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Blockade of CD28-B7, But Not CD40-CD154, Prevents Costimulation of Allogeneic Porcine and Xenogeneic Human Anti-Porcine T Cell Responses


Despite increasing use of swine in transplantation research, the ability to block costimulation of allogeneic T cell responses has not been demonstrated in swine, and the effects of costimulation blockade on xenogeneic human anti-porcine T cell responses are also not clear. We have compared the in vitro effects of anti-human CD154 mAb and human CTLA4IgG4 on allogeneic pig T cell responses and xenogeneic human anti-pig T cell responses. Both anti-CD154 mAb and CTLA4IgG4 cross-reacted on pig cells. While anti-CD154 mAb and CTLA4IgG4 both inhibited the primary allogeneic pig MLRs, CTLA4IgG4 (7.88 µg/ml) was considerably more inhibitory than anti-CD154 mAb (100 µg/ml) at optimal doses. Anti-CD154 mAb inhibited the production of IFN-γ by 75%, but did not inhibit IL-10 production, while CTLA4IgG4 completely inhibited the production of both IFN-γ and IL-10. In secondary allogeneic pig MLRs, CTLA4IgG4, but not anti-CD154 mAb, induced Ag-specific T cell anergy. CTLA4IgG4 completely blocked the indirect pathway of allore cognition, while anti-CD154 mAb blocked the indirect response by approximately 50%. The generation of porcine CTLs was inhibited by CTLA4IgG4, but not by anti-CD154 mAb. Human anti-porcine xenogeneic MLRs were blocked by CTLA4IgG4, but only minimally by anti-CD154 mAb. Finally, CTLA4IgG4 prevented secondary xenogeneic human anti-porcine T cell responses. These data indicate that blockade of the B7-CD28 pathway was more effective than blockade of the CD40-CD154 pathway in inhibiting allogeneic pig T cell responses and xenogeneic human anti-pig T cell responses in vitro. These findings have implications for inhibiting cell-mediated immune responses in pig-to-human xenotransplantation. The Journal of Immunology, 2000, 164: 3434–3444.

The effects of costimulatory blockade have been well characterized in rodents (1). However, studies examining the effects of costimulatory blockade in large animals and humans are limited. For instance, blockade of the B7-CD28 pathway in rodents induces T cell anergy in vitro (2), as does blockade of the CD40-CD154 pathway by anti-CD154 mAb (3). However, it is unclear whether blockade of either costimulatory pathway can induce T cell anergy in large animals. This question is relevant, as immunosuppressive modalities that achieve long term survival in rodents commonly prove to be ineffective in large animals and humans (4). The only report of a large animal study comparing the effects of blockade of the B7-CD28 pathway with blockade of the CD40-CD154 pathway was performed in rhesus monkeys. This study demonstrated that although CTLA4Ig, an Ig fusion protein that binds B7.1 and B7.2 (5), was more effective than anti-CD154 mAb in inhibiting T cell responses in vitro, the reverse was true in vivo treatment; CD154 mAb therapy was more effective than CTLA4Ig treatment in prolonging the survival of kidney allografts (6). The mechanism of graft prolongation mediated by anti-CD154 mAb was not addressed and remains unclear.

To our knowledge there has been no detailed study examining the effects of costimulatory blockade in swine. Furthermore, very little is known about the effects of costimulatory blockade on xenogeneic human anti-porcine T cell responses (7). This along with the fact that the pig is a potential organ donor for human xenotransplantation (8) led us to examine the in vitro effects of costimulatory blockade in allogeneic porcine and xenogeneic human anti-porcine responses. We report here that both anti-human CD154 mAb and human CTLA4IgG4 cross-reacted on pig cells and inhibited allogeneic pig T cell responses and xenogeneic human anti-pig T cell responses, but that CTLA4IgG4 was considerably more effective than anti-CD154 mAb in its ability to block costimulation. Furthermore, CTLA4IgG4, but not anti-CD154 mAb, induced T cell anergy in allogeneic pig T cell responses and completely inhibited xenogeneic human anti-pig T cell responses.
Materials and Methods

Antibodies

The purified mouse anti-human CD154 mAb, 5c8 (IgG2a), was prepared from American Type Culture Collection (Manassas, VA) hybridoma HB-10916 (9). The hybridoma was grown in DMEM with 4.5 g of glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 135 µg/ml streptomycin, and 10% FBS. The CTLA4IgG used in these studies was a human CTLA4IgG4 purified from plasmid-transformed NSO cells. CTLA4IgG4 is a fusion protein that contains the human T cell surface receptor CTLA4 with the constant region of human IgG4. The extracellular domain of the human CTLA4 was cloned as a fusion protein to hinge, CH2, and CH3 domains of a mutant hIgG4 deficient in Fc receptor binding (data not shown). This human IgG4 sequence contains L235G and G237A missense mutations. Isotype control Abs included h60.1, a “humanized” anti-CD11b IgG4 that binds human, but not porcine, CD11b; human IgG4 (Sigma, St. Louis, MO); and G155-178 (PharMingen, San Diego, CA), a mouse IgG2a, anti-trinitrophenol-keyhole limpet hemocyanin.

Animals

The MGH inbred micro pig strain used in this study has been described previously (10). Presently, inbred swine of three homozygous MHC haplotypes (swine leukocyte Ags (SLA)1, SLA2, SLA3, and SLA4) are available (11, 12). In addition, four intra-MHC recombinant haplotypes (SLAa, SLAb, SLAc, and SLAd) have been derived by spontaneous recombinant events during the breeding of heterozygotes as part of the recombination program (1). All animal care and procedures were in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animals Resources and published by the National Institutes of Health.

Preparation of PBMC

Human and pig PBMC were collected as heparinized whole blood, and the blood was diluted 2/3 with HBSS (Life Technologies, Grand Island, NY). The mononuclear cells were obtained by gradient centrifugation using Lymphocyte Separation Medium (Organon Teknika, Durham, NC). Human PBMC were isolated from healthy human volunteers. The mononuclear cells were washed once with HBSS, and contaminating red cells were lysed with ACK lysing buffer (BioWhittaker, Walkersville, MD). Cells were washed again with HBSS and resuspended in complete tissue culture medium. All cell suspensions were kept at 4°C until use.

Flow cytometry

Staining for CD154 with 5c8 and for B7 with CTLA4IgG4 on human and porcine cells was detected by indirect flow cytometry using a Becton Dickinson FACScan microfluorometer (San Jose, CA). FITC-labeled rat anti-mouse IgG2a, biotinylated mouse anti-human IgG4 (Zymed, San Francisco, CA), and PE-avidin (Becton Dickinson) were used as secondary staining reagents. Swine or human IgG was used to block FcR binding for porcine or human cells, respectively. For staining, 3–5 x 10^6 cells/tube of porcine or human cells were resuspended in 100 µl of HBSS (Life Technologies) containing 0.1% BSA and 0.1% NaN3 (FACS medium). Ten microliters of primary or isotype control Ab at approximately 1 µg/1 x 10^6 cells was added to appropriate tubes for 30 min at 4°C. After two washes, a saturating concentration of secondary Ab was added and incubated for 30 min at 4°C. Cells were washed with FACS medium twice and then analyzed by single-color flow cytometry using propidium iodide gating to exclude dead cells.

Activation of T cells

Porcine or human PBMCs (2 x 10^6 cells/ml) were incubated in 2 ml of tissue culture medium containing 0.5% PHA (Life Technologies), 10 ng/ml PMA (Sigma Chemical Co.), and 1 µg/ml ionomycin (Sigma) for 6 h at 37°C in 5% CO2. Control resting cells were incubated without mitogens. After 6 h, cells were harvested, washed, and resuspended in FACS medium for flow cytometric analysis.

Activation of B cells

Porcine or human PBMCs (10 x 10^6) were incubated in 5 ml of tissue culture medium containing LPS (Sigma) 10 µg/ml for 24 h at 37°C in 5% CO2. Cells were then harvested, washed, and resuspended in FACS medium for flow cytometric analysis.

Mixed leucocyte reactions

Responder PBMC (2–4 x 10^5) and irradiated (2500 cGy) PBMC (2–4 x 10^6) were combined in 200 µl of complete tissue culture medium in flat-bottom wells in triplicate. For pig cell assays, complete tissue culture medium for MLR assays consisted of RPMI 1640 (Life Technologies) supplemented with 10% fetal porcine sera (Sigma), 100 U/ml penicillin, 135 µg/ml streptomycin (Life Technologies), 50 µg/ml gentamicin (Life Technologies), 10 mM HEPES (Cellgro, Agawam, MA), 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (BioWhittaker), nonessential amino acids (BioWhittaker), and 5 x 10^-5 M 2-mercaptoethanol (Sigma). For assays of human cell responders, complete tissue culture medium consisted of AIM-V (Life Technologies) supplemented with 10% human AB serum (BioWhittaker), 10 µg/ml HEPES (Cellgro), and 10 µg/ml gentamicin (Life Technologies). The cultures were incubated at 37°C in humidified air containing 7% CO2 for 5 days. [3H]Thymidine (1 µCi/well; New England Nuclear, Boston, MA) was added for a 5- to 6-h period at the end of the culture. The samples were harvested onto glass-fiber filter mats, and [3H]Thymidine incorporation was measured by beta scintillation counting on a liquid scintillation counter. Results were expressed as the mean counts per minute. For inhibition assays, 1–20 µl of Ab were added to wells for the entire course of the incubation. For those assays involving anti-CD154, responder cells were preincubated with 5c8 for 30 min at 4°C. For those assays involving CTLA4IgG4, both responder and stimulator cells were cultured with Ab for 30 min at 4°C. Appropriate isotype controls were included similarly.

Secondary MLR

These assays were performed in two culture phases, termed primary and secondary MLR cultures. In the primary MLR culture (priming phase, days 0–7), either naive porcine SLA^d (F1, F2) responder PBMCs (4 x 10^6) and irradiated (2500 cGy) allogeneic stimulator SLA^d (F1, F2) PBMCs (4 x 10^6) or human responder PBMCs (4 x 10^6) and irradiated allogeneic human stimulator PBMCs (4 x 10^6) were combined in 2 ml of complete tissue culture medium and incubated for 7 days at 37°C in 5% CO2 using 24-well flat-bottom plates (Costar, Cambridge, MA). These primary MLR cultures were performed either in the presence of isotype control Ab (mouse IgG2a, 100 µg/ml, or human IgG4, 50 µg/ml) or in the presence of 5c8 (100 µg/ml) or CTLA4IgG4 (50 µg/ml) as indicated. On day 7 bulk cultures were harvested, washed once in complete medium, and reconstituted in fresh medium at 4 x 10^6 cells/ml in 25-cm^2 culture flasks (Costar) placed upright. The effector cells were allowed to rest for 3 days at 37°C in 5% CO2.

The secondary MLR culture (days 10–14) was initiated on day 10 of the assay, when the responder cells were collected, washed, and reconstituted in fresh MLR medium at appropriate concentrations. Responder cells (4 x 10^5), or if indicated, were replaced in 96-well flat-bottom plates with appropriate irradiated (2500 cGy) stimulators (2–4 x 10^5). Stimulators included autologous specific, and third party stimulators. All secondary MLR cultures in these assays were performed in the absence of Ab. Recombinant human IL-2 at 10 IU/ml (Cetus, Emeryville, CA) was added to certain wells. The secondary MLR cultures were incubated at 37°C in humidified air containing 7% CO2 for 5 days. [3H]Thymidine (1 µCi/well; New England Nuclear, Boston, MA) was added for a 5- to 6-h period at the end of the culture on appropriate days. The samples were harvested onto glass-fiber filter mats, and [3H]Thymidine incorporation was measured by beta scintillation counting on a liquid scintillation counter. Results were expressed as the mean counts per minute. For inhibition assays, 1–20 µl of Ab were added to wells for the entire course of the incubation. For those assays involving anti-CD154, responder cells were preincubated with 5c8 for 30 min at 4°C. For those assays involving CTLA4IgG4, both responder and stimulator cells were cultured with Ab for 30 min at 4°C. Appropriate isotype controls were included similarly.
scintillation counting on a liquid scintillation counter. Results were expressed as the mean counts per minute.

Cell-mediated lympholysis (CML)

Tissue culture media for CML assays were identical with MLR media, except that 6% FCS (Sigma) was used instead of fetal porcine serum. CML assays were performed as previously described (11). Briefly, MLCs containing 4 × 10^5 responder PBMC and 4 × 10^5 irradiated (2500 cGy) stimulator PBMC were incubated in 2 ml of CML medium in 24-well plates (Costar) for 6–7 days at 37°C and 5% CO_2_. On day 6 or 7 effecter cells were harvested and tested for cytolytic activity against ^51^Cr-labeled PHA (Life Technologies) targets in a 5.5-h ^51^Cr release assay. Supernatants were harvested using the Skatron collection system (Skatron, Sterling, VA), and ^51^Cr release was determined on a gamma counter (Micromedics, Huntsville, AL). The results were expressed as the percent specific lysis, calculated as % specific lysis = ([experimental release − spontaneous release]/[maxiumum release − spontaneous release]) × 100.

Secondary CML

These assays were performed in two culture phases, termed primary and secondary CML cultures. In the primary CML culture (priming phase, days 0–7) CMLs were prepared as described above for 7 days at 37°C in 7% CO_2_. Naive SLA^dd^ (I^d^, I^D^) responder PBMCs were cultured with irradiated class I-disparate SLA^cc^ (I^d^, I^D^) stimulators in 2 ml of medium and incubated for 7 days at 37°C in 7% CO_2_ using 24-well flat-bottom plates (Costar). These primary CML cultures were performed in the presence of either isotype control human IgG4 (50 μg/ml) or CTLA4IgG4 (50 μg/ml). On day 7 effector cells were harvested, washed, and reconstituted in fresh CML medium. Cells were allowed to rest for 3 days at 4 × 10^5 cells/ml in 25-cm^2_ flasks at 37°C in 7% CO_2_. The secondary CML culture (days 10–15) was initiated on day 10 of the assay, when effector cells were collected, washed, and reconstituted in fresh medium at the appropriate concentrations. Secondary CML cultures were set up in the absence of any Ab with responder (4 × 10^5) and specific irradiated (2500 cGy) class I-disparate SLA^cc^ (I^d^, I^D^) stimulators (4 × 10^6) in 2 ml of CML medium using 24-well plates (Costar) at 37°C in 7% CO_2_. On days 4 and 6 ^51^Cr release assays were performed on specific and autologous PHA lymphoblasts as described above.

Enzyme-linked immunosorbent assays

ELISA kits specific for pig IFN-γ and IL-10 were purchased from BioSource (Camarillo, CA). Supernatants harvested on days 2–3 of incubation were tested for IFN-γ and IL-10 following the manufacturer’s instructions. Appropriate standard controls were tested, and linear regression analysis was performed. Supernatants were tested in duplicate. Culture supernatants containing 5c8 were purified using protein A columns (Bio-Rad, Hercules, CA) to remove mouse IgG, as 5c8 was found to interfere with pig-specific ELISAs.

MHC class I SLA^cc^ peptides

One peptide, 25 aa in length, spanning the polymorphic region of the α1 domain (aa 3–27) of the pig class F gene PCI was purchased from Quality Controlled Biochemicals (Hopkinton, MA). Peptides were synthesized based on previously published swine class I sequences (12). The amino acid sequence of the PCI peptide (aa 3–27) was HSLRYFDTAVSRP DRRKRPRFISVGY. Peptide purity was 90% as verified by HPLC and mass spectrometry.

Immunization of pigs with MHC class I allopeptides

Five hundred micrograms of the PCI peptide (aa 3–27) in 0.25 ml of PBS was injected s.c. in the neck of an anesthetized SLAdd (I^d^, I^D^) pig in CFA (Sigma; 1/1) by volume.

In vitro peptide proliferation assay

To test for sensitization against the allogeneic PCI peptide, draining lymph nodes were harvested from the peptide-immunized pig under general anesthesia 2 wk after immunization with the PCI allogeneic peptide. Lymph nodes were processed in tissue culture medium, and lymph node cells (2–4 × 10^6_) were incubated with the same allogeneic PCI peptide (aa 3–27; 50 μg/ml) or medium alone in 96-well U-bottom plates at 37°C in 7% CO_2_. For certain wells, 5c8 (100 μg/ml), CTLA4IgG4 (50 μg/ml), control IgG4 h60.1 (50 μg/ml), or control IgG2a (100 μg/ml) was added for blocking studies. On day 5 [^3H]thymidine incorporation was measured as described above. The stimulation index (SI) was calculated according to the formula: experimental counts per minute/medium alone counts per minute.

Results

5c8- and CTLA4IgG4-stained pig cells

To find a reagent that might block costimulation in the pig, 5c8 and CTLA4IgG4 were tested for binding to pig cells using flow cytometric analysis. Fig. 2 demonstrates that resting pig and human T cells did not stain with 5c8. In contrast, pig T cells that had been activated by a combination of PHA, PMA, and ionomycin stained positively with 5c8. The level of 5c8 staining on activated pig T cell was comparable to that seen on activated human T cells (Fig. 2, C and D). Double staining revealed that both activated CD8^+ and CD4^+ pig T cells stained positively for 5c8 (data not shown). CTLA4IgG4 was tested in a similar manner using pig and human PBL. As shown in Fig. 3, CTLA4IgG4 did not stain resting human PBL. This is consistent with previous reports that resting human B cells do not express B7 constitutively, and resting APCs only express B7 at low levels (13). LPS-activated human PBM decreased in MLR and CTLA4IgG4, albeit at a low level (Fig. 3D). In contrast, both resting and activated pig PBMC stained positively with CTLA4IgG4 (Fig. 3, A and C). The differences observed in staining human and pig cells with CTLA4IgG4 may be due to higher levels of B7 expression on both resting and activated pig cells or to the possibility that CTLA4IgG4 might exhibit heteroclitic binding to pig B7 vs human B7.

CTLA4IgG4 was more effective than 5c8 in inhibiting the primary pig MLR

In vitro proliferative assays were performed to determine whether 5c8 and CTLA4IgG4 were functionally active against pig cells. To eliminate the effects of dose on the comparison of 5c8 and CTLA4IgG4 in vitro inhibitory activity, a series of dose-response analyses was performed with both reagents. As both 5c8 and
CTLA4IgG4 were specific to human ligands, we included human-human controls for direct comparison. Fig. 4 demonstrates that 5c8 exhibited optimal inhibitory effects on pig cells at a concentration of at least 100 μg/ml (Fig. 4A) and optimal inhibitory effects on human cells at concentrations as low as 10 μg/ml (Fig. 4B), although the inhibitory effects of 5c8 were only partial even at the maximal dose (~60% of the allogeneic porcine MLR and ~40% of the human MLR). Interestingly, 5c8 was more effective at inhibiting the allogeneic MLR in pig responders than in human responders. The dose-response curves for 5c8 were consistently inhibitory in multiple experiments. In contrast, the isotype control Ab was mitogenic at low concentrations (1 and 10 μg/ml) for pig and human responders (Fig. 4, A and B) and was mildly inhibitory at 100 μg/ml for pig responders (Fig. 4A). Although we do not have a clear explanation for these findings, the variability in the effects of the control Ab suggest that they were due to nonspecific mechanisms of proliferation or inhibition, possibly related to the preparation of the control Ab.

CTLA4IgG4 inhibited the allogeneic pig MLR by 90% at 7.88 μg/ml compared with that in wells without Ab (7,388/73,251 cpm; Fig. 5A). This was greater than the level of inhibition observed for allogeneic human MLRs at the same dose (50%; 28,087/55,254 cpm; Fig. 5B). The isotype control Ab was mitogenic at high concentrations in the pig allo-MLR (Fig. 5A), as increasing concentrations of control Ab led to increased proliferation against allogeneic stimulators. The reason for this mitogenicity of the control Ab is not obvious, although there was some variability in the level of stimulation among different assays, suggesting that variable contaminants in the Ab preparation may have contributed. These findings support the conclusion that CTLA4IgG4 was more effective than 5c8 in inhibiting the primary pig MLR and suggest that the differences in this inhibition were not due to differential levels of cross-reactive binding to pig cells, as the levels of inhibition were higher than those observed with human cells. To address the possibility of synergistic effects between 5c8 and CTLA4IgG4, blocking MLRs were performed using both reagents. The combination of 5c8 and CTLA4IgG4 in the pig allo-MLR did not provide any further inhibition (data not shown). Similarly, the combination of 5c8 and CTLA4IgG4 did not provide additional inhibition compared with CTLA4IgG4 alone in the human MLR (see Fig. 12D).

5c8 inhibited IFN-γ, but not IL-10, production in vitro

Previous reports have suggested that blockade of the CD40-CD154 pathway might inhibit Th1 development by inhibiting the production of IL-12, which is critical for Th1 maturation (14–16). As the
effects of anti-CD154 blockade on pig allogeneic proliferative responses were not complete, we investigated whether the production of Th1 vs Th2 cytokines was differentially affected. Using pig-specific ELISAs, we found that 5c8 inhibited IFN-γ production by 75% after allogeneic stimulation, but that IL-10 was not inhibited (Fig. 6). Since IFN-γ is produced by Th1 cells, and IL-10 is produced by Th2 cells, these results suggested that anti-CD154 blockade could preferentially suppress Th1 responses in the pig. CTLA4IgG4 completely inhibited the production of both swine IFN-γ and IL-10 (Fig. 6).

CTLA4IgG4 was more effective than 5c8 in blocking indirect allorecognition

There has been increasing evidence that indirect allorecognition is important in acute and chronic rejection and that treatment modalities that only target the direct pathway of allorecognition will probably not be sufficient to induce long term graft survival and prevent chronic rejection (17–20). To investigate whether costimulatory blockade could also inhibit T cells responding via indirect allorecognition, an SLAdd (I^d,II^d) pig was immunized s.c. in CFA with a synthetic class I peptide (aa 3–27) spanning the polymorphic regions of the a1 domain of a pig SLA cc class I gene, PC1 (12). Draining cervical lymph nodes were harvested 2 wk after immunization, and lymphocytes were tested for proliferative responses to the same allogeneic class Ic PC1 peptide (50 μg/ml) in peptide proliferation assays. Fig. 7 demonstrates that lymph node cells from the peptide-immunized SLAdd (I^d,II^d) pig demonstrated an SI of 24.9 directed against the allogeneic class I peptide compared with an SI of 1.0 when lymph node cells from an unimmunized SLAdd (I^d,II^d) pig were tested with the same allogeneic class I peptide. Thus, lymph node cells were sensitized to the PC1 a1 peptide after immunization in CFA compared with unimmunized naive controls. To test the ability of costimulatory self stimulator MLR cultures with appropriate Abs are shown for negative controls. Data are representative of two similar experiments performed in duplicate. Appropriate standards were performed in duplicate for each assay.

FIGURE 5. Dose-response curves of CTLA4IgG4 in the primary pig and human MLRs. Primary allogeneic MLRs were performed with serial dilutions of CTLA4IgG4 or isotype control Ab using pig (A) or human (B) PBMCs. Pig SLA^dd (I^d,II^d) responders were incubated with either self or allogeneic, SLA^cc (I^c,II^c; Allo-CC) stimulators. Human responders were incubated with self or allogeneic (Allo) stimulators. Tritiated thymidine incorporation was measured after 5 days of incubation. Data are representative of four similar experiments performed in triplicate cultures.

FIGURE 6. 5c8 inhibits IFN-γ, but not IL-10, production, while CTLA4IgG4 inhibits both IFN-γ and IL-10. Supernatants from primary MLR cultures containing SLA^dd (I^d,II^d) responders and SLA^cc (I^c,II^c) stimulators (Allo-CC) with no Ab, control Abs, 5c8, or CTLA4IgG4 were harvested on days 2–3 and tested for swine IFN-γ (A) and IL-10 (B) production. Supernatants from autologous self stimulator MLR cultures with appropriate Abs are shown for negative controls. Data are representative of two similar experiments performed in duplicate. Appropriate standards were performed in duplicate for each assay.

Induction of T cell anergy by CTLA4IgG4, but not 5c8, in the secondary MLR

Blockade of CD28-B7 pathway by CTLA4IgG4 was more effective in blocking the indirect pathway of allorecognition in swine than blockade of the CD40-CD154 pathway by 5c8. CTLA4IgG4 was also more effective than 5c8 in inhibiting a secondary direct alloresponse. Lymphocytes from a pig primed with a class II-disparate skin graft did not proliferate in vitro against donor cells in the presence of CTLA4IgG4, but did respond to donor cells in the presence of 5c8, albeit it was 60–70% of the control Ab response (data not shown).

Induction of T cell anergy by CTLA4IgG4, but not 5c8, in the secondary MLR

Blockade of CD28-B7 and CD40-CD154 has been shown to induce T cell anergy in rodents (3, 21). To evaluate the effects of
costimulatory blockade on anergy induction in the pig. naive SLA \textsuperscript{dd} (I\textsuperscript{d}, II\textsuperscript{d}) lymphocytes were cultured for 7 days with complete MHC-disparate SLA\textsuperscript{cc} (I\textsuperscript{c}, II\textsuperscript{c}) stimulators in a primary MLR cultures in the presence of 5c8, CTLA4igG4, both reagents, or control Ig. Responder cells were collected, washed, and rested in fresh medium for 3 days. The cells were then restimulated with the original SLA\textsuperscript{cc} stimulators in secondary MLR cultures without Ig. Pig cells primed in the presence of 5c8 showed brisk secondary proliferative responses upon restimulation, comparable to those of the Ag-specific clones were still present in the culture deletion of Ag-specific T cells during the primary MLC. To determine whether Ag-specific clones were still present in the culture, tritiated thymidine incorporation was measured on days 2–5 after restimulation (Days Post Restimulation). Data are representative of two similar experiments performed in triplicate cultures.

Absence of restimulation in these assays could be due to either functional inactivation of T cells secondary to TCR engagement in the absence of a costimulatory signal (anergy) or to the clonal deletion of Ag-specific T cells during the primary MLC. To determine whether Ag-specific clones were still present in the culture but were just not able to proliferate, we added exogenous IL-2 to the secondary MLR cultures. Fig. 9B (pig responders) and Fig. 9D (human responders) show that the Ag-specific hyporesponsiveness induced during the primary MLR cultures also led to donor-specific hyporesponsiveness compared with control Abs (data not shown). However, the level of suppression was not augmented by the presence of both reagents, suggesting no synergistic effects (data not shown). Of note, responder T cells from these cultures were more responsive to exogenous recombinant human IL-2 than responders originally cultured with CTLA4igG4 alone (data not shown).

CTLA4igG\textsubscript{4}, but not 5c8, inhibited the induction of CTLs

Given that the anti-class I MLR is considerably weaker than anti-class II MLR, we examined the effects of 5c8 and CTLA4igG4 on the inhibition of induction of CD8+ CTLs against class I-disparate stimulators. 5c8, CTLA4igG4, or both 5c8 and CTLA4igG4 were added to primary mixed allogeneic lymphocyte cultures for 6 days,
On day 6 effector lymphocytes were harvested and tested for cytotoxicity against specific PHA targets. 5c8 did not significantly prevent CTL generation across a class I barrier (Fig. 10A), nor did it inhibit the effector phase of CTL activity when added during the 51Cr release assay (Fig. 10A). Similarly, 5c8 did not prevent CTL generation in human control assays (Fig. 10C). In contrast, CTLA4IgG4 alone completely inhibited anti-class I CTL generation in a primary CML (Fig. 10B). Similar results were found using complete MHC-disparate stimulators (data not shown). As CTLA4Ig binds to B7 receptors on APCs, the effect of CTLA4Ig on effector CTLs was not examined. The effect of CTLA4IgG4 on porcine pCTLs was similar with that on human cells. CTLA4IgG4,

FIGURE 9. CTLA4IgG4 induced Ag-specific hyporesponsiveness in secondary pig and human MLRs. Primary MLR cultures were incubated for 7 days in the presence of isotype control Ab or CTLA4IgG4 using pig SLA\(\text{dd}(I^d, I^d)\); A and B) or human (C and D) responders. Responder cells were collected, washed, and rested in fresh medium for 3 days before restimulating in secondary MLRs in the absence of Ab. A, Secondary pig MLRs to self, specific SLA\(\text{cc}(I^e, I^e; \text{CC})\) or third party SLA\(\text{aa}(I^a, I^a; \text{AA})\) stimulators using pig SLA\(\text{dd}(I^d, I^d)\) responders that were originally cultured in the presence of control IgG4 or CTLA4IgG4 were performed. B, Exogenous recombinant IL-2 (10 IU/ml) was added to the secondary pig MLR. C, Human responders were also tested in secondary human MLRs to self, specific MHC-mismatched human stimulators (Hu2) and third party MHC-mismatched human stimulators (Hu3) using human responders originally cultured in the presence of control IgG4 or CTLA4IgG4. D, Exogenous recombinant IL-2 (10 IU/ml) was added to the secondary human MLR. Tritiated thymidine incorporation was measured on days 2–5 after restimulation (Days Post Restimulation). Data are representative of two similar experiments performed in triplicate cultures.

FIGURE 10. CTLA4IgG4, but not 5c8, prevents CTL induction. A, Anti-class I CMLs were performed using SLA\(\text{dd}(I^d, I^d)\) responders and SLA\(\text{gg}(I^c, I^c)\) stimulators in the presence of control IgG2a (Control IgG2a MLC) or 5c8 (5c8 MLC) at 100 \(\mu\text{g} / \text{ml}\) and tested for lysis of specific SLA\(\text{gg}(I^c, I^c)\) targets. 5c8 Ab was added either during the MLC (5c8 MLC) or during the 51Cr release assay (CRA; 5c8 + CRA) to test for blocking effector function. No lysis was detected against syngeneic targets (data not shown). B, Anti-class I primary MLCs were performed using SLA\(\text{dd}(I^d, I^d)\) responders and SLA\(\text{hh}(I^a, I^d)\) stimulators in the presence of control Ab (Control IgG4 MLC) or CTLA4IgG4 (CTLA4IgG4 MLC). 51Cr release assays were performed in the absence of any Ab and were tested against specific SLA\(\text{hh}(I^a, I^d)\) targets. No lysis was detected against syngeneic targets (data not shown). C, Complete HLA-mismatched CMLs were performed using unrelated human responder and stimulator cells in the presence of control IgG4 (Control IgG4 MLC), 5c8 (5c8 MLC), or CTLA4IgG4 (CTLA4IgG4 MLC) and were tested against specific allogeneic target. No lysis was detected against syngeneic targets (data not shown). Data are representative of two similar experiments performed in triplicate.
but not 5c8, prevented the induction of allogeneic human CTLs in vitro (Fig. 10).

We next examined whether pCTLs were made anergic after blockade of the B7-CD28 pathway with CTLA4IgG4. Secondary CMLs were performed with class I-disparate stimulators. During the primary CML culture, responder pig cells were incubated with irradiated stimulators in the presence of control Ab or CTLA4IgG4. The effector cells were rested, then were restimulated with specific Ag in the absence of Ab. Restimulated effectors were collected and tested for lysis of specific SLA\textsuperscript{h} (I\textsuperscript{p}, II\textsuperscript{p}) targets on day 4 or day 6 as indicated. No lysis was detected against syngeneic targets (data not shown). Data are representative of two similar experiments performed in triplicate.

**FIGURE 11.** CTLA4IgG4 induced anergy in secondary CMLs. Primary CML cultures were set up using SLA\textsuperscript{dd} (I\textsuperscript{d}, II\textsuperscript{d}) cells that were cultured with SLA\textsuperscript{hh} (I\textsuperscript{a}, II\textsuperscript{d}) stimulators in the presence of control IgG4 (Control IgG4) or CTLA4IgG4 (CTLA4IgG4) for 7 days. Responder cells were then collected, washed, and rested for 3 days before they were restimulated with specific class I-disparate SLA\textsuperscript{hh} (I\textsuperscript{a}, II\textsuperscript{d}) stimulators in secondary CML cultures in the absence of Ab. Restimulated effectors were collected and tested for lysis of specific SLA\textsuperscript{hh} (I\textsuperscript{a}, II\textsuperscript{d}) targets on day 4 or day 6 as indicated. Data are representative of two similar experiments performed in triplicate.

**FIGURE 12.** CTLA4IgG4, but not 5c8, inhibits the human anti-pig MLR. Human responders were tested against allogeneic human (Allo) or xenogeneic porcine SLA\textsuperscript{cc} (I\textsuperscript{c}, II\textsuperscript{c}) (Xeno) stimulators in the presence of no Ab (A), 5c8 or control IgG2a (B), CTLA4IgG4 or control IgG4 (C), or both 5c8 and CTLA4IgG4 vs both control Abs (D). Tritiated thymidine incorporation was measured 5 days after incubation. Data are representative of two similar experiments performed in triplicate.

The ability to inhibit human anti-pig T cell responses may provide an important therapeutic modality in xenogeneic transplantation. The effects of 5c8 and CTLA4IgG4 on the prevention of the human anti-pig T cell proliferative responses were examined next. Fig. 12C shows that CTLA4IgG4 significantly inhibited the human anti-pig MLR compared with control IgG4 or no Ab (Fig. 12A), while 5c8 had a mild inhibitory effect (Fig. 12B). This level of inhibition of the human anti-pig xenogeneic response was even greater than that seen for the allogeneic human MLR. The combination of 5c8 and CTLA4IgG4 showed no added benefit over CTLA4IgG4 alone (Fig. 12D). Furthermore, secondary MLRs demonstrated that CTLA4IgG4 prevented secondary proliferative responses of human T cells against specific pig stimulators, such as SLA\textsuperscript{cc} (I\textsuperscript{c}, II\textsuperscript{c}), as well as against third party pig stimulators, such as SLA\textsuperscript{aa} (I\textsuperscript{a}, II\textsuperscript{a}), while maintaining primary allogeneic responsiveness (Fig. 13B). Human responders incubated with control Ab showed primed responses to both specific xenogeneic pig SLA\textsuperscript{cc} stimulators and third party xenogeneic SLA\textsuperscript{aa} pig stimulators, with a peak of proliferation on day 2 (Fig. 13A). Naive human responders showed only a mild anti-pig proliferative response on day 2 of culture (2891 cpm) and normally displayed peak anti-pig proliferation on days 4–5 (data not shown) (24). The human allogeneic T cell response was maintained in both the control IgG4 and CTLA4IgG4 assays. To determine whether CTLA4IgG4 induced anergy in xenogeneic MLRs, IL-2 was added to secondary MLR cultures. The addition of exogenous recombinant human IL-2 enhanced proliferation against porcine stimulators (Fig. 13C). However, background responses to autologous controls were also elevated in this assay, preventing a firm conclusion as to the mechanism of CTLA4IgG4-induced xenogeneic hyporesponsiveness (Fig. 13C). Our data indicate that blockade of the B7-CD28 pathway, but not blockade of the CD40-CD154 pathway, leads to species-specific human anti-porcine unresponsiveness in in vitro assays.
blockade of the CD40-CD154 and B7-CD28 pathways on T cell responses in the pig, and it represents the first in vitro large animal study examining anergy induction through blockade of B7-CD28 vs CD40-CD154. The limited efficacy of anti-CD154 mAb in pigs contrasts with the successful results obtained with this reagent in rodents (25). This discrepancy may be explained by at least two hypotheses. First, there may be less reliance on the CD40-CD154 pathway in the pig than in rodents. Pigs might instead use other costimulatory pathways in preference to the CD45 pathway. Secondly, the anti-CD154 mAb used in this study was generated against human CD154. Thus, it is possible that an Ab specific for pig CD154 might show better efficacy in inhibiting T cell responses in the pig. The latter hypothesis seems less likely, as 5c8 was shown to stain pig cells comparably to human cells, and more importantly, 5c8 showed even better in vitro functional activity in the pig MLR than in the human MLR. Whatever the reason for the difference in efficacy of anti-CD154 blockade in the pig vs rodents, our results underscore the importance of comparing observations made in rodent models to those made in large animals.

The inefficiency of anti-CD154 Ab in blocking pig T cell responses contrasts with the apparent success of in vivo anti-CD40L therapy reported in the allogeneic rhesus monkey kidney model (6, 26). However, the rhesus monkey studies did not formally compare anti-CD154 and CTLA4Ig for their ability to induce T cell anergy. Moreover, the mechanism of graft prolongation in the monkeys treated with anti-CD154 Ab alone was not clear, as they developed vigorous anti-donor MLRs and circulating anti-donor Abs yet maintained functioning kidneys (26). It would appear that anti-CD154 Ab did not effectively induce anergy in the rhesus monkey studies, but did induce some form of immunosuppression.

The ability of CTLA4IgG4 to induce Ag-specific hyporesponsiveness in the pig is consistent with rodent and human studies (2, 27). Not only was CTLA4IgG4 effective in inducing anergy in porcine CD4+ T cells, as demonstrated by secondary MLRs, but it was also equally potent in anerizing CD8+ pCTLs, as shown in secondary CMLs. These results are consistent with findings using human cells, both in our report and in previously published reports (2). They will allow us to apply these agents of costimulatory blockade to our preclinical miniature swine model of kidney, cardiac, and stem cell transplantation (28–30).

The second conclusion from this study is that CTLA4IgG4 completely inhibits the indirect pathway of allore cognition of donor MHC peptides, while 5c8 only has a partial effect. To the best of our knowledge this is the first report demonstrating the superiority of B7-CD28 blockade over CD40-CD154 blockade in inhibiting the indirect allore cognition of donor MHC peptides. This finding may have relevance in clinical transplantation, as there has been increasing experimental evidence to support a critical role for indirect allore cognition during acute rejection of allografts (31) as well as for CTL induction (32). Furthermore, there is evidence from human recipients of cardiac and renal transplants that indirect recognition of donor MHC peptides not only occurs and is restricted to a limited T cell repertoire (33–35), but it appears likely to play a role in chronic rejection (18–20, 36). While current immunosuppressive regimens have achieved success in preventing acute rejection of vascularized allografts, similar success has not been achieved in the prevention of chronic rejection (37). This discrepancy may be secondary to the inability of current immunosuppressive agents to suppress the indirect pathway of allore cognition over time. Inhibition of the indirect pathway of allore cognition by costimulatory blockade may not only neutralize the cellular arm of chronic rejection, but it may also inhibit the humoral response to allografts, as generation of anti-donor IgG Abs.

Discussion

The primary conclusion from the present study is that blockade of CD28-B7 with CTLA4IgG4, but not anti-CD154 blockade, effectively inhibits allogeneic porcine T cell responses in vitro and induces Ag-specific hyporesponsiveness. To the best of our knowledge, this is the first study demonstrating the differential effects of
appears to be dependent on CD4 T cells primed via the indirect pathway to donor Ags (38).

The third conclusion from our investigation is that CTLA4IgG4 inhibits the human anti-porcine T cell response and prevents secondary T cell responses to porcine stimulators in vitro. Currently, costimulatory blockade with agents such as anti-CD154 mAb and CTLA4Ig is being explored in the clinical setting for autoimmunity and transplantation (39–41). Our in vitro data suggest that CTLA4Ig may be more effective than anti-CD154 mAb in suppressing xenoreactive T cells in pig-to-human transplantation despite the ability of anti-CD154 mAb to inhibit allogeneic nonhuman primate responses (26). In addition, our data suggest that this suppression of xenoreactivity is species specific, as CTLA4IgG4 rendered human T cells unresponsive to both specific SLAII porcine stimulators and third party SLAIII porcine stimulators while allogeneic reactivity was maintained. This finding may have clinical relevance, as it suggests that after CTLA4IgG4 has induced a lack of reactivity to a specific porcine organ, this lack of reactivity may extend to organs from all pig haplotypes. Of note, since human anti-porcine CTL activity involves nonspecific lysis by LAK cells and Ag-dependent cellular cytotoxicity mechanisms in vitro, we did not test costimulatory blockade in the human anti-pig CML (42, 43).

As the present study did not address T-B cell interactions, anti-CD154 mAb may be effective in preventing anti-donor humoral responses, since the CD40-CD154 interaction is required for isotype switching (44–51). Furthermore, since many different cell types express CD40 and CD154, such as endothelial cells (52–54), T cell costimulation.

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