytokine production and killing capacity, exhausted cells have altered memory maintenance since in contrast to "normal" memory T cells, they poorly respond to IL-7 and IL-15 and only survive by contact with their cognate antigen.[42] Impaired access of STAT5 to the nuclear compartment and decreased receptor expression, although not found earlier in our study, might contribute to these decreased IL-7 responses.[38,43-45] Phenotypically, exhausted T cells overexpress multiple inhibitory receptors and exhibit poor cytokine responsiveness. Recently, we demonstrated that T cells from rATG-treated kidney transplant patients have affected STAT5 phosphorylation and high levels of cell surface expression of inhibitory receptors. The latter observation strengthened our hypothesis that the impaired T cell function after T cell depletion therapy may be explained by T cell exhaustion phenomena. Clinical evidence that alemtuzumab treatment results in impaired T cell function is based on data showing that patients are prone to developing infections and malignancies.[5,42,46] A study by Peleg et al[47] showed that when alemtuzumab was given as antirejection therapy, more opportunistic infections were observed than when given as induction therapy, indicating an even more dysfunctional T cell system. Although CMV infection is associated with increased percentages of CD8+ T cells and T cell exhaustion, in this study, CMV reactivation was not associated with decreased function (data not shown).

With a population of only 12 patients, this study can only be regarded as hypothesis-generating, and by no means conclusive. Other factors should also be taken into account. In particular, all patients were not given the same maintenance immunosuppression. Most of the patients were treated with the calcineurin inhibitors tacrolimus or cyclosporine, and 1 patient was treated with the mammalian target of rapamycin inhibitor everolimus. Although we know that maintenance tacrolimus immunosuppression does not affect pSTAT5 expression, it is unknown whether the other agents have an effect on T cell exhaustion parameters.[48] The retransplanted patients were treated with rATG for rejection of their previous transplant. We do not know whether this has an effect on our study parameters. Due to very low cell numbers, the coinhibitory molecules and antigen specificity of the depletion-resistant cells were not studied. Previously published studies on the effects of T cell depletion induction therapy report less alloreactivity. It would be interesting to determine whether this is the result of depletion of alloreactive cells or of alloreactive cells becoming functionally impaired. A possible effect of intravenous immunoglobulin use should also be considered.

Because we speculate that T cell exhaustion plays an important role in decreased T cell reactivity, it would be helpful to know whether inhibitory molecules are upregulated on repopulated T cells after alemtuzumab antirejection therapy. Patients who can be treated with reduced-dose immunosuppression could potentially be identified by means of an exhaustion profile, reduced pSTAT5 capacity, and upregulation of inhibitory molecules. In addition to T cell exhaustion, decreased IL-7–induced pSTAT5 is also clinically important for monitoring and perhaps influencing T cell reconstitution. A study by Mai et al[49] showed that an allograft survival advantage in mice IL-7 signaling was blocked. Phosphorylation of STAT5 is a key step in IL-7 signaling and therefore a potential monitoring tool. The whole blood assay used here is of interest because this assesses the functionality of T cells under full immunosuppression. For our studies, we chose high concentrations of IL-2 and IL-7 to stimulate T cells because in vivo these cytokines are highly present as a result of decreased IL-7 consumption and cell lysis.[46,50]

In conclusion, the profound T cell depletion after alemtuzumab antirejection treatment observed in this small series suggests that the drug's effects are not affected by a proinflammatory environment. Repopulation of T cells appeared to arise from increased homeostatic proliferation measured by Ki-67. The reconstituted population predominantly consisted of CD8+ T cells and T cells with a memory and regulatory phenotype. Both CD4+ and CD8+ T cells, as well as Treg cells, showed diminished cytokine responsiveness which was more pronounced for IL-7 than for IL-2. The hypotheses generated by these preliminary findings require exploration in a controlled trial using protocol-specified immunosuppression with a larger study population.

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