Published in final edited form as:

Transplantation. 2006 January 15; 81(1): 109–116.

An Antagonist Mutant IL-15/Fc Promotes Transplant Tolerance

Xin Xiao Zheng¹, Wei Gao², Elina Donskoy², Manfred Neuberg², Manfred Ruediger², Terry B. Strom^{1,3}, and Thomas Moll²

¹Department of Medicine, Harvard Medical School, Division of Immunology, Beth Israel Deaconess Medical Center, Boston, MA.

²Cardion AG, Erkrath, Germany.

Abstract

Background—IL-15 is a proinflammatory and antiapoptotic T-cell growth factor that plays an important role in a variety of autoimmune disorders and transplant rejection. To inhibit IL-15 function and to target IL-15 receptor (IL-15R) bearing cells, we have generated a unique lytic antagonistic mutant IL-15/Fc fusion protein (mIL-15/Fc).

Methods—In this study, we further examined the efficacy of mIL-15/Fc in preventing allograft rejection cross minor and major histocompatibility barriers.

Results—A short-course treatment with mIL-15/Fc fusion protein is sufficient to prevent cardiac allograft rejection and induce antigen-specific tolerance in minor histocompatibility complex-mismatched recipients, and permit prolonged cardiac allograft survival in fully MHC mismatched recipients. In addition, mIL-15/Fc treatment, in combination with a suboptimal dose of anti-CD154 antibody, confers permanent cardiac allograft engraftment in a fully MHC-mismatched mouse strain combination. In a murine islet allograft model, mIL-15/Fc monotherapy is capable to permit permanent allograft survival in 50% fully MHC-mismatched recipients.

Conclusion—Immunochemistry studies demonstrated that prolonged graft survival was accompanied by reduced intra-graft mononuclear cell infiltration and pro-inflammatory cytokine gene expression in the mIL-15/Fc treated recipients. Moreover, parallel experiments employing a mutated nonlytic IgG2a Fc demonstrate that the Fc portion of mIL-15/Fc contributes to the overall efficacy of the molecule in vivo.

Keywords

Transplantation; Heart; Islet; Cytokines; Rejection

IL-15 is a member of the four α -helix bundle cytokine family with closest homology to IL-2 and IL-21 (1–3) and which, under physiological conditions, is involved in the differentiation and proliferation of NK and T cells (4, 5). Furthermore, IL-15 is an important proinflammatory cytokine that is produced by activated macrophages (1, 6). IL-15

stimulates the activation, proliferation, survival and effector functions of a variety of immune cells (3, 7–10). IL-15 is a critical growth factor for the proliferation of antigen-activated T cells in response to immunological stimuli in vivo (11). It is notable that increased IL-15 gene expression is evident during allograft rejection and in a variety of autoimmune disorders (12, 13).

To directly investigate the contribution of IL-15/IL-15R+ cells to alloimmune responses, we have constructed a recombinant fusion protein consisting of a mutated IL-15 and the constant region of murine IgG2a (14). This mIL-15/Fc fusion protein (mutant IL-15/Fc, or mIL-15/Fc) binds the IL-15 receptor alpha chain with high affinity but does not trigger IL-15R dependent signaling events. The IL-15R α chain is expressed upon activated but not resting mononuclear lymphocytes. Thus the fusion proteins act as receptor specific antagonist (14). Although the mutant IL-15 component targets the high affinity IL-15R, the Fc portion of this fusion protein confers in vivo longevity. Moreover, the Fc portion is derived from murine IgG 2a and designed to target IL-15R bearing cells, such as activated T cells and macrophages, for deletion by antibody mediated immune effector mechanisms (15). For comparison, a nonlytic IL-15/Fc, which lacks antibody mediated effector mechanisms, was also studied. Our previous studies using mIL-15/Fc have demonstrated that this fusion protein is indeed remarkably effective in blocking DTH responses and alloantigen driven T cell proliferation in vivo (14). Also, we have recently demonstrated that mIL-15/Fc prevents costimulation blockade-resistant rejection in a murine islet allograft model (16). In combination with IL-2/Fc and rapamycin, the mutant IL-15/Fc confers very potent immunosuppressive effects (17). In the present study we further explore the efficacy and functional properties of monotherapy with mIL-15/Fc in murine animal models of transplantation.

MATERIALS AND METHODS

Animals

BALB/c (H-2^d) and C57BL/6 (H-2^b) mice, 8–10 wk old, were purchased from Charles River Laboratories (Wilmington, MA). B10.A (H-2^a), CBA/Ca (H-2^k), B10.BR (H-2^k) and AKR/J (H-2^k), mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Reagents and Treatment Protocols

The design and construction of the mIL-15/Fc DNA construct has been previously described (14). Briefly, glutamine residues 101 and 108 within the fourth α helix of IL-15 were mutated to aspartic acid via site-directed and PCR-assisted mutagenesis. This mutant IL-15 was then genetically linked to murine IgG2a Fc and further cloned into an expressing vector. NS-1 cells (ATCC, Manassas, VA), or alternatively CHO-K1 cells (DMSZ, Braunschweig, Germany), were stably transfected with a plasmid carrying the construct encoding the mIL-15/Fc fusion protein (14). The transfectant cells were cloned and cultured in serum-free Ultraculture media (Bio-Whittaker Inc, Walkersville, MD) containing 100 μ g/ml Zeocin (Invitrogen, San Diego, CA). Mutant IL-15/Fc protein in the culture supernatant was purified by protein A affinity chromatography and, in some instances, ion-exchange chromatography. A nonlytic mIL-15/Fc γ 2a fusion construct was generated essentially as

described (15, 18). Briefly, oligonucleotide site-directed mutagenesis was used to replace the IgG2a C1q binding motif Glu318, Lys320, Lys322 with Ala residues. Similarly, the IgG2a residue Leu235 was replaced with Glu to inactivate the Fc γ RI binding site (15, 18). Monoclonal anti-CD154 antibody (MR-1, IgG2a) was prepared by Chimerigen Laboratories (Allston, MA). Heart and islet allograft recipients were treated daily or every second day with 1.5 μ g, 5 μ g, or 15 μ g mIL-15/Fc i.p. or 15 μ g control IgG2a i.p., respectively, for a total of 14 days, with the first treatment giving on the day of transplantation. Anti-CD154 (anti-CD40L) was single 200 μ g dose of administered i.p. on the day of transplantation.

Heart Transplantation

Abdominal heterotopic heart transplants were performed as described by Corry et al. (19). Briefly, the isolated donor heart was grafted by joining the donor aorta and pulmonary artery end to side to the recipient aorta and vena cava, respectively. Graft viability was monitored every 2 days by palpation of the beating hearts and scored on a scale of 1+ to 4+ based on strength and rate of impulse. In some instances recipients with a long term surviving grafts received a secondary cervical placed heart transplants. The basic procedures were identical to the ones used for abdominal aortic grafts, except that the second heart was grafted onto the carotid artery by side to end anastomosis with the aorta and side to end anastomosis of the pulmonary artery to the jugular vein.

Islet Transplantation

Islet transplantation was performed according to published standard procedures (16). Donor pancreata from 8–10 wk male Balb/c (H-2^d) mice were perfused in situ with 4 ml Type IV collagenase (Worthington Biochemical Corp. Freehold, NJ) through the common bile duct. The pancreata were harvested after perfusion and incubated at 37°C for 35 min. Islets were released from the pancreata by gentle vortexing and further purified on discontinuous percoll gradients, washed twice and 300 to 400 islets were transplanted under the left renal capsule of 8–10 wk old, completely MHC mismatched, C57BL/6 recipients rendered diabetic by a single i.p. injection of streptozotocin (260 mg/kg in 0.9% NaCl; Sigma Chemical Co., St. Louis, MO). Allograft function was monitored by serial blood glucose measurements (Accu-Chek III blood glucose monitor; Boehringer Mannheim, Indianapolis, IN). Primary graft function was defined as a blood glucose level below 200 mg/dl on day 3 posttransplantation, and graft rejection was defined as a rise in blood glucose exceeding 300 mg/dl following a period of satisfactory primary graft function. In order to determine whether tolerance was evident in mIL-15/Fc treated recipients, a nephrectomy to remove the islet allograft was performed on recipient mice with euglycemia for more than 120 days after primary transplantation. Removal of the left kidney bearing the islet allograft resulted in prompt hyperglycemia exceeding 300 mg/dl within 2–3 days. The second islet allografts from Balb/c or B10.A donors were transplanted under the right kidney capsule of hyperglycemic mice 4–6 days postnephrectomy. Secondary graft function was monitored by measuring the blood glucose levels of the recipient mice as described above.

Histopathology and Immunohistochemistry

Cardiac allografts were harvested at day 5 posttransplantation and divided into three parts perpendicularly to the intraventricular septum. The first one-third of the hearts was fixed in

zinc formalin for hematoxylin/eosin and immunohistochemistry (CD3 and F4/80 detection); paraffin sections were prepared from the so processed samples. One-third of the organs were imbedded in OCT and snap-frozen in liquid nitrogen to -80°C for immunohistochemistry (CD4 and CD8 detection). The last one-third were used for RT-PCR analysis (see below). After dehydration and paraffin embedding of heart tissue, 5- to 6- μm -thick sections were stained with H&E. Multiple sections of each heart were prepared and examined for the extent of rejection, myocardial damage, mononuclear cell infiltration, vasculitis and intimal proliferation. The avidin-biotin immunoperoxidase method was used for immunohistochemistry. Images were obtained using an Axioscope 2 microscope (Zeiss) equipped with a Digital Camera (SV Micro 80155) and interfaced with image analysis software (KS 300). Quantitative image analysis was performed on 10 random sections from each section of the heart stained for different cell markers (CD4 and CD8). Quantitative image analysis was performed on three hearts each from the control and treatment groups. The number of positively stained cells and total area occupied by these cells were compared for CD4 and CD8 cell markers in hearts of treated and control animals. For islet transplants the left kidneys bearing islet allografts were removed from long-term graft accepting mice and processed further. In addition, transplant-bearing kidneys from C57Bl/6 mice that had received Balb/c islet allografts were removed on day 7 posttransplantation. The kidneys were fixed in zinc formalin for hematoxylin/eosin and aldehyde-fuchsin staining and immunohistochemistry (insulin detection); paraffin sections were prepared from the so processed samples and 5- to 6- μm -thick sections of areas of islet implantation stained. Multiple sections of each kidney were prepared and examined for islet content and insulin production. The avidin-biotin immunoperoxidase method was used for immunohistochemistry, and images obtained as described for heart transplants.

RNA Isolation and Reverse Transcriptase-Assisted Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted using RNeasy 60 (Tel Test, Friendswood, TX) according to the manufacturer's instructions. RNA was then quality-controlled for traces of chromosomal DNA by PCR analysis and the concentration of the RNA samples determined with a Beckman Coulter Spectrophotometer DU 640. Two micrograms of RNA were reverse-transcribed and quality controlled for the expression of the housekeeping gene cyclophilin. Subsequently, the relative abundance of the inflammatory cytokines (IL-1 β , IL-6 and TNF α), IFN γ , and the CTL markers FasL, Granzyme B and Perforin were determined by TaqMan real-time PCR analysis with the ABI 7000 Sequence detection instrument and normalized against the housekeeping gene cyclophilin. Primers and probes for IL-1 β , IL-6 and TNF α were purchased from Applied Biosystems, primers for cyclophilin (CYC), IFN γ (IFN), FasL (FSL), Granzyme B (GRB) and Perforin (PRF) were: CYCF: GCCTGGATGCTAACAGAAGGA; CYCR: GTTCATCCCGTCGCTATGGT; CYCprobe: ATGACAAGGATGCCGGGCAAGTGT; FSLF: AATCTGTGGCTACCGGTGGTA; FSLR: GGTGGAAGAGCTGATACATTCCTA; FSLprobe: ATGGTTCCTGGTGGCTCTGGTTGGAA; GRBF: GCAAAGACTGGCTTCATATCCAT; GRBR: GCAGAAGAGGTGTTCCATTGG; GRBprobe: ACAAGGACCAGCTCTGTCCTTGGCAG; PRFF: TGCTCTTCGGGAACCAAGCT; PRFR: CAGGGTTGCTGGGCAGTGA; PRFprobe: CACCAGAGCAGTTCTCAACCTGGACAGC; IFNF:

ACAATGAACGCTACACACTGCAT; IFNR: TGGCAGTAACAGCCAGAAACA;
IFNprobe: TTGGCTTTGCAGCTCTTCCTCATGG.

Statistical Analysis

Animal survival data were analyzed using a survival curve log-rank test as provided by the Prism software (version 3.0). For comparison of survival curve between secondary same donor strain allografts and third party allografts, we represent the data as categorical and use chi square test. Histological data generated by Image Analysis were evaluated for statistical significance by using Student's two tailed *t* test at the 0.05 significance level. The Microsoft Excel data analysis tool was used to obtain mean and standard deviation as well as Student's *t* test results. Real-time PCR data were generated by analyzing each cDNA sample in triplicate by TaqMan real-time PCR. Automatic baseline determination using the ABI 7000 Sequence detection instrument was followed by manual quality control. Primary data were processed in an Excel spreadsheet format and exported into the Prism software (version 3.0) for the graphical display. Data generated were evaluated for statistical significance using a Student's two tailed *t* test.

RESULTS

Mutant IL-15/Fc, as a Monotherapy, Induces Antigen-Specific Tolerance in a Minor Histocompatibility Mismatched Cardiac Allograft Model

The efficacy of mIL-15/Fc in preventing the allograft rejection was tested in a minor histocompatibility mismatched cardiac allograft model. Treatment with 5 µg mIL-15/Fc every second day for 14 days led to permanent engraftment of B10.BR cardiac allografts in all CBA/Ca recipients. In contrast, control untreated CBA/Ca recipients promptly rejected B10.BR cardiac allografts within 13 days posttransplantation (MST = 10 days) (Fig. 1A). To test for antigen-specific tolerance, the mIL-15/Fc treated CBA/Ca recipients bearing B10.BR allografts for >100 days received secondary cardiac allografts from either same donor strain B10.BR mice or from the third-party strain AKR/J. The secondary grafts from the B10.BR donors were accepted without any further immunosuppression, whereas the grafts from the AKR/J mice were acutely rejected (Fig. 1B). For statistical analysis, we represent the data as categorical, such as 3/3 in a group received secondary same donor strain allografts vs. 0/2 in a group received secondary third party strain allografts and the *p* value is 0.0389 by using chi-square test.

Treatment with mIL-15/Fc Prolongs the Survival of Fully MHC-Mismatched Heart Allografts

The efficacy of mIL-15/Fc in preventing the rejection was further tested in a fully MHC-mismatched cardiac allograft model. As shown in Table 1, control Ig treated C57BL/6 (H-2^b) recipients acutely rejected the Balb/c (H-2^d) cardiac allografts with a MST = 7d (Table 1). Whereas treatment of C57BL/6 recipients with 1.5µg mIL-15/Fc daily for 14d led to a marginal prolongation of allograft survival (MST = 12d), treatment with 5 µg of the protein daily for 14d resulted in a significant prolongation of graft survival (MST = 26d, *P*<0.01), treatment of transplant recipients with 5 µg mIL-15/Fc every second day for 14 days (eight administrations total) led to a further prolongation of graft survival with an MST of 35 days (Table 1). Interestingly treatment with 15 µg mIL-15/Fc daily for 14 days did not

lead to a further prolongation of graft survival as compared with the 5 μ g daily treated group (Table 1). In contrast, treatment with 5 μ g mIL-15/Fc every 3 days failed to prolong cardiac allograft survival (Table 1).

Combined mIL-15/Fc and a Single Dose of Anti-CD154 Antibody Treatment Permit Permanent Engraftment of MHC-Mismatched Cardiac Allografts

As we have previously reported that mIL-15/Fc can prevent CTLA4/Fc mediated costimulation blockade resistant rejection in islet transplant models (21), we sought to determine whether blockade of the CD40/CD154 costimulation pathway would synergize with mIL-15/Fc in preventing fully MHC mismatched cardiac allograft rejection. Whereas treatment with a single dose of the anti-CD154 monoclonal antibody MR1 prolonged heart transplant survival in the Balb/c to C57/BL6 mouse strain combination, treatment was, however, insufficient to prevent rejection (Fig. 2A). In contrast, treatment with mIL-15/Fc (5 μ g/mouse every second day) for 14 days, in combination with a single dose administration of the anti-CD154 antibody, was sufficient to prevent graft rejection in all animals tested ($n = 5$) and led to permanent engraftment of the transplanted hearts (Fig. 2A).

Antibody-Mediated Cytolysis Contributes to the Efficacy of mIL-15/Fc In Vivo

To further characterize mIL-15/Fc's mode of action during the allograft response and to directly investigate the potential contribution of the IgG2a Fc domain to the overall efficacy of mIL-15/Fc, we have generated a nonlytic point-mutated variant of mIL-15/Fc (non-lytic mIL-15/Fc) with essentially diminished ADCC and CDC activities (15, 20). While the Fc sequence used in the lytic IL-15/Fc can support antibody mediated target cell destruction, the mutant Fc does not possess lytic activities. Hence the lytic protein is an IL-15R antagonist with lytic potential, while the nonlytic protein possesses only receptor antagonist properties. A short course treatment with mIL-15/Fc leads to prolonged heart allograft survival (MST = 25 days) in the Balb/c to C57/BL6 mouse strain combination, transplants in animals treated with the nonlytic mIL-15/Fc variant are rejected with kinetics comparable to control Ig-treated animals (MST = 7 days) (Fig. 2B).

Similarly, lytic mIL-15/Fc proved efficacious in preventing C57/BL6 recipients from rejection of fully MHC-mismatched Balb/c islet allografts. Treatment with 5 μ g lytic mIL-15/Fc administered every second day for 14 days led to a prolongation of islet allograft survival and permanent engraftment in 50% of the treated animals (Fig. 3A). All animals bearing functional islet allografts became diabetic after removal of the grafts (data not shown). Subsequently, two mice accepted second islet allograft from Balb/c donors without further immunosuppression, one mouse acutely rejected a third party B10.A islet graft suggesting a state of allograft tolerance (Fig. 3B). However, due to the low number of grafts no statistical analysis was performed. In contrast, non-lytic mIL-15/Fc treatment had a modest effect on islet allograft survival, all islet allograft were rejected at MST of 19 days (Fig. 3A).

Treatment with mIL-15/Fc Strongly Reduces T Cell and Macrophage Infiltration in MHC-Mismatched Cardiac Allografts

To assess the effects of lytic mIL-15/Fc on graft rejection, we studied the immunochemistry on the cardiac allografts harvested 5 days posttransplantation. The overall graft leukocyte infiltration in lytic mIL-15/Fc treated mice was reduced as compared to the control group. The leukocytes infiltrates in the cardiac allografts from lytic mIL-15/Fc treated animals were focal, and ischemic myocardial cell damage with interstitial edema and hemorrhages was also strongly reduced in comparison with that from control mice (Fig. 4). Vascular changes consisting of vasculitis and vascular endothelial cell proliferation and occlusion were also more evident in the control group (Fig. 4A and B). Immunohistological detection of leukocyte subsets on day 5 showed a strongly reduced number of CD3⁺ (data not shown), CD4⁺, CD8⁺ and F4/80⁺ cells in the allografts of lytic mIL-15/Fc treated animals (Fig. 4C–H). A quantitative image analysis performed on these samples revealed a particularly striking reduction of leukocyte infiltration for CD8⁺ T cells (panels E and F), which was at 93.5% (n = 3, *P* = 0.008). In comparison, CD4⁺ T cells in the mIL-15/Fc treated grafts were reduced by 58% (n = 3, *P* < 0.05).

Immunocytochemistry study on islet allografts harvested 7 days posttransplantation reveal a strong reduction in islet cell mass and insulin-producing cells in untreated animals, as compared to the mIL-15/Fc treated mice (Fig. 4A and B). Moreover, the structural and functional well preserved islets, as determined by aldehyde-fuchsin and insulin staining, were found in the islet allograft harvested from mIL-15/Fc treated mice with normal glycemia 120 days posttransplantation (Fig. 4C and D).

mIL-15/Fc Treatment Reduces the Expression of CTL and Inflammatory Markers as well as of Th2 Cytokines in MHC-Mismatched Heart Allografts

To further study the effects of mIL-15/Fc treatment on allogeneic transplant rejection, a real time PCR analysis of gene expression on various inflammatory cytokines (IL-1 β and TNF α), CTL effector molecules (FasL, Granzyme B and Perforin) and Th1/Th2 cytokines (IL-4 and IFN γ) was performed on the allograft samples harvested 5 days posttransplantation. Whereas the expression all of these markers was elevated in rejecting heart allografts of control-treated animals (C), treatment with m IL-15/Fc (T) led to a statistically significant reduction of expression of most of these genes in the transplanted hearts, with the notable exception of the Th2 cytokine IL-4 (Fig. 5). Similar results such as for IL-4 were also obtained for IL-5 (data not shown). Interestingly, also a reduction in IL-10 expression was observed in the mIL-15/Fc treated grafts (*P* < 0.001; data not shown), likely reflecting the strong reduction in macrophages seen in the treated grafts (Fig. 5G and H).

DISCUSSION

IL-15 is T-cell growth factor with potent proinflammatory and antiapoptotic properties. IL-15 emerges as a important cytokine that may play a multifaceted role in inflammatory and immunological disorders, such as in rheumatoid arthritis, inflammatory bowel disease and organ transplant rejection (13). It is notable that increased intra-graft IL-15 gene expression was a diagnostic marker for acute kidney (12, 21) and heart allograft rejection

(22, 23). In an earlier study, we have demonstrated that treatment with the IL-15/IL-15R blocking agent mIL-15/Fc fusion protein prevents costimulation blockade resistant transplant rejection in a murine islet allograft model (16).

In the present report we extend these observations to show that mIL-15/Fc treatment also prolongs the graft survival of fully MHC-mismatched vascularized heart transplants (Fig. 2). Treatment with lytic mIL-15/Fc reduces the graft infiltration by CD4⁺ and CD8⁺ T cells as well as macrophages. The effect of lytic mIL-15/Fc treatment upon CD8⁺ T cells is particularly striking, in that CD8⁺ T cells are almost completely absent from the grafts of mIL-15/Fc treated animals (Fig. 4A), although infiltration of CD4⁺ T cells into the grafts is also reduced (Fig. 4A). These effects are consistent with earlier reports that IL-15 acts preferentially on CD8⁺ T cells as deduced in IL-15 and IL-15R α knockout mice (4, 5). Consistent with the immunohistology results we find that mIL-15/Fc treatment reduces the expression of CTL markers in the grafts, as well as the expression of the inflammatory cytokines TNF α and IL-1 β . Interestingly, whereas mIL-15/Fc treatment leads to a reduction of Th1 cytokine expression (IFN γ and TNF α), no effect of treatment is seen on the expression of the Th2 cytokines IL-4 and IL-5. These data would indicate that IL-15 may preferentially stimulate Th1 responses, further underlining the utility of IL-15 antagonistic approaches in targeting Th1-mediated diseases, such as many autoimmune disorders and graft rejection. The dose titration experiments performed in the Balb/c to C57/BL6 mouse strain combination revealed a dose response relationship and a direct correlation between dose administered and efficacy of the treatment. Interestingly, treatment with mIL-15/Fc every second day showed an increased efficacy as compared to a daily treatment and further delayed graft rejection. We speculate that the more intensive treatment with an IL-15R antagonist fusion protein, in which the mutant IL-15 was derived from human sequence, may raise the possibility of developing neutralizing Ab that might compromise the therapeutic efficacy.

We have previously demonstrated that the deletion of activated T cells can contribute to peripheral tolerance induction, suggestive of the notion that depletion of the pool of antigen-responsive T cells may shift the balance of an immune reaction from an immunogenic to a tolerogenic response (24). In view of these earlier findings, we were interested in analyzing as to whether the IgG2a Fc portion of mIL-15/Fc would contribute to the overall efficacy of the molecule. Intriguingly, we find that the treatment with a non-lytic variant of mIL-15/Fc indeed did not prolong graft survival in the MHC mismatch transplant model. Although mIL-15/Fc is not generally lymphoablative in mice (data not shown), due to the fact that the IL-15R is only expressed on activated T cells, these results nonetheless suggest that complement and/or FcR mediated deletion of IL-15R expressing activated T cells and macrophages contributes to the overall immunoprotective effect of mIL-15/Fc. Interestingly, Smith et al. (25) reported earlier that the use of a recombinant soluble IL-15R α subunit (sIL-15R α) was ineffective in preventing graft rejection in the MHC mismatch heart transplant model, but did prolong graft survival in a minor histocompatibility mismatch mouse strain combination. An IL-15 neutralizing agent, such as sIL-15R α , would not target IL-15R bearing cells for deletion by the innate immune system. We would therefore propose that while inhibition of the IL-15/IL-15R pathway is sufficient to prevent graft rejection and induce antigen-specific tolerance in a minor histocompatibility mismatch mouse heart

transplant setting, Fc-mediated activation of the innate immune system and depletion of IL-15R bearing cells contributes to the prolonged graft survival of fully MHC mismatched heart transplants observed in this study.

In addition to prolonging graft survival, we find that a short course of mIL-15/Fc treatment can induce antigen-specific tolerance in minor histocompatibility mismatched heart transplants. Moreover, we have recently demonstrated that in combination with IL-2/Fc and rapamycin, the mutant IL-15/Fc confers very potent immunosuppressive effects to induce allograft tolerance (17). Given the inherent liabilities of the currently available immunosuppressive protocols and the need to develop improved therapeutics for the treatment of transplant recipients mIL-15/Fc may therefore represent an interesting candidate for the development of future immunomodulatory and potentially tolerogenic clinical protocols.

Acknowledgments

This work is supported by the National Institutes of Health and the Juvenile Diabetes Research Foundation (T.B.S and X.X.Z).

REFERENCES

1. Grabstein KH, Eisenman J, Shanebeck K, et al. Cloning of a T cell growth factor that interacts with the β chain of the interleukin-2 receptor. *Nature*. 1994; 264:965.
2. Parrish-Novak J, Dillon SR, Nelson A, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature*. 2000; 408(6808):57. [PubMed: 11081504]
3. Alleva DG, Kaser SB, Monroy MA, et al. IL-15 functions as a potent autocrine regulator of macrophage proinflammatory cytokine production: evidence for differential receptor subunit utilization associated with stimulation or inhibition. *J Immunol*. 1997; 159(6):2941. [PubMed: 9300718]
4. Lodolce JP, Boone DL, Chai S, et al. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity*. 1998; 9(5):669. [PubMed: 9846488]
5. Kennedy MK, Glaccum M, Brown SN, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med*. 2000; 191(5):771. [see comments]. [PubMed: 10704459]
6. Doherty TM, Seder RA, Sher A. Induction and regulation of IL-15 expression in murine macrophages. *J Immunol*. 1996; 156(2):735. [PubMed: 8543827]
7. Fehniger TA, Suzuki K, Ponnappan A, et al. Fatal Leukemia in Interleukin 15 Transgenic Mice Follows Early Expansions in Natural Killer and Memory Phenotype CD8(+) T Cells. *J Exp Med*. 2001; 193(2):219. [PubMed: 11208862]
8. Carson WE. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of IL-2 receptor. *J Exp Med*. 1994; 180:1395. [PubMed: 7523571]
9. Kanegane H, Tosato G. Activation of naive and memory T cells by interleukin-15. *Blood*. 1996; 88(1):230. [PubMed: 8704178]
10. Bulfone-Paus S, Ungureanu D, Pohl T, et al. Interleukin-15 protects from lethal apoptosis in vivo. *Nature Medicine*. 1997; 3:1124.
11. Li XC, Demirci G, Ferrari-Lacraz S, et al. IL-15 and IL-2: a matter of life and death for T cells in vivo. *Nat Med*. 2001; 7(1):114. [PubMed: 11135625]
12. Pavlakakis M, Strehlau J, Lipman M, et al. Intragraft IL-15 transcripts are increased in human renal allograft rejection. *Transplantation*. 1996; 62(4):543. [PubMed: 8781624]

13. Fehniger TA, Caligiuri MA. Interleukin 15: biology and relevance to human disease. *Blood*. 2001; 97(1):14. [PubMed: 11133738]
14. Kim YS, Maslinski W, Zheng XX, et al. Targeting the IL-15 receptor with an antagonist IL-15 mutant/Fc gamma2a protein blocks delayed-type hypersensitivity. *J Immunol*. 1998; 160(2):5742. [PubMed: 9637483]
15. Zheng XX, Steele AW, Hancock WW, et al. IL-2 receptor-targeted cytolytic IL-2/Fc fusion protein treatment blocks diabetogenic autoimmunity in nonobese diabetic mice. *J Immunol*. 1999; 163(7): 4041. [PubMed: 10491008]
16. Ferrari-Lacraz S, Zheng XX, Kim YS, et al. An antagonist IL-15/Fc protein prevents costimulation blockade-resistant rejection. *J Immunol*. 2001; 167(6):3478. [PubMed: 11544341]
17. Zheng XX, Sanchez-Fueyo A, Sho M, et al. Favorably tipping the balance between cytopathic and regulatory T cells to create transplantation tolerance. *Immunity*. 2003; 19(4):503. [PubMed: 14563315]
18. Zheng XX, Steele AW, Hancock WW, et al. A noncytolytic IL-10/Fc fusion protein prevents diabetes, blocks autoimmunity, and promotes suppressor phenomena in NOD mice. *J Immunol*. 1997; 158(9):4507. [PubMed: 9127018]
19. Corry RJ, Minn HJ, Russell PS. Primarily vascularized allograft of hearts in mice. The role of H-2D, H2-K and non-N-2 antigens in rejection. *Transplantation*. 1973; 16:343. [PubMed: 4583148]
20. Zheng XX, Steele AW, Hancock WW, et al. A Non-cytolytic IL-10/Fc fusion protein prevents diabetes, blocks autoimmunity, and promotes suppressor phenomena in NOD mice. *J Immunol*. 1997; 158:4507. [PubMed: 9127018]
21. Strehlau J, Pavlakis M, Lipman M, et al. Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. *Proc Natl Acad Sci USA*. 1997; 94:695. [PubMed: 9012847]
22. van Gelder T, Baan CC, Balk AH, et al. Blockade of the interleukin (IL)-2/IL-2 receptor pathway with a monoclonal anti-IL-2 receptor antibody (BT563) does not prevent the development of acute heart allograft rejection in humans. *Transplantation*. 1998; 65(3):405. [PubMed: 9484761]
23. Baan CC, Knoop CJ, van Gelder T, et al. Anti-CD25 therapy reveals the redundancy of the intra-graft cytokine network after clinical heart transplantation. *Transplantation*. 1999; 67(6):870. [PubMed: 10199736]
24. Li XC, Strom TB, Turka LA, Wells AD. T cell death and transplantation tolerance. *Immunity*. 2001; 14(4):407. [PubMed: 11336686]
25. Smith XG, Bolton EM, Ruchatz H, et al. Selective blockade of IL-15 by soluble IL-15 receptor alpha-chain enhances cardiac allograft survival. *J Immunol*. 2000; 165(6):3444. [PubMed: 10975865]

FIGURE 1.

Cytolytic mIL-15/Fc treatment is sufficient to induce antigen-specific tolerance in minor histocompatibility-mismatched heart allografts. (A) Minor histocompatibility-mismatched B10.BR (H-2^k) hearts were transplanted into CBA/Ca (H-2^k) recipient mice and the animals treated every second day with 5 μ g mIL-15/Fc i.p. for 14 days (15 μ g/mouse/day) and graft survival monitored. Whereas the cardiac allograft were rejected in control animals with a MST of 10 days ($n = 5$), short course treatment with mIL-15/Fc leads to permanent cardiac allograft engraftment ($n = 5$, $P = 0.0018$). (B) After more than 100 days of survival of the primary transplant, CBA/Ca animals received a secondary heterotopic cervical heart transplant using either B10.BR (H-2^k) or AKR/J (H-2^k) donor hearts without any further treatment and graft survival was monitored. Whereas B10.BR transplants ($n = 3$) are accepted without any further immunosuppressive treatment, the third party AKR/J grafts are efficiently rejected ($n = 2$, MST = 14 days, $P = 0.0389$ by a chi square analysis).

FIGURE 2.

Treatment with lytic mIL-15/Fc, but not nonlytic mIL-15/Fc, is effective at prolonging MHC-mismatched heart allograft survival and combination therapy with mIL-15/Fc and low dose antiCD154 leads to permanent engraftment of MHC-mismatched heart allografts. (A) Fully MHC-mismatched Balb/c (H-2^d) hearts were transplanted into C57/BL6 (H-2^b) recipient mice and the animals treated i.p. with either a single dose of antiCD154 (200µg, n = 5) on the day of transplantation, with mIL-15/Fc (5 µg/mouse every second day, n = 5) for 14 days, or with a combination of the two treatment protocols (mIL-15/Fc + antiCD154, n = 5), and graft survival monitored. The survival of transplants in animals treated with control IgG2a (15 µg/mouse/day, n = 5) is shown for comparison. Whereas treatment with mIL-15/Fc alone or treatment with a single dose of antiCD154 prolong allograft survival ($P < 0.005$ for both anti-anti-CD15 and mIL-15/Fc groups vs. control Ig group), but does not prevent graft rejection in the Balb/c to C57/BL6 mouse strain combination, combined treatment with both agents leads to permanent engraftment ($P < 0.005$ for mIL-15/Fc + anti-CD154 group vs. either mIL-15/Fc group or anti-CD154 group). (B) Fully MHC-mismatched Balb/c (H-2^d) hearts were transplanted into C57/BL6 (H-2^b) recipient mice and the animals treated i.p. with either control IgG2a (15 µg/mouse/day, n = 5), mIL-15/Fc (15 µg/mouse/day, n = 5) or with a nonlytic mIL-15/Fc (15 µg/mouse/day, n = 5) for 14 days and graft survival monitored. Whereas treatment with mIL-15/Fc leads to prolonged graft survival (MST = 25 days, $P < 0.05$ mIL-15/Fc group vs. IgG2a group), transplants in animals treated with nonlytic mIL-15/Fc are rejected with kinetics comparable to control-treated animals (MST = 7 days, $P < 0.05$ mIL-15/Fc group vs. nonlytic mIL-15/Fc group).

FIGURE 3.

Cytolytic mIL-15/Fc treatment induces permanent engraftment in 50% fully MHC-mismatched islet allograft recipients. Fully MHC-mismatched Balb/c (H-2^d) islets were transplanted under the kidney capsule of C57/BL6 (H-2^b) recipient mice and the animals treated every second day with 5 μ g mIL-15/Fc i.p. for 14 days and graft survival monitored. (A) Whereas the transplants are rejected in control animals with a MST of 12 days ($n = 13$), short course treatment with mIL-15/Fc leads to permanent engraftment in 50% of the treated animals ($n = 6$, $P < 0.01$ mIL-15/Fc group vs. control group). (B) After removal of the kidneys bearing longterm engrafted islet allografts, two recipients accepted secondary islet transplant from Balb/c (H-2^d) without any further immunosuppression ($n = 2$), while one recipient acutely rejected second islet allograft from third party strain B10.A (H-2^a) donor ($n = 1$), suggesting antigen-specific tolerance.

FIGURE 4.

Immunohistochemistry study on Cardiac and islet allografts. (A) Treatment with mIL-15/Fc strongly reduces T cell and macrophage infiltration in MHC-mismatched cardiac allografts. Fully MHC-mismatched Balb/c (H-2^d) hearts were transplanted into C57/BL6 (H-2^b) recipient mice and the animals treated i.p. with either control IgG2a (panels B; D; F; H; 15 μ g/mouse/day) or with mIL-15/Fc (A, C, E, G; 5 μ g/mouse/day). On day 5 posttransplantation the grafted hearts were removed and hematoxylin/eosin (A, B) as well as immunohistological stains against CD4⁺ (C, D), CD8⁺ (E, F) and F4/80 (G, H) prepared. Whereas control-treated animals show severe intimal cell proliferation and thrombosis with vessel occlusion and perivascular and tissue lymphocytic infiltrates, little infiltration is observed in the mIL-15/Fc treated animals. Also, macrophage, CD4⁺ T cell and in particular CD8⁺ T cell infiltration is strongly reduced in mIL-15/Fc treated animals. (B) In some animals the islet-bearing kidneys were removed on day 7 posttransplantation and stained for islets and insulin content. Whereas islet structures have almost completely disappeared in

untreated animals and very few and only small areas of insulin-producing cells are detected (C; immunohistological detection of insulin, HE counterstain), insulin-producing islets are preserved in mIL-15/Fc treated animals (D). Similarly, islet-bearing kidneys from long-term engrafted animals with normoglycemia showed preserved islet structures (E; aldehyde-fuchsin staining) and high insulin content (F).

FIGURE 5.

mIL-15/Fc treatment reduces the expression of CTL and inflammatory markers as well as of Th1 cytokines in MHC-mismatched heart allografts. Fully MHC-mismatched Balb/c (H-2^d) hearts were transplanted into C57/BL6 (H-2^b) recipient mice and the animals treated i.p. with either control IgG2a (C; 15 μ g/mouse/day) or with mIL-15/Fc (T; 5 μ g/mouse/day). On day 5 posttransplantation the grafted hearts were removed and the expression levels of the respective genes analyzed by reverse transcription and real time PCR analysis. Expression was normalized against the housekeeping gene cyclophilin and is given as the ratio of the expression of the respective gene vs. cyclophilin. The expression of the CTL markers Fas Ligand (FasL), perforin (Perf) and granzyme B (GrnB) as well as expression of the inflammatory cytokine markers IL-1 β and TNF α is reduced in mIL-15/Fc-treated animals. Furthermore, whereas expression of the Th1 cytokine IFN γ is reduced in mIL-15/Fc treated mice, treatment has no effect on the expression of the Th2 cytokine IL-4. Each point shown in the Figure corresponds to the results obtained from one sample from each mouse.

TABLE 1

Survival of Balb/c heart allografts in C57/BL6 recipients treated with lytic mIL-15/Fc

Donor	Recipient	Treatment	Heart graft survival (days)	P value
Balb/c (H-2d)	C57/BL6 (H-2b)	Control IgG2a 15 µg/day for 14 days	6, 7, 7, 8, 8	-
Balb/c (H-2d)	C57/BL6 (H-2b)	CRB-15 1.5 µg/day for 14 days	9, 10, 12, 12, 13	<0.005 ^a
Balb/c (H-2d)	C57/BL6 (H-2b)	CRB-15 5 µg/day for 14 days	20, 22, 26, 30, 30	<0.005 ^a
Balb/c (H-2d)	C57/BL6 (H-2b)	CRB-15 15 µg/day for 14 days	19, 22, 25, 28, 30	<0.005 ^a
Balb/c (H-2d)	C57/BL6 (H-2b)	CRB-15 5 µg/2 days for 14 days	29, 32, 35, 35, 36	<0.05 ^b
Balb/c (H-2d)	C57/BL6 (H-2b)	CRB-15 5 µg/3 days for 14 days	10, 12, 13, 14, 14	<0.005 ^b

^aGraft survival prolongation in lytic mIL-15/Fc versus control-treated animals is statistically significant (log-rank test; $P < 0.005$).

^bGraft survival versus animals treated every day.