Selective Blockade of IL-15 by Soluble IL-15 Receptor α-Chain Enhances Cardiac Allograft Survival

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IL-15 is a T cell growth factor that shares many functional similarities with IL-2 and has recently been shown to be present in tissue and organ allografts, leading to speculation that IL-15 may contribute to graft rejection. Here, we report on the in vivo use of an IL-15 antagonist, a soluble fragment of the murine IL-15R α-chain, to investigate the contribution of IL-15 to the rejection of fully vascularized cardiac allografts in a mouse experimental model. Administration of soluble fragment of the murine IL-15R α-chain (sIL-15Rα) to CBA/Ca (H-2k) recipients for 10 days completely prevented rejection of minor histocompatibility complexity-mismatched B10.BR (H-2b) heart grafts (median survival time (MST) of >100 days vs MST of 10 days for control recipients) and led to a state of donor-specific immunologic tolerance. Treatment of CBA/Ca recipients with sIL-15Rα alone had only a modest effect on the survival of fully MHC-mismatched BALB/c (H-2b) heart grafts. However, administration of sIL-15Rα together with a single dose of a nondepleting anti-CD4 mAb (YTS 177.9) delayed mononuclear cell infiltration of the grafts and markedly prolonged graft survival (MST of 60 days vs MST of 20 days for treatment with anti-CD4 alone). Prolonged graft survival was accompanied in vitro by reduced proliferation and IFN-γ production by spleen cells, whereas CTL and alloantibody levels were similar to those in animals given anti-CD4 mAb alone. These findings demonstrate that IL-15 plays an important role in the rejection of a vascularized organ allograft and that antagonists to IL-15 may be of therapeutic value in preventing allograft rejection.  

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Cytokines play a key role in regulating and amplifying the T cell-dependent immune response to an organ allograft. T cell growth factors such as IL-2, IL-4, IL-7, IL-9, and IL-15 promote differentiation and clonal expansion of activated alloreactive T cells, whereas proinflammatory cytokines such as IFN-γ and TNF-α contribute significantly to the effector mechanisms responsible for graft destruction (1, 2). The T cell-derived cytokine IL-2 was the first major T cell growth factor identified, and therapeutic Abs directed against the α-chain of the IL-2R (CD25) have recently been shown, when combined with conventional immunosuppressive agents, to reduce the incidence of acute rejection after human organ transplantation (3, 4). However, allograft rejection still occurs in the presence of IL-2/IL-2R blockade (3–5). Moreover, IL-2 gene knockout mice and IL-2/IL-2R double-knockout mice are still able to reject allografts, highlighting the redundancy in the cytokine network and suggesting a potentially important role for other immunoregulatory cytokines, such as IL-7 and IL-15, in the graft rejection process (6, 7).  

IL-15 is a recently identified cytokine that is similar in structure to IL-2 and shares with it a number of biological activities, including its ability to stimulate the proliferation and differentiation of activated T cells (8, 9). However, unlike IL-2, IL-15 mRNA is expressed in a wide range of different cell types, including activated macrophages, activated vascular endothelial cells, fibroblasts, muscle cells, epithelial cells, and T cells (10, 11). Moreover, although IL-15 and IL-2 share the same IL-2Rβ and common γ-chain receptor subunits, IL-15 uses a unique α-chain, IL-15Rα, which is expressed on a wide variety of cell types, including activated T cells, B cells, NK cells, macrophages, and some types of nonimmune cells (10–12). The biological effects of IL-15 are diverse. In addition to acting as a T cell growth factor, IL-15 stimulates the differentiation of NK cells, induces T cell chemotaxis, promotes B cell activation and isotype switching, and increases the production of proinflammatory cytokines by macrophages (10, 11). It also promotes the growth and activation of mast cells and influences activity in some types of nonimmune cell (10, 11).  

The role of IL-15 in the allograft rejection response is not clearly defined, but an important role for this cytokine has been suggested in other types of immunopathology, such as inflammatory arthritis, inflammatory bowel disease, sarcoidosis, and multiple sclerosis (13–17). Several recent studies have observed an increase in the expression of intragraft IL-15 mRNA transcripts and IL-15-expressing cells after transplantation and suggested that increased IL-15 expression correlates with acute rejection (18–22). Therefore, blockade of the IL-15/IL-15R pathway may be of value in modifying the immune response to an organ allograft. In this paper we report that administration of a soluble fragment of the murine IL-15R α-chain (sIL-15Rα)3 to neutralize IL-15 prevents rejection of fully vascularized, minor histocompatibility Ag-mismatched cardiac allografts. Furthermore, sIL-15Rα, in combination with a single dose of a nondepleting anti-CD4 mAb, markedly prolongs the survival of fully allogeneic heart allografts.
Materials and Methods

Animals
Male CBA/Ca (H-2k), B10.BR (H-2d), AKR/J (H-2b), and BALB/c (H-2d) mice were obtained from Harlan U.K. (Bicester, U.K.). All animals were maintained under conventional conditions at Central Biomedical Services, University of Cambridge (Cambridge, U.K.), and were used when they were 8–12 wk old.

Heart transplantation
Vascularized cervical heterotopic heart transplantation was performed using standard microsurgical techniques as previously described (23). The innominate artery of the donor heart was anastomosed end-to-end to the right common carotid artery of the recipient, and similarly, the donor pulmonary artery was anastomosed to the recipient external jugular vein. Cold ischemic times were <30 min. Graft function was assessed by daily palpation of the heart, and rejection was defined as the complete cessation of myocardial contraction.

Skin grafting
Tail skin grafts from adult donor mice were transplanted to the flanks of heart graft recipients, essentially as described previously (24). Dressings were removed after 7 days, and skin graft rejection was defined as complete destruction of the graft.

Histology
Heart grafts were excised, formalin fixed, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin.

sIL-15Rα
A soluble fragment of IL-15R α-chain lacking transmembrane and cytoplasmic domains was generated as described previously (14). The histidine-tagged recombinant protein was purified using nickel-agarose and dialyzed into PBS for in vivo use. The sIL-15Rα fragment used has been shown previously to inhibit rIL-15-mediated in vitro proliferation of CTLL and D10 cells, but does not bind to IL-2 or inhibit IL-2-dependent CTLL proliferation (14). Heart graft recipients received 60 μg of sIL-15Rα i.p. on the day of transplantation and daily for 9 days thereafter. This schedule was chosen on the basis that daily i.p. administration of 40 μg of sIL-15Rα for 10 days profoundly suppressed the development of murine collagen-induced arthritis (14), although it is not known whether the amount of sIL-15Rα given was sufficient to completely neutralize IL-15 for the treatment duration.

Antibodies
The rat anti-mouse CD4 mAb YTS 177.9 was provided by Dr. Steve Cobbold, Sir William Dunn School of Pathology (Oxford, U.K.) (25). Ig, purified by ammonium sulfate precipitation, was administered to recipient mice as a single dose of 1 mg given i.p. at the time of heart transplantation. The phenotype of recipient splenocytes was determined by flow cytometry, using fluorochrome-conjugated rat anti-mouse mAbs KT3 (CD3), KT15 (CD8; both from PharMingen, Becton Dickinson, Oxford, U.K.) and YTS 177.9 (CD4; Serotec, Oxford, U.K.). Mouse alloantibody levels were determined by flow cytometry after incubating recipient serum samples with donor splenocytes, followed by FITC-labelled rabbit Ab against mouse IgG plus FITC-labelled rabbit Ab against mouse IgM (P0313, Dako, Cambridge, U.K.) or against mouse IgG1, IgG2a, and IgG3 (PharMingen).

CTL activity
The CTL activity of splenocytes was determined as described previously (26). Recipient splenocytes were cocultured with irradiated donor splenocytes (20 Gy) for 5 days to generate CTL, then incubated with [3H]thymidine-labeled donor strain Con A blast target cells for 6 h to determine their cytotoxic activity. Reduced scintillation counts indicated enhanced cytotoxic activity.

Lymphocyte proliferation and cytokine production
Lymphocyte proliferation assays were performed essentially as described previously (27). Responder splenocytes (2 × 10^5/well) were cultured with irradiated (20 Gy) stimulator splenocytes (2 × 10^5/well) for 72, 96, and 120 h in 96-well plates at 37°C in 5% CO_2. Proliferation was assessed by uptake of [3H]thymidine for 6 h at the end of the culture period. Supernatant was harvested from cell culture plates and was assayed by ELISA for production of IFN-γ and IL-4 using capture and detection Abs (PharMingen) according to the manufacturer’s instructions.

Statistical analysis
Differences between groups were compared by nonparametric analysis using the Mann-Whitney U test. Values of p (two-tailed) < 0.05 were considered significant.

Results
Effect of sIL-15Rα on cardiac allograft survival
The effect of disrupting the IL-15/IL-15R pathway on cardiac allograft survival was examined by administering sIL-15Rα to recipients of either minor (mHC) or MHC Ag-mismatched heart grafts. CBA/Ca (H-2d) recipients of MHC-disparate B10.BR (H-2b) heart grafts were treated daily with an i.p. injection of 60 μg of sIL-15Rα for 10 days from the day of transplant. This treatment schedule was based on our experience of using sIL-15Rα to control murine collagen-induced arthritis (14). Control recipients rejected their heart grafts promptly, with a median survival time (MST) of 10 days, whereas treatment with sIL-15Rα extended heart graft survival well beyond the 10-day treatment schedule in all recipients, and in 70% of recipients led to long term (>100 days; p < 0.01) graft survival (Fig. 1). Histologic examination of rejected heart grafts in control recipients showed characteristic features of acute rejection, with a heavy mononuclear cell infiltrate and widespread tissue destruction (Fig. 2A). Nonrejecting hearts in sIL-15Rα-treated recipients examined at >100 days were still beating strongly and showed no histologic evidence of graft rejection (Fig. 2B).

To determine whether sIL-15Rα treatment had induced donor-specific tolerance, three sIL-15Rα-treated CBA/Ca recipients with long surviving B10.BR heart allografts (>100 days) were grafted with both donor strain (B10.BR, H-2b) and third-party (AKR/J, H-2a) skin. Third-party AKR/J skin grafts were rapidly rejected (MST, 14 days) by CBA/Ca recipients, whereas donor strain B10.BR grafts survived for >100 days, confirming the development of donor-specific immunologic tolerance.

Administration of sIL-15Rα to CBA/Ca recipients bearing fully allogeneic BALB/c (H-2d) heart grafts produced only a modest prolongation of graft survival compared with MHC-mismatched controls (MST of 11 days vs MST of 7 days, respectively; Fig. 3A).

Because sIL-15Rα alone was relatively ineffective in this fully MHC-disparate mouse strain combination, we chose in subsequent experiments to administer the antagonist with an immunosuppressive agent.
experiments to combine it with an additional immunomodulatory agent in the form of a nondepleting anti-CD4 mAb. YTS 177.9 is a rat anti-mouse CD4 mAb of the IgG2a subclass (25) that after in vivo administration induces rapid and profound modulation of CD4 from residual CD4 T cells, resulting in transient interruption of the alloimmune response, but only limited prolongation of cardiac allograft survival (28). CBA/Ca recipients of BALB/c hearts received 1 mg of YTS 177.9 given i.p. at the time of heart transplantation together with a 10-day course of sIL-15Rα. Phenotypic analysis of spleen cells from transplanted animals showed that the anti-CD4 mAb treatment schedule led to a modest reduction in CD4 T cell numbers, and this reduction was most apparent in recipients given a combination of sIL-15Rα and YTS 177.9 (Fig. 4). Cardiac allograft recipients given anti-CD4 mAb alone rejected their grafts with an MST of 15 days (Fig. 3b). In recipients treated with a combination of sIL-15Rα and anti-CD4 mAb, cardiac allograft survival was markedly prolonged (MST of 60 days vs MST of 20 days for controls; Fig. 3b). Histologic examination of heart grafts excised on day 15 from control recipients given anti-CD4 mAb showed a heavy mononuclear cell infiltrate and widespread tissue destruction (Fig. 5A). In contrast, examination of hearts excised on day 15 from sIL-15Rα+anti-CD4-treated recipients showed a moderate mononuclear cell infiltrate, but minimal graft damage (Fig. 5B).

CTL activity

Spleen cells were obtained from CBA/Ca recipients of BALB/c hearts at 10 and 15 days after transplantation, and cytotoxic T cell activity against donor target cells was determined. CTL activity against BALB/c targets was higher on day 10 than on day 15 after transplantation, but at neither time point was there any significant difference between experimental groups (Fig. 6).
Alloantibody levels

Anti-BALB/c Ab was readily detected by flow cytometry in serum obtained from anti-CD4-treated CBA/Ca recipients of BALB/c heart grafts and was tested using the F0313 rabbit anti-mouse Ig that detects both IgG and IgM. Additional treatment with sIL-15Rα had no discernible effect on the Ab titers observed (Fig. 7). Three of five recipients treated with sIL15Rα plus anti-CD4 plus showed low titers (<1/27) of the Th1-type IgG2a alloantibody and no detectable IgG3 or Th2-type IgG1 alloantibody. Control recipients given anti-CD4 mAb alone had marginally higher titers of circulating IgG alloantibody, with four of six animals having detectable IgG2a alloantibody with titers of up to 1/81, no detectable IgG3, and IgG1 titers of <1/27 (data not shown).

Lymphocyte proliferation and cytokine production

Spleen cells obtained on day 15 from CBA/Ca recipients of BALB/c heart allografts were assessed for their ability to proliferate in vitro and to produce IFN-γ in response to irradiated allogeneic stimulators. Lymphocytes from recipients treated with a combination of sIL-15Rα and anti-CD4 mAb showed a marked reduction in proliferation to donor-specific (BALB/c) stimulator cells compared with naive responder cells from normal CBA/Ca mice (p < 0.05). The level of proliferation observed was significantly lower (p < 0.02) than that shown by cells obtained from control recipients treated with anti-CD4 mAb alone (Fig. 8a). Spleen cells from animals treated with a combination of sIL-15Rα...
and anti-CD4 mAb also showed lower levels of spontaneous prolifera-
tion than cells obtained from control recipients given anti-
CD4 mAb alone (p < 0.02). Lymphocytes from recipients treated
with sIL-15Rα plus anti-CD4 mAb gave a lower proliferative re-
response to third-party (C57BL/10) stimulators compared with naive
control cells, but the residual levels of proliferation observed were
not significantly different from those shown by spleen cells from
control recipients given anti-CD4 mAb alone.

Spleen cells from CBA/Ca recipients treated with sIL-15Rα and
anti-CD4 mAb produced levels of IFN-γ comparable to those pro-
duced by naive splenocytes from normal CBA/Ca mice and sig-
nificantly less IFN-γ after 96 and 120 h of coculture than spleno-
cytes from control recipients treated with anti-CD4 mAb alone
(p < 0.05; Fig. 8b). IL-4 was not detected in any of the coculture
supernatants examined in these experiments.

Discussion

IL-15 is a member of the 4α-helix bundle family of cytokines,
initially identified through its ability to stimulate T cell growth and
differentiation, but with wide ranging effects on both lymphoid and
nonlymphoid cells (8, 9, 29). Many cell types constitutively ex-
press IL-15 mRNA, although production of IL-15 protein is subject
to considerable post-transcriptional regulation (10, 11). Recent
studies have shown that expression of IL-15 mRNA is increased in
some types of immune-mediated tissue injury, and there is con-
siderable interest in the potential contribution of this cytokine to
the pathogenesis of tissue destruction in these situations (13, 15–
22). Following organ transplantation, expression of IL-15 mRNA
is readily detectable in the graft, and increased levels of expression
have been shown to correlate with the presence of acute rejection,
leading to speculation that IL-15 may contribute to the rejection
process (18–22).

The results of the present study provide clear in vivo evidence
that IL-15 plays a role in the rejection of fully vascularized heart
allografts. In the case of multiple mHC-mismatched cardiac allo-
grafts, the contribution of IL-15 to acute rejection appears critical,
because administration of a sIL-15Rα not only prevented the re-
jection of mHC-mismatched cardiac allografts, but also led to the
development of operational donor-specific tolerance, as shown by
rejection of third-party, but not donor strain, skin grafts. It might
have been expected, in view of the considerable functional overlap
between cytokines of the T cell growth factor family, that there
would be sufficient redundancy in the cytokine network to allow
rejection of mHC-mismatched heart grafts in the presence of IL-15
antagonism (1, 30). IL-15, produced mainly by monocytes, and
IL-2, produced by T cells, have considerable functional overlap,
and both cytokines support T cell proliferation and differentiation,
augment B cell and NK cell activities, and are chemoattractant for
T cells. The extent of this functional overlap is explained in part by
sharing of common receptor subunits (30). Both IL-15 and IL-2
use the IL-2R β-chain and common γ-chain, but each has a distinct
α-chain. Interestingly the IL-15Rα-chain has such a high affinity
for IL-15 that it binds with a Ki of 10^11 M, which is 1000-fold
higher than that for IL-2Rα for IL-2 (12). In view of the functional
similarities between IL-15 and IL-2, it is important to note that the
sIL-15Rα fragment used in the present study does not bind to IL-2
in vitro and does not inhibit IL-2-dependent CTLL proliferation
(14). This argues strongly against cross-reactivity of the sIL-15Rα
with IL-2 as an explanation for our findings and points instead to
the importance of IL-15 in mediating allograft rejection.

IL-15 has a number of biological functions not shared with IL-2,
and these may arise because of the site of synthesis and regulation
of IL-15 expression, the wide tissue distribution of the IL-15R,
and the existence of another unique IL-15R (IL-15RX) (10, 11). Pro-
duction of IL-15 by dendritic cells may, for example, strongly
influence T cell activation. In addition, IL-15 stimulates the pro-
liferation of mast cells and bone marrow cells and plays a key role
in the differentiation and development of NK cells (10, 11). Nei-
ther the cell type and anatomic location of IL-15 production nor
the nature of the target cells through which IL-15 exerts its effects
on cardiac allograft rejection were directly addressed in the present
study. Many cell types express IL-15 mRNA, but there is consid-
erable post-transcriptional control of protein production as well as
regulation at the level of intracellular trafficking of IL-15 protein
(11). Activated monocytes and macrophages are one of the major
sources of IL-15 protein and large numbers of macrophages ex-
pressing IL-15 can be identified in human cardiac allografts (19).
APCs, recipient lymphocytes, monocytes, NK cells, and donor en-
thelial cells may all express receptors for IL-15 and therefore are
potential target cells for IL-15 during an alloimmune response
(11). Further studies are now needed to dissect in detail the role of
IL-15 in allograft rejection.

The rejection response to MHC-mismatched heart allografts is
considerably more powerful than that to grafts differing only in
mHC, and therefore it is not surprising that administration of

![FIGURE 8. In vitro proliferative response and cytokine production in splenocytes from sIL-15Rα-treated heart allograft recipients. Splenic lymphocytes were harvested from naive CBA/Ca mice or from CBA/Ca recipients of BALB/c heart allografts on day 15 following transplant and treatment with sIL-15Rα (or human serum albumin (HSA)) plus anti-CD4. Cells were cocultured with irradiated BALB/c (donor strain) or C57BL/10 (third-party) splenocytes for 96 h. Proliferation (a) was assessed by uptake of [3H]thymidine for 6 h at the end of the culture period. Results are the mean ± SD of three to five mice per group. *, p < 0.02 vs anti-CD4 plus HSA controls; †, p < 0.05 vs naive controls; ‡, p < 0.02 vs naive controls. IFN-γ levels (b) in supernatants were assayed by ELISA (mean ± SD of three to five mice per group).  , p < 0.05 vs HSA plus anti-CD4; †, p < 0.01 vs naive controls.](image-url)
sIL-15Rα alone induced only a modest prolongation of graft survival beyond that of control recipients. Therefore, we chose in subsequent experiments to combine sIL-15Rα treatment with a single dose of the nondepleting anti-CD4 mAb YTS 177.9. A combination of sIL-15Rα and anti-CD4 mAb prolonged survival of fully MHC-mismatched heart grafts far more effectively than anti-CD4 mAb alone, thereby demonstrating the contribution of IL-15 to rejection of MHC disparate allografts. The choice of a nondepleting anti-CD4 mAb as an additional immunomodulatory agent for these studies arose because of our long-standing interest in anti-CD4 mAbs as agents for preventing allograft rejection. However, it seems reasonable to hypothesize that combinations of sIL-15Rα and various other immunosuppressive agents might also have a synergistic effect on allograft survival.

Analysis of the effector mechanisms responsible for allograft rejection reveals that delayed-type hypersensitivity, cytotoxic T cells and alloantibody-dependent effector mechanisms may all contribute to graft destruction. In the present study administration of sIL-15Rα did not reduce the level of anti-donor CTL in the spleens of anti-CD4-treated heart graft recipients, nor did it further reduce the total serum Ig alloantibody levels below those seen in control animals given anti-CD4 mAb alone, although experimental data suggested that the anti-CD4-mediated reduction in IgG alloantibody was enhanced by additional sIL-15Rα. However, spleen cells from recipients given sIL-15Rα showed a reduction in proliferation and produced less of the proinflammatory cytokine IFN-γ in response to in vitro restimulation by donor alloantigen.

The finding that IL-15 contributes to allograft rejection is consistent with the emerging role of IL-15 in other types of T cell growth factors play an essential role in allograft rejection, as highlighted by the recent demonstration that blockade of the IL-15 receptor (IL-15Rα) inhibits cytokine production to treatment with cyclosporin (34).

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In conclusion, the results of this study provide in vivo evidence that IL-15 plays an important role in the rejection of a vascularized organ allograft and suggest that targeting IL-15-IL-15R interaction may, when combined with other chemical or biological immunosuppressive agents, be of therapeutic value in preventing allograft rejection.

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