An Antagonist IL-15/Fc Protein Prevents Costimulation Blockade-Resistant Rejection[1]

Sylvie Ferrari-Lacraz, **Xin Xiao Zheng**, **Yon Su Kim**[2], **Yongsheng Li**, **Wlodzimierz Maslinski**[3], **Xian Chang Li**, and **Terry B. Strom**[4]
Department of Medicine, Division of Immunology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215

Abstract

IL-15 is a powerful T cell growth factor (TCGF) with particular importance for the maintenance of CD8^{+} T cells. Because costimulation blockade does not result in universal tolerance, we hypothesized that "escape" from costimulation blockade might represent a CD8^{+} and IL-15/IL-15R^{+}-dependent process. For this analysis, we have used an IL-15 mutant/Fcγ2a protein, a potentially cytolytic protein that is also a high-affinity receptor site specific antagonist for the IL-15Rα receptor protein, as a therapeutic agent. The IL-15-related fusion protein was used as monotherapy or in combination with CTLA4/Fc in murine islet allograft models. As monotherapies, CTLA4/Fc and an IL-15 mutant/Fcγ2a were comparably effective in a semiallogeneic model system, and combined treatment with IL-15 mutant/Fcγ2a plus CTLA4/Fc produced universal permanent engraftment. In a fully MHC-mismatched strain combination known to be refractory to costimulation blockade treatment, combined treatment with both fusion proteins proved to be highly effective; >70% of recipients were tolerized. The analysis revealed that the IL-15 mutant/Fc treatment confers partial protection from both CD4^{+} and CD8^{+} T cell graft infiltration. In rejections occurring despite CTLA4/Fc treatment, concomitant treatment with the IL-15 mutant/Fcγ2a protein blocked a CD8^{+} T cell-dominated rejection processes. This protection was linked to a blunted proliferative response of alloreactive T cells as well silencing of CTL-related gene expression events. Hence, we have demonstrated that targeting the IL-15/IL-15R pathway represents a new and potent strategy to prevent costimulation blockade-resistant CD8^{+} T cell-driven rejection.

Blockade of B7-CD28 or CD40-CD40L costimulatory interactions blunts T cell-dependent immune activation processes (1–4) such as allograft rejection (5–8). Blockade of the CD28 T cell signal through treatment with CTLA4/Fc can suppress Th1 cytokine production in vivo (9) and deviate the alloimmune response to a Th2 pathway, which is less detrimental than Th1-type immune activation in transplant models (10). In several circumstances, however, costimulatory blockade fails to induce permanent engraftment. A CD8^{+} T cell-dependent process appears to be responsible for costimulation blockade-resistant rejection (11–15). We find that costimulation blockade does not prolong survival of islet allografts in a CD8^{+} TCR-transgenic model (our unpublished data). Honey et al. (12) have recently

[4]Address correspondence and reprint requests to Dr. Terry B. Strom, Division of Immunology, Beth Israel Deaconess Medical Center, Research North, Room 380, P.O. Box 15707, Boston, MA 02215. tstrom@caregroup.harvard.edu.
[2]Current address: Seoul National University, Boraman Hospital, 195 Shindaebangdong, Tongjak-ju, Seoul, 156-012, Korea.
[3]Current address: Department of Pathophysiology and Immunology, Institute of Rheumatology, Spartanska 1, Warsaw, Poland.

reported that anti-CD154 mAb treatment prolongs engraftment of skin allografts, but the induction of tolerance requires adjunctive treatment with anti-CD8 mAb, or other means of depleting CD8^{+} T cells (14). Trambley et al. (15) have also demonstrated the importance of GM1^{+}CD8^{+} T cells in costimulation blockade-resistant rejection.

Clearly, costimulation blockade drastically decreases expression of IL-2 posttransplantation (7, 9). Which T cell growth factors are responsible for the clonal proliferation of alloreactive T cells that cause graft rejection in some costimulation blockade-treated hosts? IL-2 expression is not a prerequisite for allograft rejection as IL-2 knockout (KO)[5] mice promptly reject islet and cardiac allografts (16, 17). Immunohistology in the IL-2 KO recipients reveals that CD4^{+} and CD8^{+} T cells infiltrate rejecting grafts while robust intragraft expression of the IL-4, IL-7, IL-15, as well as granzyme B genes is evident (Refs. 16 and 18 and our unpublished data). Moreover, mice rendered genetically deficient for both IL-2 and IL-4, the principal T cell growth factors that are produced by T cells, also readily reject allografts (17). Thus, non-T cell-derived cytokines, such as IL-15, may be actively involved in supporting allograft rejection. An analysis of biopsies taken from rejecting human renal allografts has revealed that intragraft gene expression of *IL-15* is a far more consistent marker of rejection than *IL-2* gene expression (18). Moreover, Smith et al. (19) have recently reported the prolonged survival of heart allografts in a treatment protocol consisting of soluble IL-15R chain proteins and nondepleting anti-CD4 mAb. Can expression of IL-15 be linked to CD8-dependent costimulation-resistant rejection? The phenotype of IL-15^{−/−} (20) and IL-15R [−/−] (21) mice demonstrates that IL-15 selectively supports the homeostasis and proliferation of memory CD8^{+} T cells (22, 23).

Costimulation blockade cannot act to directly decrease IL-15 expression because epithelial and endothelial cells and monocyte/macrophages, not T cells, are the primary cellular sources of this cytokine (24). Since costimulation blockade-resistant rejection may be mediated by CD8^{+} T cells and IL-15 is essential for the homeostasis and proliferation of CD8^{+} T cells, we have now tested the hypothesis that targeting the IL-15/IL-15R system in concert with costimulation blockade might provide a new tool for the induction of allograft tolerance.

The trimolecular IL-15R expressed upon T cells includes the constitutively expressed IL-2R and IL-2R common -chains shared with the IL-2R. A unique IL-15R chain (25, 26) is expressed upon activated, but not resting, mononuclear leukocytes (27). The IL-15R is expressed in lesser abundance upon activated epithelial cells, macrophages, and fibroblasts (24). We have previously reported on the specificity of a unique IL-15 mutant/Fc 2a protein possessing a prolonged circulating half-life, high-affinity IL-15R site-specific antagonist function and both complement- and Fc-related cytocidal potential against IL-15R [+] cells (28). Kim et al. (28) have demonstrated that this IL-15 mutant/Fc fusion protein specifically binds to the IL-15R ; IL-15 mutant/Fc 2a binding to activated T cells was blocked by IL-15, but not by IL-2 or anti-IL-2/IL-15R chain Abs. In this report, we also demonstrated that this IL-15 mutant/Fc protein efficiently blocks delayed-type hypersensitivity in mice (28). In the present report, the therapeutic activity of the IL-15-related protein was compared with that of a CTLA4/Fc. CTLA4/Fc treatment is known to promote engraftment and often leads to tolerance (5, 29). Combined treatment with CTLA4/Fc and IL-15 mutant/Fc proteins was also studied. Our data suggest that IL-15R^{+} cells are an important component of allograft rejection and these cells play a pivotal role in CD8^{+} T cell-mediated CTLA4/Fc blockade-resistant rejection.

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[5]Abbreviations used in this paper: KO, knockout; FasL, Fas ligand; MST, mean survival time.

Materials and Methods

Animals

BALB/c (H-2^d), DBA/2 (H-2^d), B6AF$_1$ (H-2$^{b/d.k}$), and C57BL/6 (H-2^b) mice, 8–10 wk old, were obtained from Taconic Farms (Germantown, NY). C57BL/6^{cd4tm1knw} (CD4KO, H-2^b) and C57BL/6.129S2^{cd8atm1mak} (CD8KO, H-2^b) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Islet transplantation

Allogeneic DBA/2 islet cell grafts were transplanted into 8- to 10-wk-old B6AF$_1$ recipient mice rendered diabetic by a single i.p. injection of streptozotocin (225 mg/kg; Sigma, St Louis, MO). Allogeneic BALB/c islet cell grafts were transplanted into 8- to 10-wk-old C57BL/6 recipient mice rendered diabetic by a single i.p. injection of streptozotocin (270 mg/kg). Islet cell transplantation was performed as previously described (30). Briefly, islets were isolated from donor DBA/2 or BALB/c pancreata through collagenase digestion and centrifugation on a discontinuous Ficoll gradient. The crude islet isolates containing islets, vascular tissue, ductal fragments, and lymph nodes were divided into aliquots of ~300 islets and were transplanted under the renal capsule into B6AF$_1$ or C57BL/6 recipients. By intent, we use these crude islet preparations which are more immunogenic than more pure islet preparations (our unpublished observations). Initial allograft function was verified by sequential blood glucose measurements with levels under 200 mg/dl on days 3–5 after transplantation, and graft rejection was defined as a rise in blood glucose levels exceeding 300 mg/dl following a period of primary graft function.

Treatment protocol

Murine CTLA4/Fc (31) and human IL-15 mutant/Fc 2a (28) proteins were constructed and expressed in our laboratory. CTLA4/Fc protein used in these studies bears active FcR binding and complement-binding domains (29). Treatment of islet allograft recipients with CTLA4/Fc consisted of 0.1 mg/day i.p. for 10 consecutive days after transplantation, which is the optimal treatment period in this model (our unpublished data). Islet allograft recipients received 1.5 μg of IL-15 mutant/Fc 2a/day i.p. for 21 consecutive days after transplantation. An IgG2a protein, bearing the same Fc sequence as the CTLA4/Fc and IL-15 mutant/Fc 2a proteins, was used as a control treatment at 1.5 μg/day for 10 days.

Histopathology and immunohistology

The left kidney bearing the islet graft was removed from the recipients after 8 days and embedded in OCT compound (Tissue TCK; Miles Scientific, Elkhart, IN). Cryostat sections of islets ($n = 3$/group) were fixed in paraformaldehyde-lysine-periodate for analysis of leukocyte Ags and stained by a four-layer peroxidase-antiperoxidase method involving overnight incubation with mAb, followed by mouse Ig-absorbed goat anti-rat Ig, rabbit anti-goat Ig, goat peroxidase-antiperoxidase complexes, and diaminobenzidine substrate. Rat anti-mouse mAbs and isotype-matched control mAbs were purchased from BD PharMingen (San Francisco, CA) and included mAbs to CD4$^+$ (H129.19) and CD8$^+$ (53-6.7) T cells. Sections were counterstained in hematoxylin and mounted. Isotype-matched mAbs and a control Ab were analyzed for endogenous peroxidase activity in each experiment. Samples were assigned a random number and processed and evaluated in a blinded fashion; each sample was evaluated at two to three different levels of sectioning.

PCR analysis

Intragraft mRNA analysis was performed via template RT-PCR as previously described (16). The specific primers used for hybridization to murine IL-2, TCR C , perforin, Fas

ligand (FasL), granzyme B, and GAPDH cDNA, the latter as an internal control, have been previously described (16). The PCR amplification was performed in a thermocycler (Gene Amp, PCR system 2400; PerkinElmer/Cetus, Norwalk, CT): denaturing at 94°C for 30 s, annealing at 57°C for IL-2 and FasL, at 60°C for perforin and granzyme B, at 60°C for TCR C , and at 57°C for GAPDH for 30 s, and extension at 72°C for 30 s for each cycle, for a total of 40 cycles. A negative control was included for each PCR amplification and consisted of the omission of cDNA in the PCR mixture. To further confirm data, a semiquantitative competitor template RT-PCR analysis was also performed with IL-2 using the gene-specific relative RT-PCR kit from Ambion (Austin, TX). To quantitate the relative amounts of TCR C gene transcripts between samples, each PCR was performed by coamplifying the cDNA of interest with an internal PCR control. A gene-specific competitive template cDNA was designed and the semiquantitative PCR was performed as described by Steiger et al. (16). After amplification, the samples (10 μl) were separated on ethidium bromide-stained 1.5% agarose gel and the DNA were visualized and photographed using UV transilluminator (Gel Doc 1000; Bio-Rad, Hercules, CA).

CFSE labeling and analysis of T cell proliferation in vivo

Spleen and lymph node cells from wild-type C57BL/6, $CD8^{-/-}$, or $CD4^{-/-}$ mice (C57BL/6, $H-2^b$) were harvested, processed, and labeled with CFSE (Molecular Probes, Portland, OR) as described previously (32). CFSE was dissolved in DMSO and added into the cell suspension at a final concentration of 5 nM for 3 min at room temperature. The reaction was stopped by the addition of HBSS/1% FCS. The cells were washed in HBSS/1% FCS and resuspended in the same solution before injection. Recipient BALB/c mice were sublethally irradiated (1000 rad with a GammaCell irradiator, Kanata, Ontario, Canada) before injection of the CFSE-labeled cells via the lateral tail vein. From that day, untreated recipient mice or mice receiving an i.p. injection of CTLA-4/Fc (0.1 mg/mouse) or IL-15 mutant/Fc 2a protein (1.5 μg/mouse) for 3 days were studied. Adoptive transfer experiments using syngeneic CFSE-labeled lymphocytes was also performed as a control. On day 3, recipient spleen and lymph node cells were removed and cell suspensions were processed as before. Cells were stained with anti-CD4-PE conjugate (L3T4, 2 μg/ml) or a biotinylated mAb against mouse CD8a (53-6.7, 2 μg/ml; BD PharMingen) for 30 min at 4°C. After staining, cells were washed once and resuspended in 0.5 ml of HBSS for analysis by flow cytometry using a BD Biosciences FAC-Sort equipped with CellQuest software (Mountain View, CA). Live events were collected and analyzed by gating on $CD4^+$ or $CD8^+CFSE^+$ cells.

Calculation of the frequency of proliferating T cell

Analysis of $CD4^+$ and $CD8^+$ T cell proliferation in response to alloantigen stimulation was performed according to Noorchashm et al. (33). With each round of cell division, the CFSE dye partitions equally between the daughter cells. By using the FACS acquisition software (CellQuest), the total number of cells in each generation of proliferation can be calculated and the number of precursors that generated the daughter cells was determined by using the following formula: y/n^2 (y = absolute number of cells in each peak, n = number of cell division). The calculation of the frequency of T cell proliferation was then analyzed by dividing the total number of precursors by the total CFSE-labeled cells.

Results

Survival of islet allografts in B6AF$_1$ and C57BL/6 recipients

To probe for the role of the IL-15/IL-15R network in the allograft response, partially MHC-mismatched DBA/2 ($H-2^d$) islet allografts were transplanted into B6AF$_1$ ($H-2^{b/d.k}$) mice. Untreated recipients rejected DBA/2 islet allografts with a mean survival time (MST) of 14 days (Table I) while B6AF$_1$ recipient mice treated with CTLA4/Fc or IL-15 mutant/Fc 2a

have a MST of 70 and 77 days, respectively. All B6AF$_1$ recipients of DBA/2 islet allografts treated with a combination of IL-15 mutant/Fc 2a and CTLA4/Fc were permanently engrafted. Surgical removal of the left kidney bearing the islet allograft was performed on two recipients 150 days after transplantation. Six days later, a second DBA/2 islet allograft was successfully engrafted without rejection in the absence of further immunosuppressive therapy.

To test the hypothesis that targeting the IL-15/IL-15R system would block CD8^{+}-driven CTLA4/Fc-resistant rejection, fully MHC-mismatched BALB/c (H-2^d) donor islet allografts were transplanted into C57BL/6 (H-2^b) recipient mice. In this model, the recipients are refractory to the tolerizing effects of optimal doses of CTLA4/Fc. Untreated C57BL/6 recipients rejected BALB/c islet allografts (MST, 20 days; Fig. 1*a*). As additional controls, C57BL/6 recipients were also treated with a control IgG2a protein (1.5 μg/ml). In other experiments, C57BL/6 received CTLA4/Fc (0.1 mg/ml) plus IgG2a (1.5 μg/ml). Recipients treated with IgG2a rejected rapidly their allografts (MST, 14 days; Fig. 1*a*), and the recipients treated with CTLA4/Fc and IgG2a rejected allografts at the same tempo as recipients treated with CTLA4/Fc alone (Fig. 1*a*) These data confirm that the prolonged engraftment observed with IL-15 mutant/Fc 2a fusion protein treatment is not produced solely by Fc sequences shared with IgG2a. Engraftment was prolonged in C57BL/6 recipients treated with CTLA4/Fc or with IL-15 mutant/Fc 2a protein (MST, 30 days; Fig. 1*a*); some grafts functioned permanently following monotherapy with either agent. A dramatic improvement was noted among C57BL/6 recipients treated with a combination of IL-15 mutant/Fc 2a and CTLA4/Fc (MST 120 days; Fig. 1*a*). Surgical removal of the islet allograft in each of five recipients 150 days after transplantation led to hyperglycemia, demonstrating that the euglycemic state was maintained by the islet allografts. Subsequent to removal of the primary allograft, four recipient mice were successfully engrafted with a second BALB/c islet allograft in the absence of further immunosuppressive treatment (Fig. 1*b*). One recipient received a third-party islet allograft (B10.BR, H-2^k) that was rejected at day 12 posttransplantation (Fig. 1*b*), thereby confirming that the tolerant state was donor strain specific.

T cell infiltration in the BALB/c donor allografts placed into C57BL/6 recipients

Histologic assessment of the allografts revealed a dense mononuclear leukocytic infiltrate in untreated recipients (Fig. 2*a*) and less intense infiltration was present in CTLA/Fc- or IL-15 mutant/Fc-treated recipients (Fig. 2, *b* and *c*) at day 8 posttransplantation. This day was chosen for study because it is the time at which graft infiltration by host mononuclear leukocytes peaks in untreated control recipients. In the combined treatment group, cellular infiltration was dramatically reduced and islet architecture was well preserved (Fig. 2*d*). In untreated recipients, dense graft infiltration by mononuclear leukocytes, composed predominantly of CD4^{+} and CD8^{+} T cells, was noted (Fig. 3, *a* and *b*). By comparison (and as anticipated), a decrease in graft infiltration by CD4^{+} T cells was noted in recipients treated with CTLA4/Fc (Fig. 3*c*) while infiltration by CD8^{+} T cells was similar to that detected in untreated mice (Fig. 3*d*). This result is in keeping with the hypothesis that costimulation-resistant rejection is a CD8^{+} T cell-dependent process. In contrast, in recipients treated with IL-15 mutant/Fc 2a (Fig. 3, *e* and *f*), a decrease in CD8^{+} T cell infiltration without a major change in CD4^{+} T cell infiltration was evident. Note that recipient mice treated with both CTLA4/Fc and IL-15 mutant/Fc 2a (Fig. 3, *g* and *h*) show a near absence of CD4^{+} and CD8^{+} graft-infiltrating T cells.

The effect of treatment upon intragraft IL-2 and TCR C gene expression was analyzed by RT-PCR at day 8 posttransplantation. As the constant chain of TCR C is constitutively expressed by T cells, a substantial decrease in the frequency of graft-infiltrating T cells in the allograft should be accompanied by a decrease in the intragraft TCR C gene expression.

In recipients treated with IL-15 mutant/Fc 2a or the combination of IL-15 mutant/Fc 2a protein and CTLA4/Fc, a marked decrease in expression of the IL-2 gene and TCR C gene was observed (data not shown).

To further test the hypothesis that CD8$^+$ T cells play a major role in costimulation blockade-resistant rejection in this model, we assessed CTL-selective gene expression events in islet allografts isolated 8 days posttransplantation (Fig. 4). The granzyme B, perforin, and FasL genes are uniformly expressed in tissue samples obtained from untreated recipients or from recipients treated with CTLA4/Fc (Fig. 4). In contrast, reduced expression of the CTL-selective genes was observed in recipients treated with IL-15 mutant/Fc 2a or in recipients treated with IL-15 mutant/Fc 2a plus CTLA4/Fc (Fig. 4).

IL-15 mutant/Fc treatment inhibits alloreactive proliferating CD8$^+$ T cells in vivo

To determine whether treatment with the IL-15 mutant/Fc 2a protein blunts the proliferation of alloreactive CD8$^+$ T cells in vivo, we labeled splenic lymphocytes from C57BL/6 CD4$^{-/-}$ mice with CFSE. The dye-labeled C57BL/6 CD4$^{-/-}$ lymphocytes were injected i.v. into irradiated untreated or treated (for 3 days) BALB/c mice. On the third day following adoptive cell transfer, CFSE-stained leukocytes from hosts were recovered and stained with a biotinylated anti-CD8 mAb. Since CFSE partitions equally between daughter cells following cell division, the pattern and frequency of proliferation of alloreactive CD8$^+$ T cells in vivo can be analyzed via the CFSE staining pattern (31). In untreated hosts, ~23% of CFSE-labeled allogeneic CD8$^+$ T cells proliferated in the host spleen (Fig. 5b). In CTLA4/Fc-treated hosts, CD8$^+$ T cells (~21%) divide for multiple generations (Fig. 5b). In contrast, treatment with IL-15 mutant/Fc alone or in combination with CTLA4/Fc markedly inhibited proliferation of alloreactive CD8$^+$ T cells with only 15 and 14%, respectively, of CFSE-labeled CD8$^+$ T cells proliferating in vivo (Fig. 5b). Using syngeneic controls (CFSE-labeled C57BL/6 CD4$^{-/-}$ lymphocytes injected into C57BL/6 mice) proliferation of CD8$^+$ T cells was not detected (data not shown). By contrast, when similar experiments were performed with splenic lymphocytes from C57BL/6 CD8$^{-/-}$ mice to analyze the fate of CD4$^+$ T cells, we observed that CTLA4/Fc treatment markedly decreased the frequency of proliferating CFSE-labeled alloreactive CD4$^+$ T cells in vivo (Fig. 5a). Treatment with IL-15 mutant/Fc 2a reduces the frequency of proliferating CD4$^+$ and CD8$^+$ T cells, although a lesser effect on CD4$^+$ T cells as compared with CD8$^+$ T cells was noted. Similar results were observed when wild-type C57BL/6 mice were used as donor (data not shown). IL-15 mutant/Fc 2a may represent an important therapeutic agent capable of controlling the proliferation of alloreactive CD8$^+$ (costimulation-resistant) T cells. Proliferation of CD4$^+$, but not CD8$^+$, T cells is controlled by B7 blockade agents.

Discussion

T cell costimulation blockade treatment with CTLA4/Fc or Abs against CD154 have often produced permanent engraftment and donor-specific tolerance (5–8). Unfortunately, recent reports have underlined the importance of activated CD8$^+$ T cells in a costimulation-resistant rejection process observed in various transplant models such as skin, intestine, and heart (11–15). It is notable that costimulatory blockade prolongs allograft survival in CD8 KO mice (11, 14) because in those models in which tolerance is not produced by costimulation blockade, tolerance can be achieved with provision of anti-CD8 mAb or other treatments that clear alloreactive CD8$^+$ T cells (12–15). Apparently costimulatory blockade-resistant activation of alloreactive CD8$^+$ T cells can cause allograft rejection.

Since the molecular targets of costimulation blockade are primarily expressed upon CD4$^+$, and not upon CD8$^+$ T cells, the reason that CD8$^+$ T cells play a prominent role in costimulation blockade escape rejection process is enigmatic. Studies using IL-15$^{-/-}$ mice

and IL-15R $^{-/-}$ mice (19, 20) have emphasized the role of IL-15 in support of the activation and maintenance of CD8$^+$ T cells (22). Because costimulation blockade results in diminished expression of IL-2 by CD4$^+$ T cells (7, 9), we hypothesized that escape from costimulation blockade might represent an IL-15/IL-15R-dependent process. Moreover, costimulation blockade does not target epithelial cells, the principle source of IL-15 (23). Thus, we wanted to study the effect of IL-15 mutant/Fc 2a on an islet allograft model and its potential to enhance the effectiveness of costimulatory blockade.

For our experiments, we first chose a partial MHC-mismatch combination (H-2^d–H-2$^{b/d.k}$) to examine the effect of the IL-15 mutant/Fc 2a. In this islet allograft model, the administration of IL-15 mutant/Fc 2a, a protein that targets the IL-15R chain expressed upon activated T cells, prevents allograft rejection in 50% of the recipient mice (Table I). Identical results were obtained with CTLA4/Fc administration. It is notable that combined treatment with CTLA4/Fc and IL-15 mutant/Fc 2a produced permanent engraftment and tolerance in all recipients. Next, we chose to study a fully MHC-mismatched strain combination and used a recipient strain (C57BL/6) that is refractory to costimulatory blockade therapy (15). A modest beneficial effect of CTLA4/Fc treatment was observed (MST, 30 days vs 13 days in the controls; Fig. 1*a*) and prolonged graft survival was evident in IL-15 mutant/Fc 2a-treated recipients (Fig. 1*a*). Treatment with a control IgG2a protein did not prolong engraftment (MST, 14 days). Furthermore, combined treatment with CTLA4/Fc and IgG2a does yield an improvement upon the effect of CTLA4/Fc monotherapy (Fig. 1*a*). Moreover, the bioavailability of CTLA4/Fc or IL-15 mutant/Fc 2a is not altered when the fusion proteins are administered as combined treatment (CTLA4/Fc half-life = 96 h and IL-15 mutant/Fc 2a half-life = 6–8 h). Thus, the beneficial effects observed with CTLA4/Fc and/or IL-15 mutant/Fc 2a are not dependent on solely the 2a Fc peptide sequence present in the fusion proteins. We have previously demonstrated that the IL-15 mutant/Fc 2a fusion protein specifically binds to IL-15R but not the common -chain (28). Binding of the IL-15 mutant/Fc 2a was inhibited by IL-15 but not inhibited by IL-2 or anti-common -chain Abs (28).

To begin to test our primary hypothesis concerning the role of IL-15/IL-15R-dependent CD8$^+$ processes in rejections occurring despite use of CTLA4/Fc, we treated a group of recipient mice with combined CTLA4/Fc and IL-15 mutant/Fc 2a therapy. Permanent engraftment was obtained in most treated recipients (Fig. 1*a*). Further analysis demonstrated that these permanently engrafted mice were rendered specifically tolerant to donor strain grafts (Fig. 1*b*). CTLA4/Fc treatment had a far more dramatic effect upon CD4$^+$ T cell infiltration into the graft than upon CD8$^+$ T cell infiltration. While the principal effect of IL-15 mutant/Fc 2a treatment in the combined CTLA4/Fc plus IL-15 mutant treatment group was exacted upon infiltration of CD8$^+$ T cells into the graft (Fig. 3). The prolongation of islet allograft survival in recipient mice receiving both CTLA4/Fc and IL-15 mutant/ Fc 2a was accompanied by a decrease in T cell infiltration in graft tissues (Fig. 2). Immunohistologic analysis suggests that the potential benefit of a combined treatment is related to the drastic decrease of tissue infiltration by both alloreactive CD4$^+$ and CD8$^+$ T cells (Fig. 3). Molecular markers for activated CD8$^+$ T cells, such as CTL genes, have been identified within the infiltrate of rejecting allografts (28), and expression of these CTL genes (granzyme B, FasL, perforin) has been associated with acute renal allograft rejection (18). It is thus interesting to observe that the expression of CTL genes are markedly decreased in IL-15 mutant/Fc 2a-treated mice (Fig. 4), further attesting to the effect of IL-15 mutant/ Fc 2a treatment upon alloactivated CD8$^+$/CTLs.

To further analyze the effect of costimulation blockade and IL-15 mutant/Fc 2a, we studied alloantigen-driven proliferative responses in vivo using the CFSE dye system. CTLA4/Fc treatment has no effect on the CD8$^+$ T cell proliferative response to alloantigen, whereas

IL-15 mutant/Fc γ2a has a potent inhibitory effect on the proliferation of alloreactive CD8+ T cells (Fig. 5). Because IL-15 is of essential importance for the homeostasis and proliferation of CD8+ T cells (22, 23), we cannot exclude the possibility that IL-15 mutant/Fc γ2a treatment decreases the frequency of proliferating CD8+ T cell by promoting apoptosis of these responder cells. In contrast, the alloantigen-driven response of CD4+ T cells was decreased by either CTLA4/Fc or IL-15 mutant/Fc γ2a monotherapy (Fig. 5*b*). The effect of CTLA4/Fc upon CD4+ T cells is particularly potent (Fig. 5*a*). The IL-15 mutant/Fc γ2a, but not CTLA4/Fc, treatment targets CD8+ T cells (Fig. 5). Consequently combined IL-15 mutant/Fc γ2a plus CTLA4/Fc treatment exert additive immunosuppressive effects in controlling allograft rejection (Fig. 5 and Table I).

We now confirm and extend previous observations that costimulation blockade treatment reduces the frequency of proliferating alloreactive CD4+ T cells in an allograft model (34), but is unable to inhibit the proliferation of alloreactive CD8+ T cells in vivo (13). The resistance of CD8+ T cells to costimulation blockade is related to the inability of CTLA4/Fc (35, 36) and anti-CD154 mAb (36) treatments to control alloactivated CD8+ T cells and is probably related to the restricted expression of CD28 and CD154 to CD4+ T cells (37). To obtain prolonged engraftment of heart allografts, treatment with a nondepleting anti-CD4 mAb alone is not sufficient. The successful use of soluble IL-15 receptor α-chain proteins as an adjunct to anti-CD4 treatment (19) is consistent with our hypothesis that blocking both CD4+- and IL-15/IL-15R-dependent CD8+ T cell activation is required to gain long-term graft acceptance.

IL-15 mutant/Fc γ2a treatment mediates important effects on allograft survival that are exerted, at least in part, through the inhibition of the activation and proliferation of alloreactive CD8+ T cells (Figs. 3–5 and Table I). This finding emphasizes the role of the IL-15/IL-15R pathway as an important element in the rejection process. Moreover, we have recently noted that the initial five to six waves of the T cell proliferation to alloantigens in vivo are IL-15, not IL-2, dependent (38). The induction of permanent tolerance is still an elusive goal in clinical organ transplantation. By targeting IL-15/IL-15R+ cells to prevent costimulation blockade-resistant rejection, IL-15 mutant/Fc γ2a appears to provide a promising new agent capable of interrupting allograft rejection mediated by CD8+ T cells. The combined effects of costimulation blockade plus targeting the IL-15/IL-15R pathways to target both CD4+ and CD8+ T cells may represent an effective approach to achieve permanent tolerance.

Acknowledgments

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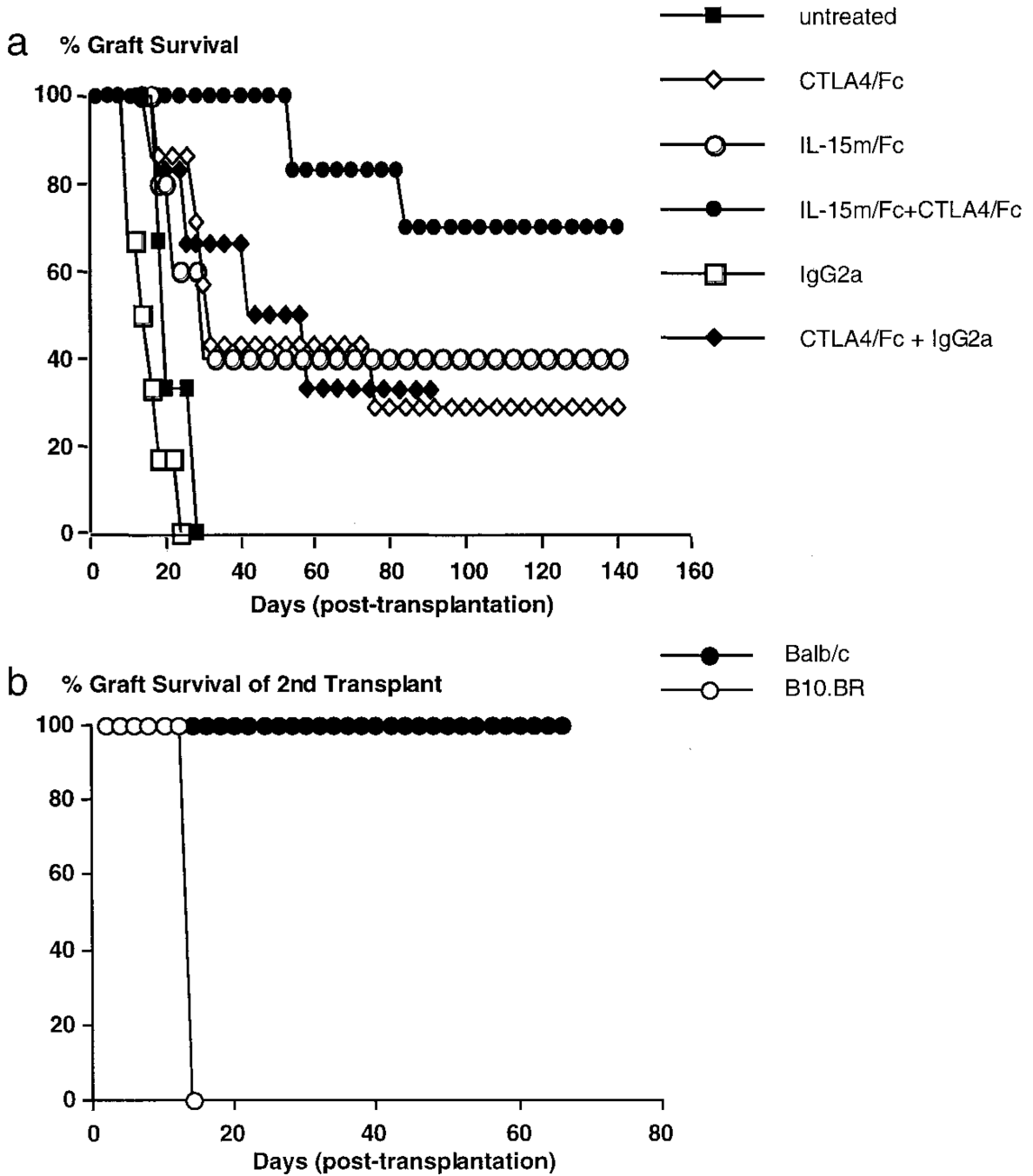
a % Graft Survival

untreated
CTLA4/Fc
IL-15m/Fc
IL-15m/Fc+CTLA4/Fc
IgG2a
CTLA4/Fc + IgG2a

b % Graft Survival of 2nd Transplant

Balb/c
B10.BR

FIGURE 1.
Survival of islet allografts with a combined treatment of CTLA4/Fc and IL-15 mutant/
Fc 2a. *a,* Islets from BALB/c (H-2^d) donors were transplanted under the renal capsule of
C57BL/6 (H-2^b) recipients. Recipients were treated with CTLA4/Fc (0.1 mg), IL-15 mutant/
Fc 2a (1.5 μg), or a combination of both proteins as indicated in *Materials and Methods*.
Untreated recipients ( ) or recipients treated with a control IgG2a protein (1.5 μg) rejected
allografts with a MST of 15 days and 14 days, respectively ( ). Graft rejection was delayed
by 30 days in recipients treated with CTLA4/Fc ( ), CTLA4/Fc plus IgG2a ( ), or IL-15 m/
Fc 2a ( ), whereas in recipients receiving the combined treatment, the MST was of 120
days ( ). *b,* Second islet transplantation. Nephrectomy of the left kidney was performed on
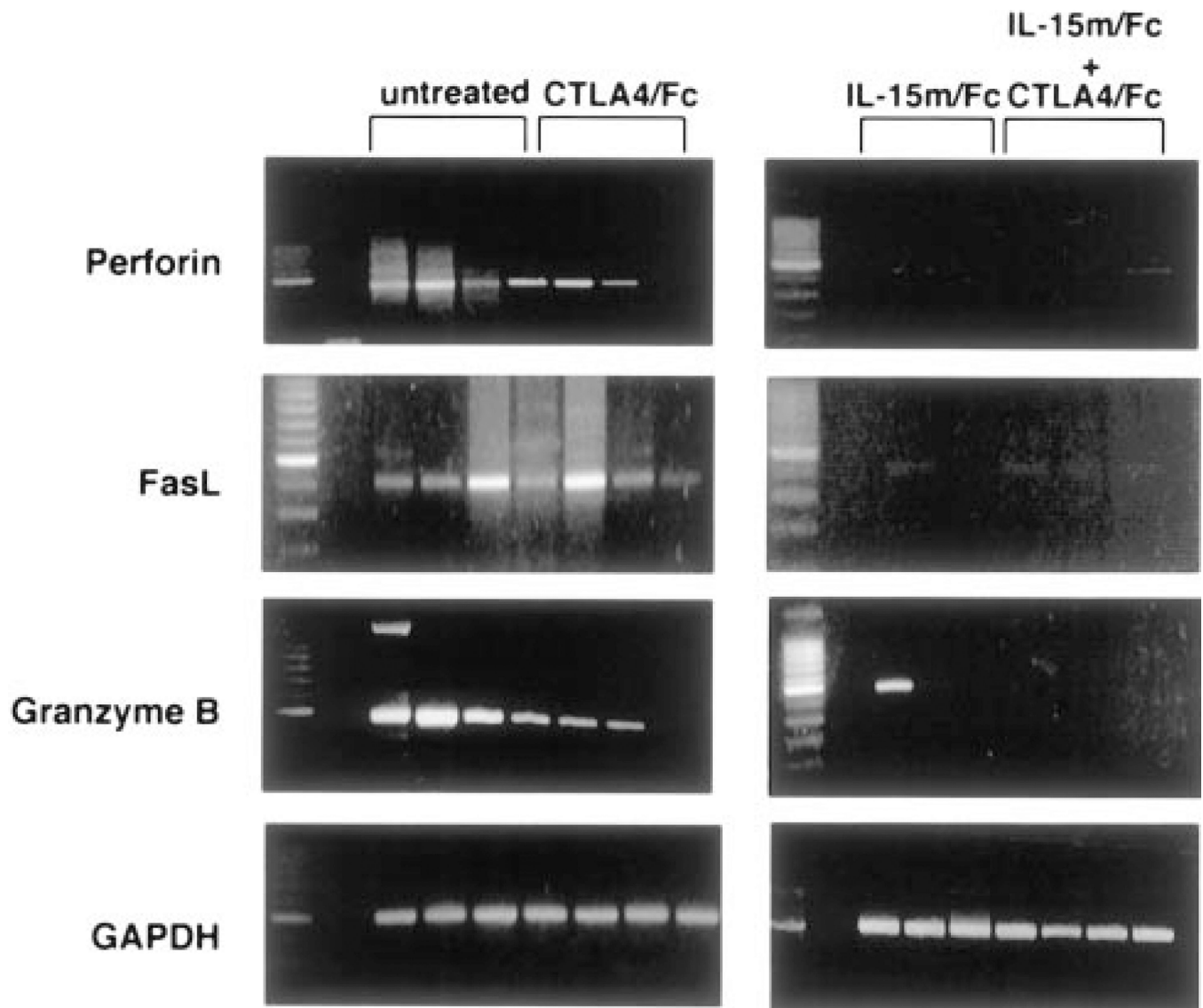
five recipients 150 days after transplantation. Then four nephrectomized mice received a second BALB/c islet allograft under the right renal capsule (following the same procedure) without further immunosuppression. All of them accepted the second transplant for more than 50 days, demonstrating a state of tolerance (   ). One mouse received a third part islet allograft (B10. BR, H-2^k;   ) which was rejected at day 12, confirming that the immune response is intact.
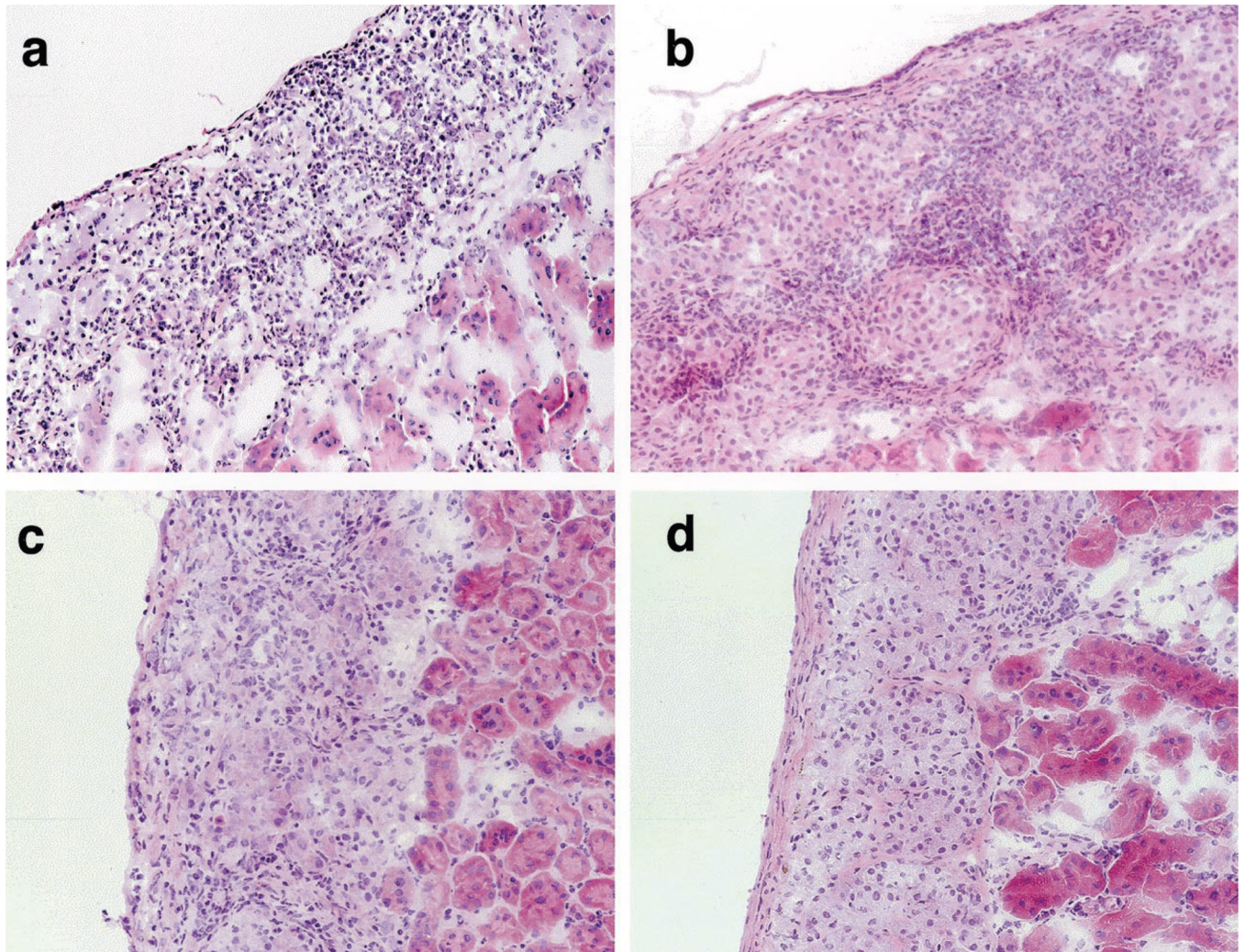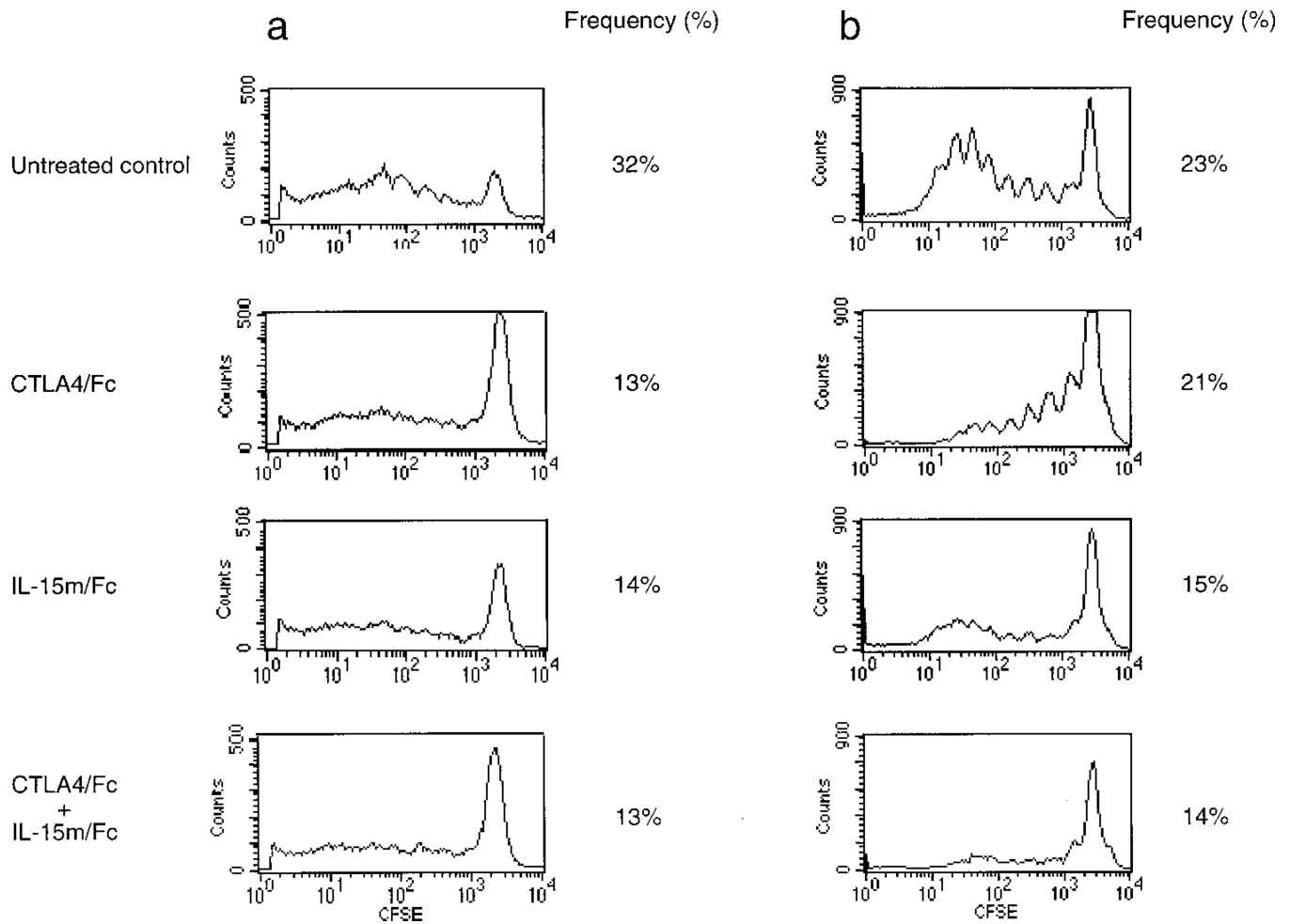
FIGURE 2.
Histology of serial sections of islet allografts isolated 8 days after islet allograft transplantation. A dense tissue infiltration by various mononuclear cells with destruction of islets is observed in untreated recipients (*a*). Recipients receiving CTLA4/Fc or IL-15 mutant/Fc 2a evidenced a decreased tissue infiltration by mononuclear leukocytes with some islets preserved (*b* and *c*, respectively). Graft sections from recipients treated with a combination of CTLA4/Fc and IL-15 mutant/Fc 2a reveal almost normal histology with minimal mononuclear cell infiltration and intact islets (*d*). Cryostat sections, hematoxylin counterstain; original magnification, ×100. These are representative sections from two allograft from each treatment group.
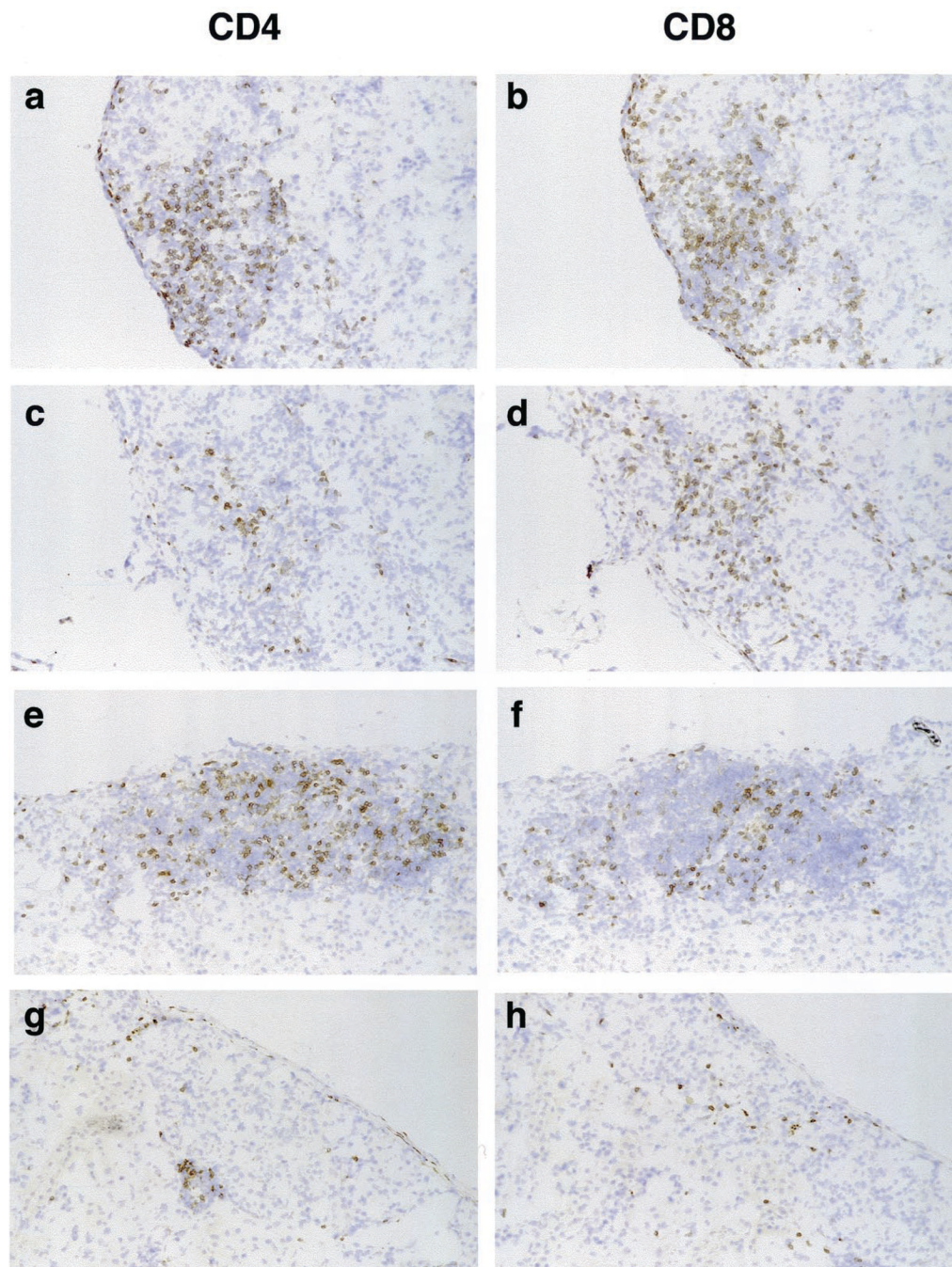
FIGURE 3.
Immunohistologic evaluation of islet allografts isolated 8 days after islet transplantation. Untreated recipients show dense tissue infiltration by mononuclear leukocytes surrounding and invading the islets with a predominance of CD4$^+$ and CD8$^+$ T cells (*a* and *b*). Recipients treated with CTLA4/Fc (*c* and *d*) or IL-15 mutant/Fc 2a (*e* and *f*) show a decreased mononuclear cell infiltrate with preservation of the tissue structure. Recipient mice receiving a combined treatment present rare CD4$^+$ and CD8$^+$ T cell infiltrates (*g* and *h*). Cryostat sections, hematoxylin counterstain; original magnification, ×100. These are representative sections from two recipients from each treatment group.

FIGURE 4.
Cytolytic gene transcripts were analyzed by RT-PCR. Tissues were isolated on day 8 after transplantation in untreated, CTLA4/Fc-treated, IL-15 mutant/Fc 2a-treated, or CTLA4/Fc plus IL-15 mutant/Fc 2a-treated recipients. Three allografts from each group were analyzed. Each lane represents an individual mouse, the first lane being the negative control. GAPDH primers were used as an internal control. Densitometric scanning quantification of gene expression for perforin, FasL, and granzyme B normalized to GAPDH was performed and the results are as follows: untreated mice: 0.733 ± 0.25, 0.362 ± 0.15, and 2.72 ± 0.57, respectively; CTLA4/Fc-treated mice: 0.98 ± 0.71, 0.56 ± 0.27, and 1.09 ± 0.24, respectively; IL-15 mutant/Fc 2a-treated mice: 0.094 ± 0.09, 0.03 ± 0.03, and 0.13 ± 0.15, respectively; CTLA4/Fc plus IL-15 mutant/Fc 2a-treated mice: 0.123 ± 0.15, 0.017 ± 0.02, and 0, respectively. Molecular weight markers are in the left part of the gel.

FIGURE 5.
Analysis of alloreactive CD4$^+$ and CD8$^+$ T cell proliferation in vivo. CFSE-labeled CD8$^{-/-}$ C57BL/6 (*a*) and CD4$^{-/-}$ C57BL/6 (*b*) lymphocytes were injected through the lateral vein into irradiated BALB/c recipient mice. Recipient mice were then untreated or treated with CTLA4/Fc (0.1 mg), IL-15 mutant/Fc 2a (1.5 μg) or a combination of both fusion proteins for 3 days. *a,* CFSE-labeled allogeneic CD4$^+$ T cells from the host spleen. *b,* CFSE-labeled allogeneic CD8$^+$ T cells from the host spleen. Data are represented as percentage of proliferation of CFSE-labeled CD4$^+$ and CD8$^+$ T cells. Data are representative of four individual experiments.

Table I

Survival of DBA/2 islet allografts in B6AF1 recipients treated with CTLA4/Fc and IL-15 mutant/Fc

Donor	Recipient	Treatment[a]	Islet Graft Survival (days)
DBA/2	B6AF$_1$	Untreated	11, 11, 11, 12, 13, 13, 15, 17, 17, 20
DBA/2	B6AF$_1$	CTLA4/Fc	20, 31, 34, 51, >120, >120, >120, >120
DBA/2	B6AF$_1$	IL-15m/Fc	15, 22, 29, 32, >120, >120, >120, >120
DBA/2	B6AF$_1$	CTLA4/Fc + IL-15m/Fc	>120, >120, >120, >120

[a]Treatment: CTLA4/Fc, 0.1 mg/day, i.p. from days 0 to 11 posttransplantation; IL-15m/Fc, 1.5 μg/day, i.p. from days 0 to 21 posttransplantation.