T-cell receptor repertoire usage after allografting differs between CD4+CD25+ regulatory T cells and their CD4+CD25- counterpart

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Funding: This work was supported by the Leukaemia Research Fund. Claudio Fozza is the recipient of the fellowship "Master and back TS 07" offered by the Region of Sardinia.

Manuscript received September 15, 2006. Accepted January 9, 2007.

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ABSTRACT

Background and Objectives

After allogeneic haematopoietic stem cell transplantation (SCT) the whole T-cell receptor (TCR) repertoire shows a markedly skewed pattern for 2-3 years. A small fraction of $CD4^+$ T cells is represented by $CD25^+$ regulatory lymphocytes (T_{reg}), which play a crucial role in modulating peripheral tolerance. To investigate their ability to react to the massive antigenic stimulation generated in an allogeneic host, which could significantly affect their pattern of reconstitution, we analyzed the TCR repertoire of T_{reg} after SCT, focusing on the degree of similarity to $CD4^+CD25^-$ conventional T cells (T_{conv}).

Design and Methods

We assessed the TCR Vβ repertoire of T_{reg} in ten patients who had received allogeneic SCT, by using complementarity determining region 3 (CDR3) spectratyping. We developed a new *similarity* score for the analysis. This score expresses the proportion of Vβ with similar profile between T_{reg} and T_{conv}.

Results

For up to 3 years after SCT the repertoires of T_{reg} and T_{conv} were characterized by several Vβ with different profiles between the two cell subsets, while they were extremely similar in patients more than 3 years post-allografting (similarity score= 0.90 vs. 0.61). The differences observed early after SCT were mainly ascribable to Vβ expressing an oligoclonal profile in T_{conv} but not in T_{reg}.

Interpretation and Conclusions

Our data show that the TCR repertoires of T_{reg} and T_{conv} are significantly different early post-SCT, while they tend to become identical with full reconstitution. This difference could reflect either a discrepancy in the *in vivo* reactivity against common antigenic stimulations or be the result of different post-transplant ontogeny.

Key words: T-cell receptor repertoire, regulatory T cells, allogeneic stem cell transplantation.

Haematologica 2007; 92:206-214

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The diversity of the T-cell receptor (TCR) repertoire is mainly determined by the complementarity determining region 3 (CDR3), which is one of the key players in the context of antigen recognition and major histocompatibility complex restriction. This diversity is due both to extensive rearrangement between V, D and J segments in the TCRα and TCRβ genes and to random junctional nucleotide insertions and deletions, which generate CDR3 regions of different lengths.[1,2] After allogeneic hematopoietic stem cell transplantation (SCT) the overall TCR repertoire is characterized by a lower diversity and a markedly skewed pattern. The TCR repertoire may start to normalize at about 6 months after transplant but most patients continue to show an abnormal profile until 2-3 years after grafting, with different kinetics between $CD4^+$ and $CD8^+$ cells.[3-5]

Natural regulatory T cells (T_{reg}) are a subpopulation of thymus-derived $CD4^+$ T cells which constitutively express the interleukin-2 receptor α chain (CD25).[6] They were initially described because of their ability to suppress autoreactive T cells in the periphery.[7,8] T_{reg} play a crucial role in the maintenance of peripheral tolerance to self and foreign antigens and modulate susceptibility to autoimmune,[9-11] infective[12] and neoplastic diseases.[13-16]

T_{reg} are characterized by a few other molecules apart from CD25, including cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR) and the Forkhead box P3 (FoxP3) gene product.[6] In contrast to murine T_{reg}, human T_{reg} are mainly confined within the fraction expressing high levels of CD25,[17] which possesses potent immunosuppressive activity and co-expresses FoxP3, whereas the subset expressing intermediate levels of CD25 contains mainly activated T cells with little or no immunosuppressive function.[18,19]

The role of T_{reg} in the induction of immunological tolerance after SCT has not been fully elucidated. Administration of these cells has a protective effect in murine models of acute graft-versus-host disease (GVHD), related to inhibition of the early expansion of alloreactive donor T cells.[20] Such an activity does not seem to interfere with stem cell engraftment or the graft-versus-leukemia effect.[21] Also the studies conducted in humans have suggested a correlation with different outcomes between GVHD and circulating T_{reg}.[22-26] Although, once activated, T_{reg} seem to perform their activity in a non-antigen-specific way,[27] they require activation via their TCR to become suppressive.[28] A better knowledge of their TCR repertoire in the post-transplant setting would offer relevant information about their ability to react to the massive antigenic stimulation generated in an allogeneic host, which could significantly affect their pattern of reconstitution. Moreover, the requirement for thymic maturation[29] could further contribute to altering the repertoire of T_{reg}, as has been found for conventional T cells.[3-5] Although it is well known that the repertoire of antigen specificities of T_{reg} in mice is as broad as that of naive T cells,[30] only two studies have been conducted in humans.[31,32] In normal subjects the TCR repertoire of T_{reg} appears to be similar to that of the $CD25^-$ counterpart, thus suggesting the recognition of a similar spectrum of antigens.[31] However, no studies focusing on the analysis of the TCR CDR3 repertoire of this cell subpopulation after allografting have been performed so far.

We examined the TCR Vβ repertoire of T_{reg} in patients who had received allogeneic SCT for chronic myeloid leukemia (CML), by using CDR3 spectratyping. We concentrated our analysis on the $CD4^+CD25^+$ subset expressing high levels of CD25, which contains the vast majority of regulatory T cells,[17] as we confirmed by FoxP3 staining. Purified T_{reg} were compared to the $CD4^+CD25^-$ conventional T (T_{conv}) counterpart, analyzing the degree of similarity within each Vβ subfamily.

Design and Methods

Patients

We performed our analysis in ten patients (five males and five females) who had undergone allogeneic SCT for chronic myeloid leukemia. As controls, we analyzed samples from seven adult healthy volunteers (three fresh blood samples and four buffy coats from single blood donations). All patients and donors had given informed consent. The study had been approved by the local ethics committee. Clinical and transplant characteristics are summarized in Table 1. The median age of the patients was 41.5 years (range 25-49). Nine had been transplanted in chronic phase and one in accelerated phase. Nine of them had received a myeloablative conditioning regimen with cyclophosphamide and total body irradiation and one had received a reduced intensity conditioning (RIC) regimen consisting of fludarabine and busulphan. Six patients had undergo *in vivo* T-cell depletion with campath-1H. All patients had received cyclosporine and methotrexate as GVHD prophylaxis, except two (including the one receiving RIC) who were given cyclosporine alone. Four and six patients had developed acute and chronic GVHD, respectively.

At the time of analysis, the median time after transplant was 37 months (range 4-171). Five out of the ten patients showed a BCR-ABL/ABL ratio ≤0.02%, indicative of molecular remission. Four of the patients had previously received donor lymphocyte infusions (DLI) for molecular relapse and two of them had achieved molecular remission. Samples were collected an average of 33 months (range 11-70) after the last infusion. Only one patient had active limited GVHD (ocular). None of the patients was or had recently been on systemic steroid treatment. One of the patients was affected by herpes zoster infection and had had a cytomegalovirus (CMV) reactivation 3 months before.

Cell separation

CD4+CD25+ and CD4+CD25- subpopulations were isolated from 40 mL of heparinized peripheral blood samples using a two-step procedure. First, CD4 negative isolation (depletion of CD8, CD16, CD19, CD36, CD56, CD66b and glycophorin A) was performed using Rosette Sep enrichment mixture (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's specifications. Next, CD25 positive selection was carried out with magnetic beads (Dynal Biotech, Oslo, Norway). The obtained CD4+CD25- cells were kept in RPMI media supplemented with 10% AB serum and 1% penicillin and streptomycin. The CD4+CD25+ cells were left overnight in supplemented media and cells and, after spontaneous detachment from the beads, were exhaustively washed in order to separate only cells expressing high levels of CD25. The numbers of CD4+CD25+ and CD4+CD25- cells isolated from each patient ranged between 0.3 and 1.5×10^6, and 1.1 and 6.9×10^6, respectively. TRIzol (Invitrogen, Paisley, UK) was added to both cell fraction pellets which were kept at -80°C until RNA extraction.

Cytofluorimetric analysis

The purity of the cell separation was assessed by staining with specific monoclonal antibodies for CD4 and CD25 and with the corresponding isotype controls (BD Pharmingen, Oxford, UK) after both the CD4 negative and the CD25 positive selection. Intracellular analysis of FoxP3 expression was performed using anti-FoxP3 antibody (clone PCH101- FITC from Ebioscience, San Diego, CA, USA). Samples were acquired using a FACSCalibur and analyzed with Cell-Quest software (BD Biosciences).

CDR3 spectratyping

The RNA was isolated from CD4+CD25+ and CD4+CD25- cells as described elsewhere.[33] For each sample, complementary DNA was synthesized from the same starting amount of total RNA (0.7 μg), by using Superscript III reverse transcriptase and random hexamer primers (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed in a volume of 25 μL comprising 1xPCR buffer, 2.5 mM $MgCl_2$, 1 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 200 μM deoxyribonucleoside triphosphate and 500 nM of one of 24 TCR Vβ primers combined with 1 Cβ primer conjugated to the fluorescent dye 6-carboxyfluorescein-amino-hexy (6-FAM).[34] The sequences of the Vβ and Cβ primers were described previously.[35] The PCR conditions were: 95°C for 10 minutes followed by 36 cycles of 94°C for 20 seconds, 55°C for 40 seconds, 72°C for 40 seconds and a final extension of 72°C for 5 minutes. The PCR fragments were then run on an ABI Prism 3100 Genetic Analyzer and data were collected and analyzed by Gene Mapper ID Software version 3.2 (Applied Biosystems, Foster City, CA, USA).

Table 1. Patients' characteristics.

Patient and disease features	
Number	10
Median age (years)	41.5 (25-49)
Median time after SCT (months)	37 (4-171)
Sex	
Male	5
Female	5
Diagnosis of chronic myeloid leukemia	
chronic phase	9
accelerated phase	1
Transplant-related factors	
Conditioning regimen	
Myeloablative	9
Non-myeloablative	1
Stem cell source	
Bone marrow	5
Peripheral blood	5
In vivo T-cell depletion	
Yes	6
No	4
Donor	
Sibling	5
Unrelated	5
HLA	
Matched	7
Mismatched	3 (minor)
Transplant-related events	
Acute GVHD	
Yes	4
No	6
Chronic GVHD	
Yes	6 (active 1)
No	4
Donor lymphocyte infusions	
Yes	4
No	6
Molecular remission	
Yes	5
No	

SCT: stem cell transplantation; GVHD: graft-versus-host disease.

Spectratyping analysis

Conventional analysis of spectratyping profiles was performed according to the following standard approaches: (i) scoring the number of peaks, in order to determine the overall complexity of the repertoire; (ii) analyzing profiles by peak area and shape, in order to establish the degree of skewing and oligoclonality. Moreover, to quantify the proportion of Vβ subfamilies that were similar between T_{reg} and T_{conv}, we developed a novel analysis method described in the result section.

The overall complexity of the TCR Vβ repertoire was determined according to the number of discrete peaks detected per Vβ subfamily, each subfamily being graded on a score from 0 to 5. Spectratypes containing five or more peaks were given a score of 5. The overall spectratype complexity score was calculated for both cell subsets in each subject, as the sum of the scores for each subfamily.[36]

Each spectratype profile was also assessed according to previously described criteria.[36] A profile was defined as normal if it showed a Gaussian bell-shaped distribution,

with discrete peaks spaced by three nucleotides. Evidence of oligoclonal expansion or skewing was assessed by calculating the relative fluorescence intensity (RI) of each peak (RI = peak area ÷ total peak area). A profile was defined as skewed if: (i) a dominant peak with a RI greater than 35% of the total peak area, corresponding to an oligoclonal profile, was observed; or (ii) two dominant peaks were present and the RI of each peak was greater than 25% of total peak area; or (iii) there were multiple peaks differing from a Gaussian pattern and the RI of the dominant peaks was greater than 25% of total peak area. The percentages of skewed and oligoclonal Vβ among the total number of Vβ analyzed were calculated.

Statistical analysis

The Student's t test was used to assess differences in the complexity score, percentage of skewed or oligoclonal Vβ subfamilies and the similarity score between different groups of subjects. Spearman's correlation coefficient was determined to evaluate the correlation between the similarity score and time after SCT. All quoted *p*-values are two-sided with values <0.05 considered statistically significant.

Results

Characterization of the selected cell subpopulations

After the first step of separation, the proportion of $CD4^+CD25^-$ cells in all samples was >93% (range 93-99%) (Figure 1A). The analysis of the $CD4^+CD25^+$ subset, performed after the immunomagnetic separation, showed a high proportion of $CD4^+$ cells expressing high levels of CD25 between 85% and 92% (Figure 1B). In order to further determine the nature of the selected cell subpopulations, intracellular analysis of FoxP3 expression was performed. The expression of FoxP3 was almost exclusively confined to the isolated $CD4^+CD25^+$ subset (Figure1C and 1D), thus conclusively confirming that this subset belongs to the regulatory lineage. The percentages of $FoxP3^+$ cells within the T_{reg} and T_{conv} subpopulations were 81.8±11% and 3.7±2%, respectively.

T_{reg} and T_{conv} exhibit a similar overall complexity and skewing in the TCR repertoire

The mean number of Vβ profiles available per sample was 21 (range, 16-24) for both T_{reg} and T_{conv} in patients as well as in controls. Figure 2 shows representative spectratyping profiles of one of the patients, whilst Table 2 summarizes the data of the TCR repertoire analysis for each patient. The overall spectratype complexity score was not different between T_{reg} and T_{conv} in both the patients (mean 106.5 vs. 105.5) and controls (mean 108.5 vs. 112.5). In the latter, T_{reg} and T_{conv} showed TCR repertoires with a predominantly Gaussian distribution and shared common patterns also in terms of percentages of skewed and oligoclonal Vβ, thus confirming previous data.[31,32]

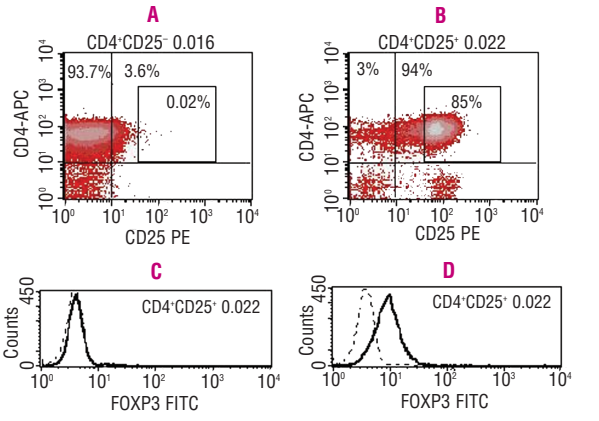

Figure 1. FoxP3 is selectively expressed on purified $CD4^+CD25^+$ cells. A. Representative profile of the $CD4^+CD25^-$ subpopulation obtained after $CD4^+$ negative isolation using the Rosette Sep enrichment mixture (depletion of CD8, CD16, CD19, CD36, CD56, CD66b and glycophorin A); B. Representative profile of $CD4^+CD25^+$ purified cells after CD25 positive selection with magnetic beads. The plots in the lower panel show the intracellular expression profile of FoxP3 in the $CD4\,CD25^-$ (C) and $CD4^+CD25^+$ (D) subsets (solid lines: FoxP3; dotted line: isotype control).

It is well documented that the $CD4^+$ TCR repertoire is not fully re-established until at least 2-3 years following allogeneic SCT.[3-5] Accordingly, we observed a significantly higher percentage of skewed Vβ in both cell subpopulations in patients whose SCT had been performed less than 3 years previously, as compared to those in whom more than 3 years had passed since their SCT (mean 41% vs. 22%, $p<0.05$, in T_{reg} and 45% vs. 15%, $p<0.05$, in T_{conv}) (Figure 3A). Moreover, the percentage of oligoclonal Vβ tended to be higher in patients closer to the SCT in both cell subpopulations (15% vs. 5%, *p*=ns, in T_{reg} and 27% vs. 4%, $p<0.05$, in T_{conv}) (Figure 3B). However, when we compared the two cell subsets of each subject following this approach, we did not identify any significant difference in the percentage of skewed and oligoclonal Vβ between T_{reg} and T_{conv}, regardless of time elapsed since the SCT. Nevertheless, it is worth noting that in patients who had undergone SCT less than 3 years previously, the percentage of oligoclonal Vβ tended to be higher in T_{conv} than in T_{reg} (27% vs. 15%, *p*=ns). When we looked at the prevalence of skewed or oligoclonal profiles in specific Vβ subfamilies, we did not find any predominant pattern, with the exception of a higher frequency of skewing in Vβ 10 and 19, which are known to be encoded by pseudogenes.

The "similarity score" detects different TCR repertoires between T_{reg} and T_{conv}

The conventional methods of analysis evaluate the overall complexity of the repertoire and are, therefore, inadequate for establishing a direct comparison within each Vβ subfamily between different cell subpopulations. To address this issue, we specifically compared the spectratyping profiles of T_{reg} and T_{conv} of each subject within each of the 24 Vβ subfamilies. In order to do this, we

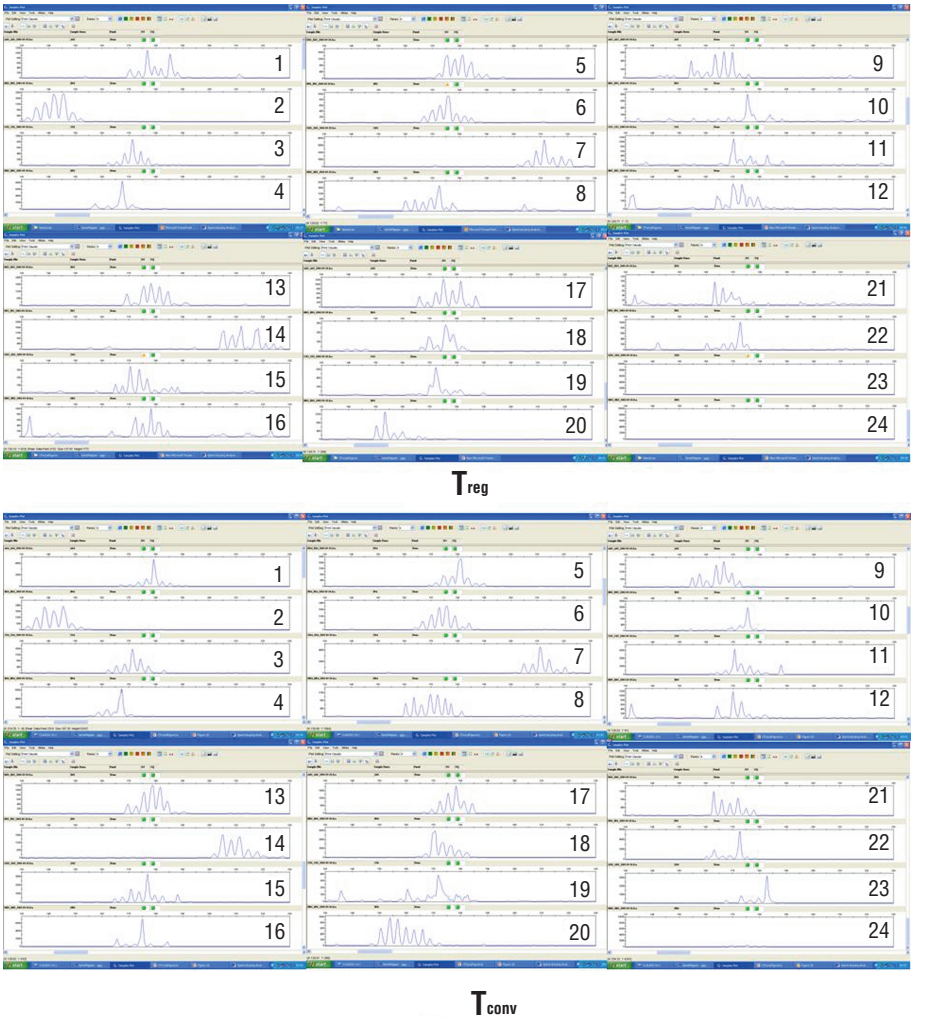

Figure 2. Representative CDR3 profiles of the 24 Vβ subfamilies in the T_{reg} and T_{conv} subpopulations. The investigation was carried out 6 months after SCT in a patient who had an active herpes zoster infection and had previously had CMV reactivation. The similarity score was 0.64, with 14 Vβ coincident between the two cell subpopulations out of 22 Vβ pairs analyzed (Vβ 23 and 24 were excluded as not detected in both subsets). Vβ 1, 5, 15, 16 and 18 expressed an oligoclonal profile only in the T_{conv} subset; Vβ 20 was oligoclonal only in the T_{reg} subpopulation and Vβ 12 and 14 displayed a skewed but non-oligoclonal profile in only one of the cell subsets.

developed a new score to express *similarity*. Within each Vβ subfamily, the spectratype profiles of T_{reg} and T_{conv} were considered to be similar if: (i) both showed a Gaussian bell-shaped distribution; or (ii) both showed the same skewed pattern, according to the criteria previously defined; or (iii) an oligoclonal peak was observed in the

Table 2. Summary of T-cell receptor repertoire pattern and transplanted related features of the analyzed patients.

Patient Number	$CD4^+CD25^+$ cells in PB (% of $CD4^+$ cells)	$CD4^+CD25^{high+}$ cells in PB (% of $CD4^+$ cells)	Skewed Vβ in T_{reg} (%)	Skewed Vβ in T_{conv} (%)	Oligoclonal Vβ in T_{reg} (%)	Oligoclonal Vβ in T_{conv} (%)	Similarity score	Months after SCT	Stem cell source	Donor type	In vivo T-cell depletion	Active aGVHD/ cGVHD	Months from previous aGVHD/ cGVHD	Active infection	Molecular remission	Months after DLI
1	31.35	2.16	50	50	6	19	0.66	22	PB	MUD	Y	N/N	ND/17	N	N	NR
2	15.39	1.05	13	13	4	4	1.00	52	PB	SIB	N	N/N	ND/45	N	N	11
3	55.65	0.66	39	76	26	52	0.35	4	BM	MUD	Y	N/N	ND/ND	N	N	NR
4	NA§	NA§	25	17	4	0	0.92	125	BM	SIB	N	N/N	122/ND	N	Y	37
5	22.42	1.81	50	56	23	43	0.64	6	PB	MUD	Y	N/N	ND/ND	Y†	N	NR
6	57.14	3.93	19	23	10	9	0.95	89	BM	MUD	Y	N/N	87/62	N	Y	70
7	38.65	1.51	22	4	9	0	0.86	171	BM	SIB	N	N/N	169/157	N	N	15
8	9.11	0.13	15	11	10	8	0.83	32	PB	MUD	Y	N/N	30/21	N	Y	NR
9	20.62	0.90	31	17	0	6	0.75	42	BM	SIB	Y	N/Y	ND/0*	N	Y	NR
10	NA§	NA§	50	32	8	15	0.64	9	PB	SIB	N	N/N	ND/ND	N	Y	NR

**Ongoing at the moment of the analysis (ocular, not on systemic steroid treatment); †herpes zoster; §sample not sufficient for flow cytometric analysis. BM: bone marrow; DLI: donor lymphocyte infusions; GVHD: graft-versus-host disease (a: acute; c: chronic); MUD: matched unrelated donor; N: no; NA: not available; ND: not developed; NR: not received; PB: peripheral blood; SCT: stem cell transplantation; SIB: sibling donor; Tconv: conventional T cells; Treg: regulatory T cells; Y: yes.*

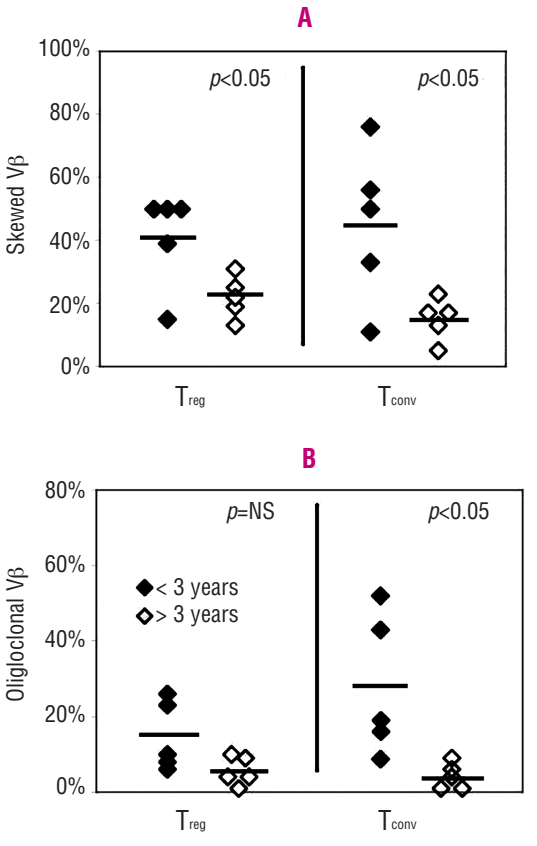

Figure 3. The percentages of both skewed and oligoclonal Vβ subfamilies are higher in patients with a shorter time between SCT and the analysis. The figures report the percentages of skewed (A) and oligoclonal (B) Vβ in the two cell subpopulations, detected in patients less (R) or more (r) than 3 years after transplantation. Horizontal bars indicate the mean for each group.

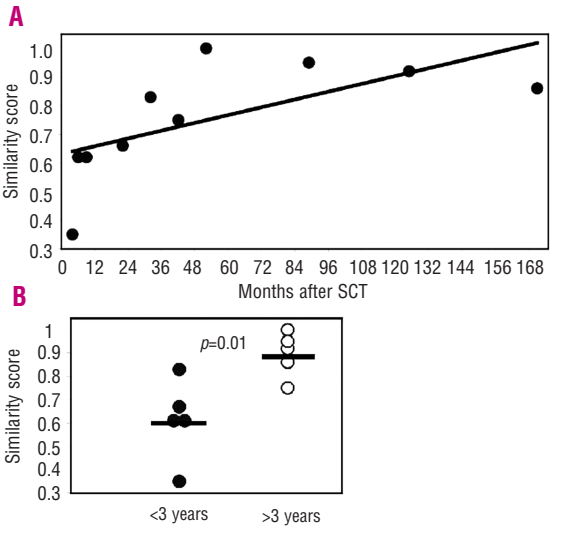

Figure 4. The TCR repertoires of T_{reg} and T_{conv} differ early after transplant while they tend to become identical more than 3 years post-SCT. A. The similarity score was calculated in each subject (?) as a ratio between the number of Vβ ?subfamilies with a profile coincident between the two cell subsets and the total number of subfamilies available in both cell subpopulations. Pearson's correlation coefficient, calculated for the relation between similarity score and time after SCT, was 0.65. B. Similarity scores in patients less (?) or more (?) than 3 years after transplantation. Horizontal bars indicate the mean for each group.

same position in both cell subpopulations. The similarity score was calculated for each subject as the ratio between the number of Vβ with coincident profiles between the two cell subpopulations and the total number of Vβ available in both cell subsets. For example, the patient whose repertoire is represented in Figure 2, who showed 14 Vβ with coincident profiles between the two cell subpopulations out of 22 Vβ available in both cell subsets, had a ratio of 14/22, equivalent to a score of 0.64.

Using this approach, we detected a positive correlation between the similarity score and time elapsed since the SCT (Pearson's correlation coefficient=0.65) (Figure 4A). A higher score was observed in patients more than 3 years after allografting, as compared to those investigated less than 3 years after SCT (mean 0.90 vs. 0.61, p=0.01) (Figure 4B). Remarkably, the similarity score in patients more 3 years after SCT was close to that detected in fresh blood samples of normal volunteers (mean 0.87).

We found four different patterns among the mismatching Vβ profiles: (i) an oligoclonal profile only in the T_{reg}; (ii) an oligoclonal profile only in the T_{conv} subpopulation; (iii) a skewed but not oligoclonal profile only in the T_{reg}, and (iv) a skewed but not oligoclonal profile only in the T_{conv} subset. Noticeably, in patients investigated less than 3 years after SCT the mismatches were very often ascribable to the detection in the same Vβ subfamily of an oligoclonal profile in the T_{conv} but not in the T_{reg} subpopulation, while the opposite condition was much less frequent (mean 52% vs. 23%, p=0.08). When considering only those patients whose SCT had been performed less than 2 years previously, this trend became statistically significant (mean 56% vs. 12%, p=0.01). Moreover, the presence of oligoclonal profiles confined to the T_{conv} subset was almost exclusively detected in patients in whom less than 3 years had elapsed since SCT (mean 52% vs. 5%, p=0.002) (Figure 5). No significant differences were shown in the group of patients who had undergone SCT more than 3 years previously, in whom the mismatches were equally distributed between the two cell subsets and more often due to a skewed profile in only one of them (*data not shown*). A clear example of selective oligoclonal profiles in the T_{conv} subpopulation early after SCT is represented in Figure 2. This patient was investigated 6 months post-SCT and had an active herpes zoster infection and a previous CMV reactivation. His repertoire was characterized by five Vβ subfamilies expressing an oligoclonal profile only in the T_{conv} subset.

Role of disease status and transplant-related factors

Patients in molecular remission at the time of analysis showed a lower percentage of both oligoclonal and skewed Vβ (*data not shown*). The percentages of skewed

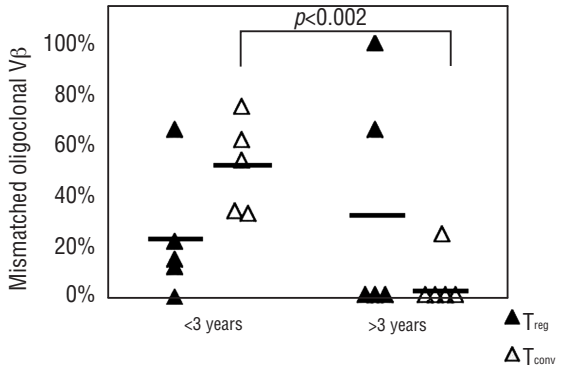

Figure 5. Oligoclonal profiles selectively confined to T_{conv} are more frequent in patients with a shorter time between SCT and the analysis. The frequency of oligoclonal profiles confined to either the T_{reg} (▲) or the T_{conv} (△) subpopulation in patients less or more than 3 years after SCT is represented. The y axis represents the percentage of Vβ showing an oligoclonal profile confined to each cell subpopulation among the overall non-similar Vβ subfamilies. Horizontal bars indicate the mean for each group.

and oligoclonal Vβ in T_{reg} were 35% and 14% vs. 28% and 6% in the non-remission and remission group, respectively. The percentages in T_{conv} were 40% and 24% vs. 20% and 8%, respectively. However, none of these differences reached a statistical significance. We did not find any difference in the similarity score between patients in remission or not.

Furthermore, the percentages of oligoclonal and skewed Vβ as well as the degree of similarity between the two cell subpopulations were not influenced by other factors such as stem cell source, donor type, HLA matching, *in vivo* T-cell depletion and previous acute or chronic GVHD (*data non shown*).

Effect of DLI

Previous treatment with DLI was associated with an increase in the amplitude of the TCR repertoire, defined on the basis of a lower percentage of oligoclonal and skewed Vβ (*data not shown*). In the T_{reg} subpopulation, the percentages of skewed Vβ in the DLI and non-DLI group were 20% and 39% (p=0.02), respectively, whilst the percentages of oligoclonal Vβ in the treated and untreated groups were 7% and 12% (p=ns). In T_{conv}, the percentages of skewed Vβ in patients who did or did not receive DLI were 14% and 40% (p=ns), respectively, whilst the percentages of oligoclonal Vβ were 3% and 24% (p<0.05). Previous treatment with DLI was strongly associated with a higher similarity score between the two cell subsets (p=0.005).

Discussion

In the last years, T_{reg} have progressively emerged as an essential component in the control and maintenance of peripheral tolerance. One of their most peculiar functional features is the requirement of activation via TCR to become suppressive,[28] notwithstanding their ability to act in a non-antigen-specific way.[27] As recent studies provided evidence for their specificity in the modulation of transplant alloreactivity,[37-39] a deeper knowledge of their TCR repertoire pattern in the post-transplant setting would offer relevant information about their ability to react to the massive antigenic stimulation generated in an allogeneic host. It is known that this cell subpopulation presents a TCR repertoire profile substantially similar to that of the $CD25^-$ counterpart in healthy subjects.[31,32] Since the overall T-cell repertoire after allogeneic SCT is greatly impaired[3-5] and naturally occurring T_{reg} require maturation in the thymus,[29] their TCR usage may be equally abnormal. We analyzed the CDR3 Vβ repertoire of purified $CD4^+CD25^+$ cells expressing high levels of CD25 in patients who had undergone allografting for chronic myeloid leukemia. This cell subpopulation contains the vast majority of cells with regulatory activity,[17] as we confirmed by FoxP3 staining. Our analysis focused on the overall complexity of the repertoire and on the degree of similarity to the T_{conv} counterpart. Our study is based on the examination of a remarkably large number of Vβ profiles in each cell subpopulation (mean 21; range 16-24), especially considering the profound lymphopenia observed after allogeneic SCT and the exiguity of the T_{reg} subset. Despite the limited number of patients analyzed, this study is the first CDR3 spectratyping analysis ever performed on the T_{reg} subset after transplantation. All previous studies have been performed either on unfractionated peripheral blood mononuclear cells[4,5,40,41] or on the $CD4^+$ and $CD8^+$ fractions.[3] Moreover, our group of patients was almost completely homogeneous in terms of disease type and conditioning regimen. None of the other transplant-related factors and post-transplant variables, apart from DLI administration, turned out to have a significant influence on the TCR repertoires of the two cell subsets in terms of both overall skewing and degree of similarity between T_{reg} and T_{conv}.

The conventional systems for spectratyping analysis, based on the determination of the overall complexity and skewing of a cell subpopulation, showed essentially similar TCR patterns between T_{reg} and T_{conv}. However, these methods are inadequate to establish a direct comparison within each Vβ subfamily between two cell subsets. We, therefore, developed a new score system, based on a quantification of the proportion of Vβ subfamilies with coincident profiles between T_{reg} and T_{conv}. This analysis revealed significant differences between the two repertoires within the first 3 years after SCT. The differences were mainly due to the frequent presence of Vβ expressing an oligoclonal profile in the T_{conv} but not in the T_{reg} subpopulation. The markedly skewed profile detected in the T_{conv} subpopulation resembled the features described early after allografting in the $CD4^+$ subset, in which the repertoire reconstitutes within 3 years.[3-5] In fact, after 3 years, the TCR Vβ profiles of the two cell subsets tended to

become identical, paralleling the T_{conv} subset recovery. The much higher degree of TCR similarity between the two cell subsets observed in patients more than 3 years after SCT as compared to those with a more recent transplant could reflect the kinetics of reconstitution of the whole T-cell compartment. On the other hand, the marked differences between the T_{conv} and T_{reg} repertoires described in recipients early after SCT seems to be ascribable to a reduced degree of skewing confined to the T_{reg} subset. The repertoire amplitude and the degree of similarity between the two cell subsets were increased in patients treated with DLI, thus corroborating its possible contribution to the re-establishment of the TCR repertoire.[42,43]

Although the intrinsic *in vitro* anergic features of T_{reg}[44] are fully consistent with the observed discrepancy, these cells have been shown to proliferate actively *in vivo* after antigenic stimulation,[45,46] especially in a lymphopenic environment.[22,47] However, from our data it appears that, in the presence of a massive antigenic stimulation, such as the one generated in an allogeneic host, there is selective expansion of T_{conv} rather than T_{reg}, producing oligoclonal profiles in their repertoire. On the other hand, the reduced degree of oligoclonality observed in the TCR repertoire of T_{reg} when compared to T_{conv}, does not necessarily mean that T_{reg} are impaired or that they have not been activated. In fact, the detection of a less restricted repertoire could be an expression of their ability to exert a suppressive effect in a non-antigen-specific manner,[27] notwithstanding the requirement of activation via TCR.[28] It is worth noting that interleukin-2[48] as well as Toll-like receptor 2 ligands of bacterial or fungal origin,[49] both of which can be largely present after SCT, are also important mediators of T_{reg} activation. Another factor that might explain the reduced similarity between the TCR repertoires of the two subpopulations early after allografting is a different origin. T_{reg} are positively selected in the thymus as a consequence of an interaction between a self-peptide and their TCR.[29] The detection of oligoclonal patterns, suggestive of antigen-driven proliferations, in T_{conv} but not in T_{reg} could imply that the TCR repertoire is shaped by a peripheral antigen-induced expansion in T_{conv} and by a broader thymic-dependent selection in T_{reg}.

Murine models[20] as well as clinical studies[22] suggest that T_{reg} of donor origin are primarily involved in transplantation tolerance. Interestingly, on one occasion we had the possibility to compare a patient's TCR profile 52 months after SCT with that of her donor (*data not shown*). We observed that the patient's repertoire was fully reconstituted, with a Gaussian profile in all the Vβ subfamilies, except for an oligoclonal pattern in Vβ20. The donor's repertoire was nearly identical, including the Vβ20, which showed the same oligoclonal profile. Noteworthy, the patient had received DLI 11 months before, which had produced a cytogenetic remission.

In conclusion, our data show that the TCR repertoires of T_{reg} and T_{conv} exhibit significant differences early after SCT, which are mainly ascribable to V? subfamilies expressing an oligoclonal profile in the T_{conv} but not in the T_{reg} subpopulation. The TCR V? profiles of the two subsets tend to become identical with full reconstitution. These different patterns may reflect a different *in vivo* reactivity against common antigenic stimulations or result from a disparity in post-transplant T-cell ontogeny.

Authors Contributions

CF performed the research, analyzed the data and wrote the manuscript; EN performed the research and provided intellectual input and critical feedback to the manuscript; ML provided intellectual input and critical feedback to the manuscript; FD designed the research, supervised all aspects of the study and reviewed the manuscript.

Conflicts of Interest

The authors reported no potential conflicts of interest.

References

1. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402.
2. Nikolich-Zugich J, Slifka MK, Messaoudi I. The many important facets of T-cell repertoire diversity. Nat Rev Immunol 2004;4:123-32.
3. Hentschke P, Omazic B, Mattsson J, Nasman-Bjork I, Lundkvist I, Gigliotti D, et al. T-cell receptor Vbeta repertoire after myeloablative and reduced intensity conditioning allogeneic haematopoietic stem cell transplantation. Scand J Immunol 2005;61:285-94.
4. Peggs KS, Verfuerth S, D'Sa S, Yong K, Mackinnon S. Assessing diversity: immune reconstitution and T-cell receptor BV spectratype analysis following stem cell transplantation. Br J Haematol 2003;120:154-65.
5. Wu CJ, Chillemi A, Alyea EP, Orsini E, Neuberg D, Soiffer RJ, et al. Reconstitution of T-cell receptor repertoire diversity following T-cell depleted allogeneic bone marrow transplantation is related to hematopoietic chimerism. Blood 2000;95:352-9.
6. Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004;22:531-62.
7. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995;155:1151-64.
8 . Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. Nat Immunol 2001;2:816-22.
9. Cao D, van Vollenhoven R, Klareskog L, Trollmo C, Malmstrom V. $CD25^{bright}CD4^{+}$ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease. Arthritis Res Ther 2004;6:R335-46.
10. Crispin JC, Martinez A, Alcocer-Varela J. Quantification of regulatory T cells in patients with systemic lupus erythematosus. J Autoimmun 2003; 21:273-6.
11. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J Exp Med 2004;200:277-85.
12. Hori S, Carvalho TL, Demengeot J. CD25+CD4+ regulatory T cells suppress CD4+ T cell-mediated pulmonary hyperinflammation driven by Pneumocystis carinii in immunodeficient mice. Eur J Immunol 2002; 32:1282-91.
13. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. Clin Cancer Res 2003;9:4404-8.
14. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, et al. Prevalence of regulatory T cells is increased in peripheral blood and

tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol 2002; 169:2756-61.
15. Viguier M, Lemaitre F, Verola O, Cho MS, Gorochov G, Dubertret L, et al. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. J Immunol 2004; 173:1444-53.
16. Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, et al. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res 2001;61:4766-72.
17. Baecher-Allan C, Wolf E, Hafler DA. Functional analysis of highly defined, FACS-isolated populations of human regulatory CD4+ CD25+ T cells. Clin Immunol 2005;115:10-8.
18. Cesana GC, DeRaffele G, Cohen S, Moroziewicz D, Mitcham J, Stoutenburg J, et al. Characterization of CD4+CD25+ regulatory T cells in patients treated with high-dose interleukin-2 for metastatic melanoma or renal cell carcinoma. J Clin Oncol 2006;24:1169-77.
19. Wing K, Ekmark A, Karlsson H, Rudin A, Suri-Payer E. Characterization of human CD25+ CD4+ T cells in thymus, cord and adult blood. Immunology 2002;106:190-9.
20. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. Nat Med 2003;9:1144-50.
21. Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, et al. Recipient-type specific CD4+CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. J Clin Invest 2003;112:1688-96.
22. Clark FJ, Gregg R, Piper K, Dunnion D, Freeman L, Griffiths M, et al. Chronic graft-versus-host disease is associated with increased numbers of peripheral blood CD4+CD25high regulatory T cells. Blood 2004; 103:2410-6.
23. Meignin V, de Latour RP, Zuber J, Regnault A, Mounier N, Lemaitre F, et al. Numbers of Foxp3-expressing CD4+CD25high T cells do not correlate with the establishment of long-term tolerance after allogeneic stem cell transplantation. Exp Hematol 2005; 33:894-900.
24. Miura Y, Thoburn CJ, Bright EC, Phelps ML, Shin T, Matsui EC, et al. Association of Foxp3 regulatory gene expression with graft-versus-host disease. Blood 2004;104:2187-93.
25. Sanchez J, Casano J, Alvarez MA, Roman-Gomez J, Martin C, Martinez F, et al. Kinetic of regulatory CD25high and activated CD134+ (OX40) T lymphocytes during acute and chronic graft-versus-host disease after allogeneic bone marrow transplantation. Br J Haematol 2004;126:697-703.
26. Zorn E, Kim HT, Lee SJ, Floyd BH, Litsa D, Arumugarajah S, et al. Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. Blood 2005;106:2903-11.
27. Thornton AM, Shevach EM. Suppressor effector function of CD4+ CD25+ immunoregulatory T cells is antigen nonspecific. J Immunol 2000; 164:183-90.
28. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 1998; 188: 287-96.
29. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, et al. Thymic selection of CD4+ CD25+ regulatory T cells induced by an agonist self-peptide. Nat Immunol 2001;2:301-6.
30. Pacholczyk R, Kraj P, Ignatowicz L. Peptide specificity of thymic selection of CD4+CD25+ T cells. J Immunol 2002;168:613-20.
31. Kasow KA, Chen X, Knowles J, Wichlan D, Handgretinger R, Riberdy JM. Human CD4+CD25+ regulatory T cells share equally complex and comparable repertoires with CD4+CD25- counterparts. J Immunol 2004; 172:6123-8.
32. Fujishima M, Hirokawa M, Fujishima N, Sawada K. TCRalphabeta repertoire diversity of human naturally occurring CD4+CD25+ regulatory T cells. Immunol Lett 2005;99:193-7.
33. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-9.
34. Chen X, Barfield R, Benaim E, Leung W, Knowles J, Lawrence D, et al. Prediction of T-cell reconstitution by assessment of T-cell receptor excision circle before allogeneic hematopoietic stem cell transplantation in pediatric patients. Blood 2005;105:886-93.
35. Gorski J, Yassai M, Zhu X, Kissela B, Kissella B, Keever C, et al. Circulating T cell repertoire complexity in normal individuals and bone marrow recipients analyzed by CDR3 size spectratyping. Correlation with immune status. J Immunol 1994; 152:5109-19.
36. Lu J, Basu A, Melenhorst JJ, Young NS, Brown KE. Analysis of T-cell repertoire in hepatitis-associated aplastic anemia. Blood 2004; 103:4588-93.
37. Albert MH, Liu Y, Anasetti C, Yu XZ. Antigen-dependent suppression of alloresponses by Foxp3-induced regulatory T cells in transplantation. Eur J Immunol 2005;35:2598-607.
38. Sanchez-Fueyo A, Sandner S, Habicht A, Mariat C, Kenny J, Degauque N, et al. Specificity of CD4+CD25+ regulatory T cell function in alloimmunity. J Immunol 2006;176:329-34.
39. Masteller EL, Tang Q, Bluestone JA. Antigen-specific regulatory T cells - ex vivo expansion and therapeutic potential. Semin Immunol 2006; 18: 103-10.
40. Liu C, He M, Rooney B, Kepler TB, Chao NJ. Longitudinal analysis of T-cell receptor variable β chain repertoire in patients with acute graft-versus-host disease after allogeneic stem cell transplantation. Biol Blood Marrow Transplant 2006; 12:335-45.
41. Tsutsumi Y, Tanaka J, Miura Y, Toubai T, Kato N, Fujisaw F, et al. Molecular analysis of T-cell repertoire in patients with graft-versus-host disease after allogeneic stem cell transplantation. Leuk Lymphoma 2004; 45:481-8.
42. Bellucci R, Alyea EP, Weller E, Chillemi A, Hochberg E, Wu CJ, et al. Immunologic effects of prophylactic donor lymphocyte infusion after allogeneic marrow transplantation for multiple myeloma. Blood 2002; 99:4610-7.
43. Claret EJ, Alyea EP, Orsini E, Pickett CC, Collins H, Wang Y, et al. Characterization of T cell repertoire in patients with graft-versus-leukemia after donor lymphocyte infusion. J Clin Invest 1997;100:855-66.
44. Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, et al. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol 1998;10:1969-80.
45. Walker LS, Chodos A, Eggena M, Dooms H, Abbas AK. Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. J Exp Med 2003;198:249-58.
46. Klein L, Khazaie K, von Boehmer H. In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. Proc Natl Acad Sci USA 2003;100:8886-91.
47. Zhang H, Chua KS, Guimond M, Kapoor V, Brown MV, Fleisher TA, et al. Lymphopenia and interleukin-2 therapy alter homeostasis of CD4+CD25+ regulatory T cells. Nat Med 2005;11:1238-43.
48. Scheffold A, Huhn J, Hofer T. Regulation of CD4+CD25+ regulatory T cell activity: it takes (IL-)two to tango. Eur J Immunol 2005;35:1336-41.
49. Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, et al. Toll-like receptor 2 controls expansion and function of regulatory T cells. J Clin Invest 2006;116:485-94.