

IL-7 receptor blockade following T cell depletion promotes long-term allograft survival

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T cell depletion is commonly used in organ transplantation for immunosuppression; however, a restoration of T cell homeostasis following depletion leads to increased memory T cells, which may promote transplant rejection. The cytokine IL-7 is important for controlling lymphopoiesis under both normal and lymphopenic conditions. Here, we investigated whether blocking IL-7 signaling with a mAb that targets IL-7 receptor α (IL-7R α) alone or following T cell depletion confers an advantage for allograft survival in murine transplant models. We found that IL-7R blockade alone induced indefinite pancreatic islet allograft survival if anti-IL-7R treatment was started 3 weeks before graft. IL-7R blockade following anti-CD4- and anti-CD8-mediated T cell depletion markedly prolonged skin allograft survival. Furthermore, IL-7 inhibition in combination with T cell depletion synergized with either CTLA-4Ig administration or suboptimal doses of tacrolimus to induce long-term skin graft acceptance in this stringent transplant model. Together, these therapies inhibited T cell reconstitution, decreased memory T cell numbers, increased the relative frequency of Tregs, and abrogated both cellular and humoral alloimmune responses. Our data suggest that IL-7R blockade following T cell depletion has potential as a robust, immunosuppressive therapy in transplantation.

Introduction

T cell depletion by antibodies is one of the most potent immunosuppressive therapies and is increasingly used as an induction therapy in organ transplantation (1). However, T cell homeostasis after depletion therapy leads to a predominance of memory T cells (1–3), which are more potent than naive T cells in mediating graft rejection and present as a major obstacle to achieving tolerance. Mice undergoing T cell homeostatic proliferation following depletion therapy rejected cardiac allograft despite costimulatory blockade by CTLA-4Ig, a treatment capable of inducing tolerance in nondepleted mice (4, 5). In human, kidney transplant patients who had received T cell depletion therapy by high-dose alemtuzumab, but no maintenance immunosuppression, uniformly developed acute rejection within the first month after transplantation (6), a period during which there was still a severe T cell lymphopenia but most of the remaining T cells were effector memory T cells (7).

T cell reconstitution after depletion therapy comprises de novo thymopoiesis and homeostatic proliferation of remaining peripheral T cells, and both processes are IL-7 dependent (8, 9). IL-7 signals through the IL-7 receptor (IL-7R) which is composed of 2 chains, the common γ chain and the α chain (IL-7R α or CD127) (10). IL-7 plays an essential, nonredundant role in lymphopoiesis, since IL-7 or IL-7R α knockout mice have severe T and B cell lymphopenia (11, 12) and infants with IL-7R α mutations have severe T cell lymphopenia necessitating bone marrow transplantation (13). IL-7 has also been shown to be necessary for the homeostatic

proliferation of both naive and memory CD4⁺ and CD8⁺ T cells in lymphopenic conditions (14–18). Therefore, in the setting of organ transplantation, the blockade of IL-7/IL-7R signaling is expected to prolong the effects of T cell depletion therapy, reduce the number of memory T cells, and increase immunoregulation, leading to better graft acceptance (19).

In this study, we investigated the role of IL-7R blockade by an anti-IL-7R α mAb, first given alone in an islet allograft model and then given after T cell depletion by a combination of anti-CD4 and anti-CD8 mAbs in a more stringent skin allograft model. We also elucidated the mechanisms underlying the therapeutic efficacy of IL-7R blockade in transplantation.

Results

IL-7R blockade reduces almost all lymphocyte subset numbers and increases Treg frequency. The anti-IL-7R α mAb (A7R34) used in our study was previously shown to block IL-7R and reduce lymphocyte numbers when given at 2 mg every other day (qod) for 2 weeks (20). In this study, we tested a lower dose of A7R34 and were able to produce similar effects. Naive BALB/c mice were injected with either PBS or A7R34 400 μ g qod for 3 weeks and sacrificed. Anti-IL-7R α -treated mice had significantly lower numbers of total lymphocytes, T cells, CD4⁺ T cells, CD8⁺ T cells, and B cells in the LNs, spleen, and peripheral blood and drastically reduced numbers of thymocytes in the thymus compared with control mice (Supplemental Figure 1, A–D; supplemental material available online with this article; doi:10.1172/JCI66287DS1). Interestingly, we found a significant increase in the percentage of CD4⁺ T cells expressing programmed death 1 (PD-1) and an increase in the percentage of CD4⁺CD25⁺FOXP3⁺ Tregs in the LNs and spleens of treated mice compared with those of control mice (Supplemental Figure 1, A and B), in concordance with recent publications (21, 22). On

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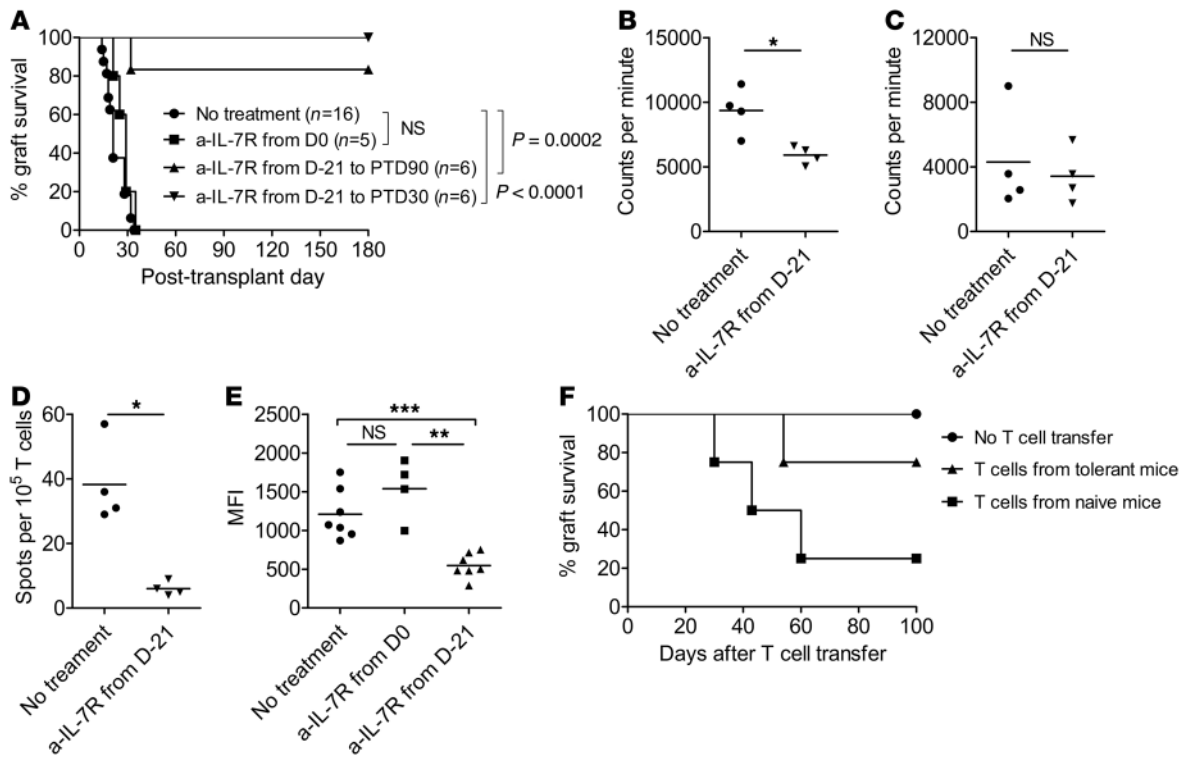


Figure 1

IL-7R blockade started 3 weeks before graft induces islet allograft tolerance and abrogates both cellular and humoral alloimmune responses. (A–E) BALB/c mice previously rendered diabetic by STZ received C57BL/6 islet graft and were treated as indicated. (A) Anti-IL-7R α mAb given from D–21 to either PTD90 or PTD30 induced indefinite graft survival in most or all of mice. (B and C) MLR with 3H-thymidine incorporation showed that the treatment of islet graft recipients with anti-IL-7R from D–21 significantly reduced T cell proliferation in response to stimulation by donor splenocytes (B), but did not alter T cell responses to third party splenocytes (C). (D) IFN- γ ELISPOT showed that mice treated with anti-IL-7R from D–21 had significantly fewer IFN- γ -secreting T cells in response to stimulation by donor splenocytes. (E) Mice treated with anti-IL-7R from D–21 had lower levels of DSAs. (F) BALB/c nude mice underwent islet grafts from C57BL/6 donors and then received 5×10^6 of either T cells from BALB/c mice that had been treated with anti-IL-7R from D–21 and become tolerant to C57BL/6 islet grafts or T cells from naive BALB/c mice ($n = 4$ for each groups). T cells from tolerant mice were less effective in inducing islet graft rejection than those from naive mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

the other hand, we found that mice sacrificed 4 days after having received A7R34 at a dose as high as 2 mg/d had not yet exhibited significant reduction in lymphocyte numbers (data not shown), indicating that A7R34 does not have important lytic activity, also in agreement with studies from other groups (21, 23).

IL-7R blockade alone started 3 weeks before graft induces islet allograft tolerance and abrogates both cellular and humoral alloimmune responses. We next investigated the effects of IL-7R blockade in a fully mismatched islet allograft model in which C57BL/6 islets were transplanted into BALB/c mice previously rendered diabetic by streptozotocin (STZ). Anti-IL-7R α mAb given at 400 μ g qod from the day of graft (D0) until rejection did not significantly prolong graft median survival time (MST) compared with no treatment (29 vs. 21 days; $P = 0.16$). Being guided by the aforementioned experiment in naive mice, we gave anti-IL-7R α mAb from 3 weeks before grafts (D–21) to posttransplant day 90 (PTD90) and obtained indefinite graft survival of more than 180 days in 5 of 6 treated mice ($P = 0.0002$ compared with no treatment). A shorter period of treatment from D–21 to PTD30 was also sufficient to induce graft tolerance in all treated mice ($P < 0.0001$ compared with no treatment) (Figure 1A). Subsequent left nephrec-

tomy to remove the islet grafts in tolerant mice led to a prompt increase in blood glucose (data not shown), confirming that the maintenance of normoglycemia was due to graft function.

Untreated and some treated mice that received anti-IL-7R from D–21 to PTD30 were sacrificed a few days after graft rejection and on PTD70, respectively, for immunological analyses. Using mixed lymphocyte reaction (MLR) with 3H-thymidine incorporation, we showed that the treatment of islet graft recipients with anti-IL-7R α mAb specifically reduced the T cell responses to donor APCs (Figure 1B), but did not alter the T cell responses to third party APCs (Figure 1C), indicating that the tolerance induced by IL-7R blockade is donor specific. Moreover, using IFN- γ ELISPOT, we demonstrated that IL-7R blockade abrogated the formation and activation of donor-specific memory T cells, as evidenced by a significantly lower number of IFN- γ -secreting T cells in response to donor APCs (Figure 1D). Of note, IFN- γ ELISPOT was negative when third-party APCs were used as stimulators, whether T cells from untreated or treated mice were used as responders (data not shown). Tolerant mice also had reduced humoral alloimmune responses as evidenced by significantly lower levels of donor-specific antibodies (DSA) (Figure 1E).

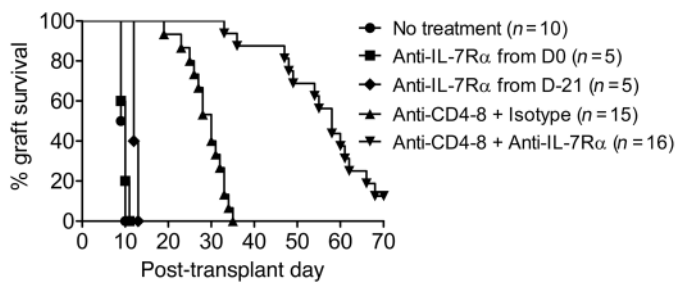


Figure 2

IL-7R blockade following T cell depletion strongly prolongs skin allograft survival. BALB/c mice received skin graft from C57BL/6 donors and were treated as indicated. T cell depletion by a combination of anti-CD4 and anti-CD8 mAbs prolonged graft survival compared with no treatment (30 vs. 9.5 days; $P < 0.0001$). The addition of IL-7R blockade to T cell depletion doubled graft MST compared with T cell depletion alone (58 vs. 30 days; $P < 0.0001$).

Lymphocyte phenotyping performed on PTD180 showed that lymphocyte reconstitution had occurred in tolerant mice (Supplemental Figure 2). In order to test whether tolerance can be transmissible, T cells purified from the spleen of tolerant mice sacrificed on PTD180 were adoptively transferred to T cell-deficient BALB/c nude mice that had received islet grafts from C57BL/6 donors. Without T cell transfer, BALB/c nude mice accepted islet allograft indefinitely. The transfer of T cells from naive wild-type BALB/c to BALB/c nude mice that had previously received C57BL/6 islet graft 2 weeks earlier led to graft rejection in 3 of 4 mice, whereas the same experiment using T cells from tolerant mice only led to graft rejection in 1 of 4 mice. However, the small sample size ($n = 4$ for each group) precluded statistical analysis (Figure 1F).

IL-7R blockade following T cell depletion strongly prolongs skin allograft survival. We next tested the effects of IL-7R blockade in a much more stringent model of fully allogeneic skin allograft from C57BL/6 donors to BALB/c recipients. Anti-IL-7R α mAb given alone either from D0 or D-21 until rejection did not result in a meaningful prolongation of graft MST compared with no treatment (10 vs. 9.5 days; $P = 0.37$ and 12 vs. 9.5 days; $P = 0.0006$, respectively) (Figure 2). Because IL-7R blockade is expected to inhibit T cell reconstitution after T cell depletion therapy, we then tested whether the addition of an anti-IL-7R α mAb to a T cell depletion protocol could prolong skin graft survival. We treated recipient mice first with a combination of 2 depleting mAbs, anti-CD4 and anti-CD8 in order to rapidly deplete T cells, followed by either isotype control or anti-IL-7R α mAb 400 μ g qod from PTD1 until rejection. Mice having received anti-CD4 and anti-CD8 followed by isotype control (referred to as “depletion alone”) had graft MST of 30 days (range: 19–35 days) ($P < 0.0001$ compared with untreated mice). Mice having received anti-CD4 and anti-CD8 followed by anti-IL-7R α mAb (referred to as “depletion plus anti-IL-7R”) had MST of 58 days (range: 36 to >90 days), which were significantly longer than that of the “depletion alone” group ($P < 0.0001$) (Figure 2).

IL-7R blockade following T cell depletion delays T cell reconstitution and favors immuno regulation. First of all, we compared the total T cell and T cell subset numbers among 4 groups of skin graft recipients having received no treatment, anti-IL-7R alone from D0, anti-IL-7R alone from D-21, and T cell depletion by anti-CD4 and anti-CD8, followed by anti-IL-7R and found that a profound reduction of T cells, including CD4⁺FOXP3⁻ effector T cells such as achieved with the last regimen was necessary for the prolongation of skin graft survival (Supplemental Figure 3).

We next focused our analyses on the 2 groups of mice treated by depletion alone and depletion plus anti-IL-7R and compared the lymphocyte subset numbers and frequencies between these 2 groups on PTD35. At this time point, mice that received deple-

tion alone had rejected their grafts, whereas grafts were well preserved in mice under the cover of depletion plus anti-IL-7R. We found that IL-7R blockade following T cell depletion profoundly inhibited T cell reconstitution. Mice with depletion plus anti-IL-7R had a 10- to 20-fold reduction in the number of total T cells, CD4⁺ and CD8⁺ T cells in the LNs, spleen, and peripheral blood compared with mice with depletion alone (all $P < 0.05$) (Figure 3, A and B, and Supplemental Figure 4, A and B). B cell numbers were also reduced but to a lesser extent. The effect of IL-7R blockade on the thymus was even more pronounced (Supplemental Figure 4C). The fact that anti-CD4 and anti-CD8 mAbs did not deplete B cells (Supplemental Figure 4D) whereas IL-7R blockade in mice reduced both T and B cell numbers explains why B cell number was reduced in the group with depletion plus anti-IL-7R compared with depletion alone.

Despite a decrease in their absolute numbers, the relative frequencies of CD4⁺FOXP3⁺ Tregs were increased in the depletion plus anti-IL-7R group compared with the depletion alone group (22.3% \pm 7.7% vs. 10.5% \pm 0.9% and 27.4% \pm 3.5% vs. 14.4% \pm 0.8% of CD4⁺ T cells in the LNs and spleen, respectively; both $P < 0.05$). As a result, the Treg/T effector ratio defined by CD3⁺CD4⁺FOXP3⁺/CD3⁺CD4⁺FOXP3⁻ was increased (0.30 \pm 0.13 vs. 0.12 \pm 0.02 and 0.38 \pm 0.07 vs. 0.17 \pm 0.01 in the LNs and spleen, respectively; both $P < 0.05$). Of note, CD4⁺FOXP3⁺ Tregs comprised CD4⁺CD25⁺FOXP3⁺ and CD4⁺CD25⁻FOXP3⁺ T cells; the percentage of both populations was increased by IL-7R blockade (Figure 3A). Similarly, the percentage of memory T cells defined as CD3⁺CD62L^{lo}CD44^{hi} among total T cells was increased in the depletion plus anti-IL-7R group; however, because of the severe overall reduction in T cell number, the absolute number of memory T cells in this group was decreased by 8-fold in the LNs (0.33 \pm 0.18 vs. 2.46 \pm 0.62 $\times 10^5$ cells; $P < 0.05$) and by 4-fold in the spleen (2.5 \pm 0.3 vs. 9.5 \pm 2.0 $\times 10^5$ cells; $P < 0.05$) compared with the depletion alone group. Interestingly, mice treated with depletion plus IL-7R blockade exhibited a 3- to 6-fold increase in the frequency of CD4⁺ T cells expressing PD-1 compared with mice treated with depletion alone (35.0% \pm 3.4% vs. 6.0% \pm 1.0% and 29.0% \pm 3.6% vs. 10.2% \pm 1.2% of CD4⁺ T cells in the LNs and in the spleen, respectively; both $P < 0.05$).

Given the striking increase in the expression of PD-1 and the increased Treg/T effector ratio, we further investigated the roles of these 2 immunoregulatory mechanisms. We performed additional skin grafts using the depletion plus anti-IL-7R treatment protocol. From PTD7, we treated recipients with either an anti-PD-L1 mAb (10F9G2) given at 200 μ g twice per week for 2 weeks to block the PD-1/PD-L1 pathway (24) or an anti-CD25 mAb (PC61) given at 500 μ g qod for a total of 4 doses to deplete Tregs (25). PD-1/PD-L1 blockade has been shown to abrogate the protective effects of

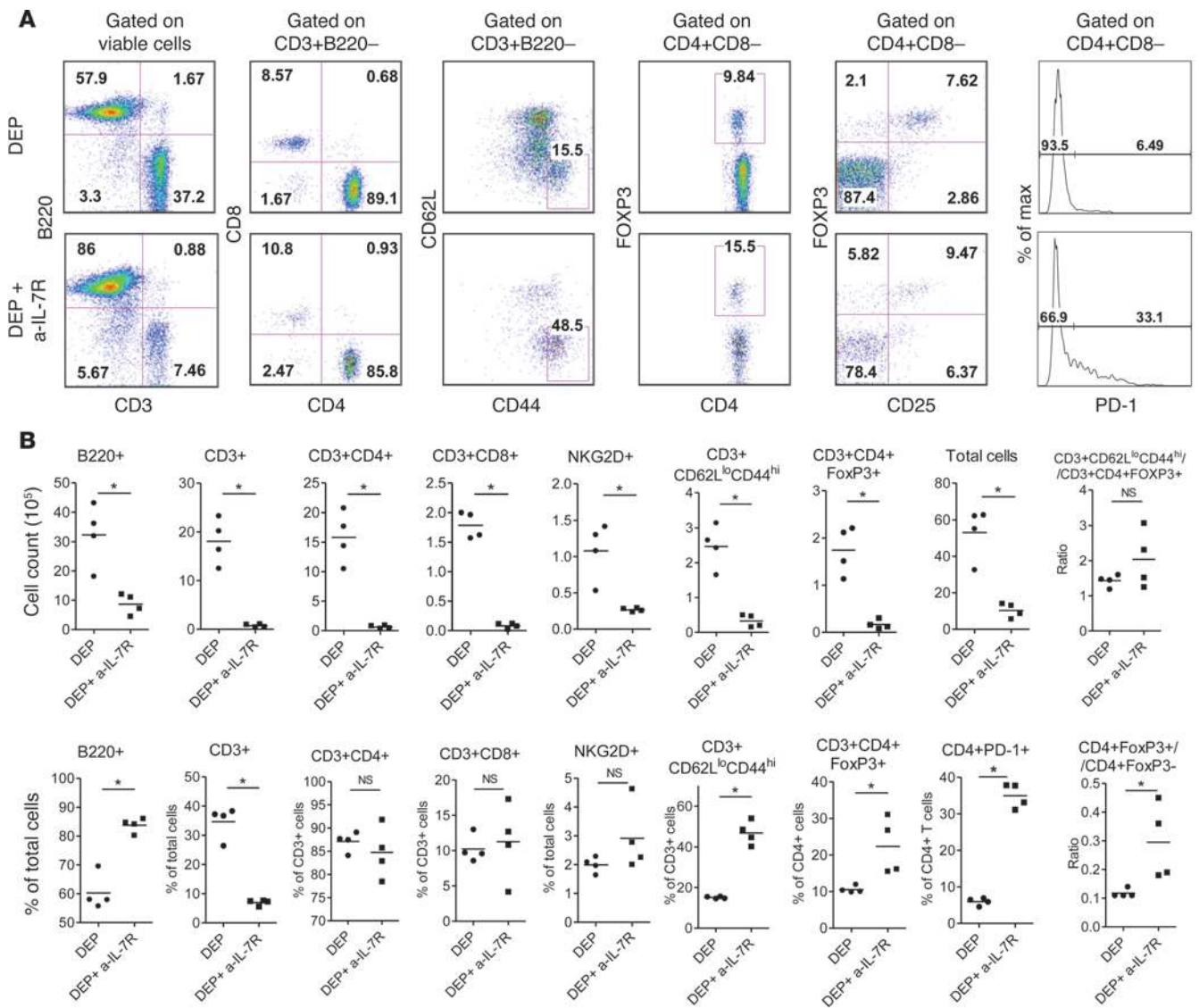


Figure 3 IL-7R blockade following T cell depletion inhibits lymphocyte reconstitution and favors immunoregulation. C57BL/6 skin was transplanted to BALB/c recipients, which were treated with T cell depletion by a combination of anti-CD4 and anti-CD8 mAbs, followed by either isotype Ig (DEP) or anti-IL-7Rα mAb (DEP + a-IL-7R) and were then sacrificed at PTD35 for lymphocyte phenotyping. (A) Representative FACS plots of the LNs in which 20,000 events in the viable cell gate were acquired for each sample. Shown are the percentages of lymphocyte subsets. (B) The absolute numbers and the percentages of different lymphocyte subpopulations in the LNs are compared between the 2 groups. The relative frequency of CD4⁺FoxP3⁺ Tregs was increased in the DEP + a-IL-7R group despite a decrease in their absolute number. See also Supplemental Figure 3 for the spleen, peripheral blood, and thymus. *P < 0.05.

anti-IL-7R treatment in an autoimmune diabetes model (21, 22) and to accelerate rejection in several transplant models (26). Surprisingly, the addition of anti-PD-L1 to our treatment protocol did not alter graft survival (Figure 4), suggesting that this pathway is not crucial for the prolongation of skin graft survival by IL-7R blockade. An alternative explanation is that, under the cover of IL-7R blockade following T cell depletion, a treatment with multiple mechanisms of action, a contributive role of the PD-1 signaling pathway may become less visible. On the other hand, Treg depletion shortened skin graft MST from 58 days to 48 days (Figure 4), although the difference only had a borderline statistical significance (P = 0.06)

perhaps due to small sample size (n = 5). This trend suggests that the increase in Treg relative frequency may be one of the underlying mechanisms of IL-7R blockade that favors skin graft survival.

IL-7R blockade following T cell depletion abrogates both cellular and humoral alloimmune responses. IFN-γ ELISPOT revealed significantly fewer donor-specific IFN-γ-secreting T cells in skin graft recipients treated with depletion plus anti-IL-7R compared with those treated with depletion alone (29 ± 26 vs. 203 ± 76 spots/10⁵ T cells; P < 0.0005) (Figure 5A). Similarly, MLR with 3H-thymidine incorporation showed a very weak donor-specific T cell response in mice treated with depletion plus anti-IL-7R, whereas there was



a strong T cell proliferation in mice treated with depletion only (245 ± 55 vs. 11410 ± 6456 counts per minute; $P < 0.0001$) (Figure 5B). Of note, unlike in the islet graft model, T cell proliferation in response to third party splenocytes was also inhibited in mice treated with depletion plus anti-IL-7R (data not shown). Cytokine measurement in the MLR supernatant by ELISA also showed a complete abrogation of IL-2, TNF, and IL-17 secretion by T cells from mice with depletion plus anti-IL-7R compared with mice with depletion only (8.0 ± 4.4 vs. 79.8 ± 38.1 , 16.8 ± 8.3 vs. 140.1 ± 25.5 , and 2.5 ± 2.2 vs. 62.0 ± 33.8 pg/ml, respectively; all $P < 0.01$) (Figure 5C). Together, these data show that T cells that have escaped depletion may become anergic to donor APCs and suggest that the effects of IL-7R blockade are not restricted to depletion. The humoral alloimmune response was also abrogated, as evidenced by a significant reduction in DSA levels in mice treated with depletion plus anti-IL-7R compared with mice treated with depletion only (616 ± 311 vs. 4101 ± 1877 ; $P < 0.0001$) (Figure 5D).

Because IL-7R blockade following T cell depletion leads to a decrease in memory T cell numbers and an inhibition of alloimmune responses, we wonder whether this treatment is effective in alloimmunized mice and whether it would also affect the antiviral immune responses acquired through previous infections or vaccinations. To address the first question, we performed skin grafts from C57BL/6 donors to BALB/c recipients without treatment; as expected, all grafts were rejected within 10 days. One month after the first graft, recipients were rechallenged with a second C57BL/6 skin graft and divided into 3 groups: depletion plus anti-IL-7R, depletion alone, and no treatment. Although we also observed a statistically significant prolongation of graft survival in the group treated by depletion plus anti-IL-7R compared with depletion alone or no treatment (MST = 15, 11, and 6 days, respectively; $P < 0.05$) (Supplemental Figure 5), graft survival was far shorter than that obtained in nonpreimmunized recipients (Figure 2), suggesting that IL-7R blockade following T cell depletion is less effective when recipients have been primed by a first graft.

To address the second question, we used a model of viral infection in which naive BALB/c mice received 2 i.m. injections of 10^{10} infectious particles of an adenovirus vector serotype 5 (AdV5) at 4 weeks apart. Two weeks after the second injection, mice received PBS for 5 weeks, anti-IL-7R α mAb for 5 weeks, or T cell depletion by 2 injections of anti-CD4 and anti-CD8 mAbs followed by anti-IL-7R α mAb for 7 weeks and then were sacrificed for analyses. ELISA showed no difference in anti-adenovirus antibody titers among the 3 groups of mice (Supplemental Figure 6A). IFN- γ ELISPOT also revealed no difference in the frequency of antiadenoviral memory T cells between the control group and the group that received anti-IL-7R alone for 5 weeks (Supplemental Figure 6B), suggesting that a short course of IL-7R blockade by itself does not impair previously acquired antiviral immunity. However, T cell depletion by anti-CD4 and anti-CD8 followed by anti-IL-7R for 7 weeks led to a decrease in the frequency of antiadenoviral memory T cells, as expected in the case of a strong immunosuppressive regimen. Therefore, IL-7R blockade following T cell depletion is likely to be more suitable for an induction rather than a long-term maintenance therapy in transplantation.

T cell targeting is critical for the efficacy of anti-IL-7R therapy in the prolongation of skin graft survival. Our aforementioned data demonstrated that IL-7R blockade exerts its effects through targeting T cells; however, at least in mice, B cells are also affected by IL-7R blockade. Therefore, we further clarify the role B cells in our treat-

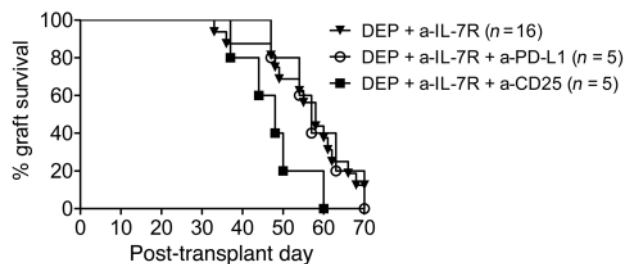


Figure 4

Treg depletion decreases skin graft survival. C57BL/6 skin was transplanted to BALB/c recipients, which were treated with T cell depletion by a combination of anti-CD4 and anti-CD8 mAbs, followed by anti-IL-7R α mAb (DEP + a-IL-7R). Some recipients were also treated with either an anti-PD-L1 mAb to block the PD-1/PD-L1 signaling pathway or an anti-CD25 mAb to deplete Tregs. As also shown in Figure 2, treatment with DEP + a-IL-7R resulted in graft MST of 58 days; the addition of anti-PD-L1 did not alter graft survival (MST = 57 days; $P = 0.89$ compared with DEP + a-IL-7R), whereas the addition of anti-CD25 shortened graft MST to 48 days ($P = 0.06$ compared with DEP + a-IL-7R).

ment protocols. We performed skin grafts from BALB/c donors to B cell-deficient muMT mice and treated recipients with the same treatment protocols used in wild-type mice to investigate whether the absence of B cells affects graft survival. In agreement with a previous report (27), untreated muMT mice rejected BALB/c skin graft as rapidly as wild-type C57BL/6 mice did (MST = 9.5 days in both cases). Anti-IL-7R α mAb given alone from D0 failed to prolong graft survival (MST = 9.5 days). On the other hand, T cell depletion by anti-CD4 and anti-CD8 mAbs prolonged graft survival to 33 days ($P = 0.002$ compared with no treatment), and the addition of anti-IL-7R α mAb to T cell depletion further prolonged skin graft MST to 52 days ($P = 0.0007$ compared with depletion alone) (Figure 6A). Indeed, under each treatment protocol, graft survival obtained in muMT mice was comparable to that obtained in wild-type mice (Figure 2), indicating that the absence of B cells does not affect the protective effects of IL-7R blockade on graft survival. Interestingly, despite the lack of B cell antigen presentation, muMT mice were able to develop a strong donor-specific memory T cell response as shown by IFN- γ ELISPOT (Figure 6B). As in wild-type mice (Figure 5A), T cell responses in muMT mice were also abrogated by the treatment with IL-7R blockade following T cell depletion (Figure 6B).

Because IL-7R blockade following T cell depletion reduced DSA levels, we wondered whether that effect is also due to the decrease in B cell number besides the diminution of T cell help. Therefore, we gave BALB/c mice an anti-mouse CD20 mAb (5D2) at 100 μ g on D-3 and D-1 to deplete B cells (Figure 6C) and then performed skin grafts from C57BL/6 donors. Although B cell depletion by anti-CD20 did not prolong graft survival (MST = 10 days), it significantly reduced DSA levels compared with those of untreated mice (Figure 6D). Taken together, our data show that the effects of IL-7R blockade on T cells are essential for the prolongation of skin allograft survival, but the decrease in B cell number associated with IL-7R blockade may contribute to the reduction in DSA.

IL-7R blockade following T cell depletion abrogates leukocyte infiltration and decreases proinflammatory gene expression in the skin graft. On PTD21, the skin grafts from mice with depletion alone were characterized by a moderate to dense leukocyte infiltrate, whereas

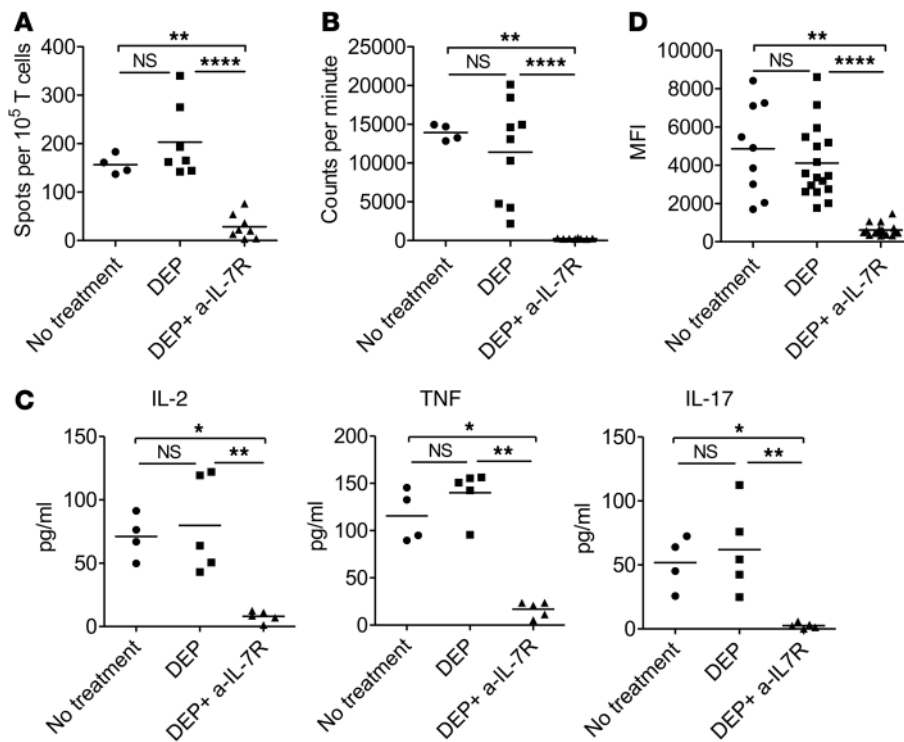


Figure 5

IL-7R blockade following T cell depletion abrogates both cellular and humoral alloimmune responses. C57BL/6 skin was transplanted to BALB/c recipients, which were untreated or treated with T cell depletion by a combination of anti-CD4 and anti-CD8 mAbs followed by either isotype Ig (DEP) or anti-IL-7R α mAb (DEP + a-IL-7R). The donor-specific alloimmune responses of these 3 groups were quantified using IFN- γ ELISPOT (A), MLR with ³H thymidine incorporation (B), cytokine measurement in the MLR supernatants by ELISA (C), and DSA measurement in the sera (D). **P* < 0.05; ***P* < 0.01; *****P* < 0.0005.

there was little inflammatory infiltration in the skin grafts from mice with depletion plus anti-IL-7R (Figure 7A). Concordantly, gene expression measurement using TaqMan Low Density Array (TLDA) revealed several-fold decreases in the expression of proinflammatory genes in the skin grafts of mice treated with depletion plus anti-IL-7R compared with those of mice treated with depletion alone. Among these genes were genes coding Th1 cytokine (IFN- γ and TNF), Th2 cytokines (IL-4, IL-5, and IL-13), chemokines (CCL-2, CCL-3, CCL-5, CXCL-10, and CXCL-11), costimulatory molecules (CD40, CD86, and ICOS), granzyme B, and perforin (Figure 7B and Table 1).

By PTD70, however, most skin grafts under treatment by depletion plus anti-IL-7R had also been rejected. Lymphocyte phenotyping at the time of graft rejection revealed that all lymphocyte subset numbers in all lymphoid organs were still very low, comparable to those observed earlier on PTD35 (Supplemental Figure 7). The skin is well known for its immunogenicity (reviewed in ref. 28) and contains abundant Langerhans cells, whose development is not IL-7 dependent (29). Therefore, skin grafts may be a preferential site for T cell homing, explaining why they are still rejected despite the persistence of lymphopenia.

We next performed immunohistology of skin grafts of mice treated by depletion plus anti-IL-7R at the time of rejection to see which types of cells participate in the rejection process. For comparison, we also did immunohistological staining of accepted skin grafts under treatment by depletion plus anti-IL-7R on PTD35 as well as rejecting skin grafts of untreated control mice. As shown in Supplemental Figure 8, accepted skin grafts of treated mice on PTD35 had little inflammatory infiltration. When rejection occurred, skin grafts of treated mice were characterized by an influx of leukocytes including macrophages, granulocytes, and T cells. However, the leukocyte infiltration in the rejecting skin grafts of treated mice was still less intense than that in untreated

mice. We also quantified the expression of inflammatory genes in rejecting and accepted skin grafts and found a similar tendency. In mice treated by IL-7R blockade following T cell depletion, skin grafts undergoing rejection displayed an increase in the expression of inflammatory gene transcripts compared with accepted skin grafts on PTD35 (Supplemental Figure 9A). However, the levels of inflammatory gene expression in rejecting skin grafts of treated mice were still significantly lower than those of untreated control mice (Supplemental Figure 9B).

IL-7R blockade synergizes with treatment protocols combining T cell depletion with either CTLA-4Ig or low-dose tacrolimus. In order to further prolong skin graft survival, we added either CTLA-4Ig (abatacept) or low-dose tacrolimus to the treatment protocols. CTLA-4Ig alone or low-dose tacrolimus alone only slightly prolonged graft MST (11 days for either treatment compared with 9.5 days for no treatment; *P* < 0.01). CTLA-4Ig combined with T cell depletion by anti-CD4 and anti-CD8 prolonged MST to 28.5 days, and the addition of anti-IL-7R α mAb to this treatment protocol further increased MST to 52.5 days (*P* < 0.05) (Figure 8A).

The synergistic effect was more pronounced when low-dose tacrolimus was used instead of CTLA4-Ig. Two-thirds of mice treated with a combination of T cell depletion, low-dose tacrolimus from the day of graft, and anti-IL-7R α mAb from PTD1 to PTD70 had skin graft survival of at least 90 days, whereas mice treated with the same protocol but without IL-7R blockade had MST of only 32.5 days (*P* < 0.001) (Figure 8B). We next investigated whether we could shorten the duration of anti-IL-7R treatment to facilitate immune reconstitution. At the same time, we also started low-dose tacrolimus from PTD12 instead of D0 to reduce immunosuppression in the early phase of lymphodepletion. We found that 80% of mice treated with IL-7R blockade following T cell depletion and low-dose tacrolimus from PTD12 had graft survival of at least 90 days whether anti-IL-7R α mAb was stopped on



PTD70 or PTD35, indicating that a short course of anti-IL-7R α mAb is sufficient to prolong graft survival in this drug combination. On the contrary, mice treated with T cell depletion and low-dose tacrolimus from PTD12 but without anti-IL-7R α mAb had skin graft MST of only 33 days ($P < 0.005$) (Figure 8B). Importantly, lymphocyte phenotyping on PTD90 showed that the shorter course of anti-IL-7R treatment was associated with a better recovery of thymic function and a higher number of T and B cells in the LNs (Figure 8C), thereby limiting the potential overimmunosuppression associated with prolonged lymphopenia.

Discussion

Inhibition of IL-7 or IL-7R by specific blocking mAbs has been successfully tested in models of autoimmune diseases (21, 22, 30–32) and graft-versus-host disease following bone marrow transplantation (33). In the context of organ transplantation, there is a single report in which an anti-mouse IL-7 polyclonal Ab had no effect when given alone, but prolonged heart graft survival when combined with CD40/CD40L costimulatory blockade (34).

Using an islet allograft model, we demonstrated that tolerance could be achieved when anti-IL-7R α mAb was given alone from 3 weeks before graft (Figure 1A). Because this blocking mAb does not have a rapid lytic effect, it must take a few weeks to obtain a significant reduction in lymphocyte numbers (Supplemental Figure 1). However, once established, tolerance persisted despite lymphocyte reconstitution after the end of anti-IL-7R treatment (Supplemental Figure 2) and tolerant mice developed donor-specific hyporesponsiveness (Figure 1, B–D). Furthermore, T cells from tolerant mice did not induce islet graft rejection in T cell-deficient nude mice as effectively as T cells from naive mice (Figure 1F). Taken together, these data indicate that immunoregulation was established after a short course of IL-7R blockade. However, this model might overestimate the efficacy of IL-7R blockade because STZ has recently been demonstrated to induce lymphopenia associated with an increase in Treg frequency and produce immunosuppressive effects (35).

Therefore, we next used a more stringent model of fully mismatched skin allograft and demonstrated that the addition of IL-7R blockade to T cell depletion therapy inhibited T cell reconstitution and doubled graft MST (Figure 2). The mechanisms underlying the prolongation of skin graft survival using this treatment protocol are multifactorial. The most evident effect of IL-7R blockade following T cell depletion is the extended reduction of lymphocyte numbers affecting the majority of lymphocyte subpopulations, including T cells, B cells, CD4⁺ T cells, CD8⁺ T cells, and especially the CD62L^{lo}CD44^{hi} memory T cells known to be associated with graft rejection (4, 5). IL-7R blockade delays postdepletional T cell recovery though both a suppression of thymopoiesis (20) and an inhibition of T cell homeostatic proliferation (14, 16, 17).

The inhibitory effect of IL-7R blockade following T cell depletion on allospecific memory T cell formation and activation as demonstrated by IFN- γ ELISPOT and MLR (Figure 5, A and B) is also a contributing mechanism. Moreover, T cells from mice that accepted their skin graft under IL-7R blockade secreted much less Th1 and Th17 cytokines (Figure 5C), a finding in concordance with previous studies using models of autoimmune diseases (21, 22, 30). Similarly, accepted skin grafts under IL-7R blockade expressed much lower levels of proinflammatory cytokines (Figure 7B and Table 1).

Table 1

List of genes significantly suppressed in the skin grafts of mice treated with depletion plus anti-IL-7R (A) compared with depletion + isotype Ig (I)

Genes	log ₂ FC (A/I)
<i>Il13</i>	-7,4
<i>Il4</i>	-6,8
<i>Il1b</i>	-5,5
<i>Ifng</i>	-4,5
<i>Il6</i>	-4,5
<i>Ptgs2</i>	-4,0
<i>Gzmb</i>	-3,9
<i>Il3</i>	-3,9
<i>Ccl3</i>	-3,7
<i>Icos</i>	-3,6
<i>Cxcl11</i>	-3,5
<i>Stat4</i>	-3,2
<i>Il2ra</i>	-3,1
<i>Csf2</i>	-3,0
<i>Ccl5</i>	-2,9
<i>Prf1</i>	-2,5
<i>CD40</i>	-2,5
<i>Il10</i>	-2,4
<i>Il5</i>	-2,2
<i>Fasl</i>	-2,1
<i>Cxcl10</i>	-2,0
<i>Ccl2</i>	-1,8
<i>Tnf</i>	-1,7
<i>H2-Ea</i>	-1,6
<i>H2-Eb1</i>	-1,5
<i>Stat1</i>	-1,2
<i>CD86</i>	-1,2

This table reports the relative expression of the genes shown in Figure 7B. FC, fold change.

The increase in Treg/T effector ratio associated with IL-7R blockade is likely to contribute to graft acceptance because Treg depletion by anti-CD25 mAb tended to shorten skin graft survival (Figure 4). The role of Tregs in the prolongation of graft survival under lymphopenic conditions is well known; for example, the adoptive transfer of exogenous Tregs to mice treated with costimulatory blockade after having undergone subtotal T cell depletion restored heart graft tolerance (5). IL-7-deprived Tregs were also shown to maintain their suppressive functions (22).

Since IL-7R blockade in mice affects both T and B cells, whereas in human, B cell number is not decreased by IL-7R α mutations, we further investigated the role of B cells in our treatment protocols. Using B cell-deficient muMT mice as skin allograft recipients, we demonstrated that IL-7R blockade following T cell depletion prolonged graft survival whether B cells were present or absent (Figure 2 and Figure 6A). On the other hand, B cell depletion by anti-CD20 mAb in wild-type mice, although not sufficient to prolong skin graft survival, led to a significant decrease in DSA levels (Figure 6D).

Taken together, our data show that IL-7R blockade after T cell depletion prolongs skin allograft survival through different mechanisms, including inhibition of T cell reconstitution, inhibition of the formation and activation of allospecific memory T cells, and induction of immunoregulation associated with an increase in Treg/T effector ratio. On the other hand, in mice, the reduction in B cell

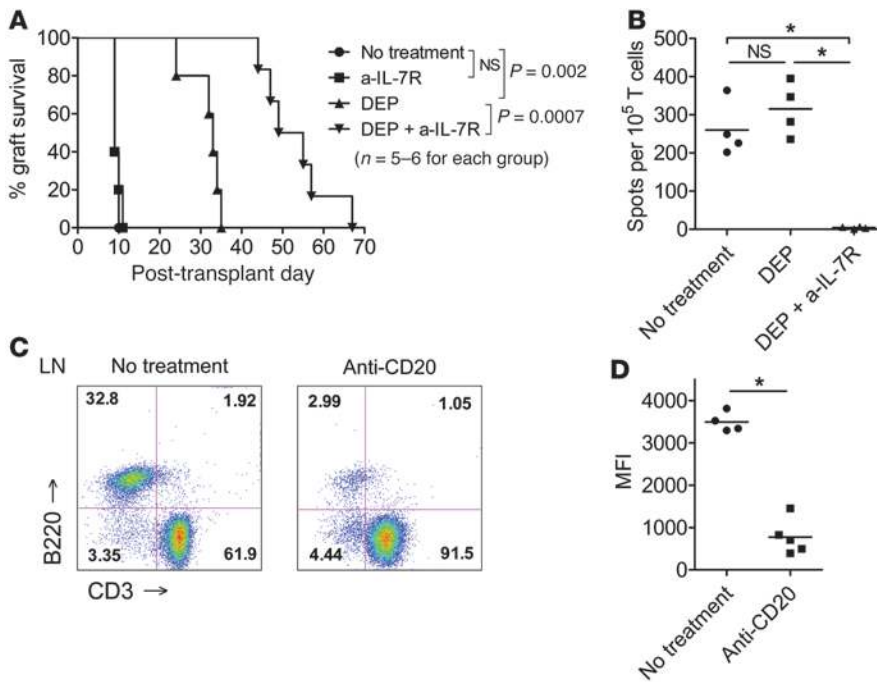


Figure 6

B cell deficiency does not affect skin graft survival, but can reduce DSA. (A and B) B cell-deficient muMT mice received BALB/c skin grafts and were treated as indicated. IL-7R blockade following T cell depletion by anti-CD4 and anti-CD8 effectively prolonged graft survival (A) and abrogated donor-specific T cell responses, as shown by IFN- γ ELISPOT (B). (C and D) BALB/c mice were treated with an anti-CD20 mAb and then received skin grafts from C57BL/6 donors. Anti-CD20 effectively depleted B cells, as shown by a representative FACS plot on PTD4 (C) and significantly reduced DSA levels on PTD14 (D), but did not prolong graft survival (MST = 10 days). * $P < 0.05$.

number caused by IL-7R blockade may contribute to the decrease in DSA and attenuate the B cell-mediated component of rejection.

Finally, combination treatment with other immunosuppressants further enhances graft survival in this stringent skin allograft model. IL-7R blockade following T cell depletion synergized with either CTLA-4Ig or suboptimal doses of tacrolimus (Figure 8, A and B). The latter protocol is likely more effective because tacrolimus is more potent than other drugs in inhibiting memory T cell activation (7), whereas memory T cells have been shown to be less dependent on costimulation for activation (36, 37).

However, lymphopoiesis in human and in mice has several differences. Under normal conditions, the maintenance of the T cell pool in adult humans and in mice is sustained almost exclusively by peripheral T cell division and by thymic output, respectively (38). But after T cell depletion therapy, the increased thymic output does contribute to immune reconstitution in adult transplant patients (1). We and others found that T cell numbers returned to normal about 3 months after a brief course of T cell depletion by anti-CD4 and anti-CD8 mAbs in mice (39), whereas it may take up to 3 years for lymphocyte numbers in the blood of kidney transplant patients treated by alemtuzumab or thymoglobulin induction to return to baseline levels (40, 41).

Nevertheless, mouse skin allograft is a predictive preclinical model frequently used in the early phase of development of new immunosuppressive drugs in transplantation (42–44). The skin graft survival obtained in our study is remarkable because, without bone marrow transplantation, not many treatment protocols could result in similar graft outcomes, especially when fully allogeneic BALB/c and C57BL/6 mice are used for donors and recipients (43, 44). Therefore, our results suggest that IL-7R blockade may present as a promising therapeutic candidate in organ transplantation. Thoroughly designed treatment protocols combining an anti-IL-7R α mAb with other immunosuppressants should harness the beneficial effects of IL-7R blockade to promote allograft acceptance.

Methods

Mice and allograft models. Eight-week-old male C57BL/6 (H-2b), BALB/c (H-2d), and SJL (H-2s) mice were purchased from Centre d'Élevage Janvier. Six-week-old male BALB/c nude (H-2d) and B cell-deficient muMT mice (B6.129S2-Ighm^{tm1Cgn}/J) on a C57BL/6 genetic background were purchased from Charles River France. To prepare for islet transplantation, BALB/c or BALB/c nude recipients received 1 i.p. injection of 250 mg/kg of STZ 5 to 10 days before graft to induce diabetes, which is defined as a nonfasting blood glucose greater than 17 mmol/l (300 mg/dl) on at least 2 occasions. Pancreases were harvested from C57BL/6 donors, digested by collagenase, and islets purified by Ficoll gradient centrifugation. Each diabetic BALB/c mouse received about 500 islets under the left renal capsule. Graft functioning and graft rejection were diagnosed when blood glucose decreased to less than 10 mmol/l (180 mg/dl) and increased again to greater than 17 mmol/l, respectively. For the skin allograft model, tail skin from donors was grafted to the dorsal trunk of recipients and protected by bandage for 6 days. The skin graft was then inspected every day and rejection was defined as graft necrosis of more than 80% of the surface.

mAbs and treatment protocols. Some mAbs were produced in our lab using the following hybridomas: rat anti-mouse IL-7R α (A7R34), CD4 (GK1.5), CD8 (2.43), and CD25 (PC61). A7R34 was a gift from Shin-Ichi Nishikawa (Laboratory for Stem Cell Biology, RIKEN Center for Developmental Biology, Kobe, Japan), and other hybridomas were purchased from LGC Standards. Rat anti-mouse PD-L1 (10F9G2) was purchased from Bio X Cell, and rat anti-mouse CD20 (5D2) was provided by Genentech. A rat anti-dinitrophenol (DNP) mAb (LO-DNP-61) was purchased from the Laboratory of Experimental Surgery, Université Catholique de Louvain (Brussels, Belgium) and used as rat IgG2a isotype control for the anti-IL-7R α mAb. Human CTLA-4Ig (Abatacept; Bristol Myers Squibb) and tacrolimus (Astellas) were purchased from the pharmacy of CHU de Nantes. All treatments were given i.p. Anti-CD4 and anti-CD8 were given in combination on days -3 and -1 before skin graft at 200 μ g per injection. CTLA-4Ig was given at 500 μ g once per week from the day of skin graft (D0) to the day of rejection or to PTD70 if there was no rejection. Low-dose tacrolimus was given at 0.5 mg/kg/d from the day of skin graft or 0.7 mg/kg/d from

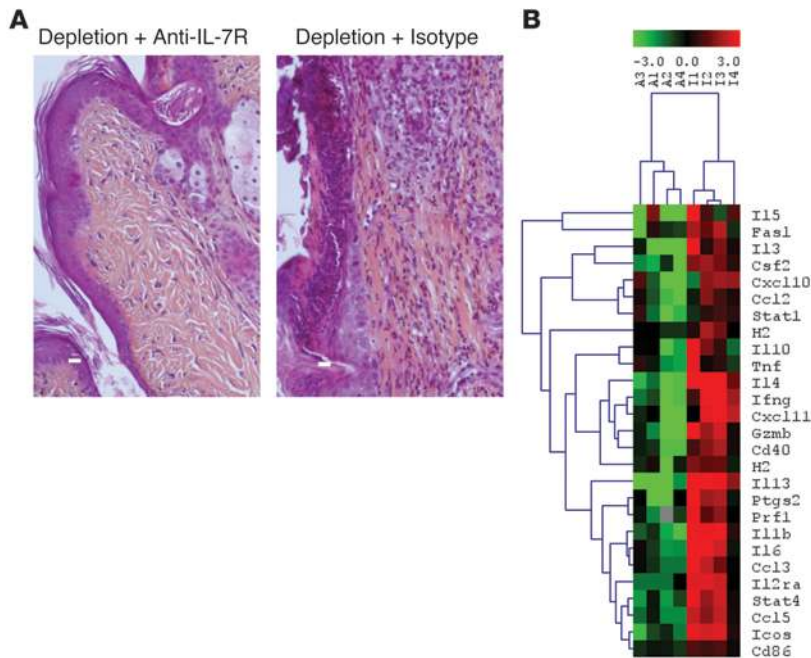


Figure 7

IL-7R blockade following T cell depletion abrogates leukocyte infiltration and decreases proinflammatory gene expression in the skin graft. C57BL/6 skin was grafted to BALB/c recipients, which were treated with T cell depletion by a combination of anti-CD4 and anti-CD8 mAbs, followed by either isotype Ig (I1 to I4) or anti-IL-7R α mAb (A1 to A4). **(A)** Representative photomicrographs of skin graft histology on PTD21 (H&E). Original magnification, $\times 200$. **(B)** Unsupervised clustering of differentially expressed genes in skin grafts on PTD21 using mouse immune panel TLDA. The heat map represents normalized and color-coded relative expression values (2^{- $\Delta\Delta$ Cq}). Red values indicate overexpression, and green values indicate underexpression.

PTD12; in either case, it was continued to the day of rejection or to the end of the experiment if there was no rejection. Anti-IL-7R α mAb was given at 400 μ g qod; the time and duration of treatment is specified for each experiment in Results. Of note, A7R34 has been effectively used for a relatively long period of time (31), suggesting that the anti-drug Abs that might develop in recipients do not abrogate its effects.

Lymphocyte phenotyping by flow cytometry. Mice were euthanized under general anesthesia. Blood was drawn from cardiac puncture; spleen, thymus, and mesenteric LNs were harvested. Whole-blood leukocyte phenotyping was performed using BD TruCOUNT Tubes (BD Biosciences) according to the manufacturer's instructions. Cells from LNs, spleen, and thymus were isolated using BD Falcon Cell Strainers (BD Biosciences). The following anti-mouse antibodies were used for flow cytometry: anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD44, anti-CD45-2, anti-CD45R (B220), anti-CD62L, anti-CD127, anti-Gr-1 (Ly-6G and Ly-6C), anti-NKG2D, anti-PD-1 and anti-FOXP3; all were purchased from BD Biosciences except anti-FOXP3, which was purchased from eBioscience, and anti-CD127 (A7R34), which was prepared in our lab. Flow cytometry was performed using a BD LSRII flow cytometer.

Measurement of DSA. Blood of recipient mice was obtained either by retro-orbital sinus sampling or by cardiac puncture in the case of sacrifice. Plasma was obtained by centrifugation and stored at -20°C for later use. Plasma of recipients was diluted at 1/20 and incubated for 30 minutes with cells freshly isolated from the spleen of a donor mouse. Cells were washed twice, incubated for 15 minutes with an FITC-conjugated goat anti-mouse IgG (Fc γ -specific) (Jackson ImmunoResearch) and hamster anti-mouse CD3, washed twice, and analyzed by flow cytometry. DSA levels were reported as MFI.

IFN- γ ELISPOT, MLR, and cytokine measurement by ELISA. Skin graft recipients were sacrificed on PTD35, whereas islet graft recipients were sacrificed as indicated in Results. Responder cells were T cells purified from splenocytes of graft recipients using the Pan-T Cell Isolation Kit II Mouse and autoMACS Pro Separator (both from Miltenyi Biotec) following the manufacturer's instructions. Stimulator cells were irradiated donor splenocytes. Third party cells were irradiated splenocytes from SJL

mice (H-2s). Responders were cocultured with either stimulators or third party; each type of cell was seeded at 10^5 cells per well (1:1 ratio) and in triplicate. IFN- γ ELISPOT was performed using a mouse IFN- γ ELISPOT kit (BD Biosciences). Plates were incubated for 16 hours, washed, and developed according to the manufacturer's instructions and read using an AID Elispot Reader System (AID GmbH). ELISPOT was considered negative when there were fewer than 8 spots per well. For MLR, plates were incubated for 72 hours, pulsed with 3H-thymidine at 1 μ Ci per well for an additional 16 hours, harvested using a Tomtec Cell Harvester (Tomtec Inc.), and read using a TopCount NXT Microplate Scintillation and Luminescence Counter (PerkinElmer). The MLR supernatants after 72 hours of culture were also stored at -20°C for later cytokine measurement using mouse BD OptEIA ELISA kit (BD Biosciences) for IL-2 and TNF and mouse IL-17 Quantikine ELISA kit (R&D Systems) for IL-17 according to the manufacturer's instructions.

Skin graft histology and gene expression assay. Skin grafts were harvested when recipients were sacrificed at different time points as indicated. One part of the skin graft was fixed in 4% formalin for histological studies and the other part cryopreserved for immunohistology and RNA extraction by the TRIzol method (Invitrogen). RNA quality and quantity were determined using an Agilent 2100 BioAnalyzer (Agilent Technologies). RNA was reverse transcribed into cDNA using a poly dT oligonucleotide and Omniscript Reverse Transcriptase kit (QIAGEN). Gene expression assay was performed using TLDA Mouse Immune Panel (Applied Biosystems) according to the manufacturer's instruction. The mean expression of Hprt1 and B2m was used for normalization, and differentially expressed genes were selected using the significance analysis of microarray (SAM) method (45) with false discovery rate below 1%. Unsupervised hierarchical clustering was performed with MultiExperiment Viewer software using uncentered Pearson's correlation with median-centered genes (46).

Evaluation of antiadenoviral immunity. E1/E3-deleted human AdV5 without transgene was obtained from Laboratoire de Therapie Genique (Nantes, France). BALB/c mice were infected with AdV5 and then received treatment as indicated. T cells and non-T cells were separated from splenocytes of AdV5-infected mice using Pan-T cell isolation kit

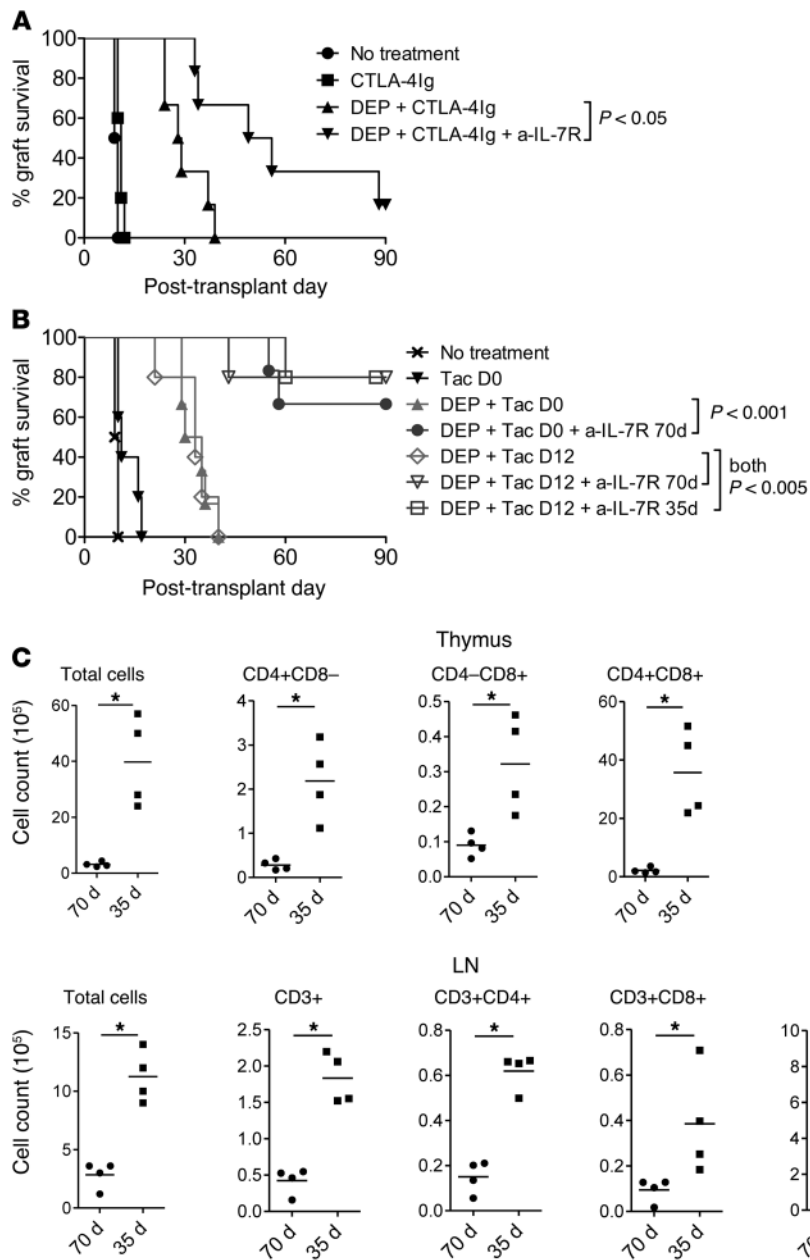


Figure 8

IL-7R blockade synergizes with treatment protocols combining T cell depletion with either CTLA-4Ig or suboptimal doses of tacrolimus. BALB/c mice received C57BL/6 skin graft and were treated as indicated ($n = 5-6$ for each group). **(A)** CTLA-4Ig alone only slightly prolonged graft MST compared with no treatment (11 vs. 9.5 days; $P < 0.01$). CTLA-4Ig combined with T cell depletion prolonged MST to 28.5 days, and the addition of IL-7R blockade to this treatment protocol further increased MST to 52.5 days ($P < 0.05$). **(B)** Low-dose tacrolimus (Tac) given from D0 only slightly prolonged graft MST compared with no treatment (11 vs. 9.5 days; $P < 0.01$). T cell depletion combined with low-dose tacrolimus given from either D0 or PTD12 prolonged MST to 32.5 or 33 days, respectively, and the addition of anti-IL-7R α mAb for a duration of either 70 days or 35 days to these 2 treatment protocols further prolonged MST to beyond 90 days in the majority of mice (see figure for direct comparisons between treatment protocols with and without anti-IL-7R α mAb). **(C)** Lymphocyte phenotyping on PTD90 showed that the shortening of the duration of anti-IL-7R treatment from 70 days to 35 days in the DEP + Tac D12 + a-IL-7R protocol was associated with a better lymphocyte recovery in the thymus and in the LNs. * $P < 0.05$.

II and autoMACS Pro Separator (Miltenyi Biotec). IFN- γ ELISPOT was performed in which each well contained 10^5 T cells, 10^5 non-T cells, and 0.3 nmol/peptide/ml of a pool of overlapping 15-mer peptides covering the complete sequence of the hexon protein of adenovirus 5 (Peptivator - AdV5; Miltenyi Biotec). To quantify antiadenoviral antibodies, ELISA plates were coated overnight with 10^9 virus particles per well, washed, and incubated consecutively with serial dilutions of mouse sera (1/500 to 1/5000), peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch), TMB substrate, and H 2 SO 4 to stop the reaction, and then read using an ELISA plate reader.

Statistics. Statistics were done using the GraphPad Prism 5.0 software (GraphPad Software). Data were presented as mean \pm SD unless otherwise stated. Graft survivals were reported as MST and analyzed by the Kaplan-Meier method using the log-rank test. Other values were compared using the Mann-Whitney test. $P < 0.05$ was considered significant.

Study approval. All animal studies were approved by the institutional review board of INSERM Unit 1064.

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