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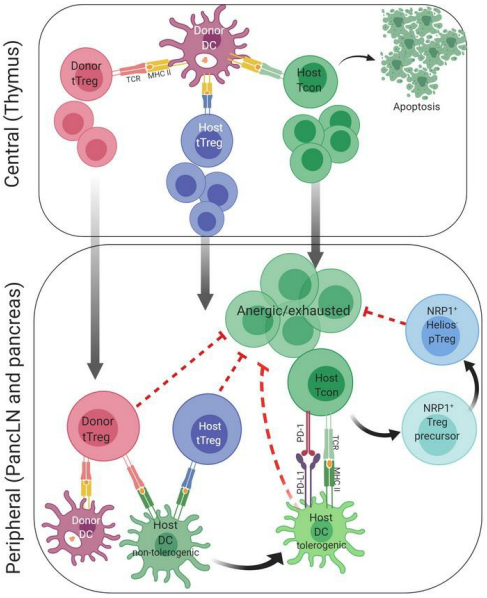
Graphical abstract



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Haploidentical mixed chimerism cures autoimmunity in established type 1 diabetic mice

Running title: Haploidentical mixed chimerism reverses autoimmunity

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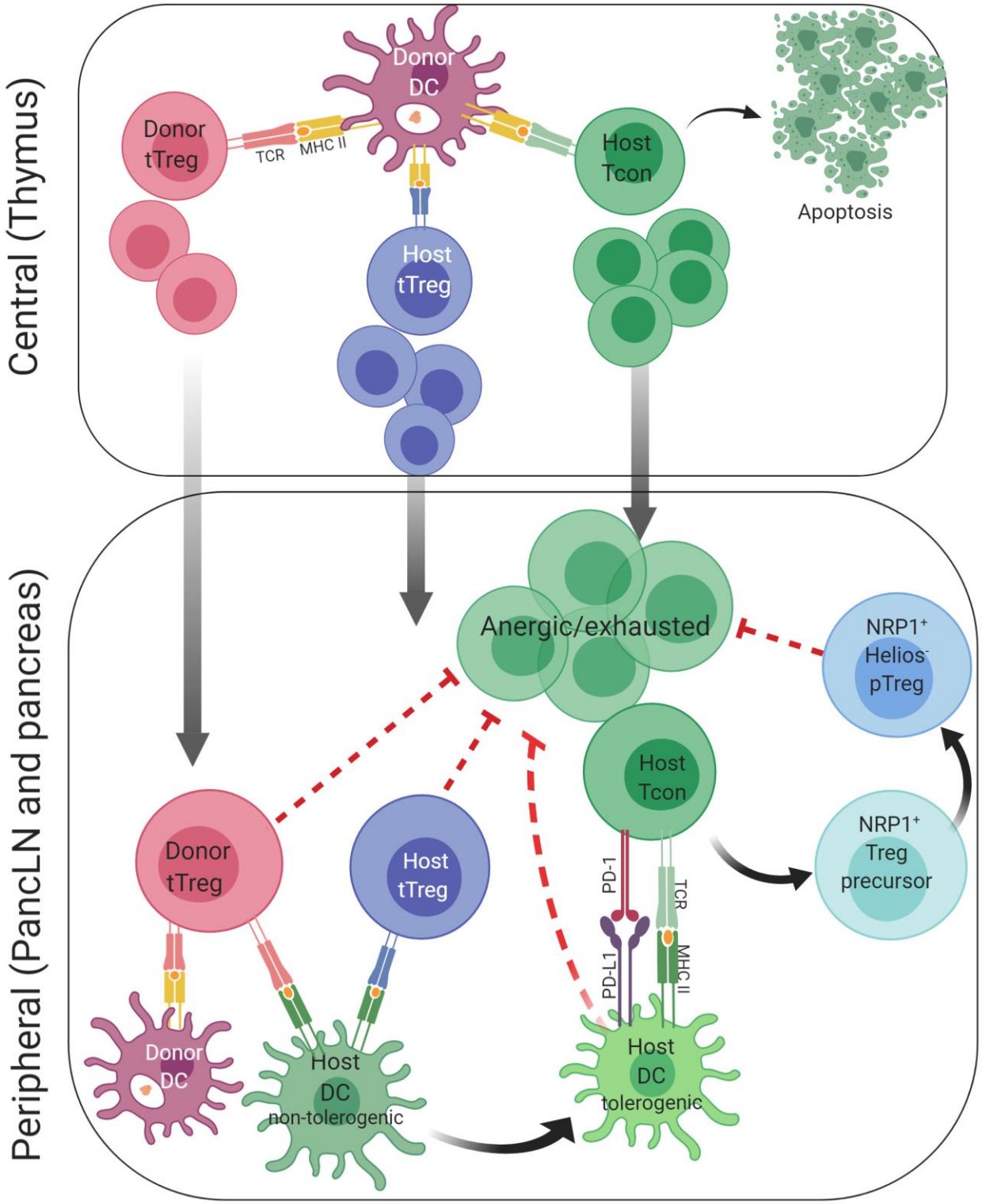
Keywords: type 1 diabetes, autoreactive T cells, mixed chimerism, Immune tolerance, Regulatory T cells, Dendritic cells, PD-L1;

Abstract

Clinical trials are currently testing whether induction of MHC-haploidentical mixed chimerism (Haplo-MC) induces organ transplantation tolerance. Whether Haplo-MC can be used to treat established autoimmune diseases remains unknown. Here, we show that established autoimmunity in euthymic and adult-thymectomized NOD (H-2^{g7}) mice was cured by induction of Haplo-MC under a non-myeloablative anti-thymocyte globulin-based conditioning regimen and infusion of $CD4^+$ T-depleted hematopoietic graft from H-$2^{b/g7}$ F1 donors that express autoimmune-resistant H-2^b or from H-$2^{s/g7}$ F1 donors that express autoimmune susceptible H-2^s. The cure was associated with enhanced thymic negative selection, increased thymic Treg (tTreg) production, and anergy or exhaustion of residual host-type autoreactive T cells in the periphery. The peripheral tolerance was accompanied with expansion of donor- and host-type $CD62L^-Helios^+$ tTreg as well as host-type $Helios^-Nrp1^+$ peripheral Treg (pTreg) and PD-$L1^{hi}$ plasmacytoid DCs (pDC). Depletion of donor- or host-type Treg cells led to reduction of host-type PD-$L1^{hi}$ pDCs and recurrence of autoimmunity; whereas PD-L1 deficiency in host-type DCs led to reduction of host-type pDCs and $Helios^-Nrp1^+$ pTreg cells. Thus, induction of Haplo-MC re-established both central and peripheral tolerance through mechanisms that depend on allo-MHC^+ donor-type DC, PD-$L1^{hi}$ host-type DCs, and the generation and persistence of donor and host-type tTreg and pTreg cells. (200 words)



Graphic abstract legend: Induction of Haplo-MC augments thymic negative selection of Tcon and production of donor- and host-type tTreg cells, leading to re-establishment of central

tolerance. In the periphery, donor- and host-type tTreg cells interact with host-type DCs such as pDCs and restore their tolerogenic features such as upregulation of PD-L1 expression. PD-L1 on DCs interact with PD-1 on activated host-type autoreactive T cells and augment the T cell differentiation into antigen-specific Treg cells. All tTreg and pTreg cells and tolerogenic DCs work together to maintain tolerance status of residual host-type autoreactive T cells.

Introduction

Haploidentical hematopoietic cell transplantation (Haplo-HCT) has been widely applied to treating hematological malignancies and non-malignant disorders (1). Induction of haploidentical mixed chimerism for organ transplantation immune tolerance is under clinical trials (NCT03292445, NCT01165762, NCT01780454, NCT02314403, NCT00801632, NCT01758042), and the results are promising (2-5). However, it remains unclear whether induction of haploidentical mixed chimerism can reverse autoimmunity, because induction of MHC-matched or HLA-matched mixed chimerism is not able to reverse autoimmunity in T1D mice or systemic lupus in humans (6-8).

The essential pathogenesis of autoimmune diseases (i.e. T1D, and lupus) lies in the abnormalities of the hematopoietic stem cells (HSC) (9, 10), because an autoimmune disease can be transferred from potential autoimmune patients into non-autoimmune patients via HLA-matched allogeneic HCT (11). The abnormalities of hematopoietic stem cells can lead to development of defective central and peripheral immune tolerance mechanisms that allow development of systemic or organ-specific autoimmune diseases including T1D, SLE, and MS (12).

NOD mouse model has provided invaluable understanding of basic immune pathogenesis, genetic and environmental risk factors, and immune targeting strategies (13, 14). HSC from NOD mice give rise to thymic medullary DCs that express I-A^{g7} that cannot mediate effective negative selection of autoreactive T cells or effective production of thymic Treg (tTreg) cells, leading to defective function of tTreg cells and loss of tolerogenic features of dendritic cells in the periphery (15, 16) including tolerogenic PD-$L1^{hi}$ plasmacytoid dendritic cells (pDCs) becoming non-tolerogenic PD-$L1^{lo}$ pDCs. Owing to these defects, co-stimulatory blockade could not induce transplantation immune tolerance in NOD mice (17).

Our series of publications with murine models have demonstrated that induction of full MHC-mismatched mixed chimerism cures established autoimmune diseases such as T1D, systemic lupus, and MS without causing graft versus host disease (GVHD) (18-22). Unfortunately, full HLA-mismatched HCT is not yet applicable in clinic. Therefore, we tested whether induction of Haplo-MC reversed established autoimmunity in T1D mice. Using a non-myeloablative conditioning regimen of anti-thymocyte globulin (ATG) + cyclophosphamide (CY) + pentostatin (PT) and infusion of donor $CD4^+$ T-depleted hematopoietic transplant, as described previously (22), we observed that induction of Haplo-MC cured established T1D in both euthymic and adult-thymectomized NOD mice through re-establishing both central and peripheral tolerance.

Results

Induction of Haplo-MC cures autoimmunity in established type 1 diabetic euthymic NOD mice.

When autoimmune-resistant H-2^{b} were backcrossed to NOD mice, the H-$2^{b/g7}$ NOD mice no longer developed T1D; but when autoimmune susceptible H-2^{s} were backcrossed to NOD mice, the H-$2^{s/g7}$ NOD mice still developed T1D (23). Therefore, we tested whether induction of haploidentical mixed chimerism (Haplo-MC) with H-$2^{b/g7}$ or H-$2^{s/g7}$ F1 donors could cure autoimmunity in both prediabetic and new-onset diabetic NOD mice.

9-12 weeks old prediabetic NOD mice were conditioned with anti-thymocyte globulin (ATG) + cyclophosphamide (CY) + pentostatin (PT), as previously described (22, 24), and transplanted with bone marrow (BM, 50 x10^{6}) and spleen cells (30x10^{6}) from H-$2^{b/g7}$ or H-$2^{s/g7}$ F1 donors, with co-injection of depleting anti-CD4 mAb (500 µg/mouse) to prevent acute GVHD, as previously described (25). Both haploidentical transplants resulted in stable Haplo-MC in blood, and the mixed chimerism was confirmed at the end of experiments at 100 days after HCT (Fig. S1). The mixed chimeras showed no signs of clinical GVHD as judged by their healthy appearance and stable bodyweight and no histopathological damage in GVHD target organs including liver and lung (Fig. S2). While 65% of NOD mice given conditioning alone developed hyperglycemia, and the residual mice without hyperglycemia showed severe insulitis, both recipients with H-$2^{b/g7}$ and H-$2^{s/g7}$ Haplo-MC showed normal glycemia for more than 100 days after HCT and showed little insulitis at the end of experiment (Fig. 1A-C). These results indicate that both H-$2^{b/g7}$ and H-$2^{s/g7}$ haplo-MC can prevent T1D development and eliminate insulitis.

Second, we induced Haplo-MC in new-onset T1D NOD mice with blood glucose >400 mg/dL for consecutive 3 days, as previously described (20). Both H-$2^{b/g7}$ and H-$2^{s/g7}$ Haplo-MC

normalized blood glucose with little insulitis in new-onset diabetic NOD mice (Fig. 1D-F). Although conditioning alone was able to normalize blood glucose in many new-onset recipients, which is consistent with previous reports (20, 26, 27), those mice still had severe insulitis (Fig. 1D-F).

Induction of Haplo-MC cures autoimmunity in adult-thymectomized NOD mice

We also tested whether functional thymus was required for preventing T1D and eliminating insulitis in Haplo-MC. Since adult (6 week old)-thymectomized NOD (Thymec-NOD) mice developed T1D (28), we tested whether induction of Haplo-MC in adult Thymec-NOD mice cured T1D. Since induction of mixed chimerism with autoimmune resistant H-$2^{b/g7}$ F1 and autoimmne susceptible H-$2^{g7/s}$ F1 donors were equally effective at curing T1D in NOD mice, we only tested induction of mixed chimerism with H-$2^{g7/s}$ F1 donors in the adult Thymec-NOD mice. The same conditioning regimen of ATG + CY + PT used for euthymic NOD mice were applied to adult Thymec-NOD mice at age ~10 weeks, that is, ~ 4 weeks after thymectomy. The mice were injected with whole bone marrow (50 x 10^6) from H-$2^{s/g7}$ F1 donors. The recipients developed stable mixed chimerism as indicated by co-existence of donor- and host-type T, B, macrophage and granulocytes in the blood, spleen and bone marrow at 80 days after HCT, the end of experiments (Fig. S4A-C). While 60% of untreated Thymc-NOD mice developed hyperglycemia, the mice given conditioning alone or given induction of Haplo-MC didn't develop T1D (Fig. S5A). The untreated mice with euglycemia still showed severe insulitis (Fig. S5 B & C). Interestingly, conditioning alone markedly reduced insulitis, and induction of Haplo-MC further cleared insulitis (Fig. S5 B & C). These results indicate that conditioning with "ATG + CY + PT" alone is able to prevent T1D development with marked reduction of insulitis in adult-thymectomized NOD mice; and induction of Haplo-MC totally eliminates residual insulitis.

Induction of Haplo-MC in lethal TBI-conditioned NOD mice prevents clinical T1D development but is not able to eliminate insulitis.

Furthermore, we tested whether induction of Haplo-MC with myeloablative total body irradiation (950 cGy TBI) conditioning and transplantation of TCD-BM, as previously described (29), could prevent T1D development. Lethal TBI-conditioned NOD mice transplanted with syngeneic NOD TCD-BM alone ($5x10^6$) were used as control. Haplo-MC was induced by transplanting TCD-BM ($5x10^6$) from NOD mice and ($7.5x10^6$) from H-$2^{b/g7}$ or H-$2^{s/g7}$ F1 donors. The recipients given H-$2^{b/g7}$ or H-$2^{s/g7}$ TCD-BM cells developed stable mixed chimerism as indicated by co-existence of donor- and host-type T, B, macrophage and granulocytes in the peripheral blood, spleen and BM (Fig. S6). While 50% (7/14) of control recipients developed T1D with hyperglycemia at ~40 days after HCT, none of the mixed chimeras developed T1D by 80 days after HCT (Fig. S7A). The residual control recipients with euglycemia had more than 60% of residual islets showing severe insulitis (Fig. S7 B & C). Surprisingly, although there was a reduction in insulitis, the mixed chimeras still had more than 30% of islets showing severe insulitis (Fig. S7 C & D). These results indicate that induction of mixed chimerism with TCD-BM is able to control T1D development, but not able to eliminate insulitis.

Taken together, the above results indicate that 1) induction of Haplo-MC via non-myeloablative conditioning with CY+ PT + ATG and transplantation with $CD4^+$ T-depleted graft cures established T1D with elimination of insulitis in prediabetic euthymic and adult-thymectomized as well as new-onset diabetic NOD mice; 2) Induction of Haplo-MC in lethal TBI-conditioned NOD mice given donor TCD-BM cells is not able to cure T1D autoimmunity with elimination of insulitis. In light of a theory proposed by Sykes and colleagues that graft versus autommune cells (GVA) activity is important for cure of autoimmunity after allogeneic HCT (12), we speculate that the lack of cure in the lethal TBI-conditioned Haplo-MC NOD mice may result from transplantation of donor TCD-BM cells that have little GVH and GVA activity; 3) The

following mechanistic studies were focused on how Haplo-MC cures autoimmunity in euthymic and thymectomized NOD mice conditioned with non-myloablative regimen of ATG + CY + PT.

Haplo-MC in euthymic NOD mice augments thymic negative selection of host-type thymocytes.

Autoimmune NOD mice have defects in thymic negative selection (30, 31). Backcross of protective H-2^{b} but not autoimmune susceptible H-2^{s} to NOD mice was able to restore negative selection (23). We tested whether Haplo-MC with H-$2^{b/g7}$ or H-$2^{s/g7}$ donors can restore thymic deletion of host-type autoreactive T cells. To avoid the confounding effects of hyperglycemia, we used prediabetic NOD mice that still have normal glycemia to evaluate the impact of Haplo-MC on thymocyte generation.

We found that the percentage of donor-type $CD4^{+}CD8^{+}$ (DP) thymocytes in the Haplo-MC NOD mice was more than 75%, similar to that of healthy donors (Fig. 2A). This normal percentage of donor-type DP thymocytes suggest that there is no GVHD damage of thymus. The percentage of host-type DP thymocytes in the NOD mice given conditioning alone was more than 80%; however, the percentage of host-type DP thymocytes in the H-$2^{b/g7}$ or H-$2^{s/g7}$ Haplo-MC was significantly reduced, the average being 51.21% and 43.70%, respectively (Fig. 2A). These results suggest that haploidentical mixed chimerism with either H-$2^{b/g7}$ or H-$2^{s/g7}$ donors can restore negative selection in the thymus.

To further test whether H-$2^{b/g7}$ or H-$2^{s/g7}$ Haplo-MC mediated deletion of autoreactive DP thymocytes, we induced Haplo-MC in BDC2.5 NOD mice as described in Fig. S3. Both H-$2^{b/g7}$ and H-$2^{s/g7}$ Haplo-MC depleted almost all DP thymocytes in BDC2.5 NOD mice (Fig. 2B). In addition, autoreactive T cells often express dual TCRα (32, 33). The Vα1Vβ4 transgenic $CD4^{+}$ T cells can express the second TCR with endogenous Vα2 ($Vα2^{+}Vβ4^{+}$) (32). We observed that the

Vβ4$^+$ transgenic CD4$^+$ T cells with endogenous Vα2$^+$ among residual CD4$^+$CD8$^-$ (SP) thymocytes were markedly reduced (Fig. 2C). These results indicate that induction of Haplo-MC augments negative selection of host-type thymocytes, including autoreactive thymocytes.

Haplo-MC in euthymic NOD mice augments thymic generation of host- and donor-type Foxp3$^+$ tTreg cells.

Augmentation of negative selection of conventional thymocytes is often accompanied by enhanced tTreg production (15). We also observed that induction of H-2$^{b/g7}$ or H-2$^{s/g7}$ Haplo-MC increased percentage of Foxp3$^+$ tTreg cells among host-type DP and CD4$^+$ SP thymocytes in WT NOD mice (Fig. 3A) and increased percentage of Foxp3$^+$ tTreg cells among CD4$^+$ SP thymocytes in transgenic BDC2.5 NOD mice (Fig. 3B). Foxp3$^+$ tTreg cells among DP thymocytes in the mixed chimeric BDC2.5 NOD mice were not measured, due to too few host-type DP thymocytes for reliable analysis as shown in Fig. 2B. Donor-type Treg production was also enhanced in the thymus of transgenic BDC2.5 NOD mice, although not in the thymus of WT NOD mice (Fig. S8). These results indicate that Haplo-MC augments thymic generation of host-type tTreg cells in NOD mice.

Donor-type DC subsets are present in the thymus of Haplo-MC mice.

There are multiple subsets of CD11c$^+$ DCs in the thymus, including CD11c$^+$B220$^+$PDCA-1$^+$ plasmacytoid DCs (pDCs), CD8$^+$SIRPα$^-$ thymus-resident DCs (tDCs), and CD8$^-$SIRPα$^+$ migratory DCs (mDCs). pDCs and tDCs augment thymic negative selection with limited impact in Treg generation; in contrast, mDCs augment both central negative selection and thymic Treg (tTreg) generation (34-37). We observed that all three subsets of donor-type DCs were present in the thymus of the wild-type NOD with Haplo-MC (Fig. 3C). As compared to control donor, there was a significant increase in CD8$^+$ tDCs, but no difference or a reduction in the percentage of pDCs and mDCs (Fig. 3C). Therefore, the increased negative selection and

augmented Treg generation in the thymus of Haplo-MC is associated with presence of donor-type DC subsets.

Haplo-MC augments reduction of host-type CD62L^{-}CD44^{hi} effector memory T cells in the periphery of both euthymic and thymectomized NOD mice.

Since H-2$^{b/g7}$ and H-2$^{s/g7}$ Haplo-MC eliminated or markedly reduced insulitis in established diabetic NOD mice (Fig. 1), we compared the percentage and yield of host-type CD62L^{-}CD44^{hi} effector memory (Tem) cells in the spleen, PancLN and pancreas of Haplo-MC WT NOD mice. Interestingly, Haplo-MC did not reduce but instead increased the percentage of CD62L^{-}CD44^{hi} CD4^{+} or CD8^{+} Tem cells in the spleen, PancLN and pancreas of WT NOD mice; however, the yield was markedly reduced (Fig. 4A-B and Fig. S10A-B). We observed similar results in adult-thymectomized NOD mice with Haplo-MC (Fig. S9).

On the other hand, both percentage and yield of host-type autoreactive CD62L^{-}CD44^{hi} CD4^{+} Tem cells in the spleen or PancLN of Haplo-MC transgenic BDC2.5 NOD mice were markedly reduced (Fig. 4C and Fig. S10C). Furthermore, we used a HIP2.5-tetramer that specifically identifies the chromogranin-proinsulin hybrid peptide-specific autoreactive CD4^{+} T cells (38) and a NRP-V7-tetramer that specifically identifies IGRP$_{206-214}$ peptide-specific autoreactive CD8^{+} T cells (39) to measure the changes of the antigen-specific autoreactive Foxp3^{-}CD4^{+} and CD8^{+} T cells in the pancreas. Tetramer^{+}CD4^{+} or CD8^{+} T cells in WT NOD mice given conditioning alone were only detectable in the pancreas but not in the spleen or PancLN, ~ 1% among Foxp3^{-}CD4^{+} T and ~10% among CD8^{+} T cells (Fig. 4D). Both H-2$^{b/g7}$ and H-2$^{s/g7}$ Haplo-MC clearly depleted the autoreactive Foxp3^{-}CD4^{+} or CD8^{+} T cells in the pancreas of Halo-MC WT NOD mice (Fig. 4D). These results indicate that Haplo-MC preferentially reduces host-type autoreactive Foxp3^{-} conventional T cells in the periphery.

Haplo-MC augments expansion of Nrp1^{+}CD73^{hi}FR4^{hi} anergic CD4^{+} T cells in the periphery of euthymic but not thymectomized NOD mice.

CD73^{hi}FR4^{hi}CD4^{+} T cells in the periphery are anergic T cells (40), and Nrp1^{+} anergic CD4^{+} T cells can be the precursors of Helios^{-}Nrp1^{+} peripheral Treg (pTreg) cells (41, 42). We also found that compared to control NOD mice, the residual CD4^{+} Tem cells in the PancLN and pancreas of Haplo-MC NOD mice contained a higher percentage of anergic CD73^{hi}FR4^{hi}CD4^{+} T cells and a higher percentage of Nrp1^{+} cells among the CD73^{hi}FR4^{hi} Tem cells (Fig. 5 A & B). We also observed that with Thymec-NOD mice, the conditioning alone increased the percentage of CD73^{hi}FR4^{hi} cells among residual host-type CD62L^{-}CD44^{hi}CD4^{+} Tem cells in the PancLN as compared to unconditioned mice, and induction of mixed chimerism did not further increase the percentage (Fig. S11). Additionally, there was no difference in the percentage of Nrp1^{+} cells among the CD73^{hi}FR4^{hi} cells in the mixed chimeras (Fig. S11). These results indicate that residual host-type CD4^{+} T cells in the pancreatic LN and pancreas of both euthymic and thymectomized Haplo-MC NOD mice have enhanced anergy status, but increase of Nrp1^{+} anergic CD4^{+} T cells is only observed in euthymic Haplo-MC NOD mice.

Haplo-MC augments expansion of host-type CD62L^{-}CD44^{hi} effector memory tTreg and Helios^{-}Nrp1^{+} pTreg cells in the PancLN and pancreas of euthymic but not thymectomized NOD mice.

Foxp3^{+} Treg cells in the periphery include thymus-derived Helios^{+} tTreg and peripheral conventional T-derived antigen-specific Helios^{-}Nrp1^{+} pTreg cells (42). tTreg and pTreg cells play important roles in regulating systemic and local autoimmunity, respectively (43). Changes of Treg cells in the spleen reflect systemic, and changes in the organ or organ-draining LN such as PancLN and pancreas reflect local regulation of immune response. Thus, we measured the changes of donor- and host-type Treg subsets in the periphery including spleen, PancLN and pancreas of Haplo-MC NOD mice. We found that total host-type Treg cells were expanded in

the pancreatic LN and pancreas of both H-$2^{b/g7}$ and H-$2^{s/g7}$ Haplo-MC, although Treg expansion in the spleen was observed only in H-$2^{b/g7}$ but not H-$2^{s/g7}$ mixed chimeras (Fig. 6A). Based on Helios and CD62L staining, we observed significant expansion of CD62L^{-}Helios^{+} effector memory tTreg cells in the pancreatic LN of both mixed chimeras as compared to NOD mice given conditioning alone (Fig. 6B).

As mentioned above, we observed expansion of Nrp1^{+}CD73^{hi}FR4^{hi}CD4^{+} T cells, the Nrp1^{+} pTreg precursors, in Haplo-MC NOD mice (Fig. 5). Thus, we compared the percentage of Nrp1^{+}Helios^{-} pTreg cells in H-$2^{b/g7}$ and H-$2^{s/g7}$ Haplo-MC. Gating on host-type Helios^{-}Foxp3^{+} pTreg cells, we found that there was an increase of Nrp1^{+} pTreg cells in the spleen and PancLN of H-$2^{b/g7}$ mixed chimeras and an increase of Nrp1^{+} pTreg cells in the pancreas of H-$2^{s/g7}$ mixed chimeras (Fig. 6C). Upregulation of ICOS, GITR and CTLA-4 expression is associated with enhanced Treg function (44-47), and consistently, host-type Treg cells in the PancLN of mixed chimeras upregulated expression of ICOS and GITR, although no difference in CTLA-4 expression was observed (Fig. S12). No difference was observed in Treg expression of ICOS, GITR or CTLA-4 in the spleen of mixed chimeras or control mice (Fig. S12).

However, compared to Thymec-NOD given conditioning alone, Thymec-NOD mice with Haplo-MC did not show significant difference in the percentage of total Treg cells or host-type Nrp1^{+}Helios^{-} pTreg cells, although they showed an increase in the percentage of Helios^{+}CD62L^{-} effector memory tTreg cells among total Treg cells (Fig. S13). Taken collectively, these results indicate that 1) Haplo-MC augments activation and expansion of host-type Helios^{+} tTreg subset in the PancLN and pancreas of NOD mice; 2) Haplo-MC also augments expansion of Helios^{-} Nrp1^{+} pTreg cells in euthymic but not thymectomized Haplo-MC NOD mice.

Haplo-MC augments expansion of donor-type CD62L^{-}CD44^{hi} effector memory tTreg in the PancLN and pancreas of euthymic and thymectomized NOD mice.

Donor-type Treg cells were present in the spleen, PancLN and pancreas of both H-2$^{b/g7}$ and H-2$^{s/g7}$ Haplo-MC. As compared to control donor mice, the percentage of total Treg of Haplo-MC was similar in the spleen and variable in the PancLN and pancreas (Fig. 7A). However, the percentage of CD62L^{-}Helios^{+} effector memory tTreg cells in the Haplo-MC was increased in both spleen and PancLN (Fig. 7B). Furthermore, donor-type Treg cells in the spleen and/or PancLN of Haplo-MC upregulated expression of CTLA-4, although expression of ICOS or GITR was variable (Fig. 7C). Similarly, as compared to donor control, there was a marked increase of donor-type total Treg and Helios^{+}CD62L^{-} effector memory tTreg cells in the PancLN of Haplo-MC Thymec-NOD mice (Fig. S14). These results indicate that Haplo-MC augments activation and expansion of donor-type tTreg cells in the periphery of both euthymic and thymectomized Haplo-MC NOD mice

Haplo-MC upregulates host-type pDC expression of PD-L1 in euthymic but not thymectomized NOD mice.

Peripheral tolerance is associated with tolerogenic DCs, especially pDCs that express high levels of PD-L1 (48, 49), and loss of tolerogenic features of pDC in the periphery plays an important role in T1D pathogenesis (50, 51). Thus, we measured changes of host-type DCs as well as their expression of PD-L1 in the spleen of mixed chimeras. We observed that among host-type DCs in both H-2$^{b/g7}$ and H-2$^{s/g7}$ Haplo-MC, there was a marked reduction in percentage of CD11c^{+}B220^{+}PDCA-1^{+} pDC among total host-type DCs, especially in the H-2$^{s/g7}$ mixed chimeras, as compared to that of control mice given conditioning alone, although we observed no significant changes in the percentage of CD8^{+} or CD11b^{+} DC subsets (Fig. 8A). In contrast, the residual pDCs in both mixed chimeras upregulated expression of PD-L1, as did CD8^{+} DC subset, but not CD11b^{+} DC subset (Fig. 8B). Interestingly, although there was a

marked reduction of pDC in the spleen of Haplo-MC of Thymec-NOD, the residual pDC did not upregulate their expression of PD-L1 as compared to conditioning alone (Fig. S15). These results indicate that induction of Haplo-MC reduces host-type pDCs in both euthymic and thymectomized NOD mice, but Haplo-MC augments the residual pDCs upregulate their expression of PD-L1 in the euthymic but not thymectomized mice.

Maintenance of peripheral tolerance of residual host-type autoreactive T cells in the euthymic Haplo-MC mice requires both donor- and host-type Foxp3^{+} Treg cells.

Since there was an expansion of donor- and host-type Treg effector memory cells in both H-2$^{b/g7}$ and H-2$^{s/g7}$ mixed chimeric NOD (Fig. 6 & 7), we tested whether those Treg cells were required for maintaining peripheral tolerance by using Foxp3^{DTR} expression in either donor- or host-type Treg cells in H-2$^{b/g7}$ mixed chimeric NOD mice, as depicted in Fig 9A. Depletion of Treg cells was induced by injection of DT every 3 days for 21 days, starting at 45-60 days after induction of mixed chimerism, as described in the materials and methods. Injection of DT specifically reduced donor-type Treg by ~95% and reduced host-type Treg by ~90% (Fig. 9B and Fig. S16). Depletion of donor-type or host-type Treg cells induced significant but moderate recurrence of insulitis, without causing hyperglycemia (Fig. 9C). Simultaneous depletion of both donor- and host-type Treg cells did not appear to significantly enhance the insulitis, but because the treatment led to rapid decline of health and the mice died or became very sick without hyperglycemia before completion of treatment, the results could not be used for comparison. Therefore, we focused on comparing depletion of donor-type versus depletion of host-type Treg cells. Depletion of donor-type Treg cells but not depletion of host-type Treg cells led to increase in percentage of host-type CD4^{+} and CD8^{+} CD62L^{-}CD44^{+} Tcon effector memory cells in the PancLN (Fig. 9D); in contrast, depletion of host-type but not donor-type Treg cells led to decrease in percentage of CD73^{hi}FR4^{hi} anergic CD4^{+} Tcon and IL-7Rα^{-}PD-1^{hi} anergic/exhausted CD8^{+} Tcon cells (Fig. 9E). These results indicate that both donor- and host-

type Treg cells contribute to maintenance of peripheral tolerance of residual autoreactive T cells, although each have a different functional effect.

Maintenance of peripheral tolerance of residual host-type autoreactive T cells requires host-hematopoietic cell expression of PD-L1.

Because host-type DCs, especially pDCs, in the H-$2^{b/g7}$ and H-$2^{s/g7}$ Haplo-MC euthymic NOD mice expressed higher levels of PD-L1 as compare to mice given conditioning alone (Fig. 8), we tested whether host DC expression of PD-L1 was required for maintaining the peripheral tolerance using H-$2^{b/g7}$ mixed chimeric NOD mice. Parenchymal cell expression of PD-L1 was reported to play a critical role in prevention of T1D in NOD mice (52). We wished to evaluate the role of host-type DC expression of PD-L1 on maintaining peripheral tolerance in the presence of host-parenchymal tissue expression of PD-L1. Accordingly, we established Haplo-MC by co-injection of donor-type TCD-BM from H-$2^{b/g7}$ F1 donor mice and host-type TCD-BM from WT or PD-$L1^{-/-}$ NOD mice into lethally irradiated WT NOD mice, as depicted in Fig. 10A. The control NOD recipients were given PD-$L1^{-/-}$-NOD TCD-BM alone.

The NOD recipients with TCD-BM from H-$2^{b/g7}$ F1 donor and TCD-BM from syngeneic WT or PD-$L1^{-/-}$ NOD mice developed stable mixed chimerism (Fig. S17). While none (0/12) of the H-$2^{b/g7}$ mixed chimeras that received PD-$L1^{+/+}$ NOD TCD-BM (PD-$L1^{+/+}$ chimeras) developed T1D or hyperglycemia, 82%(9/11) of the H-$2^{b/g7}$ mixed chimeras that received PD-$L1^{-/-}$ NOD TCD-BM (PD-$L1^{-/-}$ chimeras) developed T1D with hyperglycemia, and 94% (17/18) NOD recipients given PD-$L1^{-/-}$ NOD TCD-BM alone (PD-$L1^{-/-}$ NOD) developed T1D with hyperglycemia (Fig. 10B). Furthermore, as compared with PD-$L1^{+/+}$ mixed chimeras without T1D, PD-$L1^{-/-}$ mixed chimeras with T1D showed expansion of host-type $CD4^+$ and $CD8^+$ T effector cells in the pancreatic LN and pancreas (Fig. 10C). Those T effector cells had a decrease in percentage of anergic

$CD73^{hi}FR4^{hi}CD4^{+}$ T cells (Fig. 10D). These results indicate that host-type hematopoietic cell expression of PD-L1 is required for maintaining peripheral tolerance of residual autoreactive T cells in Haplo-MC euthymic NOD mice.

There is a mutual influence and compensatory role between donor- and host-type Treg cells in euthymic Haplo-MC NOD mice.

Both donor- and host-type Treg cells were activated in the Haplo-MC NOD mice, as indicated by the relative increase of $CD62L^{-}$ effector memory Treg cells, although they showed different changes in surface receptors: donor-type Treg cells upregulated expression of CTLA-4, but host-type Treg cells upregulated expression of ICOS and GITR (Fig. 6 & 7 and Fig. S12). Next, we evaluated whether there is a mutual influence between donor- and host-type Treg cells in the Haplo-MC NOD mice. Depletion of donor-type Treg cells led to slight increase in the percentage of host-type Treg cells and significant upregulation of expression of CTLA-4 in the spleen and PancLN (Fig. 11 A & B). However, upregulation of expression of ICOS and GITR was observed only in the spleen but not in the PancLN (Fig. 11B). In contrast, depletion of host-type Treg cells led to significant expansion of donor-type Treg cells and their upregulation of expression of CTLA-4 in the spleen but not in the PancLN. In addition, no significant changes in ICOS and GITR expression in the spleen or PancLN (Fig. 11 C & D). These results suggest that the regulatory emphasis of donor- and host-type Treg cells differs: donor-type Treg cells are more involved in regulating systemic immune response such as in the spleen, and host-type Treg cells are more involved in regulating local immune response such as in the PancLN. These observations may also provide an explanation why depletion of donor- or host-type Treg cell alone did not cause overt insulitis or hyperglycemia in the Haplo-MC NOD mice.

Donor- and host-type tTreg cells are required for upregulating host-type pDC expression of PD-L1 that augments expansion of host-type and donor-type $Nrp1^{+}Helios^{-}$ pTreg cells.

Because host-type pDCs were found to upregulate expression of PD-L1 in Haplo-MC euthymic NOD mice (Fig. 8), we analyzed the impact of depletion of Treg cells on the host-type pDC expression of PD-L1. Interestingly, depletion of either donor-type or host-type Treg cells led to a decrease in the percentage of host-type $B220^{+}PDCA\text{-}1^{+}$ pDCs (Fig. 12A) as well as their down-regulation of expression of PD-L1 (Fig. 12B). These results suggest that donor- and host-type Treg cells can augment host-type pDC expansion and their expression of PD-L1.

Furthermore, we evaluated the impact of PD-L1 expression by host-type hematopoietic cells on expansion of host-type pDC and Treg cells. We found that PD-L1 deficiency in host-type hematopoietic cells led to a marked decrease in the percentage of host-type pDCs (Fig. 12C), although no reduction in $CD8^{+}$ lymphoid or $CD11b^{+}$ myeloid DC subsets (Fig. 12D). We also observed that PD-L1 deficiency in host-type hematopoietic cells resulted in no changes in the total percentage of host- and donor-type $Foxp3^{+}$ Treg cells in the spleen, PancLN or pancreas (Fig. S18 A & B). However, the PD-L1 deficiency in host-type hematopoietic cells resulted in a marked reduction in the percentage of host-type $Helios^{-}$ pTreg cells that are predominantly $Nrp1^{+}$ in the PancLN and pancreas as well as marked reduction of donor-type $Helios^{-}$ pTreg cells in the pancreas (Fig. 12E). Additionally, we observed that expansion of antigen-specific Treg cells in the pancreas of Haplo-MC BDC2.5 NOD mice was associated with effective prevention of T1D (Fig. S19). These results indicate that 1) host-type pDC expression of PD-L1 play a critical role in expansion of host-type $Helios^{-}Nrp1^{+}$ pTreg cells in the PancLN and pancreas of Haplo-MC euthymic NOD mice; 2) autoantigen-specific pTreg cells may play an important role in controlling residual autoreactive T cells in the Haplo-MC euthymic NOD mice.

Discussion

Autoimmune T1D is associated with particular MHC (HLA) in mouse and humans (53, 54) and arises from defects in both central and peripheral tolerance mechanisms (55). We previously reported that induction of full MHC-mismatched but not MHC-matched mixed chimerism was able to reverse autoimmunity in prediabetic, new-onset and late-stage diabetic WT NOD mice (18-20); full MHC-mismtached but not matched mixed chimerism augmented thymic negative selection of autoreactive T cells and tolerized residual autoreactive T cells in the periphery of BDC2.5 NOD mice with transgenic autoreactive T cells (6, 51). However, full MHC-mismatched mixed chimerism is not yet applicable in clinic. Although haploidentical HCT is now widely used in clinic (1), whether haplo-identical mixed chimerism (Haplo-MC) could cure autoimmunity remains unknown, because MHC (HLA)-matched mixed chimerism cannot reverse autoimmunity in mice or humans (6, 7). Although we have reported that full MHC-mismatched mixed chimerism can reverse autoimmunity in WT NOD mice and augment thymic negative selection and peripheral tolerance of autoreactive T cells in transgenic BDC2.5 NOD mice, the cellular mechanisms of tolerance and how thymic Treg cells regulate peripheral DCs and pTreg cells in the mixed chimera remains unclear.

In the current studies, we have demonstrated that, with conditioning regimen of "ATG + CY + PT" and depletion of $CD4^+$ T cells in transplant, induction of Haplo-MC effectively cures the established autoimmunity with elimination of insulitis in both euthymic and adult-thymectomized NOD mice, with not only H-$2^{b/g7}$ F1 donors that possess autoimmune resistant H-2^b but also H-$2^{s/g7}$ donors that possess autoimmune susceptible H-2^s. The cure of autoimmunity in thymectomized NOD mice is associated with expansion of donor- and host-type Treg cells and anergy of residual host-type T cells. The cure of autoimmunity in euthymic NOD mice is associated with preferential augmentation of negative selection of host-type autoreactive

thymocytes and generation of tTreg cells in the thymus, as well as associated with expansion of activated tTreg cells, upregulation of pDC expression of PD-L1, and preferential expansion of host-type pTreg cells in the periphery. On the other hand, we have also found that Haplo-MC in euthymic NOD mice established with myeloablative TBI-conditioning and infusion of TCD-BM cells from the H-$2^{b/g7}$ or H-$2^{s/g7}$ donors was not able to eliminate insulitis, although it prevented clinical T1D development. These observations are novel and also support a theory proposed by Sykes and colleagues that cure of established autoimmunity by induction of mixed chimerism via allogeneic HCT requires 1) graft versus autoimmune cells (GVA) activity; 2) thymic depletion; 3) peripheral anergy and deletion of autoreactive T cells; 4) expansion of Treg cells (12).

First, GVA activity in the absence of GVHD is important. Induction of Haplo-MC without causing GVHD in recipients conditioned with non-myeloablative "ATG + CY + PT" requires infusion of $CD4^+$ T-depleted hematopoietic transplant containing donor $CD8^+$ T, NK and other cells (56). And induction of Haplo-MC in recipients conditioned with myeloablative TBI requires infusion of donor TCD-BM cells (29). We observed that the former but not the latter approach was able to eliminate insulits in Haplo-MC NOD mice, although both approaches prevented clinical T1D development. Therefore, infusion of $CD4^+$ T-depleted hematopoietic graft containing lymphocytes such as $CD8^+$ T and NK cells that mediate GVA acitivity plays an important role in eliminating residual autoreactive T cells in the mixed chimeras.

Second, Haplo-MC with donors that possess autoimmune-susceptible H-2^s is as effective as Haplo-MC with donors that possess autoimmune-resistant H-2^b in augmenting negative selection and generation of tTreg cells in the thymus. We observed that both H-$2^{b/g7}$ and H-$2^{s/g7}$ mixed chimeras showed partial depletion of host-type $CD4^+CD8^+$ (DP) thymocytes in WT NOD and near complete depletion of the DP thymocytes in BDC2.5 NOD with transgenic autoreactive

$CD4^+$ T cells. In contrast, there was a marked expansion of host-type tTreg cells among $CD4^+CD8^-$ thymocytes in both WT and BDC2.5 NOD mice with H-$2^{b/g7}$ and H-$2^{s/g7}$ chimerism. Based on the partial deletion of DP thymocytes in the thymus of WT NOD and complete deletion of DP thymocytes in the thymus of BDC2.5 NOD with transgenic autoreactive T cells, we theorize that induction of Haplo-MC preferentially augments thymic negative selection of autoreactive T cells, with augmentation of tTreg generation in NOD mice.

It is particularly interesting that autoimmune susceptible H-2^s is as effective as autoimmune-resistant H-2^b in augmenting negative selection and expansion of host-type Treg cells in the Haplo-MC NOD mice, despite being unable to augment negative selection or prevent T1D development when backcrossed to NOD mice (23). This may result from different H-2^s cell distribution in H-$2^{s/g7}$ Haplo-MC NOD mice and H-$2^{s/g7}$ NOD mice. When H-2^s is backcrossed to NOD mice, H-2^s is expressed by both thymic cortical and medullar epithelial cells and DC cells. In this case, similar to I-A^{g7}, I-A^s is involved in both positive and negative selection and manifests with defective negative selection (23). However, in the H-$2^{g7/s}$ Haplo-MC, cortical epithelial cells express I-A^{g7} without I-A^s. Donor-type DCs that express I-$A^{g7/s}$ are present in the thymic medullary. For the thymocytes positively selected by only I-A^{g7} in thymic cortex, MHCII of I-A^s expressed by donor-type DCs in the medullary is equivalent to an “allo-MHC”. TCRs have particular high binding affinity towards foreign MHC (57). The high binding affinity leads to augmentation of negative selection of host-type Tcon cells, in particular, host-type cross-reactive autoreactive Tcon cells. Our previous report showed that many autoreactive T cells are cross-reactive, and MHC-mismatched mixed chimeras preferentially deplete those cross-reactive T cells (32). On the other hand, the high binding affinity leads to augmentation of $Foxp3^+$ tTreg generation (58). In addition, augmented deletion of autoreactive T cells, especially the cross-reactive autoreactive T cells, may make the residual autoreactive T cells susceptible

to Treg suppression in the periphery. It was reported that T cells from NOD mice or T1D patients are resistant to Treg suppression (59)

Third, Haplo-MC preferentially augments deletion and induction of anergy of host-type T cells in the periphery of NOD mice. We observed that elimination of insulitis in euthymic and thymectomized WT NOD mice was associated with marked reduction in yield although not in percentage of $CD44^{hi}CD62L^{-}$ effector memory host-type T cells in the pancreatic LN and pancreas, as well as an increase in the percentage of $CD73^{hi}FR4^{hi}$ anergic cells among residual host-type T cells. Haplo-MC in the euthymic NOD mice completely deleted autoantigen-specific HIP-2.5-tetramer$^{+}CD4^{+}$ and NRP-V7-tetramer$^{+}CD8^{+}$ T cells among host-type T cells in the pancreas. Therefore, Haplo-MC can preferentially mediate deletion and anergy of host-type autoreactive T cells in the peripheral lymphoid tissues and autoimmune target organs.

Fourth, cure of autoimmunity with elimination of insulitis in euthymic and thymectomized Haplo-MC NOD mice is associated with differential expansion of tTreg and pTreg cells. T1D pathogenesis in NOD mice or T1D patients is associated with quantitative and qualitative defects in Treg cells (60, 61) as well as associated with Tcon cell resistance against Treg suppression (59, 62). We observed that cure with elimination of insulitis in the euthymic Haplo-MC was associated with expansion of both donor- and host-type $CD62L^{-}Helios^{+}$ tTreg cells as well as expansion of host-type $CD62L^{-}Helios^{-}Nrp1^{+}$ pTreg cells. In contrast, the cure in thymectomized Haplo-MC mice was only associated with expansion of both donor- and host-type $CD62L^{-}Helios^{+}$ tTreg cells. Therefore, we propose that 1) induction of Haplo-MC allows Treg cells to suppress residual autoreactive T cells; 2) activation and expansion of donor- and host-type tTreg cells are sufficient for controlling residual autoreactive T cells in thymectomized Haplo-MC, but additional expansion of host-type pTreg cells is also required for controlling residual autoreactive T cells in the euthymic Haplo-MC.

Fifth, Haplo-MC in euthymic mice restores peripheral pDC tolerance status with upregulation of PD-L1 and augments pTreg expansion. It has been reported that Foxp3$^-$CD73^{hi}FR4^{hi}Nrp1$^+$CD4$^+$ T cells can be the precursors of Foxp3$^+$ pTreg cells (41); PD-L1 interaction with PD-1 on activated Tcon cells can augment their transdifferentiation into pTreg cells (63); PD-1 signaling also stabilized Foxp3 expression in pTreg cells (64); and PD-L1 interaction with CD80 on Treg cells augmented Treg cell survival and expansion (65, 66). Consistently, Haplo-MC NOD mice showed expansion of both donor- and host-type Helios$^+$CD62L$^-$ effector memory tTreg and expansion of Helios$^-$CD62L$^-$Nrp1$^+$ pTreg cells in the spleen, pancreatic lymph nodes and pancreas. In addition, we observed that the prevention of T1D development in BDC2.5 NOD mice was associated with expansion of antigen-specific pTreg cells. Furthermore, we observed that the expansion of Helios$^-$CD62L$^-$Nrp1$^+$ pTreg cells was associated with expansion of anergic Foxp3$^-$CD73^{hi}FR4^{hi}Nrp1$^+$CD4$^+$ T cells as well as upregulation of PD-L1 by host-type pDCs.

On the other hand, depletion of either donor- or host-type Treg cells led to a marked reduction of host-type pDCs and their down-regulation of PD-L1; in contrast, PD-L1 deficiency in host-type hematopoietic cells resulted in marked reduction of host-type pDCs and severe loss of host-type pTreg cells in the PancLN and pancreas of Haplo-MC NOD mice. Therefore, we propose that donor-type and host-type tTreg cells from the thymus of Haplo-MC can restore the tolerance status of host-type peripheral pDCs by upregulating expression of PD-L1, and the PD-L1 interaction with PD-1 and CD80 on host-type autoreactive Tcon cells augments their transdifferentiation and expansion of antigen-specific pTreg cells.

In summary, we propose a systemic network of allo-MHC-expressing DCs, Treg cells and tolerogenic DCs in the Haplo-MC NOD mice. As depicted in the graphic abstract, induction of Haplo-MC allows allo-MHC expressing donor-type DC subsets to engraft in the host-thymus,

resulting in augmentation of negative selection of host-type autoreactive T cells and production of donor- and host-type tTreg cells. The tTreg cells are activated in the periphery and restore the tolerogenic features of host-type DCs (i.e. pDCs), including upregulation of their expression of PD-L1. The interactions between tolerogenic pDCs and residual autoreactive T cells via co-inhibitory receptors such as PD-L1 interaction with PD-1 augment autoreactive T cells become anergic/exhausted T cells or become antigen-specific pTreg cells. Furthermore, the Haplo-MC is a relatively stable system. Depleting either donor-type or host-type Treg cells only causes moderate and self-limiting recurrence of insulitis in the absence of clinical T1D; because depletion of donor-type Treg cells can lead to compensatory expansion of host-type Treg cells, or vice versa. Therefore, induction of Haplo-MC can restore both central and peripheral tolerance in T1D mice.

Although we have observed preferential deletion of host-type autoreactive T cells in the thymus and preferential expansion of host-type pTreg cells that suppress autoimmunity in the periphery by observing the effects of Haplo-MC in WT NOD mice and in BDC2.5 NOD mice carrying transgenic autoreactive T cells, it remains unclear whether the effect of Haplo-MC on autoimmunity in NOD mice is a result of established mixed chimerism and/or is primarily an effect of the underlying autoimmunity. Dissecting these relations may require examining anergic/Treg axis in non-autoimmune vs autoimmune mice with Haplo-MC in the future studies.

We also propose that induction of Haplo-MC using non-myeloablative conditioning of ATG + CY + PT and infusion of $CD4^+$ T-depleted hematopoietic transplant may have strong clinical potential as a curative therapy for refractory autoimmune diseases. First, induction of haplo-MC is more effective than matched-MC in reversal of autoimmunity. Induction of MHC (HLA)-matched mixed chimerism has been successfully achieved in humans for providing kidney transplantation immune tolerance (7, 67). However, induction of MHC (HLA)-matched mixed

chimerism has been reported to not prevent lupus flare in patients (7) and to not prevent T1D in mouse models (6). The current studies showed that induction of haploidentical mixed chimerism effectively "cure" T1D in both euthymic and thymectomized T1D mice, even with a donor that possesses an autoimmune susceptible MHC.

Second, the current regimen of induction of haplo-MC is likely to be applicable in clinic. Haploidentical HCT has been widely used in clinic for treating non-maligant hereditary hematological disorders (1). The current protocol for induction of Haplo-MC with conditioning regimen of "ATG + CY + PT" and infusion of donor $CD4^+$ T-depleted transplant is now under phase I safety clinical trial with sickle cell patients (NCT03249831) and we have obtained encouraging results. We have carried out trials with two sickle cell patients. We did not achieve detectable chimerism in the first patient. For the second patient, we increased CY dose during conditioning. The second patient has now reached 180 days after HCT and has developed mixed chimerism for $CD34^+$ stem cells in the bone marrow as well as mixed chimerism for T, B, NK and myeloid cells in the peripheral blood; the patient has predominantly donor-type healthy Hb with little Hbs and has total disappearance of clinical manifestation of sickle cell anemia with total absence of GVHD (Rosenthal et al: unpublished data).

Third, depletion of donor $CD4^+$ T cells in the hematopoietic transplant may be critical for induction of stable Haploidentical mixed chimerism. We understand that stable haploidentical mixed chimerism is currently difficult to achieve in humans (4, 5, 68). However, induction of stable Haplo-MC in humans may be achievable with conditioning regimen of "ATG + CY +PT" and infusion of $CD4^+$ T-depleted hematopoietic transplant, and the depletion of donor $CD4^+$ T cells may be critical. We reported that depletion of $CD4^+$ T cells allows tissue-PD-L1 to tolerize infiltrating $CD8^+$ T cells (25). It was necessary to use $CD4^+$ T-depleted donor spleen cells to induce stable mixed chimerism in mice (56). Our recent studies also showed that add-back of

donor $CD4^+$ T cells to transplants led to either graft rejection when low dose of bone marrow transplant is used or led to complete chimerism when high dose of donor bone marrow transplant is used; and the presence of donor $CD4^+$ T cells markedly reduced donor- and host-type T tolerance after HCT (Zhu et al: unpublished data). Thus, depletion of donor $CD4^+$ T cells in hematopoietic transplant may promote establishing stable Haplo-MC in non-myeloablatively conditioned recipients.

In conclusion, we have demonstrated that induction of Haplo-MC with non-myeloablative conditioning regimen of ATG + CY + PT and depletion of donor $CD4^+$ T cells in hematopoietic transplants cures established autoimmunity with elimination of insulitis in both euthymic and adult-thymectomized NOD mice. We have revealed a central and peripheral tolerance network in the Haplo-MC NOD mice. These studies provide novel insignts into the tolerance mechanisms in Haplo-MC and may help improvement of present protocols for treating patients with established autoimmune diseases. These studies have also laid a basic foundation for translating induction of Haplo-MC in clinic and for a clinical trial with autoimmune patients.

Methods:

Mice. All recipient mice were either purchased from National Cancer Institute animal production program (Frederick, Maryland, USA) or Jackson Laboratory (Bar Harbor, ME) or were bred at City of Hope Animal Research Center. Detailed information of each strain is described in table S1. All mice were housed in specific pathogen-free rooms in the City of Hope Animal Research Center.

Experimental procedures and materials. Induction of mixed chimerism with Cyclophosphamide (CY) + Pentostatin (PT) + Anti-thymocyte globulin (ATG) conditioning regimen, histopathology staining and insulitis evaluation, in vivo Treg depletion, induction of host lymphocyte PD-L1$^{-/-}$ mixed chimerism, isolation of lymphocytes from pancreas, release of dendritic cells from spleen, flow cytometry analysis including tetramer staining and detailed antibody information are described in Supplemental Methods.

Statistics. Data are displayed as mean ± SEM. Body weight and diabetes free rate in different groups were compared using log-rank test. Insulitis in different groups were compared using Chi-square test. Comparison of two means was done using unpaired 2-tailed Student's *t* test while comparison of multiple means was done using one-way ANOVA; *P* value of less than 0.05 is considered as significant.

Software. Flow cytometry data were analyzed with FlowJo™ Software version 10.5.3 (FlowJo LLC). Statistical analysis were prepared using GraphPad Prism version 8.0. Abstract figure is created with BioRender.com

Study approval. All animal procedures were approved by the IACUC of the Beckman Research Institute of City of Hope.

Author contributions:

YL designed and performed research as well as prepared the manuscript. XW, YZ, MZ, UN and SSS assisted in experiments. XZ is the home institute PhD advisor for YL and XW. ADR provided advice and financial support for the project as well as reviewed and edited manuscript. DZ designed and supervised the research and wrote the manuscript.

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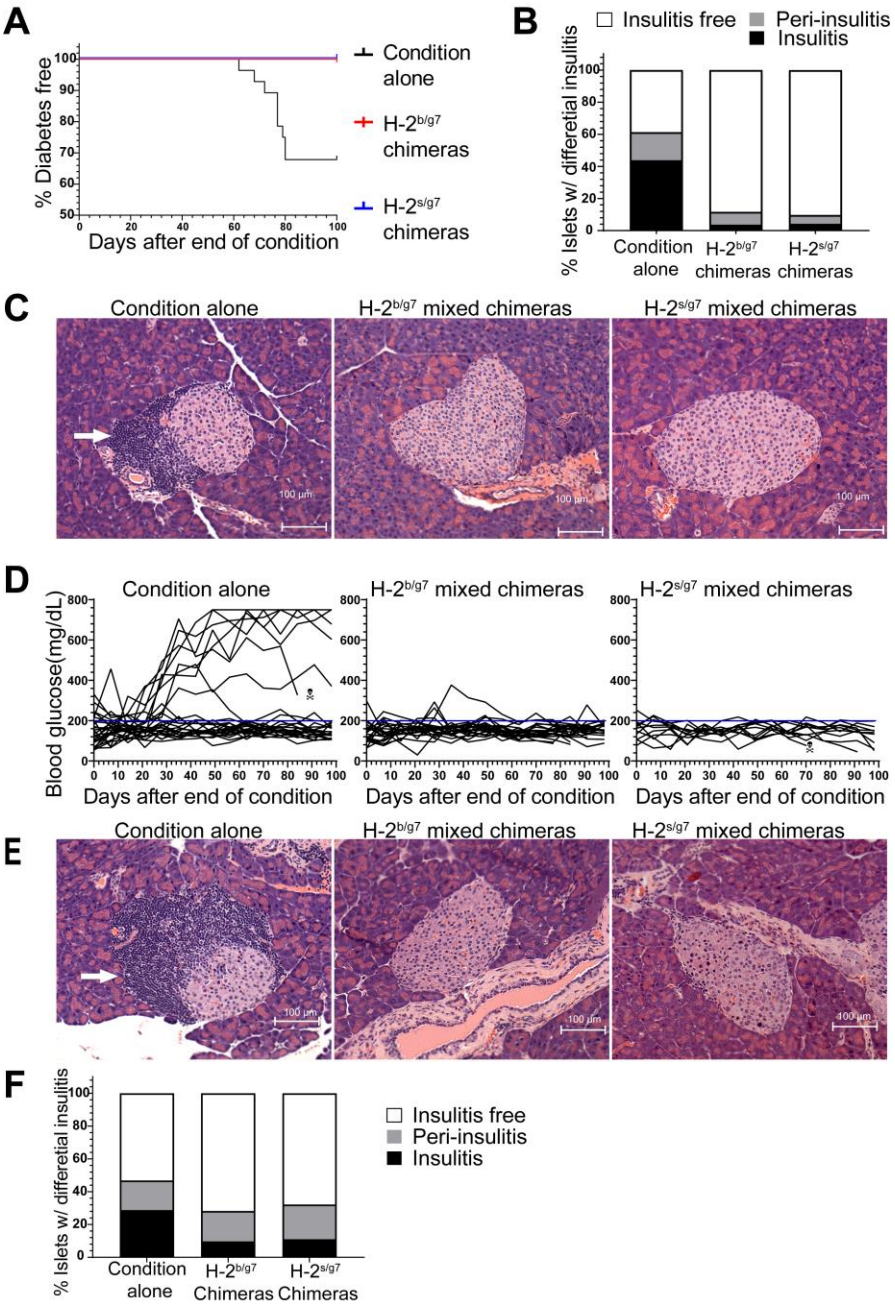

Figure 1: Induction of Haplo-MC prevents diabetes onset and reverses new-onset T1D in WT NOD mice, with clearing up insulitis. Prediabetic 9-12 weeks old NOD and new-onset diabetic NOD mice were conditioned with ATG + CY + PT, and transplanted with BM (50×10^6) and SPL cells (30×10^6) from H-$2^{b/g7}$ F1 or H-$2^{s/g7}$ F1 donors, respectively, and co-injected with depleting anti-CD4 mAb (500 µg/mouse). Recipients were monitored for diabetes development for 100 days after HCT. (**A)** T1D development curves in prediabetic NOD mice (*n*=20-37 from ≥3 experiments). *P*<0.0001 when comparing conditioning alone control to either H-2b/g7 or H-2s/g7 chimera using log-rank test**. (B and C)** 100 days after HCT, residual non-diabetic mice are subject to insulitis evaluation. Representative HE histopathology photomicrographs are shown. Summary insulitis score is shown in mean (*n*=9-12). **(D)** T1D relapse curves of new-onset diabetic NOD mice given conditioning alone or induction of either H-$2^{b/g7}$ or H-$2^{s/g7}$ Haplo-MC (*n*=12-24 from ≥3 experiments). **(E and F)** Representative photomicrographs and summary (mean) of insulitis score of recipients with normal glycemia 100 days after HCT or control mice

given conditioning alone (*n*=6-12). Statistic comparison of insulitis was completed by using Chi-square test **(B and F)** (****$P<0.0001$).

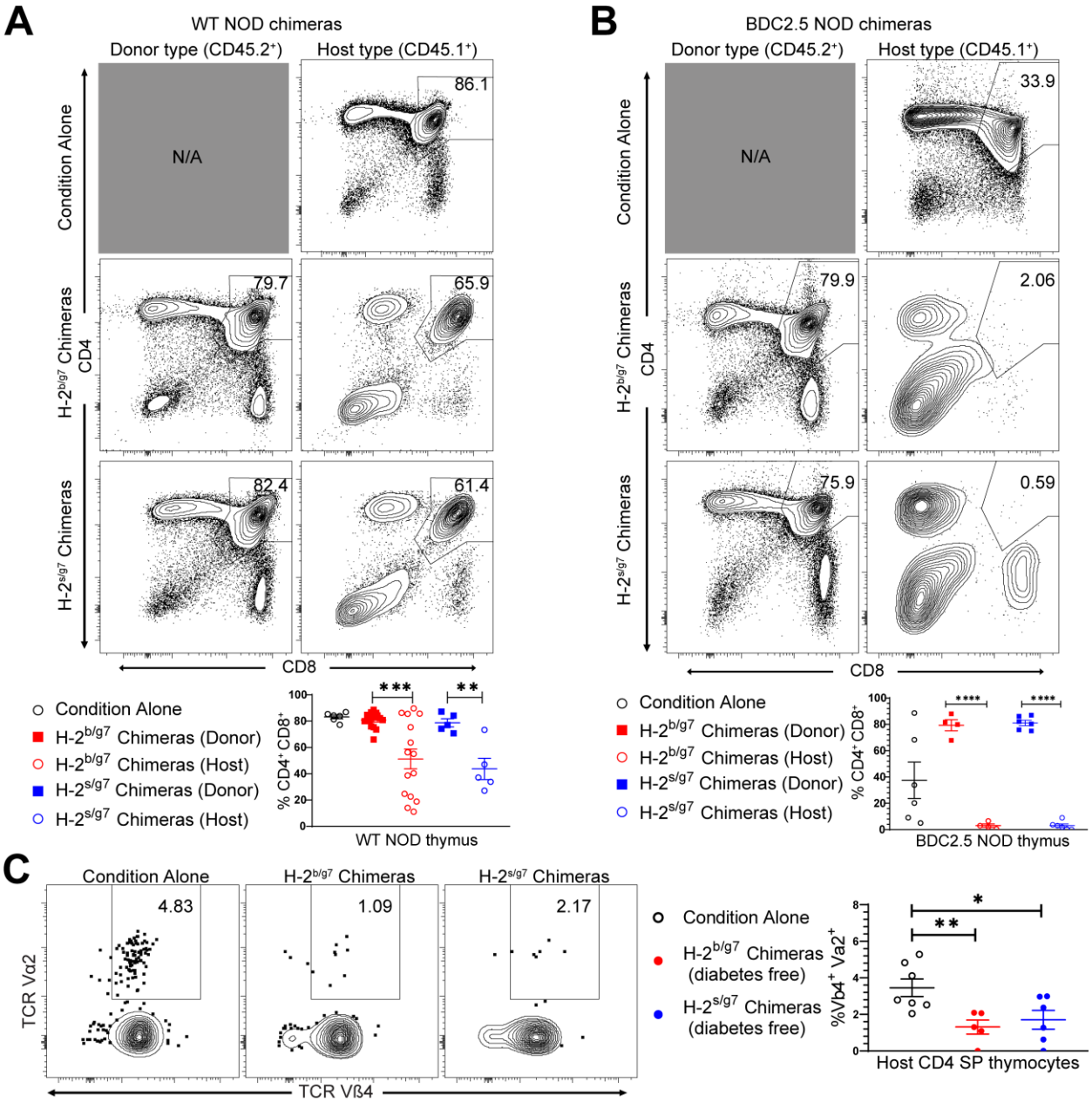

Figure 2: Haplo-MC reduces host-type CD4$^+$CD8$^+$ thymocytes and thymocytes with dual TCRs. 60 days after HCT, thymocytes from mixed chimeric WT NOD and BDC2.5 NOD or control mice given conditioning alone were analyzed for donor- and host-type CD4$^+$CD8$^+$ thymocytes. **(A and B)** Thymocytes of WT NOD and BDC2.5 NOD are shown for donor- and host-type CD4$^+$CD8$^+$, respectively, *n*=6-15. **(C)** The BDC2.5 transgenic TCR consists of Vα1 and Vß4. if a Vß4$^+$ T cell also expresses any Vα chain other than Vα1, such as Vα2, it is considered as a T cell expressing more than one set of TCR. Representative staining and summary (mean ± SEM) of % T cells with dual TCRs among host type CD4$^+$CD8$^-$ population in BDC2.5 thymus are shown, *n*=5-7. *P* values were calculated using unpaired 2-tailed Student's *t* tests **(A and B)** or one-way ANOVA **(C)** (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

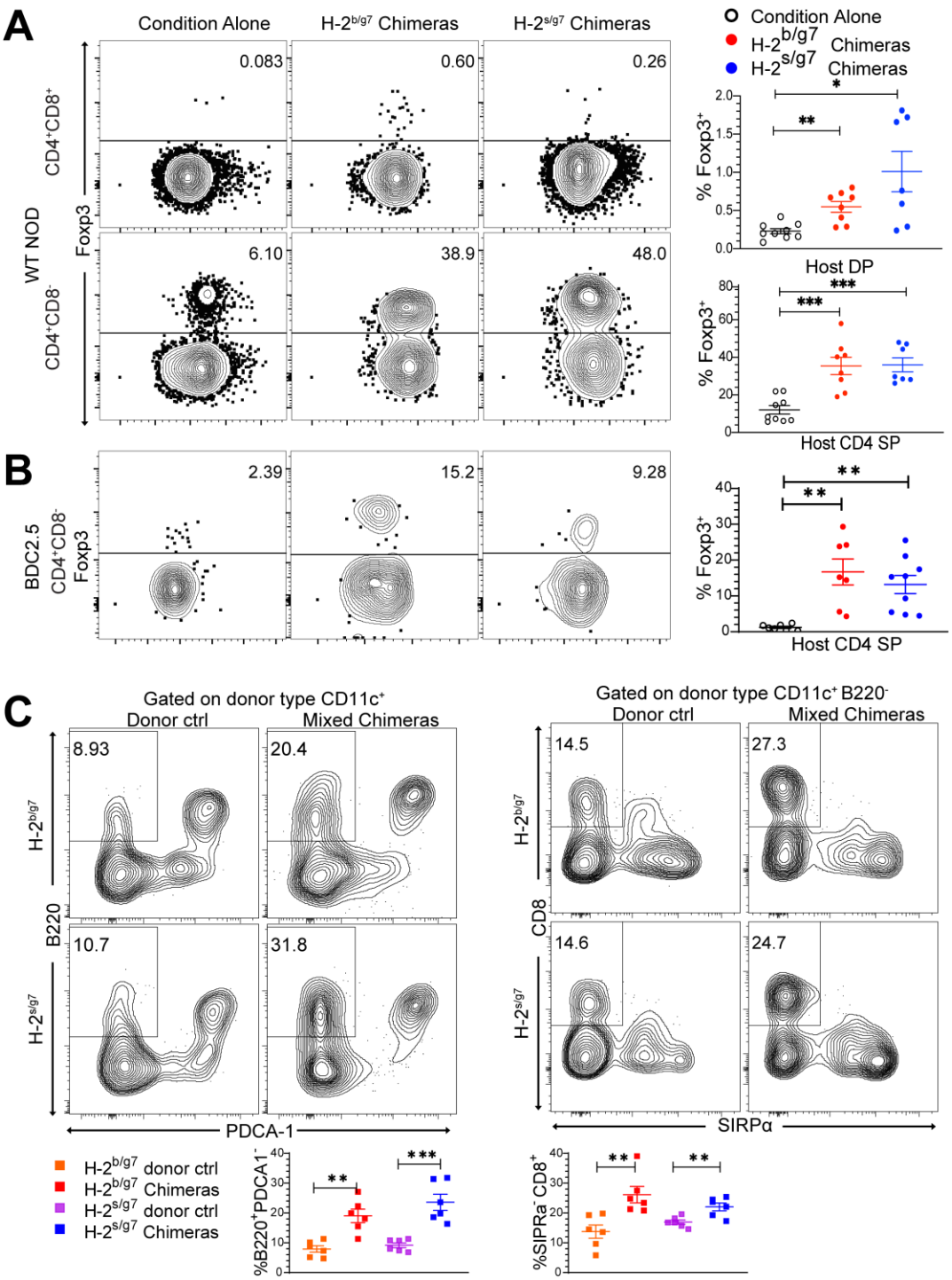

Figure 3: Haplo-MC increases Treg production in thymus, with engraftment of donor type DC subsets. 60 days after HCT, H-2$^{b/g7}$ and H-2$^{s/g7}$ Haplo-MC and control mice were measured for host-type Foxp3$^+$ Treg cells among CD4$^+$CD8$^-$ (CD4 SP) or CD4$^+$CD8$^+$ (DP) thymocytes as well as measured for donor-type DC subsets. **(A)** % Treg among host-type CD4$^+$ SP and DP thymocytes in WT NOD (*n*=7-9). **(B)** % Treg among host-type CD4$^+$ SP thymocytes in BDC2.5 NOD (*n*=7-9). **(C)** % Donor-type thymic DC subset among donor-type CD11c$^+$ DCs, in comparison to healthy donor controls of each strain, *n*=6 per group. Representative patterns and summary of mean ± SEM are shown. *P* values were calculated using unpaired 2-tailed Student's *t* tests **(C)** or one-way ANOVA **(A and B)** (*p<0.05, **p<0.01, ***p<0.001).

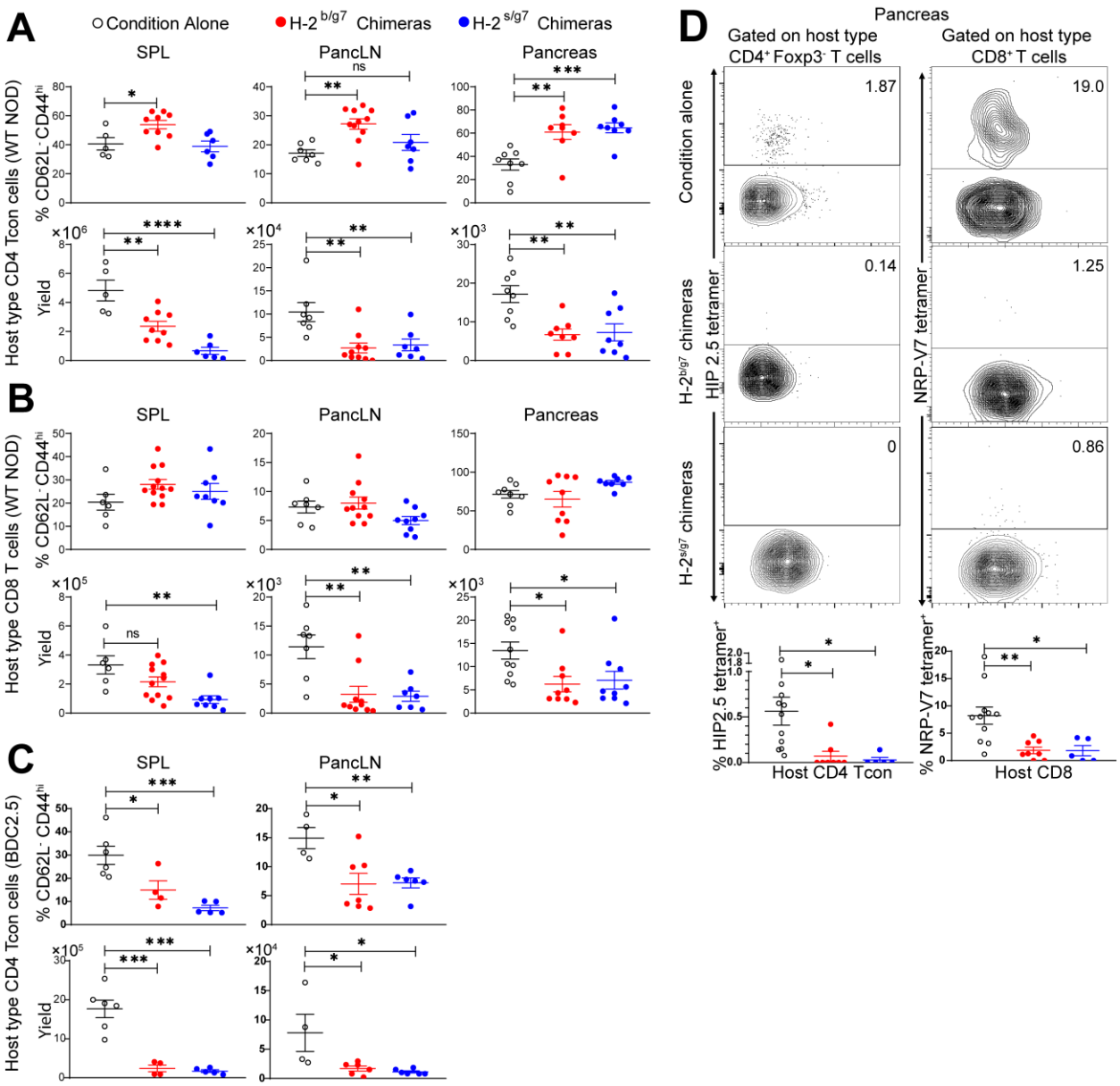

Figure 4: Haplo-MC in NOD mice reduces host-type autoreactive effector memory T cells in the pancreas of WT and BDC2.5 NOD mice. 60-80 days after HCT, mononuclear cells (MNC) of spleen, pancreatic LN and pancreas of mixed chimeric or control WT and BDC2.5 NOD mice were analyzed by flow cytometry for host-type $CD44^{hi}CD62L^{-}$ $CD4^{+}$ or $CD8^{+}$ Tem cells. Mean ± SEM of percentage and yield of $CD62L^{-}CD44^{hi}$ Tem in the Spleen (SPL), pancreatic LN (PancLN), and pancreas are shown. **(A and B)** $CD4^{+}$ and $CD8^{+}$ Tcons of WT NOD with Haplo-MC or given condintiong alone, *n*=5-12. **(C)** Percentage and yield of $CD62L^{-}CD44^{hi}CD4^{+}$ Tem cells in BDC2.5 NOD mice, *n*=4-7. **(D)** Percentage of antigen-specific autoreactive T cells in the pancreas of WT NOD mice. The pancreatic MNC of Haplo-MC or control WT NOD mice were stained with $I\text{-}A^{g7}$-HIP 2.5 tetramer to identify antigen-specific autoreactive $CD4^{+}$ T cells or $H\text{-}2^{d}$-NRP-V7 tetramer to identify autoreactive $CD8^{+}$ T cells. Representative flow cytometry patterns and mean ± SEM of percentage of $tetramer^{+}$ $CD4^{+}$ or $CD8^{+}$ T cells are shown, *n*=5-11. *P* values were calculated using one-way ANOVA (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

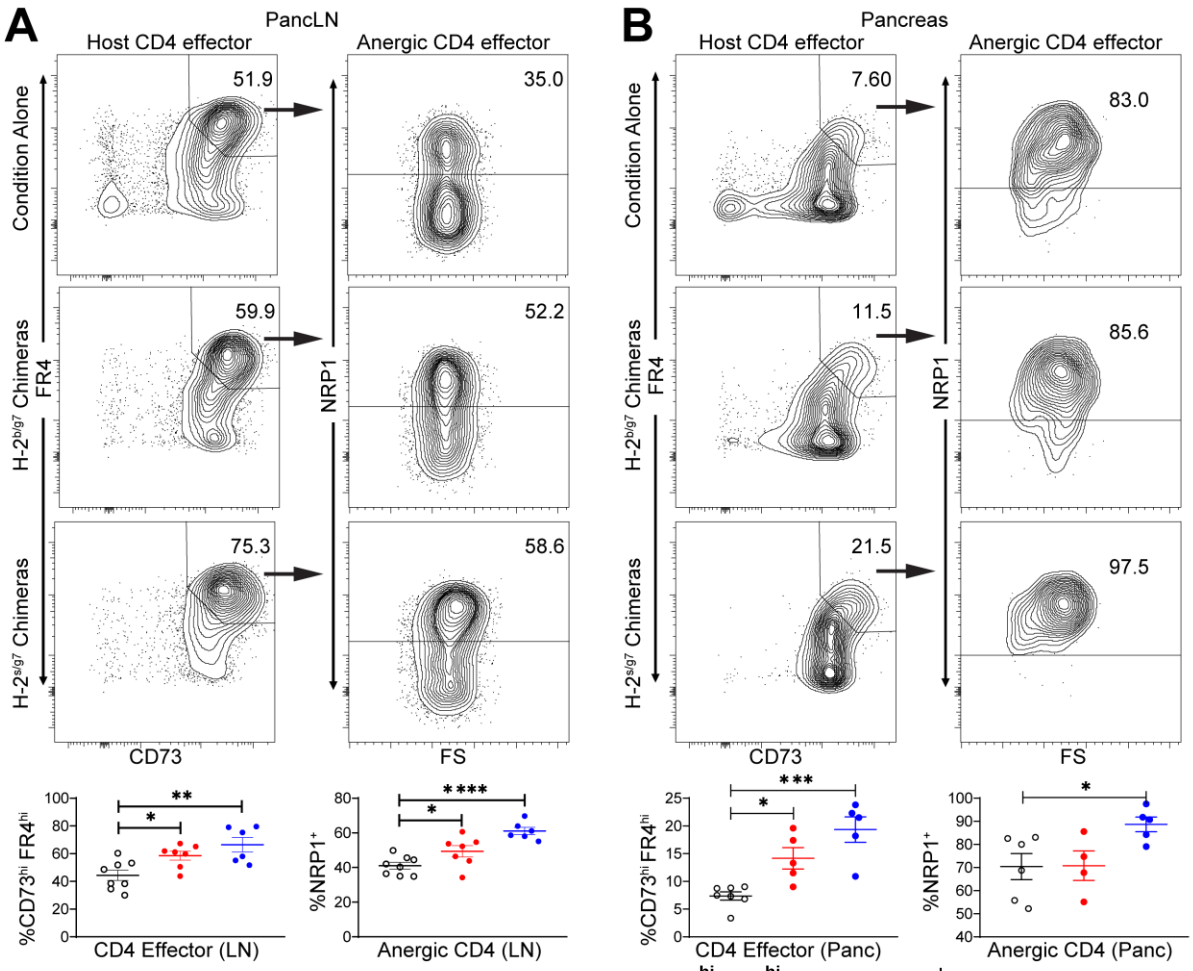

Figure 5: Haplo-MC increases percentage of total CD73^{hi}FR4^{hi}anergic CD4^{+} T cells and Nrp1^{+}CD73^{hi}FR4^{hi} anergic cells among host-type CD44^{hi}CD62L^{-}CD4^{+} Tem cells. 60-80 days after HCT, samples of pancreatic LN and pancreas MNC were analyzed by flow cytometry for their expression of CD45.2 (donor-marker), TCRβ, CD4, Foxp3, CD62L, CD44, CD73, FR4 and Nrp1. Representative patterns of flow cytometry and mean ± SEM of percentage of CD73^{hi}FR4^{hi} anergic cells among total host-type Foxp3^{-}CD62L^{-}CD44^{hi}CD4^{+} Tem cells and percentage of Nrp1^{+}CD73^{hi}FR4^{hi} cells among total CD73^{hi}FR4^{hi} anergic cells are shown, *n*=4-8. *P* values were calculated using one-way ANOVA (*p<0.05, **p<0.01, ***p<0.001, **** p<0.0001).

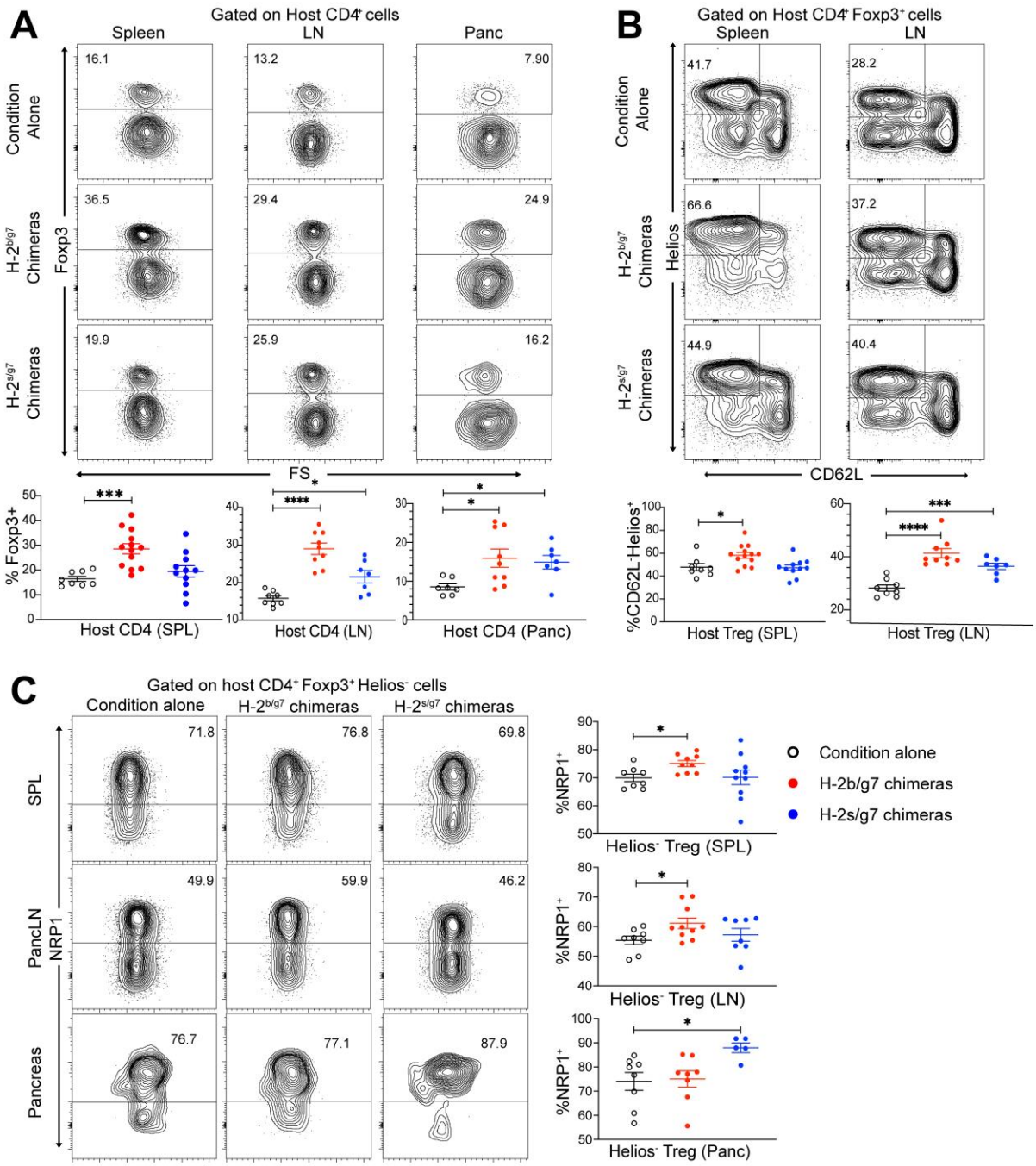

Figure 6: Haplo-MC increases CD62L$^-$Helios$^+$ effector memory Tregs and Nrp1$^+$Helios$^-$ pTreg cells. MNC from SPL, PancLN and pancreas of Haplo-MC NOD were analyzed at day 60 after HCT for CD62L$^-$ Helios$^+$ effector momory Tregs and Helios$^-$Nrp1$^+$ pTreg cells. **(A)** Representative patterns and mean ± SEM of Foxp3$^+$ Treg cells among total host-type CD4$^+$ T cells. **(B)** Representative patterns and mean ± SEM of percentage of CD62L$^-$Helios$^+$ effector memory Treg cells among total Foxp3$^+$CD4$^+$ Treg cells in the spleen, PancLN and pancreas (*n*=7-13). **(C)** Representative patterns and mean ± SEM of percentage of Nrp1$^+$ pTreg cells among host-type Helios$^-$ pTregs cells in SPL, PancLN and pancreas, *n*=5-10. *P* values were calculated using one-way ANOVA (**p*<0.05, ****p*<0.001, **** *p*<0.0001).

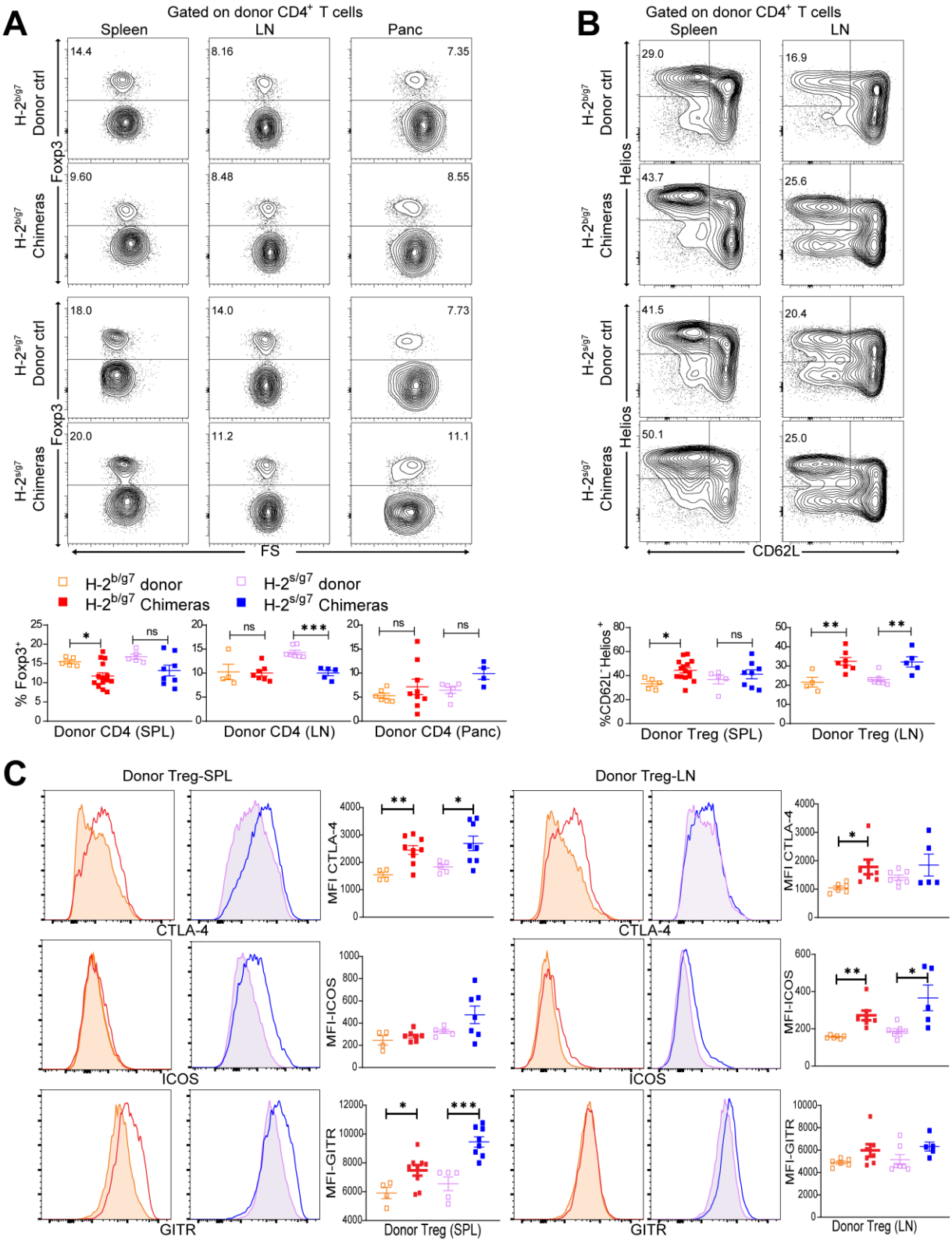

Figure 7: Haplo-MC increases percentage of donor-type CD62L$^-$ effector memory Treg cells and upregulates their CTLA-4 expression. 60 days after HCT, cells from SPL, PancLN and pancreas of

Haplo-MC NOD and control donor mice were analyzed for percentage of donor-type Treg cells among total donor-type $CD4^+$ T cells and percentage of $CD62L^-$ effector memory Treg cells among total donor-type $Foxp3^+CD4^+$ Treg cells as well as Treg cell expression of CTLA-4, ICOS, and GITR. **(A and B)** Representative patterns and mean ± SEM shows percentage of Treg cells among donor-type $CD4^+$ T cells or $CD62L^-Helios^+$ effector memory Treg cells among donor-type Treg, *n*=6-11. **(C)** Representative patterns and mean ± SEM of median fluorescent intensity (MFI) of CTLA-4, ICOS and GITR expressed by donor-type Tregs in spleen and PancLN, *n*=4-9. *P* values were calculated using unpaired 2-tailed Student's *t* tests (**p*<0.05, ***p*<0.01, ****p*<0.001).

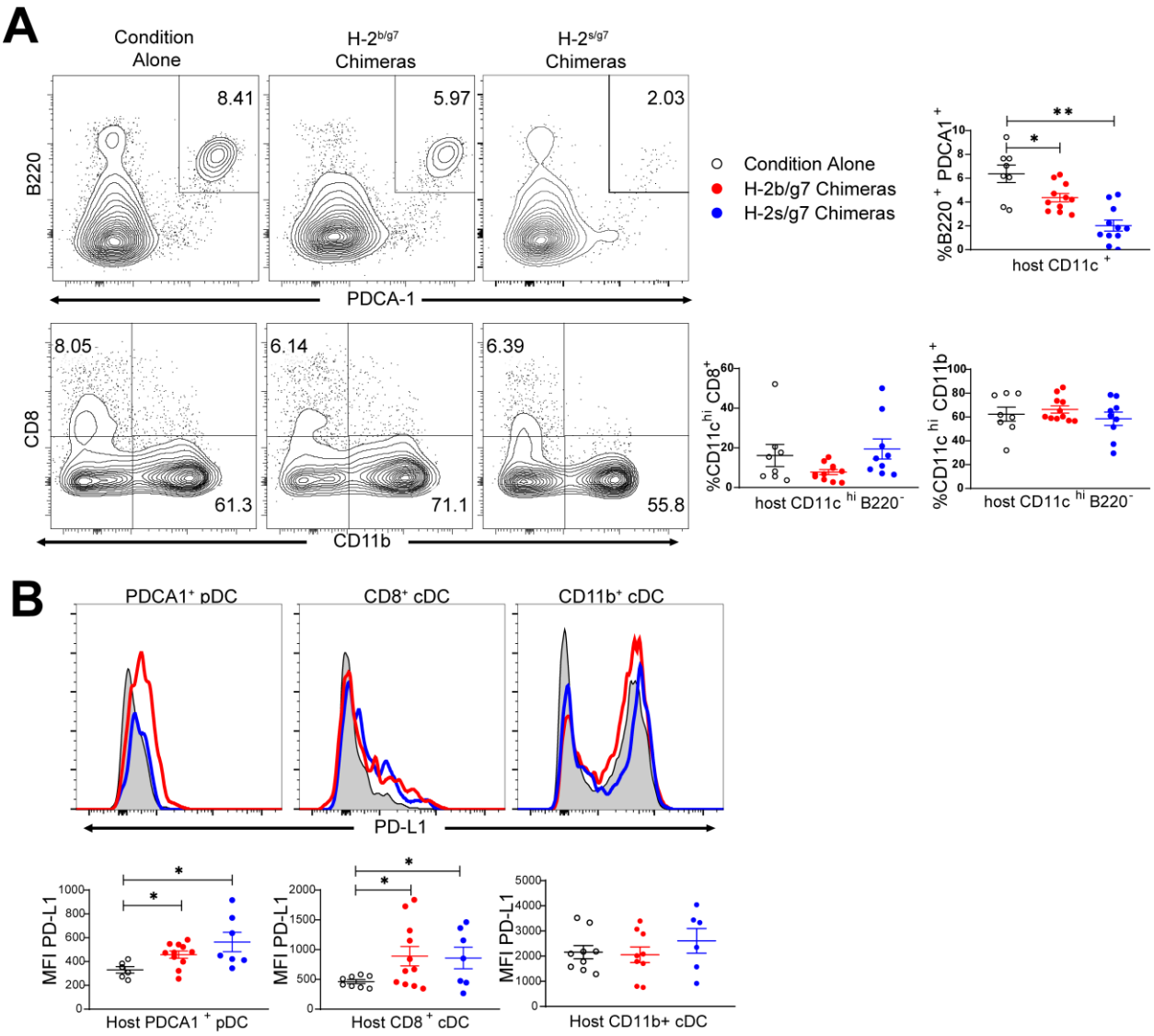

Figure 8: Haplo-MC reduces host-type pDC percentage but upregulates their PD-L1 expression. MNC from spleen of mixed chimeras and control NOD mice were analyzed at day 60 after HCT for percentage of host-type $IgM^-IgD^-CD11c^+B220^+PDCA1^+$ (pDCs), $IgM^-IgD^-CD11b^-CD11c^+CD8^+$ ($CD8^+$ DCs) and $IgM^-IgD^-CD11b^+CD11c^+$ ($CD11b^+$ DCs) subsets and their expression of PD-L1**. (A)** Representative pattern and mean ± SEM of percentage of host-type $B220^+PDCA\text{-}1^+$ pDC, $CD8^+$ DC, and $CD11b^+$ DC subsets (*n*=8-11). **(B)** Representative patterns and mean ± SEM of PD-L1 expression levels on host-type $B220^+PDCA\text{-}1^+$ pDC, $B220^-CD11b^-CD8^+$ DCs, and $B220^-CD8^-CD11b^+$ DCs, in comparison to control mice, *n*=6-11. *P* values were calculated using one-way ANOVA (**p*<0.05, ***p*<0.01).

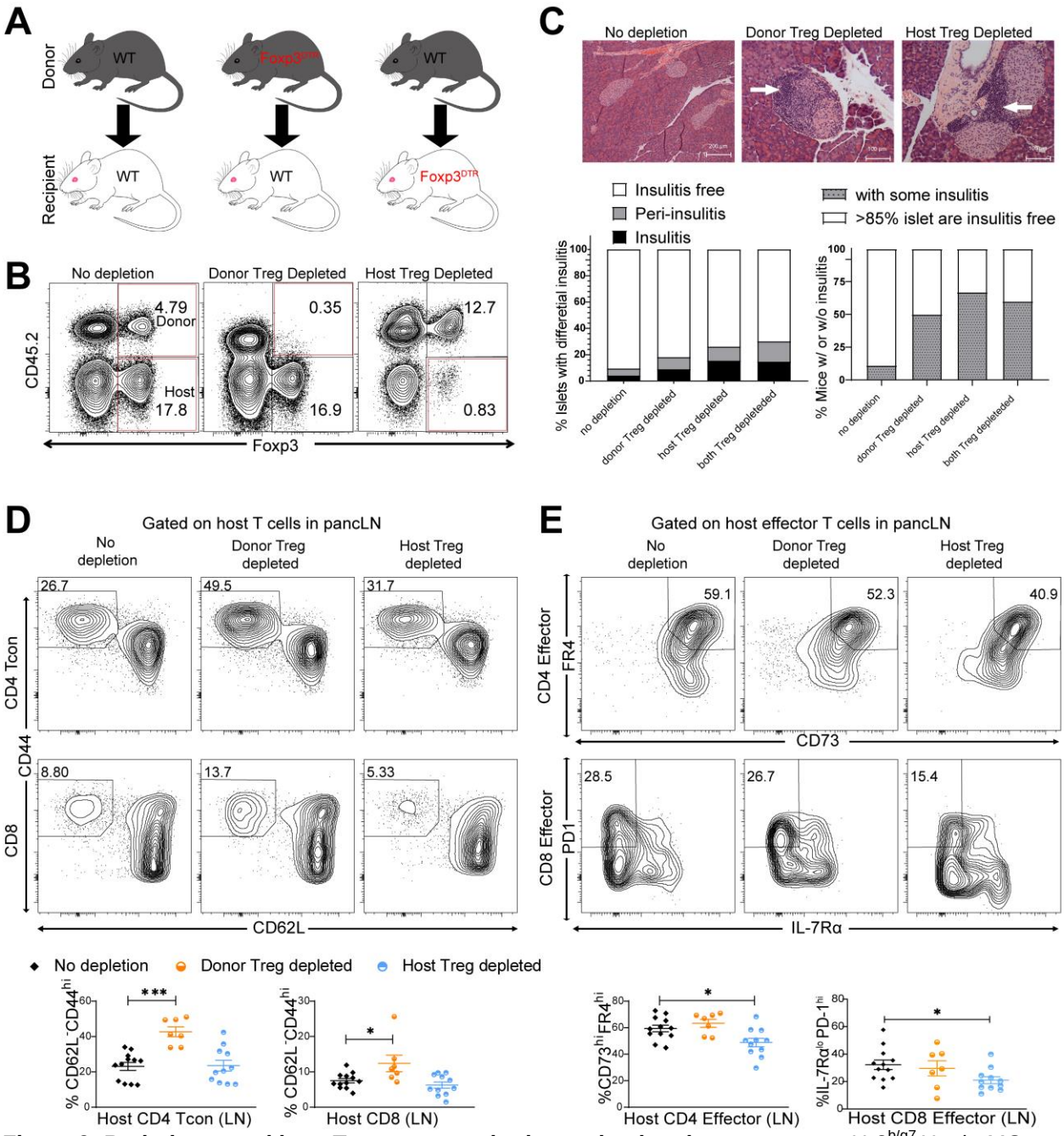

Figure 9: Both donor and host Tregs are required to maintain tolerance status. H-$2^{b/g7}$ Haplo-MC was induced using either donor- or host mice carrying $Foxp3^{DTR}$. 45-60 days after HCT, diphtheria toxin (DT) was injected to chimeric mice every 3 days for 21 days. Only $Foxp3^{+}$ Tregs cells from $Foxp3^{DTR}$ carrying mice can express DT receptor and would be depleted. **(A)** Diagram of the HCT system that allows specific *in-vivo* depletion of either donor- or host-type Treg in mixed chimeras. **(B)** Efficacy of depletion of Treg cells among spleen MNC was evaluated at day 21. **(C)** 3 weeks after the first injection, pancreas tissue from each group was collected to evaluate insulitis, (p<0.01 when comparing no depletion to host Treg depleted or both donor and host Treg depleted, p=0.17 when comparing no depletion to donor Treg depleted). Depletion of either donor- or host-type Treg led to moderate insulitis. Among WT mixed chimeras, more than 90% of mice were insulitis free in all the evaluated islets, this percentage dropped to 50% and 33% in donor-type Treg depleted or host-type Treg depleted chimeric mice, respectively. One representative was shown for 6-9 mice in each group (p<0.0001 when comparing

no depletion to any other group. **(D and E)** Representative patterns and mean ± SEM of percentage of $CD62L^{-}CD44^{hi}$ Tem cells among host-type Tcon cells and percentage of $CD73^{hi}FR4^{hi}$ anergic cells among the Tem cells in the pancLN of control Haplo-MC, Haplo-MC with depletion of donor-type Treg and Haplo-MC with depletion of host-type Treg cells, n=7-12. P values were calculated using one-way ANOVA (*p<0.05, ***p<0.001).

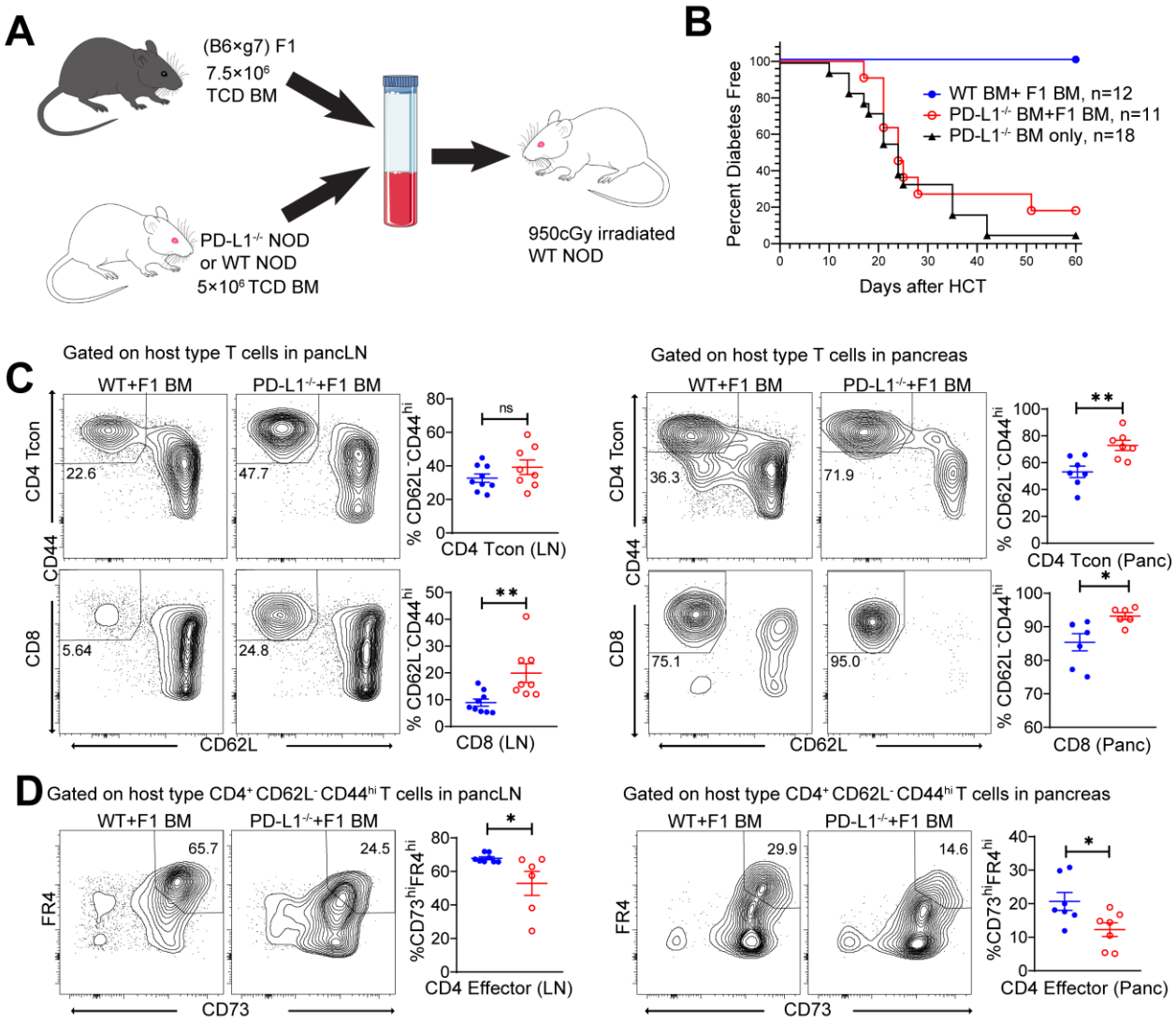

Figure 10: PD-L1 expressed on host-type hematopoietic cells is required to maintain tolerance. TCD BM cells from H-$2^{b/g7}$ F1 were mixed with TCD BM cells from either WT or PD-$L1^{-/-}$ NOD mice and injected into lethally irradiated 11-12 weeks old WT NOD mice as shown in **(A)**. **(B)** T1D development curve are shown for up to 60 days after HCT (*n*=11-18, combined from three replicate experiments). **(C)** 45-60 days after HCT, percentage of host-type $CD62L^{-}CD44^{hi}$ Tem cells among $CD4^{+}$ Tcon or $CD8^{+}$ Tcon cells in the pancreatic LN (left) and pancreas (right) were measured. Representative patterns and mean ± SEM are shown, *n*=6-9. **(D)** Percentage of anergic $CD73^{hi}FR4^{hi}CD4^{+}$ Tcon cells among $CD62L^{-}CD44^{hi}CD4^{+}$ Tem cells in the pancreatic LN (left) and pancreas (right), *n*=6-7. *P* values were calculated using unpaired 2-tailed Student's *t* tests (**p*<0.05, ***p*<0.01).

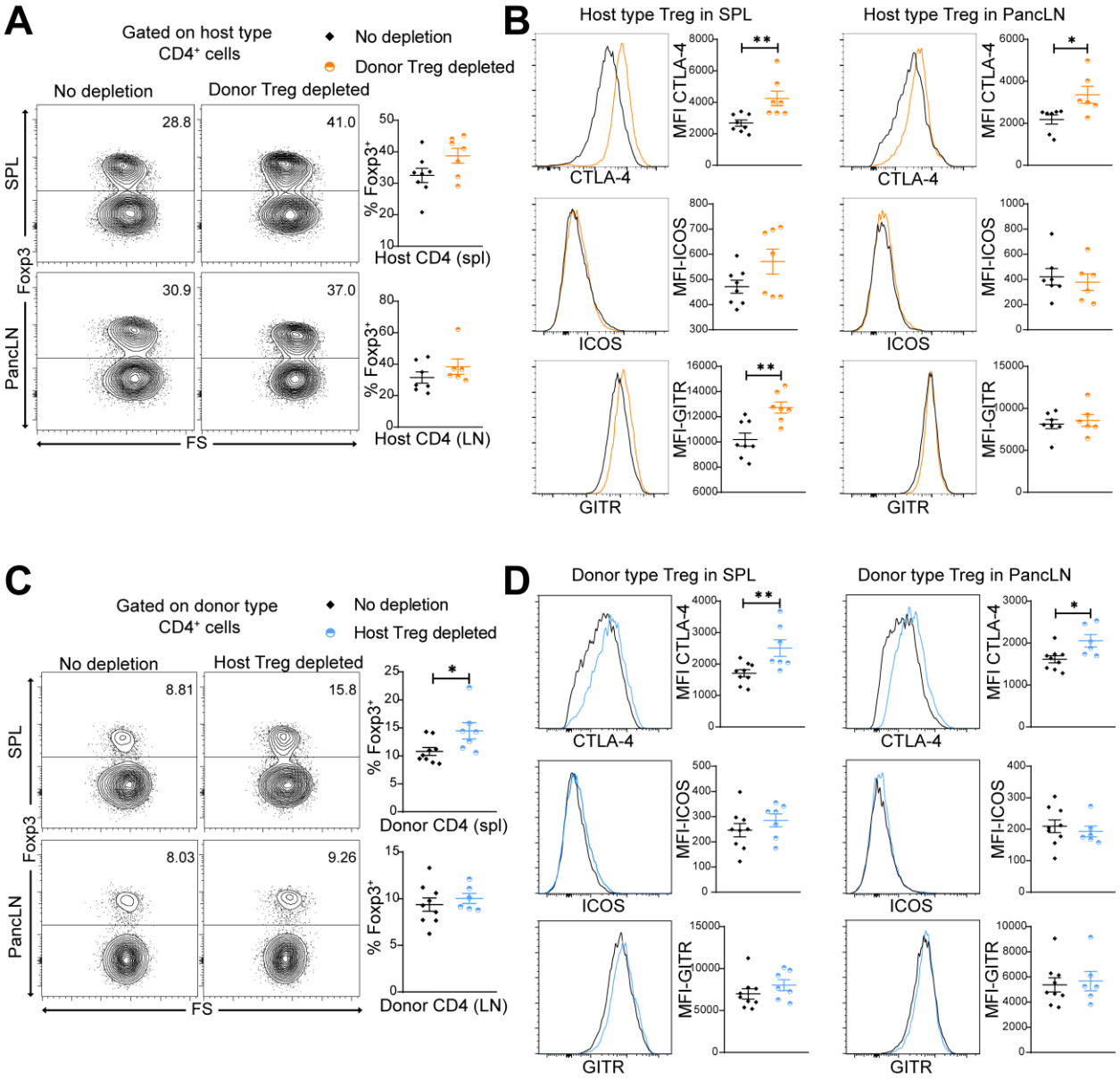

Figure 11: Percentage and surface receptor changes of donor- or host-type Treg cells after depletion of host- or donor-type Treg cells. 3 weeks after depletion of Treg cells by DT injection as described in Fig. 9, percentage and surface receptors of donor- or host-type Treg cells in the spleen and Panc LN of NOD mice with H-$2^{b/g7}$ Haplo-MC were measured. **(A and B)** Representative pattern and mean ± SEM of percentage of host-type Treg among host-type $CD4^+$ Tcon cells as well as expression levels of CTLA-4, ICOS, GITR on host-type Tregs in the spleen and PancLN of Haplo-MC NOD with or without depletion of donor-type Treg depletion, *n*=6-9. **(C and D)** Representative pattern and mean ± SEM of percentage of donor-type Treg among donor-type $CD4^+$ Tcon cells as well as expression levels of CTLA-4, ICOS, GITR on donor-type Treg cells in spleen and PancLN of Haplo-MC NOD mice with or without depletion of host-type Treg depletion, *n*=6-9. *P* values were calculated using unpaired 2-tailed Student's *t* tests (**p*<0.05, ***p*<0.01).

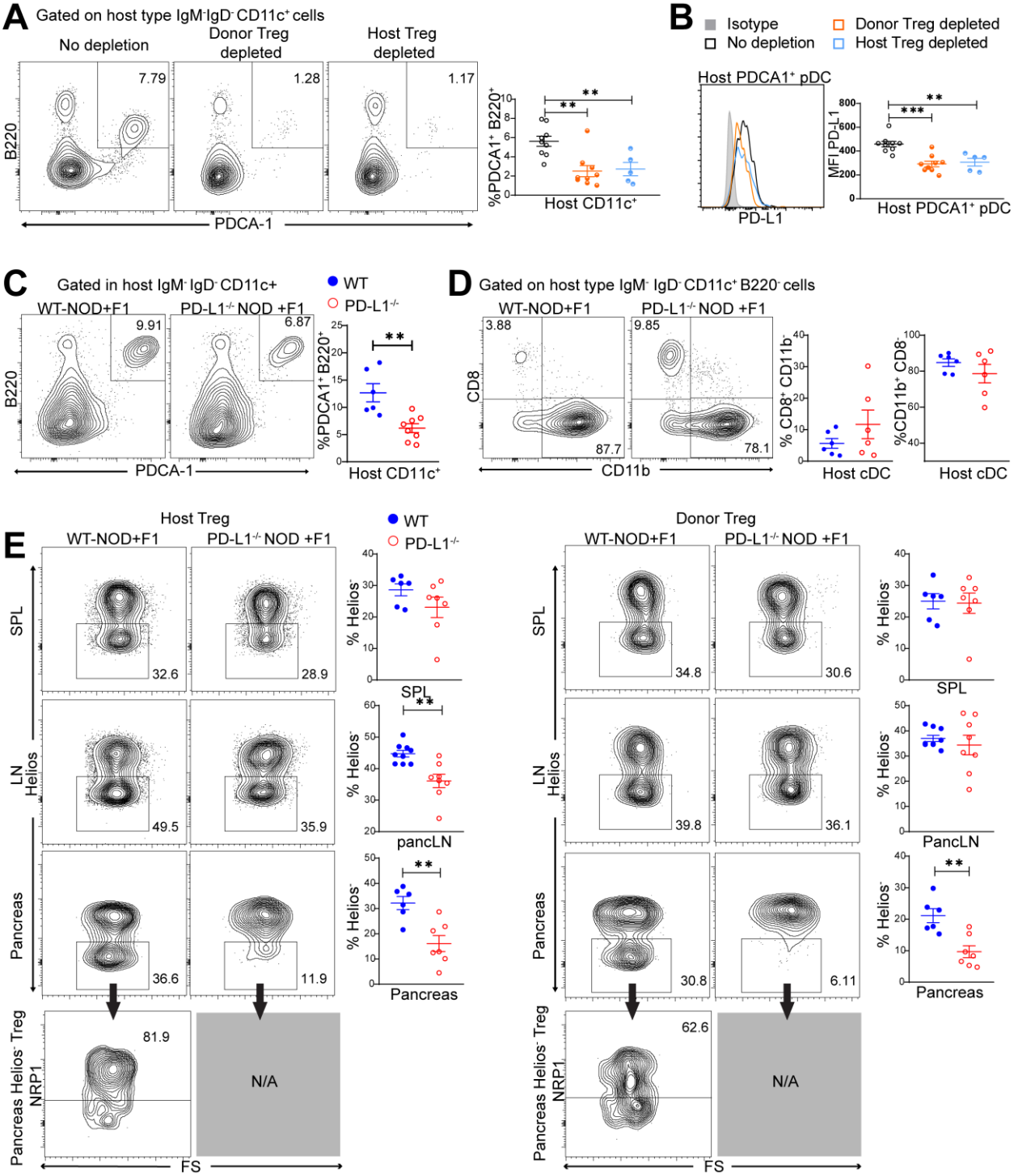

Figure 12: Interactions among donor- and host-type Treg cells and PD-L1^{hi} pDCs in the periphery of Haplo-MC NOD mice. Depletion of Treg cells in Haplo-MC NOD mice was described in Fig. 9, and establishing Haplo-MC with host-type PD-L1$^{-/-}$ hematopoietic cells was described in Fig. 10. **(A and B)** Host-type pDCs and their expression of PD-L1 in the spleen of Haplo-MC mice with or without depletion of donor- or host-type Treg cells were compared. Representative pattern and mean ± SEM of percentage of host-type B220^{+}PDCA1^{+} pDC among IgM^{-}IgD^{-}CD11c^{+} cells and their PD-L1 expression levels are shown, *n*=5-9. **(C and D)** Host-type CD220^{+}PDCA-1^{+} pDCs, CD8^{+} DC, and CD11b^{+} DC subsets in the spleen of Haplo-MC mice with or without hematopoietic cell PD-L1 deficiency were measured.

Representative pattern and mean ± SEM of DC subsets are shown, *n*=6-8. **(E)** Percentage of Helios$^-$ pTregs among host- or donor-type Tregs in the spleen, PancLN and pancreas was measured. Helios$^-$ Nrp1$^+$ pTreg cells among Helos$^-$ pTreg cells in the pancreas were also measured. Representative patterns and mean ± SEM is shown, *n*=6-9. *P* values were calculated using unpaired 2-tailed Student's *t* tests (***p*<0.01, ****p*<0.001).