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Effects of an Agonist IL-2/Fc Fusion Protein, A Mutant Antagonist IL-15Fc Fusion Protein and Sirolimus on Cardiac Allograft Survival in Non-Human Primates

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Abstract

Background—To tilt the immunologic balance towards tolerance and away from rejection, nonhuman primate recipients of cardiac allografts were treated with IL-2/Fc, mutant antagonist type (m) IL-15/Fc and sirolimus.

Methods—Heterotopic heart transplants were performed on fully mismatched cynomolgus macaques (n=8). An untreated control recipient rejected its graft by postoperative day 6. The remaining seven animals received immunosuppression with sirolimus, administered either orally or intramuscularly. A recipient treated with sirolimus alone rejected at the end of a 28 day course of immunosuppression. The remaining six monkeys also received IL-2/Fc and mIL-15/Fc intramuscularly until 28 days after transplant. In one animal, a second 28-day course of fusion protein was given starting at day 50. In these six animals, sirolimus was continued for either 28 days (n=4) or until protein levels were low (n=2).

Results—In the four monkeys treated with a 28-day course of sirolimus and fusion proteins, mean graft survival was 51.5 (range 28-76) days. The animal receiving a second course of fusion protein rejected its graft on day 177 despite detectable levels of the fusion proteins and sirolimus. The central memory, effector memory and naïve CD4+ and CD8+ T-cell populations in peripheral blood did not change significantly during the period of fusion protein administration. However, a 2.5-fold expansion in CD4+CD25+ lymphocytes occurred in recipients treated with fusion proteins and sirolimus which was not observed in the recipient treated with sirolimus alone.

Conclusions—Although IL-2/Fc, mIL-15/Fc and sirolimus administered in this manner permits modest prolongation of graft survival and expansion of CD4+CD25+ T cells, tolerance was not achieved.

Keywords

heart; transplantation; IL-15; primates; rejection

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Cardiac transplantation is the treatment of choice for end-stage heart failure, but the technique's therapeutic potential is limited by the side effects of chronic immunosuppression and the inexorable development of cardiac allograft vasculopathy. Inducing immunologic tolerance of cardiac allografts is thus extremely appealing. Long-term cardiac allograft survival in mice has been induced by various strategies, but in primates the goal remains elusive. Generalized depletion of potentially donor-reactive lymphocytes raises the risk of infection and malignancy and also may deplete regulatory lymphocytes needed for allograft tolerance. A more desirable approach is to promote apoptosis of donor-reactive cytotoxic T-lymphocytes while avoiding depletion of regulatory T cells (Tregs).

Manipulation of the interleukin 2 and interleukin 15 pathways has been proposed as a means of achieving immune tolerance (1,2). IL-2 enhances peripheral tolerance by promoting apoptosis of antigen-reactive effector T-cells (1,2) while enhancing the viability and function of Tregs (3), probably via activation of the STAT5 pathway (4). High affinity IL-2 receptors (IL-2R) are expressed by Tregs and by activated T-cells in response to alloantigens but not by resting T cells. While IL-2 promotes apoptotic death of T-effector cells (5), but not Tregs (6), IL-15 exerts an opposing function as it inhibits activation-induced cell death (AICD) and promotes the survival of $CD8^+$ memory T cells. In theory, simultaneous potentiation of the pro-AICD effects of IL-2 and blocking the anti-AICD effects of IL-15 could shift the balance of anti-donor immunity away from rejection and towards tolerance.

Murine recipients of MHC-mismatched cardiac allografts have been treated with an agonist IL-2/Fc fusion protein in an attempt to promote AICD of donor reactive effector T cells and expand the pool of donor reactive Tregs. These recipients were also treated with a mutant antagonist-type mIL-15/Fc fusion protein to prevent the proliferative and pro-survival effects of IL-15. In addition, sirolimus was employed to prevent expansion of alloreactive T cells in response to the cardiac graft and to greatly exaggerate AICD. This combined treatment led to indefinite survival of the MHC-mismatched heart grafts (6, 7).

Despite efficacy in the murine model, translation of these therapeutic agents has not been successful in nonhuman primates. Blockade of IL-15 signalling using mIL-15/Fc, whether as monotherapy or in combination with antithymocyte globulin and mycophenolate mofetil, showed no beneficial effect on the survival of renal allografts (8). Here, we extend those observations by reporting the first results of heterotopic heart transplants in fully MHC-mismatched cynomolgus macaques treated with a regimen consisting of mIL-15/Fc and lytic IL-2/Fc proteins and sirolimus.

Material and methods

Animals

Sixteen feral cynomolgus monkeys weighing 5 to 7 kg were used (Charles River BRF, Houston, TX). Recipient and donor pairs had compatible ABO blood types and mismatched cynomolgus MHC (CyLA) antigens. CyLA class I antigen disparity was determined by flow cytometry using an anti-HLA class I allele specific monoclonal antibody and CyLA class II antigen disparity was determined by mixed lymphocyte response assay. All surgical procedures and animal care complied 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” (NRC 1996). This study was also approved by the Massachusetts General Hospital Subcommittee on Research and Animal Care.

Genetic construction of human IL-2/Fc

Human IL-2 cDNA, including 20 bp of 5′ untranslated sequence and its native leader sequence was amplified by PCR from cDNA library of anti-CD3 mAb stimulated Jurkat cells (ATCC TIB 152) using synthetic oligonucleotide primers. The sense primer was designed to append a BamH I site to the 5′ end. Then antisense primer eliminated the termination codon, substituted Ser, for the unpaired Cys residue, and changed the codon usage for the terminal Thr residue from ACT to ACG to provide the first base of a BamH I site appended to the 3′ end. The human Fcv1 were generated from mRNA extracted from an IgG1 secreting hybridoma (American Type Culture Collection (ATCC) HB8177, Rockville, Maryland), in a standard technique using reverse transcriptase MMLV-RT (Gibco BRL, Grand Island, NY) with a synthetic oligo-dT(12-18) oligonucleotide (Gibco BRL). Ligation of hIL-2 and Fcγ1components in the correct translational reading frame at the Bam HI site yielded a 1190 bp long open reading frame encoding a single 385 amino acid polypeptide. The plasmid was transfected into CHO cells and selected by G418. The high yield clones were selected and cultured in a serum free medium. The IL-2/Fc fusion protein produced was then purified by protein A sepharose affinity chromatography followed by dialysis against PBS and 0.22 μm filter sterilization. Purified proteins were stored at -20°C before use (9).

Generation of mIL-15/Fc

The design and construction of the human mutant antagonist type IL-15 construct has been previously described (10). Briefly, site-directed and PCR assisted mutagenesis was used to replace glutamine residues 101 and 108 of human IL-15 with asparatic acid. A poor efficiency of natural signal peptides was observed in our attempt to produce secreted fusion protein, which may represented one of the mechanisms involved in the control of IL-15 secretion (data not shown). Therefore, we replaced the natural signal peptides of human IL-15 with human CD5 signal peptides. The mutant human IL-15 gene sequence encoding mature IL-15 protein was genetically linked to human CD1 leading sequence and human IgG1 Fc and then cloned into an expresson vector. The resulting gene was transfected into CHO cells. The transfectant cells were cloned and cultured in serum-free media; mIL-15/Fc protein in the culture supernatant was purified by protein a affinity chromatography or ion-exchange chromatography.

Cardiac Transplantation

All procedures were performed under general endotracheal anesthesia using isofluorane. Donor hearts were procured through a median sternotomy. All recipients underwent a laparotomy with exposure of the infrarenal aorta and inferior vena cava (IVC). The donor pulmonary artery was anastomosed end-to-side to the recipient IVC and the donor ascending aorta was anastomosed end-to-side to the recipient aorta. Postoperatively, heart function was monitored by transabdominal palpation by two observers. Absence of ventricular contraction was confirmed by open biopsy followed by histologic analysis.

Adminstration of fusion proteins

On day -1 and day 0, 0.5mg/kg of both fusion proteins were given intravenously and on day 2 onward, 0.2mg/kg of both proteins were given by subcutaneously. Maintenance therapy consisted of three subcutaneous injections during the first week at a dose of 0.2mg/kg. Then, subcutaneous injections of both proteins at 0.2mg/kg were given twice a week until day 28. In one recipient, a second course of fusion protein was administered from day 50 to 78. Circulating levels of the fusion proteins were monitored twice a week by dual probe ELISA. The target serum level of fusion protein was 0.5 optical density (OD) (Strom, T, personal communication).

Immunosuppression

Two recipients were treated with sirolimus by gavage. A dose of 2mg/kg was given on day -2, day -1 and day 0 and a dose of 1mg/kg from day 0 to day 28. All other recipients were treated with daily intramuscular injections of sirolimus, starting with a dose of 0.2 mg/kg and then titrated to achieve target blood levels between 15-20 ng/μl from day 0-28. Serum sirolimus level was monitored twice a week with a commercial muparticle enzyme immunoassay.

Histopathology

Cardiac biopsies and necropsy tissue were fixed in 10% formalin and evaluated using hematoxylin and eosin (H&E) stain and van Gieson's elastin stain. The specimens were then scored under light muscopy by a blinded observer to determine the severity of acute rejection and degree of intimal proliferation. Scoring of interstitial rejection in the heart allograft was based on the 2007 revision of the International Society for Heart and Lung Transplantation classification system (11). Cardiac allograft vasculopathy was determined by the presence or absence of intimal thickening as assessed by an experienced cardiac pathologist blinded to the origin of the samples.

FACS assays

Peripheral blood mononucleocytes were obtained 15, 50 and 100 days after heart transplantation. Cell surface antigens were analyzed by multicolor flow cytometry. Phenotypic markers for memory and naïve T cells in cynomolgus monkeys were chosen based on the studies by Pitcher et al. (12). PBMC were directly interacted with the following monoclonal antibodies purchased from BD Pharmingen (San Digeo, CA): CD3 PerCP (SP 34-2), CD4 PerCP (L-200), CD8 PerCP (RPA-T8), CD8 APC (RPA-T8), CD16 (NKP15), CD25 FITC (M-A251), CD20 PE (2H7), CD95 FITC (DX2), CD95 APC (DX2) CD28 PE (CD28.2) and CD28 FITC (CD28.2). The fluorescence of the stained samples was analyzed using FACS Calibur and FACS Scan flow cytometers and Cell Quest Software (BD Immunocytometry Systems, San Jose, CA). Lymphocytes were gated on the forward and side light scatter and 3,000-5,000 events were collected.

ELISPOT assay

Ninety-six-well spot enzyme-linked immunosorbent (ELISPOT) plates (Polyfiltronics, Rockland, MA) were coated with either anti-IFN-γ or anti-IL-2 capture mAbs (Pharmingen) overnight. The plates were washed with RPMI media supplemented with sterile PBS and blocked with 10% normal monkey plasma. Responder cells were added to wells containing irradiated stimulator cells and incubated for 44 hours for IFN-γ and IL-4, and 18 hours for IL-2 (MABTECH USA, Cincinnati, OH). The plates were washed with PBS, and then the appropriate biotinylated detection mAbs were added to the media for 2 hours at room temperature. After washing with PBS, streptavidin-HRP (MABTECH USA, Cincinnati, OH) was added for one hour. Four washes with PBS were performed before addition of the developing solution. Spots were counted and analyzed on a computer-assisted ELISPOT image analyzer (Cellular Technology Ltd, Shaker Heights, OH).

Results

Graft survival and function

Cardiac allograft survival times are shown in Table 1. One animal (M5708) received a heterotopic heart transplant without treatment and its graft was lost due to acute cellular rejection on post-operative day 6. Two animals, M1808 and M1510, were treated with a 28-day course of sirolimus by intramuscular injection. One monkey (M1808) maintained an

adequate therapeutic level (mean 15.1±4.2 ng/μL) of sirolimus for 22 days. A biopsy on day 14 demonstrated ISHLT 1R rejection and the graft was lost due to ACR on day 28, at which time the sirolimus serum level was 4.0 ng/μL. A much higher sirolimus level (mean 57.8±5.7 ng/μL) was reached in M1510, however its graft stopped beating on post-transplant day 22.

Two monkeys (M4205 and M3605) were treated with IL2/Fc, mIL15/Fc and daily oral sirolimus for 28 days post-transplant. In these animals, cardiac contractility was absent by day 28 (M4205) and day 40 (M3605), respectively. In both cases, sirolimus levels were below the therapeutic target of 15-20ng/μL (M4205 mean level = 3.7±1.7ng/μL; M3605 mean level = 8.6±3.5 ng/μL). Increased dosing was used in M3605, but was limited by the development of severe diarrhea. In M4205, the mean fusion protein level while the heart graft was in place was above the initial target of 0.5 O.D. but below 1.0 O.D (Table 2). In M3605, a higher fusion protein level was reached by delaying transplant until the target level had been reached (Figure 1, M3605 data only)

In order to achieve adequate sirolimus levels without gastrointestinal toxicity, an intramuscular preparation of the drug was used in subsequent experiments. Two monkeys (M206 and M8507) were treated with IL2/Fc, mIL15/Fc and daily IM sirolimus for 28 days post-transplant. In these animals, cardiac contractility ceased by day 62 (M206) and day 51 (M8507) respectively (Table 1). Target levels of sirolimus were achieved more readily with IM than with oral sirolimus (M206 mean level = 21.4±2.3ng/μL; M8507 mean level = 16.8±6.7ng/μL) and persisted transiently after cessation of the drug (Figure 1A, M206 data only). In M206, rejection occurred despite the presence of target levels of the fusion proteins (Table 2; Figure 1B). In M8507 reliable fusion protein levels could not be obtained due to a technical failure.

In a final pair of animals, the course of immunosuppression was lengthened. In M1106, intramuscular sirolimus was continued until fusion protein levels fell below 0.5 O.D. (61 days of sirolimus; mean level = 18.9 ± 4.8 ng/μL). Graft contractility was absent by day 76, at which time the sirolimus level was 11.5ng/μL. In M5606, a second course of IL2/Fc and mIL15/Fc was administered from day 50 to day 78 post-transplant and levels remained above 1.0 O.D. on day 177; at which time graft contractility was lost due to acute cellular rejection. The target sirolimus level (mean level = 19.5 ± 4.0 ng/μL) was maintained throughout this course.

Histology and pathology

Cardiac allografts were explanted at the time of rejection and stained with hematoxylin and eosin. Grossly, the explanted grafts in fusion-protein treated animals demonstrated thickened, stiff myocardium which did not bleed easily and demonstrated no contractility (Figure 2A, D). Muscopic examination of the myocardium demonstrated abundant lymphocytic infiltrates with extensive myocyte necrosis (Figure 2 B, E). The small coronary vessels demonstrated intimal thickening characteristic of cardiac allograft vasculopathy (Figure 2C, F).

Flow cytometry

To determine whether administration of the fusion protein/sirolimus mix promoted the expansion of regulatory T cells, we analyzed peripheral blood mononuclear leukocytes at 15 and 50 days post-transplantation using anti-CD4 and anti-CD25 mAbs to identify putative Tregs. There was significant variation in the initial percentage of CD4+CD25+ T cells (1.8% - 6.2%) but in each case a 200-300% expansion of CD4+CD25+ T cells was observed

(mean increase = 250% ± 80%; Figure 3). Hence the therapeutic regimen did enable an expansion of CD4+CD25+ T cells.

Peripheral mononucleocyte populations were analyzed by flow cytometry to determine whether the proportion of CD4+ and CD8+ T-lymphocytes having a naïve (CD95-CD28+), central memory (CD95+CD28+) or effector memory (C D95+CD28-) phenotype changed as a result of fusion protein administration (12). Regardless of the route of sirolimus administration or the duration of fusion protein treatment, we did not detect a consistent reduction in the proportion of peripheral T-lymphocytes having a central memory or effector memory phenotype (Figure 4).

Mixed lymphocyte reaction

The anti-donor response was assessed 14, 50 and 100 days after heart transplantation using the ELISPOT assay (Figure 5). In the animals treated with fusion proteins and oral sirolimus for 28 days (Fig 5A,B), production of IL-2 and IFN-γ on day 14 by recipient lymphocytes in response to stimulation with frozen donor lymphocytes was unchanged. Anti-donor sensitization was evident by day 50, and markedly amplified by day 100. The same pattern was seen in the animal treated with 28 days of fusion proteins and intramuscular sirolimus (Fig 5C). In the animals treated with a prolonged course of IL-2/Fc, mIL-15/Fc and sirolimus, the anti-donor response remained flat at day 50, at which time sirolimus was still being administered. In recipient M1106 (Fig 5D), sirolimus was discontinued on day 61 and evidence of increasing reactivity was detected by ELISPOT assay on day 100. Sirolimus was continued until day 177 in recipient M5606, and predictably no sensitization was detectable on day 100 (Fig 5E). Although treatment with IL-2/Fc, mIL-15/Fc and sirolimus was sufficient to prevent a mixed lymphocyte response, it is not clear that this assay detected an effect beyond that achieved by administration of sirolimus alone.

Discussion

Although tolerance of solid organ allografts has been successfully induced in mice using various techniques, the goal of inducing tolerance in primates has remained elusive. We report the first results of treating nonhuman primates of whole organ allografts with a regimen comprising an agonist-type fusion protein targeting IL-2 and an antagonist-type fusion protein targeting IL-15 in combination with sirolimus. In this admittedly very small series the regimen proved well tolerated and successful at modestly prolonging cardiac allograft survival beyond that achieved with sirolimus monotherapy and an expansion in the pool of recipient $CD4^+CD25^+$ T cells was observed. The role of IL-2 in inducing naïve CD4+CD25- cells to differentiate toward a suppressor phenotype has been described previously (13) and we speculate that treatment of these monkeys with a long-lived IL-2 agonist is responsible for the observed result. Although this finding would be expected if an expansion in peripheral regulatory T cells had occurred, interpretation of the finding is limited by the fact that we could not obtain sufficient cell samples to test for the presence of the Treg marker FoxP3 or to perform functional assays on these $CD4^+CD25^+$ lymphocytes.

In contrast to the effects noted in mouse models, neither marked contraction of the pool of effector T cells nor tolerance was achieved despite the achievement of target levels of each of the therapeutic agents. Although IL-15 is a survival factor for CD8+ memory lymphocytes (14), we did not observe any decline in the relative contribution of memory lymphocytes to the CD8+ subset as a consequence of mIL-15/Fc administration. The induction of transplant tolerance is inhibited in the presence of donor-reactive CD8+ effector memory T-lymphocytes (15). Although tolerance to renal allografts has been induced in previously transplanted animals through the addition of a depleting antibody against CD8 to a regimen based on costimulatory blockade, this agent carries a high risk of infectious and

malignant complications. In mice transplant models, mIL-15/Fc has proven efficacy against costimulation-resistant CD8+ T-lymphocytes (16). We plan to test whether that fusion protein is useful as an alternative to nonselective depletion of all $CD8^+$ lymphocytes.

It should be emphasized that the young mice used in the previous studies were housed in a clean and controlled environment. Consequently, those mice did bear a large cohort of memory T cells. In contrast, monkeys typically used in these experiments are often exposed to a wide variety of pathogens and bear a larger population of memory T cells (17). We suspect that this expanded population of memory T cells in the primates as compared to mice likely contains T cells that cross react with donor alloantigens and were responsible for the failure to obtain prolonged drug free engraftment in this study.

In this small pilot study, the benefical effects of Il-2/Fc and mIL-15/Fc fusion protein, if any, are difficult to separate from the survival-prolonging effects of increased dose and duration of sirolimus. Nevertheless, the proposed mechanism of these well-tolerated proteins suggests that there are potential applications for these agents in combination with other therapeutic strategies. For example, homeostatic proliferation of memory lymphocytes in response to lymphopenia is a potential pitfall of lymphocyte-depleting regimens and may lead to accelerated graft rejection (18). Proliferating CD8+ memory T-cells demonstrate increased responsive to IL-15 (19). Blockade of IL-15/Fc might therefore be useful in combination with lymphodepletion.

In summary, while the regimen of IL-2/Fc, mIL15-Fc and sirolimus we have tested in nonhuman primate recipients was associated with an expansion of T cells with a CD4+CD25+ phenotype resembling Tregs, it did not lead to a reduction in memory-type CD8 T cells or induce tolerance to MHC-mismatched cardiac allografts. Although modestly prolonged survival was achieved, rejection occurred in some hosts with circulating levels of the therapeutic proteins similar to what was observed when mIL-15/Fc was administered with or without antithymocyte globulin and mycophenolate mofetil in nonhuman primate recipients of renal allografts (20). We suspect that the failure to reduce the burden of memory type CD8+ cells was responsible, at least in part, for the failure to achieve drug free engraftment and tolerance.

Acknowledgments

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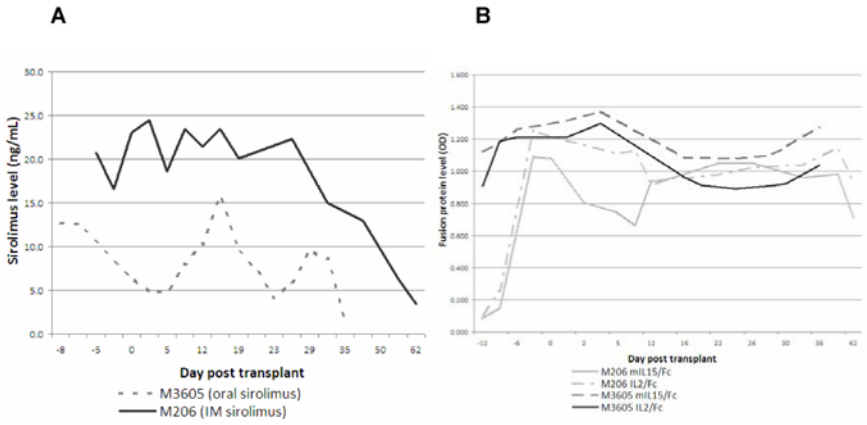

Figure 1.

Representative sirolimus and fusion protein levels from a recipient treated with oral (M3605) and intramuscular sirolimus (M206). **A** Oral administration of sirolimus (1.0-2.0 mg/kg) was associated with low serum drug levels (dark line, mean level = 8.6±3.5) whereas intramuscular administration at a lower dose (0.05-0.3mg/kg) led to higher, prolonged serum levels (light line, mean level = 21.4±2.3). **B** Fusion proteins were administered by intramuscular injection in both cases. The target level of 0.5 O.D, was maintained throughout the life of the graft. A pre-transplant loading period was used in all animals other than M4205.

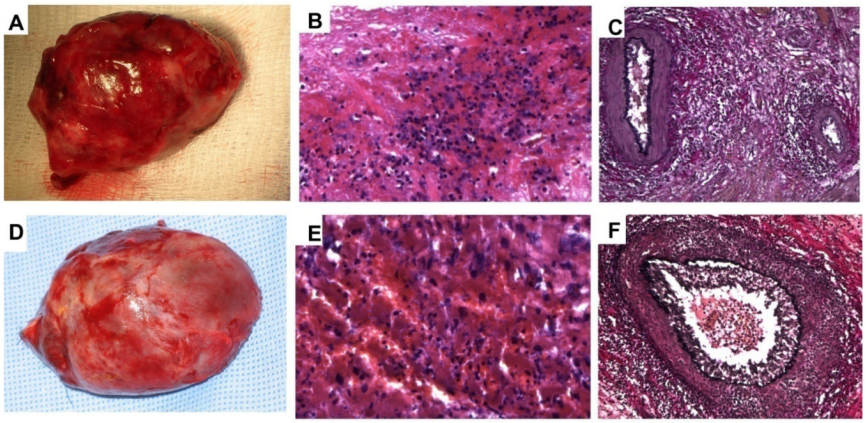

Figure 2.
Gross and muscopic appearance of cardiac allografts at time of explantation after treatment with fusion proteins and either oral (A-C; M3605) or IM (D-F; M206) sirolimus. Acute cellular rejection was present in both sets grafts at time of explantation (B, E) and cardiac allograft vasculopathy was present in the longer-surviving grafts (C, F).

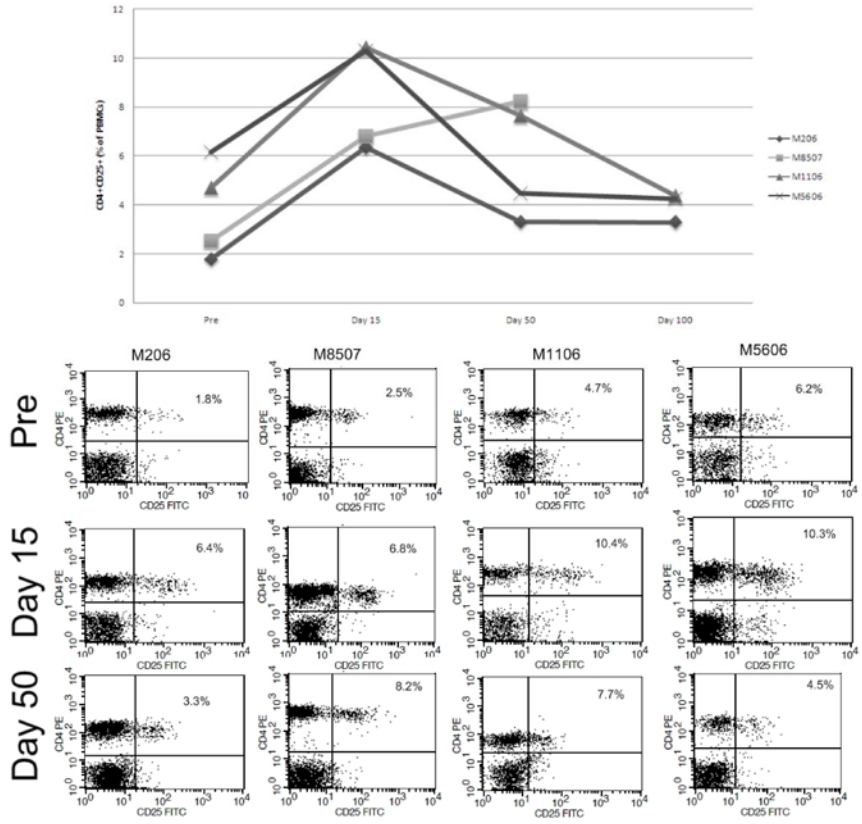

Figure 3.

Expansion of $CD4^{+}CD25^{+}$ lymphocytes occurred during the first two weeks of combined IL2/Fc and mIL15/FC and sirolimus administration.

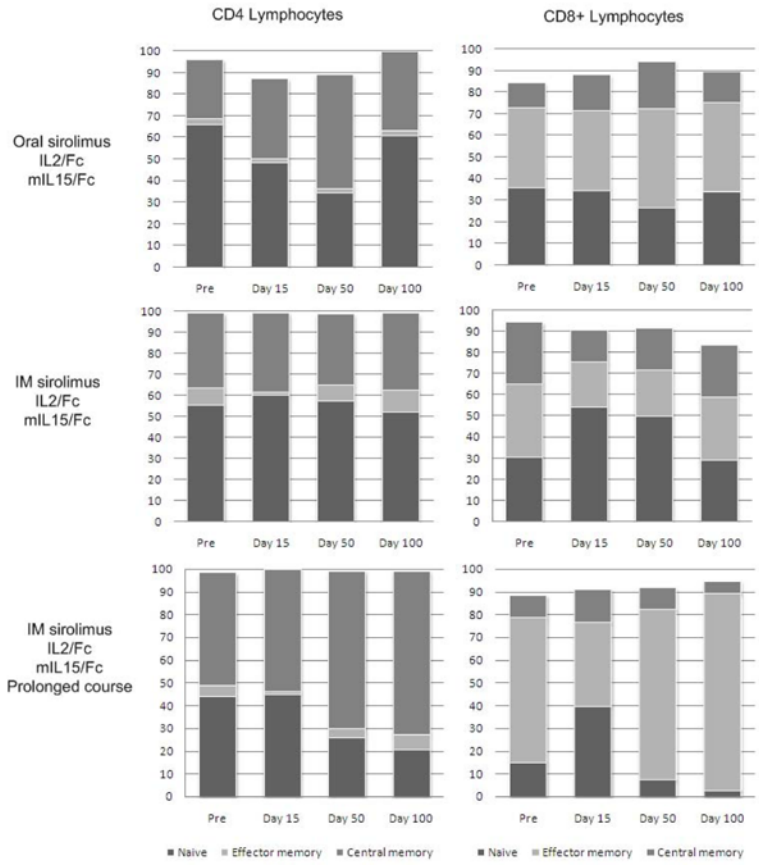

Figure 4.

Changes in the proportion of naïve ($CD28^{+}CD95$-), effector ($CD95^{+}CD28$-) and central ($CD95^{+}CD28^{+}$) memory cells during sirolimus and fusion protein administration. There was no consistent reduction in CD8+ memory T-cells.

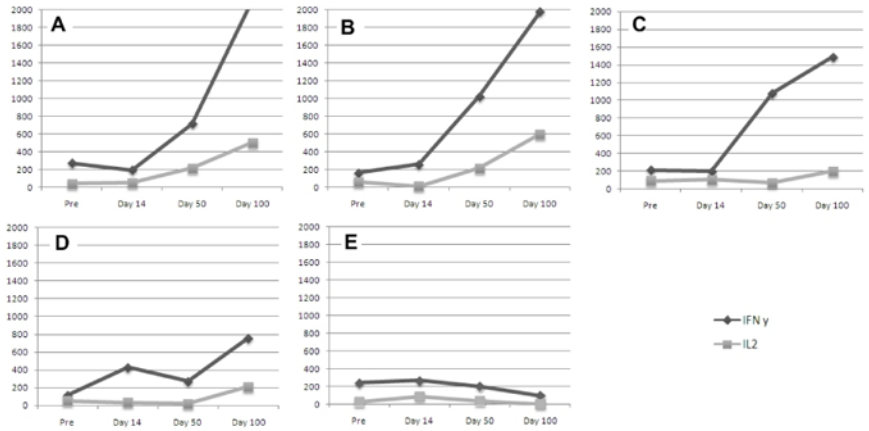

Figure 5.

Anti-donor sensitization assessed using the ELISPOT assay. **A, B** Animals treated with 28 days of fusion proteins and oral sirolimus (M3605, A; M4205 B), increasing anti-donor sensitization was present on day 50 and 100. **C** Animal treated with 28 days of fusion proteins and intramuscular sirolimus (M206). **D, E** Animals treated with a prolonged course of fusion

Table 1

Outcomes after cardiac transplantation with no treatment (M5708), sirolimus alone (M1808) or sirolimus plus fusion proteins.

Monkeys	Sirolimus	IL-2/mIL-15	Survival
M5708	*None*	*NONE*	*6*
M1808	*IM, 28 days*	*NONE*	*28*
M1510	*IM, 28 days*	*NONE*	*22*
M4205	**Oral**, 28 days	**Day -2 to day 28**	**28**
M3605	**Oral**, 28 days	**Day -29 to day 28**	**40**
M206	**IM, 28 days**	**Day -13 to day 28**	**62**
M8507	**IM, 28 days**	**Day -16 to 28**	**51**
M1106	**IM, 61 days**	**Day -23 to day 28**	**76**
M5606	**IM, 177 days**	**Day -14 to day 28 and day 50 to day 78**	**177**

Table 2

Mean serum levels of sirolimus and fusion proteins during the post-transplant period of administration.

Monkey	Mean Sirolimus level	Mean IL2/Fc	Mean mIL15/Fc
M4205	3.7 ± 1.7 ng/μL	0.7 ± 0.5 O.D.	0.8 ± 0.4 O.D.
M3605	8.6 ± 3.5 ng/μμL	1.0 ± 0.2 O.D.	1.2 ± 0.1 O.D.
M206	21.4 ± 2.3 ng/μL	1.1 ± 0.1 O.D.	0.9 ± 0.1 O.D.
M8507	16.8 ± 6.7 ng/μL	*	*
M1106	18.9 ± 4.8 ng/μL	0.8 ± 0.1 O.D.	0.7 ± 0.1 O.D.
M5606	19.5 ± 4.0 ng/μL	1.4 ± 0.1 O.D.	1.4 ± 0.1 O.D.

* No data obtained due to technical error.