Association of rapamycin and co-stimulation blockade using anti-B7 antibodies in renal allotransplantation in baboons

Gwénoila Boulday, Joanna Ashton-Chess, Pierre Bernard, Georges Karam, Henri Vié, Anne Moreau, David Minault, Katrien Lorre, Jean-Paul Soulillou and Gilles Blancho

1INSERM U437 ‘Immunointervention en Allo et Xénotransplantation’ and Institut de Transplantation et de Recherche en Transplantation and 3Service d’Anatomopathologie, CHU Hôtel-Dieu, Nantes cedex, 2INSERM U463, Nantes, France and 4Innogenetics, Gent, Belgium

Abstract

Background. Co-stimulation blockade has already been shown to induce transplantation tolerance in rodents, but until now has failed in large animal models. We therefore sought to investigate whether the addition of rapamycin to a co-stimulation blockade regimen could induce tolerance in baboon recipients of a renal allograft and to characterize the immunological characteristics of rejection.

Methods. Two baboons were used for a pharmacological and toxicological analysis and received anti-B7.1 and anti-B7 antibodies every other day for 60 days. Three groups of baboons underwent classical heterotopic renal allotransplantation; the first group received no treatment (control group; n = 2), the second received a combination of anti-CD80 and anti-CD86 monoclonal antibodies (mAbs) (B7 group; n = 4), and the third received the anti-B7 antibody treatment as above with an additional treatment of rapamycin (B7-Rapa; n = 4). Graft survival as well as immunological analyses were performed.

Results. Anti-B7 mAb monotherapy prolonged allograft survival in three out of four of the animals, one of whom survived rejection free for 87 days but died from a pulmonary embolism; the fourth animal died without rejection. The addition of rapamycin to the regimen did not prolong survival further; three of the four animals underwent early rejection whereas the fourth survived long term but eventually rejected at day 114. Whereas alloimmunization only occurred in this latter animal, rejection was always characterized by a substantial lymphocyte and monocyte infiltration, associated with a strong pro-inflammatory/cytotoxic mRNA accumulation in the anti-B7-treated animals, but to a lesser extent in the B7-Rapa group. T cells extracted and cloned from a biopsy taken at a stable post-transplant time showed a lower frequency of anti-donor alloreactivity in vitro than those extracted from a rejected tissue. Nevertheless, these non-responding clones failed to show regulatory activity in vitro.

Conclusions. We thus confirm that blocking the CD28/B7 pathway by anti-B7 mAbs could prolong graft survival in baboons, but the addition of rapamycin was insufficient to induce tolerance.

Keywords: anti-B7 antibodies; baboons; co-stimulation; rapamycin; renal allotransplantation

Introduction

T lymphocytes require two different signals in order to be fully activated. The first consists of the presentation of antigen-derived peptides by major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs), to the T-cell receptor. The second, or co-stimulatory signal, occurs simultaneously and involves the interaction of accessory molecules between APCs and T cells. One of the major pathways of this co-stimulatory signal is controlled by the interaction between the two molecules B7.1 (CD80) and B7.2 (CD86) on APCs, and CD28 on T cells [1].

In the context of transplantation, co-stimulation blockade currently is being investigated extensively, since this strategy has already been shown to be sufficient in preventing allograft rejection, especially in rodent models [2,3]. One approach consists of the inhibition of the B7–CD28 pathway using the CTLA4–Ig molecule, a fusion protein of the extracellular domain of CTLA4 and a fragment of the Fc domain of an IgG, that binds
to B7 with a higher affinity than CD28, and results in the blockade of T-cell activation [4]. Another approach consists of blocking the CD28–B7 co-stimulation signal via anti-B7 antibodies. It has been shown in rodents that CTLA4–Ig, as well as anti-CD80 and anti-CD86 antibodies, are potent immunosuppressants in prolonging allograft survival [5–7]. Moreover, some of these co-stimulatory blockade protocols can lead to tolerance induction [7,8].

However, it is now becoming clear that tolerance in large animal models such as primates will be more difficult to obtain than in rodent models [9]. A previous study in vivo has also shown that the combination of anti-CD80 and anti-CD86 monoclonal antibodies (mAbs), with or without cyclosporin A (CsA) can prolong renal allograft survival in rhesus monkeys [10]. Furthermore, two recent studies have demonstrated the beneficial effect of anti-B7 antibody treatment in allotransplantation in macaques, although tolerance was not achieved [11,12].

Thus, although co-stimulation blockade in primates has given promising results, the addition of adjunctive conventional immunosuppression will almost certainly be necessary if tolerance is to be achieved. However, the choice of the adjunctive immunosuppressive treatment is likely to be crucial since, in rodents, CsA has been shown to hinder rather than to facilitate tolerance induction [13,14]. In the latter study, however, the immunosuppressive drug rapamycin was shown to promote permanent engraftment. Rapamycin shows potentially interesting properties for tolerance induction. First, Wells et al. [15] found that alloreactive T-cell apoptosis was a prerequisite for the induction of transplantation tolerance via co-stimulatory blockade, and that rapamycin, although an inhibitor of interleukin-2 (IL-2)-dependent T-cell proliferation, does not inhibit IL-2 priming for activation-induced cell death, thus allowing apoptosis to occur. Secondly, rapamycin has been shown not to inhibit the development of regulatory T cells that could be implicated in the process of tolerance [16]. Moreover, rapamycin has been shown to prolong renal allograft survival in primates when given as a monotherapy [17].

In this study, we investigated the effect of an anti-B7.1 and anti-B7.2 mAb treatment for a time period of 60 days with or without rapamycin in a renal allotransplantation model in baboons. Since we completed this study, which had not been performed in primates before, Birsan et al. [18] published a study in macaques concerning the effect of rapamycin and anti-B7 antibodies on survival prolongation in which they showed a significant survival prolongation compared with rapamycin monotherapy. However, they did not observe any tolerance induction since all animals rejected their grafts following treatment cessation.

In our study, which is the first to be performed in baboons, we looked at survival prolongation and we additionally undertook to explore the immunological processes (cellular, molecular and humoral) occurring at rejection as well as at a stable post-transplant time.

Materials and methods

In vivo experiments

All experiments were performed in accordance with our institutional ethical guidelines. The study was carried out in baboons (Papio anubis) allocated to four groups. The first group (n = 2) was used for a pharmacokinetic and toxicity analysis where an mAb cocktail consisting of 0.7 mg/kg of anti-B7.1 (Innogenetics; clone M24, murine IgG2a; Kd = 1–2 × 10^−9 M) and 0.7 mg/kg anti-B7.2 (Innogenetics; clone IG10, murine IgG2a, Kd = 2 × 10^−9 M) was given every other day for 60 days. The half-life of each antibody was 48 h. The three remaining groups underwent classical heterotopic renal allotransplantation, followed by a bilateral nephrectomy. In the first transplanted group (control group; n = 2), the baboons did not receive any treatment. In the second transplanted group (B7 group; n = 4), the baboons received a treatment consisting of a combination of anti-CD80 and anti-CD86 mAbs (Innogenetics, Gent, Belgium), injected intravenously on the day of transplantation [day 0 (D0); 1 h before declamping] and every 2 days thereafter until D60 at 0.7 mg/kg per antibody, to give trough levels of ≥20 μg/ml, with no additional immunosuppressive treatment. This dose was based on the previous study performed in macaques where the same antibodies induced a significant prolongation of renal allograft graft survival [10], and on plasma levels of the circulating antibodies as determined in our preliminary pharmacokinetic analysis. This dose corresponded to twice that used in vitro to inhibit a mixed lymphocyte reaction (MLR) by 90% and saturated B7 sites on peripheral blood mononuclear cells (PBMCs) in vitro as determined by a preliminary fluorescence-activated cell sorting (FACS) analysis. In the third transplanted group (B7-Rapa; n = 4), the baboons received the anti-B7 mAb treatment as above with an additional treatment of rapamycin at a daily oral dose of 0.5 mg/kg/day (Rapamune®, Wyeth Europa Ltd., Berkshire, UK) from D0 to D60. Since the technology required to measure rapamycin blood levels was unavailable in our institution at the time of the study, we based our dose on classical doses used in primates in the literature [17] as well as on unpublished pre-clinical primate pharmacokinetic data from Wyeth. The donor–recipient combinations were chosen on the basis of blood group compatibility and MHC incompatibility as assessed by a positive MLR.

Monitoring and in vivo sampling

The transplanted animals were monitored daily for creatinine and urea, and weekly for haematology and clinical chemistry. The blood trough levels of both administered mAbs, as well as the baboon anti-mouse antibody response, was monitored by specific enzyme-linked immunosorbent assays (ELISAs; provided by Innogenetics) to detect any immunization against the mAb treatment [10]. Biopsies and serum samples were taken from recipient baboons at different times post-transplantation for further histological and immunological analyses. Surgical biopsies were performed after 2 weeks, when rejection was suspected following a 30% increase in blood creatinine levels in the absence of other causes, and just prior to euthanasia. Biopsies were either fixed in 10% formalin (for histological analysis), snap-frozen in liquid nitrogen and stored at −80°C (for RNA extraction) or
surrounded in Tissue Tek (Miles, Elkhart, IN) before being snap-frozen as above (for immunohistochemical analysis). No post-mortem biopsies were included for RNA analyses.

Histology and immunohistochemistry

Histology on serial surgical biopsies was performed on fixed renal samples embedded in paraffin, sectioned at 5 μm and stained with haematoxylin–eosin–safran.

Immunostaining was performed using a standard three-step indirect immunoperoxidase technique on 6 μm biopsy sections. Sections were fixed in acetone, blocked with H2O2, saturated with 2% baboon serum and stained with the primary antibodies: CD3 and CD4 (DAKO, Glostrup, Denmark), CD8 (BD Biosciences, Heidelberg, Germany), CD16 (3G8, American Type Culture Collection, Manassas, VA) and CD11b (Immunotech, Marseille, France), followed by a biotin-conjugated F(ab')2 anti-primary antibody (Immunotech, France) revealed by avidin-conjugated hors eradish peroxidase (Vector Laboratories Inc., Burlingame, CA) and Peroxidase Substrate Kit Vector® VIP (Vector Laboratories Inc.). Sections were counterstained with haematoxylin and lithium carbonate.

mRNA transcription

RNA was extracted from biopsies according to the method described by Chomczynski, and real-time quantitative reverse transcription–polymerase chain reaction (RT–PCR) was then performed as previously described in detail [19], in an ABI Prism 7700-Perkin Elmer Sequence Detection System (Perkin Elmer, Foster City, CA). Amplifications were performed for hypoxanthine phosphoribosyltransferase (HPRT), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-6, IL-8, IL-10, IL-2, IL-13, Granzyme B and FasL. Briefly, 100 ng aliquots of reverse-transcribed RNA from samples were amplified in duplicate in SYBR® Green PCR Core Reagents (Perkin Elmer). The PCR started with an initial step of 2 min at 55°C, followed by 10 min at 95°C, and then 40 cycles of 15s at 95°C followed by 1 min at 60°C. Direct detection of PCR products was monitored by measuring the intensity of fluorescence. The exact number of copies was deduced by comparison of the measured fluorescence with the standard curve. The results were expressed as the following ratio: the transcript number of the target sequence/the housekeeping gene HPRT. The data from biopsies are depicted as an n-fold increase compared with a mean of four normal kidneys (ratio target sequence/HPRT = 1).

Expansion of T lymphocytes from graft biopsies and MLR

Kidney graft specimens were washed at least twice in RPMI containing 10% pooled human sera, 1% l-glutamine (2 mmol/l) and 50 μg/ml gentamycin, and then cultured in a 24-well plate (Nunclon, Copenhagen, Denmark) in the same medium supplemented with recombinant IL-2 (rIL-2, 300 BRMP U/ml). Cultures were kept at 37°C in a 5% CO2 atmosphere. In the cases presented, T-cell proliferation started as early as day 1 for the biopsy from the graft undergoing a rejection and at day 4 for the non-rejected tissue. To generate a panel of clones from each kidney biopsy T-cell line, one responder T cell was seeded in every three culture wells in 96-microwell round bottom culture plates together with pooled allogeneic feeder cells (5 x 104 peripheral blood lymphocytes and 5 x 103 B lymphoblastoid cell line, irradiated at 30 Gy) in the presence of rIL-2 (300 BRMP U/ml). Clone viability was confirmed by a classical dye exclusion test. MLRs were performed using the clones stimulated by irradiated donor or third party PBMCs, in 96-well round bottom plates in RPMI (RPMI 1640; Sigma, Saint Quentin Fallavier, France) supplemented with 100 U of penicillin (Sigma), 100 μg/ml streptomycin (Sigma), 2 mM l-glutamine (Sigma), 1 mM sodium pyruvate, 1% non-essential amino acids and 10% heat-inactivated fetal calf serum. Co-cultures were incubated for 5 days at 37°C and harvested after overnight pulsing with 1 μCi of [3H]Tdr (Amersham, Les Illis, France). Cells were harvested onto glass fibre filters and [3H]Tdr incorporation measured using standard scintillation procedures (Packard Institute, Meriden, CT).

Analysis of the anti-donor humoral response

Recipient serum samples were tested for the presence of anti-donor antibodies by flow cytometry. Briefly, thawed donor splenocytes were incubated with recipient serum diluted 1:2. The presence of anti-donor antibodies was detected by a second incubation with specific biotin-conjugated anti-baboon IgG or IgM mAbs (Pharmingen, San Diego, CA) followed by revelation with a streptavidin–phycoerythrin conjugate (Immunotech, Marseille, France). For the negative control, the serum incubation step was omitted. Fluorescence was measured using a FACScalibur (Becton Dickinson, Mountain View, CA).

Results

Pharmacokinetic and toxicity analysis

Two non-transplanted animals served to confirm that the administration of both antibodies at the dose of 0.7 mg/kg every 2 days was adequate since the therapeutic objective of trough levels superior to 20 μg/ml was reached. Moreover, no toxic or particular side effects were noted during the 60 days of treatment.

Anti-B7.1 and -B7.2 mAb treatment, with or without rapamycin, prolonged graft survival

The outcome of the three transplanted groups is summarized in Table 1. In the two control non-treated animals, cellular rejection occurred at D5. To analyse the effect of co-stimulation blockade on graft survival, a second group was set up (B7 group, n = 4) in which baboons were treated with the combination of the anti-B7.1 and anti-B7.2 mAbs, without any additional treatment. In this group, two animals underwent renal failure associated with histological signs of cellular rejection at D16 and D22. The two remaining animals died without any histological signs of rejection. One died due to a haemolytic and uraemic syndrome (HUS)
at D7, the precise cause of which was not obvious. Although HUS does not seem to have been reported in the literature in the case of co-stimulation blockade, we cannot rule out that in this particular case it was related to the anti-B7 antibodies, since no other obvious classical cause was found. The other animal survived for 87 days, 3 weeks after discontinuation of the mAb treatment. Unfortunately, this latter animal died of a pulmonary embolism secondary to a sural vein phlebitis, probably due to multiple sural vein perforations, with a fairly normal renal function and a creatinine level at 110 μmol/l (Figure 1). Since this mAb monotherapy led to a long prolongation of graft survival in only one animal, a further series of transplantations was performed in which rapamycin was administered in addition to the anti-B7 treatment described above. Surprisingly, in this B7-Rapa group (n = 4), three out of four animals rejected their graft rapidly (D6, D6 and D19; Table 1). However, the fourth animal had the longest survival of the study (D114), but had to be sacrificed because a late rejection, that had probably started around D90, was reflected by an increase in creatinine levels which could not be reversed by steroid boluses initiated at D100 (Figure 1).

Rejection was always characterized by a large cellular infiltrate

Histological analysis of biopsies taken at the time of rejection for all animals revealed a moderate tubulopathy and a substantial lymphocyte infiltration with an absence of severe vascular lesions, indicating a pure cellular rejection, as opposed to the two baboons who died through causes other than rejection (PA974 and PA982; B7 group). We then analysed the composition of this strong cellular infiltrate via immunostaining (Figure 2). In all animals, at the time of rejection, we observed an extensive and diffuse cellular infiltrate composed of monocytes (CD11b) and T lymphocytes (CD3, CD4 and CD8; Figure 2A), whereas a biopsy from an animal with stable renal function as well as the late biopsy (D83) from the long-term survivor (LTS) with stable renal function taken just 4 days prior to the pulmonary embolism at D87 (PA974; B7 group) showed moderate infiltration (Figure 2B and C, respectively). A small number of scattered natural killer cells (CD16) was present in all three groups (data not shown).

Rejection was not linked to a T helper 1 (Th1) or Th2 cytokine profile within the transplanted organs

To characterize further the cellular infiltrate observed in the grafts, which consisted predominantly of macrophages and T lymphocytes, we explored the environment created by the infiltrating cell population in terms of cytokines and cytolytic enzymes by quantitative RT-PCR. We observed a pro-inflammatory profile of cytokine accumulation in all grafts from the B7 group. In this group, the cytokines TNF-α, IFN-γ and IL-6 were strongly induced 2 weeks after transplantation, as compared with cytokine levels measured in four normal kidneys used as controls (data not shown). When comparing biopsies taken at rejection for the two treatment groups with the B7 group LTS with stable renal function, some interesting differences could also be noted. As illustrated in Figure 3, IL-6 and IL-8 were both much lower in the LTS than in rejecting animals from the same group, whereas IFN-γ was at similar
levels. Likewise, in the B7-Rapa group at rejection, we observed a strong inhibition of the inflammatory cytokine induction compared with the B7 group, for IFN-γ, IL-6 and IL-8 (Figure 3A–C). Interestingly, no upregulation of IL-10 was observed in the LTS in contrast to those rejecting their grafts in the same group (Figure 3D). Again, rejecting biopsies from rapamycin-treated animals had baseline levels of IL-10 similar to the LTS with stable function (Figure 4D). Finally, we observed no induction of IL-2 or IL-13 (data not shown). We thus observed no Th1 or Th2 deviation between rejecting and non-rejecting animals.

On exploring the cytotoxic activity of the cellular infiltrate within the grafts, we were unable to detect transcription of FasL in any of the biopsies (data not shown). In contrast, we observed a strong induction of Granzyme B in rejecting animals from the B7 group, which was lower in the LTS (Figure 4D) and again, in the B7-Rapa group, Granzyme B induction was strongly inhibited.

Alloreactivity of graft infiltrating cells

To understand further the properties of the infiltrating cells in rejecting and non-rejecting animals, T cells were extracted and cloned from renal biopsies from two animals of the B7-Rapa group; one animal undergoing rejection (K936A; D15) and one at the same post-transplant time with stable renal function (V9627, the LTS; D15). Interestingly, cellular proliferation was observed in vitro as soon as day 1 of culture from the rejected tissue as opposed to day 4 for the accepted tissue. Among the 14 clones obtained from the biopsy from the rejecting animal, 11 reacted against the donor cells, whereas amongst the 12 derived from the LTS biopsy at stable post-transplant time, only four reacted against the donor (Figure 4A and B, respectively). Furthermore, the level of proliferation observed for the clones derived from the biopsy with stable function tended to be much lower than that observed for rejected biopsy-derived clones. Among the eight non-reacting clones from the non-rejected biopsy, some did not respond to donor or phytohaemagglutinin (PHA) stimulation and some proliferated in response to donor cells even less than the level of spontaneous proliferation, suggesting that they could be anergic or regulatory clones. When we analysed the donor specificity of two of the non-responding clones (nos 4 and 6) and two of the strong responding clones (nos 13 and 15), we found the latter to be donor specific whereas the former were not (Figure 4C).
of the two non-responding clones, these latter clones were co-cultured with the donor-specific clones but failed to inhibit their proliferation in response to irradiated donor PBMCs, suggesting that they were not regulatory but rather anergic T cells (Figure 4D).

Absence of a humoral response

To determine the role of the humoral component in graft failure, we analysed the presence of alloantibodies in recipient sera taken at various time points post-transplantation. We did not detect any anti-donor IgG or IgM in the sera of recipients from all groups, except for baboon V9627, the LTS of the B7-Rapa group. For V9627, a low level of anti-donor IgG was observed in the serum from D90 until rejection (D114), without any detection of anti-donor IgM (data not shown).

Discussion

The ultimate goal for organ transplantation is the induction of tolerance. To this end, many protocols have been developed in rodents to block the CD28/B7 and/or CD40/CD40 ligand co-stimulation pathways. However, although several such studies in rodents, using this type of strategy, have led to graft survival prolongation or even tolerance [7,8,20], the use of similar approaches in primates has so far only led to graft prolongation, with tolerance remaining elusive [9,21]. It is thus clear that for tolerance to be attained in primates, co-stimulation inhibition will have to be combined with other immunomodulating manoeuvres. We therefore endeavoured in this study to explore the possibility of inducing tolerance in a model of renal allotransplantation in baboons by combining co-stimulation blockade with the immunosuppressor rapamycin.
Untreated animals rejected their allografts in 5 days, as described in macaques [10]. Using an anti-CD80 and anti-CD86 mAb treatment, without any additional immunosuppression, we observed a prolongation of allograft survival in three of the four animals, the fourth animal dying early (D7) without rejection through other causes. Interestingly, one animal survived rejection free for 87 days, but eventually succumbed to a pulmonary embolism. The survivals we observed (16, 22 and 87 days, excluding the animal who died through unrelated causes at D7) were within a similar range if slightly shorter than those obtained by Kirk et al. [11] using other murine anti-B7 antibodies in rhesus monkeys at higher doses, where survivals of 25, 42, 77 and 227 days were obtained. Using the same antibodies as those used here, but at lower doses, Ossevoort et al. observed a more homogenous and significant survival prolongation of 21, 22, 34 and 35 days [10]. On addition of rapamycin to the anti-B7 treatment regimen, we did not observe any further prolongation of allograft survival. Surprisingly, two animals rejected their grafts with a similar time frame to the control, non-treated animals. However, one animal showed a moderate prolongation and the other had the longest survival in the study (114 days), but eventually underwent rejection. We show, therefore, that, as for macaques [10], co-stimulation blockade in baboons can prolong survival and that the addition of rapamycin was insufficient for tolerance induction [18]. This latter study by Birsan et al. was the first to use this treatment combination in primates on a larger scale. Our work presented here confirms their results and provides an additional mechanistic point of view concerning the immunological processes occurring under this treatment, in particular by comparing non-rejected and rejected biopsies at the same post-transplant time and under the same treatment.

Biopsies from the allografts of all animals that underwent rejection showed extensive cellular infiltration characteristic of cellular rejection, in contrast to the biopsies taken at early time points from kidneys with stable function or from the B7 group LTS who did not undergo rejection, where infiltration was also present but moderate. Thus, neither treatment was able to inhibit the cellular infiltration, which appeared to play an essential role in the rejection process.

Nevertheless, we did observe some interesting differences between the anti-B7 treatment alone and that combined with rapamycin in terms of the pro-inflammatory and cytotoxic environment detected within the graft at the time of rejection. The association of rapamycin with the anti-B7 cocktail appeared to decrease both the intra-graft pro-inflammatory and cytotoxic environment, in particular by decreasing levels of IFN-γ, IL-8, IL-6 and Granzyme B. Furthermore, the cytokine transcription in non-rejecting
and rejecting baboons was not associated with a shift from a Th1 to a Th2 profile. These data are in accordance with data from Woodward et al., who did not observe any Th1–Th2 cytokine profile in a cardiac allograft model following anti-B7 treatment [6]. Thus, despite the apparent beneficial effect of rapamycin in decreasing the pro-inflammatory and cytotoxic graft environment, its inability to prolong survival beyond that obtained with the anti-B7 protocol alone suggests that a low level of inflammation/cytotoxicity may be sufficient to precipitate early rejection, although late rejection a long time after immunosuppression discontinuation may have an additional humoral component.

Given the presence of a cellular infiltrate in biopsies from both rejecting and non-rejecting animals, we focused on intra-graft reactivity by performing MLR using T cells extracted and cloned from a biopsy from one animal at a post-transplant time with stable renal function and one from an animal at rejection at the same post-transplant time. We found that the majority of the T-cell clones from the biopsy with stable function did not react against the donor cells, in contrast to those extracted from the biopsy taken at rejection. Nevertheless, the fact that these non-responding clones failed to show suppressive activity in vitro indicates that they were not regulatory T cells, but rather anergic T cells. This is in accordance with the well-known in vitro evidence that the recognition of alloantigens in the absence of co-stimulation leads to anergy [22]. The differential frequency of reactive/non-reactive clones and the subsequent differences in graft outcome could be explained by differences in concentration of anti-B7 antibodies at the local tissue level, leading to varying degrees of B7 saturation despite similar blood trough levels of anti-B7 antibody being obtained. It may therefore be crucial in future to monitor tissue levels of anti-B7 antibodies in addition to the classical blood trough level measurement.

We next determined whether alloimmunization participated in the rejection process, and we found that alloantibodies were absent in all recipients, except for the long-term survivor of the B7-Rapa group where a low level of serum anti-donor IgG was observed at rejection. These data are in accordance with those from Kirk et al. who detected anti-donor-specific IgG in long-term surviving rhesus monkey recipients of renal allografts treated by anti-B7 mAbs [11]. In fact, the general absence of alloimmunization we observed could be explained by the early death of the majority of animals still under treatment, before any immunization could occur. Furthermore, the blockade of the B-cell response seemed to be prolonged with these agents given that the LTS PA974 had no alloantibodies at day 83 and alloantibodies only became detectable from day 90 in the LTS V9627.

The inability of this treatment regimen to induce tolerance in primates is in contradiction to the results from a murine model [14] where the addition of rapamycin to a co-stimulation blockade regimen facilitated allograft tolerance whereas the opposite was true for CsA. There may be many reasons for this discrepancy. First, primate models are known to be much less permissive and, in fact, very few models of tolerance induction have been translated from rodent models to primates. Secondly, it is possible that higher doses of rapamycin are necessary in primates to induce tolerance; this could explain why we and Birsen et al. observed some cases of rejection under rapamycin treatment. The doses we used were chosen according to the literature at the time of our study, but it now seems clear that the adjustment of rapamycin dosage has to be based on individual blood levels, that we were unfortunately unable to measure due to the lack of the necessary mass spectrometer in our institution.

In conclusion, we confirm that co-stimulation blockade alone in primates using a combination of anti-CD80 and anti-CD86 mAb treatment can prolong renal allograft survival by an immunosuppressive effect only, rather than by inducing tolerance. Furthermore, we show, this time in baboons, that the addition of rapamycin to this anti-B7 treatment, although leading to a reduction in cellular reactivity as well as the intra-graft pro-inflammatory/cytotoxic environment, does not lead to the induction of tolerance.

We now aim to test higher doses of anti-B7 antibodies and rapamycin as well as associations with other tolerance-inducing procedures such as donor-specific transfusion, immature dendritic cells and other immunosuppressive treatments.

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Conflict of interest statement. K. Lorre holds stock in Innogenetics, the makers of anti-B7 antibodies and at the time of research the company sponsored this study; the company has currently no further interest in commercial development of the product.

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