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Title page

Blockade of CD27/CD70 pathway to reduce the generation of memory T cells and markedly prolong the survival of heart allografts in presensitized mice

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Running title: Blockade of CD27/CD70 pathway to achieve long-term survival

Abstract

Background. Alloreactive memory T cells are a major obstacle to transplantation acceptance due to their capacity for accelerated rejection.

Methods. C57BL/6 mice that had rejected BALB/c skin grafts 4 weeks earlier were used as recipients. The recipient mice were treated with anti-CD154/LFA-1 with or without anti-CD70 during the primary skin transplantation and anti-CD154/LFA-1 or not during the secondary transplantation of BALB/c heart. We evaluated the impact of combinations of antibody-mediated blockade on the generation of memory T cells and graft survival after fully MHC-mismatched transplantations.

Results. One month after the primary skin transplantation, the proportions of $CD4^+$ memory T cells/$CD4^+$ T cells and $CD8^+$memory T cells/$CD8^+$ T cells in the anti-CD154/LFA-1 combination group were 47.32±4.28% and 23.18±2.77%, respectively. In the group that included anti-CD70 treatment, the proportions were reduced to 34.10±2.71% and 12.19±3.52% ($P<0.05$ when comparing the proportion of memory T cells between the two groups). The addition of anti-CD70 to the treatment regimen prolonged the mean survival time following secondary heart transplantation from 10 days to more than 90 days ($P<0.001$). Furthermore, allogenic proliferation of recipient splenic T cells and graft-infiltrating lymphocytes were significantly decreased. Meanwhile, the proportion of regulatory T cells were increased to 9.46±1.48% on day 100 post-transplantation($P<0.05$).

Conclusions. The addition of anti-CD70 to the anti-CD154/LFA-1 combination given during the primary transplantation reduced the generation of memory T cells. This therapy regimen provided a potential means to alleviate the accelerated rejection mediated by memory T cells during secondary heart transplantation and **markedly prolong the survival of heart allografts**.

Keywords. costimulatory blockade; anti-CD70;memory T cells; heart transplantation.

Introduction

Transplant patients may develop alloreactive memory T cells after exposure to alloantigens during previous transplantations, blood transfusions, pregnancies, or due to continuous exposure to bacterial and viral pathogens[1,2]. In adult humans, 40–50% of T cells circulating in the peripheral blood have memory phenotypes[3,4]. Memory T cells, including $CD4^+$ and $CD8^+$ subsets, play a key role in accelerated rejection. Furthermore, there is a growing body of evidence that B-cell production of alloantibodies is also an important element in the accelerated rejection[5]. Therefore, inhibiting the generation of memory T cells and alloantibodies is a critical step in the prevention of rejection.

A variety of methods to induce memory cells tolerance in mouse models have been identified, such as the use of antilymphocyte serum and antithymocyte globulin for general clearance of T and B lymphocytes [6], anti-CD122 to deplete $CD8^+CD122^+$ memory T cells [7,8], and anti-CD20 to deplete B-lymphocytes during treatment of autoimmune diseases [9,10]. Here, we developed a protocol designed to minimize the generation of memory T cells and production of alloantibodies during primary transplantation in order to significantly prolong the secondary allograft survival.

The CD40/CD154 and LFA-1/ICAM-1 pathways have been shown to play an important role in the activation of T cells [11-13]. The CD40/CD154 pathway also provides important signals regulating B lymphocytes clonal expansion, antibody production and isotype switching, as well as the development of humoral memory[14,15]. CD70 (CD27 ligand) is a type II transmembrane glycoprotein belonging to the TNF family that is induced upon T and B cell activation [16]. CD27/CD70 engagement has been implicated in T-cell development, T-cell activation, and T-cell-dependent antibody production by B cells [17,18]. CD70 blockade can inhibit clonal expansion of $CD8^+$ T cells and reduce the generation of memory $CD8^+$ T cells [19,20].

In this study, we attempted to reduce the generation of memory T cells and alloantibodies by treating mice with anti-CD70 added in combination with an anti-CD154/LFA-1 regimen during primary transplantation. We provided evidence that this approach could alleviate the accelerated rejection mediated by memory T cells during

secondary heart transplantation, and we explored the possible mechanism of the obviously prolonged survival.

MATERIALS AND METHODS

Animals

Female C57BL/6 (B6, H-2^b) and BALB/c(H-2^d) mice (8–12 weeks old) were purchased from Slac Laboratory Animal Co. Ltd. (Shanghai, China) and used as graft recipients and donors, respectively. All animals were maintained and bred in a pathogen-free facility, and all procedures were performed according to the Institutional Animal Care and Use Committee (IACUC) guidelines.

Antibodies

All administered antibodies were produced by Bioexpress (West Lebanon, NH, USA), including anti-CD70 (FR-70), anti-CD154 (MR-1), anti-LFA-1(M17/4), and their respective isotype controls. Antibodies used for flow cytometric analysis, including FITC-anti-CD4 (GK1.5), FITC-anti-CD8 (53-6.7), PE-anti-CD44 (IM7), PECy5-anti-CD62L (MEL14), PE-anti-IgM (RMM-1), FITC-anti-IgG1 (RMG1-1), FITC-anti-IgG2a (RMG2a-62), and their isotype controls were purchased from Biolegend (San Diego, CA, USA). $CD4^+Foxp3^+$ regulatory T cells (Tregs) were detected using the Mouse Regulatory T cell Staining Kit from eBioscience (San Diego, CA, USA).

Skin transplantation

Full-thickness skin grafts were prepared from the lateral thoracic skin of BALB/c mice, cut into circular pieces (~1.2 cm^2 in area), and engrafted onto the lumbar region of B6 mice. After the fully MHC-mismatched transplantation, the B6 mice received a 2-antibody treatment regimen consisting of 0.25 mg of anti-CD154 and 0.1 mg of anti-LFA-1, or a 3-antibody treatment regimen consisting of 0.25 mg of anti-CD154, 0.1 mg of anti-LFA-1, and 0.25 mg of anti-CD70. Control group mice were treated

with isotype antibodies (Table 1). The drugs were intraperitoneally (i.p.) administered on days 0 and 2 post-transplantation.

Alloantigen-primed heart transplantation model

Four weeks after skin grafting, the B6 mice were defined as alloantigen-primed mice. Vascularized heterotopic heart transplantations from BALB/c donors to B6 recipients were performed with anastomosis to the vessels of the neck using a non-suture cuff technique as described previously [21]. The mice were treated with 0.25 mg anti-CD154 and 0.1 mg of anti-LFA-1 or isotype controls on days 0, 2, 4, and 6 post-transplantation (Table 1). Graft survival was monitored by daily palpation. Rejection was defined as complete loss of palpable heart beat.

Mixed lymphocyte reactions (MLR)

T lymphocytes were isolated from spleens of B6 mice using nylon wool columns (Wako, Osaka, Japan) and used as responder cells. Donor spleen cells were used as stimulator cells and treated with mitomycin(40 lg/ml, Amresco, Solon, OH, USA) before used in the MLR assay. For proliferation assays, 10^5 stimulator cells were cultured with 5×10^5 responder cells in RPMI1640 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in 96-well plates. Three separate wells were dedicated to each responder-stimulator combination and each experiment was repeated three times. Cells were incubated for 72 h at 37ºC in 95% humidified air mixed with 5% carbon dioxide. After 72 h of culture, cell proliferation was quantified using a BrdU kit (Roche Applied Science, Mannheim, Germany).

Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed using commercially available kits (NeoBioscience Technology Limited Company, Shenzhen, China) to detect the concentrations of IL-2, IFN-γ, IL-10, and TGF- β in the supernatants from MLR according to the manufacturer's instructions. A standard curve was generated using known amounts of

purified recombinant murine cytokines.

Graft pathological analysis

The heart allografts were resected from the recipient mice on day 7 or day 100 post-transplantation. Tissues were fixed in 10% buffered formalin solution, embedded in paraffin, cut into 5 μm sections and stained with hematoxylin and eosin (H&E). Graft rejection was graded on the extent of infiltration and the anatomical localization of inflammatory cells according to the International Society of Heart and Lung Transplantation (ISHLT) standard [22,23].

qRT-PCR

RNA was isolated from the heart allografts using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription and qRT-PCR were performed using commercially available reagents (TOYOBO, Osaka, Japan) with the StepOne Real-Time PCR System (ABI, Foster, UK). Syber Green I was used to detect amplification and β-actin was used as a normalizing control. Calculations were performed using the $2^{-\Delta\Delta CT}$ method. Each reaction was carried out in triplicate. The primer sequences used for the qRT-PCR were listed in Table 2.

Extraction of lymphocytes from heart allografts

The harvested heart allografts were minced with a sterile blade and incubated in 10 ml buffered saline with 2% bovine serum albumin (BSA) and 2 mg/ml collagenase at 37°C for 2 h. The cells were strained through a 70 μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA). Lymphocytes were isolated from these cells using EZ^{-SepTM} Mouse lymphocyte separation medium (Dakewe Biotech Company, shenzhen, China) with centrifugation for 20 min at 1600 rpm. After washed twice in RPMI 1640, lymphocytes were resuspended in phosphate buffered saline (PBS) with 10% fetal bovine serum for the Flow cytometry.

Flow cytometry

A total of 30 μl of sera from different mice in each group was collected following rejection of the allografts in the control group mice. BALB/c spleen cells (1×10^6 cells/100 μl) were incubated with 10 μl of recipient sera at 4ºC for 30 min. After washing twice in PBS, the cells were incubated with PE-IgM and FITC-IgG1 or IgG2a antibodies at 4ºC for 30 min. Approximately 1×10^6 T lymphocytes from recipients and heart grafts were stained using fluorescent antibodies according to the manufacturer's instructions. At last, all of the positive cells were analyzed with a FACS instrument (Partec Co, Germany). Data were analyzed with Flow Jo 7.5 software.

Statistical analyses

The mean survival times (MST) of the six groups were analyzed by the Kaplan-Meier method and Log-rank test. All other data were analyzed by one-way analysis of variance (ANOVA). Because multiple comparisons were made during the analysis, a Bonferroni correction was calculated and applied. A P value<0.05 was considered statistically significant; P<0.01 and P<0.001 indicated highly significant differences. All analyses were performed using GraphPad Prism® (GraphPad, Inc., La Jolla, CA, USA) software.

Results

Addition of anti-CD70 to anti-CD154/LFA-1 combination treatment during primary skin transplantation reduced the generation of memory T cells

In order to determine whether anti-CD70 treatment would impact the generation of alloreactive effector memory T cells ($CD44^{high}CD62L^{low}$), we isolated B6 mouse splenic T lymphocytes four weeks after skin transplantation and analyzed CD4, CD8, CD44, and CD62L expression using flow cytometry (Fig. 1). The proportions of

$CD4^+$ memory T cells/$CD4^+$ T cells and $CD8^+$ memory T cells/$CD8^+$ T cells in the S0 group mice following skin transplantation were 57.71±3.25% and 31.04±1.06%, respectively. In the S2 treated group, the proportions were 47.32±4.28% and 23.18±2.77%, respectively. Finally, in the S3 group, which had anti-CD70 included in the combination treatment, the proportions were reduced to 34.10±2.71% and 12.19±3.52%, respectively (Fig. 1). $P<0.001$ when comparing the proportion of $CD4^+$ and $CD8^+$memory T cells between treatment group S3 to S0 group. $P<0.05$ when comparing the proportion of $CD4^+$ and $CD8^+$ memory T cells between S2 group to S0 and S3 groups.

Evaluations of groups without treatment during secondary heart transplantation

To determine the impact of the reduction of effector memory T cells observed in the S3 group on secondary transplantation, we selected B6 mice which came from the three groups (S0, S2, S3) as recipients for heart transplantation. Mice from all three groups were treated with isotype control antibodies during the secondary heart transplantation. The mean survival times following heart transplantation were 3.5 d for the S0H0 control group, 4.5 d for the S2H0 treated group, and 6.5 d for the S3H0 treated group. The P value for the S3H0 vs. the S0H0 and S2H0 groups was less than 0.001. The P value for the S2H0 group vs. the S0H0 group was less than 0.05. (Fig. 2A). Spleens from recipient mice were removed on day 3.5 post-transplantation following identification of rejection in the control S0H0 group. The values of MLR in the S3H0 and S2H0 group were reduced compared to the S0H0 control group (Fig. 2B, $P < 0.05$). There were no statistical differences in IL-2 concentrations between groups. The concentration of IFN-γ was reduced (Fig. 2B, $P < 0.001$). The proportions of $CD4^+$ memory T cells/$CD4^+$ T cells and $CD8^+$ memory T cells/$CD8^+$ T cells were reduced, as was previously observed following primary skin transplantation. $P<0.001$ when comparing the proportion of $CD4^+$ and $CD8^+$memory T cells between treatment groups (S2H0 and S3H0) to positive control group (S0H0). $P<0.05$ when comparing the proportion of $CD4^+$ and $CD8^+$ memory T cells between S2H0 group to S3H0 group (Fig. 2C). Splenic Tregs were not significantly different between groups. The

alloantibodies IgG1 and IgG2a of recipient sera were nearly 100% in the control group. However, there were no IgG1 and IgG2a in the S2H0 and S3H0 groups, and alloantibodies IgM were not different between the groups.

Addition of anti-CD70 to combination treatment during primary skin transplantation markedly prolonged survival when anti-CD154/LFA-1 were also given during secondary heart transplantation.

Four weeks following primary skin transplantation, B6 mice selected as recipients for heart transplantation were treated with anti-CD154/LFA-1. The MST for group S3H2 was greater than 90 days, as compared to 6.5 days for group S0H2 and 10.0 days for group S2H2. These results indicated that only mice from group S3H2 which had received primary skin transplantation with anti-CD70 treatment could markedly prolong survival. The P value between each group was less than 0.001 (Fig. 3A).

In order to discover the mechanism of obviously prolonged allografts survival induced by the treatment including anti-CD70, we performed functional tests on the immune cells of the recipient mice. First, we examined the proliferative responses of recipient splenic T cells to donor cells. Recipient splenic T cells were isolated at day 7 after heart transplantation. MLR results showed that mice that received anti-CD70 treatment during primary skin transplantation had lower cell proliferative responses than mice that did not receive anti-CD70 treatment(Fig. 3B, $P<0.05$). Furthermore, cell proliferation in the S3H2 group mice was further reduced on day 100 post-transplantation (Fig. 3B, $P<0.05$). TGF-β concentrations in the MLR supernatants were increased in the S3H2 mice compared to the S2H2 mice that did not receive anti-CD70 (Fig. 3B, $P<0.05$). By contrast, IL-10 expression was not different between the groups in the supernatants. IL-2 concentrations in the MLR supernatants were reduced in the S3H2 and S2H2 mice compared to the S0H2 mice (Fig. 3B, $P<0.05$). The concentrations of IFN-γ in the MLR supernatants were lower in the S3H2 mice compared to the S2H2 mice (Fig. 3B, $P<0.05$). Flow cytometric analysis of the splenic cell populations showed that the S3H2 100d group mice had a higher proportion of Tregs than mice in the S0H2, S2H2 and S3H2 groups (Fig. 3C, $P<0.05$). Furthermore, Treg populations in the S3H2 mice were increased to 9.46±1.48% on day 100 post-transplantation. $P<0.001$ when comparing the proportion

of $CD4^+$ memory T cells between S3H2 group to S0H2 and S2H2 groups. $P<0.01$ when comparing the proportion of $CD8^+$ memory T cells between S2H2 group to S0H2 group (Fig. 3C). Finally, the levels of alloantibodies IgG1, IgG2a and IgM detected in all groups were low.

Addition of anti-CD70 to combination treatment during primary skin transplantation effectively prevented lymphocyte infiltration and induced high Foxp3 gene expression in the graft

As shown in Figure 4A, heart allografts tissue from mice in the S3H2 group showed low levels of lymphocytic infiltration and fewer changes in myocardial structural integrity compared to S2H2 groups ($P<0.01$). This result was consistent with the detection of low levels of graft-infiltrating lymphocytes by flow cytometry. The proportion of $CD8^+$ T cells/graft-infiltrated cells was 43.38±4.83% in the S2H2 group that had not receive anti-CD70 treatment. In contrast, the proportion was 23.39±2.78% in the S3H2 group mice on day 7 post-transplantation and was further reduced to 13.44±2.99% by day 100 post-transplantation. $P<0.01$ when comparing the proportion of $CD8^+$ T cells/graft-infiltrated cells between S3H2 group to S2H2 group. $P<0.001$ when comparing the proportion of $CD8^+$ T cells/graft-infiltrated cells between S3H2 100d group to S2H2 group. (Fig. 4B). There were very little $CD4^+$ T cells infiltration detected in the grafts (Fig. 4B). The intragraft immune response was dominated by effector T cells (data not shown). In order to determine the mechanism of graft protection, we examined graft rejection and tolerance-related gene expression. Compared with the no treatment group, the inflammatory factors (IL-2, IFN-γ) in S3H2 group were significantly suppressed (Fig. 4C, $P<0.001$). Treatment with anti-CD70 induced higher Foxp3 gene expression (Fig. 4C, $P<0.001$) and TGF-β expression in the graft, although there was no difference in IL-10 expression between groups. TGF-β levels remained high in the S3H2 group on day 100 post-transplantation (Fig. 4C).

Discussion

Although the generation of memory T cells specific for pathogens is beneficial in providing protective immunity, memory T cells specific for alloantigens can be deleterious to the recipient of a transplanted organ. Graft rejection is accelerated and

allograft acceptance is more difficult to induce in hosts whose immune repertoires contain donor-reactive memory T cells [2,24].

Nicolls et al. demonstrated that combining monoclonal antibody therapies targeting CD154 and LFA-1 induced transplantation acceptance to pancreatic islet allografts [25]. Other studies had shown that anti-CD154 or anti-CD154/CD28 treatment could prevent the occurrence of anti-donor antibodies following xenogeneic transplantation [26,27]. Here, we showed that anti-CD154/LFA-1 and anti-CD70 combination treatment during primary skin transplantation reduced the generation of memory T cells. $CD8^+$ memory T cells in particular were maintained close to the level observed in naïve mice (Fig. 1). This was consistent with previous studies showing that blocking the CD27/70 pathway effectively inhibited the function and generation of memory T cells [28,29]. Furthermore, we found that this treatment regimen abrogated the production of IgG1/IgG2a alloantibodies in recipient mice, were consistent with a prior study by Nathan et al. demonstrating that anti-CD154 treatment could abrogate the production of alloantibodies [30]. CD154 is a key molecule in the contact-mediated signaling required for B cell activation and differentiation and can induce Ig isotype switching [31,32], while the CD27/CD70 pathway is important in stimulating B cell activation, plasma cell formation, and Ab production [33]. Therefore, the anti-CD154/LFA-1 and anti-CD70 regimen following primary skin transplantation provided the benefit of abrogating alloantibodies production along with reducing the generation of memory T cells.

Treatment with anti-CD154/LFA-1 and anti-CD70 during primary skin transplantation prolonged secondary graft survival compared to the control group, but the survival time was not very long (Fig. 2A). In view of these results, we treated mice with anti-CD154/LFA-1 during secondary heart transplantation to suppress effector T cell function, markedly prolong survival in presensitized mice.

The results obtained from the S0H2 group mice are comparable to transplantation patients who have a history of previous transplantations, blood transfusions, or pregnancies, as well as continuous exposure to bacterial and viral pathogens. These patients have higher levels of memory T cells and alloreactive antibodies in vivo which are barriers to secondary transplantation. In contrast, the S2H2 group mice are comparable to primary transplant patients given conventional

treatment, and the S3H2 group mice are comparable to primary transplant patients given treatments to decrease production of allogenic memory T cells. Repeated transplantation from the same donor would not be performed in human transplantation. However, our model could research the alloreactive memory T cells conveniently. Our study was performed in pathogen free mice and therefore did not study alloreactivity by viral specific memory T-cells. Therefore, it would be particularly useful to know if this regimen could also prolong survival in pathogen exposed mice. This could be particularly useful for our future work. Based on survival curves, mice in the S3H2 group which had anti-CD70 treatment combined with anti-CD154/LFA-1 during the primary skin transplantation could markedly prolong allograft survival (Fig. 3A). Nicolls et al. found that combined anti-CD154/LFA-1 therapy induced "dominant" transplantation acceptance in naïve mice and that this combination therapy did not deplete lymphocytes or require the long-term deletion of donor-reactive T lymphocytes to maintain allograft survival [25]. Here, we explored an effective approach designed to markedly prolong allograft survival in previous transplant recipients. Our novel method used anti-CD70 treatment combined with anti-CD154/LFA-1 treatment during primary skin transplantation to reduce the generation of memory T cells, and anti-CD154/LFA-1 treatment during secondary heart transplantation to inhibit the function of effector T cells.

To explore the mechanisms of the obviously prolonged allograft survival associated with our treatment regimen, we examined the proliferative response of recipient splenic T cells to donor antigens in the presensitized mice. Mice treated with anti-CD70 during primary skin transplantation had lower cell proliferation than mice that did not receive anti-CD70. Furthermore, cell proliferation remained low in the anti-CD70 treated group at day 100 post-transplantation (Fig. 3B). These results showed that anti-CD70 treatment effectively suppressed the recipient cellular immune response *in vivo*. As shown in Figure 3C, the group treated with anti-CD70 exhibited increased memory T cells and Tregs production on day of 100 post-transplantation. Moreover, the group treated with anti-CD70 had fewer graft-infiltrating $CD8^+$ T cells (Fig. 4B). Through these results, we speculated that the majority of T cells existed in the spleen as effector memory T cells [34] and were kept in balance with the Tregs population.

As we can see from the pathological analysis of allografts, the group treated with anti-CD70 showed only a small amount of lymphocytic infiltration and few changes in myocardial structural integrity compared to mice not treated with anti-CD70 (Fig. 4A, $P<0.01$). This was consistent with flow cytometic analysis of graft-infiltrating lymphocytes. These results showed that anti-CD70 treatment could reduce T cells infiltration to the allografts. We found few graft-infiltrating $CD4^+$ T cells, suggesting that $CD4^+$T cells play a key role in secondary lymphoid organs. This was consistent with previous results published by Yalai et al.[35]. The Foxp3 gene expression was higher in the group treated with anti-CD70 (Fig. 4C, $P<0.001$). Moreover, the anti-CD70 treatment group exhibited higher levels of TGF-β expression but not IL-10 expression in the grafts that were maintained until at least day 100 post-transplantation (Fig. 4C). This result, in combination with the high ratio of Tregs in the spleen, showed that mice treated with anti-CD70 might protect allografts using Th3-type Tregs. A few studies had shown that T-cell-derived IL-10 was not essential for induction of allograft acceptance in mice treated with a combined CTLA-4Ig/anti-CD154/LFA-1 blockade, but this treatment limited T-cell expansion in the recipients. These results further indicated that allograft acceptance was maintained by intragraft Tregs [36]. Arefanian et al. found that the combination of anti-LFA-1 and anti-CD154 monoclonal antibodies induced allograft acceptance to neonatal porcine islet xenografts maintained by Tregs [37]. As with allograft acceptance induced by anti-CD154/LFA-1 in naïve mice, our obviously prolonged allograft survival in pre-sensitized mice demonstrated in this study might be also maintained by intragraft Tregs. We will further examine the differences and similarities between the two in future studies.

In summary, we had markedly prolonged allograft survival in presensitized mice by addition of anti-CD70 to the anti-CD154/LFA-1 combination treatment during primary skin transplantation. Our regimen reduced the generation of memory T cells during primary transplantation and inhibited effector T cells function during secondary heart transplantation. This obviously prolonged allograft survival induction program inhibited the proliferative response of recipient splenic T cells to donor antigens, greatly reduced the infiltration of T lymphocytes to the grafts, and might successfully protect the allografts by Tregs. The program can provide a reference for clinical organ transplantation and show that minimizing allogeneic memory T cells

and allogeneic antibodies in primary transplantation patients could allow treatment with conventional therapy during secondary transplantation.

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Table 1.The meanings of abbreviation in this study

abbreviation	treatment	days post-transplantation
S0	Skin transplantation+isotype antibodies	at day 0 and 2
S2	Skin transplantation+anti-CD154/LFA-1	at day 0 and 2
S3	Skin transplantation+ anti-CD154/LFA-1/CD70	at day 0 and 2
H0	Heart transplantation+ isotype antibodies	at day 0, 2, 4 and 6
H2	Heart transplantation+ anti-CD154/LFA-1	at day 0, 2, 4 and 6

Table legend: mAbs were pooled and administered i.p. after the transplantation procedure as follows: 0.25 mg/dose anti-CD154, 0.1 mg/dose anti-LFA-1, and 0.25 mg/dose anti-CD70. Heart transplantations were performed one month after primary skin transplantation.

Table 2. Primer sequences used for qRT-PCR

Primer name	Sequences
β-actin	forward 5′-CATCCGTAAAGACCTCTATGCCAAC-3′,
	reverse 5′-ATGGAGCCACCGATCCACA-3′
IFN-γ	forward 5′-CGGCACAGTCATTGAAAGCCTA-3′,
	reverse 5′-GTTGCTGATGGCCTGATTGTC-3′

IL-2	forward 5′-GGAGCAGCTGTTGATGGACCTAC-3′,
	reverse 5′-AATCCAGAACATGCCGCAGAG-3′
IL-10	forward 5′-GACCAGCTGGACAACATACTGCTAA-3′,
	reverse 5′-GATAAGGCTTGGCAACCCAAGTAA-3′
Foxp3	forward 5'- CAGCTCTGCTGGCGAAAGTG -3′,
	reverse 5′-TCG.TCTGAAGGCAGAGTCAGGA -3′
TGF-β	forward 5′- TGACGTCACTGGAGTTGTACGG -3′,
	reverse 5′- GGTTCATGTCATGGATGGTGC -3′

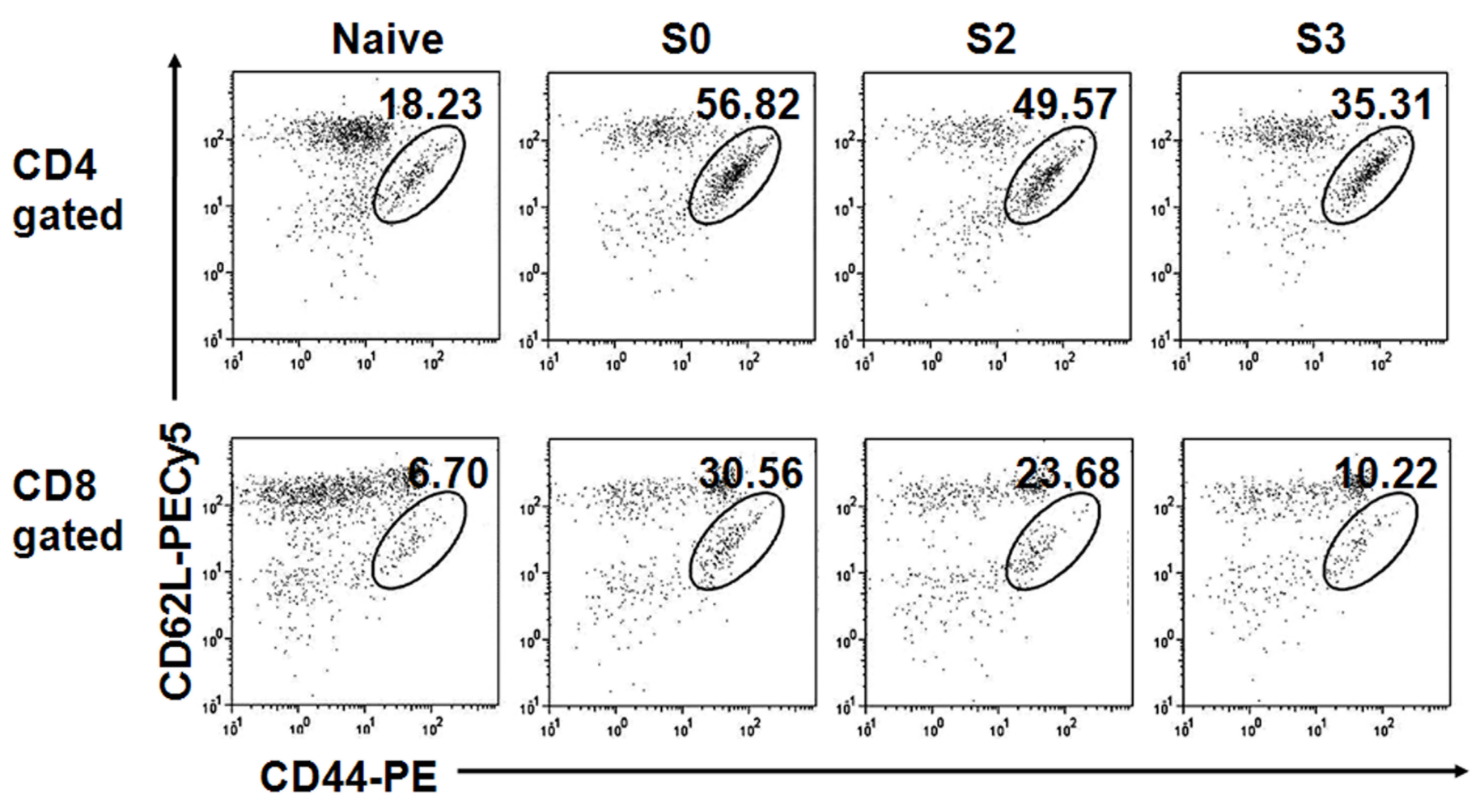
Figure 1. Changes in the proportion of memory T cells in each treatment group following skin transplantation. Proportions of $CD4^+$ memory T cells/$CD4^+$ T cells (top) and $CD8^+$ memory T cells/$CD8^+$ T cells (bottom) were analyzed using flow cytometry. Naïve B6 mice were used as negative controls(n=3 mice/group). The abbreviation of S0 represented mice received isotype antibodies only at the day 0 and 2 post skin transplantation. S2 represented mice received a 2-antibody treatment regimen consisting of anti-CD154/LFA-1 at the day 0 and 2 post skin transplantation, S3 represented mice received a 3-antibody treatment regimen consisting of anti-CD154/LFA-1/CD70 at the day 0 and 2 post skin transplantation. Data are represented of three separate experiments with similar results.

Figure 2. Graft survival time, recipient splenic lymphocyte function, and serum alloantibody levels in mice that received no treatment during secondary heart transplantation. Spleens and sera were recovered from recipient mice at day 3.5 post-transplantation for analysis. (A) Graft survival were showed by Kaplan-Meier method and compared by Log-rank test(n=6 mice/group). (B) MLR assays were used to test the proliferative responses of recipient splenic T cells to donor BALB/c antigens (top; n=3 mice/group; * $P < 0.05$). Three separate wells were dedicated to each responder-stimulator combination and each experiment was repeated three times. Concentrations of IL-2 and IFN-γ in the supernatant from MLR were detected by EISA (bottom; n=3 mice/group;*** $P<0.001$). Each reaction was carried out in triplicate. (C) The proportions of $CD4^+$ memory T cells/$CD4^+$ T cells, $CD8^+$ memory T cells/$CD8^+$T cells, and Tregs ($CD4^+Foxp3^+$) were analyzed by flow cytometry taking into account the proportion of splenic lymphocytes (n=3 mice/group). (D) Alloantibodies in recipient sera were detected by flow cytometry (n =3 mice/group).

Data are represented of three separate experiments with similar results.

Figure 3. Graft survival time, recipient spleen lymphocyte function, and serum alloantibody levels in mice treated with anti-CD154/LFA-1 during secondary heart transplantation. Spleens and sera were recovered from recipient mice at day 7 post-transplantation for analysis. Mice in the S3H2 group were sacrificed at either day 7 or 100 post-transplantation. (A) Graft survival were showed by Kaplan-Meier method and compared by Log-rank test. (B) MLR assays were used to test the proliferative responses of recipient splenic T cells to donor BALB/c antigens (top; n=3 mice/group; $*P < 0.05$). Three separate wells were dedicated to each responder-stimulator combination and each experiment was repeated three times. Concentrations of IL-2, IFN-γ, IL-10, and TGF-β in the supernatant from MLR were detected by ELISA (bottom; n=3 mice/group; $*P < 0.05$; $**P < 0.01$). Each reaction was carried out in triplicate. (C) The proportions of $CD4^+$ memory T cells/$CD4^+$T cells, $CD8^+$ memory T cells/$CD8^+$T cells, and Tregs ($CD4^+Foxp3^+$) were analyzed by flow cytometry taking into account the proportion of splenic lymphocytes (n=3 mice/group). (D) Alloantibodies in recipient sera were detected by flow cytometry (n=3 mice/group). Data are represented of three separate experiments with similar results.

Figure 4. Pathological analysis of grafts, detection of graft-infiltrating lymphocytes, and determination of rejection/tolerance levels associated with cytokines in grafts from mice treated with anti-CD154/LFA-1 during secondary heart transplantation. Recipient mice were sacrificed at day 7 post-transplantation. Mice from the S3H2 group were sacrificed at either day of 7 or 100 post-transplantation. (A) H&E staining of grafts and the ISHLT score. Each point represents the score from one slice. The horizontal line represents the mean for each group (n=3 mice/group; $**P < 0.01$; $*** P < 0.001$). (B) Flow cytometric analysis of graft-infiltrating lymphocytes. (C) Gene expression levels of IL-2, IFN-γ, IL-10, Foxp3, and TGF-β detected by qRT-PCR. Each reaction was carried out in triplicate. Data are representative of three separate experiments (n=3 mice/group; $*P < 0.05$; $***P < 0.001$).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S2
S3
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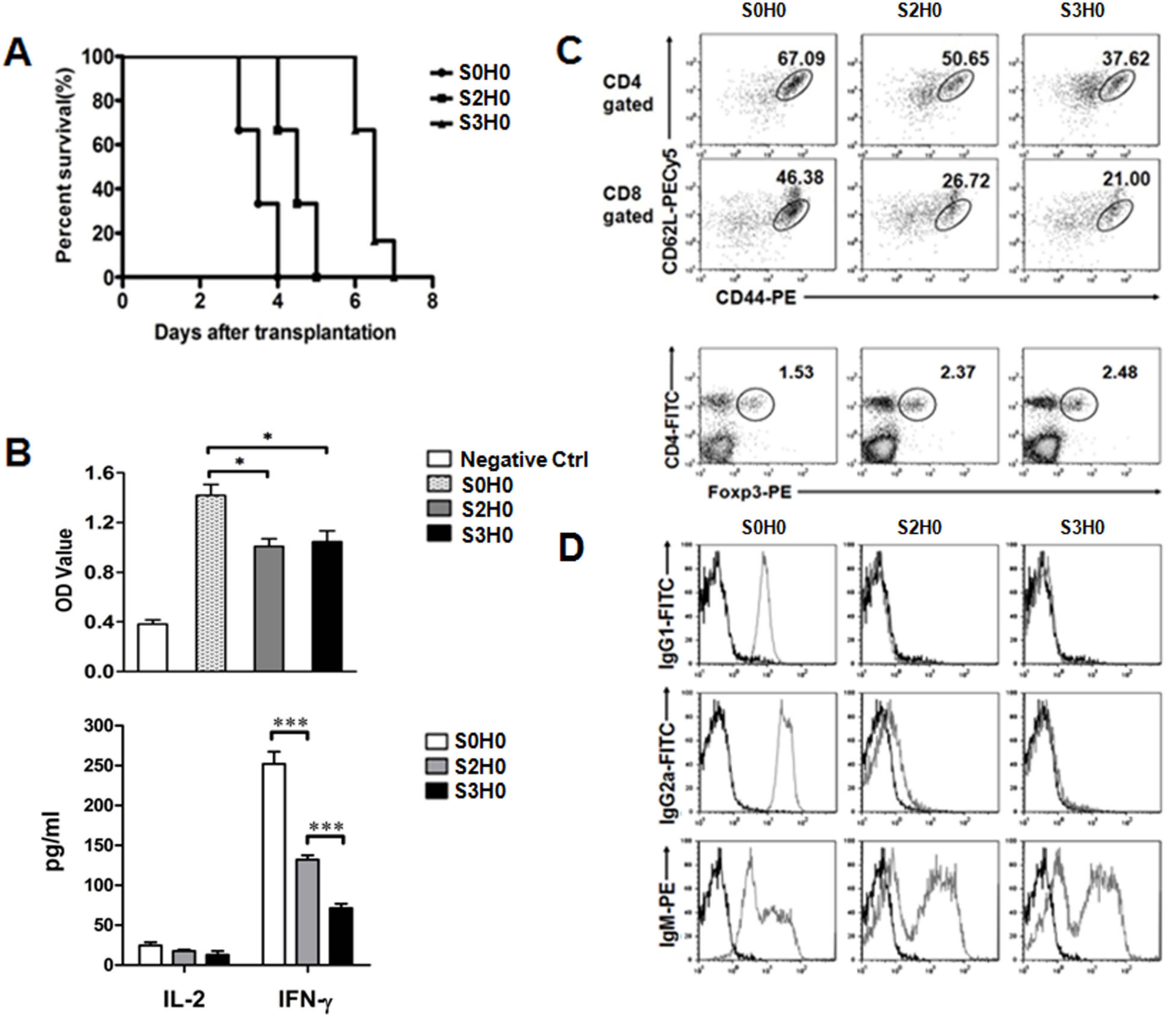
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Percent survival(%)
Days after transplantation
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B
OD Value
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pg/ml
IL-2
IFN-γ
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S2H0
S3H0
CD4 gated
CD8 gated
CD62L-PECy5
CD44-PE
67.09
50.65
37.62
46.38
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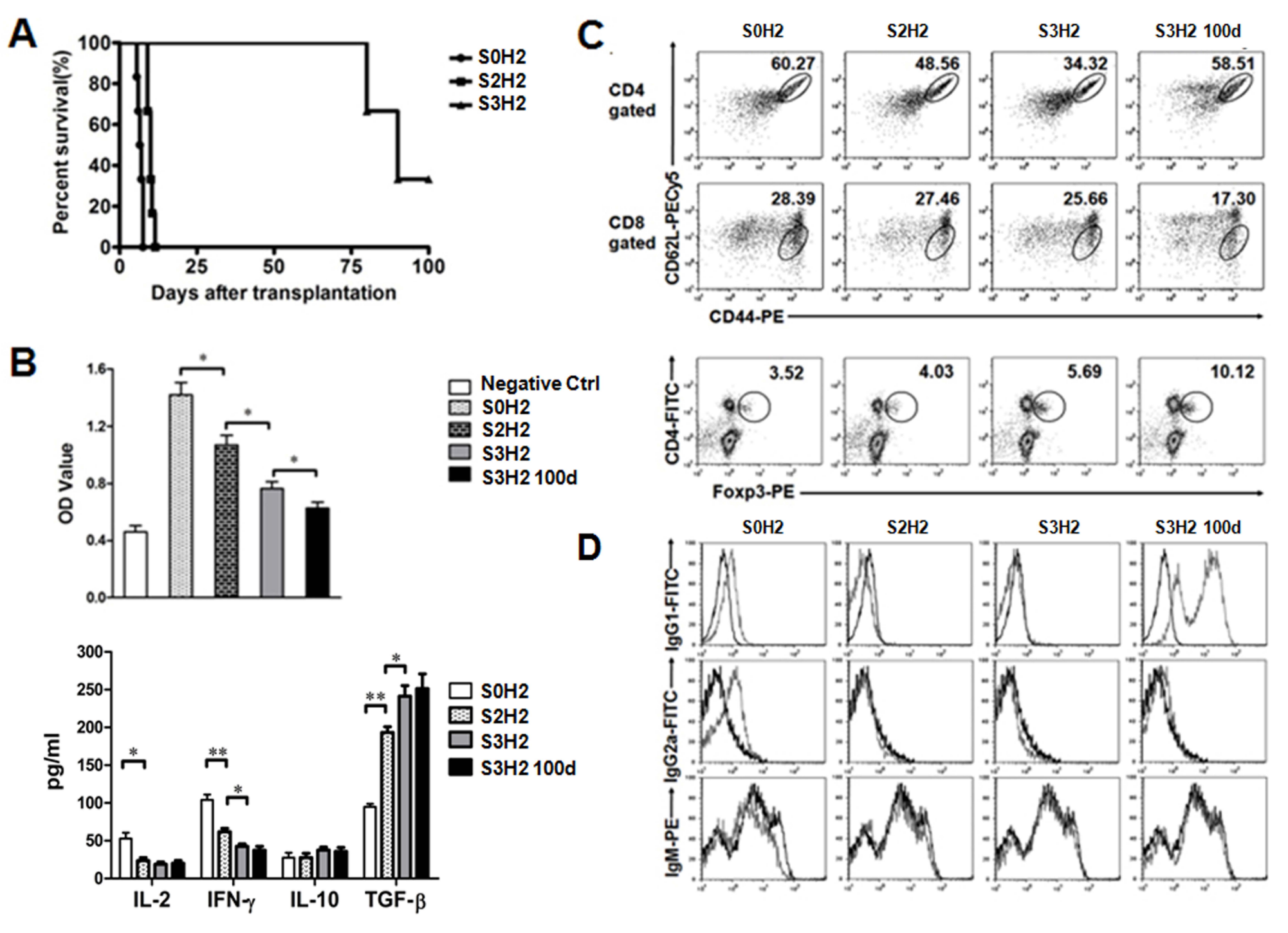
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Percent survival(%)
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CD4 gated
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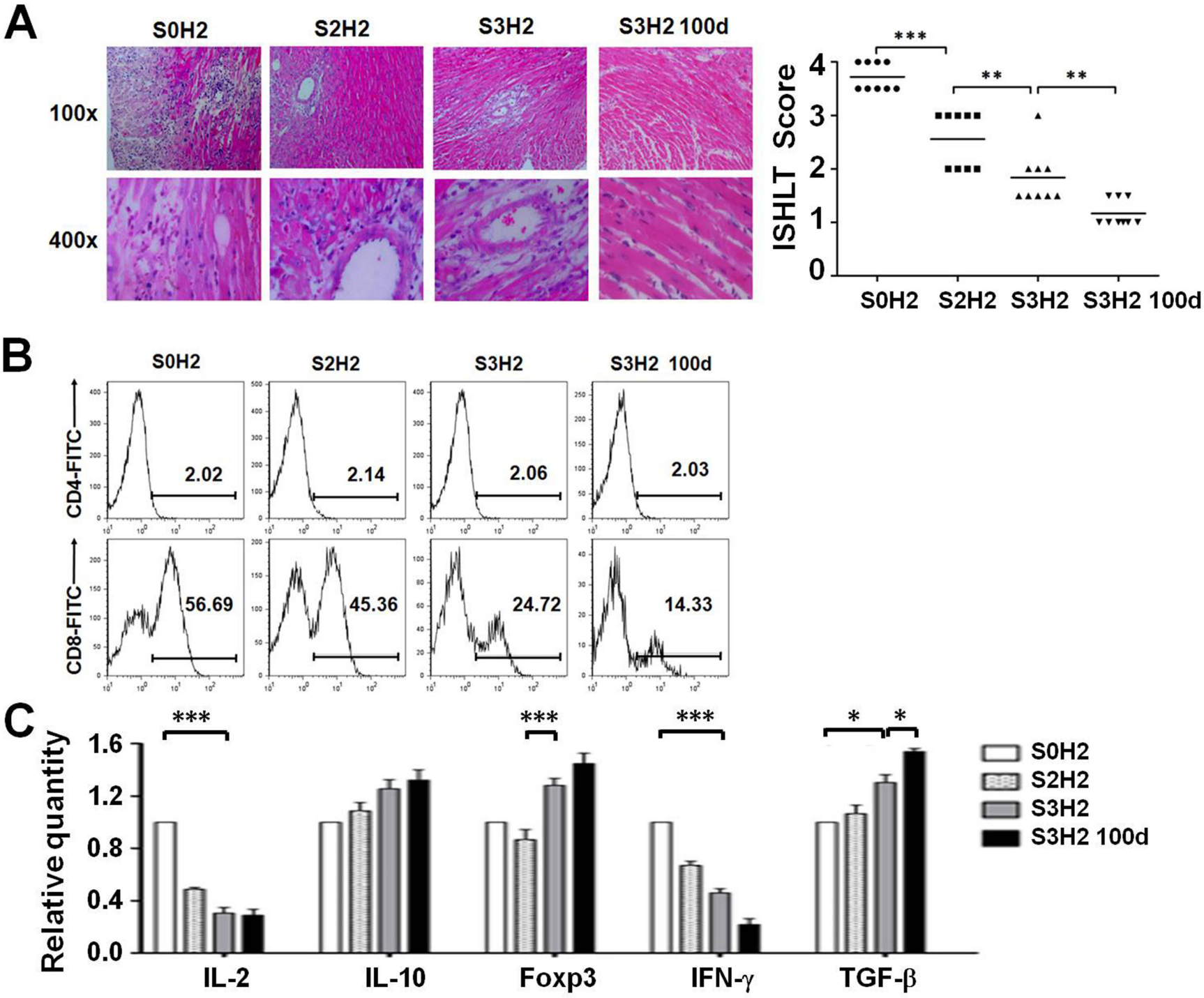
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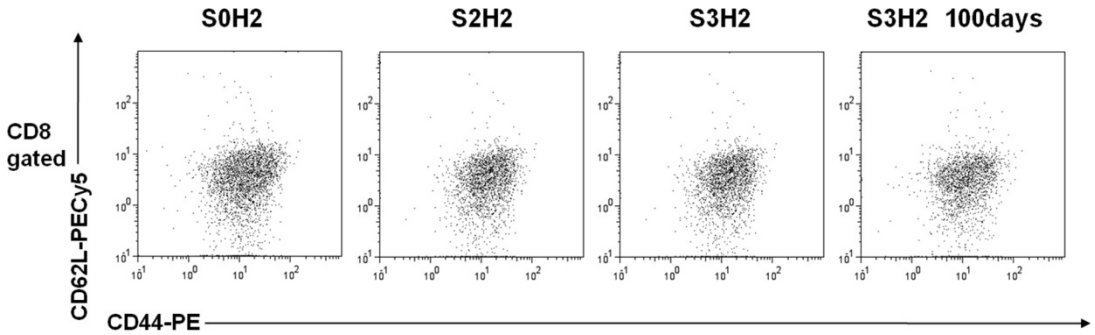

Supplement figure1. The character of CD8$^+$ T in the grafts after secondary tranplantation. The lymphocytes from the recipients' grafts were analyzed by flow cytometry. CD8$^+$ Tcells were effector/memory T cells. Data are representative of three independent experiments.