Induction of Immunological Tolerance/Hyporesponsiveness in Baboons with a Nondepleting CD4 Antibody

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Tolerance induction with anti-CD4 Abs is well established in rodent transplant and autoimmune disease models, but has yet to be demonstrated in non-human primates or in clinical studies. In retrospect, failure of anti-CD4 Abs to induce tolerance in primates may be technical, a consequence of insufficient dosing and Ab properties influencing immunogenicity and cell depletion. To circumvent these possible limitations, we constructed a novel anti-CD4 mAb, TRX1, humanized to reduce immunogenicity and Fc-modified to prevent cell depletion. Using equine immune globulin (equine Ig) as a model Ag, we examined the tolerance-inducing capacity of TRX1 in baboons. During the induction phase, TRX1 inhibited the humoral response to equine Ig in a dose-dependent manner, with complete suppression of response at the highest dose tested (40 mg/kg). Upon challenge, anti-equine Ig responses were generated in baboons treated with 1 and 10 mg/kg doses of TRX1 and in control animals. In higher dosing cohorts (20 and 40 mg/kg), however, the immune response to equine Ig was modulated in seven of nine animals, including complete unresponsiveness to Ag challenges in two animals. Five of nine were hyporesponsive to equine Ig, generating titers 50- to 250-fold lower than control groups. Repeated challenge resulted in titers falling to baseline or near baseline, with two of five hyporesponsive animals becoming unresponsive to Ag. All animals responded to neoantigen immunization, indicating that the modified response to equine Ig was Ag specific. These studies demonstrate that anti-CD4 Ab-mediated, Ag-specific tolerance can be achieved in baboons without long term immune suppression. *The Journal of Immunology,* 2004, 173: 4715–4723.

Nondepleting Abs directed against the CD4 coreceptor have proven to be exceptionally effective at inducing durable, Ag-specific tolerance to soluble proteins (1–3) and tissue and organ transplants (4, 5) and at re-establishing self-tolerance in rodent models of autoimmune disease (6–9). Tolerance so induced with anti-CD4 Abs is generated in the periphery (3, 10, 11) and is mediated, at least in part, by Ag-specific CD4$^+$ regulatory T cells (10, 12, 13) capable of suppressing both naive and primed CD4$^+$ and CD8$^+$ T cells (4, 5, 12–15) and also guiding the development of naive T cells toward tolerance, a process known as infectious tolerance (13). Challenge with Ag has been shown to maintain and in some instances boost tolerance induced with anti-CD4 Abs, demonstrating that once established, tolerance could be maintained by Ag alone (16).

Yet despite the success in rodents, tolerance induction with anti-CD4 Abs has yet to be demonstrated in primates. Although several anti-CD4 Abs have been evaluated in preclinical non-human primate models of transplant (17, 18) and autoimmune disease (19, 20) as well as in a number of clinical studies (21–32), their therapeutic effectiveness was modest at best, of short duration, and most likely the consequence of transient immunosuppression. In retrospect, the failure of anti-CD4 Abs to induce a more robust and durable response in primates may be attributed to technical factors relating to both Ab properties and dose. For example, early clinical studies used mouse (25, 26, 28–31) and later chimeric (21, 23, 24, 27, 32) anti-CD4 mAbs that were in many instances immunogenic

(28, 33) and, therefore, elicited neutralizing human anti-mouse Ab (HAMA)[2] and human anti-chimeric Ab (HACA) responses against the Abs leading to their rapid clearance. In addition, the posology of anti-CD4 Ab-mediated tolerance induction from rodent studies indicated a need for high doses of Ab, if only for a short time (16). Clinical studies did not achieve, and in most cases did not approach, comparable dosing levels due to adverse side effects or the depleting nature of the Abs. In fact, many previous clinical studies failed to recognize the advantages of a nondepleting anti-CD4 Ab, although it is now clear that this is preferable because immune reconstitution in adults is limited (34–36), and the major regulatory T cell population mediating such tolerance is itself CD4$^+$ (3, 10, 12).

To circumvent these proposed limitations we constructed a novel anti-CD4 mAb, TRX1, humanized to reduce immunogenicity and further modified in the Fc region to eliminate FcR interactions and complement binding. This would avert CD4$^+$ cell depletion and enable us to dose at levels predicted to be efficacious from rodent models. We tested the ability of TRX1 to induce tolerance in baboons to an immunogenic biologic, antivenin, or equine immune serum globulin (equine Ig) and report that durable Ag-specific tolerance can indeed be induced in primates with a nondepleting anti-CD4 mAb and without long term immune suppression or dose-limiting side effects.

Materials and Methods

Equine Ig as a source of Ag

Antivenin (Crotalidae polyvalent; Fort Dodge Laboratories, Overland Park, KS) was reconstituted with diluent provided by the manufacturer and was used as the source of equine Ig. The solution was passed through a 2-μm pore size syringe filter and aggregated by diluting to 25 mg/ml in 0.9%

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[2] Abbreviations used in this paper: HAMA, human anti-mouse Ab; CBC, complete blood count; CHO, Chinese hamster ovary; HACA, human anti-chimeric Ab; MCF, mean channel fluorescence.

saline and incubating at 64°C for 35 min, followed by overnight incubation on ice. The material was stored at −80°C until use. The amount of aggregated material in each lot was determined by HPLC size exclusion chromatography and ranged from 21.2 to 29.9% of total protein.

TRX1 production and purification

TRX1 is derived from the mouse anti-human CD4 hybridoma, NSM 4.7.2.4 (H. Waldmann, unpublished observations). The parental H and L chain cDNA were cloned from an NSM 4.7.2.4 cDNA library by cross-hybridization with rat H and L chain gene cDNA probes using standard molecular biology techniques. Sequence analysis of the cDNA derived from NSM 4.7.2.4 confirmed the H chain isotype to be γ1 and the L chain isotype to be κ. The NSM 4.7.2.4 mouse V_H and V_L regions were reshaped to human V_H and V_L regions using best-fit or human frameworks with the highest sequence similarity to that of the mouse V_H and V_L (M. Frewin, S. Gorman, and H. Waldmann, unpublished observations). For the L chain, Ab HSIGKAW (EMBL accession no. M29467) with a sequence similarity of 79% was used as the framework source. For the H chain, Ab A32483 (PIR accession no. A32483) with a sequence similarity of 74% was used. Humanization was performed by site-directed mutagenesis of the mouse cDNA clones. To eliminate Ab binding to FcRs as well as complement fixation, a single amino acid substitution was introduced in the Fc region at amino acid position 297 of the γ1 H chain constant region by site-directed mutagenesis eliminating the site of *N*-linked glycosylation.

TRX1 Ab was produced at the Therapeutic Antibody Centre (Oxford, U.K.) by hollow fiber fermentation of Chinese hamster ovary (CHO) cell transfectants. The Ab was purified from culture supernatant by protein A affinity chromatography, followed by cation/anion exchange, nanofiltration, and size exclusion chromatography. The purified material was formulated in PBS and stored at −80°C.

Tolerance induction and challenge protocol

All baboon work was performed at the Southwest Foundation for Biomedical Research (San Antonio, TX) under an Institutional Animal Care and Use Committee-approved protocol. Seven to 21 days before study, animals were screened by physical examination, complete blood count (CBC), and serum chemistries. Lymphocyte subset numbers and CD4 expression level on CD3^{+} cells were determined for baseline values. A second set of baseline values was collected on day −1 before the first TRX1 or saline infusion. Animals were sedated with a single dose of 10 mg/kg ketamine plus 5 mg of diazepam as needed. Infusions were administered i.v. at 30 ml/h. Temperature, blood pressure, and respiration were monitored during and after infusions. Animals were examined for skin rashes and lymphadenopathy at the time of each infusion and serum sample collection. In addition, animals were monitored daily for signs of discomfort, malaise, arthralgia, and gastrointestinal complications. The first dose of Ag (equine Ig) was given on day 0 as a 10 mg/kg i.v. bolus. All other doses of Ag (days 4, 8, 68, 95, and 130) were given as a 10 mg/kg s.c. bolus, except for the last challenge on day 130, which was a 1 mg/kg s.c. bolus.

Animals were immunized with SRBC (HemoStat Laboratories, Dixon, CA) to demonstrate immunocompetence to a neo-Ag after TRX1 exposure. All animals received a single i.v. injection of a 10% SRBC solution in 0.9% sterile saline at a dose of 1.7 ml/kg on day 68 of the study.

TRX1 serum concentration

The concentration of TRX1 in serum was determined by ELISA. Fifty microliters of a 5 μg/ml solution of soluble CD4 in PBS (provided by Therapeutic Antibody Centre) was dispensed into 96-well plates and incubated overnight at 2–8°C. After three washes with PBS containing 0.05% Tween 20 (wash buffer), plates were blocked with 1% BSA/0.05% Tween 20 in PBS (blocking buffer) for 1 h at 37°C and stored at 2–8°C. Immediately before use, plates were washed three times with wash buffer. Baboon serum samples were prepared from a 1/10 or 1/100 starting dilution in blocking buffer, followed by serial 1/10 dilutions and transfer of 50 μl of diluted sample to the soluble CD4-coated plates. A standard curve included on each plate was prepared from a 1 μg/ml solution of unconjugated TRX1 serially diluted 1/4. After a 2-h incubation at 37°C, plates were washed three times, and 50 μl of a peroxidase-conjugated donkey anti-human IgG (0.08 μg/ml in blocking buffer) was added to each well. Plates were incubated for 1 h at room temperature, washed three times, and developed. TRX1 serum concentrations were calculated from all OD values falling within the linear portion of the TRX1 standard curve.

Immune response to equine Ig

Baboon antiglobulin responses to equine Ig were determined by ELISA. Ninety-six-well plates coated with 50 μl/well of a 10 μg/ml solution of

antivenin in carbonate buffer were incubated overnight at 4°C. Plates were then washed three times and blocked for 2 h at 37°C. After the blocking step, plates were washed three times, and baboon serum samples were added to wells (50 μl/well) using a 3-fold serial dilution scheme beginning with a 1/10 dilution and incubated for 2 h at room temperature.

After three washes, peroxidase-conjugated, rabbit anti-human IgG/IgM Ab (diluted 1/10,000) was added to each well (50 μl/well) and incubated for 1 h at room temperature. Plates were washed three times and developed for 8 min at room temperature. The assay was standardized by including on each plate a positive control serum from a previously immunized animal. The positive control was used in all assays at a 1/25,000 dilution. Titer is defined as the reciprocal of the dilution resulting in an OD value equivalent to twice the OD value of the diluted standard.

SRBC hemolysis assay

The immune response to SRBC was assessed by hemolysis. Serum samples were heat-inactivated at 56°C for 30 min, followed by preparation of a 2-fold dilution series starting from a 1/10 dilution in PBS plus 0.1% BSA. One hundred microliters of diluted serum was combined with an equal volume of 1% SRBC solution, followed by the addition of 100 μl of guinea pig complement (Sigma-Aldrich, St. Louis, MO) preabsorbed with SRBC diluted 1/10 in PBS. The plates were incubated at 37°C for 30 min. Titer is defined as the reciprocal of the highest dilution of serum that did not cause obvious hemolysis.

Abs and flow cytometry

Normal donkey serum, donkey anti-human IgG-biotin, donkey anti-human IgG F(ab′)$_2$-biotin, donkey anti-human IgG-peroxidase, donkey IgG-biotin, rabbit anti-human IgG/IgM, and human IgG-biotin were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). FITC-conjugated mouse anti-human CD4, clone M-T441, and FITC-conjugated mouse IgG2b, clone BPC 4, were purchased from Ancell (Bayport, MN). Mouse anti-human CD3-FITC, clone SP34, mouse IgG3-FITC, and mouse anti-human CD45RA-PE were purchased from BD Biosciences Pharmingen (San Diego, CA). Mouse anti-human CD8-PerCP and mouse IgG1–PerCP were purchased from BD Biosciences Immunocytometry Systems (San Jose, CA). Streptavidin-Quantum Red was purchased from Sigma-Aldrich, and FITC- and Cy5-conjugated standard beads were obtained from Bangs Laboratories (Fishers, IN).

CD4 saturation was determined as a function of free CD4 sites on circulating lymphocytes. One hundred microliters of heparinized whole blood was pelleted by centrifugation, and plasma was removed by aspiration. Cells were resuspended in 100 μl of a 1.0 μg/ml solution of biotinylated TRX1 or biotinylated human IgG. After a 20-min incubation on ice, cells were washed with 1 ml of wash buffer and incubated with 50 μl of streptavidin-Quantum Red (1/5 dilution of stock) for 20 min on ice. RBC were then lysed by the addition of 2 ml of lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 100 μM disodium EDTA). Samples were vortexed and incubated at room temperature until clear (∼10 min). RBC debris was removed by centrifugation and washing with 1 ml of wash buffer. Cells were fixed by the addition of PBS/0.1% Formalin. Intraday fluorescence sensitivity variation was controlled using FITC- and Cy5-conjugated standard beads.

CD4^{+} lymphocyte counts

The number of CD4^{+} lymphocytes in peripheral blood was determined by multiplying the absolute lymphocyte count obtained from CBC by the percentage of CD4^{+} lymphocytes. The percentage of CD4^{+} lymphocytes in whole blood was determined by flow cytometry as the percentage of CD4^{+} cells in the lymphocyte gate staining with FITC-conjugated M-T441, a mouse Ab recognizing domain 2 of CD4 that does not compete with TRX1 binding.

Results

Tolerance induction protocol

TRX1 is a humanized IgG1 Ab recognizing domain 1 of human CD4 further modified by introducing a single amino acid substitution (Asn to Ala) at position 297 in the H chain constant region, thus eliminating a major glycosylation site necessary for high affinity FcR interactions and complement binding (37–39). To identify a model species in which to test tolerance induction with TRX1, we screened several non-human primate species, including African green monkey, cynomolgus and rhesus macaque, baboon, and chimpanzee, for cross-reactivity with TRX1. All showed some

degree of immunoreactivity, but the binding affinity was comparable to human only in chimpanzee and baboon. Baboon was selected as the model species.

As a target Ag for tolerance induction, we sought a simple, yet clinically relevant, model Ag. This would allow us to test for Ag-specific tolerance as well as to optimize the induction protocol before evaluating TRX1 in more complex models of transplant and autoimmunity. We selected a well-characterized immunogenic biologic antivenin or anti-venom, a commercial preparation of equine immune serum globulins (equine Ig) isolated from horses immunized with pit viper venoms (40, 41). To ensure immunogenicity, the antivenin was heat-aggregated, and the preparation was tested in a pilot experiment to determine a dose and route of administration that would generate a robust immune response before use in our tolerance induction protocol (not shown).

To investigate the feasibility of tolerance induction with TRX1 in baboons, we designed an experimental protocol divided into three phases: induction, washout, and challenge (Fig. 1A). Twenty-one baboons (*Papio cynocephalus anubis*) were assigned to one of seven groups (three animals per group) including four experimental and three control groups (Fig. 1B). The experimental arm of the induction phase included four TRX1 dosing cohorts of 1, 10, 20, or 40 mg/kg/dose infused four times over 13 days on days −1, 3 or 4, 8, and 12. A 10 mg/kg i.v. bolus of heat-aggregated Ag (equine Ig) was delivered on day 0, followed on days 4 and 8 with an s.c. bolus of the same dose. In the control arm, animals in control group I (Ag only) were infused with an equivalent volume of saline rather than TRX1 at each time point, exactly as animals in the experimental groups. Control group II (TRX1 only) was comprised of two cohorts, 20 and 40 mg/kg TRX1, treated on the same schedule as the experimental groups, but receiving saline instead of equine Ig during the tolerization phase. TRX1 serum concentrations were determined 24 h after the first dose of Ab and

immediately before the three subsequent doses as well as weekly thereafter. Serum levels of TRX1 and equine Ig were monitored until they were no longer detectable (washout phase), at which time all animals were challenged by s.c. injection with Ag (challenge phase).

TRX1 suppresses the humoral response during induction without depletion of T cells

A dose-dependent increase in TRX1 serum concentration was evident 24 h after the first dose, ranging from a mean of 15.6 ± 4.1 μg/ml ($n = 3$) in animals receiving 1 mg/kg up to a mean of 542.5 ± 138.1 μg/ml ($n = 6$) in those receiving 40 mg/kg (Fig. 2A). Serum concentrations of TRX1 determined immediately before subsequent doses indicated a dose accumulation of TRX1 in the 20 and 40 mg/kg treated animals, with mean trough level concentrations increasing after each dose. Minimum TRX1 serum concentrations occurred between the first and second doses of Ab and ranged from a mean of 39.4 ± 18.0 μg/ml ($n = 6$) for 20 mg/kg TRX1-treated animals up to a mean of 162 ± 63.3 μg/ml ($n = 6$) for those receiving 40 mg/kg TRX1. There was no dose accumulation of TRX1 in animals receiving 1 or 10 mg/kg TRX1, because trough level concentrations determined immediately before the last three doses of Ab were below the limit of detection of the assay (0.2 ng/ml) as were those in control group I animals, i.e., those receiving Ag only. A protocol deviation at the time of the second TRX1 infusion eliminated one animal (no. 16250) from further study in the 20 mg/kg TRX1 only control group II.

TRX1 was detected by flow cytometry on $CD3^+$ lymphocytes using biotinylated $F(ab')_2$ donkey anti-human IgG. Twenty-four hours after the first infusion, mean channel fluorescence (MCF) values were well above baseline values and remained so throughout the treatment period, beginning a return to baseline levels by day 27. TRX1 was undetectable on cells by day 48 (data not shown). To determine the level of CD4 saturation by TRX1, biotinylated TRX1 was added to whole blood samples, and cell staining was assessed by flow cytometry (Fig. 2B). As expected from the TRX1 serum concentration data, free CD4 sites were readily detected in the 1 mg/kg TRX1 group. Except for the initial 24 h point, MCF values determined for samples obtained just before TRX1 treatment on days 3, 8, and 12 in the 1 mg/kg group were only slightly less than baseline values, averaging 89.5% of baseline (range, 86.0–92.9%), or 10.5% saturated. Free binding sites were also detected in the 10 mg/kg TRX1 group from samples taken just before TRX1 treatment on days 3, 8, and 12, with an average MCF value of 25.8% of baseline (range, 19.3–33.4%) during the induction phase, indicating that 74.2% of the sites were saturated. The 20 mg/kg group averaged 14.9% of baseline MCF staining (range, 10.2–18.2%), or 85.1% saturated, during the induction phase, whereas the 40 mg/kg group averaged MCF values 9.5% of baseline (range, 8.1–10.7%), or 90.5% saturated. By day 20, 1 wk after the last dose of TRX1, MCF values for both 1 and 10 mg/kg TRX1 groups had returned to baseline, whereas staining from the 20 mg/kg TRX1 group indicated the number of free CD4 sites to be ~25% of baseline. The 40 mg/kg TRX1 group maintained maximum saturation on day 20, but free CD4 sites were detected on day 27 with average MCF values at 24.7% of baseline, reflecting 75.3% saturation. By day 48 MCF values had returned to baseline for both the 20 and 40 mg/kg TRX1 groups. Reappearance of free CD4 sites correlated with the reduction in TRX1 serum concentrations during the washout phase with biotinylated TRX1 staining; they first began to increase once TRX1 serum levels dropped below ~10 μg/ml.

One animal in the 20 mg/kg TRX1 experimental group (no. 15983) showed a more rapid return to baseline of free CD4 sites as
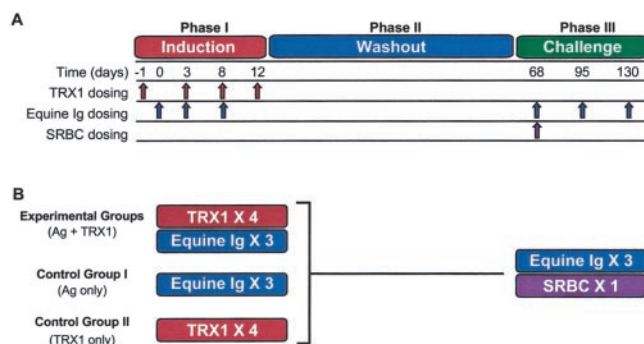

FIGURE 1. Schematic overview of the tolerance induction and Ag challenge protocol. *A*, The protocol was divided into three phases: induction, washout, and challenge. During the induction phase, TRX1 Ab or saline was infused on days −1, 3 or 4, 8, and 12. Ag (equine Ig), or saline was administered on days 0, 3, and 8. The induction phase was followed by a washout phase, during which serum levels of TRX1 and equine Ig were monitored until no longer detectable. The challenge phase was initiated on day 68 by treating all animals with equine Ig as well as a neoantigen, SRBC. Additional equine Ig challenges were administered on days 95 and 130. *B*, Treatment groups consisted of four experimental TRX1 dosing cohorts and two control groups. The experimental groups received four infusions of TRX1 at 1, 10, 20, or 40 mg/kg and three doses of Ag. Control group I (Ag only), received four infusions of saline and three doses of Ag. Control group II (TRX1 only) was comprised of two cohorts with animals receiving four infusions of TRX1 at 20 or 40 mg/kg plus three doses of saline rather than Ag. All animals were challenged three times with equine Ig and received a single immunization with SRBC at the time of the first Ag challenge.
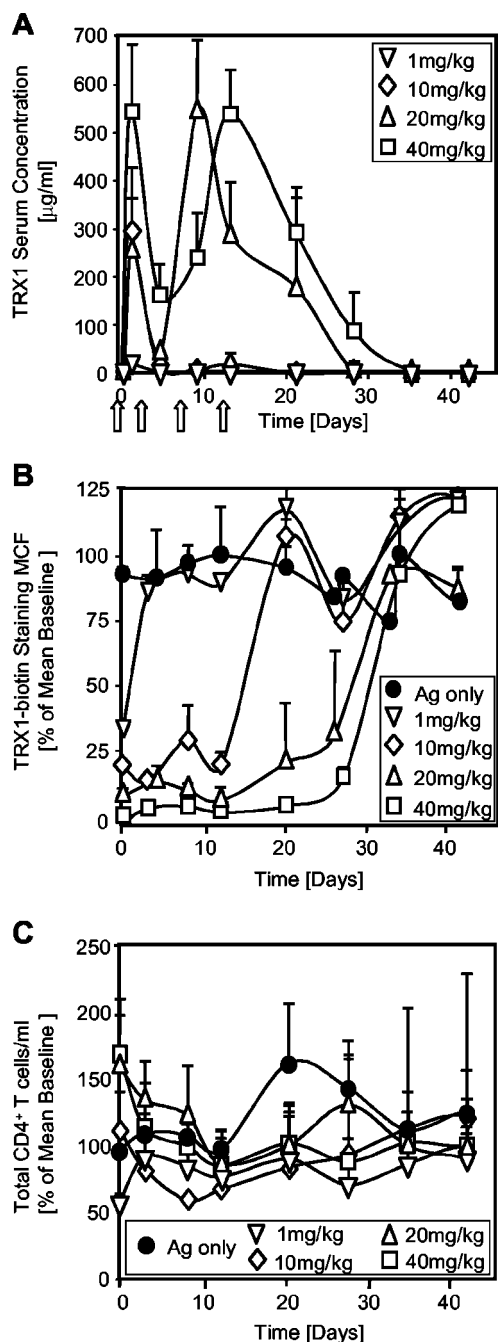
FIGURE 2. Pharmacokinetics and pharmacodynamics of TRX1 during the induction and washout phases. *A*, Group mean TRX1 serum concentrations (micrograms per milliliter). Experimental and control group II animals receiving equivalent TRX1 doses (20 and 40 mg/kg) are combined. The arrows indicate treatment with TRX1. ▽, ◇, △, and □, Animals grouped according to the dose of TRX1 received: 1 mg/kg (*n* = 3), 10 mg/kg (*n* = 3), 20 mg/kg (*n* = 4), or 40 mg/kg (*n* = 6). *B*, Saturation of CD4 sites on CD3^{+} cells in peripheral blood during induction and washout phases. Free CD4 sites were detected by TRX1-biotin staining of whole blood. The mean MCF value for each group is represented as a percentage of the mean baseline value. ●, Control group I (*n* = 3); ▽, ◇, △, and □, animals grouped according to the dose of TRX1 received: 1 mg/kg (*n* = 3), 10 mg/kg (*n* = 3), 20 mg/kg, and 40 mg/kg (*n* = 6). *C*, Total CD4^{+} T cells per milliliter of blood. Group mean absolute CD4^{+} lymphocyte counts as a percentage of the mean baseline values. CD4^{+} cells were detected with a domain 2-specific mAb, and absolute values were calculated as described in *Materials and Methods*. ●, Control group I (*n* = 3); ▽, ◇, △, and □, animals grouped according to the dose of TRX1 received: 1 mg/kg (*n* = 3), 10 mg/kg (*n* = 3), 20 mg/kg (*n* = 4), and 40 mg/kg (*n* = 6).

well as a more rapid clearance of TRX1 from serum. We suspected that this was due to the development of an immune response against TRX1, which we subsequently confirmed by ELISA. Of note, this animal had the lowest TRX1 serum concentration trough level of all animals in the 20 mg/kg TRX1 group (13.4 μg/ml on day 4) between the first and second doses of Ab. All other animals in this group had TRX1 serum concentrations ≥35.0 μg/ml. Data from this animal are not included in the 20 mg/kg group mean calculations. All animals in the 1 mg/kg (three of three) and 10 mg/kg (three of three) TRX1 experimental groups mounted an immune response to TRX1, which was detectable by ELISA 7–10 days after the first dose of Ab (not shown). Only one other animal (no. 16313) made a detectable immune response to TRX1; this occurred in the 40 mg/kg TRX1 control group II. However, this response was not detectable until day 27, >2 wk after the last dose of TRX1.

We observed no treatment-related adverse events during infusions or at any time after TRX1 treatment for the duration of the study.[3] CBCs and flow cytometry data showed no apparent depletion of CD4^{+} lymphocytes at any dose. Although day-to-day variability in lymphocyte counts was evident, no significant differences between TRX1-treated animals and those receiving saline were observed, nor were any dose-dependent differences evident among the TRX1-treated animals (Fig. 2*C*). Similar to our in vitro assessment, we observed only modest CD4 modulation from the cell surface (not shown).

Administration of TRX1 did result in a dose-dependent inhibition of the humoral response to equine Ig during the induction and washout phases (Fig. 3*A* and supplementary Table V*A*). We detected no immune response to equine Ig in any animal in the 40 mg/kg TRX1 experimental group throughout this period. However, an elevation in the group mean titers against equine Ig was evident for the 20 mg/kg TRX1 experimental group. Two of three animals in this group (no. 16276 and 16096) responded with maximum peak titers of <10-fold above baseline; this occurred on day 27, followed by a return to baseline by day 48. Animal 15983, the same animal in which we observed an immune response to TRX1, mounted a larger and more sustained response to equine Ig during the induction and washout phases, peaking on day 41 at >25-fold above baseline and remaining >10-fold above baseline through the washout phase. Higher titers were also evident in both the 1 and 10 mg/kg TRX1 experimental groups as well as in control group I (Ag only). Surprisingly, mean titers for the 1 mg/kg TRX1 experimental group were ~10- to 15-fold above those for control group I. One explanation for this apparently enhanced response may be priming to human Ig epitopes cross-reactive with equine Ig.

TRX1 induces Ag-specific hyporesponsiveness and tolerance

Once TRX1 serum levels fell below the limit of detection, we assessed tolerance to equine Ig by challenging animals with Ag and measuring the resulting specific humoral immune response. Animals were first challenged by s.c. administration of 10 mg/kg equine Ig on day 68. All animals in the 1 and 10 mg/kg TRX1 dose groups generated a robust secondary immune response to the Ag, with group mean Ab titers closely matching that of control group I (Fig. 3*B* and supplementary Table V*A*). The response was characterized by a rapid rise in Ab titer as well as higher maximum titers compared with the response observed in these groups during the tolerization phase. Showing no evidence of tolerance to equine Ig, animals from the 1 and 10 mg/kg TRX1 experimental groups
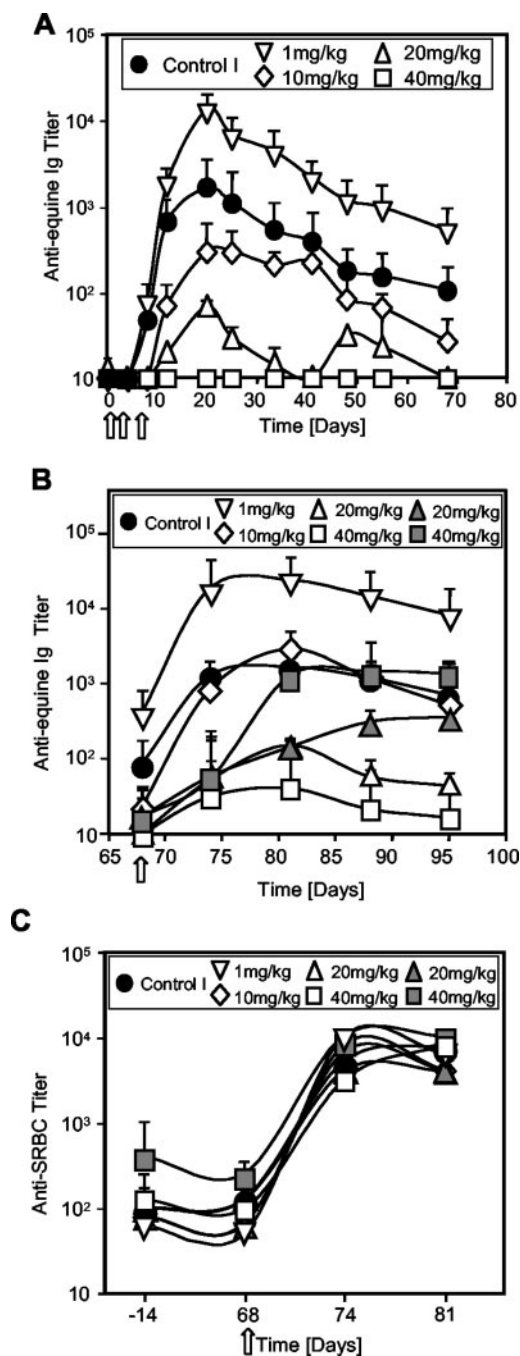
FIGURE 3. Immune response during induction and first challenge. *A*, Group mean Ab titers generated against equine Ig during the induction phase. Animals received three doses of Ag, as indicated by arrows. Titer is defined as the reciprocal of the serum dilution resulting in an OD value equivalent to twice the OD value of a positive control standard. ●, Control group I (*n* = 3); ▽, ◇, △, and □, TRX1 experimental dosing cohorts: 1 mg/kg TRX1 (*n* = 3), 10 mg/kg TRX1 (*n* = 3), 20 mg/kg TRX1 (*n* = 2), and 40 mg/kg TRX1 (*n* = 3). *B*, Group mean Ab titers generated against equine Ig after the first challenge given on day 68 (arrow). ●, Control group I (*n* = 3); ▲ and ■, control group II cohorts: 20 mg/kg TRX1 (*n* = 2); and 40 mg/kg (*n* = 3); ▽, ◇, △, and □, TRX1 experimental dosing cohorts: 1 mg/kg (*n* = 3), 10 mg/kg (*n* = 3), 20 mg/kg (*n* = 3), and 40 mg/kg (*n* = 3). *C*, Immune response to the neo-Ag, SRBC, administered at the time of first challenge on day 68 (arrow) and measured by hemolysis of SRBC. ●, Group mean Ab titers for control group I (*n* = 3); ▲ and ■, control group II cohorts: 20 mg/kg TRX1 (*n* = 2) and 40 mg/kg (*n* = 3); ▽, ◇, △, and □, TRX1 experimental dosing cohorts: 1 mg/kg (*n* = 3), 10 mg/kg (*n* = 3), 20 mg/kg (*n* = 3), and 40 mg/kg (*n* = 3). Titer is defined as the reciprocal of the highest dilution of serum that did not cause hemolysis.

were released from study after the first challenge. Control group II, receiving Ag for the first time on day 68, responded with a group mean Ab titer to equine Ig rising more slowly than the recall response in control group I (Fig. 3*B* and supplementary Table V*A*), as would be expected of a primary response. Group mean titers for the 20 and 40 mg/kg TRX1 experimental groups also increased in response to challenge, but with significantly reduced (50- to 250-fold) peak titers compared with control group I (Fig. 3*B* and supplementary Table V*A*). One of three animals in the 20 mg/kg TRX1 experimental group responded to challenge with a rise in titer similar to that in the control group I; this occurred in animal 15983, which had also generated an immune response to TRX1 during the induction and washout phases. The two other animals in this group (no. 16276 and 16096) were hyporesponsive to Ag challenge, with a maximum mean peak response 10-fold less than that in control group I. In the 40 mg/kg TRX1 experimental group, one animal (no. 16192) was similarly hyporesponsive to challenge, with the two other animals in this group (no. 16178 and 16286) showing no response to challenge.

To demonstrate that the absence of a vigorous immune response to equine Ig challenge in five of six animals in the combined 20 and 40 mg/kg TRX1 experimental groups was Ag specific and not the consequence of treatment-related immune suppression, we assessed immunocompetence by immunizing all animals with a third-party Ag, SRBC, at the time of first challenge on day 68. All groups mounted an essentially equivalent anti-SRBC hemolytic response to this challenge (Fig. 3*C*), which we confirmed to be predominately IgG by ELISA (not shown).

Control groups I and II as well as the 20 and 40 mg/kg TRX1 experimental groups were rechallenged with equine Ig on day 95 and again on day 130 (Fig. 4*A* and supplementary Table V*A*). All control groups showed a similar boost in the humoral response to Ag challenge, demonstrating that TRX1 treatment alone did not induce long-standing immune suppression. However, group mean titers for the 20 and 40 mg/kg TRX1 experimental groups failed to rise above the maximum peak titers of the first challenge even with repeated challenges. For animals in the 20 mg/kg TRX1 experimental group, excluding animal 15983, maximum titers occurred after the first challenge, with peak titers of 269 and 145 for animals 16096 and 16276, respectively. Peak responses then diminished upon repeated challenge to 35 and 92, respectively, after the third challenge. Group mean titers in the 40 mg/kg TRX1 experimental group were consistently lower than those in the 20 mg/kg group, with a single animal (no. 16192) accounting for essentially all the response, with a maximum peak titer of 313 after the first challenge. Similar to animals in the 20 mg/kg TRX1 group, the peak response to each subsequent challenge was lower than for the previous challenge, with the response in 16192 response declining to a peak titer of only 39 after the third challenge with Ag (Fig. 4*B* and supplementary Table V*A*). The two other animals in the 40 mg/kg TRX1 experimental group (no. 16178 and 16286) generated virtually no detectable immune response to equine Ig upon repeated challenge.

We performed a second study (three animals per group) with the 20 mg/kg TRX1 dose, reducing the number of TRX1 doses from four to three, but administering them every other day on days −1, 1, and 3. A control group (control group I) was also included with animals receiving saline infusions in place of TRX1. Equine Ig treatment was unchanged, with the animals receiving three doses of 10 mg/kg on days 0, 3, and 8. As in the first study, TRX1 administration resulted in a suppression of the humoral response to equine Ig during the induction and washout phases compared with control group I, with one animal (no. 16224) accounting for essentially all the detectable response (Fig. 5*A* and supplementary
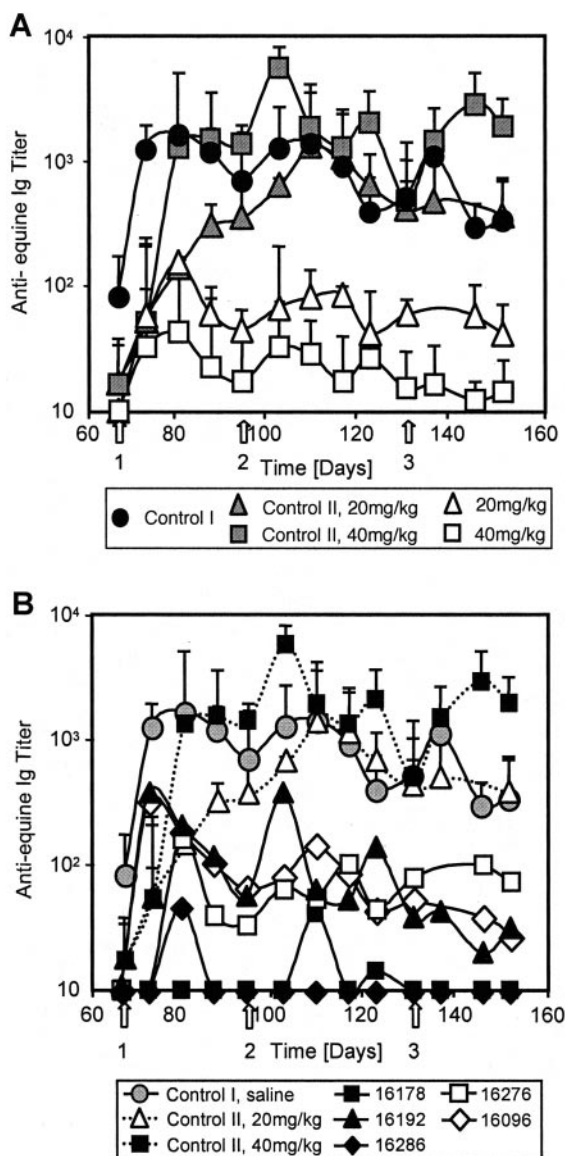
FIGURE 4. Immune response to equine Ig after multiple challenges. *A*, Group mean Ab titers for Control group I (*n* = 3), ●; Control group II cohorts: △ and ■, 20 mg/kg TRX1 (*n* = 2) and 40 mg/kg TRX1 (*n* = 3); △ and □, TRX1 experimental group cohorts: 20 mg/kg (*n* = 2) and 40 mg/kg (*n* = 3). *B*, Ab titers to equine Ig of individual animals in the TRX1 experimental groups: 20 mg/kg (animals 16276 and 16096; □ and ◇, solid lines) and 40 mg/kg (animals 16178, 16192, and 16286, ■, ▲, and ◆, solid lines) cohorts plotted with the group mean Ab titers to equine Ig for control group I ◐, solid line) and control group II, 20 mg/kg (△, dotted line; *n* = 2) and 40 mg/kg (■, dotted line; *n* = 3) cohorts.

FIGURE 5. Immune response to equine Ig with modified TRX1 dosing. ●, Control group I (*n* = 3); △, 20 mg/kg TRX1 experimental group (*n* = 2). *A*, Group mean Ab titers generated against equine Ig during the induction phase. Animals received three doses of TRX1, indicated by arrows, on day −1, 1, and 3. Equine Ig was administered on days 0, 4, and 8. *B*, Group mean Ab titers generated against equine Ig during the challenge phase. Animals were challenged s.c. with 10 mg/kg Ag on days 68 and 97 and with 1 mg/kg Ag on day 133.

to a third challenge with Ag. As in the previous study, all animals responded to SRBC neoantigen immunization at the time of first challenge on day 68 (not shown).

Discussion

We postulated that the failure of previous anti-CD4 Abs to induce tolerance in non-human primates or to produce long term clinical benefit in man may be technical in nature due to characteristics of the Abs, in particular, immunogenicity and $CD4^+$ cell depletion, as well as inadequate dosing. We engineered a novel anti-CD4 Ab, TRX1, humanized to reduce immunogenicity and Fc-modified to eliminate effector functions and thus avert depletion of $CD4^+$ cells. These modifications enabled us to administer TRX1 at predicted tolerogenic doses based on previous studies in rodents (16). The two low dose cohorts, in which four doses of 1 or 10 mg/kg TRX1 were administered over 13 days, did not result in tolerance or hyporesponsiveness to our model Ag, equine Ig, although the 10 mg/kg cohort did exhibit a slight suppression of the humoral response during the induction phase. By increasing the TRX1 dose to 20 mg/kg, we were able to induce hyporesponsiveness in two of

Table V*B*). On day 68 with serum levels of TRX1 below detectable levels, animals were challenged with equine Ig. Control group I responded as expected with a rapid and robust rise in titers to a mean peak response of 7652. In the 20 mg/kg TRX1-treated group, animal 16224 showed a rapid rise in titer similar to control group animals, with a maximum peak titer of 6139. However, two other animals in the group (no. 12093 and 16130) were hyporesponsive to challenge, generating peak titers of 37 and 161, respectively, for a mean peak response of 78. A second challenge on day 97 produced only a slight rise in titer to 20 and 26 for animals 12093 and 16130, respectively, which fell rapidly to baseline. These two animals showed no response
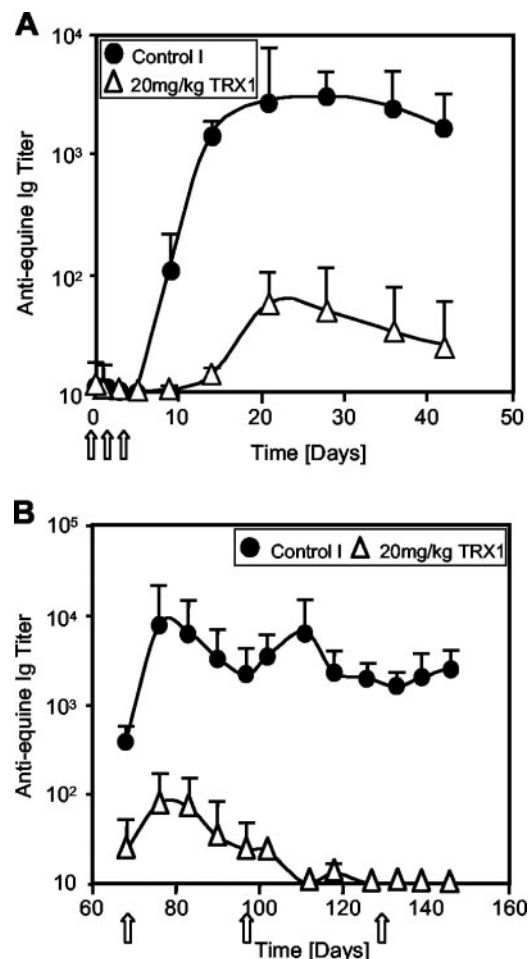
three animals, with the maximum response titer diminishing after each subsequent challenge. With doses of 40 mg/kg, two of three animals were completely nonresponsive to multiple Ag challenges, and the third was hyporesponsive to Ag, with peak response titers again declining with each Ag challenge. The amount of TRX1 administered in the two high dose cohorts (20 and 40 mg/kg) that resulted in modulation of the humoral response to equine Ig is consistent with the effective doses of nondepleting anti-CD4 Abs used in rodents to generate tolerance to soluble proteins and allografts (1–5, 10, 12) and is well above those used in most previous non-human primate and clinical studies with anti-CD4 Abs (21–32). In fact, the highest Ab doses administered in most previous clinical studies with anti-CD4 Abs, both cumulative and on a per weight basis, fall below the amount administered in our lowest TRX1 dose cohort. Studies in mice have demonstrated that three doses of 20–25 mg/kg of a non-depleting anti-CD4 Ab administered every other day were sufficient to induce tolerance, although the time required for tolerance to become evident was approximately 1 mo after dose initiation (16). We modified the 20 mg/kg TRX1 treatment, administering three doses, one every other day, beginning 1 day before Ag administration. With this modification two of three animals became completely unresponsive to Ag challenge after an initial period of hyporesponsiveness. Although difficult to conclude definitively without formal head-to-head comparisons, the results with TRX1 suggest the likelihood that immunogenicity, depletion and dose were among the key factors underlying the limited effectiveness of previous anti-CD4 Abs.

The mechanism by which TRX1 induces hyporesponsiveness or tolerance to equine Ig in baboons is unresolved. In mice, tolerance induced with nondepleting anti-CD4 Abs is mediated by Ag-specific $CD4^+$ regulatory T cells generated in the periphery (3, 10, 11, 42). Although these cells have features in common with thymic-derived $CD4^+CD25^+$ regulatory T cells, they appear to represent a distinct population (42–45). Despite recent progress, anti-CD4 Ab-induced regulatory T cells remain poorly defined in terms of their specificity, phenotype, and origin, although sufficient numbers reside in the spleens of tolerant mice to impart Ag-specific tolerance to naive recipients upon adoptive transfer. Such cell transfer experiments, which provide key information in mouse models, are not possible in baboons. However, recent studies with anti-CD4 Abs in mice have shown that regulatory T cells accumulate and persist in tolerated grafts (46, 47). Analysis of graft biopsies from baboon transplant studies with TRX1 may, therefore, be informative. These studies are in progress.

We recognize that the dosing regimens resulting in hyporesponsiveness and nonresponsiveness require substantial amounts of Ab. However, we have not determined a minimal efficacious dose in baboons, nor have we fully optimized the dosing regimen for either Ab or Ag. In man, reduced immunogenicity and improved pharmacokinetics may support a lower efficacious dose of TRX1. For example, all baboons receiving only a single dose of TRX1 ($n = 9$) generated an immune response against the Ab, but we detected no immune response to TRX1 after a single dose of the Ab in man ($n = 9$; our unpublished observations). Furthermore, a 2.5-fold increase in the serum half-life of TRX1 in man should allow for sustained CD4 coverage with less Ab compared with that in baboon.

We observed no acute adverse events with any dose of TRX1, and those treatment regimens that resulted in hyporesponsiveness and tolerance, whereas clearly immunosuppressive during the induction phase, were not associated with any clinical or histopathologic side effects. TRX1-treated animals were not housed in isolation or in germfree or specific pathogen-free conditions. Despite virtually complete saturation of CD4 sites on peripheral lymphocytes of at least 21 days, we could find no evidence for increased prevalence of opportunistic bacterial, fungal, or viral infections or recrudescence of endogenous virus during TRX1 treatment or at any time thereafter.

A concern with tolerance induction therapies is the inadvertent induction of tolerance to pathogenic organisms. Although certainly a formal possibility, we believe it is much more likely that infection will abrogate tolerance induction, as has been shown in several other tolerance models. For example, viral infection has been shown to abrogate transplant tolerance induced by anti-CD154-plus donor-specific cells in mice by preventing deletion of $CD8^+$ T cells (48). Influenza virus infection at the time of nasal administration of protein that normally leads to tolerance instead results in the generation of a Th1 response against the protein (49). Similarly, helminth infection at the time of oral tolerance induction prevents tolerance to the fed Ag and instead results in immune deviation toward a Th2 response to the Ag (50). Other mechanisms by which infection, particularly with pathogens, may abrogate tolerance induction have been described recently, including activation of the TLR pathway, which blocks the suppressive effects of regulatory T cells (51). This block of suppressor activity was shown to be dependent in part on IL-6, which was induced by TLRs upon recognition of microbial products. Other work has demonstrated that IL-6 can replace and may perhaps mediate the effect of CD40 ligation in ablating the tolerogenic activity of $CD8^+$ dendritic cells (52). We suspect that failure of TRX1 to induce self-tolerance in the control group II animal 16313 may be due to acute infection during the tolerance induction phase with SA8 virus, an α herpesvirus prevalent in the baboon colony. Animal 16313 became seropositve to SA8 during the induction phase, whereas all other animals were either seropositive before the study or remained seronegative throughout the study.

We have used polyclonal antivenin as a model Ag in this study because it is a convenient source of clinical grade Ag suitable for such studies. However, such heterologous immune globulins still have an important place as therapeutic agents and are primarily used to neutralize venoms of poisonous animals and insects as well as in some transplant settings as a component of induction protocols or to treat allograft rejection. During the 19th century and early part of the 20th century, immune serum therapy was used to treat a variety of infectious diseases, with the frequent side effect of serum sickness developing as a consequence of immunogenicity of the therapeutic product. With the discovery of antibiotics to treat infectious diseases, serum therapy was largely abandoned for these safer and more effective alternatives. More recently, mAb technology has similarly replaced most polyclonal antisera preparations with recombinant mAb products, at least in the developed world. However, the emergence of new pathogens and antibiotic-resistant microorganisms and the threat of biowarfare have sparked renewed interest in the use of polyclonal heterologous antisera to treat infectious diseases (53, 54). There are clinical circumstances, such as the treatment of snakebites, where polyclonal antisera cannot be replaced with mAbs, because each venom contains many individual toxins.

The need for high doses of CD4 Ab is not only related to needs for saturating CD4 sites, but also for providing sufficient Ig to act as a tolerogen (2). The capacity of CD4 Abs to tolerize is not limited to a naive immune response, because tolerance can also be demonstrated in mice previously primed to transplanted tissues (4,

5, 12–15). This suggests that reprogramming of the immune system in CD4 T cell-mediated autoimmune diseases should be considered a viable therapeutic option for Abs such as TRX1. However, in situations of past priming and in transplantation, other subsets of lymphocytes may become involved, requiring that additional immunosuppressive agents curtailing CD8^{+} T cell or B cell activity might also be required to obtain the full benefits of CD4 Ab therapy.

Acknowledgments

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