Chapter 2

Prevention of kidney allograft rejection using anti-CD40 and anti-CD86 in primates

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Abstract

**Background** Costimulation blockade has been proposed to induce allograft tolerance. We combined an antagonist anti-CD40 monoclonal antibody (mAb) with an antagonist anti-CD86 mAb in a rhesus monkey kidney allograft model. We chose this combination because it leaves CD80-CD152 signalling unimpaired, allowing for the down-regulatory effect of CD152 signalling to take place through this pathway.

**Methods** Rhesus monkeys underwent transplantation with a major histocompatibility complex (MHC)-mismatched kidney. One group of animals received anti-CD40 alone, and a second group received the combination of anti-CD40 and anti-CD86, twice weekly for 56 days.

**Results** Three animals with low levels of anti-CD40 rejected the transplanted kidney while still receiving treatment. Three animals with high levels of anti-CD40 rejected at days 91, 134, and 217 with signs of chronic rejection. Animals treated with the combination of anti-CD40 and CD86 mAbs rejected their kidneys at days 61, 75 and 78, shortly after cessation of treatment. Two animals were killed on days 71 and 116 with a blocked ureter. These animals developed virtually no signs of tubulitis or interstitial infiltration during treatment and no donor-specific alloantibodies.

**Conclusion** Both treatment protocols prevented rejection for the duration of the treatment in most animals. Blocking costimulation by anti-CD40 or by anti-CD40 plus anti-CD86 may be an effective method to prevent graft rejection and may obviate the need for other immunosuppressive drugs, especially in the immediate post-transplantation period.
Introduction

It was recently demonstrated in non-human primates (NHP) that a combination of anti-CD80 and anti-CD86 treatment in renal allograft recipients does not lead to the induction of tolerance. Treatment with the parent murine mAbs prolonged graft survival after cessation of treatment [1], but even humanised mAbs were not able to induce stable tolerance in all recipients [2, 3]. Treatment with CTLA4-Ig also blocks CD80 and CD86, but treatment with CTLA4-Ig alone was only partially effective in monkeys [4]. The addition of anti-CD154 mAb to CTLA4-Ig treatment was as effective as treatment with anti-CD154 mAb alone. Because anti-CD154 alone is already effective, no benefit of adding CTLA4-Ig could be demonstrated.

A number of studies were performed using a humanised antagonistic anti-CD154 mAb (clone hu5c8) alone or in combination with CTLA4-Ig or CD80 and CD86 in kidney [4, 5], heart [6], or pancreatic islet transplantation [7] in rhesus monkeys or in pancreatic islet transplantation in baboons [8]. All protocols led to long graft survival times in most monkeys, in many cases even long-term rejection-free survival after cessation of treatment. Trials with hu5c8 in human renal allograft recipients were aborted after reports of thromboembolic events in patients with systemic lupus erythematosus who were treated with the same antibody [9]. This could be explained by the fact that activated platelets also express CD154. In addition, the hu5c8 seemed less effective in human kidney recipients than in NHP. However, it should be noted that in this small trial, the hu5c8 was combined with standard immunosuppression (Mycophenolate mofetil) [9]. Treatment of rhesus monkey kidney allograft recipients with a combination of anti-CD80, anti-CD86 and anti-CD154 did not significantly prolong graft survival, when compared with anti-CD154 treatment alone [10].

In this study we have used a different approach, using an antagonist chimeric anti-CD40 mAb (clone ch5D12) [11] to block the CD40-CD154 pathway in a rhesus monkey kidney allograft model. This antibody reduces allogeneic T-cell proliferation in vitro [12, 13]. It prevents antigen presenting cell (APC) activation, thereby reducing IL-12 and IFN-γ production and preventing upregulation of CD80 and CD86 on the APC [14]. As a consequence, CD154 is not upregulated on the T-cell and T-cells are not skewed to a T helper 1 (Th1) phenotype [15]. To further inhibit T-cell activation, the anti-CD40 mAb was combined with an antagonist chimeric anti-CD86 mAb (clone chFun-1) [16] in a second treatment group. In vitro, this antibody blocks proliferation, IL-2 production and cytolytic activity completely, when combined with 5D12 [17]. The treatment with anti-CD40 and anti-CD86 leaves CD80-CD152 signalling unimpaired, allowing for the downregulatory effect of CD152 signalling to take place through this pathway [18, 19], which could further enhance the induction of allospecific tolerance. Binding avidity and dissociation rates of CD80/CD86 with CD28/CD152 suggest specific roles for CD86-CD28 on the one hand and CD80-CD152 on the other [20]. Chai et al. [19] demonstrated that CD80-CTLA4 interaction is required to induce in vitro unresponsiveness. Judge et al. [21] demonstrated in mice that CD80-deficient donor hearts are rejected under the immunosuppressive treatment of CTLA4-Ig, whereas hearts from CD80+ donors show prolonged graft
survival. Yamada et al. [22] demonstrated that blockade of CD80 or CD152 accelerates allogeneic heterotopic heart rejection in CD28-deficient mice, whereas CD86 blockade significantly prolongs graft survival. Moreover, CD28 is constitutively expressed on T-cells, whereas CD152 expression is upregulated late after T-cell activation suggesting that CD86 is a preferable ligand for CD28, whereas CD80 is a preferable ligand for CD152 [20]. We therefore speculated that using anti-CD86 without anti-CD80 might be more favourable by leaving a downregulatory interaction between CD80 and CD152 unimpaired.

Materials and methods

Animals

Naive, captive-bred 4- to 6-kg rhesus monkeys (Macaca mulatta) were either born and raised at the BPRC or purchased from a licensed breeder. All procedures were performed in accordance with guidelines of the Animal Care and Use Committee installed by Dutch law. All animals were typed for Mamu-A, B and DR antigens by serology [23]. Disparity for DR locus antigens was confirmed by DRB typing [24]. Recipients were mismatched for one or two Mamu-DR antigens, and had at least one Mamu-A and -B mismatched antigen with the donor. The recipient-donor pairs were compatible for ABO antigens [25]. In addition, selected monkeys had a stimulation index (SI) of more than three in a one-way mixed lymphocyte reaction (MLR) of the recipient cells directed against the donor antigens.

Antibody treatment

Chimeric anti-human anti-CD40 (ch5D12) and anti-CD86 (chFun-1) were provided by Tanox Pharma BV, Amsterdam, The Netherlands (a subsidiary of Tanox Inc., Houston, TX). The mAbs were administered intravenously. One group (n=7) was treated with ch5D12 alone and a second group (n=6) was treated with a combination of ch5D12 and chFun-1. Two animals in both groups received two initial doses of 10 mg/kg for each antibody on days -1 and 0, followed by 5 mg/kg on days 4, 7, 11, and 14 and 5 mg/kg weekly thereafter until day 56. Subsequent animals were treated with a doubling of the dosing schedule: 20 mg/kg on days -1 and 0; 10 mg/kg on days 4, 7, 11, and 14; and 5 mg/kg twice weekly thereafter until day 56. No additional immunosuppression was given.

Kidney transplantation

Heterotopic kidney allotransplantation with bilateral nephrectomy was performed as described previously [1, 26]. The clinical condition of the animals was monitored by daily visual inspection and by frequent haematologic and clinical chemistry blood values determined in a local clinical laboratory (SSDZ, Delft) or at the BPRC. Graft function was monitored by serum creatinine and urea levels. In addition, 18-gauge needle biopsies were taken from the kidney at regular time intervals.
Costimulation blockade for prevention of kidney allograft rejection

Rejection episodes were not treated. When the serum creatinine level showed a significant rise or when the clinical condition began to deteriorate, the animals were killed and necropsy was performed. For histologic examination, biopsy material and tissues from the necropsy were formalin fixed and paraplast embedded. Four-micron-thick sections were stained with hematoxylin-eosin, periodic acid Schiff, and a silver impregnation stain (Jones). Histomorphologic evaluation of allograft rejection was performed according to the Banff classification [27].

Determinations of therapeutic antibody levels

Serum samples were collected at regular time points, before and after transplantation. Microtiter plates (96 well) were coated with 100 ng per well CD40-IgG or CD86-IgG (Tanox Pharma BV) and incubated. After washing, plates were blocked with bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The plates were emptied and incubated for 2 hr at 37 °C with serum dilutions and with known concentrations of ch5D12 and chFun-1 to create standard curves. Plates were washed again and incubated for 1 hr at 37 °C with a 1:4000 dilution of an alkaline phosphatase-labelled goat anti-human \( \kappa \) light chain antibody (Southern Biotechnology Associates, Inc., UK). Plates were washed, substrate (p-nitrophenyl phosphate) was added, and absorbance was measured at 405 nm. Antibody levels were calculated using the standard curves.

Rhesus Anti-Chimeric-Antibody (RACA) responses

Serum samples were collected to screen animals for preexisting RACAs and at regular time points before and after transplantation. Microtiter plates (96 well) were coated with murine 5D12 or murine Fun-1 (Tanox Pharma BV). The plates were coated and incubated overnight with 100 ng per well and 500 ng per well for determining the IgG RACA response and the IgM RACA response, respectively. After washing, plates were blocked with BSA in PBS. The plates were emptied and incubated with serially diluted serum. After washing, plates were incubated with alkaline phosphatase-labelled rabbit anti-monkey-IgG (Sigma, The Netherlands) or goat anti-monkey-IgM (Rockland, Gilbertsville, PA). Plates were washed again, substrate (p-nitrophenyl phosphate) was added and absorbance was measured at 405 nm. For IgG, the first dilution to be higher than three times the pretransplantation value was taken as the absolute titer. IgM antibodies were expressed as index of posttransplantation values divided by pretransplantation values. This was done because pretransplantation values showed considerable high background and interanimal variation. An IgM RACA is considered positive when the index is 1.5 or higher on two or more consecutive time points.

FACS analysis

Subset analyses were performed at regular time points using whole EDTA blood. The blood was washed with FACS buffer (0.5% BSA, 0.05% NaN₃ in PBS) to remove circulating free antibody, present in the serum. The samples were incubated with
either a FITC-labeled ch5D12 or chFun-1 to detect in vivo coating of the cells or with either a non-crossblocking anti-CD40 or anti-CD86 mAb to detect the percentage of positive cells for CD40 and CD86. A FITC labeled anti-CD40 (clone 26 [Tanox Pharma BV]) and a PE-labeled anti-CD86, (clone IT2.2 [Becton Dickinson Pharmingen, San Diego, CA]) were used. The cells were incubated for 30 min at 4 °C. The red blood cells were lysed using FACS Lysing Solution (Becton Dickinson), for 10 min at room temperature. Cells were washed and fixed using formaldehyde. Fluorescence was measured within 48 hr. Analysis was performed using CellQuest software (Becton Dickinson). Lymphocytes were analysed for CD40 and CD86 coating in vivo and for CD40, CD80, and CD86 expression.

Anti-donor antibody responses
Anti-donor antibodies were assessed by incubating donor spleen cells with serum taken from the recipient before and after transplantation. Because circulating chimeric antibodies in the recipient serum bound to donor spleen cells, which interfered with the detection antibody, rabbit anti-human IgG or IgM, donor spleen cells were preincubated with mouse anti-human 5D12 (CD40) and Fun-1 (CD86) mAbs provided by Tanox Pharma BV. Donor spleen cells were also preincubated with 50 μl of 1:20 diluted rabbit anti-human Ig (DAKO, Glostrup, Denmark), to block aspecific antibody binding. All incubations were performed for 30 min at 4 °C. Cells were washed with FACS buffer. Cells were then incubated with 25 μl of recipient serum. Cells were washed again and incubated with rabbit anti-human IgG- or IgM-FITC F(ab’)_2 (DAKO; dilution 1:20). Cells were washed and fixed with formaldehyde and resuspended in PBS. Cells were analysed on a FACScan (Becton Dickinson, Mountain View, CA) using standard settings for lymphocyte analysis.

Results
Graft survival
A summary of the kidney graft survival times of the animals is given in Table 2.1. Results obtained in both treatment groups were compared with two control groups: group 1 did not receive any treatment and group 4 received Cyclosporin A (CsA) for 35 days. Both groups were previously reported by Ossevoort et al. [1]. The mean kidney allograft survival time of animals without immunosuppressive treatment (n=4) was 6 ± 0.8 days. Animals receiving ch5D12 alone showed graft survivals between 8 and 217 days. Three animals rejected while still on treatment. One animal had a blocked ureter and was killed on day 12. This animal showed minimal signs of graft rejection. The other three animals rejected well after antibody treatment was discontinued at days 91, 135, and 217. These animals rejected their kidneys with histologic signs of both acute and chronic rejection.

One of the animals receiving the combination of ch5D12 and chFun-1 was killed on day 7 because of a cytomegalovirus (CMV) infection, without histologic signs of rejection. Prophylactic ganciclovir treatment was started in subsequent animals and
no evidence of CMV infections was found thereafter. Three animals rejected their kidney shortly after treatment was stopped on days 61, 75, and 78 with a grade II to III acute + grade 0 to I chronic, grade I acute, and grade I to II acute + grade 0 to I chronic rejections, respectively. In the animal that finally rejected on day 75, signs of rejection became apparent during treatment, as evidenced by rising serum urea and creatinine levels starting approximately day 40 (from ±100 μmol/l to ±200 μmol/l, which stayed in this range until around day 70, with borderline rejection seen in a biopsy specimen taken at day 70). The two remaining animals were killed on days 71 and 116 both with a blocked ureter and mild acute rejection (borderline to grade I and grade I to II acute rejections, respectively). Animals treated with CsA rejected between days 25 and 312.

### RACA responses

RACAs against ch5D12 and chFun-1 were determined in all animals (Fig. 2.1). Three animals were killed before any RACA response could be detected (on days 8 and 12, respectively, in group 2 and on day 7 in group 3). Two animals from group 2, with graft survival times of 42 and 217 days, had positive anti-ch5D12 IgM RACAs, starting on days 14 and 11, respectively (data not shown). This reactions persisted for more than a week. None of the animals from group 3 had a positive anti-ch5D12 IgM response (data not shown). Three animals had rather low, but positive, anti-Fun-1 IgM indexes, all starting on day 11 (survival times 61, 75, and 78 days) (data not shown).

In both groups animals developed anti-ch5D12 IgG responses. Animals in group 2 that rejected during treatment had high titers within four weeks after start of the treatment (Fig. 2.1A). Because of this anti-ch5D12 RACA development, these two animals had rapidly declining levels of ch5D12 (see below) and rejected early. Another animal from group 2 developed a relatively low anti-ch5D12 IgG titer, more than 10 days after the last injection on day 56, and had a graft survival of 91 days. The other two animals in this group (graft survivals of 135 and 217 days) did not develop

Table 2.1: Identification of groups and survival times of all animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Graft survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>5, 6, 6, 7</td>
</tr>
<tr>
<td>2</td>
<td>ch5D12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>low</td>
<td>8, 30</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>12&lt;sup&gt;a&lt;/sup&gt;, 42, 91, 135, 217</td>
</tr>
<tr>
<td>3</td>
<td>ch5D12 + chFun-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>low</td>
<td>(7&lt;sup&gt;b&lt;/sup&gt;), 71&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>61, 75, 78, 116</td>
</tr>
<tr>
<td>4</td>
<td>CsA, 35 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25, 69, 74, 266, 312</td>
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</tbody>
</table>

<sup>a</sup> borderline to grade I rejection, ureter obstruction, showing inflammation.

<sup>b</sup> CMV infection, no rejection.
Figure 2.1: RACAs of IgG isotype. (A) Against ch5D12 of animals in group 2. (B) Against ch5D12 of animals in group 3. (C) Against chFun1 of animals in group 3.

RACA. Two animals from group 3 developed anti-ch5D12 IgG antibodies immediately after treatment was stopped and these animals rejected on days 71 (grade I) and 78 (grade I-II) (Fig. 2.1B). One animal developed a lower anti-ch5D12 IgG titer and rejected on day 116. Two animals did not develop a RACA response against ch5D12. These animals rejected on days 61 (grade II-III acute + grade 0-I chronic) and 75 (grade I). Two of the three animals that developed anti-ch5D12 IgG antibodies also developed anti-chFun-1 IgG antibodies (rejection at days 78 and 116) (Fig. 2.1C). Animals that developed an anti-ch5D12 IgG titer earlier after transplantation rejected earlier than animals that developed a titer later after transplantation. Animals that
Costimulation blockade for prevention of kidney allograft rejection

did not develop any anti-ch5D12 IgG titer generally survived longer than animals that did develop an anti-ch5D12 IgG RACA response.

**mAb serum levels**

mAb trough levels were determined in all animals. In the first two animals in each group (these animals received half the dose of subsequent animals), the circulating mAb levels at the time of maintenance therapy were below 100 μg/ml serum. Subsequent animals treated with higher and more frequent doses of the mAbs showed circulating mAb levels above 100 μg/ml serum throughout the treatment period. In addition, mAb levels were also influenced by the development of RACA responses. One animal in group 2 with high-dose treatment (survival 42 days) developed a RACA response against ch5D12 by day 25 and ch5D12 levels were already less than 100 μg/ml with rapidly declining levels thereafter. Ch5D12 levels were more comparable to the low dose treatment group. Therefore group 2 could be subdivided into a group with low levels of circulating ch5D12 levels (group 2a) (survival 8, 30 and 42 days) and a group with high circulating ch5D12 levels (group 2b) (survival 91, 135 and 217 days). Survival analyses with the log-rank test of groups 2a, 2b, and 3 were performed based on the first day of creatinine rise. All groups differed significantly from each other. Figure 2.2A shows the mean ch5D12 levels of all animals of both groups with a low-dose treatment or with early RACA and for all animals receiving a high-dose treatment. Figure 2.2B shows chFun-1 levels for group 3.

**Histologic examination**

Kidney biopsy specimens were taken at several time points, and rejection was scored by histologic examination according to Banff criteria (Table 2.2). A comparison of the scores was made between the two groups and with group 4, which has been reported earlier by Ossevoort et al. [1]. Rejection scores of group 2 seem to be higher in biopsy specimens taken at day 21 and especially at day 42 than rejection scores of group 3. However, both groups showed lower scores compared with group 4 evaluated on day 35 after transplantation. The effect is most pronounced for tubulitis ("t") and mononuclear cell interstitial inflammation ("i"). Almost all animals of group 3 started to reject after discontinuation of treatment, and kidney biopsy specimens taken at day 70 show more infiltrates than biopsy specimens from animals of group 2b taken at day 70. Initial high rejection scores, with a decline at day 70, are seen in animals of group 2b and in CsA-treated animals (group 4). Rejection scores at the time of rejection did not differ between both antibody-treated groups, nor did they differ from the scores seen in animals of group 4 at the time of rejection. Day 70 biopsy specimens taken from animals that survived for more than 100 days all showed fewer infiltrates, irrespective of the treatment.

**Effect of antibody treatment on lymphocytes**

To investigate the systemic effects of the mAb treatments, lymphocyte subset analyses were performed. Figure 2.3A shows the CD40 expression in all animals of groups
<table>
<thead>
<tr>
<th>Group</th>
<th>Survival</th>
<th>Day 21</th>
<th>Day 42</th>
<th>Day 70</th>
<th>Grading at euthanasia</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>g</td>
<td>i</td>
<td>t</td>
<td>v</td>
</tr>
<tr>
<td>2a ch5D12</td>
<td>8</td>
<td>0-1</td>
<td>1-2</td>
<td>1</td>
<td>0</td>
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<tr>
<td>low</td>
<td>30</td>
<td></td>
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<td></td>
<td>42</td>
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<tr>
<td>2b ch5D12</td>
<td>12</td>
<td>0</td>
<td>0-1</td>
<td>1</td>
<td>0</td>
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<tr>
<td>high</td>
<td>91</td>
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<td>134</td>
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<td></td>
<td>217</td>
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<td></td>
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<tr>
<td>3 ch5D12</td>
<td>7</td>
<td>0</td>
<td>0-1</td>
<td>1</td>
<td>0</td>
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<tr>
<td>chFun-1</td>
<td>61</td>
<td></td>
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<td>116</td>
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<td>4 CsA for 35 days</td>
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<td></td>
<td>312</td>
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</table>

Table 2.2: Banff scores of biopsies, taken on days 21, 42, and 70 and grading of kidneys at euthanasia. * No evaluation of biopsy possible for this parameter.
Figure 2.2: The mean mAb concentration (μg/ml) ± SEM (μg/ml). Filled squares represent animals with low levels, either because of low dosing or early RACA development. Open rounds represent all animals receiving a high dose of the antibodies. (A) Mean ch5D12 levels of animals in groups 2 and 3. (B) Mean chFun-1 levels of animals in group 3.

2 and 3. During treatment, cells could not be stained using 5D12/FITC, but were detectable using another, non-competing anti-CD40 mAb. This indicates that CD40 was completely coated with ch5D12 in vivo, but that CD40+ cells were not removed from the circulation, although a small decrease in CD40+ cells can be seen from day 7 until day 28. Figure 2.3B shows the CD86 expression on the cells of the animals treated with the combination of ch5D12 and chFun-1. The anti-CD86 mAb stained more cells than Fun-1. As for ch5D12, no cells could be stained by Fun-1/FITC and a decrease in CD86+ cells was observed, indicating both a complete coating of CD86 and downregulation of the number of CD86+ cells. CD3+, CD4+, CD8+, and CD20+ cell populations did not change during the time of treatment (data not shown).
Donor-specific alloantibodies

IgG alloantibodies were measured by incubating donor spleen cells with serum of the recipient taken at several time points. Donor-specific antibodies developed in three animals of group 2 (ch5D12), which had graft survival times of 30, 134 and 217 days, respectively (Fig. 2.4A). In all these cases the antibodies only reached significant levels at the day of rejection. Even then, the percentage of donor cells stained was lower than 20%. None of the animals of group 3 (ch5D12 + chFun-1) developed anti-donor antibodies (Fig. 2.4B). In contrast, all animals of group 4 (CsA) developed significant anti-donor antibody levels (Fig. 2.4C).
Figure 2.4: Percentage of donor cells stained for donor-specific IgG alloantibodies for (A) animals treated with ch5D12 alone (group 2), (B) animals treated with ch5D12 and chFun-1 (group 3), and (C) animals treated with CsA for 35 days (group 4).
Discussion

To become fully activated, T-cells need an antigen-specific signal via MHC class I or II and, in addition to that, a costimulatory signal. CD40 on the APC must bind to CD154 on the T-cell before the T-cell can respond to the antigen [28]. Blocking the CD40-CD154 (CD40L) pathway will lead to antigen presentation by APCs to T-cells in the absence of costimulation, and this may result in the induction of a tolerant state of the T-cell in vitro [12, 13] and long-term graft survival in NHP in vivo [5]. When CD40 signalling was inhibited by an antagonistic chimeric anti-CD40 (ch5D12), rejection of a mismatched kidney in a life-supporting kidney allograft model in the rhesus monkey was prevented, given that circulating antibody levels are sufficiently high. When serum levels of at least 100 μg/ml during maintenance therapy (day 14 until day 56) were reached, rejection was delayed, even until after cessation of treatment.

CD40-CD154 interaction is also needed for the upregulation of the B7 molecules (CD80 and CD86) on APCs [28]. Binding of B7 to CD28 on the T-cell fully activates T-cells. The B7 molecules can also bind to CD152 on the T-cell, which serves as a downregulatory signal for the T-cell. There is evidence to support a specific role for CD80-mediated negative signalling by CD152 [19–21, 29]. We combined the anti-CD40 antibody with an antagonistic chimeric anti-CD86 (chFun-1), leaving CD80-CD152 signalling intact. No rejection was seen until treatment was stopped (group 3). One animal had lower levels of anti-CD40 (and anti-CD86), but this did not result in accelerated rejection, which may indicate an additive immunosuppressive effect of the anti-CD86 mAb.

Although no rejection was observed during treatment, animals treated with anti-CD40 and anti-CD86 did not survive longer than animals receiving high-dose anti-CD40. Biopsy specimens taken on days 21 and 42 showed only few graft-infiltrating cells and tubulitis in animals receiving the two antibodies. This is in agreement with the notion that T-cells are prevented from becoming activated under costimulation blockade. However, after cessation of treatment (day 56), biopsy specimens taken on day 70 showed a rise in Banff scores just before graft rejection. This is in contrast to biopsy specimens taken from animals treated with anti-CD40 alone or with CsA. Graft-infiltrating cells were seen during therapy, whereas on day 70 fewer graft-infiltrating cells were seen. It is striking that these animals showed a longer graft survival, and it might be that a certain degree of graft pathology is beneficial for the induction of anergy. It can be speculated that these graft-infiltrating cells have entered a (reversible) anergic state and that these cells are present in the graft as long as anti-CD40 immunosuppression is given. When no anti-CD40 is present anymore, these anergic cells could disappear and rejection could occur. At a later stage, new donor-reactive cells may have entered the graft and rejection occurred. Because no infiltrating cells are present in grafts of animals with combined antibody treatment, such cells cannot exert a regulatory role. Once immunosuppression is stopped, alloreactive cells enter the graft and cause rejection of the graft.

A possible explanation for the fact that anti-CD40 treatment might render T-cells anergic, and anti-CD40 plus anti-CD86 treatment does not, could be that ei-
ther CD80, or CD152, or both are not as strongly upregulated under the influence of CD86 blockade. CD86 knockout mice produce reduced amounts of IFN-γ and IL-4 [30], which are involved in the upregulation of CD80. If CD80-CD152 interaction cannot take place because of insufficient upregulation of either one of the molecules, no negative T-cell signalling can take place and recipients may reject the allograft as soon as immunosuppressive treatment is stopped, as was the case for the group with combined treatment.

None of the animals developed significant anti-donor antibodies. Only three animals developed a low donor-specific response at the time of rejection. This is in contrast to CsA-treated animals, which all made significant levels of anti-donor antibodies before rejection. The fact that virtually no anti-donor antibodies are formed is a unique capacity of the anti-CD40 mAb. With only anti-CD154 [5], or only anti-CD80/CD86 [2] alloantibodies are formed in all animals, although they do not seem to have any immediate effects on graft function or survival. Only after combining anti-CD80, anti-CD86 and anti-CD154 were anti-donor antibodies not formed within the first year after transplantation [10]. The production of antibodies to the anti-CD40 antibody was also prevented in most cases. In two animals of the ch5D12 low group, high anti-ch5D12 RACAs were detected. The anti-CD40 level might have been too low to prevent the formation of RACAs. In all other animals no anti-ch5D12 RACAs were detected or were detected only after cessation of treatment. When ch5D12 levels are low, the chimeric ch5D12 is probably more immunogenic to rhesus monkeys than to humans. Although we have no explanation for the fact that antibodies against the therapeutic antibodies are more easily formed than against the donor, we have also seen this in another study [31].

The usage of costimulation blockade prevents graft rejection without using calcineurin inhibitors and other immunosuppressive agents with their known side effects. Although the mAbs described here do not result in prolonged drug-free graft survival as has been described for anti-CD40L antibody treatment, the treatment with either anti-CD40 alone or in combination with anti-CD86 has not shown any serious side effects. The advantage of using anti-CD40 rather than anti-CD154 is that CD154 is present on activated platelets, possibly causing undesired side effects [9]. Thus treatment with anti-CD40 or anti-CD40 plus anti-CD86 could be used as steroid or calcineurin sparing treatment, to be followed with a low dose of conventional immunosuppressive treatment.

Acknowledgements

The authors thank the veterinary staff and the animal caretakers for their expert care and technical assistance. The authors also thank Bert ’t Hart for critical reading of the manuscript, Henk van Westbroek for help in preparing the figures, and Ed Remarque for help with the statistical analyses.
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