Patients and Methods

Study design and populations

We analysed a prospective cohort of 566 consecutive RTR having received a kidney transplant at the transplant unit of the University hospital of Besançon between January 1993 and December 2009. The ethic committee of Franche Comté has approved the study.

All the patients received a quadruple sequential immunosuppression. Induction consisted of either ATG (n=347, 61%) [ATG Fresenius® (day 0: 9 mg/kg; days 1-4: 3 mg/kg/d, n= 221, 64%) or Thymoglobulin® (Genzyme) (day 0: 2 mg/kg; days 1-4: 1 mg/kg/d, n= 126, 36%)] or monoclonal anti-CD25 antibody (n=219, 39%) [Simulect® (Novartis) (day 0: 20mg, day 4: 20mg)].

ATG was used in all patients until April 1998 (n=118). From May 1998 to May 2004, Simulect use was reserved for patients older than 59 years (n=215). From June 2005, ATG was restricted to second transplant and patients with PRA > 20% regardless of age (n=233).

The same maintenance immunosuppressive treatments were used including Cyclosporine (January 1993-July 2001) or Tacrolimus (August 2001-December 2009), Azathioprine (January 1993-October 2000) or Mycophenolate Mofetil (November 2000-December 2009), and steroids.

All the patients except CMV seronegative recipients of a CMV seronegative donor received CMV prophylaxis with Valaciclovir in the first three months following transplantation. Antiviral prophylaxis dose was adapted to renal function. All patients received Pneumocystis antimicrobial prophylaxis with trimethoprim-sulfamethoxazole.

Characteristics of the study population are described in tables 1 and 2.

CM infection and disease

CMV serology (ELISA) was performed before transplantation. Donor CMV serology was assessed through medical records. CMV PCR were performed weekly until months three post-transplant, monthly until six months post-transplant, and each year during follow-up.

Patients were considered to have CMV infection in any case of positive PCR. CMV disease was defined by the need of treatment in a patient with viral replication. CMV exposure was defined by a positive pre-transplant CMV serology and/or post-transplant CMV infection or disease.

Confounding factors

Age, gender, weight, size, hemodialysis duration before transplantation, pre-transplant cardiovascular disease, diabetes mellitus, hypercholesterolemia, hypertension, body mass index, smoking status, panel reactive antibody, rank of transplantation (first vs iterative), donor type, and immunosuppressive treatment (type of induction, cyclosporine vs tacrolimus, azathioprine vs mycophenolate mofetil) were assessed.

Atherosclerotic events

Coronary heart disease: Myocardial infarction documented by serial 12-lead electrocardiogram evidence or Q-wave infarction and appropriate myocardial enzyme elevations; coronary revascularization including coronary artery bypass surgery or percutaneous transluminal coronary angioplasty; typical history of angina with abnormal coronarography.

Stroke/cerebrovascular disease: Both nonhemorrhagic and hemorrhagic strokes confirmed by neurologic examination findings consistent with new onset focal neurologic deficits, with or without computed tomography or magnetic resonance imaging evidence of cerebral infarction; symptomatic extracranial artery stenosis resulting in carotid endarteriectomy.

Abdominal aortic or lower extremity arterial disease: Abdominal aortic repair; lower extremity revascularization via bypass surgery or angioplasty; lower extremity amputation; new onset of intermittent claudication confirmed by doppler or arteriography findings.

Two physicians independent of the study were responsible for diagnostic ascertainment. This analysis was performed without knowledge of baseline characteristics.

Lymphocyte subsets

Lymphocyte subsets were studied in 62 patients included in the ORLY-EST study (*Influence de l'**O**rientation de la **R**éponse **LY**mphocytaire dans l'athérosclérose post-transplantation*). Briefly, ORLY-EST, started in November 2008, is an observational study including all incident RTR in seven French transplants centers (Strasbourg, Nancy, Reims, Dijon, Clermont-Ferrand, Kremlin-Bicêtre, Besançon). Blood samples are collected at transplant and one year after transplantation, and sent with written consent to the Biomonitoring Plateform (CIC-BT506, EFS Besançon, France) for processing and storage. To date, five hundred patients have been included in this study. Sixty-two patients were extracted from the main cohort to explore immune exhaustion. The ethic committee of Franche-Comté approved the study (2008).

Absolute numbers of circulating B and T cells ($CD4^+$ and $CD8^+$) were determined on FC500 cytometer (Beckman Coulter, Villepinte, France) as previously described (8, 9). Naive $CD4^+$ T cells were also assessed as $CD45RA^+$, $CD62L^+$, $CD45RO^-$ $CD4^+$ $CD3^+$ T cells using the following antibodies: FITC-conjugated CD45RA (clone HI100), phycoerythrin-CD62L (Dreg56) (BD Biosciences, Le Pont de Claix, France), ECD-CD45RO (UCHL1), PC7-CD4 (13B8.2) and allophycocyanin-CD3 (UCHT1) (Beckman Coulter) (9). Exhausted T cells were assessed as $CD57^+CD28^-$ using the following antibodies: FITC-conjugated CD57 (NC1) (Beckman Coulter), PerCP/Cy5.5 CD28 (L293) (BD Biosciences). These T cell subsets were analyzed on FACS CANTO II (BD Biosciences) flow cytometer.

Statistical analysis

Baseline characteristics

Arithmetic mean was calculated and expressed as ± SD.

We separated patients who had received ATG from those who had received anti-CD25 mab. We first compared baseline characteristics using two-sample t-tests, Wilcoxon rank-sum test, or chi-2 test as appropriate.

Propensity score analyses

As this was a nonrandomized study, we supposed that there were inherent differences between the two groups. To overcome these limitations, we used propensity scores to parsimoniously adjust for confounding factors and to address potential confounding by indication. By using propensity scores, a better control for the likelihood of being assigned to a group is expected and occult biases are reduced (23). We calculated the propensity score of receiving ATG after fitting a multivariable logistic regression model with ATG use as the dependent variable. Data that could affect the choice of immunosuppressive agents were used to calculate the propensity score and included age, pre-transplant history of malignancy, duration of dialysis prior to transplant, pre-transplant anti-HLA antibodies, number of HLA mismatches, donor type, cold ischemia time, period of transplant (1993-2001 / 2001-2004 / 2004-2008). Pre-transplant traditional cardiovascular risk factors (gender, previous cardiovascular disease, hypercholesterolemia, hypertension, BMI, smoking status, diabetes) were also included in the model. We matched 1 ATG-treated patient to 1 non-ATG-treated patient by their propensity score + 0.02 (0.1 x standard deviation of the propensity score) to generate a sub-cohort of 159 ATG-treated patients and 159 non-ATG-treated patients.

Survival analyses

Using log rank tests on Kaplan Meier nonparametric estimates of the survival without death-censored AE distribution, we selected variables with a *p* value lower than, or equal to, 0.20. The selected variables were included into a Cox proportional hazards model, and a backward stepwise selection process was performed, this time at a classical $\alpha=0.05$. Gender and age being potential confounding variables, they were also entered into the Cox model, no matter the significance of their relationships with death. Tobacco consumption was accounted for as currently smoking versus non-smoking definition variables. Because indications for the use of ATG differed during the study period, we defined 3 periods (1993-2001/2001-2004/2004-2008) corresponding to different clinical practices.

Propensity score was used in two ways. First, propensity score was forced in a second Cox model in order to reduce selection bias. Second, survival analysis was performed in a sub-cohort of ATG-treated and non-ATG-treated patients matched by their propensity score. Results are expressed as hazard ratio (HR) and 95 % confidence interval (CI), with a *p* value testing the null hypothesis: HR=1. Therefore when *p* value is less than 0.05, HR is significantly different from 1, either greater than 1 (i.e. risk of death is increased) or less than 1 (i.e. risk of death is decreased). Assumptions of Cox models (log-linearity, proportionality of risk in time) were met in this analysis.