The contribution of Fc effector mechanisms in the efficacy of anti-CD154 immunotherapy depends on the nature of the immune challenge

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Abstract

Blockade of the CD154–CD40 co-stimulatory pathway with anti-CD154 mAbs has shown impressive efficacy in models of autoimmunity and allotransplantation. Clinical benefit was also demonstrated in systemic lupus erythematosus (SLE) and idiopathic thrombocytopenia patients with the humanized anti-CD154 mAb, 5C8 (hu5C8). However, thromboembolic complications that occurred during the course of the hu5C8 clinical trials have proven to be a major setback to the field and safe alternative therapeutics targeting the CD154–CD40 pathway are of great interest. Recently, effector mechanisms have been shown to have a role in anti-CD154 mAb-induced transplant rejection in murine models, while this issue remains unresolved for humoral-mediated models. Herein, aglycosyl anti-CD154 mAbs with reduced binding to FcγR and complement were used as a novel means to test the role of effector mechanisms in non-human primate and murine models not amenable to gene knockout technology. While aglycosyl hu5C8 mAb was relatively ineffective in rhesus renal and islet allotransplantation, it inhibited primary and secondary humoral responses to a protein immunogen in cynomolgus monkeys. Moreover, an aglycosyl, chimeric MR1 mAb (muMR1) prolonged survival and inhibited pathogenic auto-antibody production in a murine model of SLE. Thus, the mechanisms required for efficacy of anti-CD154 mAbs depend on the nature of the immune challenge.

Introduction

Signaling by CD154 through its receptor, CD40, initiates a cascade of events resulting in the activation of the CD40 receptor-bearing cells and CD4 T cell co-stimulation (1). This interaction promotes B cell differentiation into antibody-secreting and memory cells, activation of macrophages and dendritic cells and generation of NK cells and CTL. The role of CD154 in regulating the function of both humoral and cell-mediated responses has provoked great interest in the use of inhibitors of this pathway for therapeutic immunomodulation, and anti-CD154 antibodies have been shown to be beneficial in a wide variety of models of autoimmunity and allotransplantation (2). Furthermore, clinical benefit was evident in man from interim data collected in systemic lupus erythematosus (SLE) and idiopathic thrombocytopenia trials (3–7). However, clinical use of the humanized anti-CD154 mAb, 5C8 (hu5C8), was halted due to thromboembolic events whose cause remains unresolved but could potentially be related to FcγR interactions. New clinical approaches targeting this pathway are of great interest (8).

Since humans and mice lacking CD154 have impaired humoral and cell-mediated immunity (9, 10), and CD154-deficient mice show prolonged acceptance of allogeneic transplants (11–16), the efficacy of anti-CD154 mAbs may be mediated by simple blockade of signaling through CD40. However, their efficacy may also depend on effector functions such as deletion of activated T cells through FcγR or complement as suggested by recent observations made with knockout mice (17, 18). Other FcγR-dependent mechanisms, such as the enhancement of binding avidity by the formation of a cell-surface scaffold or by promoting access of the mAb to its site of...
Fc effector mechanisms in anti-CD154 immunotherapy

action, may also increase efficacy. In order to assess whether such secondary interactions are critical to the activity of anti-CD154 antibodies, we utilized an aglycosyl form of anti-CD154 mAb in which binding to FcγR and the complement protein C1q is greatly reduced by elimination of the conserved N-linked glycosylation site in the CH2 domain of the mAb’s Fc region (19–27). Since serum half-life and bivalent binding to antigen are not affected in the aglycosyl form (19, 21, 23, 24, 28), while the in vivo capacity for effector function is greatly reduced (29), it provides a specific means of reducing effector function in animal models.

In this paper we demonstrate that the importance of anti-CD154 mAb effector function for immune modulation is dependent on the immune challenge. Specifically, immune responses in transplanted animals are fully inhibited only by the normal glycosylated form, whereas inhibition of a humoral response to a protein immunogen and efficacy in a model of systemic autoimmunity are relatively independent of Fc effector mechanisms.

Methods

Anti-CD154 mAbs

The selection (30), cloning and humanization (31) of hu5C8 have been described previously. The heavy-chain glycosylation site mutation N297Q (Kabat EU-numbering scheme) was made by unique site elimination mutagenesis using a kit from Amersham-Pharmacia Biotech (Piscataway, NJ, USA). Aglycosyl hu5C8 was stably expressed in N50 myeloma cells and purified by Protein A and gel filtration chromatography. The MR1 hybridoma, which produces hamster anti-murine CD154, and the P1.17 myeloma cell line, which produces a murine IgG2a (mulgG2a) mAb, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The heavy- and light-chain variable domains of the MR1 hybridoma were cloned by reverse transcription-PCR and fused to muIgG2a and murine kappa constant region cDNAs. The heavy-chain glycosylation mutation N297Q was made by site-directed mutagenesis. The expression vectors for the chimeric mAbs, muMR1 and aglycosyl muMR1, were stably transfected into N50 cells and the mAbs were purified by Protein A and gel filtration chromatography.

Human CD154-binding assay

A competitive binding assay was performed on human CD154+ D1.1 cells (ATCC). The binding of biotinylated hu5C8 to D1.1 cells was competed with titrations of hu5C8, detected with streptavidin–PE (BD-PharMingen, San Diego, CA, USA) and analyzed on a FACScan equipped with Cellquest software (Becton-Dickinson). Relative binding affinities were inferred from the IC50 values of four-parameter curve fits.

Murine CD154-binding assay

A competitive binding assay was performed on murine CD154+ 12D4 hybridoma cells (gift from Brigette Huber, Tufts University, Boston, MA, USA). The binding of MR1 PE (BD-PharMingen) was competed with titrations of muMR1 and aglycosyl muMR1 and analyzed on a FACScan equipped with Cellquest software (Becton-Dickinson). Relative binding affinities were inferred from the IC50 values of four-parameter curve fits.

Binding of anti-human CD154 mAbs to FcγR

FcγR-binding capabilities were measured with assays based on the ability of the antibody to form a ‘bridge’ between antigen and an FcγR-bearing cell. The FcγRII (CD64) bridging assays were performed on 96-well Maxisorb ELISA plates (Nalge-Nunc, Rochester, NY, USA) coated with human recombinant soluble CD154 (rsCD40L) (32) with measurement of the mAb-dependent binding of fluorescently labeled CD64+CD32+ U937 cells (ATCC). Specificity for CD64 binding in this assay was demonstrated in experiments where anti-CD64 (clone 10.1) inhibited binding and anti-CD32 (clone FL18.26) had no effect (supplementary data are available at International Immunology Online). The FcγRII (CD32) and FcγRII (CD16) bridging assays were performed using a monolayer of CD154-transfected CHO grown in 96-well tissue culture plates, with measurement of the mAb-dependent binding of fluorescently labeled human FcγR+ cells: CD32b+ Daudi cells (ATCC), CD32a+ K562 cells (ATCC) and CD16a-transfected Jurkat cells (gift from Paul Anderson, Dana Farber Cancer Institute, Boston, MA, USA). In all assays, the FcγR+ cells were labeled with 2',7'-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein acetoxyethyl ester (Molecular Probes, Eugene, OR, USA) and 1 × 10⁶ labeled cells were added to each well. Unbound FcγR+ cells were removed by washing several times and plates were read on a Cytometer Z350 fluorescent microplate reader (Millipore Corporation, Bedford, MA, USA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Binding of anti-murine CD154 mAbs to FcγR

MuMR1 and aglycosyl muMR1 were bound to murine FcγR+ P388D1 cells (ATCC), detected with goat anti-mouse IgG PE (Jackson ImmunoResearch, West Grove, PA, USA) and analyzed on a FACScan equipped with Cellquest software (Becton-Dickinson).

Binding of anti-CD154 mAbs to human C1q

Complement activity was evaluated by binding purified human C1q (Sigma–Aldrich Corp., St Louis, MO, USA) to titrations of anti-CD154 mAbs (hu5C8, aglycosyl hu5C8, muMR1, and aglycosyl muMR1) immobilized on Maxisorb ELISA plates (Nalge-Nunc). Bound C1q was detected with chicken anti-C1q (Cedarlane Laboratories, Ontario, Canada) followed by a donkey F(ab’2) anti-chicken IgY–HRP conjugate (Jackson ImmunoResearch) and development with 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Plates were read at 450 nm using a SpectraMax plate reader equipped with SoftmaxPro software (Molecular Devices, Sunnyvale, CA, USA) for data analysis.

ELISAs for hu5C8, anti-tetanus toxoid and anti-hu5C8

Serum levels of hu5C8 were measured by capture on human rsCD154-coated Nunc Maxisorb ELISA plates, detection with donkey anti-human IgG–HRP (Jackson ImmunoResearch) and quantification by interpolation to an hu5C8 standard curve.
Anti-tetanus toxoid (TT) antibodies in serum were captured on TT (Massachusetts Public Health Biologic Laboratories, Boston, MA, USA) coated plates and detected with rabbit anti-monkey IgG-HRP (Cappel-Organon Teknika, Durham, NC, USA). Anti-hu5C8 antibodies in serum were measured by capture on hu5C8-coated plates and detected with biotinylated hu5C8 followed by streptavidin–HRP (Pierce Biotechnology, Rockford, IL, USA). All ELISA plates were developed with TMB substrate and read at 450 nm. Antibody titers were defined as the reciprocal of the dilution which yielded >0.100 optical density (OD) units over pre-bleed values.

Membrane transfer assay to detect components of hu5C8/anti-hu5C8 immune complexes

In addition to the ELISA methods described, a novel method capable of detecting the components of immune complexes was established (Y.-M. Hsu et al., in preparation) and utilized for the detection of hu5C8 and anti-hu5C8. Serum samples were serially diluted into 100 mM citrate buffer, pH 3.0, incubated for 15 min at 37°C to denature immune complexes and then captured onto PROTRAN brand nitrocellulose (Schleicher & Schuell, Keene, NH, USA) using a slot blot filtration manifold (Hoeffer Pharmacia Biotech, San Francisco, CA, USA). The membrane-bound proteins were re-natured by a 1-h incubation in 2% milk/PBS. The membrane was then probed for 2 h with 0.5 μg ml⁻¹ of biotinylated hu5C8 (Biogen Idec, Cambridge, MA, USA) or biotinylated rsCD40L (Biogen Idec) to bind anti-hu5C8 or hu5C8, respectively. The bound biotinylated reagents were detected by a 1-h incubation with streptavidin–HRP (Pierce Biotechnology). The blot was developed using the ECL western blotting detection reagents (Amersham-Pharmacia Biotech) and the results were captured onto film.

Humoral immune response to TT in cynomolgus monkeys

Two independent studies in cynomolgus monkeys were conducted at Primedica Corporation (Worcester, MA, USA). Serum samples from each study were collected by Primedica and shipped frozen to Biogen Idec for analysis. All animals received an intramuscular dose of 5 Lf of adsorbed TT 4 h after treatment with saline or anti-CD154 mAbs on day 1 in each study. The aglycosyl hu5C8 study comprised two treatment groups for evaluation of a primary immune response to TT. Group 1 (n = 8) received saline and Group 2 (n = 4) received a single 20-mg kg⁻¹ i.v. dose of aglycosyl hu5C8 on day 1. Blood was collected both pre- and post-treatment on day 1 and on selected days up to 85 days post-dosing. Lymph node biopsy samples were taken on day 15. Several months (230 days) after the primary TT challenge, the saline control animals (Group 1) were re-challenged with TT to evaluate the ability of aglycosyl hu5C8 to inhibit a secondary immune response. Group 1A (n = 4) served as untreated controls while Group 1B (n = 4) received a single 20-mg kg⁻¹ i.v. dose of aglycosyl hu5C8 on day 1. Blood was collected both pre- and post-treatment on day 1 and on selected days up to 85 days post-dosing. The aglycosylated hu5C8 study was previously reported (33). The historical data from two groups in this study were compared with the data obtained in the aglycosyl hu5C8 primary TT challenge study, a group that received saline (untreated controls, n = 3) and a group that received a single 20-mg kg⁻¹ i.v. dose of glycosylated hu5C8 (n = 3). To allow comparability of these two independent studies, selected serum samples were analyzed side-by-side in the anti-TT ELISA. Immune responses were calculated using non-compartmental analysis of the anti-TT antibody responses. Pharmacokinetic analysis was performed using a two-compartment model with a first-order elimination rate constant (WinNonLin Professional Software v3.1, Pharsight Corp., Cary, NC, USA).

Isole cell allograft transplant in rhesus monkeys

These studies were performed at the Diabetes Research Institute (Miami, FL, USA) as described previously (34). In brief, alloreactive donor–recipient pairs of rhesus monkeys were chosen based on positive mixed lymphocyte culture reactivity. Recipients underwent complete pancreatectomy and intraportal, allogeneic islet transplantation on day 0. Animals were dosed with 20 mg kg⁻¹ of mAb on days −1, 0, 3, 10 and 18 followed by one 20-mg kg⁻¹ dose per month starting on day 28. Isole graft function was monitored daily through fasting and post-prandial blood glucose levels.

Renal allograft transplant in rhesus monkeys

These studies were performed according to the methods previously described (35) at the National Institutes of Health (Bethesda, MD, USA). In brief, MHC-mismatched donor–recipient pairs of rhesus monkeys were selected based on non-identity at both class I and class II MHC loci. Renal allograft transplantation was performed on recipients subjected to bilateral nephrectomy. Animals were dosed with 20 mg kg⁻¹ of mAb on day 0 pre- and post-operatively and on days 3, 10 and 18. Serum creatinine was measured every other day until stable and weekly thereafter. Animals were euthanized at the time of anuria in accordance with the American Association for the Accreditation of Laboratory Animal Care standards.

Pharmacokinetics of chimeric MR1 mAbs in BALB/c mice

BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). A single 100-μg intra-peritoneal (i.p.) dose of muMR1 or aglycosyl muMR1 was administered and blood samples were collected after 4 h and on days 1, 2, 4, 7, 9, 11 and 14. Two mice were used for each time point. Serum levels of the chimeric MR1 mAbs were measured by capture on murine rsCD154-coated Nunc Maxisorb ELISA plates followed by detection with anti-mouse IgG2a–HRP (Southern Biotech, Birmingham, AL, USA) and quantification by interpolation to a muMR1 standard curve.

Model of lupus nephritis in (SWR × NZB)F1, hybrid mice

SWR and NZB mice were purchased from The Jackson Laboratory. (SWR × NZB)F1 hybrids (SNF1) were bred in the animal facility at Biogen Idec under conventional barrier conditions. Female SNF1 mice were randomly enrolled into studies when the animals were ~5.0–5.5 months of age with established mild nephritis as measured by proteinuria (PU) levels between 30 and 100 mg dl⁻¹ (1⁺ to 2⁺). The lupus studies consisted of three treatment groups, receiving the P1.17 mAb (n = 9), glycosylated muMR1 (n = 14) or aglycosyl muMR1 (n = 15). mAbs were given
Fc effector mechanisms in anti-CD154 immunotherapy

as i.p. injections of 500 μg weekly for the first 6 weeks, followed by a single injection of 500 μg monthly until termination of the studies at 9.5 or 13 months of age. Serum samples were collected monthly for auto-antibody analysis. Anti-DNA ELISAs were performed as previously described (36) and titers were defined as the reciprocal dilution at 0.1 OD units over background. The urine of each mouse was monitored weekly with Albustix (Bayer Corp., Tarrytown, NY, USA) to measure proteinuria. The level was scored as follows: 0.5+, 15–30 mg dl⁻¹; 1+, 30 mg dl⁻¹; 2+, 100 mg dl⁻¹; 3+, 300 mg dl⁻¹; and 4+, >2000 mg dl⁻¹. Mice surviving up to 12–13 months of age were sacrificed and kidneys were obtained for histopathological analysis.

Assessment of renal pathology in SNF1 mice

Formalin-fixed, paraffin-embedded kidney tissues were stained with hematoxylin and eosin for inflammatory infiltration, Masson trichrome for fibrosis and periodic acid Schiff for basement membrane thickening. The stained tissue sections were scored blindly by a veterinary pathologist. The overall score for histopathological grading of lupus nephritis was based on glomerular, interstitial and tubular changes. The grades 0–4⁺ are based on percent involvement of the structure being examined (i.e. glomeruli and vessels) and are as follows: 0, no significant lesions; 1⁺, 1–30% of architecture affected; 2⁺, 30–60% of architecture affected; 3⁺, >60% of architecture affected to some degree; and 4⁺, >60% of architecture severely affected.

All the in vivo experiments described in this article were conducted according to the principles set forth in the ‘Guide for the Care and Use of Laboratory Animals’, Institute of Laboratory Animals Resources, National Research Council, United States Department of Health and Human Services, Pub. No. (NIH) 86-23 (1985).

Results

Engineering and characterization of aglycosyl hu5C8

In order to reduce the effector function of hu5C8 an aglycosyl form was created by changing the canonical N-linked Asn site in the heavy-chain CH2 domain to a Gln residue. A competitive binding assay demonstrated that binding of aglycosyl hu5C8 to cell-surface human CD154 was unaltered from the glycosylated form (Fig. 1A). The reduction in effector function was measured in vitro using a bridging assay format for FcγRI binding and an ELISA for C1q binding. The relative binding capacity of aglycosyl hu5C8 to FcγRI is diminished ~25-fold when compared with glycosylated hu5C8 based on the EC₅₀ (effective concentration 50%) values (Fig. 1B). No binding of the aglycosyl hu5C8 to FcγRIIa, FcγRIIib or FcγRIIla could be demonstrated at microgram per milliliter concentrations, while the normal glycosylated hu5C8 gave an EC₅₀ in the nanogram per milliliter range in the same assay format (Fig. 1C–E). Additionally, aglycosyl hu5C8 has greatly reduced binding capacity for C1q compared with glycosylated hu5C8 (Fig. 1F). These results demonstrate that the aglycosyl form provides a specific means of testing the importance of effector receptor binding while not impacting the other properties of the mAb, although the remaining weak binding capacity may allow some residual effector activity.

Aglycosyl anti-CD154 inhibits a primary humoral response to TT antigen in cynomolgus monkeys

A single 20-mg kg⁻¹ dose of aglycosyl and glycosylated hu5C8 gave an EC₅₀ in the nanogram per milliliter range in the same assay format (Fig. 1B). Additionally, aglycosyl and glycosylated hu5C8 were found to be comparable after a single 20-mg kg⁻¹ dose (Fig. 2B). Histological analyses of inguinal lymph node biopsies showed that germinal centers in the aglycosyl hu5C8-treated animals were rare and small, while sections from control animals had marked reactive secondary follicles and germinal centers with many blasts (data not shown). The moderate-to-severe degree of lymph node hypoplasia is consistent with that previously observed with glycosylated hu5C8 (33) and is consistent with the pharmacological action of anti-CD154 mAbs. In addition, aglycosyl hu5C8 did not provoke a significant antibody response in cynomolgus monkeys. Low anti-hu5C8 titers developed in three of the four animals after the anti-CD154 mAb was cleared from circulation (Fig. 2C) similar to what was observed with the glycosylated form in earlier studies.

Aglycosyl anti-CD154 inhibits a secondary humoral response to TT in cynomolgus monkeys

The ability of a single 20-mg kg⁻¹ dose of aglycosyl hu5C8 to inhibit a secondary immune response was evaluated by giving a second TT challenge to the eight saline control animals in the previous experiment several months after their primary immunization. Prior to the TT challenge, four animals received 20 mg kg⁻¹ of aglycosyl hu5C8 (Group 1B) and four received saline (Group 1A). Secondary immune responses are of quicker onset and higher magnitude than primary immune responses; therefore, the magnitude of the secondary response relative to the primary response for individual animals was calculated (Table 1). TT challenge in the saline controls (Group 1A) produced a secondary antibody response that was 6.5 times higher (range, 4.2–18.8) than their primary response, whereas animals receiving aglycosyl hu5C8 (Group 1B) produced a secondary antibody response that was only 2.0 times higher (range, 1.6–2.2), equating a 70% reduction in the magnitude of the secondary antibody response as compared with saline controls. Unfortunately, we do not have a matched data set to compare the efficacy of aglycosyl hu5C8 with glycosylated hu5C8 in the inhibition of a secondary immune response.

Aglycosyl anti-CD154 is relatively ineffective in preventing islet allograft rejection

We have previously demonstrated that glycosylated hu5C8 reproducibly prolongs islet allograft acceptance in rhesus monkeys (34); all six animals tested accepted islets without rejection and were insulin independent until at least 125 days and some for as long as 476 days post-transplant whereas an untreated control monkey exhibited acute rejection on
day 6 as evidenced by persistent hyperglycemia and a lack of c-peptide production (a product of endogenous insulin production). Two aglycosyl hu5C8-treated monkeys experienced acute rejection on day 7 indicated by fasting blood glucose levels $>100$ mg dl$^{-1}$. Treatments with aglycosyl hu5C8 were continued as scheduled on days 10, 18 and 28. Exogenous insulin was required to maintain normal blood glucose levels in one animal starting on day 7 and rejection was later confirmed based on the lack of serum c-peptide at this time point. The second monkey was treated with insulin starting on day 13 and partial function of the allograft was exhibited as measured by the presence of c-peptide through day 28. However, this monkey was c-peptide negative on day 39, indicating complete rejection. Similar serum levels were observed with the glycosylated and aglycosyl forms of hu5C8 in this model (Fig. 3A) and anti-aglycosyl hu5C8 antibodies were not
detected in these animals, indicating that the difference in efficacy is not due to a difference in the pharmacokinetics of the anti-CD154 mAbs.

**Effector functions of anti-CD154 are required for renal allograft survival**

Previous studies have also shown reliable long-term renal allograft acceptance in rhesus monkeys treated with glycosylated hu5C8; all nine rhesus monkeys treated with a 20-mg kg\(^{-1}\) induction/maintenance-dosing regime maintained graft function without a rejection episode during the 6-month dosing period, while untreated monkeys promptly rejected their transplants within 8 days (35, 37). In contrast to the glycosylated hu5C8, two monkeys that received aglycosyl hu5C8 promptly rejected their allografts within 8 days as indicated by elevated serum creatinine levels. In an attempted rescue from an acute rejection episode, one of the animals (AT5D) was given 40 mg kg\(^{-1}\) of glycosylated hu5C8 on day 8 and 20 mg kg\(^{-1}\) on day 12. During both rescue attempts, this monkey experienced severe infusion reactions resembling serum sickness and was sacrificed on day 16. The second monkey (AW34) was sacrificed on day 9 without a rescue attempt. Autopsies performed on both monkeys confirmed the acute allograft rejection. Serum levels of aglycosyl hu5C8 were comparable to those obtained in glycosylated hu5C8-treated monkeys at early time points but by days 9–12 aglycosyl hu5C8 was undetectable in the ELISA (Fig. 3B). This result was surprising especially for animal AT5D, who received 40 mg kg\(^{-1}\) of glycosylated hu5C8 on day 8 and 20 mg kg\(^{-1}\) on day 12 during rescue attempts. Since it is known that the presence of neutralizing anti-hu5C8 antibodies prevents binding of hu5C8 to rsCD154 in the ELISA format, we utilized a membrane transfer assay capable of detecting the components of denatured immune complexes. Using this method it was evident that high titers of neutralizing anti-hu5C8 antibodies were present in the serum of aglycosyl hu5C8-treated animals (Fig. 4) but none was detected in serum from animals treated with glycosylated hu5C8 (data not shown). These data indicate aglycosyl hu5C8 clearance by anti-hu5C8 antibodies, and the circulating immune complexes probably led to the serum sickness reaction observed in one animal. Thus, in contrast with the ability of aglycosyl hu5C8 to block the antibody response to itself in the TT immunization and islet transplant systems, Fc-dependent effector mechanisms are required to block its own immunogenicity in the context of renal transplantation. Consequently, in the face of limiting drug, the allograft is briskly rejected.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Group 1A (saline–saline)(^a)</th>
<th>Group 1B (saline–aglycosyl hu5C8)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal no.</td>
<td>1A-1  1A-2  1A-3  1A-4  1B-1  1B-2  1B-3  1B-4</td>
<td>6.5  2.0</td>
</tr>
<tr>
<td>Primary (E_{\text{AUC}}) ((\times 10^5))</td>
<td>2.1  1.1  2.0  0.3  3.9  0.9  1.6  2.6</td>
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</tr>
<tr>
<td>Secondary (E_{\text{AUC}}) ((\times 10^5))</td>
<td>8.7  5.2  9.6  6.0  8.3  1.5  3.3  5.5</td>
<td></td>
</tr>
<tr>
<td>Magnitude(^d)</td>
<td>4.2  4.8  4.8  18.8  2.1  1.6  2.1  2.2</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Group 1A animals received saline prior to both the primary and the secondary TT challenge. \(^b\)Group 1B animals received saline prior to the primary TT challenge and aglycosyl hu5C8 prior to the secondary TT challenge. \(^c\)The overall immune response \(E_{\text{AUC}}\) is represented by the area under the curve of TT titers from days 1 to 85. \(^d\)The magnitude of the secondary response is represented by the ratio of secondary \(E_{\text{AUC}}\) to primary \(E_{\text{AUC}}\).
binding data are in accord with the results for the human antibody; however, binding of aglycosyl muMR1 to human C1q is only modestly diminished (Fig. 5D). Murine C1q is not available for a comparable binding assay. Despite the difference in FcR interactions, both these mAbs delayed the onset of severe nephritis as measured by PU levels. Compared with the mulgG2a isotype control treated groups, anti-CD154-treated groups, 6–9 months old, had lower PU values, but by ~10 months of age all groups had high PU values indicative of disease progression (Fig. 6A). Importantly, muMR1 and aglycosyl muMR1 clearly prolonged survival of SNF1 mice in both studies (Fig. 6B and C) and the mechanisms underlying the prolonged survival in both anti-CD154-treated groups were therefore investigated. Treatment with muMR1 or aglycosyl muMR1 greatly diminished the auto-antibody response to both double-stranded DNA and single-stranded DNA as compared with control animals (Fig. 7A and B). A comparison of the overall histopathological scores of kidneys obtained from the remaining mice at 12.5–13 months of age (Fig. 7C) indicated that both anti-CD154-treated groups exhibited markedly less renal structural damage as compared with the mulgG2a control group wherein the two surviving animals had severe (>3+) end-stage nephropathy involving both glomerular and tubulointerstitial changes. Three other mulgG2a control mice died at 7–12 months of age before tissue could be obtained. All animals treated with muMR1 (n = 6) and most of the animals treated with aglycosyl muMR1 (n = 6) had mild (1+) early glomerular changes and little or no significant tubulointerstitial changes. However, two animals in the aglycosyl muMR1 group had mild-to-moderate (1+ to 2+) glomerulonephritids, suggesting a slight loss of efficacy as compared with the glycosylated mAb. Although mean disease scores for the glycosylated form were slightly lower, they were not statistically significant.

Discussion

The similar functional properties of aglycosyl human IgG1 mAb and the glycosylated form, except for binding to FcγRs and complement C1q, make it a useful tool for testing the role of effector function in models that are not amenable to gene knockout approaches. A striking result in our studies in non-human primate models was that the aglycosyl form of hu5C8 inhibited humoral immunity to a protein immunogen but was not effective in the context of allotransplantation. The aglycosyl form was comparable to the glycosylated form in inhibiting a primary humoral response to TT and suppressing germinal center formation. Aglycosyl hu5C8 was also efficacious in the inhibition of a secondary humoral response to TT. In contrast, aglycosyl hu5C8 was relatively ineffective in two different transplant settings.

In the islet cell transplant model a transient inhibition of rejection could be found in only one of the two aglycosyl hu5C8-treated animals, while in previous studies with the glycosylated form graft acceptance was found in all six animals tested. The comparable pharmacokinetics of the aglycosyl form to the glycosylated form in the islet study indicates that neither initial dosage nor clearance by anti-aglycosyl hu5C8 antibodies was a factor in the decreased efficacy and implicates that effector function is required for islet allograft acceptance. In the renal allograft model, rejection appeared to be as rapid and intense

Aglycosyl anti-CD154 delays disease progression in the SNF1 mouse model of lupus

The efficacies of an aglycosyl and a glycosylated anti-murine CD154 were compared in the SNF1 model of SLE, a spontaneously arising autoimmune disease characterized by the production of pathogenic anti-nuclear auto-antibodies, their deposition in the kidney and subsequent effector-mediated damage. The PK profiles of the anti-murine CD154 mAbs muMR1 and aglycosyl muMR1 are comparable to each other in BALB/c mice (Fig. 5A) and their CD154-binding activity is similar by competitive binding assay in vitro (Fig. 5B). No binding of the aglycosyl muMR1 to murine FcγRII P388D1 cells could be demonstrated at concentrations up to 400 μg ml⁻¹, while the normal glycosylated MR1 gave an EC₅₀ of 10 μg ml⁻¹ in the same assay format (Fig. 5C). The Fcγ-
in animals treated with aglycosyl hu5C8 as in the untreated controls. This is in contrast with previous experience with the glycosylated form in which long-term acceptance has been achieved without exception in nine animals. It should be emphasized that the failure to prolong allograft acceptance in the renal transplant model was accompanied by the development of a neutralizing antibody response to the aglycosyl mAb that removed it from circulation. It is therefore difficult to determine whether renal allograft rejection was due to lack of effector function or the unavailability of anti-CD154. Thus, even the ability of the aglycosyl form to inhibit a humoral response is compromised in the renal transplant setting and may contribute to allograft rejection.

The difference in efficacy in the three non-human primate model systems suggests that the mechanistic requirements depend on critical differences in the nature and intensity of the immune response. In the humoral setting, TT was given to cynomolgus monkeys as a thymus-dependent antigen, adsorbed to alum to act as a mild immune stimulant. In contrast, in the islet transplant studies the rhesus monkeys were mismatched based on in vitro alloreactivity and in the renal transplant studies they were fully MHC mismatched thereby involving the activation of a higher number of lymphocytes. Furthermore, the renal transplant surgery would be expected to cause a significant inflammatory response, which might make the ensuing cellular and humoral responses less dependent on CD154. Thus, in order to predict the clinical utility of non-effector forms of anti-CD154 mAbs in complex disease contexts such as SLE, we engineered an aglycosyl form of an anti-murine CD154 mAb and tested its effectiveness in a murine

**Fig. 4.** A membrane transfer assay capable of detecting the components of immune complexes was utilized to further analyze the serum from two aglycosyl hu5C8-treated rhesus monkeys that underwent renal transplant. Denatured immune complexes were immobilized on duplicate membranes and probed with biotinylated rsCD40L to measure hu5C8 or with biotinylated hu5C8 to detect anti-hu5C8. This method was validated by immobilized hu5C8 and rabbit anti-hu5C8 included as controls for each detection method. Serum samples obtained pre- or post-dose are as indicated. Anti-hu5C8 antibodies were detected in AT5D at days 12 and 16 and while hu5C8 was undetected by ELISA at day 12, it was readily detected here. Anti-hu5C8 antibodies were also detected in serum from AW34 on day 9.
lupus model. In this model, both forms of the anti-CD154 inhibited auto-antibody production and prolonged survival, although there were indications in the histopathological grading that the aglycosyl form was somewhat less potent. These results indicate that efficacy of anti-CD154 in SLE is largely independent of FcR interactions, although residual binding to C1q might play a role in the mechanism of action. These results are particularly promising given the recently reported clinical success of hu5C8 in lupus nephritis (5, 38) and they elucidate the potential utility of an aglycosyl anti-CD154 mAb in this and other autoimmune indications.

Our findings in allotransplantation are in agreement with those of others who recently reported Fc-dependent mechanisms of action of anti-CD154 mAbs in murine models. Sanchez-Fueyo et al. (17) demonstrated a dependence on complement for the efficacy of an anti-CD154 mAb given with donor-specific transfusion (DST) in a murine islet transplant model. Similarly, Monk et al. (18) have recently reported that an anti-CD154 mAb fails to delay rejection in a skin allograft model in complement- or FcR-deficient mice. They argue that the mAb must act through depletion of activated T cells in the wild-type mice, although no direct evidence for depletion is presented. In the primate models described here, preliminary investigations have not found evidence for T cell depletion in the humoral or transplant studies (34, 35), but this may be due to the low percentage of activated T cells involved. In addition to effector-induced cell depletion, other, non-ablative, effector mechanisms could also enhance the potency of the normal glycosylated mAb, including FcR-dependent distribution to sites of inflammation or scaffolding of the mAb to increase binding avidity. The high level of glycosylated hu5C8 required for efficacy in the humoral response (5–20 mg kg$^{-1}$) and the even higher level required in the transplant, relative to the antibody’s $K_d$ of interaction with CD154, may be suggestive of the need for scaffolding rather than cell ablation.

The requirement for effector interactions in some contexts is surprising given the previous information on CD154 biology. CD154-deficient humans (9) and mice (10) display cellular as well as humoral immune insufficiencies. In some transplant settings partial or complete engraftment has been achieved in CD40 and CD154 knockout mice (11–16). This demonstrates that interference with CD40–CD154 interactions can be adequate for graft acceptance under some conditions, perhaps by inducing regulatory mechanisms leading to T cell apoptosis (39–42) or enhancement of the effectiveness of regulatory T cells (43). Consistent with this, the aglycosyl anti-murine CD154 antibody is able to inhibit rejection of skin grafts when given in combination with DST (A. Rossini, personal communication).

The results of these studies highlight the complexities of the mechanisms of action of a therapeutic mAb and suggest that...
more than one mechanism may determine efficacy dependent on the disease context. Simple co-stimulatory blockade by an anti-CD154 mAb appears sufficient for inhibition of humoral-based diseases; yet, additional mechanisms are required for prolonged allograft survival. Thus, the efficacy profile of the aglycosyl anti-CD154 mAbs is attractive as it may provide safe immunomodulation in the contexts of autoimmune disease, but in the case of transplantation, concomitant blockade of other co-stimulatory pathways, reduction of inflammation or administration of anti-CD154 with DST may enable the aglycosyl form to promote full graft acceptance.
Supplementary data

Supplementary data are available at International Immunology Online.

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>DST</td>
<td>donor-specific transfection</td>
</tr>
<tr>
<td>hu5C8</td>
<td>humanized anti-CD154 mAb, 5C8</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>mulgG2a</td>
<td>murine IgG2a</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>PU</td>
<td>proteinuria</td>
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<tr>
<td>rsCD154</td>
<td>recombinant soluble human CD154</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SNF</td>
<td>(SWR x NZB)f1 hybrids</td>
</tr>
<tr>
<td>TMB</td>
<td>3',5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>TT</td>
<td>tetanus toxoid</td>
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References


