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# The number of circulating recent thymic emigrants is severely reduced 1 year after a single dose of alemtuzumab in renal transplant recipients

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## Summary

To better understand the kinetics of the delayed reconstitution of peripheral CD4+ T-cells after depletion with a single administration of alemtuzumab (AL) for renal transplantation, we evaluated in these patients the percentage and absolute number of recent thymic emigrants (RTEs) CD4+ T cells, together with naive and memory subsets, defined by the analysis of CD31, CD45RA and CCR7 expression, and compared with patients treated with a nondepleting protocol based on basiliximab, and with healthy controls. In AL-treated patients, the number of circulating CD4+ T cells was greatly reduced 1 year after the infusion ($P < 0.01$), but the proportions of central memory, effector memory and terminally differentiated effector memory subsets among CD4+ cells were significantly increased. On the contrary, the proportion and the absolute number of naïve CD4+ T cells, although progressively increasing with time, were severely reduced. In particular, the absolute number of RTEs had only very slight increase with time ($P = 0.049$) and was dramatically low 1 year after the therapy ($P < 0.01$ vs. healthy controls; $P < 0.05$ vs. basiliximab-treated transplant recipients). These data suggest that a prolonged defective thymic output after AL therapy in renal transplant recipients is one of the main causes of the persistent CD4+ T-cell lymphopenia observed in these patients.

## Introduction

Alemtuzumab (Campath-1H; AL) is a humanized monoclonal antibody (mAb) binding CD52, a cell surface glycoprotein expressed by T and B lymphocytes, natural killer (NK) cells, monocytes, and dendritic cells. It has been used in the therapy of immune-mediated diseases including autoimmune disorders, chronic lymphocytic leukemia, graft-versus-host disease after bone marrow transplantation, and transplants of solid organs, including kidney [1–7].

*In vivo* infusion of AL causes a profound cytopenia by antibody-dependent cellular cytotoxicity, as well as through complement fixation and activation. It has been reported that B cells and monocytes numbers recover to baseline values by 5 and 2 months, respectively [8]. Peripheral T-cell depletion is profound (almost complete at the outset), but also long-lasting. In fact, CD4 and CD8 T lymphocytes maximal recovery were seen to be 17.5% and 50% respectively of normal values at 2 months and these levels did not increase further during the study period of 18 months [8]. In another study, 36 months after infusion of AL, T cells recovered only to approximately 50% of baseline value, and a persistently decreased CD4/CD8 ratio was observed [9]. Interestingly, the dominant T-lymphocyte population in the first few months following cell depletion with AL was represented by memory cells [10,11].

Memory CD4+ and CD8+ T cells are generally CD45RA-negative and comprise at least two functionally

distinct subsets: (i) nonpolarized 'central memory' T cells (TCMs), which express the chemokine receptor CCR7 that allows them to home to the T-cell areas of secondary lymphoid organs; and (ii) polarized 'effector memory' T cells (TEMs), which have lost the expression of CCR7 and have acquired the capacity to migrate to nonlymphoid tissues [12–14]. Therefore, the evaluation of CCR7 together with CD45RA protein expression is now considered the best available way to characterize TCMs and TEMs. A third subset of memory T cells expresses CD45RA as do naive cells, but not CCR7. Variously termed, these are highly differentiated cells with persisting effectors functions ['terminally differentiated effector memory' (TTDEM)]. In healthy state, these cells accumulate with age in the CD8+ T-cell compartment but represent also a minor CD4+ T-cell subset [15] and are mainly found in nonlymphoid tissues and to some degree in the spleen [12,16].

The aim of this study was to better understand the kinetics of the delayed T-cell reconstitution after depleting therapy in patients treated with AL for renal transplantation. For this purpose, we have evaluated not only the number of circulating memory and naïve T lymphocytes with different phenotypes, but we also studied the expression of the adhesion molecule CD31 (PECAM-1), which has been proposed as a marker of T cells that have recently emigrated from thymus (recent thymic emigrants; RTEs) [17,18].

## Patients and methods

### Patients and immunosuppressive protocol

All patients gave their informed consent prior to their inclusion in the study, which was reviewed by the local ethics committee, and performed in accordance with Declaration of Helsinki.

Between May 2006 and October 2007, 48 consecutive patients (median age 54 years; 10th-90th percentile: 34–64; 11 female, 37 male subjects) undergoing kidney transplantation from deceased donors were enrolled into a study to investigate the effectiveness and safety of AL induction therapy for renal transplantation, in a steroid-free maintenance regimen. Recipients with previous renal transplants or panel reactive antibody >50% were excluded. Taking into account the recipient weight (<60 kg or >60 kg), the dosage of 20 or 30 mg AL was administered intraoperatively before kidney reperfusion. The premedication was with 500 mg of methylprednisolone, paracetamol 1 g and clorfenamine 1 mg. Immunosuppression maintenance consisted of tacrolimus and sirolimus. Tacrolimus was started 12–24 h post-transplant and the dose adjusted to achieve a trough level of 8–10 ng/ml during the first month and 5–7 ng/ml thereafter. Sirolimus was started 36–48 h post-transplant and the dose adjusted to achieve a trough level of 5–10 ng/ml. Steroid therapy was introduced and maintained in the event of rejection being observed based on the histological finding. No patients received cytomegalovirus prophylaxis, yet a pre-emptive strategy was performed.

As a control group, we evaluated 15 patients (Table 1) who received induction therapy with the anti-CD25 mAb basiliximab 20 mg at day 0 and day 4 and methylprednisolone 500 mg, 200 mg, 100 mg, 50 mg, 20 mg at day 0, 1, 2, 3, and 4, respectively post-transplantation. Subsequently, these patients received maintenance scheme with tacrolimus (trough level of 5–10 ng/ml), or, in few cases, cyclosporine (2-h postdose levels (C2) of 400–600 ng/ml), plus mycophenolate mofetil (500 mg twice daily) and methylprednisolone gradually tapered to 4 mg/day after 3 months and stopped after 6–8 months. In two patients, the steroid was stopped at day 5 post-transplantation.

Fifteen healthy volunteers of comparable age (median: 48 years; 10–90th percentile: 34–51; 10 female, 5 male subjects) were recruited among laboratory personnel and served as healthy controls.

### Leukocyte phenotype analysis

Whole peripheral blood was taken in K-EDTA-test tube at the moment of transplantation ($t = 0$), and at days +1, +5, +10, +30, then monthly till day +180, and, later, every 3 months till day +360. Leukocyte surface membrane expression was analysed by flow cytometry (Cytomics FC-500, Beckman Coulter Inc., Fullerton, CA, USA). Absolute cell count was determined by single-platform analysis using Flow-Count beads (Beckman Coulter). T-cell lymphocyte populations were numbered using mAbs conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-TxRed (ECD), PE-Cyanin 5.1 (PC5) Tetrachrome CD45/CD4/CD8/CD3 from Beckman Coulter. B cells were determined using CD19 (Beckman Coulter). NK lymphocytes and monocytes were recognized using CD45/CD14/CD16 and side-scatter analysis.

In 21 consecutive patients, an in-depth monitoring of lymphocyte subsets was performed at days +90, +180, +270, +360, to identify naïve (N), TCMs, TEMs, TTDEMs, lymphocytes subpopulations using PE-anti-CCR7 (R&D Systems Inc., Minneapolis, MN, USA), FITC-CD45RA, ECD-CD4 and PC5-CD3 mAb (Beckman Coulter). At the same time, RTEs were evaluated using FITC-CD31, PE-CD45RA, ECD-CD4, and PC5-CD3 (Beckman Coulter). Main clinical and laboratory data of these 21 patients are summarized in the Table 1. Their characteristics did not differ from the entire cohort of 48 patients treated with AL. These lymphocyte populations were evaluated also in the healthy individuals and in the

 

**Table 1.** Main demographic and clinical features of 21 patients treated with alemtuzumab.

| | Alemtuzumab | Basiliximab |
|---|---|---|
| No. patients | 21 | 15 |
| Mean donor age (year) | 47 ± 16 | 48 ± 15 |
| Mean recipient age (year) | 51 ± 14 | 54 ± 7 |
| Recipient gender | 6 F/15 M | 5 F/10 M |
| Panel reactive antibody >20% | 0 | 1 (7%) |
| HLA mismatch AB | 1.8 ± 1.0 | 1.5 ± 0.8 |
| HLA mismatch DR | 1.1 ± 0.7 | 0.9 ± 0.6 |
| Delayed graft function | 19% | 20% |
| Mean follow-up (year) | 1.7 ± 0.4 | 2.2 ± 0.4 |
| Mean creatininemia (mg/dl) | 1.5 ± 0.6 | 1.3 ± 0.5 |
| Patient survival | 100% | 93% |
| Graft survival | 95% | 100% |
| Rejection | 1 (5%) | 2 (13%) |
| Incidence of CMV infection | 1 (5%) | 3 (20%) |
| Incidence of BK nephritis | 2 (10%) | 1 (7%) |
| Sepsis | 1 (5%) | 0 |
| Diabetes | 2 (10%) | 2 (13%) |
| Antibody-related adverse events | 0 | 0 |

control group of patients treated with basiliximab-based protocol. It should be considered, however, that in this latter group, the evaluation of CD4+ subsets was not longitudinal, but only cross-sectional, and that it was performed at a mean time of 2.2 years after transplantation, significantly longer than the last evaluation done in the AL-treated patients.

### Statistical analysis

If not otherwise indicated, data are expressed as the median (10–90th percentile). The comparisons between various groups were assessed by the nonparametric Dunn's multiple comparison test. The variations of cell populations over time were evaluated by standard one-way analysis of variance.

## Results

The absolute numbers of leukocytes and main lymphocytes subsets in 48 patients treated with AL at $t = 0$ were not significantly different from those observed in healthy controls and in 15 patients treated with a basiliximab-based protocol. The reconstitution of these populations is shown in Tables 2 and 3. After AL infusion, a transient neutrophilia appeared at day +1 and normalized at day +5. The depletion of monocytes was not complete, and absolute number recovered at data not significantly different from baseline value at day +60. A profound depletion of circulating lymphocytes was observed, but while NK and B cells returned to baseline value at day +60 and day +120 respectively, total T-cell number was still markedly decreased at day +360 (22% of its baseline value). At this date, the absolute number of CD8+ T cells was only 40% of its baseline value and it was also significantly lower than that in healthy controls (Table 2; $P < 0.01$). The absolute number of CD4+ T cells was even more severely reduced (12% of its baseline value; $P < 0.01$).

On the contrary, basiliximab-treated patients showed a much less profound and more transient lymphopenia (likely attributable to glucocorticoids boluses), which normalized already at day +10 (Tables 2 and 3). Accordingly, at day +360, T-cell number was still markedly lower in patients treated with AL than in patients treated with basiliximab ($P < 0.01$).

As depletion of CD4+ was the most significant alteration after AL therapy, we evaluated in depth the phenotype of CD4+ T cells in 21 consecutive patients treated with AL, and compared them with 15 healthy controls of comparable age and 15 patients treated with basiliximab-based protocol. As shown in Fig. 1, the proportion of naïve (CD45RA+CCR7+) T cells progressively increased with time within the CD4+ subsets ($F = 2.92$, $P = 0.045$). However, at day +360, this proportion was still severely reduced as compared with normal controls (median: 12.1% vs. 43%; $P < 0.05$). Moreover, resulting from the persistence of CD4+ T-cell cytopenia, the absolute number of naïve T cells also was dramatically reduced (median: 26 per μl vs. 319 per μl in normal controls; $P < 0.01$) at day +360. Accordingly, the proportion of RTEs within the CD4+ T cells had a very marginal, not statistically significant increase with time ($F = 2.08$; $P = 0.12$), and it was still below normal values at day +360 (median: 8.3% vs. 25.0%; $P < 0.05$). Therefore, their absolute number, although increasing with time ($F = 2.58$; $P = 0.049$), was still dramatically low at day +360 (median: 13 per μl vs. 182 in normal controls; $P < 0.01$). The absolute number of circulating RTEs at day +360 was inversely correlated with the age of transplant recipient ($r = -0.53$; $P = 0.003$).

The reconstitution of memory CD4+ T lymphocytes is shown in Fig. 2. We found a marked and persisting predominance of TEM (CD45RA−CCR7−) cells. At day +90 this, subset reached a maximal proportional value (80% of all CD4+ cells), after which period, it progressively declined ($F = 3.94$, $P = 0.015$), but at day +360, it still represented 50% of total CD4+ cells, a significantly higher proportion than in healthy controls ($P < 0.01$). Similarly, the proportion of TTDEM cells (CD45RA+CCR7−), another effector CD4+ population, was significantly higher than in healthy controls at day +360 (median: 3.5% vs. 0.7%; $P < 0.01$). Conversely, the relative percentage of TCM+ (CD45RA−CCR7−) cells was still lower than in healthy people at this date (median: 21.8%

**Table 2.** Kinetics of proportions of leukocyte and main lymphocyte subsets in patients treated with alemtuzumab or basiliximab.

| Cell line | T = 0 | T = 1 day | T = 5 days | T = 10 days | T = 30 days | T = 60 days | T = 90 days | T = 120 days | T = 150 days | T = 180 days | T = 270 days | T = 360 days | Healthy donors |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Alemtuzumab | | | | | | | | | | | | | |
| Granulocytes (% of total WBC) | 59 (45–71) | 98 (95–99) | 92 (85–95) | 91 (85–94) | 82 (75–86) | 75 (59–80) | 69 (54–80) | 69 (54–78) | 68 (52–77) | 68 (56–77) | 67 (52–79) | 64 (52–73) | 55 (40–71) |
| Monocytes (% of total WBC) | 9 (6–13) | 1 (0–3) | 6 (3–13) | 6 (3–10) | 11 (7–14) | 12 (7–17) | 12 (8–17) | 11 (7–15) | 11 (7–15) | 11 (7–15) | 11 (8–13) | 12 (7–17) | 8 (4–12) |
| Lymphocytes (% of total WBC) | 28 (17–38) | 0 (0–0) | 1 (0–2) | 1 (1–3) | 5 (3–9) | 11 (7–21) | 14 (8–32) | 17 (11–29) | 17 (11–34) | 16 (12–27) | 19 (11–28) | 20 (11–30) | 30 (19–44) |
| CD3 (% of total lym) | 80 (61–87) | 34 (11–58) | 18 (7–41) | 9 (3–37) | 12 (5–25) | 20 (9–44) | 31 (13–60) | 41 (20–59) | 35 (22–54) | 35 (20–60) | 43 (22–61) | 45 (26–61) | 76 (66–80) |
| CD4 (% of total lym) | 52 (37–66) | 11 (0–25) | 3 (1–9) | 1 (0–3) | 3 (1–9) | 6 (1–16) | 11 (5–17) | 11 (8–21) | 13 (9–26) | 13 (8–23) | 17 (10–27) | 18 (11–28) | 43 (38–52) |
| CD8 (% of total lym) | 22 (12–30) | 13 (2–26) | 11 (4–33) | 4 (1–31) | 6 (2–15) | 11 (3–26) | 14 (5–43) | 17 (8–45) | 17 (10–37) | 17 (6–41) | 19 (8–32) | 20 (9–34) | 26 (21–30) |
| NK (% of total lym) | 13 (6–27) | 38 (13–57) | 63 (45–81) | 82 (52–89) | 80 (62–92) | 66 (35–80) | 49 (27–70) | 41 (24–66) | 40 (26–60) | 41 (25–72) | 38 (16–60) | 33 (13–57) | 13 (6–27) |
| CD19 (% of total lym) | 8 (4–15) | 6 (2–19) | 2 (1–4) | 1 (0–2) | 1 (1–4) | 10 (3–30) | 14 (6–30) | 16 (7–35) | 20 (7–36) | 18 (10–37) | 20 (9–37) | 21 (13–36) | 13 (7–19) |
| Basiliximab | | | | | | | | | | | | | |
| Granulocytes (% of total WBC) | 68 (59–85) | 93 (88–95)† | 80 (66–85)† | 69 (63–77)† | 58 (44–72)† | 62 (49–72) | 56 (50–72) | 52 (29–70) | 54 (41–63) | 58 (48–66) | 59 (41–75) | 57 (46–70) | 55 (40–71) |
| Monocytes (% of total WBC) | 8 (6–13) | 4 (1–9)* | 9 (6–13) | 10 (7–12) | 11 (6–13) | 8 (1–10)* | 9 (5–12) | 10 (7–14) | 10 (7–11) | 8 (5–11) | 10 (6–11) | 9 (7–12) | 8 (4–12) |
| Lymphocytes (% of total WBC) | 23 (13–31) | 4 (2–6)* | 11 (5–20)* | 18 (12–24)* | 28 (16–42)* | 27 (15–43)* | 30 (18–36)* | 37 (17–59)* | 36 (25–42)* | 29 (22–48)* | 27 (13–46)* | 31 (14–41) | 30 (19–44) |
| CD3 (% of total lym) | 79 (66–83) | 57 (39–77)* | 76 (56–85)* | 79 (71–86)* | 80 (65–90)* | 82 (68–85)* | 82 (74–89)* | 84 (74–91)* | 84 (74–93)* | 84 (76–92)* | 86 (75–92)* | 84 (49–90)* | 76 (66–80) |
| CD4 (% of total lym) | 42 (34–58) | 29 (19–39)* | 51 (32–60)* | 57 (36–66)* | 53 (29–61)* | 55 (31–60)* | 45 (29–60)* | 41 (29–58)* | 49 (28–53)* | 47 (24–52)* | 45 (28–50)* | 31 (24–49)* | 43 (38–52) |
| CD8 (% of total lym) | 26 (16–34) | 25 (12–31)* | 23 (14–34)* | 20 (14–31)* | 25 (19–37)* | 28 (17–33)* | 29 (21–43)* | 31 (22–46)* | 33 (26–53)* | 33 (24–62)* | 35 (28–49)* | 35 (23–55)* | 26 (21–30) |
| NK (% of total lym) | 15 (9–23) | 32 (19–35) | 11 (6–14)† | 5 (3–11)† | 11 (7–27)† | 10 (3–19)† | 10 (4–17)† | 10 (6–18)† | 9 (4–19)† | 8 (3–18)† | 11 (4–19)† | 10 (4–29) | 13 (6–27) |
| CD19 (% of total lym) | 9 (1–15) | 14 (5–34)† | 16 (8–28)† | 15 (5–24)† | 8 (4–19)† | 10 (4–19) | 7 (2–15) | 6 (2–11)* | 4 (2–13)* | 6 (2–10)* | 8 (3–10)* | 7 (3–14)* | 13 (7–19) |

*$P < 0.05$ Alemtuzumab vs. Basiliximab.
†Alemtuzumab > Basiliximab: $P < 0.05$.

**Table 3.** Kinetics of absolute numbers of leukocyte and main lymphocyte subsets in patients treated with alemtuzumab or basiliximab.

| Cell line | T = 0 | T = 1 day | T = 5 days | T = 10 days | T = 30 days | T = 60 days | T = 90 days | T = 120 days | T = 150 days | T = 180 days | T = 270 days | T = 360 days | Healthy donors |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Alemtuzumab | | | | | | | | | | | | | |
| Granulocytes | 3900 (2254–6275) | 11 380 (3870–18 092) | 3592 (2083–5626) | 4055 (2619–6998) | 2739 (1340–4831) | 2203 (1324–3996) | 2331 (1415–3849) | 3238 (1846–3872) | 2654 (1871–3888) | 2729 (1661–3781) | 2537 (1562–4178) | 2319 (1475–3768) | 4107 (2212–5503) |
| Monocytes | 570 (317–1012) | 134 (46–369) | 214 (104–662) | 273 (144–606) | 318 (228–566) | 378 (186–844) | 391 (268–684) | 445 (303–737) | 431 (308–682) | 429 (257–645) | 438 (249–669) | 398 (215–551) | 630 (244–911) |
| Lymphocytes | 1798 (918–3061) | 11 (2–21) | 26 (7–81) | 75 (38–132) | 172 (80–263) | 354 (186–734) | 503 (256–1229) | 650 (420–1395) | 676 (357–1219) | 638 (392–1366) | 669 (375–1344) | 709 (444–1139) | 1994 (1079–2311) |
| CD3 | 1435 (740–2140) | 3 (0–9) | 4 (1–13) | 6 (2–32) | 19 (8–39) | 64 (20–246) | 158 (34–499) | 283 (85–811) | 223 (68–642) | 213 (97–594) | 286 (98–607) | 317 (118–621) | 1370 (850–1862) |
| CD4 | 938 (486–1524) | 1 (0–5) | 1 (0–3) | 1 (0–3) | 4 (2–10) | 35 (4–81) | 51 (17–127) | 100 (34–144) | 98 (49–240) | 87 (41–184) | 121 (63–193) | 118 (61–240) | 789 (474–1068) |
| CD8 | 379 (152–800) | 1 (0–4) | 2 (1–10) | 3 (0–29) | 7 (2–27) | 50 (10–189) | 71 (13–356) | 145 (37–459) | 128 (39–390) | 85 (33–374) | 141 (31–367) | 151 (54–317) | 501 (280–623) |
| NK | 189 (23–507) | 3 (0–8) | 14 (4–59) | 56 (26–102) | 142 (61–235) | 255 (101–463) | 234 (111–466) | 281 (152–528) | 276 (183–493) | 211 (156–549) | 219 (133–424) | 191 (105–430) | 187 (129–366) |
| CD19 | 133 (40–343) | 1 (0–4) | 0 (0–1) | 1 (0–2) | 1 (1–4) | 39 (6–131) | 84 (27–205) | 113 (43–251) | 124 (31–312) | 133 (52–293) | 140 (66–430) | 186 (112–609) | 201 (99–428) |
| Basiliximab | | | | | | | | | | | | | |
| Granulocytes | 4351 (3080–5772) | 10 858 (8994–12 693) | 6939* (4560–11 350) | 6224* (4684–9257) | 4005 (2087–5196) | 5078* (2117–6190) | 4058* (1765–6714) | 3068 (1193–6840) | 2779 (1803–3404) | 1850 (564–4884) | 2996 (2027–5945) | 3273 (1925–4509) | 4212 (2123–5025) |
| Monocytes | 612 (370–1020) | 282 (125–646) | 934* (407–1546) | 807* (583–1144) | 618* (390–1022) | 405 (102–702) | 504 (414–1000) | 196 (116–662) | 530 (310–645) | 294 (55–943) | 544 (234–974) | 485 (256–884) | 604 (283–880) |
| Lymphocytes | 1382 (923–2458) | 427* (248–587) | 945* (677–1477) | 1483* (1063–2985) | 1727* (1241–2242) | 1593* (1030–2903) | 1452* (1184–3110) | 1298* (1011–1744) | 1630* (1146–2552) | 1815* (1080–2690) | 1292* (701–3076) | 1568* (863–3426) | 1994 (1079–2311) |
| CD3 | 978 (678–1913) | 177* (109–335) | 677* (486–1032) | 1139* (859–2055) | 1229* (671–1827) | 1001* (751–2095) | 1185* (793–2668) | 1064* (792–1374) | 1226* (811–2356) | 1536* (831–2382) | 1198* (496–2720) | 1387* (725–2932) | 1370 (850–1862) |
| CD4 | 492 (371–1124) | 91* (54–181) | 393* (216–823) | 682* (604–1250) | 810* (320–1219) | 542* (371–1038) | 675* (385–1330) | 636* (413–818) | 666* (334–1361) | 637* (312–1234) | 469* (209–965) | 746* (308–1073) | 789 (474–1068) |
| CD8 | 317 (229–419) | 74* (50–114) | 213* (133–285) | 282* (221–443) | 357* (291–499) | 325* (265–380) | 374* (297–1089) | 372* (281–643) | 478* (335–1106) | 400* (264–1408) | 375* (222–866) | 545* (318–1595) | 501 (280–623) |
| NK | 220 (131–269) | 133* (75–246) | 93* (58–136) | 86 (40–188) | 220 (53–311) | 127 (65–247) | 136 (61–217) | 117† (76–234) | 134 (83–268) | 94 (57–300) | 121 (75–280) | 133 (90–263) | 187 (129–366) |
| CD19 | 118 (8–394) | 58* (14–138) | 110* (63–357) | 224* (68–567) | 139* (36–314) | 202* (58–381) | 109 (37–224) | 89 (16–223) | 119 (20–272) | 119 (20–239) | 127 (19–208) | 122 (47–223) | 201 (99–428) |

*Basiliximab > Alemtuzumab: $P < 0.05$.

†Alemtuzumab > Basiliximab: $P < 0.05$.

 

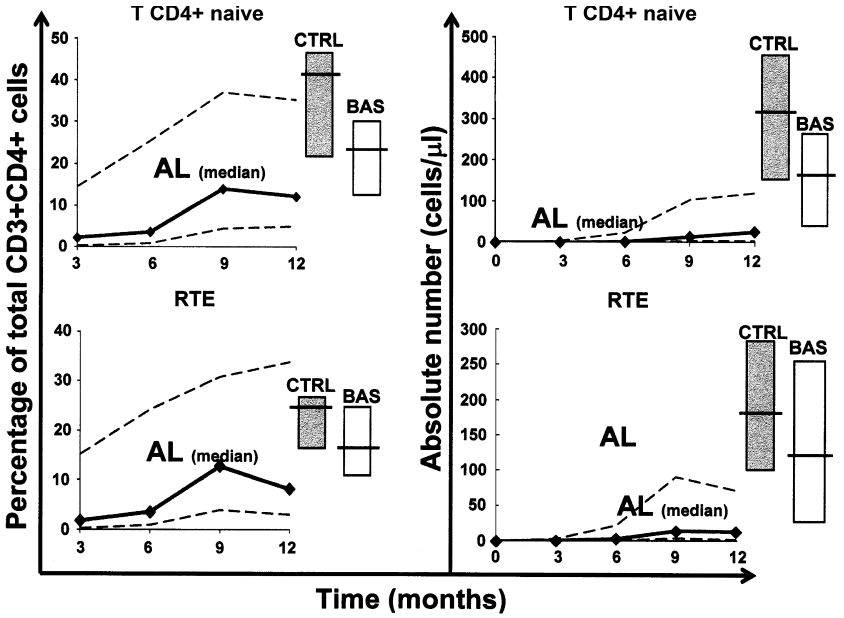


**Figure 1** Evolution of percentages and absolute numbers of naive CD3+CD4+ T cells and recent thymic emigrants in 21 patients treated with alemtuzumab (median and 10–90th percentile). Boxes indicate the median and 10–90th percentile in 15 healthy controls (CTRL; gray box) and 15 patients treated with basiliximab (BAS; white box).

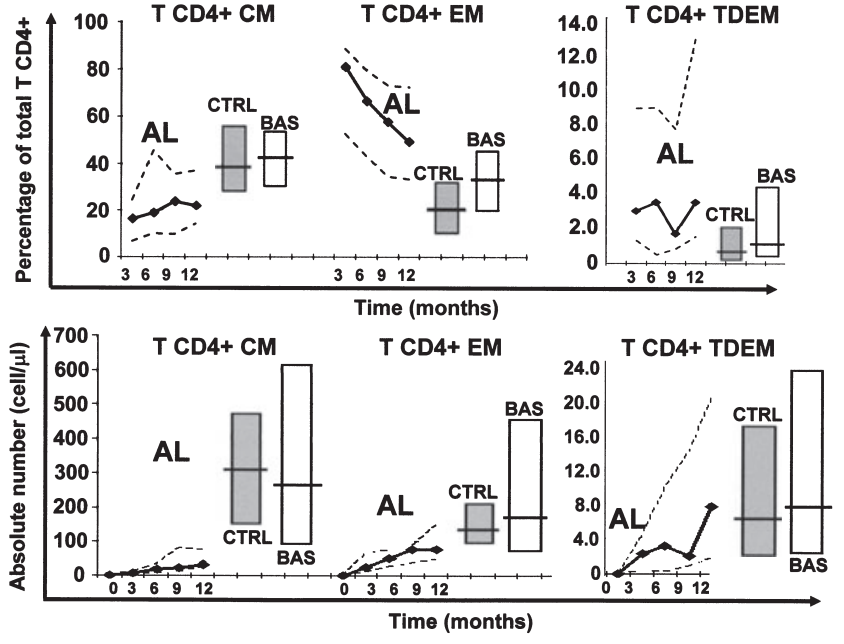


**Figure 2** Evolution of percentages and absolute numbers of central memory, effector memory and terminally differentiated effector memory CD3+CD4+ T cells in 21 patients treated with alemtuzumab (median and 10–90th percentile). Boxes indicate the median and 10–90th percentile in 15 healthy controls (CTRL; gray box) and 15 patients treated with basiliximab (BAS; white box).

vs. 38.7%; $P < 0.01$). As a result of the persisting CD4+ cytopenia, at day +360, the absolute cell numbers of all the memory subsets but TTDEM cells were significantly lower than that in healthy individuals.

Patients treated with basiliximab-based protocols had only minor differences from healthy controls: the percentages and absolute numbers of naïve CD4+ T cells were lower (Fig. 1; medians: 23.4% vs. 41.3%, $P < 0.01$, and 162 cells/μl vs. 319, $P < 0.05$, respectively) and the percentages of TEM were higher (32.9% vs. 20.3% $P < 0.01$). The absolute numbers of RTEs and all other CD4+ lymphocyte subsets were significantly higher ($P < 0.05$) in patients treated with basiliximab based-protocols than those observed in AL-based protocol at day +360 (Figs 1 and 2).

It should be acknowledged, however, that at the moment of the phenotypic analysis of CD4+ lymphocyte subsets, the time since transplantation was significantly longer in the basiliximab group (2.2 years) than in the AL group.

The CD3+CD4− subset is accounted for almost exclusively by CD8+ T cells, even if the occasional possible presence of minor CD4−CD8− T-cell populations cannot be excluded. In normal individuals, the TTDEM subset is much more represented within the CD3+CD4− than CD4+ T cells. In AL-treated patients, this cell population was predominant at all time points evaluated after depletion therapy and was still higher than that in healthy controls at day +360 (median: 54.3% vs. 29.2%; $P < 0.05$). The proportion of TEM and TCM did not change significantly with time after treatment with AL, but that of TCM was significantly lower than in healthy controls at most time points evaluated. The proportion of naïve (CD45RA+CCR7+) cells, that was very low at day +90 (1.3%) tended to rise with time, but not significantly, and was still slightly lower than in healthy controls at day +360, although this difference was not statistically significant (12.0% vs. 20.4%). Absolute count showed a progressive rise of all the CD3+CD4− subsets, but only the TTDEM population was comparable with normal individuals at day +360, while the other subsets were still significantly reduced.

## Discussion

Alemtuzumab is a powerful lytic agent for T and B lymphocytes, but not bone marrow stem cells that was originally used in the treatment of rejection in renal transplant recipients. Subsequent studies have evaluated its application in the prophylaxis of rejection. In fact, AL has been used without any other immunosuppression as an induction therapy in renal transplant recipients [2]. In this setting, however, a monocyte-mediated rejection was detected in all the seven patients evaluated, showing that lymphocyte depletion alone does not induce full tolerance in humans [2]. This condition was therefore described as 'almost' (or prope) tolerance [19]. Nowadays, the concept of AL as a 'magic bullet' to achieve tolerance has been abandoned [20]. However, maintenance immunosuppression after AL, with reduced intensity therapies, such as low-dose cyclosporine [1,6], tacrolimus [21], or sirolimus [4], has allowed acceptable rates of rejections, with relatively low incidence of viral infection and lymphoproliferative disorders.

Although used since many years in clinical practice, the influence of AL on immune system remains to be determined more in depth. To understand the characteristics of repopulating lymphocytes in AL-treated renal transplant recipients might better clarify this issue.

The degree of AL-mediated killing of leukocytes and the dynamics of their repopulation seems to be inversely related to the degree of CD52 surface expression [22]. This is higher in lymphocytes than monocytes and granulocytes. The brighter CD52 expression by CD4+ T lymphocytes in peripheral blood, as compared with CD8+ T cells, might explain the prolonged CD4/CD8 ratio inversion and the selective advantage of CD8+ lymphocytes, as carrying more CD52 implies more antibody-binding, and hence more efficient killing by complement-mediated lysis and slower reconstitution [22].

We have therefore focused our study mainly on the CD4+ populations. Previous data deriving from observations in a limited number of patients ($n = 5$) indicated that in the first 3 weeks after treatment with AL the large majority of circulating lymphocytes was represented by memory (CD45RA−CD62L−) CD4+ T cells with an activated (DR+) phenotype [10]. In three cases, these cells were evaluated for the expression of CCR7 and were found to be uniformly negative, consistent with a TEM phenotype [10]. Interestingly, these cells were relatively resistant to *in vitro* AL-mediated cell depletion and could preferentially survive because of the reduced intensity of CD52 surface expression [11]. Other studies showed that they produce high levels of IL-2 and IFN-gamma when stimulated *in vitro* [10]. *In vivo,* their number may increase in the periphery before rejection episodes and they can be detected within the allograft [10,11], indicating their role in the recipient immune response against graft antigens. However, these cells can be inhibited *in vitro* by calcineurin inhibitors [10]. This latter observation may explain the need of maintenance immunosuppression after depleting treatment with AL in the prevention of allograft rejection, which is generally planned for long-term. In fact, a study with longer follow-up showed persistently high proportions of TEM and TCM within the CD4+ population, and low naïve cell proportion up to 6 months after therapy with AL [11].

Our data confirm and extend these findings, demonstrating that even 1 year after a single infusion of AL, the proportion of the TEM CD4+ population is higher than the same in normal individuals. Moreover, the other effector population, previously poorly evaluated in this context [10], and herein called TTDEM [15], is increased during the first year after treatment. This subset, variously termed (TEMRA, CD45RA+ memory, terminally differentiated, or persisting effectors), includes highly differentiated cells which have generally lost CCR7, CD62L, CD27, and CD28 expression, and represents a major component of the CD8+ T-cell compartment with increasing age [23], but also a minor CD4+ T-cell subset [24] in the healthy individuals.

The relative sparing of the effector T-cell subsets (particularly among CD8+ cells), after depleting therapy might explain the relatively low infective risk in patients treated in this way (see Table 1 and [21]). In this respect, we have observed few cases of BK polyomavirus infection,

not dissimilar from the rate reported by others who used anti-thymocyte globulin as depleting agents, or daclizumab in transplant induction [25]. The low incidence of CMV infection in our group of AL-treated patients might be influenced also by the use of sirolimus in the maintenance regimen, as it appears that this drug is associated with lower rates of CMV infection or reactivation [26].

The most relevant result of our study is the observation that a profound defect of T cells with naïve phenotype persists up to at least 1 year after a single infusion with AL. A similar depleting effect on T cells, particularly on naïve CD4+ lymphocytes, can be observed also after induction therapy with anti-thymocyte globulin [27], but this appeared to be less profound and of shorter duration, as at 26 weeks the number of CD4+ T cells recovered was at 30% of baseline values.

Poor T-cell reconstitution after depleting therapy could result either from reduced *de novo* T-cell production by the thymus or from poor peripheral expansion of residual T cells (which is the result of the balance between cell death, and proliferation with activation resulting from the encounter with cognate antigen or bystander stimulation). To better understand this kinetics, we have evaluated the thymic output, using the combination of CD45RA and CD31 as markers of RTEs. CD31 is a surface protein expressed preferentially by naive T cells that have undergone a low number of cell divisions and is well correlated with the presence of T-cell receptor excision circles (TRECs), which, despite some limitations [28] are generally considered the best markers for RTEs [17]. *In vitro*, CD31 is lost upon T-cell receptor engagement, and is therefore not expressed after peripheral post-thymic expansion [17]. In fact, a progressive decrease of percentages and absolute numbers of naive CD31+ CD45RA+CD4+ T cells has been found associated with aging, while absolute numbers of naive CD31− CD45RA+CD4+ T cells remain fairly stable throughout life [18].

Our data herein suggest therefore that the prolonged defective thymic output after AL therapy in renal transplant recipients is one of the main causes of the persistent T-lymphopenia observed in these patients. The depleting action of AL on thymic output is well accounted for by the intense CD52 expression by virtually all the thymocytes [29], whereas CD52 is not expressed by human thymic epithelial cells [30]. Indeed, the thymus is essential for the restoration of the T-cell repertoire, but during aging, thymic function declines and is unable to meet the demand for peripheral T cells. Therefore, the recovery after a single thymus depleting insult performed by AL in aged renal recipients might be very difficult. It is of note that the number of circulating RTEs was not reduced in the control group of patients who received the nondepleting mAb basiliximab as induction therapy. Although we acknowledge that time from transplantation to RTE evaluation in this group was longer than that in the AL group, the recovery of total CD4+ T-cell number in basiliximab-treated patients was very prompt, being not different from baseline already at day +10 (see Table 3). On the other hand, the rate of RTE recovery during the first year after AL was so slow that it is difficult to hypothesize that the difference of circulating RTE numbers between basiliximab- and AL-treated patients was solely attributable to the different time interval at which the study was performed. As basiliximab-treated patients received a maintenance immunosuppressive protocol that was similar to or even more intense than AL-treated patients, we suggest that depletion of RTEs was not an aspecific effect of immunosuppression but rather that it was attributable to the action of AL. It is likely that other depleting agents such as anti-thymocyte globulin might exert a similar effect on T-cell production by the thymus [27].

An important consequence of the reduced T-cell production by the thymus after AL depletion might be a restriction of the T-cell repertoire. Interestingly, a highly restricted T-cell repertoire was in fact observed, especially in CD4+ cells, following treatment with AL in patients with B-cell chronic lymphocytic leukemia [31]. This may contribute to trigger T-cell-mediated autoimmunity. Many cases have indeed been observed, months to years after exposure to AL [32–34], mostly targeting the thyroid or blood components. However, the pattern of lymphocyte recovery in patients who received AL without further immunosuppressive co-medication might be different from that observed in kidney graft recipients. Indeed, in these patients autoimmune diseases are described but are rather rare, probably because the immunosuppressive co-medication received after transplantation modulates the recovery and the function of T cells. In this light, it should be considered also that the immunologic actions of AL might not be limited to their depleting role, as CD52 provides a costimulatory signal which can contribute to induce CD4+ T regulatory (Treg) cells. It has been shown that *in vitro* CD52-stimulated CD4+ T cells can suppress allogeneic responses of CD4+ and CD8+ T cells stimulated with mature dendritic cells [35]. In fact, the proportion of Treg (CD4+CD25+FoxP3+) was reported to be higher among patients treated with AL than among recipients not treated with AL or healthy controls [36,37].

The increase of this population is not explained by a selective sparing of Treg by AL [38] and is independent of maintenance immunosuppression [38], but can be observed after treatment with other depleting agents like anti-thymocyte globulin [27]. It has therefore been suggested that it is the result of *de novo* generation of Treg

 

from TEM [38]. These Treg may govern allogeneic immune responses by effector T cells. It is therefore interesting that the rise in Treg percentages in the peripheral blood of AL-treated patients gradually decrease if patients are maintained with calcineurin inhibitors, but not with sirolimus [38,39]. These observations have led to pilot uncontrolled studies aimed at minimizing the immunosuppressive maintenance after AL therapy [38,39]. The final goal would be to design individualized immunosuppression minimization and withdrawal programs, possibly based on monitoring of the immune system. Our demonstration of a long-term reduced thymic output, particularly in elderly patients, provides another pathophysiological piece in the complex framework of immune reconstitution after AL therapy for renal transplantation.

In conclusion, we have observed a very severe and prolonged reduction of the number of circulating RTEs after a single infusion of AL. The prolonged defective thymic output is likely to be one of the main causes of the persistent CD4+ T-lymphopenia observed in renal transplant recipients treated in this way and it might have important clinical consequences. This should be considered in the design of future studies aimed at better tailoring immunosuppressive therapies.

## Authorship

MS, SS, and PA: study conception; MS, FM, and PA: study design; MS, NB, and FV: data collection; MS: data analysis; NB, FV, and SS: clinical care; FM and PA manuscript redaction.

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