

American Journal of
Transplantation

**Co-transplantation of pre-activated mesenchymal stem cells
improves intraportal engraftment of islets by inhibiting liver
natural killer cells in mice**

Journal:	<i>American Journal of Transplantation</i>
Manuscript ID	AJT-O-18-00930.R1
Wiley - Manuscript type:	O - Original Article
Date Submitted by the Author:	10-Feb-2019
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Keywords - Scope:	translational research / science, basic (laboratory) research / science
Keywords - Discipline:	islet transplantation, immunosuppression / immune modulation
Keywords - Focus:	innate immunity, natural killer (NK) cells / NK receptors, immune regulation, graft survival

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5 **Co-transplantation of pre-activated mesenchymal stem cells improves intraportal**
6 **engraftment of islets by inhibiting liver natural killer cells in mice**
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Abbreviations:

Cas9, CRISPR-associated protein 9; COX-2, cyclooxygenase 2; CRISPR, clustered regularly interspaced short palindromic repeat; CXC-motif ligand, CXCL10; EDTA, ethylenediaminetetraacetic acid; IBMIR, instant blood-mediated inflammatory reaction; ICAM-1, intercellular adhesion molecule-1; IFN- γ , interferon- γ ; IL-1 β , interleukin-1 β ; KO, knockout; MSCs, mesenchymal stem cells; NK, natural killer; NKT, natural killer T; PGE2, prostaglandin E2; TGF- β , transforming growth factor- β ; TNF- α , tumor necrosis factor- α ; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor

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Abstract

The activation of natural killer (NK) cells in the liver inhibits engraftment of intraportally transplanted islets. We attempted to modulate the activity of NK cells by co-transplanting mesenchymal stem cells (MSCs) with islets in mice. We first investigated the ability of MSCs to secrete prostaglandin E2 (PGE2), a predominant inhibitor of NK cell function, in various combinations of inflammatory cytokines. Notably, we found that PGE2 production was partially delayed in MSCs activated by inflammatory cytokines *in vitro*, whereas liver NK cells were activated early after islet transplantation *in vivo*. Accordingly, pre-activated MSCs, but not naive MSCs, substantially suppressed the expression of activation markers in liver NK cells after co-transplantation with islets. Similarly, co-transplantation with pre-activated MSCs, but not naive MSCs, markedly improved the survival of islet grafts. These results highlight MSC co-transplantation as an effective and clinically feasible method for enhancing engraftment efficiency.

1 | Introduction

Islet transplantation is an approach used to reduce hyperglycemia and minimize episodes of severe hypoglycemia in patients with diabetes mellitus type 1.¹ However, multiple islet transplantations are typically required owing to a low efficiency of engraftment efficiency.² Indeed, islets are rapidly degraded after intraportal islet transplantation for various reasons, including hypoxia,³ mechanical damage from embolization of the portal vein,⁴ and a nonspecific inflammatory and thrombotic reaction termed the instant blood-mediated inflammatory reaction (IBMIR).^{5,6} In addition, the innate immune response immediately following islet transplantation has also been considered a major obstacle. To date, immune cells such as macrophages,⁷ granulocytes,^{8,9} natural killer T (NKT) cells,^{8,10} and natural killer (NK) cells¹¹ have been implicated in islet graft degradation.

NK cells in the liver have properties distinct from those in the peripheral blood, i.e., NK cells are more abundant among mononuclear cells in the liver, and have higher cytotoxicity.¹²⁻¹⁴ In the mouse, two distinct subsets of liver NK cells have been identified: DX5⁻ and DX5⁺ NK cells.¹⁵ The former are predominantly found in the liver, and have higher cytotoxicity than the latter.¹⁶ In addition, liver NK cells include a subset lacking Ly-49-inhibitory receptors that recognize self-major histocompatibility complex class I molecules and, thus, may even attack self-hepatocytes.¹⁷ Accordingly, graft survival is enhanced after syngeneic intraportal islet transplantation into mice depleted of NK cells.^{11,18} Therefore, the suppression of liver-resident NK cells after intraportal islet transplantation may improve islet graft survival.

To date, mesenchymal stem cells (MSCs) have been shown in several experimental models of islet transplantation to elicit angiogenesis, prevent apoptosis, and suppress allograft rejection.¹⁹⁻²³ Moreover, MSCs were demonstrated to suppress various immune

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5 cells such as NK cells,²⁴⁻²⁷ macrophages,^{28,29} neutrophils,³⁰ NKT cells,³¹ and T cells³²
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7 through a variety of mechanisms. However, whether MSCs also suppress liver-resident
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9 NK cells exhibiting vigorous cytotoxicity, even against syngeneic inocula, has not been
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11 investigated.

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14 Intraportal islet transplantation elicits an IBMIR that is accompanied by the release of
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16 inflammatory cytokines such as interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α),
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18 and interleukin-1 β (IL-1 β).^{7,8} These cytokines were reported to activate MSCs, and
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20 induce the production of various soluble factors, including nitric oxide, prostaglandin E2
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22 (PGE2), transforming growth factor- β (TGF- β), indoleamine 2,3-dioxygenase, TNF-
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24 inducible gene 6 protein, and several other chemokines. These cytokines elicit the
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26 expression of surface molecules such as programmed cell death-ligand 1, intercellular
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28 adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) on the
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30 activated MSCs.^{26,32-37}

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34 Hence, we hypothesized that MSCs co-transplanted with islets would be activated by
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36 inflammatory cytokines after intraportal administration, thereby suppressing immunity. In
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38 addition, data from recent studies revealed the effectiveness of MSC pre-activation by
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40 inflammatory cytokines before administration.³⁸ That is, the possibility exists that the
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42 effect of MSCs can be more effectively elicited by cytokine stimulation *in vitro* prior to
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44 administration than by endogenous cytokine stimulation *in vivo*. Related to this
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46 hypothesis, previous *in vitro* studies have shown that PGE2, TGF- β , and indoleamine 2,3-
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48 dioxygenase promote NK cell inhibition through MSCs, with PGE2 appearing to be most
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50 important.^{24-27,39} These observations prompted us to investigate temporal changes in
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52 PGE2 output from MSCs stimulated with inflammatory cytokines to effectively harness
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54 the ability of these cells to suppress NK cells.
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5 In this study, we have demonstrated that co-transplantation of islets and pre-activated
6 MSCs suppressed NK cells and improved graft survival in mice. These results highlight
7 MSC co-transplantation as a promising approach to calm the innate immune responses
8 mediated by NK cells following islet transplantation.
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2 | Materials and methods

2.1 | Mice

C57BL/6J mice were obtained from Clea Japan, Inc. (Osaka, Japan), and housed under specific pathogen-free conditions, and used at 8–12 weeks of age. All experiments were approved by the institutional review board of Hiroshima University and were conducted in accordance with the guidelines of the National Institutes of Health (Publication no. 86–23, revised 1996).

2.2 | Mesenchymal stem cells

Bone marrow-derived MSCs from C57BL/6 mice at ≤ 8 weeks of age were obtained from Thermo Fisher Scientific (Waltham, MA, USA) (catalog number S1502-100). The manufacturer's data sheet indicates that CD29, CD34, CD44, and Sca-1 were expressed by $>70\%$ of the MSCs, and that CD117 was expressed by $<5\%$. We also founded that the surface antigens CD34, CD44, and Sca-1 were detectable on MSCs, but that CD11b, CD14, CD40, CD45, CD80, CD86, H-2Db, and IAb were not (Fig S1). Although CD34 is a hematopoietic stem cell marker that is often regarded as a negative marker of MSCs, high expression of CD34 has been reported on bone marrow MSCs derived from C57BL/6 mice.^{40,41}

2.3 | Cell culture

MSCs were cultured at 37 °C and 5 % CO₂ in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 10 % heat-inactivated fetal calf

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5 serum (Sanko Chemical Co., Tokyo, Japan) and 50 U/mL penicillin (Thermo Fisher
6 Scientific). Sub-confluent MSCs were re-seeded at a density of 5,000 cell/cm² and used
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8 between passages 9 and 15.
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15 **2.4 | Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-** 16 **associated protein 9 (Cas9)-induced gene knockout(KO) of cyclooxygenase 2 (COX-** 17 **2)** 18 19 20 21

22 COX-2 KO was performed in MSCs, using the CRISPR/Cas9 KO plasmid (Santa Cruz
23 Biotechnology, Dallas, TX, USA) according to the manufacturer's protocol. The COX-2
24 HDR plasmid (Santa Cruz Biotechnology) was used to repair Cas9-induced DNA
25 cleavage and insert a puromycin-resistance gene to enable the selection of stable COX-2
26 knockouts. A control CRISPR/Cas9 plasmid (Santa Cruz Biotechnology) containing a
27 single scrambled gRNA sequence that does not bind to genomic target DNA was used to
28 generate control cells. Briefly, 2×10^5 MSCs were seeded into 6-well cultured plates in 3
29 ml of Plasmid-Transfection Medium (Santa Cruz Biotechnology), 24 hours prior to
30 transfection. MSCs were transfected with 1 μ g each of the CRISPR/Cas9 KO plasmid
31 and COX-2 HDR plasmid using the UltraCruz[®] Transfection Reagent (Santa Cruz
32 Biotechnology) and incubated at 37 °C and 5 % CO₂ for 24 h. Three days after
33 transfection, the cells were selected for 2 days in a medium containing 7.5 μ g/ml
34 puromycin (Santa Cruz Biotechnology). The puromycin-resistant cells were expanded for
35 ten days and used as stable COX-2-knockout cells in subsequent experiments.
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55 **2.5 | Diabetes induction** 56 57 58 59 60

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5 Diabetes was induced by intraperitoneal injection of 200 mg/kg streptozotocin (Sigma–
6 Aldrich, St. Louis, MO, USA). After injection, non-fasting blood glucose exceeded 450
7 mg/dL by day 3, as measured with a GT-1830 glucose analyzer (Arkray, Tokyo, Japan),
8 and the mice remained hyperglycemic until islet transplantation. Islet transplantation was
9 performed 7 days after streptozotocin injection. Blood glucose levels that decreased to
10 less than 250 mg/dL for consecutive measurements after transplantation were considered
11 to indicate reversal of diabetes.
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23 **2.6 | Islet isolation**

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26 Islets were isolated from C57BL/6 mice and used for transplantation. Briefly, the
27 pancreas was distended by infusing it with 1 mg/mL Collagenase P (Roche Diagnostics,
28 Basel, Switzerland) and incubating it at 37 °C for 17 min. The tissue was then washed
29 several times in Hank's balanced salt solution (HBSS, Thermo Fisher Scientific), and
30 islets were purified using density gradient centrifugation in Percoll PLUS (GE
31 Healthcare, Little Chalfont, UK). Islets with a diameter of 100–200 µm were handpicked,
32 suspended in HBSS, and kept on ice before transplantation.
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45 **2.7 | Preparation of MSCs**

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47 For *in vitro* experiments, MSCs were cultured with 20 ng/mL IFN- γ , 20 ng/mL TNF- α ,
48 and 10 ng/mL IL-1 β (R&D Systems, Minneapolis, MN, USA), either individually or in
49 combination, for the indicated number of h. Subsequently, the medium was aspirated
50 from the culture dishes, and each dish was rinsed with PBS. After adding pre-warmed
51 0.05% trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA; Thermo Fisher
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5 Scientific) and incubating the cells at 37°C for 2 min, the detached cells were collected
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7 and used for flow cytometric analysis.
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10 For *in vivo* experiments (co-transplantation with islets), MSCs were cultured with
11 cytokines (20 ng/mL IFN- γ , 20 ng/mL TNF- α , and 10 ng/mL IL-1 β) or without
12 cytokines. After 24 h, MSCs were collected and transferred to a 50-ml tube. After
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14 washing the cells 3 times, they were suspended in HBSS for injection in *in vivo*
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16 experiments. For co-transplantation with islets, suspended MSCs were mixed with the
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18 solution containing islets before transplantation.
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26 | **2.8 | *In vivo* transplantation via the portal vein (MSC transplantation alone, islets 27 28 transplantation alone, or MSC and islet co-transplantation)**

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31 Before transplantation, 2×10^3 – 5×10^5 MSCs, 300 islets, or a mixture of 300 islets and 2
32 $\times 10^4$ naive or pre-activated MSCs were suspended in 200 μ l HBSS in a 0.5 ml tube and
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34 placed on ice. Under anesthesia with intraperitoneal injection of xylazine (5 mg/kg body
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36 weight) and ketamine (100 mg/kg body weight), a 2-cm midline incision was made below
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38 the xiphoid process. The intestines were gently pulled out to the left side from the
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40 peritoneal cavity onto dampened gauze to expose the portal vein. After aspirating the
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42 200 μ l HBSS containing MSCs, islets, or islet–MSC mixture from a 0.5-ml tube with a 1-
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44 ml syringe and a 27-gauge needle, the needle was inserted into the portal vein through fat
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46 tissue, and the solution was slowly injected over 3 min. After all the injection was
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48 completed, blood was quickly refluxed into the syringe and was re-injected into the portal
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50 vein to ensure that the remaining islets enter the portal vein. After removing the needle
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52 from portal vein, a cotton swab was gently placed over the punctured part of the vein
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54 until bleeding stopped. Then the peritoneal cavity was closed using 5-0 polydioxanone.
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2.9 | Isolation of liver leukocytes

Under anesthesia, liver leukocytes were isolated from experimental and control mice, as previously described.⁴² Briefly, the portal vein was pre-perfused with 0.5 mL phosphate-buffered saline (PBS) containing 10% heparin. The liver was then perfused with 50 mL PBS supplemented with 0.1% EDTA (Miltenyi Biotec, Bergisch Gladbach, Germany). The perfusate was collected, and cleared of erythrocytes, using an ammonium chloride/potassium solution. Cell counts were performed on a hemocytometer with trypan blue exclusion.

2.10 | Antibodies and flow cytometric analysis

Cells were analyzed by flow cytometry with a fluorescence-activated cell sorter, either a Canto II or a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA). Analysis was performed using FlowJo software (Tree Star Software, Ashland, OR, USA). The following antibodies were used for analyzing cell surface antigen expression on MSCs: fluorescein isothiocyanate (FITC)-conjugated anti-CD80 (diluted 1:40), FITC-conjugated anti-CD86 (1:10), FITC-conjugated anti-CD40 (1:40), FITC-conjugated anti-CD 45 (1:10), FITC-conjugated anti-Sca-1 (1:2), phycoerythrin (PE)-conjugated anti-CD14 (1:10), biotin-conjugated anti-CD34 (1:10), biotin-conjugated anti-CD11b (1:160), biotin-conjugated anti-CD44 (1:10), biotin-conjugated anti-H2Db (1:10), and biotin-conjugated anti-IAb (1:10). Cells labeled with different biotin-conjugated antibodies were subsequently stained with PE-conjugated streptavidin (1:160). These reagents were purchased from BD Biosciences. To analyze intracellular COX-2 expression, MSCs were fixed, permeabilized, and stained with an FITC-conjugated anti-COX-2 polyclonal

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5 antibody (1:150; Cayman Chemical, Ann Arbor, MI, USA). The following antibodies
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7 were used to analyze liver leukocytes by flow cytometry: FITC-conjugated anti-DX5
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9 (1:32; BD Biosciences), allophycocyanin (APC)-conjugated anti-NK1.1 (1:32; BD
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11 Biosciences), APC-Cy7-conjugated anti-T cell receptor (TCR)- β (1:32; BD Biosciences),
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13 PE-conjugated anti-CD69 (1:16; BD Biosciences), PE-conjugated anti-tumor necrosis
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15 factor-related apoptosis-inducing ligand (TRAIL) (1:8; Thermo Fisher Scientific), and
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17 PE-conjugated anti-CXCR3 (1:16; BD Biosciences). Nonspecific Fc γ R binding of labeled
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19 mAbs was blocked using an anti-CD16/32 antibody (2.4G2) (BD Biosciences). Dead
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21 cells were excluded from the analysis based on light-scattering and/or propidium iodide
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23 analysis.
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30 **2.11 | Immunohistochemistry staining**

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32 The liver samples obtained from mice with intraportally transplanted islets were fixed
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34 with 4%-paraformaldehyde, paraffin-embedded and sectioned. After dehydration, antigen
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36 retrieval was performed by microwave heating in Tris/EDTA-based buffer (Epitope
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38 Retrieval Solution pH 9; Leica Biosystems, Nussloch, Germany) for 30 min. The sections
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40 were treated with 3% H₂O₂ in methanol to block non-specific antibody-binding sites. The
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42 slides were incubated with the NK 1.1 Mouse Monoclonal Antibody (1:100; Thermo
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44 Fisher Scientific) for 1 h, followed by incubation with EnVision+ anti-mouse peroxidase
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46 for 1h. For color reaction, the sections were incubated with the DAB substrate-
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48 chromogen solution (Agilent, Santa Clara, CA, USA) for 10 min. The sections were
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50 counterstained with 0.1% hematoxylin. All slides were examined using a BZ-9000
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52 microscope (Keyence, Osaka, Japan).
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2.12 | Statistical analysis

GraphPad Prism 7.0 software (GraphPad, La Jolla, CA, USA) was used for statistical analysis. Two groups were evaluated using Student's two-tailed test, and data obtained from 3 or more groups were analyzed with one-way analysis of variance and Tukey's multi-comparison tests. Data are presented as the mean \pm standard deviation (SD). A value of $p < 0.05$ was considered to indicate statistical significance.

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3 | Results

3.1 | Stimulation with inflammatory cytokines induces intracellular COX-2 expression in MSCs

Pre-activation of MSCs by inflammatory cytokines is a key strategy for promoting MSC functions both in *in vivo* and *in vitro* studies.³⁸ PGE2 released from MSCs was previously reported to mediate the suppression of NK cells.^{24-27,39} Thus, we first investigated the activation of the COX-2/PGE2 pathway in MSCs *in vitro* by IFN- γ , TNF- α , and IL-1 β , either individually or in combination. The working dosage of the stimuli was determined from the results of previous studies.^{26,33,37} COX-2 expression was assessed from 6 to 144 h after stimulation. COX-2 expression gradually increased until 96 h in cells stimulated with a combination of all three cytokines (Figure 1A, B). In contrast, the cells stimulated with TNF- α and IL-1 β expressed high levels of COX-2 within 24 h, although the expression was not sustained thereafter (Figure 1B). COX-2 expression was not governed solely by IFN- γ , TNF- α , and IL-1 β stimulation, and diminished at 144 h, regardless of the stimulus (Figure 1B). These results suggested that, although the COX-2-expression rate of MSCs activated by these three cytokines was low, it is possible that *ex vivo* pre-activation by the three cytokines reduced the lead time required to activate MSCs and maintained high-level COX-2 expression in MSCs for a relatively long period after co-transplantation with islets.

3.2 | Co-transplantation of pre-activated MSCs suppresses liver NK cells after islet transplantation

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Next, we examined NK cell activity after co-transplantation of islets and MSCs into syngeneic mice. To avoid interference with immune cells by streptozotocin, diabetes was not induced in mice receiving islets in this study. A preliminary dose-escalation study (MSC transplantation alone) revealed that intraportal administration of 2×10^4 MSCs did not cause hepatic infarction, but that of 2×10^5 MSCs did (Figure S2). Hence, mice in one group were given 300 intraportally transplanted islets only, while those in other groups were co-transplanted with 300 islets and 2×10^4 naive or pre-activated MSCs. Pre-activated cells were prepared by stimulation for 24 h with IFN- γ , TNF- α , and IL-1 β at the same doses used in the COX-2-expression study described above. We collected liver leukocytes from each mouse 1, 3, and 5 days after intraportal inoculation and analyzed expression of the activation marker CD69, the cytotoxic ligand TRAIL, and the chemokine receptor CXCR3 on NK cells, as well as within the DX5⁻ and DX5⁺ subsets. These molecules were more abundantly expressed in the DX5⁻ subset (Figure 2A), as previously reported.¹⁵ TRAIL on DX5⁻ NK cells and its receptors on islets are involved in a mechanism by which NK cells injure islets.¹¹

On day 1, the proportion of DX5⁻ NK cells increased in all groups (Figure 2C). Co-transplantation of islets and pre-activated MSCs significantly inhibited the increase in the proportion of DX5⁻ NK cells on day 3, whereas co-transplantation with naive MSCs did not (Figure 2B, C). Consistent with these results, the expression of TRAIL, CD69, and CXCR3 on liver NK cells was substantially suppressed on day 3 in mice co-transplanted with islets and pre-activated MSCs (Figure. 2D–F). However, no significant inhibitory effects were observed on expression of these markers on the exclusive DX5⁻ NK cells on day 3 (Figure. S3). Thus, the overall activity of liver NK cells was suppressed, mainly by inhibiting the increase in the proportion of DX5⁻ NK cells, which are highly active. A considerable inhibitory effect was observed on the expression of markers on DX5⁺ NK cells (Figure S3); however, these effects were minimal compared to the inhibitory effects

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5 on the overall activity of liver NK cells. In addition, the absolute number of NK cells was
6 significantly suppressed on day 3 in mice co-transplanted with islets and pre-activated
7 MSCs (Figure 2G), although the absolute number of leukocytes was comparable among
8 the groups (Figure 2H). These results revealed that pre-activated MSCs suppressed NK
9 cells most effectively on day 3, among the three observation periods studied after
10 transplantation. We also attempted to investigate the form of the islets transplanted into
11 the liver of mice at three days post-transplantation, either alone or in combination with
12 pre-activated MSCs. Well-formed islets were observed in the liver sections of both the
13 groups. In addition, necrotic islets were observed in both the groups; however,
14 inflammatory cells including NK 1.1 positive cells were rarely observed around the
15 necrotic islets in the mice transplanted with pre-activated MSCs (Figure S4). This result
16 suggests that pre-activated MSCs inhibited the migration of inflammatory cells to
17 transplanted islets.
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33 We examined whether pre-activated MSCs displayed inhibitory effects on NK cells
34 three days after transplantation, even in mice with streptozotocin-induced diabetes.
35 Consistent with the findings of a previous study that both streptozotocin and acute
36 hyperglycemia have inhibitory effects on lymphocytes,⁴³ the number of NK cells in the
37 liver decreased, and the expression of TRAIL on DX5⁻ NK cells decreased in mice
38 administered streptozotocin in the present study (Figures S3 and S5). However, the
39 proportion of DX5⁻ NK cells among whole NK cells and the expression of CD69 and
40 CXCR3 on DX5⁻ NK cells remained constant even in mice with streptozotocin-induced
41 diabetes (Figure S5). Islet transplantation alone resulted in the increased proportion of
42 CD69⁺, CXCR3⁺, and DX5⁻ NK cells among whole liver NK cells, while co-
43 transplantation of pre-activated MSCs significantly inhibited such consequences (Figure
44 S5).
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3.3 | Co-transplantation of pre-activated MSCs improves islet graft survival

We also examined whether co-transplantation of islets and pre-activated MSCs would improve islet graft survival in mice with streptozotocin-induced diabetes, as reflected by plasma glucose levels. Normalization of blood glucose levels was not observed in mice transplanted with islets alone. Similarly, co-transplantation of islets and naive MSCs did not improve islet graft survival. However, co-transplantation of islets and pre-activated MSCs significantly improved islet graft survival (Figure 3A–E). Additionally, histological analysis was performed in the mice 45 days after transplanting islets alone, or in combination with pre-activated MSCs. Co-transplantation with pre-activated MSCs resulted in well-formed islets, whereas transplantation of islets alone showed a loss of islet structure (Figure S6).

After monitoring blood glucose level for 45 days, mice were euthanized to collect liver leukocytes and analyze phenotypic alterations in NK cells. The differences in the proportion of DX5⁺ NK cells, and in the expression levels of TRAIL, CD69, and CXCR3 on liver NK cells were no longer observed among the experimental groups at this point (Figure 4A–D). In addition, the absolute number of NK cells and leukocytes was comparable between the three groups (Figure 4E, F). Hence, the inhibitory effects of intraportal inoculation with pre-activated MSCs on liver NK cells were not long-lasting, and timely inhibition of NK cell activity soon after islet transplantation may be important for improving islet engraftment.

3.4 | PGE2 from MSCs suppresses NK cells after islet transplantation, and subsequently improves islet graft survival

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5 To examine whether PGE2 suppresses NK cells, islets were co-transplanted with MSCs
6 transfected with a COX-2-KO CRISPR/Cas9 plasmid or a control CRISPR/Cas9 plasmid.
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8 COX-2 expression was sufficiently diminished in KO cells even after 24 h of stimulation
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10 with inflammatory cytokines (Figure 5A). One group of mice was transplanted with 300
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12 islets and 2×10^4 MSCs transfected with the control plasmid. The other group was
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14 transplanted with 300 islets and 2×10^4 COX-2-KO MSCs. For both groups, MSCs were
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16 activated prior to transplantation by exposure to IFN- γ , TNF- α , and IL-1 β for 24 h. The
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18 phenotype and numbers of NK cells and leukocytes in each mouse were analyzed three
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20 days after transplantation. A higher proportion of DX5⁻ cells was observed in mice
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22 receiving COX-2 KO-MSCs, compared with that in mice receiving control MSCs (Figure
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24 5B). The expression of TRAIL and CXCR3 on NK cells was also substantially higher in
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26 the mice receiving COX-2-KO MSCs (Figure 5D, E), although CD69 expression and the
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28 numbers of NK cells and leukocytes showed no significant difference between the groups
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30 (Figure 5C, F, G).
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36 We further examined whether PGE2 improves islet graft survival in mice with
37 streptozotocin-induced diabetes, as reflected by plasma glucose levels. Normalization of
38 blood glucose levels significantly decreased in mice transplanted with islets and COX-2-
39 KO MSCs, whereas co-transplantation of islets and pre-activated MSCs transfected with
40 the control plasmid improved islet graft survival (Figure 6A–D). These results indicate
41 that PGE2 from MSCs suppressed NK cells after islet transplantation, and subsequently
42 improved islet graft survival.
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4 | Discussion

The mechanism by which MSCs improve islet graft survival has not been fully elucidated, although co-transplantation with islets, usually into the kidney capsule or the anterior of the eye,^{20-23,44-46} has been examined in several studies. In the case of co-transplantation into the liver, which has been less commonly attempted, even in experimental settings,^{19,22} the impact of MSCs on liver immune cells may need to be further elucidated if this approach is to be feasible in clinical settings. Hence, we investigated the potential modulation of liver NK cell activity by co-transplantation with MSCs using intraportal islet transplantation in mice. We found that pre-activated MSCs, but not naive MSCs, suppressed NK cell activation immediately after co-transplantation. We further found that PGE2 mediates the suppression of NK cells, as previously observed *in vitro*.^{24,26,27,39} PGE2 is also known to mediate the inhibitory activity of MSCs against NKT cells and macrophages.^{28,29,31} Considering that NKT cells and macrophages interact with NK cells,⁴⁷⁻⁵⁰ it is likely that transplanted MSCs directly inhibit NK cells, or do so indirectly by inhibiting NKT cells and/or macrophages in a PGE2-mediated fashion. However, we did not assess macrophages and NKT cells in this study.

We showed that PGE2 from pre-activated MSCs plays a key role in suppressing NK cells. Since PGE2 is a soluble, highly diffusible factor with a short half-life, it is unlikely that systemically administered PGE2 efficiently and persistently influences liver-resident immune-cells. In this regard, engraftment into the liver of MSCs actively producing PGE2 seems to have an advantage for constant delivery of PGE2 to a specific site, leading to sustained suppression of intrahepatic NK cells. In addition to PGE2, other factors are potentially involved in activated MSC-mediated NK cell suppression. Inflammatory cytokines elicit the production of PGE2, chemokines, and adhesion molecules such as ICAM-1 and VCAM-1.³² Hence, MSCs may attract NK cells by

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5 releasing chemokines, and trapping them via these adhesion molecules, thereby
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7 enhancing suppression by cell–cell contact. Indeed, Ren et al. reported that ICAM-1 and
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9 VCAM-1 are critical for the inhibitory activity of MSCs against T cells,³² as is CXCR3-
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11 mediated chemotaxis.^{33,35} Such a chemokine/adhesion molecule-mediated factor may also
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13 support the inhibitory activity of PGE2 on NK cells. This possibility remains to be
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15 examined in future studies.
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19 In the mouse, resident DX5⁻ NK cells in the liver are phenotypically and functionally
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21 distinct from DX5⁺ cells in the peripheral blood.^{15,16} Similarly, liver CD56^{bright} NK cells
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23 are distinct from peripheral blood CD56^{dim} NK cells, in humans.⁵¹ Murine DX5⁻ cells and
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25 human CD56^{bright} cells share many other similarities.⁵² We found that DX5⁻ cell
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27 populations expanded after islet transplantation unless pre-activated MSCs were co-
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29 transplanted to suppress them. Transplanted MSCs appeared to suppress the overall
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31 activity of liver NK cells by suppressing the expansion of the highly active DX5⁻ cell
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33 population. The possibility exists that pre-activated MSCs not only suppress the
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35 proliferation of DX5⁻ NK cells, but also induce apoptosis in DX5⁻ NK cells. This issue
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37 remained to be elucidated. Nevertheless, such suppressive effects of MSCs on NK cells in
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39 mice may be relevant to clinical islet transplantation in humans. In support of this
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41 possibility, a previous *in vitro* study using human MSCs and NK cells demonstrated that
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43 MSCs inhibited increased proliferation without inducing cell death and cytokine
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45 production by IL-15-activated CD56^{bright} cells more than that by IL-15-activated CD56^{dim}
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47 cells.²⁴ Notably, human MSCs stimulated with IFN- γ showed more resistance than non-
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49 stimulated cells to cytolysis by activated human NK cells.^{24,27} Hence, pre-activated MSCs
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51 may optimize their own engraftment, even in humans.
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55 We demonstrated that pre-activated, but not naive, MSCs improve islet graft survival,
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57 possibly by suppressing NK cells. In addition to such an immunosuppressive factor, other
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5 non-immunological factors may be also involved. For example, Ito et al.¹⁹ showed that
6 even naive MSCs improved islet graft survival in intraportally transplanted rats by
7 promoting revascularization. Indeed, expression of vascular endothelial growth factor
8 (VEGF), which mediates angiogenesis by MSCs, is enhanced by exposure to TNF- α
9 exposure, as is that of other growth factors such as insulin-like growth factor-1, basic
10 fibroblast growth factor, and hepatocyte growth factor.⁵³ Additionally, the multi-
11 differentiation properties of MSCs might be involved in the improvement of the graft
12 survival. Several studies have reported that MSCs have the capacity to differentiate into
13 insulin-producing cells when co-cultured or co-transplanted with islets.⁵⁴⁻⁵⁶ These
14 findings should be validated further to improve our knowledge on the contribution of
15 MSCs to the success of islet transplant outcomes.

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As with our approach, several preconditioning strategies to increase the advantageous biological properties of MSCs have been reported for improving islet engraftment. Hypoxia preconditioning is known to enhance the secretion of various growth factors by MSCs.³⁸ Xiang et al.⁵⁷ reported co-transplantation with hypoxia pre-treated MSCs improved mouse islet graft engraftment to the kidney capsules. In addition to utilizing the intrinsic properties of MSCs, genetically engineered MSCs have also been reported as an efficient method for enhancing the therapeutic effect.⁵⁸ Genetic engineering can constitutively enhance a specific property of MSCs, regardless of their environment. For example, Hajizadeh-Saffar et al.⁵⁹ reported that enhanced expression of VEGF by genetically modified MSCs improved islet engraftment. Our approach is based on the analysis of the activation kinetics of MSCs in the presence of cytokines. From the perspective of efficiency, constitutive COX-2 gene expression in genetically mediated MSCs could more effectively suppress liver NK cells after intraportal transplantation without prior pre-activation, provided that the expression level of COX-2 due to genetic modification is comparable to that induced by cytokine stimulation.

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5 In summary, our research indicates that pre-activated MSCs suppress NK cells
6 following intraportal islet transplantation, and that this improves islet graft survival. Thus,
7 co-transplantation of these cells is a new and clinically relevant strategy for improving
8 engraftment outcomes.
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Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research Support Foundation from the Ministry of Education, Culture, Sports, Science and Technology. This work was carried out at the Research Facilities for Laboratory Animal Science, Natural Science Center for Basic Research and Development (N-BARD), Hiroshima University.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Figure legends

Figure 1. Intracellular COX-2 expression in MSCs measured by flow cytometry. A: Representative histograms showing the gradual increase in COX-2 expression 24 to 96 h after cells were stimulated with IL-1 β , TNF- α , and IFN- γ . B: Relative mean fluorescence intensity (MFI), calculated as the ratio of the MFI of activated cells to that of naive cells (dotted line) at each time point. Data are shown as the mean \pm SD from four independent experiments; n = 4 per group at each time point; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure 2. Phenotypic alterations of liver NK cells, as assessed by flow cytometry. A: Representative analysis of liver NK cells derived from a non-treated mouse. Liver NK cells were identified as TCR- β^- NK1.1 $^+$ cells, and stratified by DX5 expression. B: Representative flow cytometry for liver leukocytes collected three days after transplantation of islets, alone or in combination with pre-activated MSCs. Samples were analyzed for DX5 expression on NK cells. C: Proportion of DX5 $^-$ NK cells, as assessed by flow cytometry at the indicated time points after transplanting islets, alone or in combination with naive or pre-activated MSCs. D–F: Time course of CD69, TRAIL, and CXCR3 expression on liver NK cells. G, H: Number of NK cells and leukocytes obtained from whole livers at the indicated time points. Each dot represents an individual animal, and bars show the mean \pm SD from 5–7 independent experiments, with one mouse per group at each time point. * $p < 0.05$; ** $p < 0.01$

Figure 3. A–C: Graft survival in mice with streptozotocin-induced diabetes, as assessed by measuring plasma glucose levels after transplantation of 300 syngeneic islets into the liver, alone (A, n = 6) or in combination with naive (B, n = 5) or pre-activated MSCs (C,

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5 n = 6). The lines represent non-fasting plasma glucose levels in individual mice from six
6 independent experiments with one mouse per group. D. The mean blood glucose level
7 was 429 ± 130 mg/dL in the group transplanted with islets alone, 453 ± 132 mg/dL in the
8 group co-transplanted with islets and naive MSCs, and 234 ± 133 mg/dL in the group co-
9 transplanted with islets and pre-activated MSCs. E: Cumulative diabetes-reversal curve
10 after islet transplantation. Normoglycemia was defined as < 250 mg glucose/dL on
11 consecutive days. Statistical analysis was performed using the log-rank test. Significance
12 for all graphs: $**p < 0.01$; $***p < 0.001$
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26 Figure 4. Effect of MSCs on liver NK cells 45 days after transplantation of islets, alone (n
27 = 6) or in combination with naive (n = 5) or pre-activated MSCs (n = 6). A: Proportion of
28 DX5⁻ NK cells in liver NK cells, as assessed by flow cytometry. B–D: Expression of
29 CD69, TRAIL, and CXCR3 on liver NK cells. E, F: Number of liver NK cells and
30 leukocytes from whole livers. Each dot represents an individual animal, and the bars
31 represent the mean \pm SD from six independent experiments with one mouse per group.
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39 ns, not significant
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45 Figure 5. Effect of COX-2-KO MSCs on liver NK cells. A: Representative histograms of
46 COX-2 expression in cells transfected with a control or COX-2 KO plasmid, and
47 stimulated for 24 h with IFN- γ , TNF- α , and IL-1 β . B: Proportion of DX5⁻ cells in liver
48 NK cells after co-transplantation. C–E: Expression of CD69, TRAIL, and CXCR3 on
49 liver NK cells after co-transplantation. F, G: Number of NK cells and leukocytes from
50 whole livers. Each dot represents an individual animal, and bars represent the mean \pm SD
51 from seven independent experiments performed with one mouse per group. $*p < 0.05$
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8 Figure 6. A, B: Graft survival in mice with streptozotocin-induced diabetes, as assessed
9 by measuring plasma glucose levels after islet transplantation of 300 syngeneic islets into
10 the liver in combination with pre-activated control MSCs (A, n = 11) or pre-activated
11 COX-2-KO MSCs (B, n = 11). The lines represent non-fasting plasma glucose levels in
12 individual mice from 11 independent experiments with one mouse per group. C: The
13 mean blood glucose level was 272 ± 137 mg/dL when islets were co-transplanted with
14 pre-activated control MSCs or 369 ± 171 mg/dL after co-transplantation with pre-
15 activated COX-2-KO MSCs. D: Cumulative diabetes-reversal curve after islet
16 transplantation. Normoglycemia was defined as < 250 mg glucose/dL on consecutive
17 days. Statistical analysis was performed using the log-rank test. Significance for all
18 graphs: $**p < 0.01$; $***p < 0.001$
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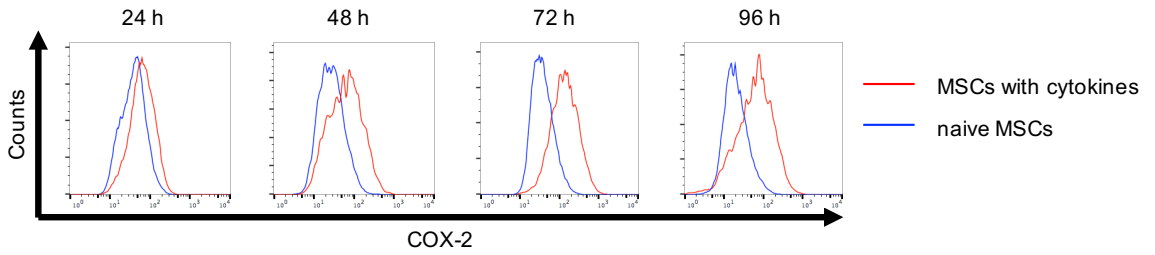
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Figure 1

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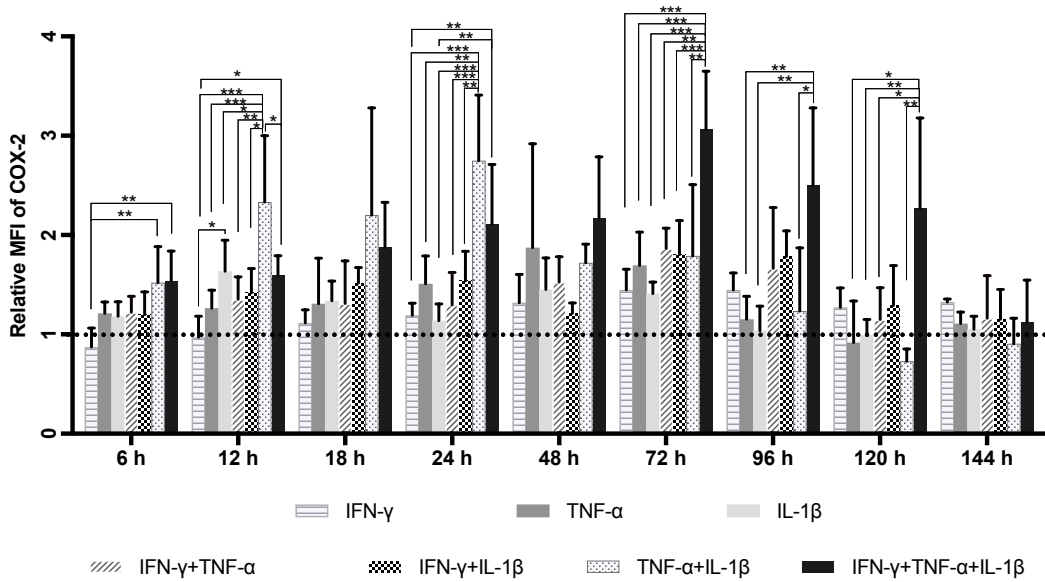


Figure 2

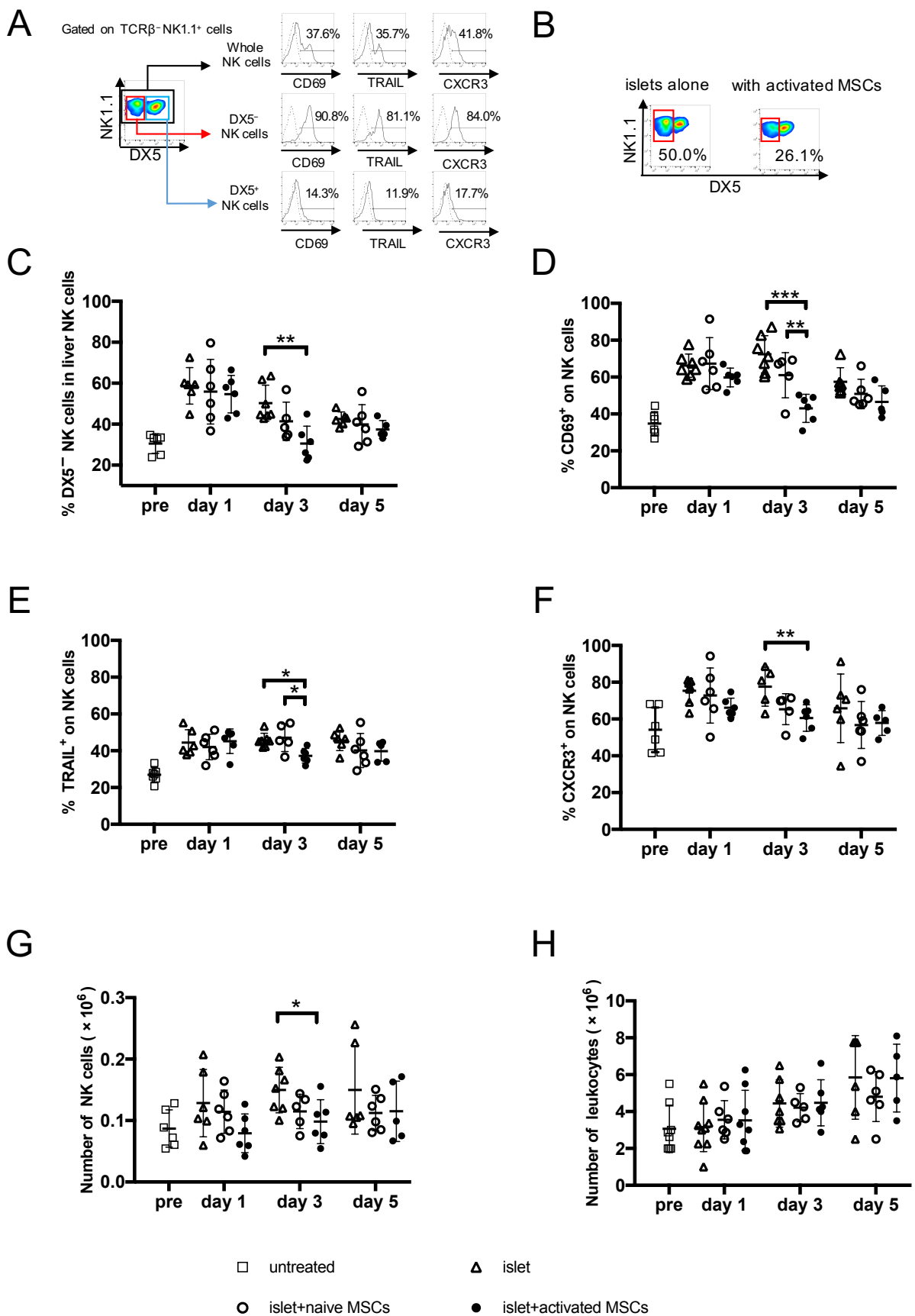


Figure 4

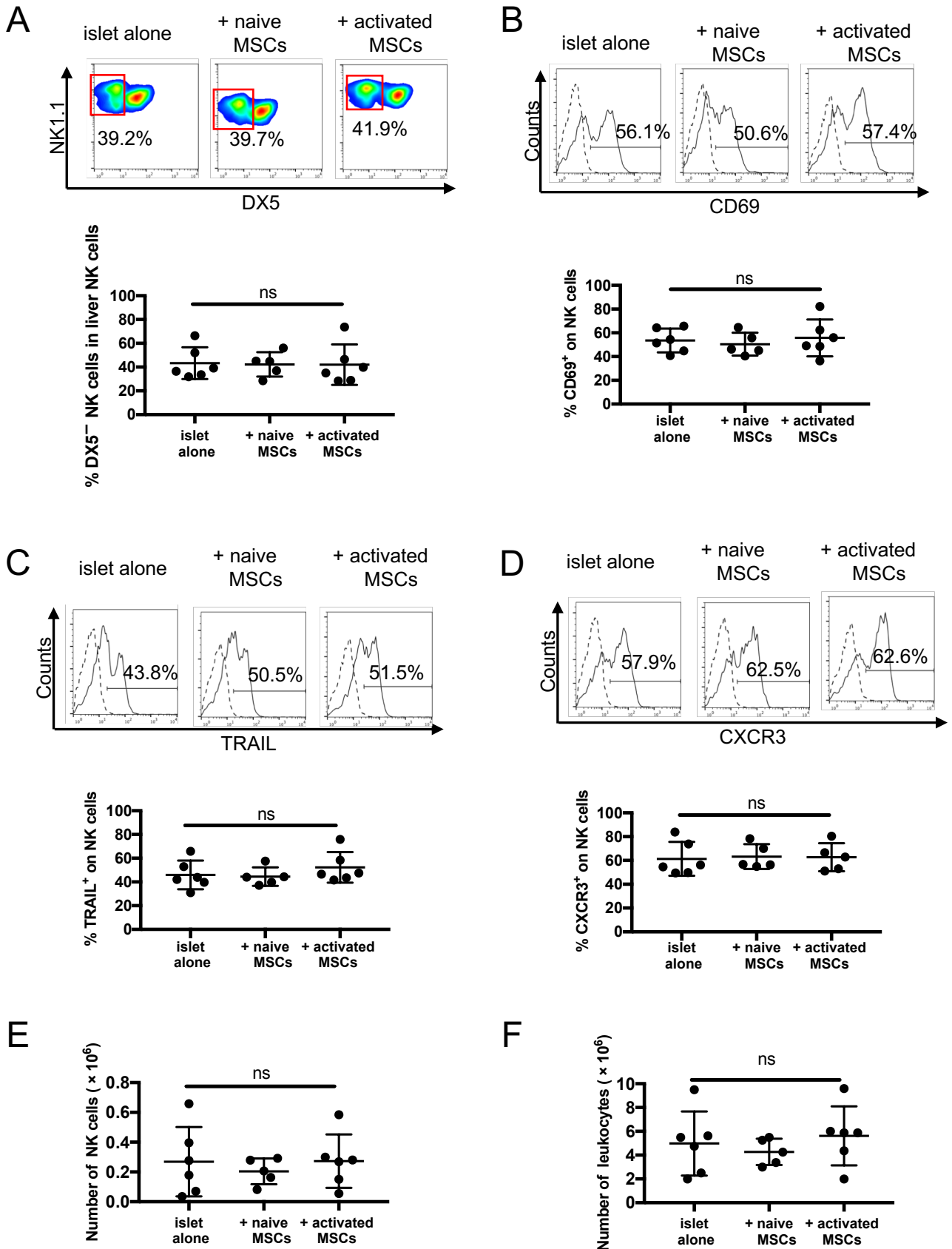
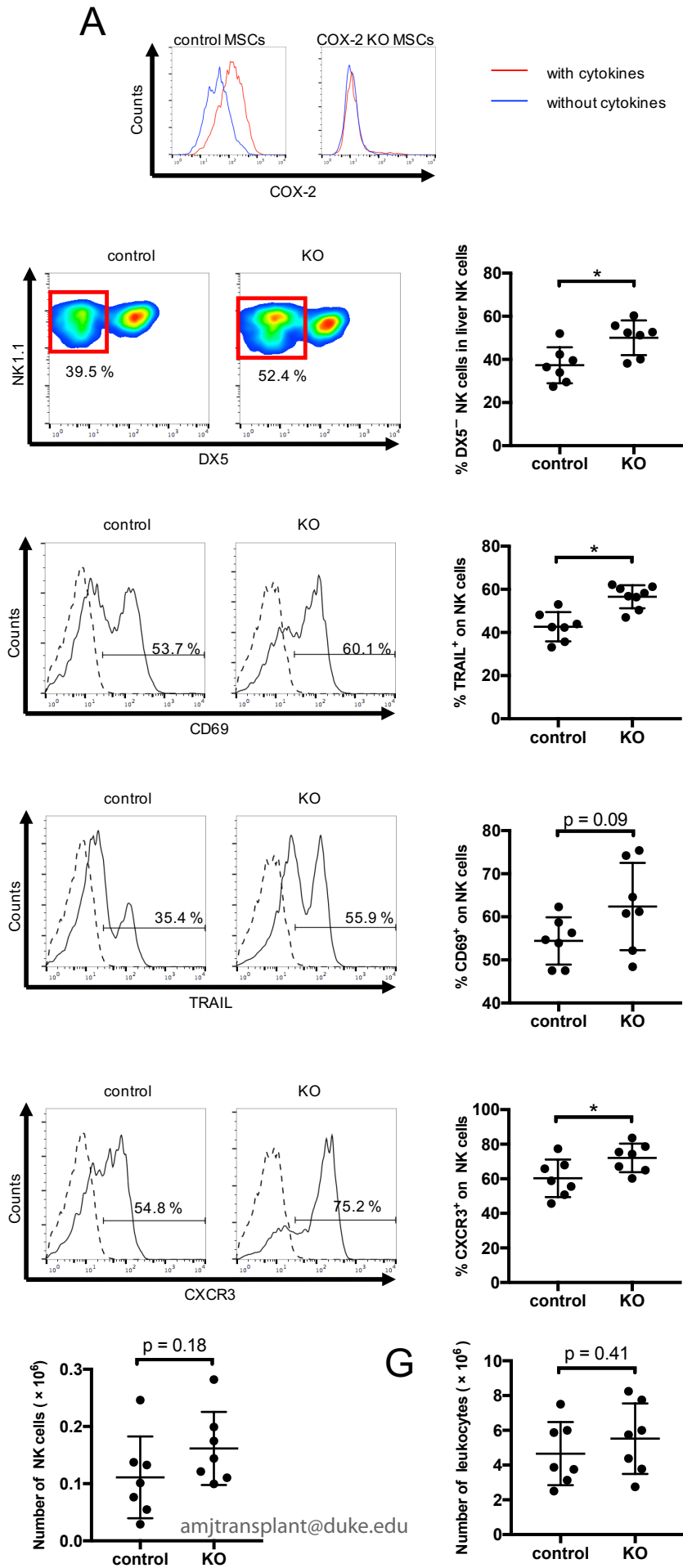
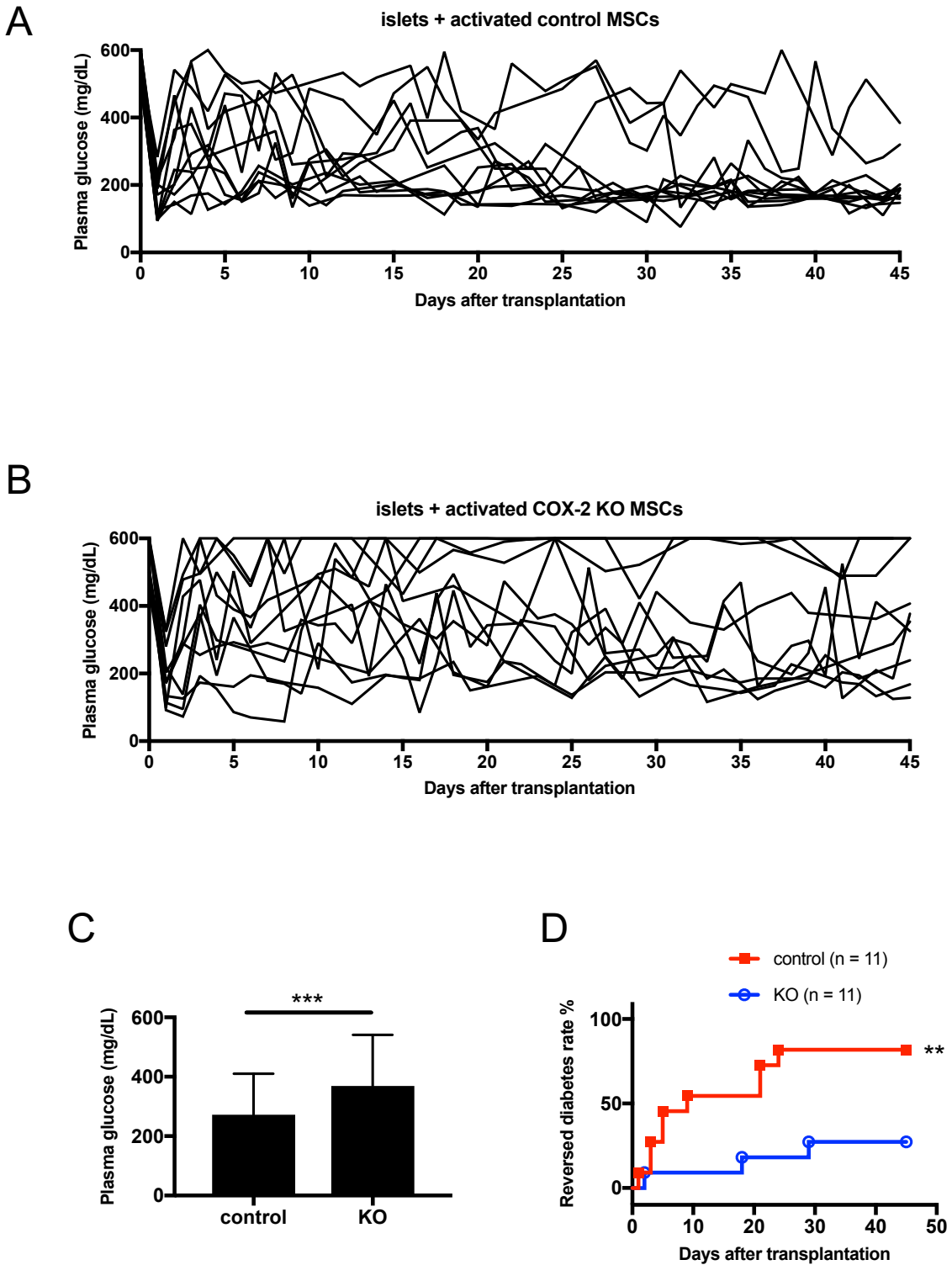


Figure 5



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Figure 6



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SUPPLEMENTARY FIGURES

Figure S1. Phenotypic analysis of MSCs used in this study. Black lines represent the expression levels of a specific markers on MSCs. Dotted lines represent isotype controls.

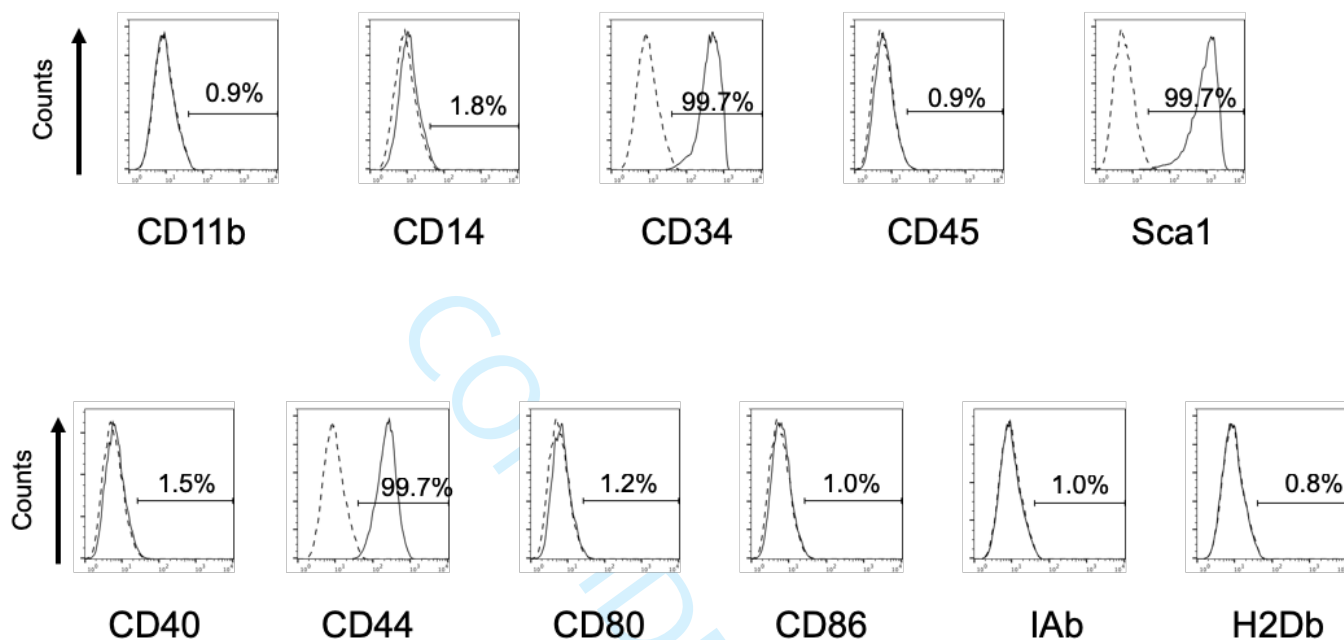
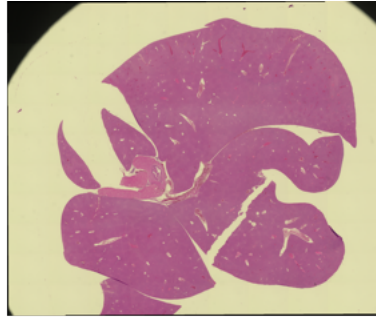
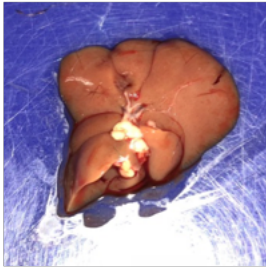
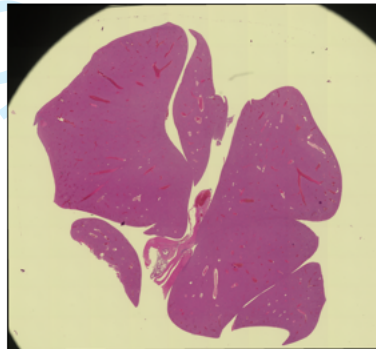
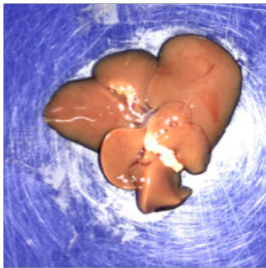


Figure S2. A–D: Macroscopic appearance and histological findings of the liver three days after intraportal administration of 2×10^3 MSCs (A), 2×10^4 MSCs (B), 2×10^5 MSCs (C), or 5×10^5 MSCs (D). Liver tissue sections were stained with hematoxylin and eosin. Arrows indicate infarcted areas. Enlarged regions are from the circled areas. Administration of 2×10^3 MSCs or 2×10^4 MSCs did not cause hepatic infarction. Representative images are from two independent experiments, performed with one mouse per group.

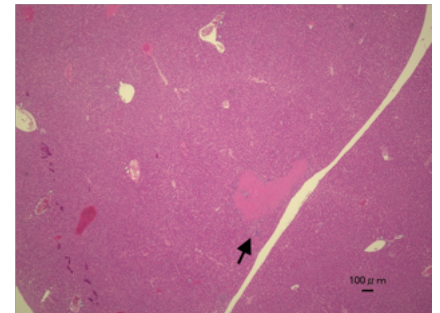
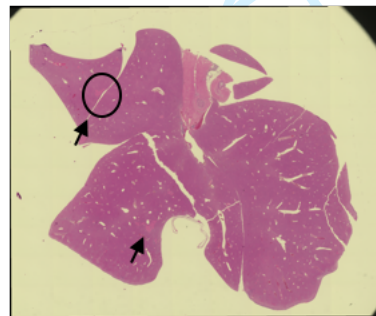
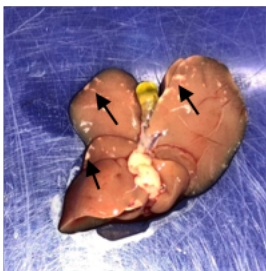
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B



C



D

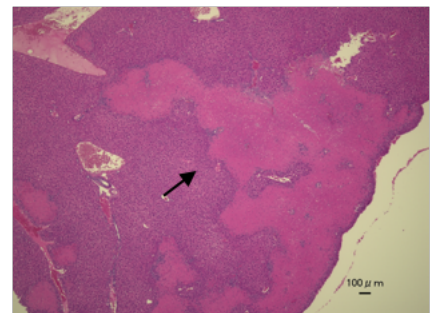
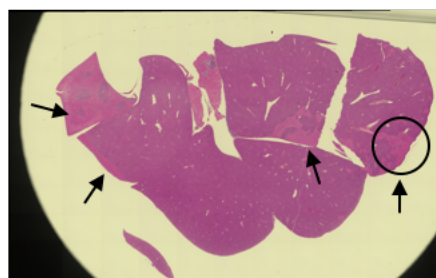
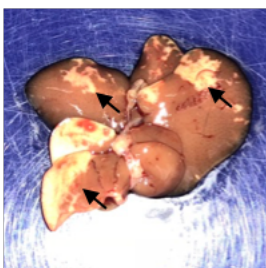
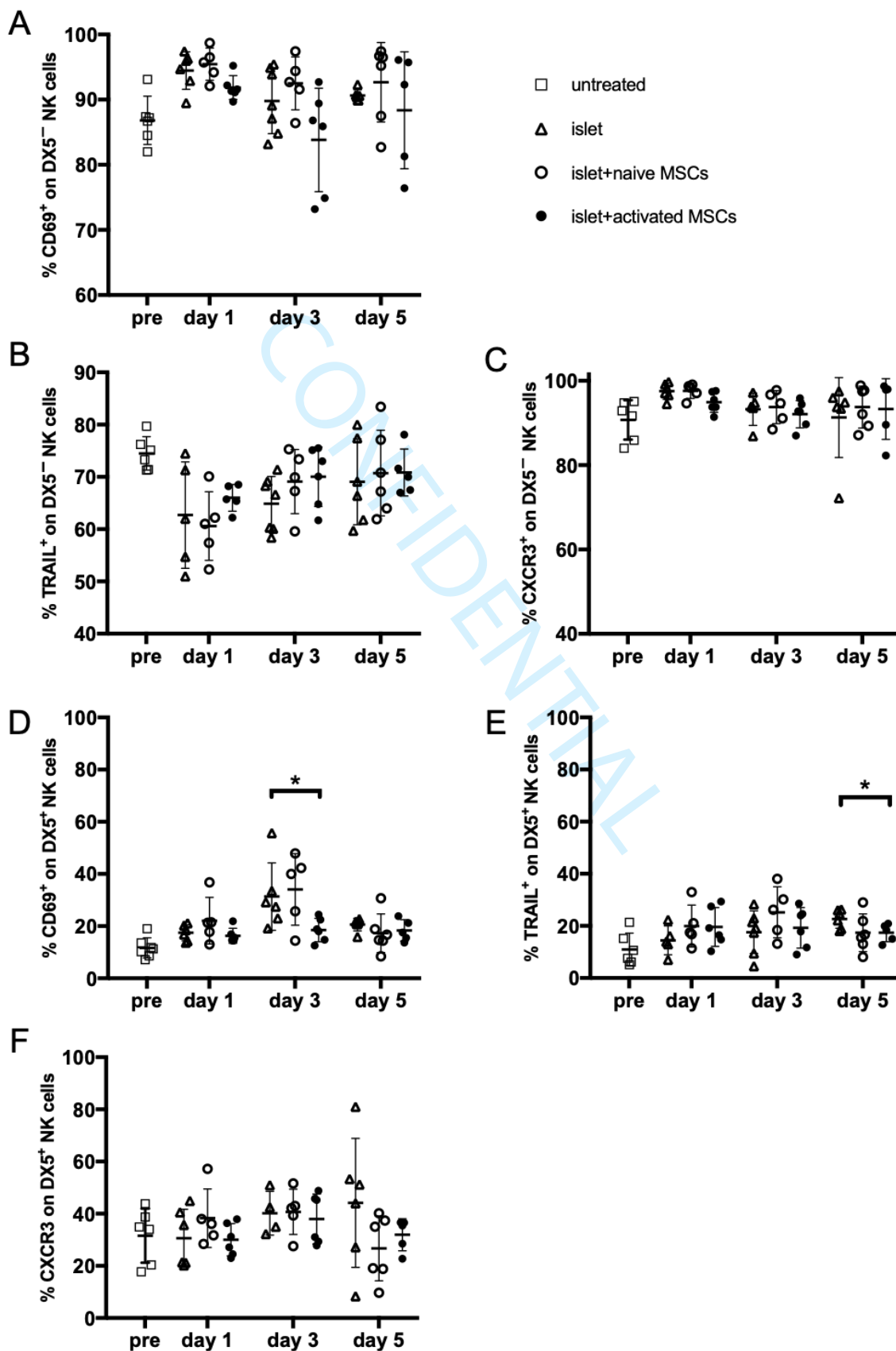


Figure S3. Phenotypic alterations in liver NK cells, as assessed by flow cytometry. Time course of CD69, TRAIL, and CXCR3 expression in liver DX5⁻-gated NK cells (A–C), and DX5⁺-gated NK cells (D–F). Each dot represents an individual animal, and the bars indicate the mean ± SD from 5–7 independent experiments with one mouse per group at each time point. **p* < 0.01



1 **Figure S4.** Islet morphology in the liver three days after transplantation with islets alone or in combination with pre-activated
2 MSCs. Well-formed islets (upper panels) and necrotic islets (middle panels) were found in both the groups by hematoxylin
3 and eosin staining. Immunostaining was performed with anti-NK1.1 antibody (lower panels). NK1.1 is expressed on NK and
4 NKT cells. Arrowheads indicate NK1.1 positive lymphocytes. A representative image from two independent experiments
5 with one mouse per group is shown.
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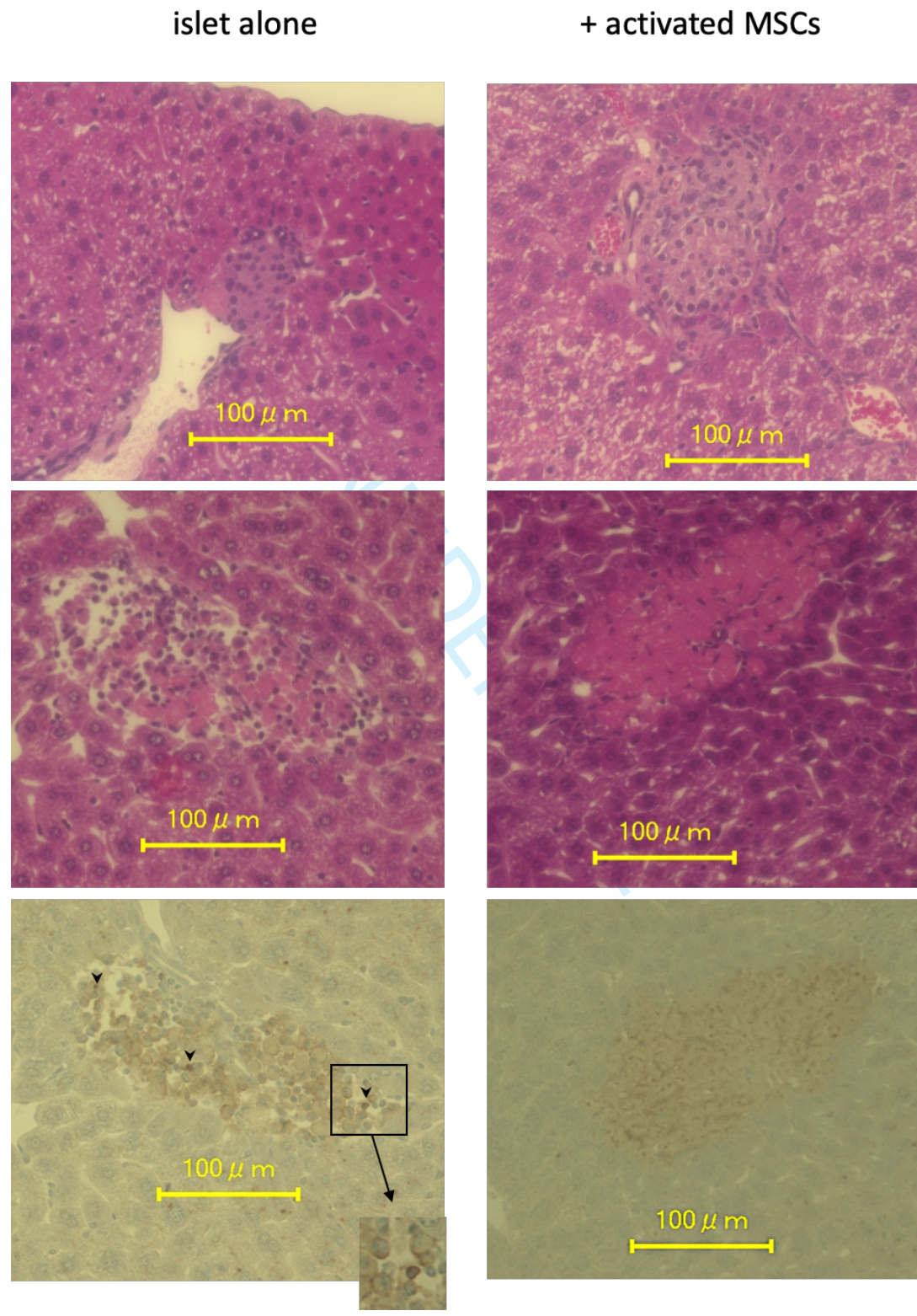


Figure S5. Effect of naive or pre-activated MSCs on liver NK cells in mice with streptozotocin induced-diabetes. Seven days after intraportal injection of streptozotocin, islet transplantation was performed. The phenotype of liver NK cells in each mouse was analyzed three days after transplantation. As a control, 10 days after intraportal injection of streptozotocin, the phenotype was analyzed (STZ-DM). Open circles show naive mice that were not administered streptozotocin (naive). A, B: Number of leukocytes and NK cells from whole livers. C: Proportion of DX5⁻ cells in liver NK cells after co-transplantation. D–F: Expression of CD69, TRAIL, and CXCR3 in liver NK cells after co-transplantation. G–I: Expression of CD69, TRAIL, and CXCR3 expression in liver DX5⁻-gated NK cells. J–L: Expression of CD69, TRAIL, and CXCR3 expression in liver DX5⁺-gated NK cells. Each dot represents an individual animal, and the bars represent the mean \pm SD from five independent experiment with one or two mice per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

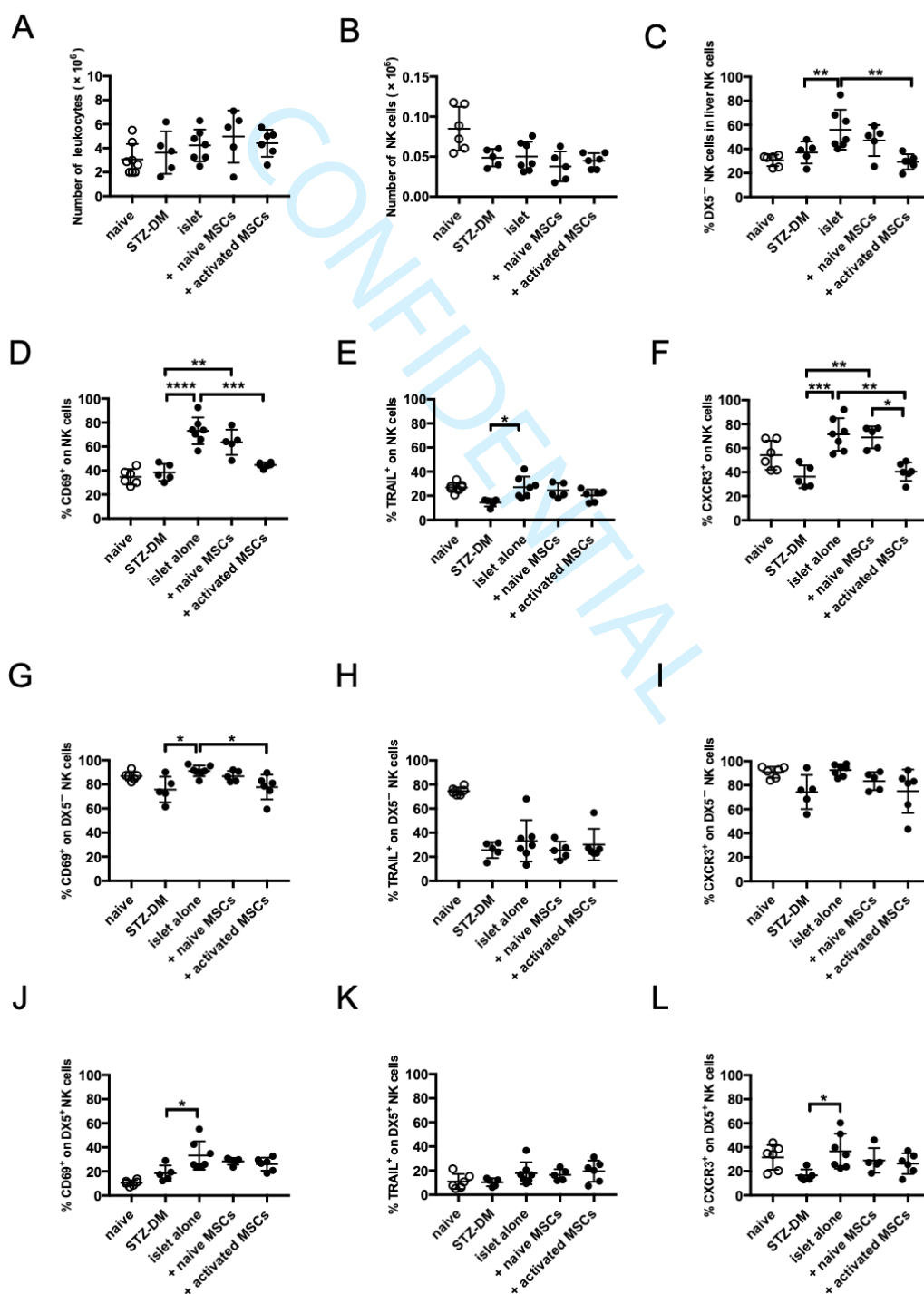
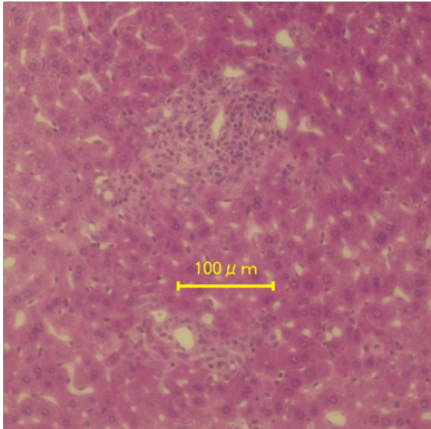
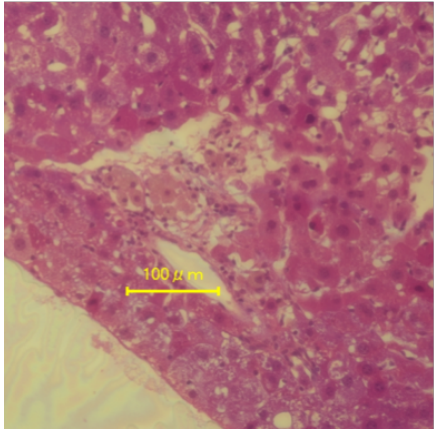


Figure S6. Hematoxylin and eosin staining of islets in the liver 45 days after transplantation of islets, alone or in combination with pre-activated MSCs. A representative image from two independent experiments with one mouse per group is shown.

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islet alone

+ activated MSCs



CONFIDENTIAL