Original Research Paper

Administration of Donor Splenocytes via the Respiratory Tract Generates CD8α$^+$ Regulatory Dendritic Cells and Induces Hyporesponsiveness to Fully Allogeneic Cardiac Grafts

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Authors' contributions

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D. Iwami, O. Aramaki, N. Shirasugi, and M. Niimi participated in the research study.

D. Iwami and N. Shinohara participated in data analysis.

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Abbreviations

DC: dendritic cells, ELISA: enzyme-linked immunosorbent assay, IL: interleukin, ITD: intratracheal delivery, LPS: lipopolysaccharide, mAb: monoclonal antibody, MHC: major histocompatibility complex, MST: median survival time, PBS: phosphate-buffered saline, pLN: paratracheal lymph nodes, RT: room temperature, Tregs: regulatory T cells

Abstract

Background: We previously showed that pretreatment with intratracheal delivery (ITD) of alloantigen induced prolonged cardiac allograft survival and generated regulatory T cells (Tregs) in mice. In this study, we examined the role of splenic dendritic cells (DCs) in the ITD model.

Methods: CBA mice were treated with ITD from C57BL/10 splenocytes and 7 days later received transplantation of C57BL/10 hearts. In adoptive transfer studies, splenic DCs from ITD-treated mice were transferred into naïve CBA recipients that received C57BL/10 hearts immediately after the transfer. In addition, to determine the role of splenic DCs isolated from ITD-treated mice, the cells were incubated under stimulation with lipopolysaccharide (LPS).

Results: ITD-treated CBA recipients had markedly prolonged allograft survival (median survival time [MST], 67 days) while naïve recipients rejected allografts acutely (MST, 8 days). In adoptive transfer studies, CBA recipients of the transfer of splenic DCs from ITD-treated mice had prolonged allograft survival (MST, 85 days), while CBA recipients of the transfer of splenic DCs from naïve mice did not have prolonged allograft survival (MST, 8 days). In another transfer study, CBA recipients of the transfer of splenic CD8α^+ DCs

from ITD-treated mice had prolonged allograft survival (MST, 79 days), while those receiving splenic CD8α$^-$ DCs from ITD-treated mice did not have prolonged allograft survival (MST, 8 days). *In vitro* studies showed that ITD-treated splenic DCs produced more IL-10 and less IL-12 than naïve splenic DCs under stimulation with LPS.

Conclusions: ITD pretreatment induces regulatory DCs, which produce high amounts of IL-10 resulting in the prolongation of graft survival in our model.

Keywords: regulatory T cell, trachea, transplantation, regulatory dendritic cell, mouse

1. Introduction

Active suppression mediated by regulatory cells is important for the maintenance of transplant tolerance. We have previously shown that pretreatment with the intratracheal delivery (ITD) of alloantigen induced hyporesponsiveness to fully mismatched cardiac allografts and induced donor-specific regulatory cells including $CD4^+CD25^+$ regulatory T cells (Tregs) in the spleen within 7 days [1-3]. Various costimulatory pathways [4-6] and IL-10 are essential [7] for the generation of regulatory cells after ITD. Moreover, we showed that intratracheally delivered donor splenocytes were not detected in paratracheal lymph nodes (pLN) or spleen implying these delivered cells were immediately digested and processed without migrating out of the lung [1, 3]. In addition, the depletion of alveolar macrophages, which regulate the reaction against exogenous antigen, impaired the induction of Tregs leading to the abrogation of graft survival prolongation [3]. However, the exact mechanism of Tregs induction in the spleen, especially the initial events in the respiratory system after ITD, remains unknown.

Dendritic cells (DCs), specialized antigen-presenting cells, are integral for the generation of Tregs [8]. Thus, DCs with tolerogenic potential might be a candidate for the generation of Tregs, which protect allografts in our ITD model.

2. Objectives

In this study, we demonstrated that ITD-treated DCs induce fully mismatched cardiac allograft survival, that regulatory DCs induced by ITD treatment secreted high amounts of IL-10 and induced Tregs *in vivo,* and that $CD8\alpha^+$ DCs were the major subpopulation of the regulatory DCs.

3. Materials and Methods

3.1. Mice

Inbred male C57BL/10 ($H\text{-}2^b$, donors) and CBA ($H\text{-}2^k$, recipients) mice at 8–12 weeks of age were purchased from Sankyo Ltd. (Tokyo, Japan), housed in conventional facilities in the Biomedical Service Unit of Teikyo University, and used in accordance with protocols for animal experiments approved by the Animal Care and Use Committee of Teikyo University.

3.2. Reagents

Lipopolysaccharide (LPS) was purchased from Sigma Chemical Co. (St. Louis, MO). IL-10 recombinant standard, capture monoclonal antibody (mAb) (JES5-2A5), and

detection mAb (JES5-16E3) for enzyme-linked immunosorbent assays (ELISA) were purchased from BD Biosciences (San Jose, CA). The capture and detection mAbs for IL-12 p40 (C15.6 and C17.8, respectively) and recombinant IL-12 p40 were from PeproTech (London, UK). For flow cytometry, fluorochrome-conjugated mAbs for CD11c (HL3), CD8α (53-6.7), CD80 (16-10A1), CD86 (GL1), and CBA MHC class II I-E^k (17-3-3) were purchased from BD Biosciences; F4/80 (BM8) and CD103 (2E7) were purchased from BioLegend (San Diego, CA); CD205 (DEC205) was purchased from Miltenyi Biotec (Teterow, Germany); CD68 (FA-11) was purchased from Bio-Rad (Oxford, UK); and CD207 (eBioL31) was purchased from eBioscience (San Diego, CA).

3.3. Intratracheal Delivery of Alloantigen

C57BL/10 splenocytes were used as alloantigens and ITD was performed as previously described [1]. In brief, CBA mice were placed under general anesthesia, the trachea was exposed by dissection of the overlying muscles, and 1×10^7 C57BL/10 splenocytes in 100 μl phosphate-buffered saline (PBS) were injected directly into the trachea through a 30 G needle (Fig. 1A).

3.4. Heart Transplantation

CBA mice were given ITD of donor splenocytes and 7 days later, the CBA mice

underwent transplantation of fully vascularized heterotopic heart grafts from C57BL/10 mice with microsurgical techniques [9]. Graft rejection was defined as complete cessation of heartbeat and confirmed by direct visualization of the graft as well as its histology.

3.5. Adoptive Transfer Studies

Adoptive transfer studies were conducted to determine whether regulatory cells were generated after ITD and which subsets were involved in the regulatory function *in vivo*. As shown in Figure 1B, splenocytes were obtained from CBA mice that had been given ITD of C57BL/10 splenocytes (1×10^7 cells) 7 days previously. Naïve CBA mice intravenously injected with whole splenocytes (5×10^7 cells in 100 µl of PBS) from ITD-treated mice then received the transplantation of C57BL/10 hearts on the same day. In some groups, $CD11c^+$ DCs, $CD8\alpha^+CD11c^+$ or $CD8\alpha^-CD11c^+$ DCs were isolated from naïve or ITD-treated (7 days previously) CBA mice spleens by AUTOMACS (Miltenyi Biotec Inc., Auburn, CA) and the cells were transferred into naïve CBA recipients (1×10^6 cells per mouse) that received cardiac transplantation.

In addition, to determine the function of splenic DCs to induce Tregs *in vivo*, a sequential adoptive transfer study was performed (Fig. 1C). In an additional study, splenic DCs (1×10^6 cells) from ITD-treated (7 days previously) CBA mice were transferred into

naïve CBA recipients (primary recipients) with C57BL/10 heart transplantation. Fourteen days after the transplant with adoptive DC transfer, splenic $CD4^+$ T cells (2×10^7 cells) were isolated from the DC-transferred CBA recipients with cardiac allografts and adoptively transferred into another group of naive CBA recipients (secondary recipients) that received transplantation of C57BL/10 hearts.

3.6. Isolation of Splenic DCs

DCs were isolated from the mouse spleen of naïve, ITD-treated, or heart transplant recipients. Briefly, spleens were digested with collagenase D (1 mg/mL; Boehringer Mannheim, Roche, Germany), and mononuclear cells were isolated by Histopaque 1083 (Sigma-Aldrich Inc., Saint Louis, MI) gradient centrifugation. Then, $CD11c^+$ cells were isolated using AUTOMACS and a mouse pan Dendritic Cell Isolation Kit (Miltenyi Biotec Inc.). Purity was >95% as determined by microscopy. Splenic $CD8\alpha^+$ DCs and $CD8\alpha^-$ DCs were also isolated by AUTOMACS and a mouse $CD8\alpha^+$ Dendritic Cell Isolation Kit (Miltenyi Biotec Inc.).

3.7. Flow Cytometry

All staining reactions were performed at 4°C. First, cells were incubated with Fcγ receptor II/III mAb (2.4G2; BD Biosciences) to reduce nonspecific binding. To detect

splenic DCs, single cell suspensions were stained with fluorochrome-labeled anti-CD11c and anti-CD8α mAbs. $CD8\alpha^+$ and $CD8\alpha^-$ splenic DCs were further stained with anti-DEC205, F4/80, CD68, CD11b, CD103, CD207 and MHC II ($I\text{-}E^k$) mAbs [10]. Fluorescence was analyzed by FACSCalibur and FACSCanto II (BD Bioscience, San Jose, CA).

3.8. Cytokine Assays

To determine the function of DCs to secrete cytokines *in vitro*, splenic $CD11c^+$ DCs isolated by AUTOMACS from naïve CBA or ITD-treated CBA mice were stimulated by LPS and concentrations of IL-10 and IL-12 in the culture media were assessed by ELISA. For stimulation, splenic DCs were seeded at 2×10^5 cells/well in 96-well culture plates and stimulated with LPS (1 or 10 μg/ml) or unstimulated for 24 hours [11]. After stimulation, cell-free supernatants were collected and analyzed by ELISA as described previously [12].

3.9. Statistical Analysis

Survival times for cardiac allografts in different groups of mice were compared by the log-rank test. The results of ELISA and flow cytometric studies were compared by unpaired Student's *t*-test or analysis of variance (ANOVA) with a post *ad hoc* Tukey-Kramer method. Analysis was performed using GraphPad Prism ver. 6.0 (GraphPad Software Inc., La Jolla,

CA). Values of $p < 0.05$ were considered statistically significant differences between two groups.

4. Results

4.1. Intratracheal Delivery of Alloantigen Prolongs Allograft Survival and Induces Regulatory Cells in Recipients

As we previously reported [1]**,** the ITD of C57BL/10 splenocytes into CBA recipients 7 days before transplantation significantly prolonged the survival of cardiac allografts (MST, 67 days) compared with untreated (naïve) recipients (MST, 8 days, $p<0.0001$, Fig. 2A). The ITD of syngeneic (CBA) splenocytes did not prolong C57BL/10 cardiac allograft survival in CBA recipients (MST, 8 days, Fig. 2A). The optimal number of splenocytes for ITD treatment and the optimal time for cardiac transplant after ITD treatment were initially determined based on additional experiments. First, we administered various numbers of splenocytes to recipients for ITD treatment before cardiac transplantation. We divided mice into 4 groups of cardiac transplant recipients with ITD pretreatment of 1×10^5, 1×10^6, 1×10^7, or 1×10^8 splenocytes 7 days before transplant. Their MSTs were 12, 36, 84, and 78 days, respectively. These data suggest that the effect on prolongation of allograft survival induced

by ITD treatment is dose (cell number)-dependent and that the optimal cell number for ITD treatment is 1×10^7. Then, we divided mice into 5 groups to determine the optimal time after ITD treatment for cardiac transplantation. We performed cardiac transplantation at 1, 3, 7, 14, and 28 days after ITD treatment. MSTs in these groups were 9, 11, 84, 23, and 11 days, respectively, suggesting that the optimal time for cardiac transplantation is 7 days after ITD treatment.

To determine the presence of regulatory cells after ITD, we adoptively transferred splenocytes into naïve CBA recipients that received cardiac transplantation (Fig. 1B). As we reported previously [6], the adoptive transfer of splenocytes into naïve CBA recipients from CBA mice treated with the ITD of C57BL/10 splenocytes 7 days previously, significantly prolonged the survival of C57BL/10 cardiac allografts (MST, 72.5 days). In contrast, CBA recipients adoptively transferred splenocytes from naive CBA mice rejected C57BL/10 hearts (MST, 9 days, $p<0.0001$ vs. adoptive transfer of ITD-treated splenocytes, Fig. 2B). These results suggested that the ITD of allogeneic splenocytes induced regulatory cells in recipient spleens. The optimal number of splenocytes and optimal timing for the adoptive transfer after ITD pretreatment were also determined based on additional experiments using recipient groups with various timings for the adoptive transfer of

splenocytes from ITD-treated mice. In the experiment, 5×10^7 splenocytes were isolated from ITD-treated mice at 1, 3, 7, 14, and 28 days after ITD. The splenocytes were transferred into naïve CBA mice that underwent cardiac transplant at the same time as the adoptive transfer. Graft survival was markedly prolonged when splenocytes were harvested for transfer 7 days after ITD (MST, 68 days), while graft survival was modestly or barely prolonged with when harvested at 1, 3, 14, and 28 days after ITD treatment (MSTs, 12, 24, 28 and 14 days, respectively). These results indicate that 7 days is the optimal time for the generation of regulatory cells after the intratracheal delivery of splenocytes. Furthermore, we assessed the optimal dose for adoptive transfer after ITD of donor splenocytes. Naive CBA mice were adoptively transferred 5×10^5, 5×10^6, or 5×10^7 splenocytes, from CBA mice pretreated 7 days earlier with ITD of 1×10^7 C57BL/10 splenocytes. Immediately after the adoptive transfer, CBA mice underwent transplantation of C57BL/10 hearts. The CBA recipients receiving the adoptive transfer of 5×10^6 splenocytes had moderate increases in the survival of C57BL/10 grafts (MST, 32 days), compared with recipients adoptively transferred 5×10^7 splenocytes from ITD-treated mice (MST, 68 days). When CBA recipients were adoptively transferred 5×10^5 splenocytes from CBA mice pretreated 7 days

earlier with ITD, C57BL/10 cardiac grafts were rejected acutely (MST, 12 days). Thus, 5×10^7 splenocytes is the optimal dose for adoptive transfer in our ITD model.

We previously showed that regulatory cells induced by ITD included $CD4^+CD25^+$ Tregs [3]. Moreover, the results from our preliminary study of the adoptive transfer of splenic DCs suggested the possible existence of a DC population in the regulatory cells [3]. To confirm our preliminary results, we isolated $CD11c^+$ DCs from the spleens of ITD-treated or untreated (naïve) mice by AUTOMACS and then transferred them into CBA recipients (Fig. 1B). Cardiac graft survival was significantly prolonged compared with the secondary recipients transferred with splenic DCs from naïve mice (MST, 85 and 9 days, respectively, $p<0.01$, Fig. 2C).

These results indicated that one population of regulatory cells induced by ITD is $CD11c^+$ cells (i.e. regulatory DCs).

4.2. Regulatory DCs induced by ITD induce Tregs *in vivo*

To determine the regulatory function of splenic DCs from ITD-treated mice (regulatory DCs induced by ITD) to prolong allograft survival, we performed a sequential adoptive transfer study. As shown in Figure 1C, C57BL/10 splenocytes were intratracheally delivered into naive CBA mice. Seven days later, splenic DCs from ITD-treated mice were

adoptively transferred into primary CBA recipients that subsequently received C57BL/10 hearts. Then, 14 days later, splenic $CD4^+$ T cells from primary recipients were transferred into naïve CBA recipients (secondary recipients) that received a C57BL/10 cardiac allograft immediately. Secondary recipients had a markedly prolonged allograft survival compared with recipients transferred with splenic $CD4^+$ T cells from naïve mice (MST, >100 and 12 days, respectively, $p<0.01$, Fig. 3A). These results suggested that the adoptive transfer of splenic DCs from ITD-treated mice induced $CD4^+$ Tregs in secondary recipients as well as prolonging graft survival.

To determine the characteristics of regulatory DCs *in vitro*, splenic DCs from ITD-treated mice or naïve CBA mice were incubated with LPS (1 μg/ml or 10 μg/ml) or without LPS. Naïve DCs produced a small amount of IL-10 either with or without LPS stimulation (untreated, 18±4 pg/ml; 1 μg/ml of LPS, 25±3 pg/ml; 10 μg/ml of LPS, 22±5 pg/ml, respectively, Fig. 3B). In contrast, DCs from ITD-treated mice produced significantly higher amounts of IL-10 compared with naïve splenic DCs and LPS stimulation upregulated IL-10 production in a dose-dependent manner (untreated, 152±35 pg/ml; 1 μg/ml of LPS, 524±87 pg/ml; 10 μg/ml of LPS, 2189±516 pg/ml, respectively, $p<0.05$ compared with naive counterparts, Fig. 3B). Naïve DCs produced a small amount of

IL-12 without stimulation and LPS upregulated IL-12 in a dose-dependent manner (untreated, 118.7±17.3 ng/ml; 1 μg/ml of LPS, 2309.4±265.2 ng/ml; 10 μg/ml of LPS, 3587.9±953.1 ng/ml, respectively, Fig. 3C). In contrast, ITD-treated DCs did not produce IL-12 when unstimulated or stimulated with LPS (untreated, 86±13.9 ng/ml; 1 μg/ml LPS, 261.8±98.7 ng/ml; 10 μg/ml LPS, 168.3±72.6 ng/ml, respectively, Fig. 3C). These results indicated that ITD-treated DCs have the potential to produce more IL-10 compared with naïve DCs or unstimulated ITD-DCs.

4.3. Regulatory DCs from ITD-treated mice are positive for CD8α

To determine which subpopulation of ITD-treated splenic DCs has regulatory function, splenic $CD8\alpha^+$ or $CD8\alpha^-$ DCs from ITD-treated mice were adoptively transferred into naïve CBA recipients that received C57BL/10 hearts immediately. $CD8\alpha^+$ DCs significantly prolonged allograft survival compared with $CD8\alpha^-$ DCs (MST, 79 and 9 days, respectively, $p<0.0001$, Fig. 4A), suggesting that a major population of regulatory DCs are $CD8\alpha^+CD11c^+$.

Flow cytometric analysis showed that the frequency of $CD8\alpha^+$ DCs was slightly but significantly higher in ITD-treated DCs in the spleen compared with naïve DCs (ITD-DC, 28.7±4.4%; naïve DCs, 19.7±1.7%, respectively, Fig. 4B, $p<0.05$). To determine their

surface molecule expression, $CD8\alpha^+$ or $CD8\alpha^-$ DCs from ITD-treated mice were analyzed for the expression of CD207 (Langerin), CD103 (αE-integrin), CD205 (DEC205), CD80, and CD86 by flow cytometry. In ITD-treated mice, $CD8\alpha^+$DCs showed a higher expression of CD80, CD86, CD103, and CD205 compared with $CD8\alpha^-$ DCs (Fig. 4C).

5. Discussion

In the current study, we demonstrated that the administration of donor antigen (i.e. donor splenocytes in this study) via the trachea induced the prolongation of fully allogeneic graft survival and generated both $CD4^+$ Tregs and DCs with regulatory function in a murine cardiac transplantation model. Our data also suggested that the major subset of regulatory DCs that induced $CD4^+$ Tregs was $CD8\alpha^+$. We previously reported that IL-10 was necessary for inducing regulatory cells by the ITD of donor cells [7]. In the current experiments, ITD-treated DCs upregulated IL-10 production upon stimulation. Taken together, this suggests that $CD8\alpha^+$ regulatory DCs are responsible for the IL-10-mediated induction of Tregs in ITD-treated mice.

Although intratracheal delivery seems an unusual route of administration, the induction of tolerance and regulatory cells by means of the respiratory mucosa has been described in previous studies of autoimmune and allergic diseases [13, 14]. Staines et al. demonstrated that the nasal inhalation of type II collagen-specific peptides reduced the mean disease severity in a rat collagen-induced arthritis model [13]. The presentation of soluble innocuous antigens to the respiratory and intestinal immune systems constantly induces antigen-specific unresponsiveness termed mucosal tolerance. Because intratracheal

administration was superior to the intravenous administration of donor splenocytes in terms of prolonging allograft survival (MST, 81 and 13 days, respectively), the route of administration has a critical role in defining the fate of allografts [15].

DCs are specialized antigen-presenting cells that have a dual role in inducing adaptive immune responses that eliminate harmful antigens and inducing immunological tolerance to innocuous antigens [16, 17]. At steady state, DCs are generally immature and are poorly immunogenic because they express only modest levels of MHC molecules and little or no co-stimulatory molecules and proinflammatory cytokines, resulting in the generation of anergic T cells and Tregs [18].

According to a review by Shortman [19], $CD8\alpha^+$ DCs represent around 20% of splenic DCs. It was also reported that $CD8\alpha^+$ DCs in peripheral lymphoid tissues are involved in preventing self-reactive responses [20]. Although splenic $CD8\alpha^+$ DCs have a similar expression of co-stimulatory molecules with splenic $CD8\alpha^-$ DCs, they induce less proliferation of both $CD4^+$ and $CD8^+$ T cells compared with splenic $CD8\alpha^-$ DCs [19, 21-24]. Splenic $CD8\alpha^+$ DCs are generally at steady state and do not produce sufficient cytokines for the sustained proliferation of T cells, resulting in their death by cytokine deprivation [21]. Yamazaki et al. also showed that splenic $CD8\alpha^+CD205^+$ DCs are more effective at

inducing Foxp3$^+$ Tregs compared with splenic CD8α^- DCs both *in vitro* and *in vivo* [25]. Consistent with their data, CD8α^+ regulatory DCs in our model also expressed CD205. In addition, it was shown that splenic DCs pretreated with ITD produced low levels of IL-12 and high levels of the regulatory cytokine, IL-10, compared with naïve splenic DCs. Their cytokine profile may lead to reduced T cell alloimmune responses and the induction of Tregs.

CD103, the α_E integrin subunit, pairs with β_7 to generate a functional integrin that binds to E-cadherin [26], and this interaction enables human and mouse intestinal leukocytes to adhere to epithelial cells [27]. In the intestine, CD103$^+$ DCs among other intestinal DCs are the critical DC subset for the induction of Tregs [28]. In the spleen, CD8α^+ DCs are positive for CD103 and CD205 and are crucial for the induction of allogeneic Tregs [29]. Qiu et al. also reported that the depletion of CD8α^+CD103$^+$ DCs in the spleen impaired tolerance induction to self-antigens [30]. Consistent with their results, it is likely that the major population of splenic regulatory DCs after ITD treatment is CD8α^+. Furthermore, CD103 might be another marker for regulatory DCs in our model.

In conclusion, the data presented showed that ITD treatment prolonged the survival of MHC-mismatched cardiac allografts and induced regulatory DCs. We found that regulatory

DCs induced by ITD treatment secreted high amounts of IL-10, induced Tregs *in vivo,* and that $CD8\alpha^+$ DCs were the major subpopulation of the regulatory DCs. We previously reported that the intratracheal administration of donor-MHC class I complex-derived peptide corresponding to the complementarity-determining region also prolonged allograft survival in mice [31]. These results suggest that both donor splenocytes and single MHC class I K^b peptide was sufficient as the door antigen for ITD pretreatment to induce the prolongation of allograft survival [31, 32]. Therefore, in patients undergoing transplantation, especially in the setting of living donor transplant in which pretreatment is possible, intratracheal delivery would be as easy to use as the oral route to induce donor-specific hyporesponsiveness. When a suitable HLA peptide is identified, the hyporesponsiveness and generation of regulatory cells induced by intratracheal delivery should be applicable to clinical transplantation. Thus, our findings may have significant implications for designing novel strategies for immunomodulatory treatments in transplantation. In clinical transplantation, the development of donor-specific regulatory cells has an advantage of being physiologic without inducing pan-immune suppression leading to infectious diseases. Several types of regulatory cells produced endogenously by the immune system might be applicable to the clinic to establish hyporesponsiveness or tolerance to alloantigens.

However, the precise mechanisms of the generation of regulatory cells remain unclear and require additional investigation.

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Figure Legends

Figure 1. Schema of the intratracheal delivery of splenocytes for single or sequential adoptive transfer study. (A) **Intratracheal delivery of alloantigen.** Donor splenocytes as the donor antigen were administered into the trachea under direct vision and 7 days later, donor heart transplantation was performed. (B) **Adoptive transfer study.** Whole splenocytes, whole splenic DCs, splenic $CD8\alpha^+$ DCs or splenic $CD8\alpha^-$ DCs from ITD-treated mice were intravenously administered into naive recipients that received allogeneic heart transplant simultaneously. (C) **Sequential adoptive transfer study.** Splenic DCs from ITD-treated CBA mice were transferred into another group of naïve CBA mice (primary recipients) with C57BL/10 heart transplantation. Then, splenic $CD4^+$ T cells were isolated from the secondary recipients and adoptively transferred into naive CBA mice (secondary recipients) that were transplanted with C57BL/10 hearts. DCs: dendritic cells, ITD: intratracheal delivery.

Figure 2. Survival of cardiac allografts in ITD-treated recipients and recipients with adoptive transfer. (A) Survival of C57BL/10 cardiac grafts in recipients with ITD pretreatment. C57BL/10 or CBA splenocytes were injected into the trachea of CBA

recipients. C57BL/10 hearts were transplanted 7 days later. (B, C) Survival of C57BL/10 cardiac grafts in recipients with the adoptive transfer of splenocytes from ITD-treated mice. (B) C57BL/10 splenocytes were intratracheally injected into CBA mice. Seven days later, whole splenocytes (5×10^7 cells) from ITD-treated mice were adoptively transferred into naïve CBA recipients that subsequently received the transplantation of C57BL/10 hearts. (C) Splenic DCs (1×10^6 cells) from naïve or ITD-treated CBA mice were isolated 7 days after ITD and adoptively transferred into naïve CBA recipients that received C57BL/10 (donor-specific) heart transplant on the same day. DC: dendritic cells, ITD: intratracheal delivery, MST: median survival time. *p<0.01, **p<0.001, ***p<0.0001 for differences between two groups.

Figure 3. (A) Cardiac allograft survival in a sequential adoptive transfer study. As shown in Figure 1C, splenic DCs were isolated from CBA mice pretreated with ITD 7 days previously, and were adoptively transferred into CBA recipients (primary recipients) that subsequently received C57BL/10 hearts. An additional 14 days later, splenic $CD4^+$ T cells from the primary recipients were adoptively transferred into the secondary recipients that received cardiac transplant from C57BL/10 mice. In the control group, splenic DCs from

naïve CBA mice were adoptively transferred into the primary CBA recipients and an additional 14 days later, splenic $CD4^+$ T cells from primary recipients were adoptively transferred into the secondary CBA recipients that received C57BL/10 heart transplant immediately. (B) Splenic DCs isolated from naïve or ITD-treated CBA mice were cultured untreated or stimulated with LPS (1 μg/ml or 10 μg/ml) and the concentrations of IL-10 and IL-12 were determined. Similar results were obtained in three independent experiments. *p<0.01, **p<0.001, ***p<0.0001 for differences between two groups. DCs: dendritic cells, IL: interleukin, ITD: intratracheal delivery. LPS: lipopolysaccharide, MST: medial survival time, N.S.: not significant.

Figure 4. Phenotypic analyses of splenic $CD8\alpha^+$ DCs and their regulatory function to prolong allograft survival. Splenic $CD8\alpha^+$ or $CD8\alpha^-$ DCs were isolated from ITD-treated CBA mice 7 days after ITD and were adoptively transferred into naïve CBA recipients that received C57BL/10 heart transplant immediately (A). Gating Strategy of $CD8\alpha^+$/$CD8\alpha^-$ DCs and their frequency in the spleen. Splenocytes from naïve or ITD-treated CBA mice were stained for CD11c and CD8α. The frequency of $CD8\alpha^+$ and $CD8\alpha^-$ DCs was determined (B). Characterization of surface phenotypic molecules of $CD8\alpha^+$/$CD8\alpha^-$ DCs in

the spleens of ITD-treated mice. Splenocytes of ITD-treated mice were evaluated for the expression of various surface molecules by flow cytometry. Representative data of the histograms are shown in the left 2 columns (dark grey represents isotype control) and the frequency of DCs positive for the markers are shown in the right column (C). DC: dendritic cells, ITD: intratracheal delivery, mAb: monoclonal antibody, MST: median survival time. *p<0.01, **p<0.001, ***p<0.0001 for differences between two groups.

Figure 1A

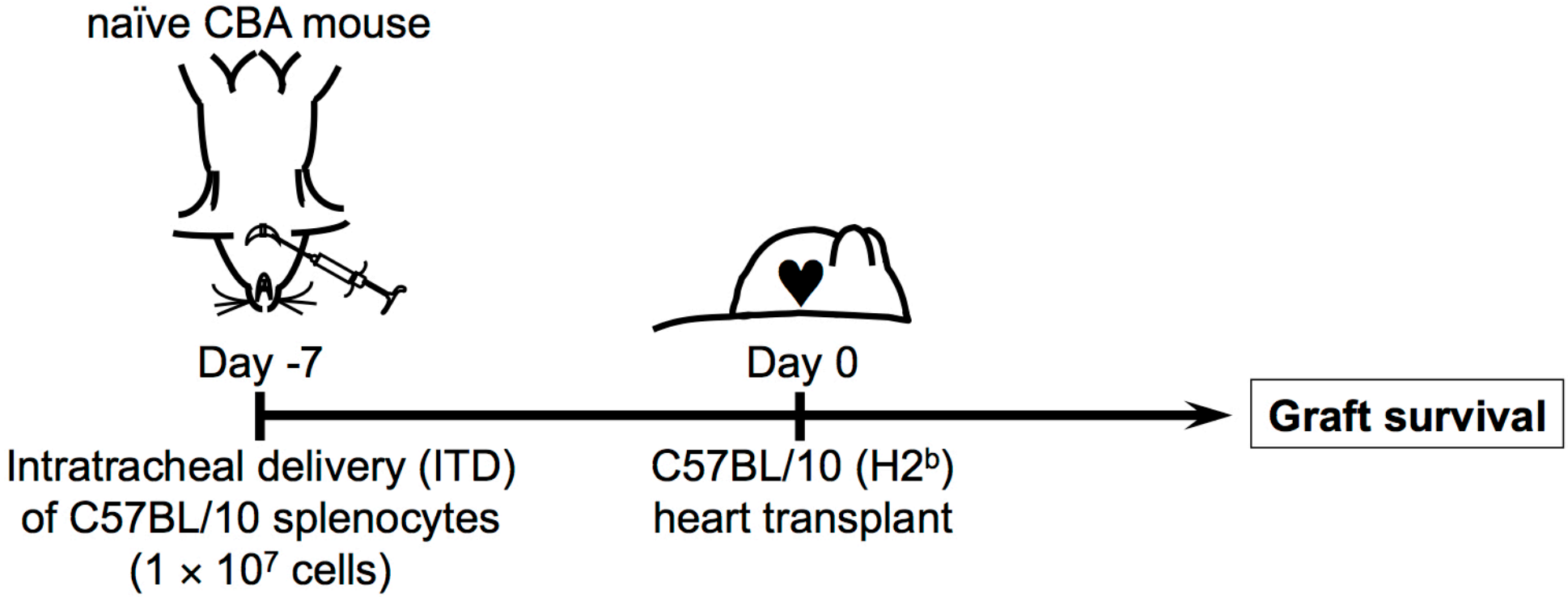

naïve CBA mouse
Day -7
Day 0
Graft survival
Intratracheal delivery (ITD)
of C57BL/10 splenocytes
(1 × 10^7 cells)
C57BL/10 (H2^b)
heart transplant

Figure 1B

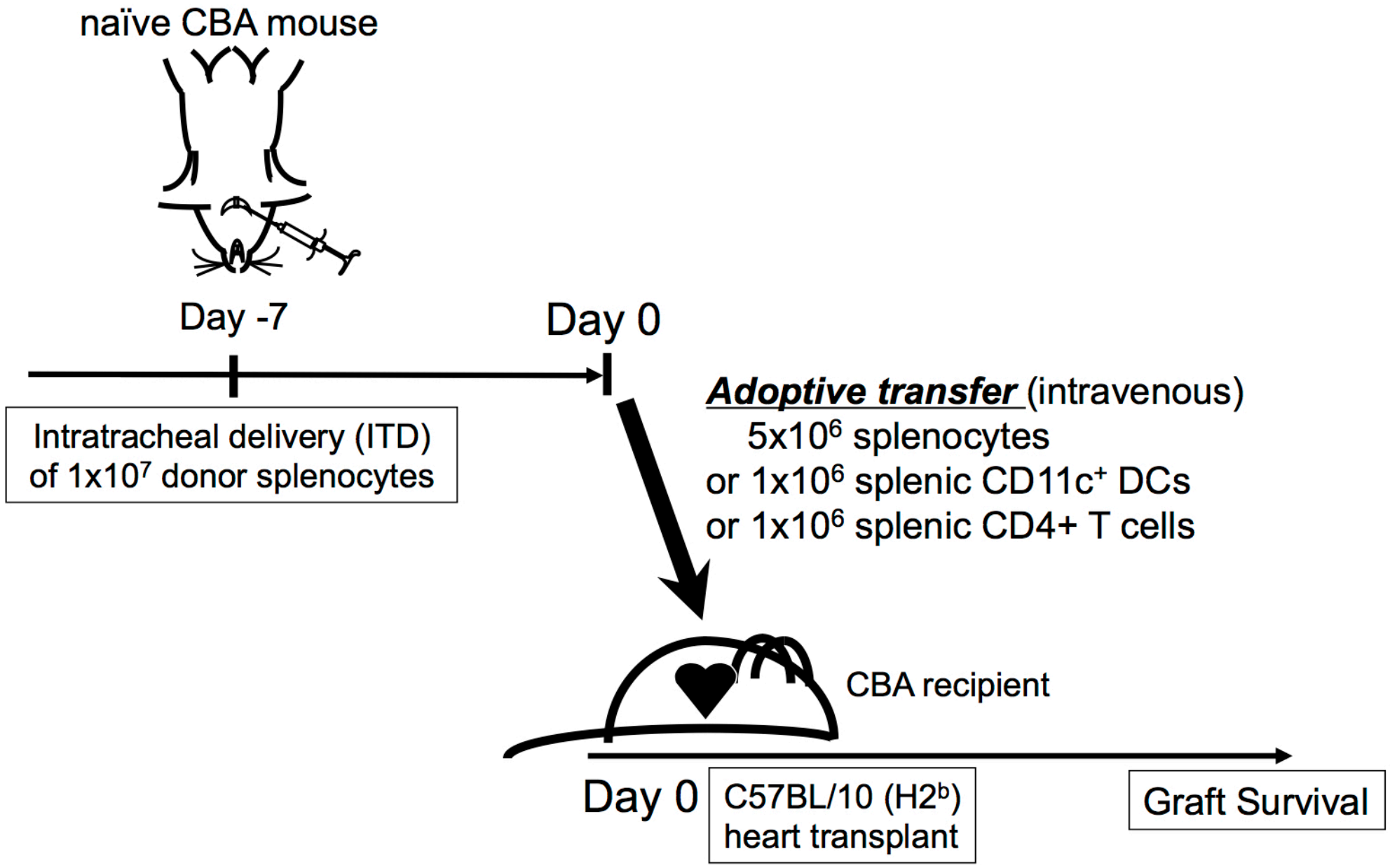

naïve CBA mouse
Day -7
Day 0
Intratracheal delivery (ITD)
of 1x10^7 donor splenocytes
Adoptive transfer (intravenous)
5x10^6 splenocytes
or 1x10^6 splenic CD11c+ DCs
or 1x10^6 splenic CD4+ T cells
CBA recipient
Day 0
C57BL/10 (H2^b)
heart transplant
Graft Survival

Figure 1C

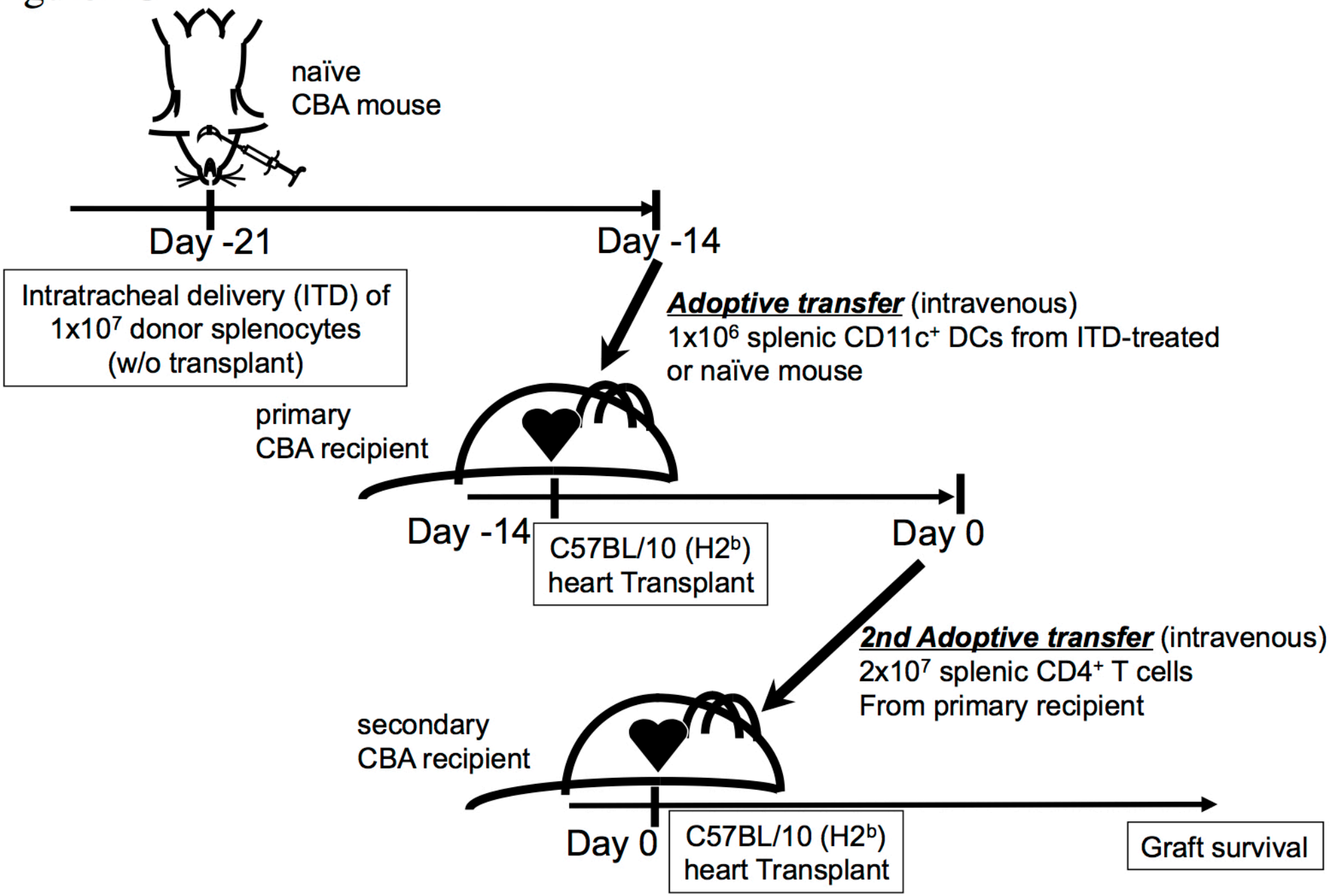

naïve
CBA mouse
Day -21
Day -14
Intratracheal delivery (ITD) of
1x10⁷ donor splenocytes
(w/o transplant)
Adoptive transfer (intravenous)
1x10⁶ splenic CD11c⁺ DCs from ITD-treated
or naïve mouse
primary
CBA recipient
Day -14
C57BL/10 (H2ᵇ)
heart Transplant
Day 0
2nd Adoptive transfer (intravenous)
2x10⁷ splenic CD4⁺ T cells
From primary recipient
secondary
CBA recipient
Day 0
C57BL/10 (H2ᵇ)
heart Transplant
Graft survival

Figure 2A

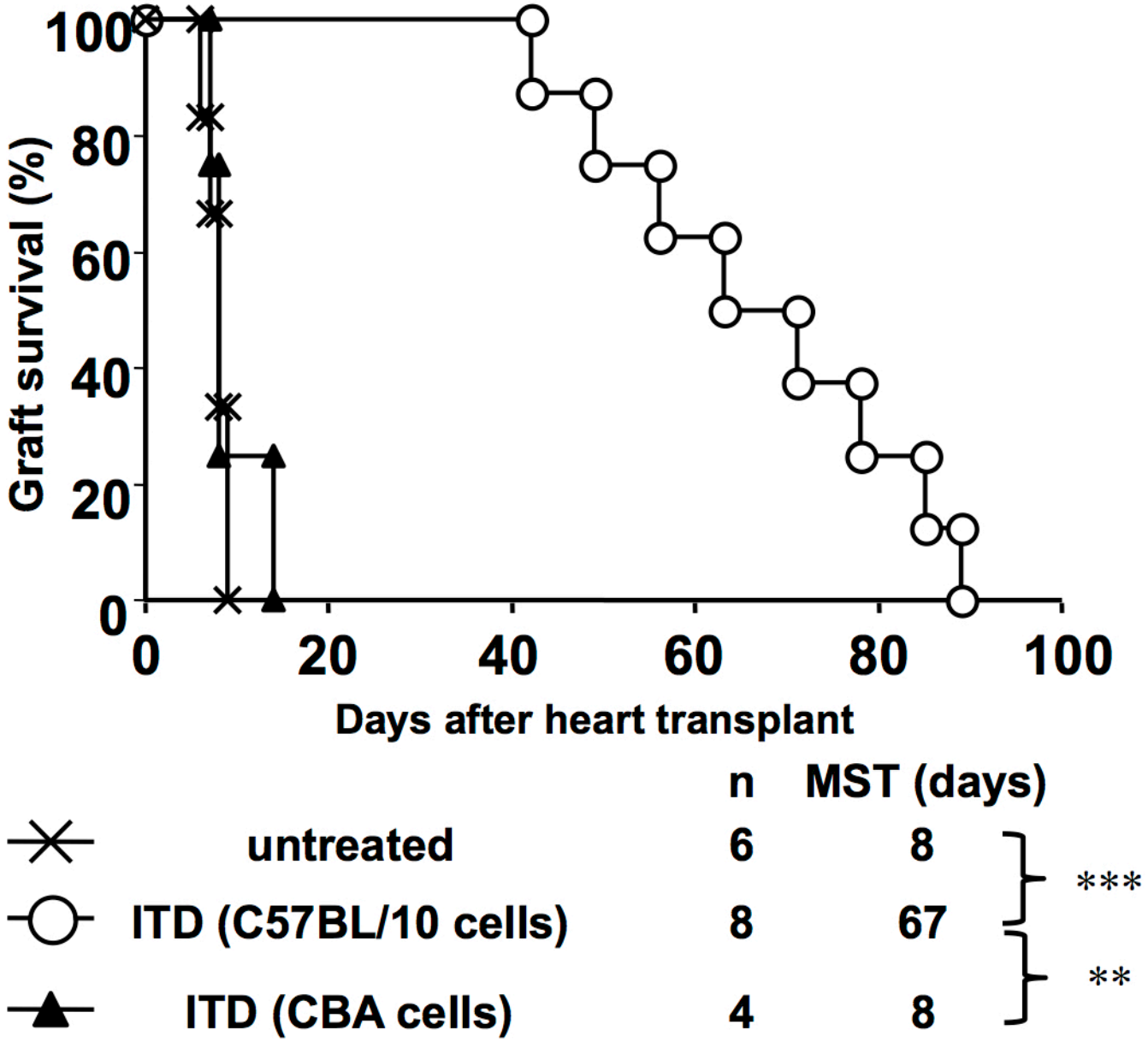

100
80
60
40
20
0
Graft survival (%)
0
20
40
60
80
100
Days after heart transplant
n
MST (days)
untreated
6
8
ITD (C57BL/10 cells)
8
67
***
ITD (CBA cells)
4
8
**

Figure 2B

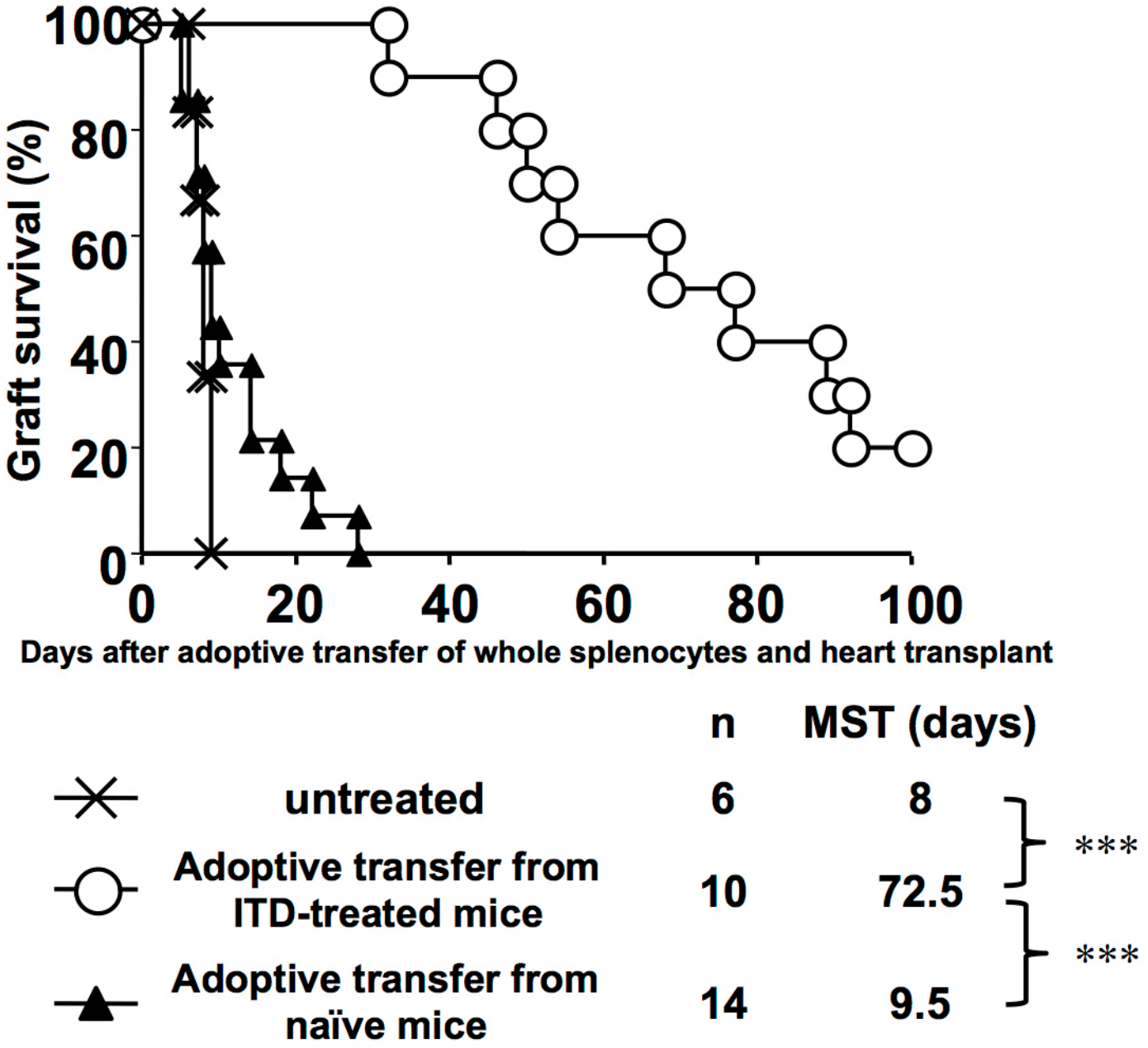

Graft survival (%)
100
80
60
40
20
0
0
20
40
60
80
100
Days after adoptive transfer of whole splenocytes and heart transplant
n
MST (days)
untreated
6
8
Adoptive transfer from ITD-treated mice
10
72.5
***
Adoptive transfer from naïve mice
14
9.5
***

Figure 2C

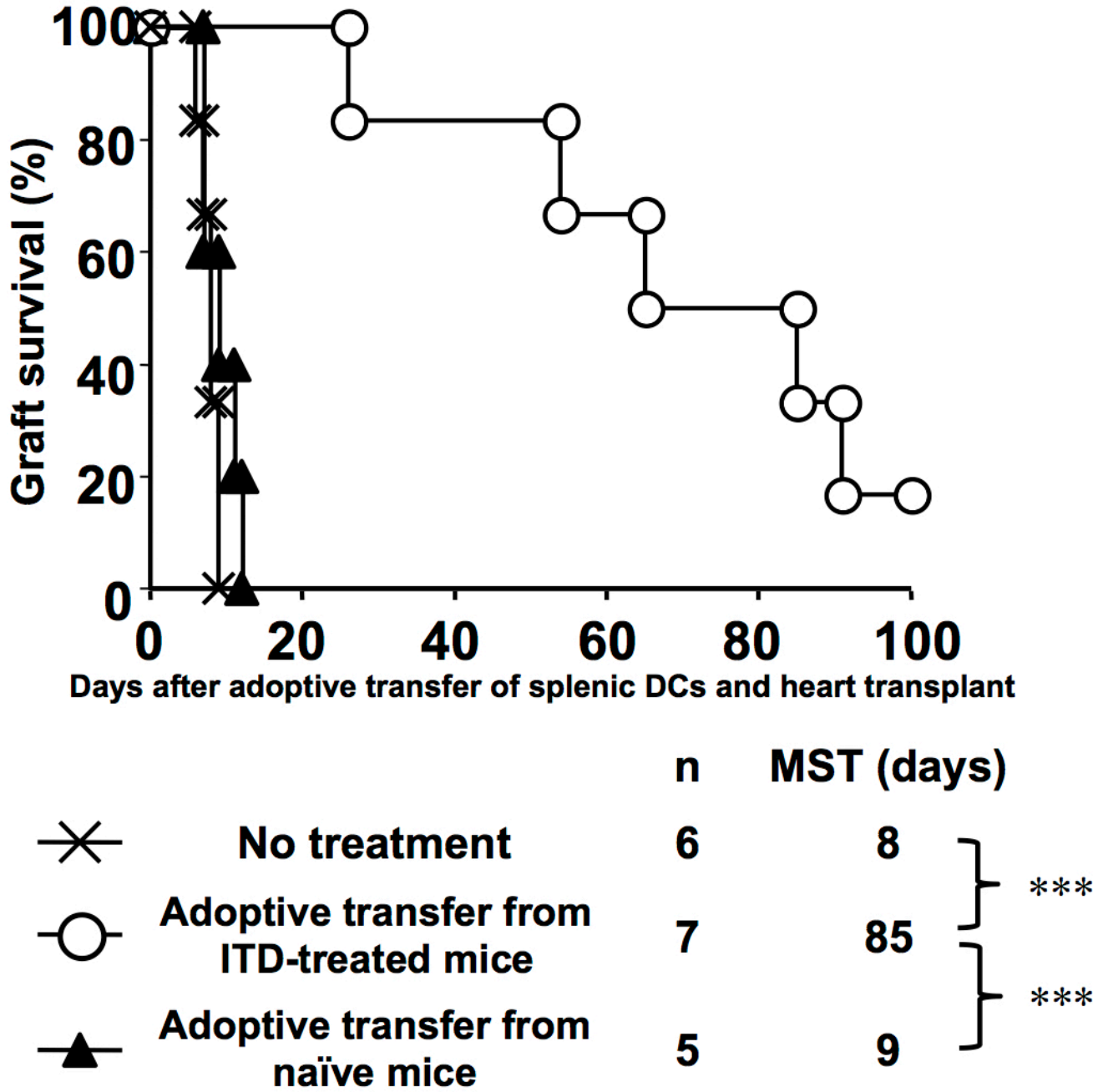

Graft survival (%)
100
80
60
40
20
0
0
20
40
60
80
100
Days after adoptive transfer of splenic DCs and heart transplant
n
MST (days)
No treatment
6
8
***
Adoptive transfer from ITD-treated mice
7
85
***
Adoptive transfer from naïve mice
5
9

Figure 3A

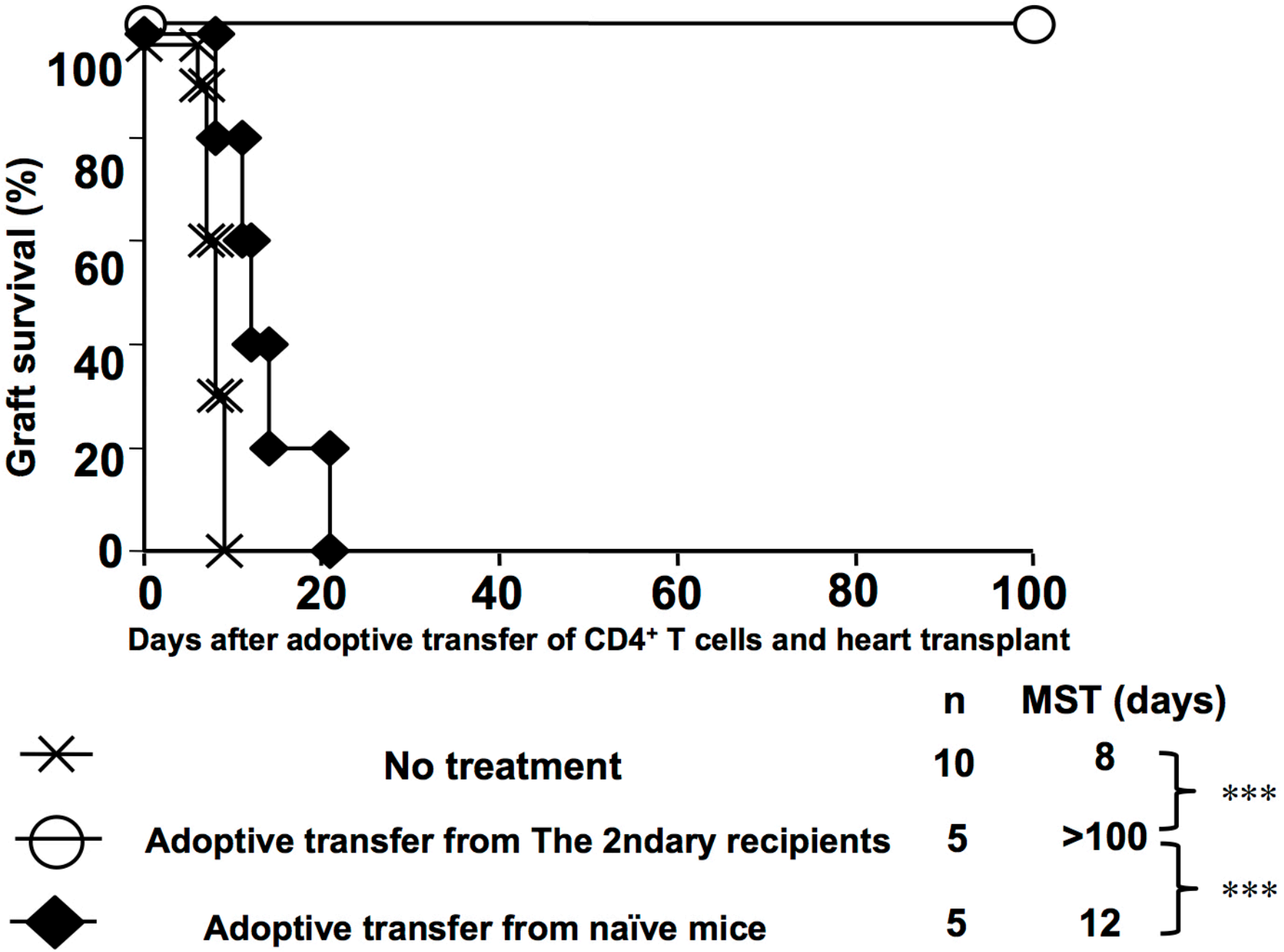

Graft survival (%)
100
80
60
40
20
0
0
20
40
60
80
100
Days after adoptive transfer of CD4+ T cells and heart transplant
n
MST (days)
No treatment
10
8
***
Adoptive transfer from The 2ndary recipients
5
>100
***
Adoptive transfer from naïve mice
5
12

Figure 3B

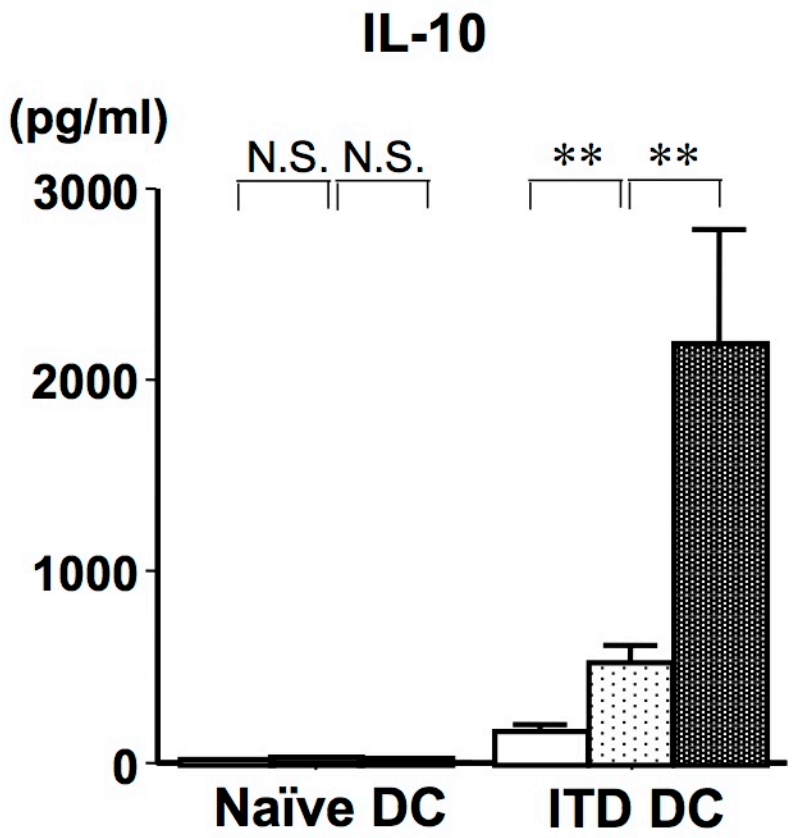
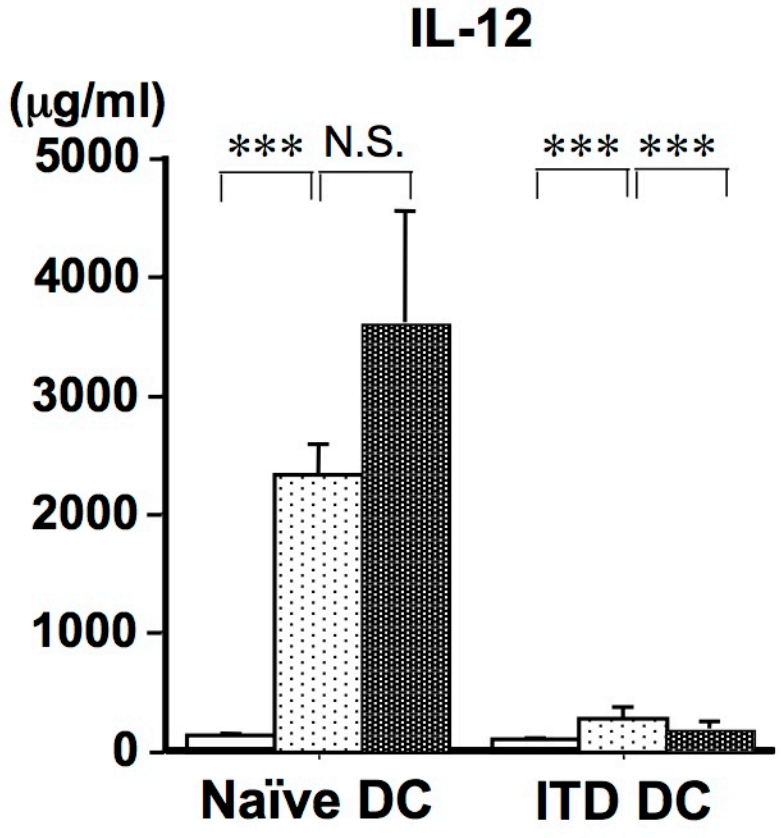


Figure 3C



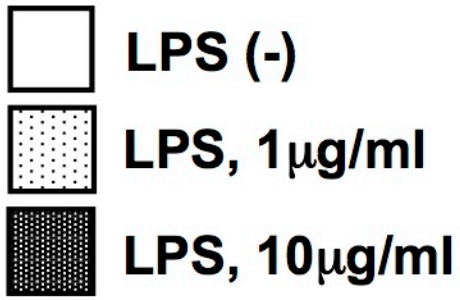


Figure 4A

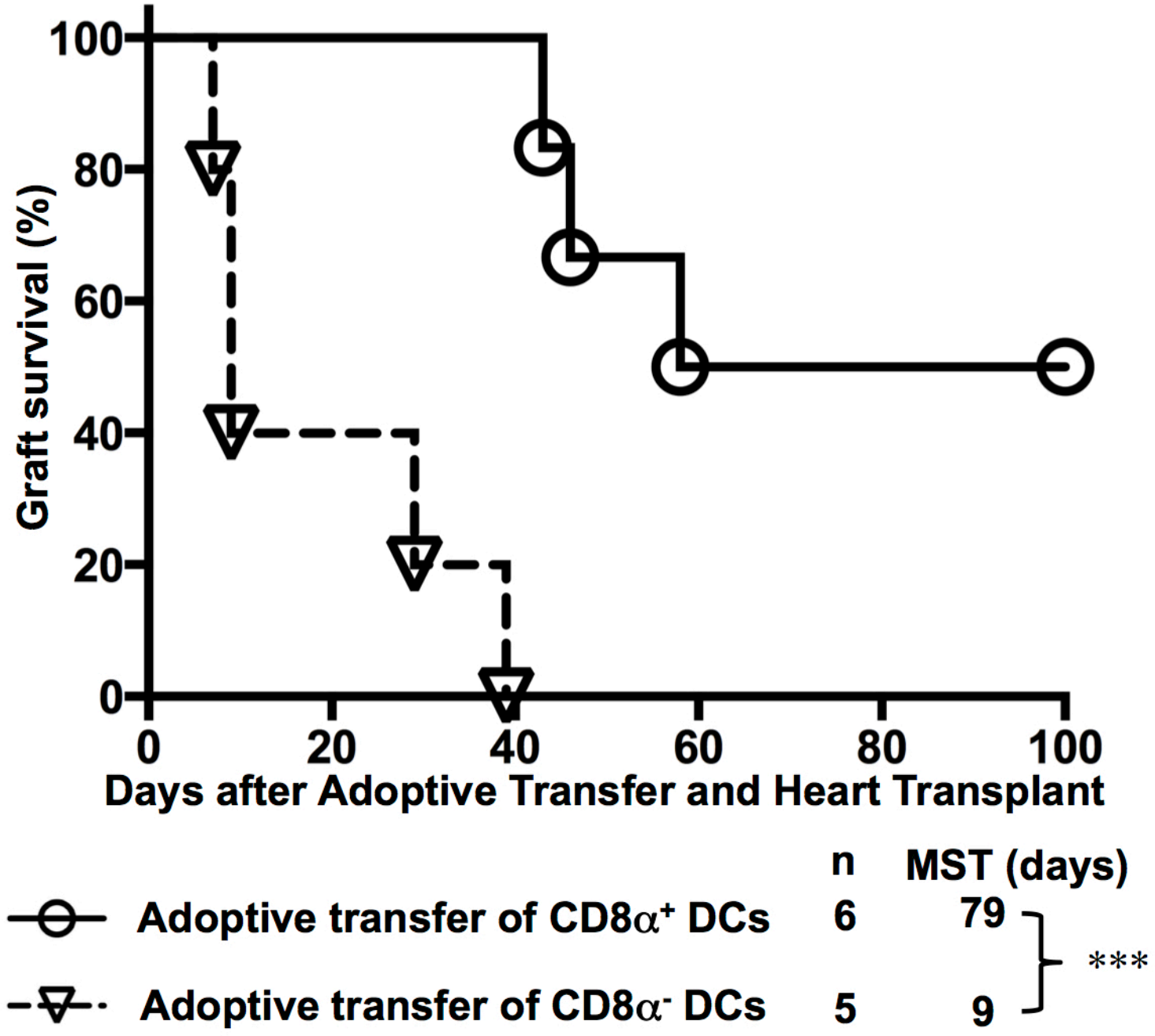

100
80
60
40
20
0
Graft survival (%)
0
20
40
60
80
100
Days after Adoptive Transfer and Heart Transplant
n
MST (days)
Adoptive transfer of CD8α+ DCs
6
79
Adoptive transfer of CD8α- DCs
5
9
***

Figure 4B

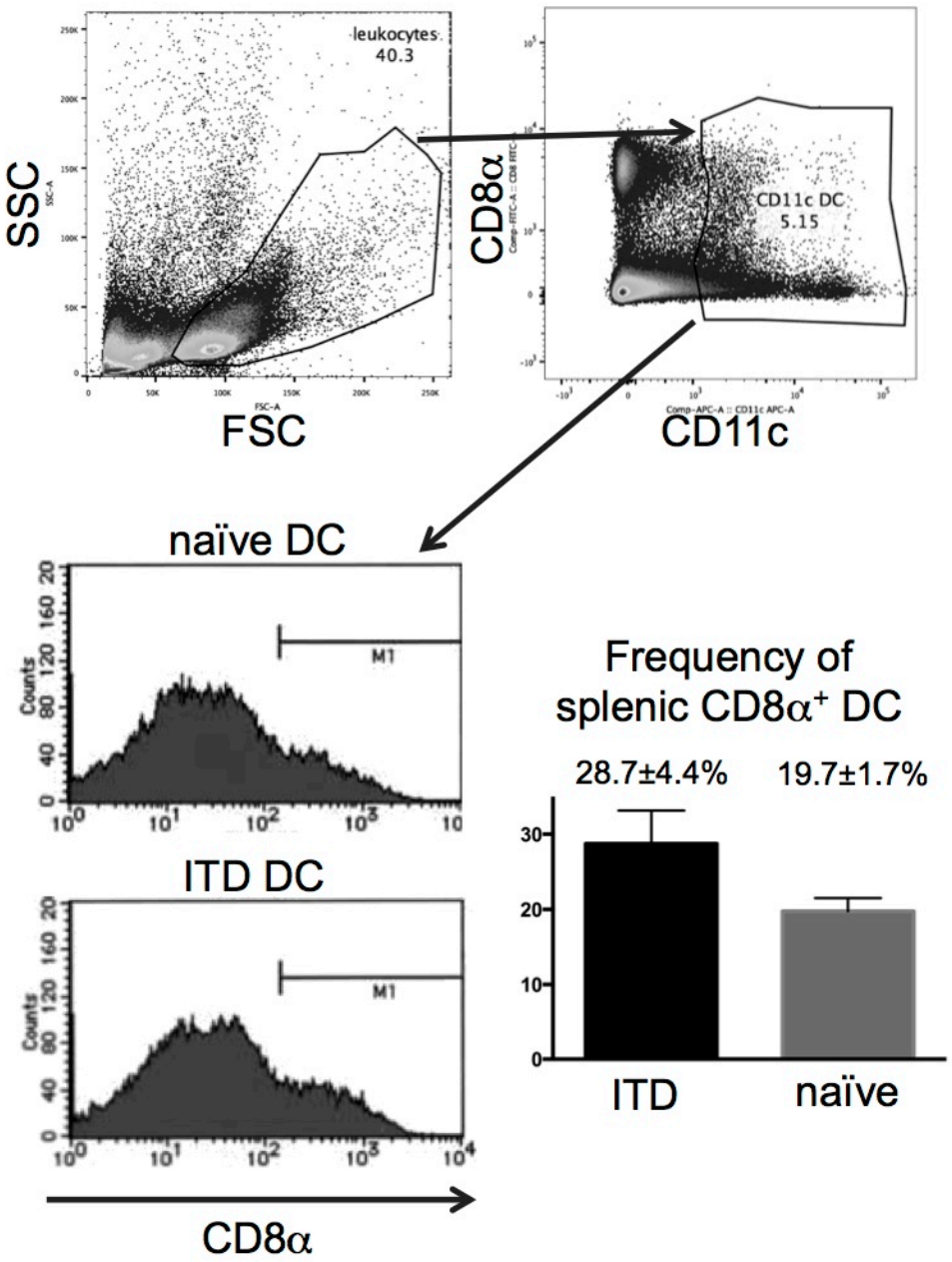


Figure 4C