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Preemptive CD20+ B cell depletion attenuates cardiac allograft vasculopathy in CD154-treated monkeys

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Abstract

Background—Anti-CD154 monotherapy is associated with anti-donor alloantibody (Ab) elaboration, cardiac allograft vasculopathy (CAV), and allograft failure in preclinical primate cell and organ transplant models. In the context of calcineurin inhibition (CNI), these pathogenic phenomena are delayed by preemptive “induction” B-cell depletion.

Methods—IDEC-131(αCD154)-treated cynomolgus monkey heart allograft recipients were given peritransplant rituximab (αCD20) alone or with rabbit anti-human thymocyte globulin (rATG).

Results—Relative to previously reported reference groups, αCD20 significantly prolonged survival, delayed Ab detection, and attenuated CAV within 3 months in αCD154-treated recipients (αCD154+αCD20 graft median survival time (MST) >90d, n=7, vs 28d for αCD154 alone (IDEC-131), n=21; p=0.05). Addition of rATG to αCD154 (n=6) or αCD154+αCD20 (n=10) improved graft protection from graft rejection and failure during treatment, but was associated with significant morbidity in 8 of 16 recipients (6 infections, 2 drug-related complications). In αCD20-treated animals, detection of anti-donor Ab and relatively severe CAV were anticipated by appearance of CD20⁺ cells (>1% of lymphocytes) in peripheral blood, and were associated with low αCD154 trough levels (below 100 μg/ml).

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The authors declare no conflicts of interest.

Conclusions—These observations support the hypothesis that efficient preemptive ‘induction’ CD20⁺ B-cell depletion consistently modulates pathogenic alloimmunity and attenuates CAV in this translational model, extending our prior findings with CNIs to the context of CD154 blockade.

Introduction

A growing body of evidence indicates that B-cells provide an important source of donor-specific antigen presentation. Thus peritransplant depletion of B-cells may remove an efficient source of donor antigen-specific costimulation, one potentially pivotal to long-term graft fate. In support of this paradigm, recent reports in cynomolgus monkey models show that pre-emptive “induction” B-cell depletion delayed the onset or attenuate the severity of chronic heart allograft rejection¹ and facilitated prevalent long-term islet allograft survival.² These studies and related antecedent work in several other models suggest that B-cells play a pivotal and non-redundant role proximal to alloantibody elaboration in the alloimmune response, as in autoimmunity.³ This evidence has informed two prospective randomized clinical trials evaluating B-cell depletion with rituximab (Rituxan®, Genentech, South San Francisco, CA) for perioperative ‘induction’ in kidney transplant recipients,^{4,5} both showed a trend toward improved outcomes with rituximab. In addition, CD20⁺ B-cell depletion has been evaluated at the time that alloantibody is initially detected in renal allograft recipients (CT0T-2/CCTPT-02: NCT00307125; study enrolment completed).

Here we report that, when combined with selective CD154 inhibition, preemptive “induction” CD20⁺ B-cell depletion attenuates alloantibody elaboration and inhibits CAV in a preclinical cardiac allograft model. Data are presented in the context of relevant reference groups that have been previously reported.^{1,6}

Methods

General methods used for this work have previously been described in detail.¹ αCD154 monotherapy, αCD154+rATG,⁶ and αCD20 monotherapy^{1,7} groups have previously been reported, and are included here for comparison, by permission.

Cynomolgus Monkeys

Captive-bred and wild-caught cynomolgus monkeys (*Macaca fascicularis*) of Chinese and Indonesian origin were utilized for this study. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine and were conducted in compliance with National Institutes of Health guidelines for the care and use of laboratory animals. Males and females weighing 2.8–5.5kg were selected as organ recipients of ABO blood type-compatible donors of either sex. Stimulation index >3 assured that each donor-recipient pair was MHC class II-mismatched, and pairings were arranged so as to maximize mixed lymphocyte reaction response (median 18, range 5.8–73).

Surgical Procedures

Five intended recipients (of 10 in the αCD154+ATG+αCD20 group) underwent endoscopically assisted thymectomy over 2 weeks prior to transplant. Complete thymectomy

was confirmed at subsequent necropsy. As previously reported,¹ some animals treated with α CD154 (5 of 21) or α CD154+rATG (3 of 6) also receive intrathymic or intravenous donor bone marrow on the day of transplant.

All recipient animals underwent heterotopic intraabdominal cardiac transplantation, as described previously.^{6,26} Graft function and core temperature were assessed at least once daily by telemetry (D70-PCTP, Data Sciences International; implanted at the time of transplantation) until graft explant. Signs of graft dysfunction (decline in heart rate or developed pressure within the graft of >20% from that recipient's stable postoperative baseline) prompted transabdominal ultrasound and biopsy and/or empiric treatment for presumed rejection.¹ Primary graft survival was defined as time to the first rejection diagnosis and/or treatment. In some animals, suspected or biopsy-confirmed rejection was treated with methylprednisolone (Solu-Medrol, Henry Schein, Melville, NY Cat# 9086745, 40mg/kg IV once followed by 20mg/kg daily for 2 days) and rATG (M51, M327, MB621) or α CD20 (M348). Secondary graft failure was defined by further decline in graft function after previous rejection treatment. Open cardiac biopsies were performed by protocol on postoperative days 14, 28, and monthly thereafter until graft explant whenever clinical condition allowed. Biopsies were occasionally omitted in case of recipient anemia or malaise, which in this series was attributable to a transient parvovirus epidemic,⁹ and which had resolved prior to the current study. Molecular monitoring for CMV was not performed, and antiviral medications were not used. Surviving grafts were explanted at the protocol-defined study endpoint of 3 months (90–98 days) or at the time of graft failure (M10670, d112; M9412, d114) after cessation of treatment on d84. Two recipients of grafts implanted before adoption of telemetric monitoring had shrunken, fibrotic grafts removed at exploration on postimplant d230 (M167) and d269 (M116), respectively.

Experimental Groups and Drug Immunosuppression Dosing

Seventeen cardiac allograft recipients were treated with “high-dose” α CD154 treatment as defined previously⁶ (IDEC-131, 30 mg/kg d0, 3, 7, 14; 20mg/kg d21, 28, 56 and 84, and chimeric α CD20 antibody (rituximab (Rituxan®), kind gift of Genentech, San Francisco, CA, or purchased commercially; (α CD20); 20mg/kg IV on the day before transplantation or on the day of surgery, and on postoperative days 7, 14, and 21. The efficiency of this regimen to deplete peripheral blood and secondary lymphoid tissue B-cells in this species has been described previously.⁷ Ten recipients were additionally treated with three doses of rATG (5–20 mg/kg; Nashville rabbit anti-human thymocyte immune globulin ATG, a kind gift of Tennessee Donor Services; or Thymoglobulin, kind gifts of Sangstat and Genzyme). rATG was administered every 1–3 days within the first week after transplant with antihistamine premedication (diphenhydramine, Benadryl). Previously reported recipients that received no immunosuppression (n=5)^{6,26}; α CD20 monotherapy (n=2);¹ α CD154 (IDEC-131) at “high-dose” alone (M51, M147, M62, M350, M643, M327, M10538, M10682, M18, M10670) or with induction ATG (n=6); or “low-dose” IDEC-131 (n=11)⁶ are included as relevant reference groups.

Trough α CD154 levels were checked retrospectively by ELISA and confirmed that therapeutic target IDEC-131 levels (>100 μ g/ml) were achieved within the first months after

transplant in most study subjects unless otherwise noted (“Lo” IDEC coverage, Table 1). An assay to detect anti-idiotypic antibody against IDEC-131 was not available to us to investigate why some IDEC-131-treated animals exhibited “low” titers during the second month after transplant.

Blood Sampling and Flow Cytometry Staining of Peripheral Lymphocytes

Cell blood counts (CBC) and B and T lymphocyte subsets were measured by protocol at regular intervals in freshly collected EDTA-blood using an automated cell counter (Hemavet) and flow cytometry, respectively, as previously described.¹ All antibodies used for flow cytometry (clones SP34, M5E2, and L243) were obtained from BD Pharmingen (San Diego, CA).

Detection of Anti-donor Alloantibody

Alloantibodies were measured retrospectively by flow cytometry using archived frozen donor splenocytes and recipient sera as described previously.^{1,6,27} Alloantibody reactivity was defined as an increase, consistently detected, of more than 10% in the proportion of IgM- or IgG- positive donor CD3⁺CD20⁻ T cells relative to donor serum before transplant; levels repeatedly above 20% were interpreted as unequivocal evidence of anti-donor alloantibody elaboration.

Histology and Cardiac Allograft Vasculopathy (CAV) Quantification

Biopsy and explant tissue specimens were fixed with 10% formalin, processed routinely for paraffin embedding, and sections were stained with hematoxylin and eosin. Pericardial granulation tissue was excluded from the rejection and CAV assessments, and biopsies with poor tissue preservation or absence of identifiable myocardial tissue were classified as nondiagnostic (ND). Classification of intramyocardial cellular infiltrates was done according to the 2005 International Society of Heart and Lung Transplantation (ISHLT) revised criteria for cardiac allograft rejection.²⁸ CAV severity was scored in myocardial biopsies and all explanted hearts. All endothelium-lined cardiac vascular structures were scored in each biopsy at multiple levels. In explants a minimum of 20 and up to 50 epicardial arteries and intramyocardial arterioles were evaluated by morphology on H&E. Three independent evaluators (S.K. or L.B, T.Z., and R.N.P.) were blinded with respect to treatment group. CAV was graded and adjudicated as previously.¹ Immunohistochemistry staining for B-cells was performed on formalin-fixed paraffin-embedded tissue sections using an automated method and B-cell staining was scored by two blinded investigators (AMA, AH) on a scale of 0 to 4 as previously¹.

BAFF levels

Serum levels of B-cell-activating factor (BAFF) were measured on archived samples using Human BAFF/BLyS/TNFSF13B Quantikine ELISA Kit (R&D System, Minneapolis, MN).

Statistical Analysis

Nominal variables (incidence of early rejection) were compared using a contingency table and the Fischer Exact test. Continuous variables (CAV score) were expressed as the mean

plus standard deviation unless otherwise indicated and were compared using the 2-tail unpaired t-test. P-values less than 0.05 were considered statistically significant. All statistical analyses were performed on a personal computer with GraphPad InStat (version 3.01, GraphPad Software, San Diego, CA, USA).

Results

Administration of four weekly doses of rituximab efficiently depleted B-cells in peripheral blood in all treated animals (Suppl. Figure 1), as in our previous reports.^{1,7} B-cells typically comprised <1% of peripheral blood lymphocytes for at least 30 days after cessation of treatment, and absolute B-cell numbers typically remained <50 per μ l. T cell numbers in peripheral blood were not decreased by α CD20 treatment as expected based on our previous experience¹. In contrast, T cells numbers were dramatically decreased by additional rATG therapy and remained low for about 2 months thereafter they slowly recovered (Suppl. Figure 1).

Addition of α CD20 to α CD154 Prolongs Primary Graft Survival

Four of 7 animals treated with α CD154+ α CD20 exhibited graft survival with preserved function to 90 days (MST>90 days; range 46–>98) (Figure1, Table1). In contrast, 3 of 20 evaluable α CD154-treated recipients (MST 28 days, range 6–>90; $p=0.05$ vs CD154+ α CD20) (1 censored for anemia on d21) and 1 of 3 evaluable α CD154+ATG-treated animals (MST 74 days, range >25–>90; $p=1.0$ vs α CD154+ α CD20; $p=0.45$ vs α CD154) (3 censored for infectious death) exhibited graft survival to day 90. Addition of rATG to α CD154+ α CD20 was associated with survival to elective explant around 90 days in 3 of the 4 evaluable grafts in this group ($p=0.35$ vs α CD154; $p=0.49$ vs α CD154+rATG; 6 other animals treated with this regimen died with histologically normal grafts due to rATG reaction (2); surgical complications (1); or infection (3) (detailed above). Considered in aggregate, treatment with α CD20 was associated with significantly higher incidence of survival to day 90 (7/10) relative to α CD154+/-rATG without α CD20 (4/23) ($p=0.006$).

Effect of α CD20 on ISHLT Cellular Rejection Score

As previously reported, without immunosuppression or with α CD154 or α CD20 monotherapy, failed grafts revealed severe acute rejection, with hemorrhagic necrosis and variable degrees of cellular infiltration at explant (Table 1, ISHLT columns).^{1,6} With CD154 monotherapy, all early explants (11 of 11 removed within 30 days) displayed acute rejection (2 mild, 9 severe), and 9 of 10 grafts that survived beyond 1 month displayed mild (1), moderate (1), or severe (7) acute rejection on biopsy or at explant. With α CD154+rATG treatment severe rejection was present at explant in 2 of 3 functioning grafts removed by POD 41, and mild (1R) or moderate (2R) rejection was observed in 2 of 3 electively explanted grafts from this group.

In contrast, α CD20+ α CD154 was associated with a significantly reduced incidence of severe acute rejection (>1R) within 30 (0% vs. 52% with α CD154 alone; $n=7$ and 21 respectively; $p=0.023$) and 60 days (17% vs. 75% with α CD154 alone; $n=6$ and 20, respectively; $p=0.018$) [1 graft in each group was censored between 30 and 60 days due to

recipient death with a beating graft]. Only 1 α CD154+ α CD20 graft was lost to acute rejection (MB621, ATG-resistant), and rejection was absent (4) or mild (1) within the first 2 months in the other 5 recipients in this group. With α CD154+ α CD20+rATG, only 1 animal of 10 (MA021) exhibited graft loss due to acute rejection within 2 months. With α CD154+ α CD20, with or without rATG, ISHLT rejection scores at functioning graft explant or recipient demise revealed absent or mild graft rejection (ISHLT score 0-1R) in 6 of 10 animals with recipient and graft survival >1 month, and in 12 of 14 protocol biopsies.

Effect of α CD20 on Anti-Donor Alloantibody Elaboration

As previously reported, α CD154 monotherapy is associated with prevalent elaboration of anti-donor antibody.⁶ Alloantibody was detected in all α CD154 monotherapy animals with graft failure by 21 days (not illustrated here for clarity; see reference 6). Among the subset of recipients in this group with grafts surviving >21 days, 11 of 12 treated with α CD154 exhibited anti-donor antibody, with most (10 of 12) mounting unequivocally elevated titers within the first months after transplant (Figure 2, top row).

In 3 of the 7 α CD154+ α CD20-treated recipients anti-donor IgM and IgG alloantibody was consistently detected beginning between 35 and 80 days after transplant; 3 of the remaining 4 animals exhibited no detectable anti-donor antibody by elective sacrifice at 90 days, with transient low levels (~10%) of anti-donor IgG (only) detected in MB301 shortly prior to elective graft explant. (Figure 2, second row) In contrast, 4 of 5 α CD154+rATG-treated recipients that were evaluable (M19 did not have viable donor lymphocytes for study) elaborated anti-donor antibody before day 56 (Figure 2, third row), with 3 of 5 mounting unequivocally elevated titers within the first month. Six of 10 recipients treated with α CD154+ α CD20+rATG exhibited an unexplained, transient early peak in IgM and IgG, not seen with rATG or α CD20, or in our prior report with CsA+ α CD20 (1) (Figure 2, fourth row). Addition of rATG to α CD154+ α CD20 was associated with anti-donor alloantibody detection in only 1 (MA021) of 4 animals available for assessment beyond the first month.

Association between trough α CD154 levels, alloantibody elaboration, and graft outcomes

Low IDEC-131 trough levels between 21 and 60 days correlated closely with appearance of anti-donor antibody with α CD154+ α CD20 treatment (MB621, DJ2F1, DL1K2) and with additional rATG (MA021). All 3 (of 7) α CD154+ α CD20-treated recipients with a trough IDEC-131 level <100 μ g/mL elaborated anti-donor IgM and IgG >20% before day 90; 2 of these grafts failed in association with acute rejection and CAV before 90 days (MB621, DJ2F1), and the third (DL1K2) exhibited mild acute rejection (1R) and moderately severe CAV (1.8) at graft explant. (Table 1) In contrast, none of the animals in this group with trough levels consistently >100 μ g/mL had detectable anti-donor antibody, and all their grafts survived >90 days. In MB301, with trough IDEC-131 levels consistently >100 μ g/mL, low levels of anti-donor IgG (only) appeared transiently during the second month, and were detected again (<20%) at elective sacrifice on d93.

Uniquely in the α CD154+ α CD20+rATG group, MA021 developed rapidly rising anti-donor antibody titers on d46 in the setting of low IDEC-131 levels (<10 μ g/mL) and graft dysfunction, with severe acute rejection (3R) and moderate CAV (1.8) on histology. Anti-

donor antibody was not detected in other α CD154+ α CD20+rATG-treated animals with trough IDEC-131 levels above (M9412) or intermittently below (M9387: 30–68; MB030: 57–68) our target trough of 100 μ g/mL.

Effect of α CD20 on graft CAV

With α CD154 monotherapy, 18 of 21 evaluable heart allografts exhibited acute (8) or acute and chronic rejection (10) before day 90, with severe CAV (>2.0) or graft fibrosis at explant in 2 of the remaining 3 grafts in this group. (Figure 3, Table 1, CAV columns) α CD154+rATG treatment was associated with moderate (1.3, M19) to severe (2.1–2.7) CAV in every graft surviving beyond 1 month (3 animals were euthanized for parvovirus-associated anemia by d41).

CAV lesions were less severe within 3 months after transplantation in association with addition of CD20 depletion to α CD154 (mean CAV score 1.2 ± 0.9 in 6 grafts surviving at least 1 month; median 1.55; range 0–2.2), significantly lower than was seen among 11 grafts surviving at least 1 month with α CD154 alone (2.2 ± 0.7 ; median 2.3; range 0.1–2.8; $p=0.03$ vs α CD154+ α CD20) or in 5 recipients treated with α CD154+rATG (2.3 ± 0.6 ; median 2.6; range 1.3–2.7; $p=0.05$ vs α CD154+ α CD20) (Table 1).

When “therapeutic” trough CD154 levels were achieved and anti-donor antibody was not detected, α CD154+ α CD20 was associated with minimal CAV at graft explant (0–0.2 in MA985, MA008, MA053). In MB301 mild-moderate CAV (1.3) at elective graft explant on d93 was associated with moderate acute cellular rejection, trough IDEC-131 >100 , and low but increasing titers of anti-donor IgG. With α CD154+ α CD20+rATG, moderate CAV (1.8) was present in MA021’s graft that was acutely rejected in the context of high and rising anti-donor antibody levels. Mild-moderate CAV (1.2) was present in M9412, explanted in the context of deteriorating graft function and mild acute cellular rejection on d114, 30 days after the last dose of IDEC-131. CAV was minimal in the other grafts in this group, including 2 long-term surviving grafts that were electively explanted at 3 months (MB9387, 0.5; MB030, 0).

Detection of circulating B-cells anticipates detection of alloantibody

Initial B-cell depletion was efficient ($>95\%$) in all recipients treated with α CD20 (Suppl. Figure 1). In some recipients treated with α CD154+ α CD20, peripheral blood B-cell counts, as a percent of circulating lymphocytes, recovered to $>5\%$ of peripheral blood lymphocytes between d40 (MB621, 5–9%) and d84 (DL1K2, 7%; DJ2F1, 30%) by the time alloantibody was detected on d42, d56, and d90, respectively. In each instance (MB621, DL1K2, DJ2F1) intra-graft B-cells were also detected (Figure 4). B-cell counts $>2\%$ anticipated alloantibody detection by 2–4 weeks in both animals where an informative flow cytometric analysis was available (MB621, d27; DL1K2, d48). Similarly, B-cell recovery to 2.5% (d27) and 14% (d46) was detected in MA021 (α CD154+ α CD20+rATG), and anticipated first detection of alloantibody on d42. In contrast, peripheral B-cell counts remained consistently $<2\%$ until d90 in all α CD154+ α CD20 and α CD154+ α CD20+rATG-treated animals in that anti-donor antibody was not detected, including MB301 (0.6% at d93), and intra-graft B-cell infiltration was absent or rare in all other graft explants evaluated (Figure 4).

Effect of α CD20 on BAFF levels

Serum BAFF levels consistently increased within 2 weeks following B depletion, whether α CD20 was given alone, combined with α CD154, or α CD154+rATG (Figure 5, right panels), whereas BAFF levels rose only modestly and inconsistently in the context of control regimens lacking α CD20 (Figure 5, left panels). The profile of BAFF levels in B cell-depleted animals was very heterogeneous, and no obvious correlation was found between levels or kinetics of BAFF and alloantibody or CAV.

Surgical Complications and Side-Effects

B-cell depletion combined with α CD154 was generally well tolerated, without clinical evidence of unusual susceptibility to infection despite omission of antiviral prophylaxis.^{2,8} Posttransplant lymphoproliferative disease, which is common with intense immunosuppression in macaque species, was not observed in any animal in this series. In contrast, addition of rATG to α CD154+ α CD20 was associated with a 50% incidence of treatment-attributed morbidity in 5 of 10 recipients treated with this regimen: acute pulmonary edema was temporally associated with rATG infusion in 2 recipients [M9389, M9399]; 2 lung infections were identified at necropsy, 1 ascribed to CMV [MA094], and the other with no identified organism [M9397]; severe anemia associated with epidemic simian parvovirus infection⁹ triggered euthanasia by protocol in 1 recipient [MA036]. (Table 1, clinical notes) The incidence of symptomatic viral infection α CD154+ α CD20+rATG (3 of 7 at risk) was similar to that observed with α CD154+ATG (3 acute primary parvovirus infections that were associated with severe anemia, of 6 at risk). In 1 α CD154+ α CD20 animal (MA985) of 7 in this group, CMV was diagnosed incidentally on lung histology after euthanasia for isolated severe anemia due to documented acute parvovirus infection. Epidemic parvovirus acute infection with severe anemia required sacrifice in 1 animal (M62) with a functioning graft treated with α CD154 alone, as previously reported.⁹

Two experiments were terminated due to surgical complications: 1 animal (MA015) was euthanized due to small bowel obstruction associated with a retained surgical gauze on d12; another was sacrificed after incurring a small bowel injury at exploration for planned biopsy (MA008). One animal (M922) with a histologically normal functioning graft died on d85 following anesthesia with no pathology identified at necropsy.

Discussion

Impressive improvements in the efficacy of organ transplantation over the past 60 years are attributable in large measure to availability of an expanding array of immunosuppressive agents, combined with the use of lymphocyte infiltration in graft biopsies as the principal barometer of pathogenic alloimmunity, 1 that guides adjustment of immunosuppression intensity. However during the last 2 decades, serologic, immunohistologic, and molecular tools with improved sensitivity and specificity have strongly implicated anti-donor alloantibody in the pathogenesis of both acute and chronic clinical allograft rejection.^{10,11} Detection of alloantibody in association with graft pathology implicates donor antigen-specific B-cells as an important contributor to graft injury and identifies them as a potential

therapeutic target. B-cell depletion has been used safely for other indications in oncology,¹² autoimmunity,¹³ and in presensitized transplant patients.^{14,15}

Here we extend our cardinal observation in a preclinical solid organ allograft model,¹ confirming for the context of CD154 blockade that B-cells exert a pivotal influence during the initiation of alloimmunity by showing that their efficient depletion around the time of transplant is associated with significant protection from acute and chronic rejection. This observation confirms and extends similar findings in rodent heart transplant recipients^{16,17} and in a monkey islet allograft model.²

Intensity of CD154 treatment (IDEC-131 trough level, and by inference CD40/154 signaling blockade) influenced efficacy, since only those animals with “low” trough IDEC-131 levels (<100 µg/ml) elaborated detectable alloAb and manifested relatively accelerated CAV while being treated with αCD154 antibody. Variability in outcomes within the αCD154+αCD20 (+/-rATG) groups also appeared to be associated with early re-appearance of circulating B-cells in peripheral blood, which in turn anticipated elaboration of anti-donor alloantibody and CAV. These associations – similar to those we previously described for CsA+αCD20 – add further support to the mechanistically plausible hypothesis that humoral alloimmunity is pathogenic and causally related to clinical chronic rejection. Further, they implicate both B-cell-driven APC function and incomplete CD154 costimulation pathway inhibition in CAV’s pathogenesis. Finally, we hypothesize that B-cell monitoring may reliably identify an immunologic “window of opportunity” for intervening in the immune response to alloantigens after B cell-depleting “induction” treatment. Serum BAFF levels were consistently increased at higher levels in αCD20 depleted groups compared to control regimens, suggesting BAFF as a marker for B cell depletion as reported in autoimmune diseases^{29–31}. Strategies that do prevent subsequent elaboration of alloantibody should be evident within a few months after B-cell recovery, and would be expected to delay or even prevent subsequent CAV in our model. If confirmed in humans, detection of B-cells could be used to efficiently evaluate candidate strategies designed to redirect the emerging immune response to prevent elaboration of pathogenic alloantibody, and eventually toward induction of durable tolerance. Each of these conclusions has important implications as the basis for logical, testable strategies to inhibit alloantibody elaboration and perhaps improve outcomes in clinical heart transplantation, and perhaps other organ and cell transplants.

Side effects and toxicities associated CNI therapy motivate efforts to develop CNI-free alternative regimens, including costimulation pathway blocking agents. Our current observations predict that combining B-cell depletion with costimulation pathway blockade – with either belatacept^{18–19}, 1 of the emerging αCD40/CD154 reagents,²⁰ or selective, nonactivating CD28 blockade^{21,22} -- will prove safe and efficacious in reducing the risk of early acute rejection in humans. On the other hand our data raises concerns that viral infections are likely to be problematic if additional anti-T cell (ATG) or more broadly depleting agents (eg, alemtuzumab) were added to 1 of these regimens. Meanwhile, the safety profile of an αCD154 Fab PEG to treat autoimmune disease is being evaluated in a Phase 1 clinical trial (NCT01764594), underscoring the potential clinical relevance of our ongoing work on this pathway.²³

Detection of evanescent anti-donor IgM and IgG in the majority of recipients treated with α CD154+ α CD20+rATG was unexpected. Absence on the day of transplant, rapid disappearance after 14 days, and sustained “negative” results in 3 of 4 animals with long-term follow-up suggest that the “positive” signal does not reflect transient elaboration of anti-donor antibody. False positive cross-match results in humans treated with α CD20 have been ascribed to Fc γ -receptor binding by α CD20, particularly in the setting of autoimmunity.²⁴ We speculate that high recipient serum levels of rATG and α CD20, residual in the context of the associated profound recipient lymphopenia, may mimic autoimmunity (Type III systemic immune complexes) when recipient serum is then exposed to donor leukocytes. Presence of preformed macaque anti-rabbit antibody (which would bind to rabbit ATG bound to donor lymphocytes) only in these particular recipients is possible, but seems unlikely.

We have previously reviewed the role of B cells in the onset and progression of CAV lesions in various other transplant models.¹ B cell depletion may operate primarily by altering the anatomy of primate secondary lymphoid organs,⁷ by “incidentally” depleting high-affinity donor-reactive B cells, or by a combination of both mechanisms: our current observations remain compatible with either model. In addition, it may regulate nonredundant antigen presenting functions previously ascribed mainly to other “professional” antigen presenting cells, either directly, or through the production of alloantibody acting as opsonins. The paucity of informative tools available to apply in much larger numbers of experimental subjects continues to recommend rodent models and human clinical trials for determining their relative importance.

In summary, preemptive B cell depletion combined with CD154 blockade attenuates both T and B cell-mediated pathogenic immunity, and delays or prevents chronic rejection in those experimental subjects who exhibit efficient, sustained B cell clearance. Our current observation confirms that the immune response to an allograft is particularly susceptible to B cell depletion, and that B cells play a pivotal role at a proximal point in the evolution of the pathogenic immune response to an allograft. Extending our prior findings with α CD20 treatment in the context of CNi-based immunosuppression, the current data underscore the potential value of preemptive B cell depletion as a novel adjunct to delay or prevent chronic rejection after transplantation of the heart, which prove salient to other solid organ and cellular allografts. Until a clinically feasible approach to specifically target donor-reactive B-cells can be devised,²⁵ preemptive “induction” CD20⁺ B cell depletion represents a clinically applicable strategy to delay or potentially prevent anti-donor alloantibody elaboration and CAV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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abbreviations

ABO	ABO carbohydrate blood group antigens
αCD20	anti-CD20 monoclonal antibody (rituximab)
APC	antigen presenting cells
BAFF	B cell-activating factor
CAV	cardiac allograft vasculopathy
CsA	cyclosporine A
EDTA	ethylenediaminetetraacetic acid
IDECD 131	αCD154 monoclonal antibody
IHC	immunohistochemistry
ISHLT	International Society of Heart and Lung Transplantation
IT	intrathymic
IV	intravenous
MLR	mixed lymphocyte reaction
MST	median survival time
POD	postoperative day
rATG	rabbit anti-thymocyte globulin

Reference List

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Figure 1. Primary graft survival by treatment regimen

Cynomolgus monkey recipients of MHC- mismatched heterotopic cardiac allografts were treated with α CD154 alone (blue line) or with additional rATG (green line), or α CD20 (red line), or both antibody preparations (black line). Graft function was monitored by telemetric ECG and pressure waveforms. Historical controls that were either untreated or given α CD20 alone are not shown (see Table 1, Refs 6 and 26). Clinical rejection was treated in some animals as indicated in Table 1. Addition of α CD20 to α CD154 prolonged rejection-free primary graft survival ($P < 0.05$ vs. α CD154 alone). Numbers in parentheses indicate grafts without clinical rejection by 90 days/uncensored grafts at risk.

Figure 2. Alloantibody production in various treatment groups

IgM (left) and IgG (right) anti-donor antibodies were measured by flow cytometry in serum collected serially (weekly for a month, bi-weekly thereafter) after cardiac allotransplantation as described in Methods. With addition of α CD20, alloantibody was detected (levels repeatedly above 20%) only when α CD154 antibody levels were low and/or B cell depletion less durable.

Figure 3. B cell depletion inhibits cardiac allograft vasculopathy (CAV)

A. Representative pictures from cardiac allografts explanted at graft failure (M350, M643, α CD154 therapy, original magnification $\times 100$) or at approximately day 90 (MA008, MB301, α CD154+ α CD20 therapy, original magnification $\times 200$) and stained with H&E. Vascular intimal proliferative lesions are prominent with α CD154 monotherapy, but not with additional α CD20. Perivascular cellular infiltration and some degree of hemorrhage and endothelial activation were detected in rejected allografts, consistent with cellular and humoral immune injury. B. Median quantitative severity scores are shown for explanted

grafts surviving for >21 days that were treated with α CD154 (n=11) and rATG (n=5), α CD20 (n=6), or α CD20+rATG. *p<0.05 vs α CD154 alone.

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Figure 4. Immunohistochemistry analysis of graft-infiltrating B cells

Explanted allograft specimens were stained for CD20 and scored as previously⁶. A. Representative pictures for explanted control (M364, M10670) or αCD20-treated grafts (DJ2F1, MA008). Presence of intra-graft B cells at d84 in DJ2F1 correlated with graft failure and CAV lesions (score: 1.8), detectable alloantibody and blood B cells (30% of baseline on d84) while absence of intra-graft B cells at d90 in MA008 was associated with graft protection (CAV score: 0), absence of alloantibody, and low blood B cell levels (3.6% of baseline, 39/μl). Original magnification ×100. B. CD20 intra-graft score across treatment

regimens for all specimens evaluated. Each dot represents the average score for 1 cardiac allograft; horizontal bar, group median. * $P<0.05$ vs. untreated controls.

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Figure 5. B cell depletion increased serum BAFF levels

Serum BAFF levels were measured at d0, 7, 14, 21, 28, 35, ~50, 64, 84 and 90 in recipients of a cardiac transplantation. BAFF levels increased after transplantation in all groups, but the increase was more modest in untreated controls (3.8 ± 1.8 fold on d7 vs. baseline, $n=5$) compared to αCD20 monotherapy (11.3 ± 4.5 , $n=2$, $p=0.95$). BAFF increased to similar levels as untreated controls in graft recipients treated with αCD154 with or without ATG (3.4 ± 0.8 fold on d28 vs. baseline, $n=5$), whereas levels were dramatically increased with αCD20-based regimens (12.8 ± 7.7 , $n=7$, $p=0.03$). The longitudinal profile of BAFF levels in

B cell-depleted animals was very heterogeneous, and no obvious correlation was found between levels or kinetics of BAFF and alloantibody or CAV. Therefore, B cell depletion was associated with an increase in BAFF levels compared to reference groups.

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Summary Study Outcomes by Treatment Group

Table 1

Treatment Group	Animal ID	A1° survival (days)	B2° survival (days)	Clinical Reject	C _{ISHLT} Bx/Exp <30d	ISHLT Biopsy 30–90d	ISHLT Final >90d/Exp	CAV Bx/Exp <30d	CAV Biopsy 30–90d	pCAV Final >90d/Exp	αCD154 trough levels	Additional Procedures or Rejection Rx	Clinical Notes
Untreated Azimzadeh 2006	M360	6		AR	2R			0.7					
	M364	6		AR	2R			0.2					
	M20	6.5		AR	3R			0.2					
	M278	7		AR	3R			0.7					
	M342	7		AR	3R			0.8					
αCD20 Kelishadi 2010	Median	6.5											
	MDL41	9		AR	3R			0.5					
	MDL1X	8		AR	3R			0.4					
αCD154 Azimzadeh 2006	Median	8.5											
	M983	6		AR	3R			0.4				Donor BM IV	
	M007	8		AR	3R			0				Donor BM IT	
	M10604	8		AR	3R			0.5				Donor BM IT	
	M51	9	53	CAV	1R	1	1	0.8	1.5	2.4		Donor BM IT rATG d9	rATG reversed AR
	M147	>9		AR	3R			0.8				Euthanized	III/failing graft
	M53	10		AR	3R			0.2					
	M10687	12		AR	3R			1.9				Donor BM IT	
	M9557	13		AR	3R			2.1					
	M1050	15		AR	3R			1.3					
	M62	>21		AR CAV	1R			0.6					Anemia Parvovirus
	M350	28		AR CAV	3R			0.6		2.7			
	M643	35		AR CAV	3R			0		2.3			
	M327	35	42	AR CAV	1		3R	0.2		2.3		rATG d35	(Failed Rejection Rx)
	M9604	38		AR CAV	3R		3R	0		2.3		Donor BM IV	
	M10538	41		AR CAV	1		3R	0.5		2.1		Donor BM IT	
	M10682	43		AR CAV	ND		3R	ND		2.7			

Treatment Group	Animal ID	A1° survival (days)	B2° survival (days)	Clinical Reject	C ¹ ISHLT Bx/Exp <30d	ISHLT Biopsy 30-90d	ISHLT Final >90d/Exp	CAV Bx/Exp <30d	CAV Biopsy 30-90d	D ¹ CAV Final >90d/Exp	αCD154 trough levels	Additional Procedures or Rejection Rx	Clinical Notes
αCD154+rATG	M18	58		AR CAV	IR		3R	0.7		2.8			
	M348	75	82	AR CAV	1	IR	2R	0		2.1		αCD20 Rx	(Failed Rejection Rx)
	M922	>85		Functioning	0	0	1	0	0	0.1			Anes. compl. Resp. arrest.
	M10670	112		AR CAV	0	IR	3R	0.5	1.2	2.1			
	M1116	<269		AR CAV	0	IR	ND	0.2	1	ND			Fibrotic graft (CR)
	Median	28											
Azimzadeh 2006	M15	>25		Functioning	0		0	0.1					Anemia Parvovirus
	M19	>41		Functioning	0		3R		1.3			Donor BM IT	Anemia Parvovirus
	M192	>41		Functioning	0		3R	0.2	2.6			Donor BM IT	Anemia Parvovirus
	M191	56	93	CAV	0		Infarct	0.5	2.7			rATG Rx	Fibrotic graft (CR)
	M10	74		CAV	ND		0	ND	0	2.7		Donor BM IT	Fibrotic graft (CR)
	M167	<230		CAV	0	IR	0	0.2	0.3	2.1			Fibrotic graft (CR)
αCD154+αCD20	Median	74⁰											
	MA985	>21		Functioning			0	0.2			Tt>100		Anemia Parvo+CMV
	MB621	43	48	AR CAV	0	2R	3R			2.2	Lo 21,42	rATG Rx	Anti-donor Ab/Fail Rx
	DJ2F1	84		AR CAV	0	0	3R	0	0	1.8	Lo 36,58		Anti-donor Ab
	MA008	>90		Functioning	0	0	0	0	0	0	Tt>100		Tech/SB injury Bx
	MA053	>92		Functioning	0	0	0	0		0.2	Tt>100	Elective Exp	
	MB301	>93		Functioning	0	1R	2R	0	0.5	1.3	Tt>100	Elective Exp	
	DL1K2	>98		CAV	0		IR	0		1.8	Lo 42,77	Elective Exp	Anti-donor Ab
	Median	>90⁰											

Treatment Group	Animal ID	A1° survival (days)	B2° survival (days)	Clinical Reject	C ⁱ ISHLT Bx/Exp <30d	ISHLT Biopsy 30-90d	ISHLT Final >90d/Exp	CAV Bx/Exp <30d	CAV Biopsy 30-90d	D ^b CAV Final >90d/Exp	αCD154 trough levels	Additional Procedures or Rejection Rx	Clinical Notes
αCD154+αCD20+rATG	M9389	>7		Functioning	0			0			Tt>100		rATG reaction
	M9399	>7		Functioning	0			0			Tt>100		rATG reaction
	MAA015	>12		Functioning	0			0				Recip thy	SBO (gauze)
	M9397	>14		Functioning	0			0.5			Tt>100	Recip thy	Pneumonia
	MAA094	>14		Functioning	0			0					CMV Pulm. Infect
	MAA036	>24		Functioning	0			0			Tt>100		Anemia Parvovirus
	MAA021	46		AR	0		3R	0.3		1.8	Lo<10	Recip thy	Anti-donor Ab
	M9387	>90		Functioning	0		0	0		0.5	Lo 30,68	Recip thy/Elec Exp	
	M9412	114		Func(CAV)	0		1R	0		1.2	Tt>100	Recip thy/Elec Exp	
	MB030	>91		Functioning	ND		0	ND		0	Lo 57,68	Elective Exp	Fibrotic graft (poor fix.)
Median		>90⁰											