Differences for T-Cell Subtypes in Aspiration Biopsies of Patients With Kidney Transplant Under Polyclonal and Monoclonal Immunosuppressive Treatments

P. Xavier[a,*], M. Magalhães[b], S. Sampaio-Norton[c], T. Guimarães[b], and J.G. Oliveira[d]

[a]Portuguese Institute of Blood and Transplantation, Porto, Portugal; [b]Department of Clinical Pathology, S. João Hospital Center, Porto, Portugal; [c]Department of Nephrology, S. João Hospital Center, Porto, Portugal; and [d]CINTESIS/Department of Medicine, Faculty of Medicine, University of Porto, Portugal

ABSTRACT

Thymoglobulin, or antithymocyte globulin (ATG), and anti-interleukin 2α (IL-2α) chain receptor antibody (IL-2αRAb) achieve comparable good results in kidney transplantation notwithstanding different actions on immune cells. Previously, we reported the usefulness of flow cytometry (FC) analysis of lymphocyte subsets present in peripheral blood sample (PBL) and fine-needle aspiration biopsies (FNABs) for clinical surveillance, as, FC reaches very high predictive positive values for acute rejection diagnosis. Now we report an FC study on 2 kidney transplantation (KT) groups under ATG (n = 19) and IL-2αRAb (n = 24) treatment. Both groups were further treated with calcineurin inhibitors mycophenolate mofetil (MMF) and prednisone.

PBL and FNAB samples were collected on day 7 post-KT, stained for several T- and B-lymphocyte subsets, and acquired using FACScan. Statistical analysis were done by Mann-Whitney *U* test.

FNAB results showed a significant downregulation by ATG of CD3 ($P < .001$), CD4 ($P = .009$), CD4CD29 ($P = .003$), and CD2 ($P \leq .001$) and significant upregulation of death receptor (DR) ($P = .03$), CD3CD69 ($P < .001$), and CD3CD25 ($P < .0001$) as compared to groups treated with IL-2αRAb. For PBL, the same trend was seen for CD3, CD4, CD2, CD3CD25, CD3CD69, CD4CD29, and DR plus a downregulation of CD45RO ($P = .001$) and an upregulation of CD4CD45RA ($P < .0001$) in IL-2αRAb.

This study shows that among stable KTs, ATG as compared to IL-2αRAb induces a significant downregulation of a subset of T-memory (CD4CD29) cells but an upregulation of antigen-experienced cells (CD45RO). Further, ATG decreases CD2, CD3, CD4, and naïve (CD45RA) and stimulates T cells as translated by CD3CD69 and DR. As it should be expected from an IL-2αRAb agent, CD25 cells were virtually eliminated.

KIDNEY transplantation (KT) constitutes the best therapeutic option for almost all cases of renal failure and has achieved impressive success rates. The improvements in immunosuppressive therapies have contributed to a great part of this success and several options are available, sometimes with quite different mechanisms of action. Thymoglobulin (Genzyme, Cambridge, MA, United States), or antithymocyte globulin (ATG), is a polyclonal agent directed against a multitude of surface molecules involved in intercellular communications and activation steps [1, 2], including CD2, CD3, CD4, CD8, CD11a, CD25, CD45, and death receptor (DR), at least. Thymoglobulin has secured an almost consensual place among highly reactive KTs as induction therapy as well as antirejection therapy.

Interleukin 2 (IL-2) receptor antagonists are monoclonal antibodies targeting the IL-2 receptor, a cytokine whose role

*Address correspondence to Paula Xavier, Portuguese Institute of Blood and Transplantation, Rua Dr. Roberto Frias – Pavilha, o Maria Fernanda, Porto 4200-465, Portugal. E-mail: paula.xavier@ipst.min-saude.pt

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is indisputable in the activation and proliferation of T lymphocytes once bound to its receptor [3] but that is also involved in the differentiation of regulatory T cells [4]. Other effects may be attributed to anti-IL-2α chain receptor antibody (IL-2αRAb); we reported that it can modulate toll-like receptors 4 and 9 [5].

A consensus has been reached that the immunosuppressive ability of thymoglobulin is superior as compared to αIL-2RAb. While the former has an important place for acute rejection treatment, the use of αIL-2RAb is advised for induction therapy in candidates of low immunologic transplant risk.

Previously, we have reported the usefulness of flow cytometry (FC) analysis of fine-needle aspirates in (1) detecting differences in various T-cell subsets present in peripheral blood and inside the allograft and (2) accurately diagnosing acute rejection in patients with a history of KT [6,7] by looking at the subsets present inside the graft.

The aim of our study is to compare these 2 induction treatment molecules in the modulation of T-cell subsets in a group of patients with KT, focusing our study on (1) those T subsets that we showed were most discriminatory between stable and acute rejection cases and (2) subsets to which quite different but important tasks are attributed [6,7], namely CD4, CD8, CD3CD8, CD8CD45RA, CD4CD29, CD3DR, CD8DR, CD3CD69, and CD69.

MATERIALS AND METHODS

This study included 43 patients with history of KT from brain-death cadaver donors and every patient admitted to this study enjoyed a minimum of 6 months of a rejection-free period post-KT.

Patients were divided into two groups: (1) group 1 (G1) comprised 24 patients who received two intravenous (IV) doses of αIL-2RAb (20 mg) on days 0 and 4 post-KT and (2) group 2 (G2), 19 patients who received polyclonal antibodies (ATG) with a perfusion dose of 1.25-2 mg/kg per day for 3 to 5 days post-KT guided by a target lymphocyte count of 200 elements/mm^3. Every patient was also treated with methylprednisolone and mycophenolate mofetil (MMF) in combination. A calcineurin inhibitor, tacrolimus, was added from the outset in G1 and started by day 7 post-KT in G2.

The demographic characteristics of patients in this population, age, sex, renal failure etiology, number of mismatches, infectious complications, delayed graft function, and graft loss, were not significantly different when comparing the two groups. However, a major demographic difference was noted, while patients in G1 were first-transplant recipients, those of G2 were second-transplant recipients or more.

Each patient provided adequate fine-needle aspiration biopsy (FNAB) on day 7 post-KT and concurrently a blood sample was also collected. FNAB was carried out 2 hours after the morning dose of oral drug administration and less than 2 days following the last thymoglobulin perfusion. None of patients from G2 had started a calcineurin inhibitor when FNAB was performed. All FNAB samples were observed and analyzed at the microscope after a modified Rapi-Diff Romanowski stain (HD Supplies, Atlanta, GA, United States) to ensure that it was appropriate, according to published reports [8].

Table 1. Flow Cytometry Analysis of Lymphocytes Subsets Expressed in Fine-Needle Aspiration Biopsy Samples

Biomarkers	rATG	IL-2αRAb	*P* Value*
CD2	61.9 ± 15.4	85.2 ± 7.5	<.0001
CD3	44.2 ± 24.3	72.6 ± 11.7	.0002
CD4	28.4 ± 16.9	45.1 ± 10.9	.009
CD8	27.6 ± 16.0	31.0 ± 10.8	.570
CD8CD57	6.2 ± 3.7	9.4 ± 5.7	.16
CD25	23.1 ± 10.9	0	< .0001
CD3CD25	12.4 ± 6.8	0	< .0001
CD4CD25	13.0 ± 31.1	0	< .0001
CD69	23.5 ± 21.7	6.1 ± 6.2	.0001
CD3CD69	13.1 ± 16.9	3.9 ± 5.1	.0004
DR	32.2 ± 18.6	19.9 ± 13.4	.034
CD3DR	8.4 ± 6.3	9.3 ± 10.8	.660
CD8DR	6.6 ± 6.6	7.8 ± 10	.44
CD4CD45RA	10.8 ± 7.7	13.9 ± 7.8	.13
CD8CD45RA	20.4 ± 10.9	17.5 ± 8.8	.19
CD45RA	59.1 ± 19.5	56.3 ± 9.8	.11
CD8CD45RO	11.2 ± 8.6	12.5 ± 8.3	.11
CD45RO	34.7 ± 19.8	40.8 ± 10.1	.62
CD29	40.2 ± 21.6	54.4 ± 15.4	.03
CD4CD29	14.4 ± 9.1	26.1 ± 8.9	.003
CD54	37.6 ± 16.6	21.5 ± 22.9	.20
CD2CD54	8.3 ± 5.4	15.3 ± 19.7	.70
CD19	25.9 ± 14.5	12.9 ± 8	.12

Note. Values are given in median ± SD unless otherwise specified.
Abbreviations: IL-2αRAb, anti-interleukin 2α chain receptor antibody; rATG, rabbit antithymocyte globulin.
*Performed using Mann-Whitney *U* test.

One milliliter of FNAB aspirate was incubated for 10 minutes at room temperature with either 100 μL or 50 μL of monoclonal antibodies from Becton Dickinson and Coulter, and acquired in a FACScan (Becton Dickinson, Franklin Lakes, NJ, United States). The same methods were applied using whole-blood samples and the following subsets were studied: CD2, CD2CD54, CD54, DR, CD3, CD3DR, CD8DR, CD3CD25, CD25, CD3CD69, CD69, CD8, CD8CD45RO, CD8CD45RA, CD8CD57, CD4, CD4CD25, CD4CD45RA, CD45RA, CD45RO, CD4CD29, CD29, and CD19.

Data for the lymphocyte subsets were expressed as mean and SD and the statistical analysis of T-cell subsets were performed using a Mann-Whitney *U* test from Statistica (Statsoft, Tulsa, Okla, United States).

This study was approved by the local Committee of Ethics, and informed consent was obtained in all cases.

RESULTS

No significant complication was observed resulting from the FNAB procedure and every aspirate was suitable for FC analysis. The number of cells present in FNAB samples varied between 0.3 and 1.2 x 10^6 cells/mL. The calcineurin inhibitors blood levels were within the target (6-12 ηg/mL).

Tables 1 and 2 present the results of FC analysis of FNAB and PBL samples, respectively. Within the 23 phenotypes analyzed, several differences were observed when comparing lymphocyte subsets in G1 with G2. In FNAB samples, 11 subsets showed significant differences when comparing both drugs. ATG downregulated CD2 ($P \leq .0001$), CD3 ($P = .0002$), CD4 ($P = .009$), and

Table 2. Flow Cytometry Analysis of Lymphocyte Subsets Expressed in Peripheral Blood Samples

Biomarker	ATG	IL-2α	*P* Value*
CD2	59.5 ± 23.2	83.7 ± 8.5	.16
CD3	43.5 ± 19.5	72.3 ± 9.7	< .0001
CD4	21.2 ± 12.8	47.2 ± 9.8	< .0001
CD8	23.3 ± 14.4	29.7 ± 10.4	.10
CD8CD57	5.5 ± 4.0	6.6 ± 4.0	.40
CD25	20.9 ± 7.9	0	< .0001
CD3CD25	10.1 ± 5.8	0	< .0001
CD4CD25	3.6 ± 3.8	0	< .0001
CD69	7.2 ± 6.2	3.0 ± 3.9	.02
CD3CD69	4.2 ± 3.3	1.5 ± 1.8	.01
DR	48.0 ± 23.3	21.8 ± 12.6	.002
CD3DR	5.3 ± 4.3	8.7 ± 8.8	.61
CD8DR	3.1 ± 2.8	7.5 ± 8.7	1.00
CD4CD45RA	9.2 ± 7.3	19.9 ± 8.8	< .0001
CD8CD45RA	20.2 ± 15.9	17.9 ± 7.4	.09
CD45RA	71.4 ± 11.9	65.1 ± 7.9	.019
CD8CD45RO	6.3 ± 5.0	10.3 ± 7.2	.47
CD45RO	29.8 ± 14.8	41.0 ± 11.5	.001
CD29	54.8 ± 9.9	59.1 ± 12.6	.02
CD4CD29	19.4 ±8.5	29.2 ± 8.1	.0003
CD54	34.5 ±14.4	23.9 ± 16.7	.40
CD2CD54	9.3 ± 5.5	14.7 ± 13.2	.16
CD19	50.1 ± 31.9	12.2 ± 6.6	.07

Note. Values are given in median ± SD unless otherwise specified.
Abbreviations: ATG, antithymocyte globulin; IL-2α, interleukin 2α.
*Performed using the Mann-Whitney *U* test.

CD4CD29 ($P = .003$) while DR ($P = .03$), CD3CD69 ($P < .001$), and CD3CD25 ($P < .0001$) were upregulated as compared to αIL-2RAb. Comparative PBL results were similar to those of the FNAB although showing higher significant differences. T-cell subsets of CD3 ($P < .001$), CD4 ($P = .0001$), and CD4CD29 ($P = .0003$) were also downregulated with ATG and DR ($P = .002$) and CD3CD69 ($P = .01$) were upregulated as compared to αIL-2RAb. Moreover, on PBL samples, we observed a downregulation of CD45RO ($P = .001$) and an upregulation of CD45RA ($P = .019$). On the contrary, we observed that the FNAB sample CD2 subset did not show significance on peripheral blood testing.

Also, in FNAB and in blood samples, 2 markers for cell activation were consistently upregulated in G2, namely DR ($P = .03$; $P = .002$), and CD69 ($P = .0001$; $P = .02$), respectively. Other T subsets showed differences that did not reach significance, namely CD8CD69 and CD54, both higher in ATG samples.

Either FNAB or PBL showed a conspicuous absence of CD25 expression in G1, confirming the effectiveness of the used antibody.

DISCUSSION

Our results confirm our expectations of significant differences when comparing several T-lymphocyte subsets both in peripheral circulation and infiltrating kidney allografts procured by FNAB in recipients of KT treated with polyclonal antilymphocyte preparation vs those treated with αIL-2RAb. Actually, close to half of the studied subsets showed significant differences. Some observations deserve a brief commentary.

While CD2, CD3, and CD4 were downregulated with ATG compared to αIL-2RAb, CD8 − although lowered by ATG − displayed a stronger resistance to the polyclonal antibody. ATG also induced a significant downregulation on a T-memory subset (CD4CD29) together with a down-modulation on antigen-experienced cells (CD45RO), which was something unexpected according to previous reports of resistance of experienced niched T cells to suppression [9,10].

Of interest was the effect on CD69 expression, another marker of activation and member of the natural killer-gene complex, which was significantly upregulated with ATG. We surmise this to be a mirror of the earlier steps of activation of the cell cycle, known to occur with ATG without late acute rejection; this early activation was acknowledged, but its importance is not completely understood.

It is noteworthy the ratio between CD3CD25 over CD4CD25, which was close to 1 in FNAB samples and close to 3 in PBL samples; we therefore surmise that ATG allows and even enhances the accumulation of potential T-regulatory cells inside the graft as compared to the periphery. However, the abolition of CD25 expression by αIL-2RAb was sustained in every sample, preventing any comparison.

Previously, we have reported that in FNAB, two lymphocyte subsets display a strong association with the immune response following transplantation, namely, CD3DR and CD8DR, and when these percentages combined are higher than 8.5 points, it is associated very strongly with acute rejection [7]. Here in G2, this sum reached an average of 13.1 while in G1 it was 11.7, thus, both higher than the previous cut-off set, notwithstanding the fact that both groups were rejection-free. This means that our cut-off must be redefined among kidney recipients under both therapies.

Unexpectedly, although αIL-2RAb is supposed to act on only αIL-2R expression, which was thoroughly confirmed by its absence on samples from our patients, both at the peripheral blood level and aspiration samples, the end result of an "activation status" (includes other T-cell subsets as we defined in other studies [6,7]) was not significantly different. In fact, the end result even showed a lower expression as compared to that observed with ATG, pointing to a broader-than-suspected action of αIL-2R. We currently report a cut-off for diagnosis of acute rejection, which includes CD4, CD4CD29, CD3DR, CD8DR, DR, and CD3CD69, multiplied by different coefficients. This reaches 310 points for ATG and 292 points for αIL-2RAb (not significant).

STUDY LIMITATIONS

We acknowledge the limitations of our study, both concerning the number of samples and the restriction to rejection-free cases. Also, some of the differences we have

found may be partly related to the calcineurin inhibitor effect present only among one group.

CONCLUSIONS

We conclude that ATG and αIL-2RAb treatment of rejection-free kidney transplant recipients is associated with several differences in lymphocyte subsets observed either in blood circulation or inside the graft, and may be associated with a different picture once an acute rejection is declared as compared with current triple immunosuppressive therapies.

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