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PD-L1 Prevents the Development of Autoimmune Heart Disease in Graft-vs-Host Disease

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Abstract

Effector memory T cells (T_{EM}) are less capable of inducing graft-versus-host disease (GVHD) compared to naïve T cells (T_N). Previously, in the TS1 T cell receptor transgenic model of GVHD, wherein TS1 CD4 cells specific for a model minor histocompatibility antigen (miHA) induce GVHD in miHA-positive recipients, we found that cell-intrinsic properties of TS1 T_{EM} reduced their GVHD potency relative to TS1 T_N. Post-transplant, TS1 T_{EM} progeny expressed higher levels of PD-1 than did TS1 T_N progeny, leading us to test the hypothesis that T_{EM} induce less GVHD due to increased sensitivity to PD-ligands. Here we tested this hypothesis and found that indeed TS1 T_{EM} induced more severe skin and liver GVHD in the absence of PD-ligands. However, lack of PD-ligands did not result in early weight loss and colon GVHD comparable to that induced by TS1 T_N, indicating that additional pathways restrain alloreactive T_{EM}. TS1 T_N also caused more severe GVHD without PD-ligands. The absence of PD-ligands on donor bone marrow (BM) was sufficient to augment GVHD caused by either T_{EM} or T_N, indicating that donor PD-ligand expressing antigen presenting cells (APCs) critically regulate GVHD. In the absence of PD-ligands, both TS1 T_{EM} and T_N induced late onset myocarditis. Surprisingly, this was an autoimmune manifestation, as its development required non-TS1 polyclonal $CD8^+$ T cells.

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Author Contributions

KWJ designed and performed experiments, analyzed data and wrote the paper. FS designed and performed experiments and analyzed data. CZ performed experiments. KR interpreted echocardiography results. AS created the PD-L1/2$^{-/-}$ mice. JM and AD scored histopathology. WDS and MJS conceived the project, designed experiments, analyzed data and wrote the paper.

Myocarditis development also required donor BM to be PD-ligand-deficient, demonstrating the importance of donor APC regulatory function. In sum, PD-ligands both suppress miHA-directed GVHD and the development of alloimmunity-induced autoimmunity post allogeneic hematopoietic transplantation.

Introduction

Allogeneic hematopoietic stem cell transplantation (alloSCT) can cure hematological malignancies and nonmalignant inherited and acquired disorders of blood cells. Mature allograft αβT cells promote engraftment, contribute to immune reconstitution, and can attack malignant cells, mediating the graft-versus-leukemia (GVL) effect. However, alloreactive T cells also attack recipient nonmalignant cells, causing graft-versus-host disease (GVHD). Because of GVHD, all patients receive some form of immunosuppression to diminish its incidence and severity. A primary goal of alloSCT research is to understand and differentiate mechanisms of GVHD and GVL in order to maximize the positive effects of donor T cells while minimizing GVHD.

Towards this end, others and we discovered in mouse models that naïve T cells (T_N) induce severe GVHD, while effector memory T cells (T_{EM}) fail to induce sustained GVHD, but engraft and can mediate GVL(1–5). These data support the selective depletion of T_N as a method of GVHD prevention. This has shown promise in humans, where T_N-depletion reduced chronic GVHD, without an apparent increase in risk of relapse(6). Further efforts to understand the mechanisms behind this effect could help to further optimize this approach in humans.

However, why memory T cells (T_M), and in particular T_{EM}, fail to induce GVHD is incompletely understood. Deciphering the mechanisms might provide strategies to similarly impair GVHD-inducing T_N. We initially considered that T_{EM} may be less capable of causing GVHD because they are relatively restricted from key sites of priming such as lymph nodes, but this proved incorrect(7). To test whether T_{EM} fail to induce GVHD solely due to their having a less alloreactive TCR repertoire and/or whether repertoire-independent properties also reduce their ability to cause GVHD, we developed a T cell receptor (TCR)-transgenic GVHD model that enabled direct comparison of T_N and T_{EM} with identical TCRs. In this model, BALB/c $CD4^+$ TCR-transgenic T cells (TS1 T cells), specific for the hemagglutinin (HA)-derived S1 peptide 110-119 (SFERFEIFPK) presented by $I\text{-}E^d$, were combined with BALB/c bone marrow (BM) and transferred into irradiated BALB/c recipients that express HA at a low level in all cells (HA104 mice; (8)). TS1 T_N induced severe acute GVHD, characterized by weight loss and typical GVHD pathology of skin, liver and colon. In contrast, TS1 T_{EM} only induced transient disease, demonstrating that T_{EM} have TCR repertoire-independent limitations.

Although TS1 T_N and T_{EM} caused very different degrees of GVHD, they nonetheless proliferated and accumulated to a similar extent in secondary lymphoid tissues early post-transplant (3). However, compared to T_N, TS1 T_{EM} progeny produced less IFN-γ and accumulated to a lesser degree in the colon, a major site of GVHD in the model. In addition, although progeny of both T_N and T_{EM} upregulated PD-1 post-transplant, PD1 expression

was higher on the progeny of TS1 T_{EM} relative to that of TS1 T_N in both secondary lymphoid tissues and colon(3).

Here we have investigated whether the higher level of PD-1 expression on TS1 T_{EM} progeny leads to greater inhibition, which in turn would reduce their capacity to cause GVHD. We found that PD-ligands regulate GVHD directly mediated by TS1 T_N and T_{EM}. Surprisingly and contrary to our expectations, PD-ligands were also critical to prevent the emergence of autoimmune myocarditis triggered by alloimmune TS1. Together these data indicate that PD-ligands not only restrain alloreactive TS1 T_{EM}, but are also critical for blocking subsequent GVHD-dependent autoimmunity, which could masquerade clinically as GVHD and be more difficult to cure.

Materials and Methods

Mice

BALB/c mice were from the NCI (Frederick) or the Jackson Laboratory. After being obtained from the following sources, gene-modified mice were bred at Yale and the University of Pittsburgh: BALB/c RAG2$^{-/-}$ (Taconic); BALB/c PD-L1/2$^{-/-}$ and BALB/c PD-L1$^{-/-}$ (Arlene Sharpe); BALB/c TS1(9) (Adam Adler, University of Connecticut); and BALB/c HA104(10) (Wistar Institute). These strains were intercrossed to produce the following strains: TS1 RAG2$^{-/-}$, PD-L1/2$^{-/-}$ HA104, PD-L1$^{-/-}$ HA104, PD-L1/2$^{-/-}$ RAG$^{-/-}$ HA104 and RAG$^{-/-}$ HA104. For all experiments, mice were heterozygous for the HA and TS1 alleles. All TS1 cells were RAG2$^{-/-}$ and are referred to as “TS1”.

TS1 memory cell generation

TS1 CD4$^+$ T cells were enriched from splenocytes to greater than 80% purity using a CD4$^+$ T cell negative selection (StemCell Technologies). TS1 cells (10^6 cells/mL) were cultured with HA S1 peptide 110-119 (SFERFEIFPK, 10ug/mL, Keck Facility, Yale University) and T cell-depleted, irradiated splenocytes (3×10^6 cells/mL) for 3 days. 5×10^6 TS1 cells were transferred intravenously into BALB/c RAG2$^{-/-}$ recipients. TS1 T_{EM} were harvested from these “memory mice” for use 8-14 weeks later.

Antibodies

Antibodies were from the following sources: CD4 Alexa 488 or Alexa 405 (GK1.5), TS1 Alexa 647 (6.5) and F4/80 Alexa 488 (HB-198) were lab-prepared. CD25 (R-phycoerythrin (PE) (7D4)) was from Southern Biotech. FOXP3 (150D), CD62L PE-Cy7 (Mel-14), PD-1 PE (29F.1A12), CD8± PE (53-6.7) and CD44 APC-Cy7 (1M7) were from BioLegend. Gr-1 PE (RB6-8C5) and CD11b PE (MI/70) were from eBioscience. CD4 PE (RM4-5; BD) and CD8 PE (YTSI56.7.7; Biolegend), which could bind in the presence of the depleting antibodies, were used for confirmation of in vivo T cell depletions. Ethidium monoazide (EMA, Invitrogen) or propidium iodide were used to exclude dead cells.

Cell sorting

TS1 T_N (CD62L$^+$ CD44^{dim} CD25$^-$) were sorted from unmanipulated TS1 mice and TS1 T_{EM} (CD62L$^-$ CD44$^+$ CD25$^-$) were sorted from memory mice. To avoid staining cells with

anti-CD4 antibody, which we found to reduce engraftment, TS1 cells were enriched via exclusion of $CD11b^+$, $F4/80^+$, $CD8\pm^+$, and $CD25^+$ cells. Sorted cells were 40%-60% $CD4^+TS1^+$, with no contaminating $CD8^+$ $TS1^+$ cells. Purities of $CD4^+$ T_N and T_{EM} amongst TS1 cells were greater than 99%.

GVHD protocol

Seven to 12-week-old HA104 BALB/c hosts were irradiated with 750 cGy via a cesium irradiator and transplanted with 8×10^6 bone marrow (BM) cells alone or in combination with 1000 sorted TS1 T_N or T_{EM}, except as otherwise noted in the text. Recipients received wet food and sulfatrim treated water for two weeks post-transplant. Weights were taken 2-3 times per week. Mice that lost 20% of their body weight were euthanized and scored as dead. For euthanized mice, the last recorded weight was carried forward to subsequent timepoints. All transplants were performed in accordance with Yale University or the University of Pittsburgh IACUC regulations.

Histopathology

Skin, liver, and colon histology were scored as described previously (3). Cardiac histology was scored as follows: 0: no inflammation or necrosis; 0.5 minimal inflammation and necrosis involving <5% of the total cross-sectional area of the left and right ventricles; 1: mild inflammation and focal necrosis involving >5 – 33%; 2: moderate inflammation and focal necrosis involving >33% -66%; 3: severe inflammation and focal necrosis involving >66%. Tissues were taken for histopathologic analysis when mice dropped below 80% of their original body weight (between 30 and 60 days post-transplant). Histopathology for mice that maintained body weight above 80% was taken at the end of each experiment.

Cell extractions

Livers were perfused with PBS, excised, minced, passed through a cell strainer and incubated in 0.02% collagenase IV (Sigma) at 37°C for 40 minutes. Lymphocytes were separated on a 25% Optiprep gradient (Accurate Chemical & Scientific). To perfuse the heart, a cut was first made in the right atrium and PBS with 1% heparin was then injected into the left ventricle until flow of blood ran clear. The heart was then, excised, minced and incubated with 20 ug/mL DNAse (Sigma) and 0.1 mg/mL Liberase TM (Roche) at 37°C for 30 minutes. The resulting cell suspension was passed through a 40 micron cell strainer. Lymphocytes were isolated by centrifugation over a Percoll gradient (GE Healthcare Bio-Sciences AB).

Flow cytometry and intracellular cytokine staining

Flow cytometric and cell sorting were performed using a LSRII or Fortessa (BD) and a FACS Aria II (BD), respectively. Data were analyzed using Flowjo software (Tree Star). For intracellular cytokine staining, splenocytes were cultured for 1 hour in the presence of 20 ng/mL PMA (VWR Scientific) and 750 ng/mL ionomycin (VWR Scientific) and for an additional 2 hours in the presence of 10 ug/mL brefeldin A (Biolegend). Cells were treated with DNAse (Sigma), surface stained for CD4 and TS1 and treated with Cytofix/Cytoperm (BD). Perm/Wash (BD) was used for intracellular staining with IFN-γ PE (XMG1.2;

Biolegend). For FOXP3 staining, cells were surface stained for CD4 and TS1, treated with FOXP3 Fix/Perm buffer (Biolegend) and stained for FOXP3 in FOXP3 perm buffer (Biolegend).

In vivo CD4 and CD8-depletion

To deplete $CD8^+$ T cells mice received 200ug TIB105 (BioXcell) i.p. on days −6 and −4. Mice were transplanted on day 0 and injected with 100 ug of TIB105 on days 6, 13, 20, 27, 34 and 41. To deplete CD4 cells mice received 150 ug of GK1.5 (lab-made) on days 27, 31, 35, 39, 42 and 46 post-transplant. Additional groups received both anti-CD4 and anti-CD8 or control rat IgG2a (BioXcell).

In vivo measurements of cardiac function

Echocardiographic assessments of cardiac structure and function were performed 38 days post-transplant using a 40-MHz probe and a Vevo2100 Ultrasound system (FUJIFILM, VisualSonics, Toronto, Canada). Mice were lightly anesthetized with 0.5-1.0% isoflurane with continuous monitoring of heart rate, body temperature (kept at normal by a heated platform), and respiratory rate. Two-dimensionally guided M-mode images of the left ventricle were acquired in addition to 2D evaluation of left ventricular size and function. The data were collected in both short and long axis views at heart rates > 400 BPM. A total of 3-5 cardiac cycles/measurement were averaged in each view to obtain functional and anatomical data.

Statistics

The significance of weight change was calculated by Student t test. The significance of survival differences was calculated by logrank test. The significance of all other comparisons (pathology, cell numbers, percentage of IFN-γ^+ cells and IFN-γ MFI) were calculated by the Mann-Whitney U test.

Results

PD-1 ligands inhibit TS1 T_{EM}-dependent GVHD of skin, liver and heart

To investigate whether PD1 regulates TS1-induced GVHD, we modified the TS1 GVHD model to employ BM donors and HA104 hosts that lacked both PD-L1 and PD-L2. We chose to create an environment without PD-ligands instead of using PD-$1^{-/-}$ TS1 T_{EM} to avoid the confounding effects the absence of PD-1 might have on TS1 development and T_{EM} generation. As reported previously(3), transplantation of wild type (WT) BM and TS1 T_{EM} into WT HA104 recipients caused no death and only mild, transient weight loss (Figure 1, A-B). In contrast, the transfer of TS1 T_{EM} and PD-L1/$2^{-/-}$ BM into PD-L1/$2^{-/-}$ HA104 hosts caused severe weight loss and death (Figure 1, A-B), but with delayed kinetics compared to what is observed with TS1 T_N with intact PD-ligands(3) (Figure 2A). Weight loss began 35 days post-transplant and rapidly progressed, with the majority of T_{EM} recipients dying by day 50. Histological analysis revealed that T_{EM}, in the absence of PD-ligands, induced more severe skin and liver GVHD relative to when PD-ligands were intact (Figure 1, C-D). In particular, liver GVHD was more severe than was induced by TS1 T_N in a PD-ligand sufficient environment (Figure 2; P=0.0064). In contrast, the absence of PD-

ligands did not increase colon GVHD (Figure 1E), which is a dominant feature of TS1 T_N-mediated disease ((3) and Figure 2). Very mild liver disease was also observed in PD-L1/2$^{-/-}$ HA104 recipients of PD-L1/2$^{-/-}$ BM without TS1 T_{EM}, indicating that some aspects of liver disease are TS1-independent (Figure 1D).

To better pinpoint the cause of clinical disease, we performed repeat experiments (weight loss and survival, Supplemental Figure 1) in which we examined kidney, lung, stomach, tongue and small intestine and found no significant pathology (data not shown). However, in the absence of PD-L1/2, TS1 T_{EM} recipients developed cardiac pathology (Figure 1F), manifested by a myocardial inflammatory infiltrate with cardiomyocyte necrosis (Supplemental Figure 2). Notably, severe heart disease did not develop in PD-L1/2$^{-/-}$ BM controls.

This inflammation had apparent functional significance. Approximately 5 weeks post-transplant, in the absence of PD-ligands, TS1 recipients had a reduction in heart rate (Figure 1, G-I). Two of 5 such mice had complete heart block and a third had an ectopic atrial focus, consistent with conduction system damage. Echocardiograms demonstrated reduced cardiac output and diastolic and systolic stroke volumes, even in mice without conduction abnormalities, further evidence that the myocarditis was likely to be functionally significant and may have been a cause of death (Figure 1, G-I).

Taken together, these data indicate that PD-ligands limit some, but not all, aspects of T_{EM} function post-transplant, as in their absence TS1 T_{EM} induce skin, liver and late-onset heart disease. However, in the absence of PD-ligands TS1 T_{EM} fail to recapitulate the severity of GVHD caused by TS1 T_N, particularly colon GVHD, indicating that pathways in addition to those downstream of PD-1 distinguish T_{EM} and T_N function post-transplantation.

TS1 T_N disease is inhibited by PD-ligands

To determine the role of PD-ligands in regulating GVHD mediated by TS1 T_N, irradiated WT HA104 or PD-L1/2$^{-/-}$ HA104 recipients were reconstituted with WT or PD-L1/2$^{-/-}$ BM (respectively), with or without TS1 T_N. TS1 T_N caused more early weight loss and death in the absence of PD-L1/2 (Figure 2, A-B), consistent with prior reports in GVHD models(11–13). This contrasts with what was observed with TS1 T_{EM} in a PD-ligand deficient environment wherein the increases in weight loss and death were delayed relative to what was observed with T_N (compare Figure 1A and 1B to Figure 2A and 2B; for weight loss, $P \leq 0.003$ from day +6 onward; for survival, $P=0.0002$). Nonetheless, TS1 T_N recipients developed a second wave of weight loss and death that shared the kinetics seen with TS1 T_{EM}. In the absence of PD-L1/2, TS1 T_N caused slightly increased skin disease and markedly more severe liver disease, but colon disease was similar to when PD-ligands were present (Figure 2, C-E). Consistent with our prior report (3), in a PD-ligand sufficient environment, TS1 T_N induced more severe histologic liver and colon GVHD than did TS1 T_{EM} ($P \leq 0.0024$). However, in PD-ligand deficient environments, TS1 T_{EM} and T_N caused similar liver GVHD.

Additional experiments were performed to evaluate heart disease (weight and survival are shown in Figure 3, A-B). TS1 T_N caused very mild heart disease when PD-L1/2 were intact;

but, importantly, they induced severe heart disease when PD-L1/2 were absent (Figure 2F; representative histology, Supplemental Figure 3). These data indicate that PD-L1/2 limit TS1 T_N-induced skin, liver and heart disease, similar to what was observed in TS1 T_{EM} recipients. However, unlike with TS1 T_{EM}, PD-L1/2 also limit early TS1 T_N-induced weight loss and death.

PD-ligands on recipient and donor cells restrict TS1-dependent GVHD

We next dissected the individual roles of PD-ligands on recipient and donor-derived cells in modulating TS1-induced GVHD. We transferred TS1 T_N or T_{EM} and WT BM into PD-L1/2$^{-/-}$ HA104 hosts and, reciprocally, TS1 T_N or T_{EM} and PD-L1/2$^{-/-}$ BM into WT HA104 hosts. Compared to WT HA104 recipients of TS1 T_N and WT BM, the absence of *host* PD-L1/2 alone did not increase skin, liver or heart disease, but did increase mortality. In contrast, the absence of PD-L1/2 on *donor BM* alone did not increase mortality but exacerbated skin, liver and heart disease, with scores as severe as those when PD-L1/2 were lacking on both donor BM and host cells (Figure 3, A-E). Notably, in this experiment there was less early death than typically seen when donor and host lack PD-ligands. In contrast to the case with T_N-mediated GVHD, neither clinical nor histopathologic TS1 T_{EM}-induced GVHD was increased when only the recipients lacked PD-ligands (Figure 3, F-J). However, the absence of PD-L1/2 on only donor BM increased weight loss and liver and heart pathology. Nonetheless, GVHD was less severe than when PD-ligands were absent on both the recipient and the donor BM, indicating that PD-L1/2 on both donor BM and host cells modulate TS1 T_{EM}-induced GVHD.

Roles of PD-L1/2 on host stromal and hematopoietic cells

We next determined the contributions of PD-L1/2 on host hematopoietic and radioresistant, primarily nonhematopoietic, cells. To do so, we created WT HA104→PD-L1/2$^{-/-}$ HA104 and PD-L1/2$^{-/-}$ HA104→WT HA104 BM chimeras and used these as recipients in a second transplant in which GVHD was induced by TS1 T_{EM}. As controls, WT HA104→WT HA104 and PD-L1/2$^{-/-}$ HA104→PD-L1/2$^{-/-}$ HA104 chimeras were also transplanted. Because the absence of PD-L1/2 on host cells alone does not enable TS1 T_{EM}-mediated GVHD (Figure 3), all chimeras received PD-L1/2$^{-/-}$ BM in the second GVHD-inducing transplant. As expected, transfer of TS1 T_{EM} and PD-L1/2$^{-/-}$ BM caused mild heart disease in WT→WT chimeras and severe weight loss, death and heart disease in PD-L1/2$^{-/-}$→PD-L1/2$^{-/-}$ chimeras. In comparison to PD-L1/2$^{-/-}$→PD-L1/2$^{-/-}$ chimeras, disease was similar in WT→PD-L1/2$^{-/-}$ chimeras but was reduced in PD-L1/2$^{-/-}$→WT chimeras (Figure 4, A-E). Therefore, PD-L1/2 on donor BM and host stromal cells, but not recipient hematopoietic cells, inhibits TS1 T_{EM}-dependent cardiac disease.

Roles of PD-L1 and PD-L2

Whereas PD-L1 is widely expressed and can be induced by IFN-γ, PD-L2 is expressed primarily on hematopoietic cells(14). To test if PD-L1 is sufficient to modulate TS1-mediated GVHD, TS1 T_{EM} and PD-L1$^{-/-}$ BM were transferred into PD-L1$^{-/-}$ HA104 hosts. GVHD in the absence of PD-L1 alone phenocopied TS1 T_{EM}-mediated GVHD in the absence of both PD-L1 and PD-L2 (Figure 5) indicating that PD-L1 is sufficient for GVHD suppression.

Effect of PD-L1/2 on TS1 T_{EM} accumulation and IFN-γ production

Despite increased TS1 T_{EM}-induced GVHD in the absence of PD-L1/2, TS1 numbers in the spleen and the percentage of IFN-γ^+ TS1 at days +14 or +42 were similar (Figure 6). However, in the absence of PD-L1/2, splenic TS1 at day 42 produced more IFN-γ/cell measured by intracellular cytokine staining (Figure 6C). In the liver, TS1 numbers were increased in the absence of PD-ligands only on day +42 (Figure 6A), consistent with the increased liver GVHD scores. In sum these data indicate that PD-L1/2 diminishes IFN-γ production by splenic TS1 cells(15), which could result in fewer pathogenic TS1 cells infiltrating GVHD target organs. Alternatively, or in addition, PD-L1/2 could suppress TS1 cells locally.

Non-TS1 T cells are required for cardiac disease

Unexpectedly, the absence of PD-L1/2 reduced the number of TS1 cells in the heart (Figure 6D). However, non-TS1 $CD4^+$ and $CD8^+$ T cells were increased 3 and 6-fold, respectively (Figure 6, E-F). This increase only occurred in TS1 T_{EM} recipients and not in PD-L1/$2^{-/-}$ BM alone controls. These data suggested a model wherein TS1 cells are required for the initiation of a process that leads to heart disease, but that non-TS1 T cells are directly pathogenic. These polyclonal T cells could be donor or recipient BM-derived, radioresistant recipient mature T cells or a combination of these.

To test the hypothesis that polyclonal T cells contribute to heart disease, we developed a system that lacked all non-TS1 T cells. PD-L1/$2^{-/-}$ HA104 or PD-L1/$2^{-/-}$ $RAG2^{-/-}$ (triple knockout; TKO) HA104 mice were transplanted with PD-L1/$2^{-/-}$ or TKO BM (respectively) with no T cells or with TS1 T_{EM} or TS1 T_N. TKO HA104 recipients of TS1 T_{EM} and TKO BM developed early weight loss and liver disease similar to controls; however, TKO recipients suffered less late-onset weight loss and death, and strikingly, no heart disease (Figure 7, A-D). Likewise, both RAG2-sufficient and RAG2-deficient TS1 T_N groups developed GVHD, manifested by weight loss (Figure 7E). Initial weight loss was more severe in $RAG2^{-/-}$ recipients. However, after regaining some weight, the RAG2-sufficient TS1 T_N group developed a second wave of weight loss beginning around day +40. We harvested hearts from both groups beginning on day +47. Strikingly, there was severe heart disease in RAG2-intact TS1 T_N recipients whereas cardiac pathology scores in TKO TS1 T_N recipients were indistinguishable from negative control PD-L1/$2^{-/-}$ $RAG2^{+/+}$ BM alone mice (Figure 7F; representative histology, Supplemental Figure 4). Consistent with a role for polyclonal CD8 cells in mediating heart disease, they were readily detectable in the spleen (Figure 7G). Therefore, non-TS1 T cells are required for TS1-induced heart disease, but not for other aspects of GVHD. We again observed low level liver histopathology in PD-L1/$2^{-/-}$ recipients of PD-L1/$2^{-/-}$ BM without TS1, but not when donors and recipients were also $RAG2^{-/-}$. This makes it likely that such disease is caused by polyclonal donor- or recipient-derived T cells.

Residual recipient polyclonal T cells are sufficient for autoimmune cardiomyositis

To determine whether recipient T cells were sufficient to cause cardiac disease, we performed GVHD experiments with TKO donor BM and $RAG2^{+/+}$ PD-L1/$2^{-/-}$ HA104 recipients. Compared to recipients of TS1 T_{EM} and PD-L1/$2^{-/-}$ $RAG2^{+/+}$ BM, recipients of

TS1 T_{EM} and TKO BM developed similar weight loss and liver disease while mortality and heart disease were increased (Figure 8), indicating that recipient polyclonal T cells are sufficient for myocarditis, whereas donor BM derived T cells are not required and might be protective. Unexpectedly, heart disease also developed in a small but significant number of PD-L1/2$^{-/-}$ HA104 recipients of TKO BM that did not receive TS1 T_{EM}, but not in TKO HA104 recipients of TKO BM without TS1 T_{EM} (Figure 7D). Host CD4 and CD8 cells were readily detected in spleens of PD-L1/2$^{-/-}$ recipients of TKO BM, with or without TS1 T_{EM}, at day 60 (Figure 8F), consistent with a role for host polyclonal T cells in mediating heart disease in both groups. Although PD-L1/2$^{-/-}$ HA104 recipients of PD-L1/2$^{-/-}$ BM alone did not develop severe heart disease, there were also occasional mice with mild disease (see Figures 1L, 3E and 3J).

Taken together, these results support the idea that the absence of PD-ligands during T cell reconstitution in the alloSCT setting is permissive for myocarditis mediated by autoreactive polyclonal T cells. Alloreactive TS1 cells reproducibly trigger this autoimmunity whereas donor BM-derived polyclonal T cells may suppress it.

Treg reconstitution in the presence and absence of PD-ligands

If regulatory T cell (Treg) reconstitution in the absence of PD-ligands were diminished, this could have contributed to autoimmune myocarditis. We therefore enumerated Tregs in irradiated WT or PD-L1/2$^{-/-}$ HA104 mice that were reconstituted with WT or PD-L1/2$^{-/-}$ BM (respectively), with or without TS1 T_{EM} (Figure 9). On day 14 post-transplant, with or without TS1 T_{EM}, PD-L1/2$^{-/-}$ recipients of PD-L1/L2$^{-/-}$ BM had higher percentages and total numbers of Tregs; however, there was no difference in the ratios of TS1 to Tregs. On day 42 post-transplant, a higher percentage of splenocytes were Tregs in the absence of PD-L1/2, though neither the total numbers nor the Treg:TS1 ratios differed. In the liver on day +42, PD-L1/2$^{-/-}$ recipients of PDL1/2$^{-/-}$ BM with and without TS1 T_{EM} had a greater number and percentage of Tregs, but again without a significant increase in the Treg:TS1 ratio. In sum these data make it unlikely that autoimmunity developed in the absence of PD-L1/L2 due to poor Treg reconstitution.

Polyclonal CD8$^+$ T cells contribute to myocarditis

To determine the individual roles of polyclonal CD4$^+$ and CD8$^+$ T cells in TS1 T_{EM}-induced cardiac disease we used depleting antibodies in PD-L1/2$^{-/-}$ HA104 recipients of TS1 T_{EM} and PD-L1/2$^{-/-}$ BM. CD8 cells were depleted with anti-CD8 twice in the week prior to transplant and then weekly thereafter. To avoid depleting TS1 cells during GVHD development, we began CD4 depletion on day +27, prior to the onset of clinical cardiac disease (manifest by weight loss).

CD4-depletion paradoxically increased mortality and weight loss but had no impact on heart disease (Figure 10, A-C). In contrast, CD8-depletion alone and combined CD8 and CD4 depletion decreased both mortality and heart disease. Enumeration of splenic CD4$^+$ and CD8$^+$ T cells revealed that anti-CD4 treatment slightly decreased CD8$^+$ T cell numbers but drastically reduced TS1 and non-TS1 CD4$^+$ T cells numbers (Figure 10D). Anti-CD8 treatment had no effect on CD4$^+$ T cell numbers but reduced CD8$^+$ T cells. Taken together,

these data indicate that polyclonal CD8 cells, and not CD4 cells, are essential for heart disease. The persistence of heart disease even after profound TS1 depletion suggests that once autoimmune cardiac disease is established, it is independent of further actions by alloimmune cells.

Discussion

It has long been known that T_{EM} cause less GVHD than do T_N, a phenomenon which we and others found to be independent of TCR repertoire differences(3, 16). The difference between T_N and T_M is clinically relevant, as a recent human trial has suggested a benefit with T_N-depletion(6). However, the mechanisms that restrain the GVHD-inducing potential of T_M have not been elucidated. In the present work, we found that PD-ligands are one component that limits alloreactive T_{EM}. PD-ligands inhibited TS1 T_{EM}-mediated GVHD through two main mechanisms. First, they suppressed HA-directed GVHD in the liver and skin. However, neither colon histopathology, a dominant feature of TS1 T_N-induced GVHD, nor early weight loss, were restored to that induced by TS1 T_N in a WT or PD-L1/2$^{-/-}$ environment. Therefore, additional properties of TS1 T_{EM} must diminish their ability to cause GVHD relative to TS1 T_N. Second, and contrary to expectations, PD-ligands maintained tolerance of polyclonal nonalloreactive T cells which, in the absence of PD-ligands, was reproducibly broken by alloreactive TS1 T_{EM}. TS1 T_N-HA-directed GVHD was also more severe in the absence of PD-ligands. And as we observed with TS1 T_{EM}, in the absence of PD-ligands and in the presence of polyclonal T cells, TS1 T_N induced myocarditis and late onset weight loss and death.

The roles of PD-ligands in modifying HA-directed GVHD were complex and context-dependent, in part owing to their expression on hematopoietic as well as parenchymal cells. Both TS1 T_{EM}- and T_N-induced liver GVHD was were suppressed by PD-ligands. TS1 T_{EM} were particularly sensitive to PD-1 suppression as liver scores in the absence of PD-ligands were similar to scores in TS1 T_N recipients, with or without PD-ligands. On the other hand, colon GVHD was not increased in the absence of PD-ligands in TS1 T_N and T_{EM} recipients, which suggests the possibility that PD-ligands do not regulate colon GVHD directly. For T_N-mediated GVHD, the absence of PD-ligands on only donor BM-derived cells was necessary and sufficient for maximal acute GVHD intensification. These features were not modified when hosts also lacked PD-L1/2$^{-/-}$. The absence of PD-ligands on only donor BM was also sufficient to increase TS1 T_{EM}-induced HA-directed liver GVHD; however, skin disease and maximal liver GVHD required that PD-ligands be absent from both donor BM and recipient. The dominant role of PD-ligands on donor BM-derived cells emphasizes the importance of indirect presentation of miHAs (the HA-derived S1 peptide in this case) by donor APCs to donor CD4 cells(17, 18). Although indirect presentation is commonly thought of as being stimulatory and disease-promoting, our data show that indirect miHA presentation by PD-ligand-expressing donor APCs suppresses GVHD. This regulation could occur in secondary lymphoid tissues, peripheral GVHD target organs, or both. PD-ligands on hematopoietic APCs may be especially important in the regulation of CD4-mediated immunopathology, as CD4-mediated GVHD in miHA-disparate MHC-matched models *can* occur when nonhematopoietic cells are MHCII^{-}(19). Therefore, hematopoietic cells could be the only

cells, or at least the most common cell, capable of engaging CD4 cells by their TCRs while presenting PD-ligands.

In contrast to our studies, prior work on PD-ligands in GVHD found a dominant role for host PD-ligands, and when examined, on host radioresistant nonhematopoietic cells(12). In these studies, GVHD was in MHC-mismatched models. As such, direct recognition of recipient MHC likely dominated the alloimmune response, which would dictate less of a role for indirect miHA presentation by donor APCs. Also, recipient nonhematopoietic PD-L1 would be expected to be dominant in models with CD8 cells as CD8 cells must make TCR:MHCI contact with nonhematopoietic cells to cause GVHD(19).

Surprisingly, genetic and depletion studies demonstrated that TS1-induced cardiac disease in the absence of PD-ligands required polyclonal, $CD8^+$ non-TS1 T cells. Commensurately, infiltrating T cells were predominantly not TS1 clonotype, with approximately 2-fold more $CD8^+$ than $CD4^+$ T cells. That CD4 depletion, which also profoundly reduced TS1 T_{EM} progeny, did not diminish myocarditis suggests that once TS1 cells break tolerance, they are no longer required to maintain heart disease. Surprisingly, CD4 depletion, but not dual CD4 and CD8 depletion, accelerated weight loss and death. Anti-CD4 treatment likely depleted $CD4^+$ Tregs, which may suppress autoimmune CD8 cells.

Of note, there was mild but significant heart disease in the absence of TS1 cells when donors were $RAG2^{-/-}$PDL-$L1/2^{-/-}$ and recipients were $RAG2^{+/+}$PD-$L1/2^{-/-}$. These data further support the notion that PD-ligands suppress the development of autoreactive polyclonal T cells post transplantation even without GVHD and that this autoreactivity is reproducibly triggered by alloreactive T cells. It is tempting to speculate that alloimmune attack, as in the system we studied, is just one example of an inflammatory condition that would trigger autoimmunity when PD-ligand engagement is suboptimal. Other examples might include chronic infection or toxic injury, both settings that are associated with autoimmune induction in general.

How do PD-ligands regulate self-tolerance in the context of alloSCT? A major clue is that the absence of PD-L1/2 on the donor BM is sufficient for myocarditis. Therefore parenchymal expression of PD-L1 suppressing effector cells, as invoked in PD-ligand-dependent tumor immune resistance settings(20), cannot be the only mechanism by which PD-ligands regulate autoimmunity. Instead, the absence of donor hematopoietic PD-L1/2 could lead to the peripheral activation of self-reactive polyclonal T cells, be they mature T cells that survived radiation, donor allograft T cells or T cells derived from hematopoietic progenitors. In this connection, presentation of self-antigen by APCs is thought to be a natural means of maintaining tolerance(21–23). In the absence of PD-ligands, such toleragenic APCs could become stimulatory instead in a background of immune system activation by alloreactive CD4 cells.

The absence of PD-L1/2 on recipient nonhematopoietic tissues promoted even more extensive TS1 T_{EM}-triggered myocarditis. PD-$L1/2^{-/-}$ thymic parenchyma may have been permissive for the selection of self-reactive T cells. PD-L1 has been demonstrated on cardiac endothelia(24, 25), and therefore PD-ligands could suppress both the generation of

autoreactive CD8 cells and their effector activities. Resolving the mechanisms that account for TS1-induced autoimmunity would require both thymus and cardiac transplantation, which can be addressed in future work.

An unanswered question is why we observed only autoimmune heart disease and not autoimmune disease in other organs. One possibility is that, for unknown reasons, autoreactive CD8 cells were reactive only against cardiac-specific antigens. On the other hand, autoreactive CD8 cells could recognize antigens with a broader tissue distribution. However, we might not have readily been able to detect their contribution to disease in tissues wherein TS1 already cause significant immunopathology. For example, in the absence of PD-ligands, but without TS1, there was low level liver histopathology, which was not present when donor and recipient were $RAG2^{-/-}$. Such mild changes could easily have been masked by HA-directed alloimmune damage.

The PD-1 pathway has not been implicated in protecting the heart in polyclonal models of GVHD. In most of these reports, impairment of the PD-1 pathway led to early death such that later onset myocarditis could not have been evaluated. It will be of interest to determine whether autoimmune myocarditis develops in the absence of PD-ligands in polyclonal GVHD models, which will require establishing conditions such that PD-1 blockade does not result in early lethality, but still intensifies GVHD.

Outside the GVHD literature, there is evidence that PD-1 and its ligands protect the heart against autoimmunity. $PD\text{-}1^{-/-}$ BALB/c mice develop fatal dilated cardiomyopathy, though this was found to be antibody-mediated(26, 27) whereas disease in our model was caused by $CD8^{+}$ T cells. PD-1 or PD-L1 deficient MRL-lpr mice developed cardiomyositis with a mixed inflammatory infiltrate along with anti-cardiac antibodies(24, 28). PD-1 and PD-L1 also suppressed experimental myocarditis directed against a model antigen(25, 29), which differs substantially from the autoimmune disease we describe.

Recent reports describe rare cases of myocarditis with checkpoint inhibitor therapies, especially when CTLA-4 and PD-1 were combined(30–32). PD-1 or PD-L1 inhibition is being considered in patients with relapsed hematologic malignancies post alloSCT and has been applied in a small number of patients, mostly with Hodgkin’s lymphoma relapsed post alloSCT (33, 34). While prior mouse work clearly demonstrated the risk for increased GVHD with such therapy(11–13, 35), our results raise additional concerns that autoimmune manifestations, including heart disease, could be precipitated.

When cardiac dysfunction occurs post alloSCT, it is frequently in the setting of complex medical problems that can include GVHD. Heart pathology is rarely examined, such that cardiac GVHD could be underappreciated. Nonetheless, cardiac GVHD has been reported. Diagnoses included heart block (as we observed), myocarditis, cardiomyopathy and coronary artery disease, with instances of patients improving with immunosuppressants(36, 37).

Our results bear on the long-standing hypothesis that chronic GVHD has an autoimmune component(38–41). Acute GVHD is the single greatest risk factor for chronic GVHD, which suggests that alloimmunity can beget autoimmunity. Our data directly demonstrate that this

can occur and that it is important for pathogenesis. It is of a “chronic” nature in that it occurs only later in the course, after the initial wave of TS1-mediated allorecognition that leads to acute disease. Multiple non-exclusive mechanisms have been suggested for GVHD-associated autoimmunity in other contexts(41–44). Our data suggest that if cGVHD is at least in-part autoimmune, PD-ligands may be an additional mechanism that promotes self-tolerance. In our experiments, residual recipient T cells were sufficient to cause myocarditis. Even with ablative conditioning, a substantial number of patients have residual circulating host-derived T cells post-transplant (45–51). Our results suggest that these T cells could be functionally autoimmune with insufficient PD-ligand engagement.

In any case, our results demonstrate a clear role for PD-ligands in regulating miHA-directed GVHD mediated by T_{EM} and T_N, and that this occurs at multiple levels including by donor APCs that indirectly present alloantigens. Most intriguingly, we also reveal a clear PD-ligand-dependent regulation of autoimmunity that is triggered by initial alloimmunity. This could explain chronicity of GVHD in some cases and in fact why chronic GVHD can be unremitting. While we have used genetic knockouts to elicit strong phenotypes, differences in the strength of PD-ligand signals—perhaps genetically encoded—could underlie susceptibility to chronic GVHD, which itself could be partly caused by autoimmune induction downstream of alloimmune-mediated tissue damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References Cited

1. Anderson BE, McNiff J, Yan J, Doyle H, Mamula M, Shlomchik MJ, Shlomchik WD. Memory CD4+ T cells do not induce graft-versus-host disease. J Clin Invest. 2003; 112:101–108. [PubMed: 12840064]
2. Zheng H, Matte-Martone C, Li H, Anderson BE, Venketesan S, Sheng Tan H, Jain D, McNiff J, Shlomchik WD. Effector memory CD4+ T cells mediate graft-versus-leukemia without inducing graft-versus-host disease. Blood. 2008; 111:2476–2484. [PubMed: 18045967]
3. Juchem KW, Anderson BE, Zhang C, McNiff JM, Demetris AJ, Farber DL, Caton AJ, Shlomchik WD, Shlomchik MJ. A repertoire-independent and cell-intrinsic defect in murine GVHD induction by effector memory T cells. Blood. 2011; 118:6209–6219. [PubMed: 21768295]
4. Chen BJ, Cui X, Sempowski GD, Liu C, Chao NJ. Transfer of allogeneic CD62L- memory T cells without graft-versus-host disease. Blood. 2004; 103:1534–1541. [PubMed: 14551132]
5. Chen BJ, Deoliveira D, Cui X, Le NT, Son J, Whitesides JF, Chao NJ. Inability of memory T cells to induce graft-versus-host disease is a result of an abortive alloresponse. Blood. 2007; 109:3115–3123. [PubMed: 17148592]
6. Bleakley M, Heimfeld S, Loeb KR, Jones LA, Chaney C, Seropian S, Gooley T, Sommermeyer F, Riddell SR, Shlomchik WD. Outcomes of high risk leukemia patients transplanted with stem cell grafts depleted of naïve T cells. Journal of Clinical Investigation. 2015 In Press.

7. Anderson BE, Taylor PA, McNiff JM, Jain D, Demetris AJ, Panoskaltsis-Mortari A, Ager A, Blazar BR, Shlomchik WD, Shlomchik MJ. Effects of donor T-cell trafficking and priming site on graft-versus-host disease induction by naive and memory phenotype CD4 T cells. Blood. 2008; 111:5242–5251. [PubMed: 18285547]
8. Shih FF, Cerasoli DM, Caton AJ. A major T cell determinant from the influenza virus hemagglutinin (HA) can be a cryptic self peptide in HA transgenic mice. Int Immunol. 1997; 9:249–261. [PubMed: 9040007]
9. Kirberg J, Baron A, Jakob S, Rolink A, Karjalainen K, von Boehmer H. Thymic selection of CD8+ single positive cells with a class II major histocompatibility complex-restricted receptor. Journal of Experimental Medicine. 1994; 180:25–34. [PubMed: 8006585]
10. Caton AJ, Swartzentruber JR, Kuhl AL, Carding SR, Stark SE. Activation and negative selection of functionally distinct subsets of antibody-secreting cells by influenza hemagglutinin as a viral and a neo-self antigen. J Exp Med. 1996; 183:13–26. [PubMed: 8551216]
11. Blazar BR, Carreno BM, Panoskaltsis-Mortari A, Carter L, Iwai Y, Yagita H, Nishimura H, Taylor PA. Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-gamma-dependent mechanism. J Immunol. 2003; 171:1272–1277. [PubMed: 12874215]
12. Saha A, Aoyama K, Taylor PA, Koehn BH, Veenstra RG, Panoskaltsis-Mortari A, Munn DH, Murphy WJ, Azuma M, Yagita H, Fife BT, Sayegh MH, Najafian N, Socie G, Ahmed R, Freeman GJ, Sharpe AH, Blazar BR. Host programmed death ligand 1 is dominant over programmed death ligand 2 expression in regulating graft-versus-host disease lethality. Blood. 2013; 122:3062–3073. [PubMed: 24030385]
13. Deng R, Cassady K, Li X, Yao S, Zhang M, Racine J, Lin J, Chen L, Zeng D. B7H1/CD80 interaction augments PD-1-dependent T cell apoptosis and ameliorates graft-versus-host disease. J Immunol. 2015; 194:560–574. [PubMed: 25488990]
14. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol. 2008; 26:677–704. [PubMed: 18173375]
15. Wherry EJ. T cell exhaustion. Nat Immunol. 2011; 12:492–499. [PubMed: 21739672]
16. Zhang P, Wu J, Deoliveira D, Chao NJ, Chen BJ. Allospecific CD4(+) effector memory T cells do not induce graft-versus-host disease in mice. Biol Blood Marrow Transplant. 2012; 18:1488–1499. [PubMed: 22809867]
17. Markey KA, Banovic T, Kuns RD, Olver SD, Don AL, Raffelt NC, Wilson YA, Raggatt LJ, Pettit AR, Bromberg JS, Hill GR, MacDonald KP. Conventional dendritic cells are the critical donor APC presenting alloantigen after experimental bone marrow transplantation. Blood. 2009; 113:5644–5649. [PubMed: 19336758]
18. Anderson BE, McNiff JM, Jain D, Blazar BR, Shlomchik WD, Shlomchik MJ. Distinct roles for donor- and host-derived antigen-presenting cells and costimulatory molecules in murine chronic graft-versus-host disease: requirements depend on target organ. Blood. 2005; 105:2227–2234. [PubMed: 15522961]
19. Matte-Martone C, Liu J, Jain D, McNiff J, Shlomchik WD. CD8+ but not CD4+ T cells require cognate interactions with target tissues to mediate GVHD across only minor H antigens, whereas both CD4+ and CD8+ T cells require direct leukemic contact to mediate GVL. Blood. 2008; 111:3884–3892. [PubMed: 18223170]
20. Chen L, Han X. Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. J Clin Invest. 2015; 125:3384–3391. [PubMed: 26325035]
21. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu Rev Immunol. 2003; 21:685–711. [PubMed: 12615891]
22. Lechler R, Ng WF, Steinman RM. Dendritic cells in transplantation–friend or foe? Immunity. 2001; 14:357–368. [PubMed: 11336681]
23. Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M, Ravetch JV, Steinman RM, Nussenzweig MC. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med. 2001; 194:769–779. [PubMed: 11560993]

24. Lucas JA, Menke J, Rabacal WA, Schoen FJ, Sharpe AH, Kelley VR. Programmed death ligand 1 regulates a critical checkpoint for autoimmune myocarditis and pneumonitis in MRL mice. J Immunol. 2008; 181:2513–2521. [PubMed: 18684942]
25. Grabie N, Gotsman I, DaCosta R, Pang H, Stavrakis G, Butte MJ, Keir ME, Freeman GJ, Sharpe AH, Lichtman AH. Endothelial programmed death-1 ligand 1 (PD-L1) regulates CD8+ T-cell mediated injury in the heart. Circulation. 2007; 116:2062–2071. [PubMed: 17938288]
26. Okazaki T, Tanaka Y, Nishio R, Mitsuiye T, Mizoguchi A, Wang J, Ishida M, Hiai H, Matsumori A, Minato N, Honjo T. Autoantibodies against cardiac troponin I are responsible for dilated cardiomyopathy in PD-1-deficient mice. Nat Med. 2003; 9:1477–1483. [PubMed: 14595408]
27. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, Sasayama S, Mizoguchi A, Hiai H, Minato N, Honjo T. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science. 2001; 291:319–322. [PubMed: 11209085]
28. Wang J I, Okazaki M, Yoshida T, Chikuma S, Kato Y, Nakaki F, Hiai H, Honjo T, Okazaki T. PD-1 deficiency results in the development of fatal myocarditis in MRL mice. Int Immunol. 2010; 22:443–452. [PubMed: 20410257]
29. Tarrio ML, Grabie N, Bu DX, Sharpe AH, Lichtman AH. PD-1 protects against inflammation and myocyte damage in T cell-mediated myocarditis. J Immunol. 2012; 188:4876–4884. [PubMed: 22491251]
30. Heinzerling L, Ott PA, Hodi FS, Husain AN, Tajmir-Riahi A, Tawbi H, Pauschinger M, Gajewski TF, Lipson EJ, Luke JJ. Cardiotoxicity associated with CTLA4 and PD1 blocking immunotherapy. J Immunother Cancer. 2016; 4:50. [PubMed: 27532025]
31. Laubli H, Balmelli C, Bossard M, Pfister O, Glatz K, Zippelius A. Acute heart failure due to autoimmune myocarditis under pembrolizumab treatment for metastatic melanoma. J Immunother Cancer. 2015; 3:11. [PubMed: 25901283]
32. Johnson DB, Balko JM, Compton ML, Chalkias S, Gorham J, Xu Y, Hicks M, Puzanov I, Alexander MR, Bloomer TL, Becker JR, Slosky DA, Phillips EJ, Pilkinton MA, Craig-Owens L, Kola N, Plautz G, Reshef DS, Deutsch JS, Deering RP, Olenchock BA, Lichtman AH, Roden DM, Seidman CE, Koralnik IJ, Seidman JG, Hoffman RD, Taube JM, Diaz LA Jr, Anders RA, Sosman JA, Moslehi JJ. Fulminant Myocarditis with Combination Immune Checkpoint Blockade. N Engl J Med. 2016; 375:1749–1755. [PubMed: 27806233]
33. Haverkos BM, Abbott D, Hamadani M, Armand P, Flowers ME, Merryman R, Kamdar M, Kanate AS, Saad A, Mehta A, Ganguly S, Fenske TS, Hari P, Lowsky R, Andritsos L, Jagasia M, Bashey A, Brown S, Bachanova V, Stephens D, Mineishi S, Nakamura R, Chen YB, Blazar BR, Gutman J, Devine SM. PD-1 blockade for relapsed lymphoma post allogeneic hematopoietic cell transplant: high response rate but frequent GVHD. Blood. 2017
34. Herbaux C, Gauthier J, Brice P, Drumez E, Ysebaert L, Doyen H, Fornecker L, Bouabdallah K, Manson G, Ghesquieres H, Tabrizi R, Hermet E, Lazarovici J, Thiebaut-Bertrand A, Chauchet A, Demarquette H, Boyle E, Houot R, Yakoub-Agha I, Morschhauser F. Efficacy and tolerability of nivolumab after allogeneic transplantation for relapsed Hodgkin lymphoma. Blood. 2017; 129:2471–2478. [PubMed: 28270452]
35. Fujiwara H, Maeda Y, Kobayashi K, Nishimori H, Matsuoka K, Fujii N, Kondo E, Tanaka T, Chen L, Azuma M, Yagita H, Tanimoto M. Programmed death-1 pathway in host tissues ameliorates Th17/Th1-mediated experimental chronic graft-versus-host disease. J Immunol. 2014; 193:2565–2573. [PubMed: 25080485]
36. Rackley C, Schultz KR, Goldman FD, Chan KW, Serrano A, Hulse JE, Gilman AL. Cardiac manifestations of graft-versus-host disease. Biol Blood Marrow Transplant. 2005; 11:773–780. [PubMed: 16182178]
37. Ahn JS, Cho SH, Kim YK, Yang DH, Bae WK, Shim HJ, Lee JJ, Chung IJ, Juhng SW, Kim HJ. Polymyositis and myocarditis after donor lymphocyte infusion. Int J Hematol. 2009; 90:113–116. [PubMed: 19472035]
38. Parkman R. Clonal analysis of murine graft-vs-host disease. I. Phenotypic and functional analysis of T lymphocyte clones. Journal of Immunology. 1986; 136:3543–3548.
39. Parkman R. Is chronic graft versus host disease an autoimmune disease? Curr Opin Immunol. 1993; 5:800–803. [PubMed: 8240743]

40. Chen, W., Gress, R., Shlomchik, WD. Animal Models of Chronic Graft-versus-Host Disese. In: Vogelsang, G., Pavletic, S., editors. Chronic Graft-Versus-Host Disease. Cambridge University Press; 2009. p. 31-45.
41. Wu T, Young JS, Johnston H, Ni X, Deng R, Racine J, Wang M, Wang A, Todorov I, Wang J, Zeng D. Thymic damage, impaired negative selection, and development of chronic graft-versus-host disease caused by donor CD4+ and CD8+ T cells. J Immunol. 2013; 191:488–499. [PubMed: 23709681]
42. Zhang Y, Hexner E, Frank D, Emerson SG. CD4+ T cells generated de novo from donor hemopoietic stem cells mediate the evolution from acute to chronic graft-versus-host disease. J Immunol. 2007; 179:3305–3314. [PubMed: 17709547]
43. Tivol E, Komorowski R, Drobyski WR. Emergent autoimmunity in graft-versus-host disease. Blood. 2005; 105:4885–4891. [PubMed: 15746077]
44. Matsuoka K, Kim HT, McDonough S, Bascug G, Warshauer B, Koreth J, Cutler C, Ho VT, Alyea EP, Antin JH, Soiffer RJ, Ritz J. Altered regulatory T cell homeostasis in patients with CD4+ lymphopenia following allogeneic hematopoietic stem cell transplantation. J Clin Invest. 2010; 120:1479–1493. [PubMed: 20389017]
45. Doney K, Loken M, Bryant E, Smith A, Appelbaum F. Lack of utility of chimerism studies obtained 2-3 months after myeloablative hematopoietic cell transplantation for ALL. Bone Marrow Transplant. 2008; 42:271–274. [PubMed: 18500370]
46. Mossallam GI, Kamel AM, Storer B, Martin PJ. Prognostic utility of routine chimerism testing at 2 to 6 months after allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transplant. 2009; 15:352–359. [PubMed: 19203726]
47. Saito B, Fukuda T, Yokoyama H, Kurosawa S, Takahashi T, Fuji S, Takahashi N, Tajima K, Kim SW, Mori S, Tanosaki R, Takaue Y, Heike Y. Impact of T cell chimerism on clinical outcome in 117 patients who underwent allogeneic stem cell transplantation with a busulfan-containing reduced-intensity conditioning regimen. Biol Blood Marrow Transplant. 2008; 14:1148–1155. [PubMed: 18804045]
48. Lee HC, Saliba RM, Rondon G, Chen J, Charafeddine Y, Medeiros LJ, Alatrash G, Andersson BS, Popat U, Kebriaei P, Ciurea S, Oran B, Shpall E, Champlin R. Mixed T Lymphocyte Chimerism after Allogeneic Hematopoietic Transplantation Is Predictive for Relapse of Acute Myeloid Leukemia and Myelodysplastic Syndromes. Biol Blood Marrow Transplant. 2015; 21:1948–1954. [PubMed: 26183077]
49. Reshef R, Hexner EO, Loren AW, Frey NV, Stadtmauer EA, Luger SM, Mangan JK, Gill SI, Vassilev P, Lafferty KA, Smith J, Van Deerlin VM, Mick R, Porter DL. Early donor chimerism levels predict relapse and survival after allogeneic stem cell transplantation with reduced-intensity conditioning. Biol Blood Marrow Transplant. 2014; 20:1758–1766. [PubMed: 25016197]
50. Koreth J, Kim HT, Nikiforow S, Milford EL, Armand P, Cutler C, Glotzbecker B, Ho VT, Antin JH, Soiffer RJ, Ritz J, Alyea EP 3rd. Donor chimerism early after reduced-intensity conditioning hematopoietic stem cell transplantation predicts relapse and survival. Biol Blood Marrow Transplant. 2014; 20:1516–1521. [PubMed: 24907627]
51. Bleakley M, Heimfeld S, Loeb KR, Jones LA, Chaney C, Seropian S, Gooley TA, Sommermeyer F, Riddell SR, Shlomchik WD. Outcomes of acute leukemia patients transplanted with naive T cell-depleted stem cell grafts. J Clin Invest. 2015; 125:2677–2689. [PubMed: 26053664]

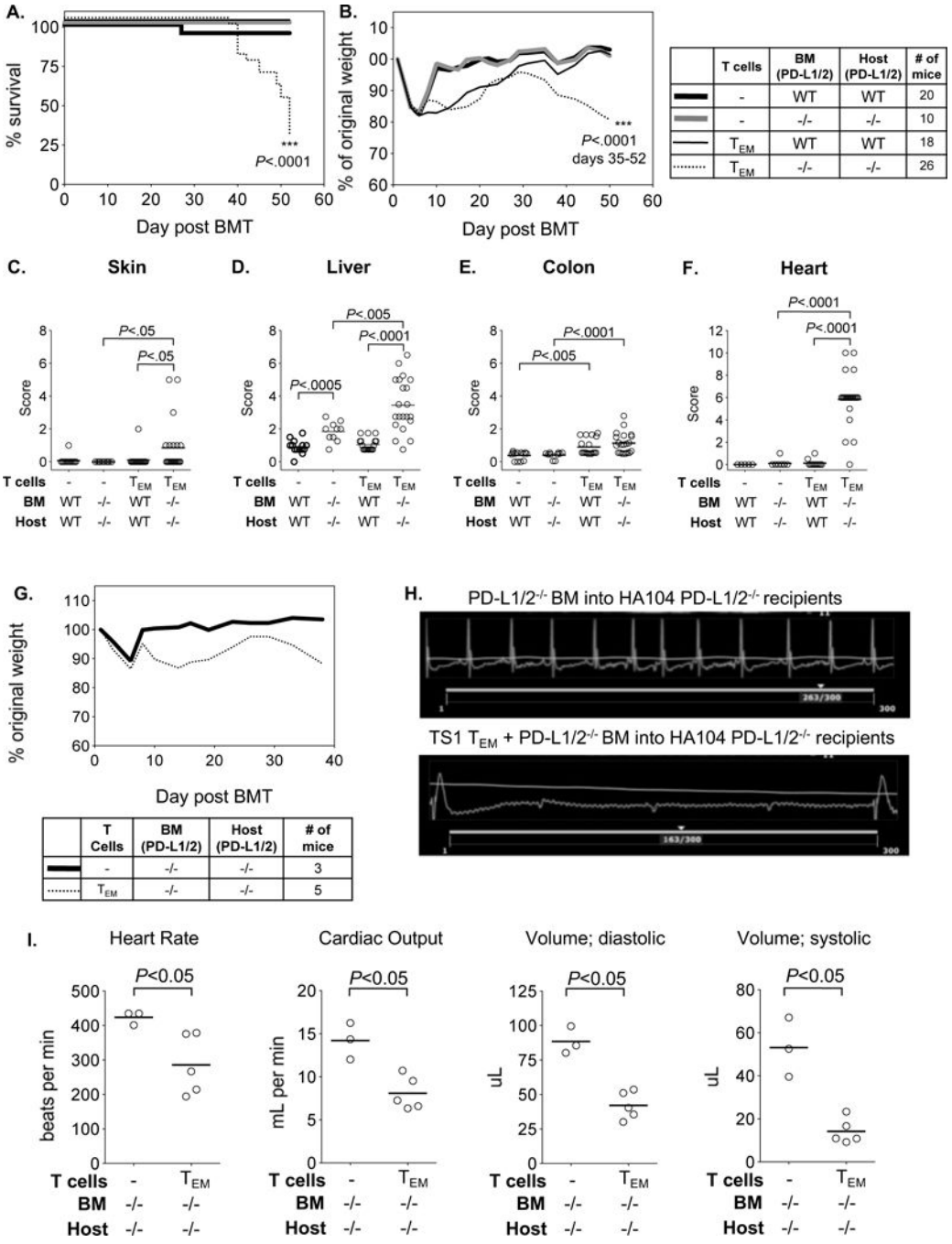


Figure 1. PD-L1/2 limit TS1 T_{EM}-mediated disease in the skin, liver and heart but not the colon

(A–F) WT or PD-L1/L2$^{-/-}$ BM was transferred into irradiated WT or PD-L1/2$^{-/-}$ HA104 mice, respectively, with or without 1000 TS1 T_{EM}. Shown are survival (A), weight loss (B) and pathology scores for the skin (C), liver (D), colon (E) and heart (F). Data from (A-E) were combined from 2 independent experiments. Data from (F) were combined from two additional independent experiments, for which weight and survival curves are shown in Supplemental Figure 1. *P* values for survival and weight curves are relative to WT HA104 recipients of WT BM and TS1 $T_{EM.}$ (G-I) PD-L1/2$^{-/-}$ BM with or without TS1 T_{EM} were

transferred into irradiated HA104 PD-L1/2$^{-/-}$ hosts. Thirty-eight days post-transplant, mice were anesthetized and underwent echocardiography. Weights are shown in (G). ECG tracing for one BM ctrl and one TS1 T_{EM} recipient is shown in (H). The ECG tracing for the TS1 T_{EM} recipient was representative of two out of five mice. Heart rate, cardiac output, diastolic volume and systolic volume are shown in (I). Data are from one experiment.

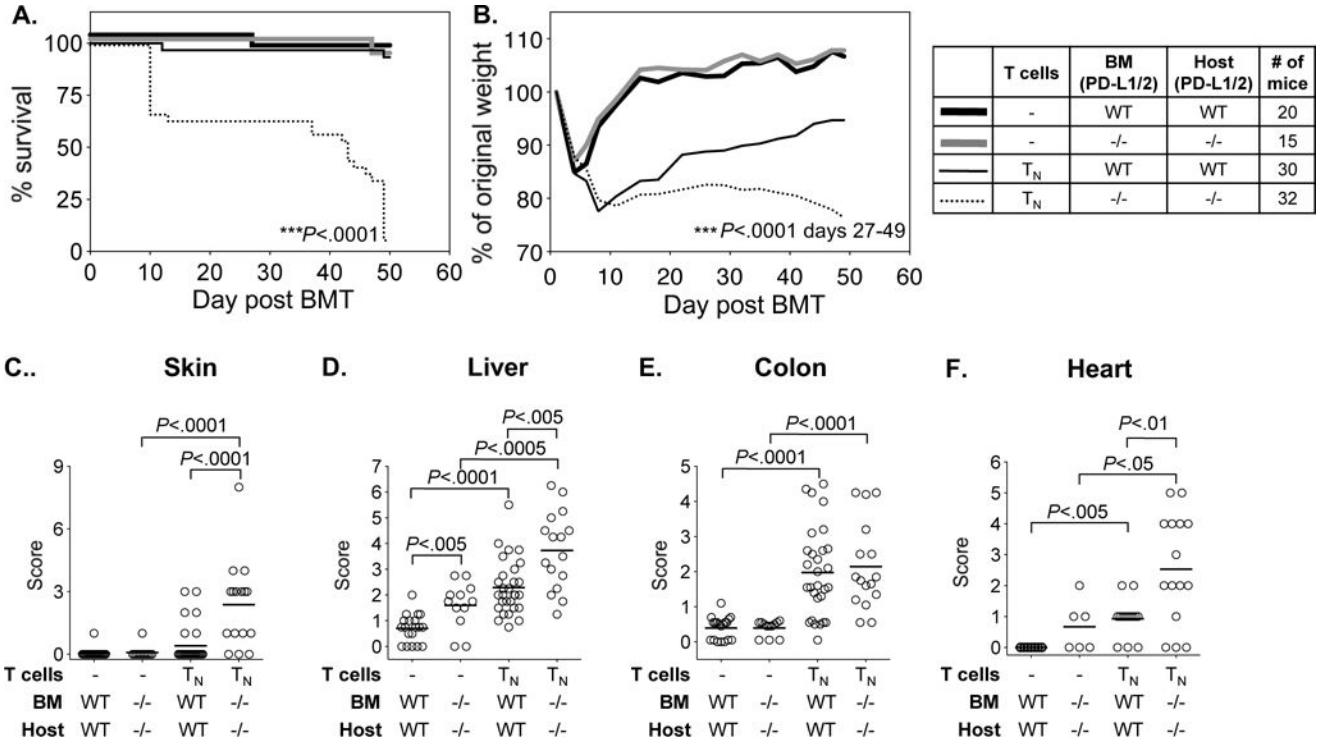


Figure 2. PD-L1/2 limit TS1 T_N-mediated disease in the skin, liver and heart but not the colon

(A–F) WT or PD-L1/2$^{-/-}$ BM was transferred into irradiated WT or PD-L1/2$^{-/-}$ HA104 mice, respectively, with or without 1000 TS1 T_N. Shown are survival (A), weight loss (B) and pathology scores for the skin (C), liver (D), colon (E) and heart (F). Data from (A-E) were combined from 3 independent experiments. Data from (F) were combined from two additional independent experiments, for which survival and weight curves are shown in Figure 3 (A–B). *P* values for survival and weight curves are relative to WT HA104 recipients of WT BM and TS1 T_N.

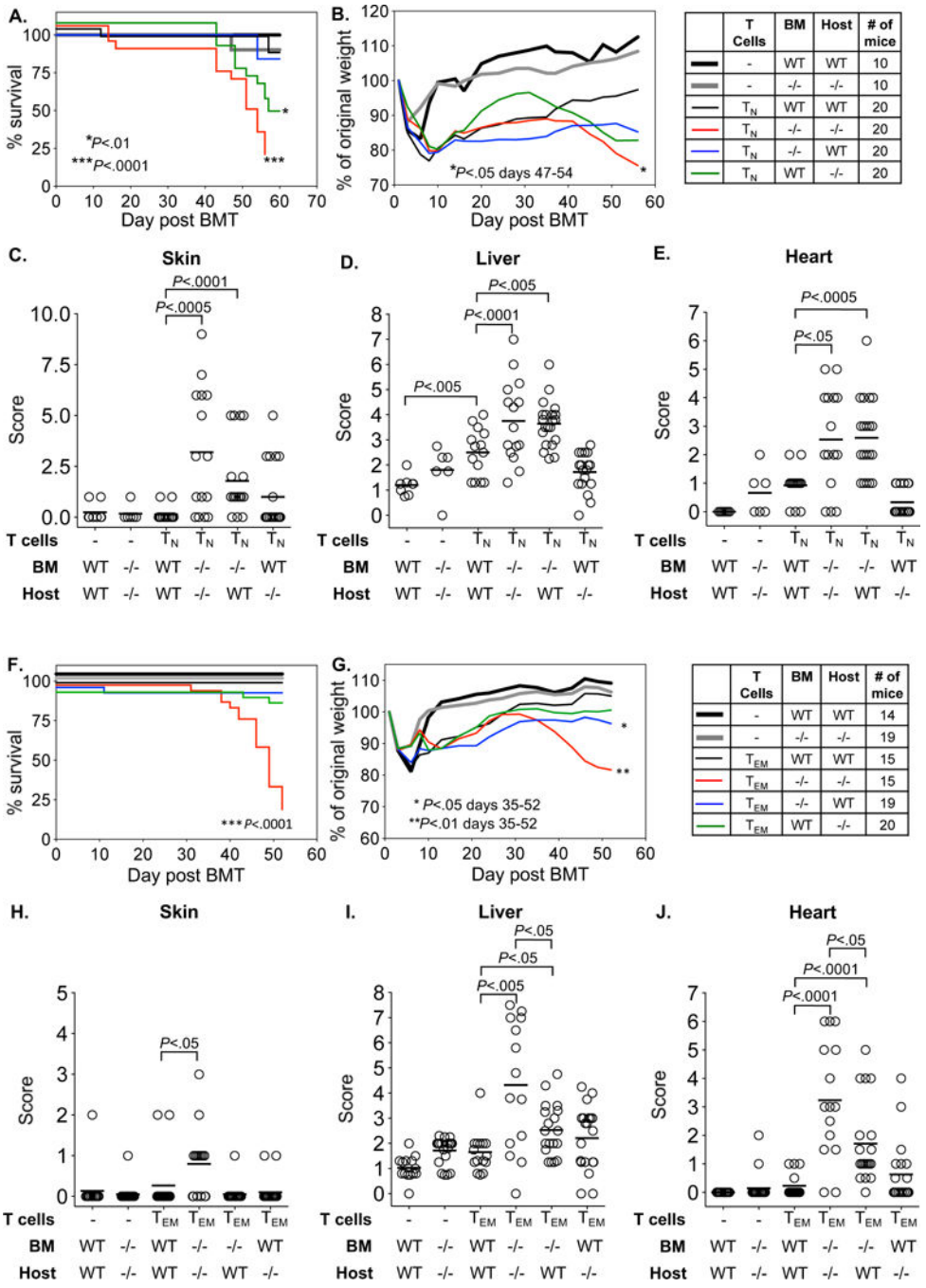


Figure 3. Role of PD-L1/2 on donor BM and host cells

(A–E) TS1 T_N and WT BM were transferred into irradiated PD-L1/2$^{-/-}$ HA104 hosts and TS1 T_N and PD-L1/2$^{-/-}$ BM were transplanted into irradiated WT HA104 hosts. As controls, WT BM into WT HA104 hosts and PD-L1/2$^{-/-}$ BM into PD-L1/2$^{-/-}$ HA104 hosts, with or without TS1 T_N, were also generated. Survival is shown in (A), weight loss in (B) and pathology scores for skin, liver and heart are shown in (C), (D) and (E), respectively. Data for Figure 2F and Figure 3E were generated in the same experiment (2 repetitions, data combined) in order to reduce mouse numbers. Data in Figure 3E from groups in which

donor and recipient were both PD-L1/2$^{+/+}$ or PD-L1/2$^{-/-}$ are therefore the same as in Figure 2F. (F-J) Mice were transplanted as in (A-E), except T_N were replaced with T_{EM}. Survival is shown in (F), weight loss in (G) and pathology scores for skin, liver and heart are shown in (H), (I), and (J), respectively. T_N and T_{EM} results were each combined from two independent experiments. *P* values for survival and weight curves are relative to HA104 WT recipients of WT BM and TS1 T_{EM} or T_N.

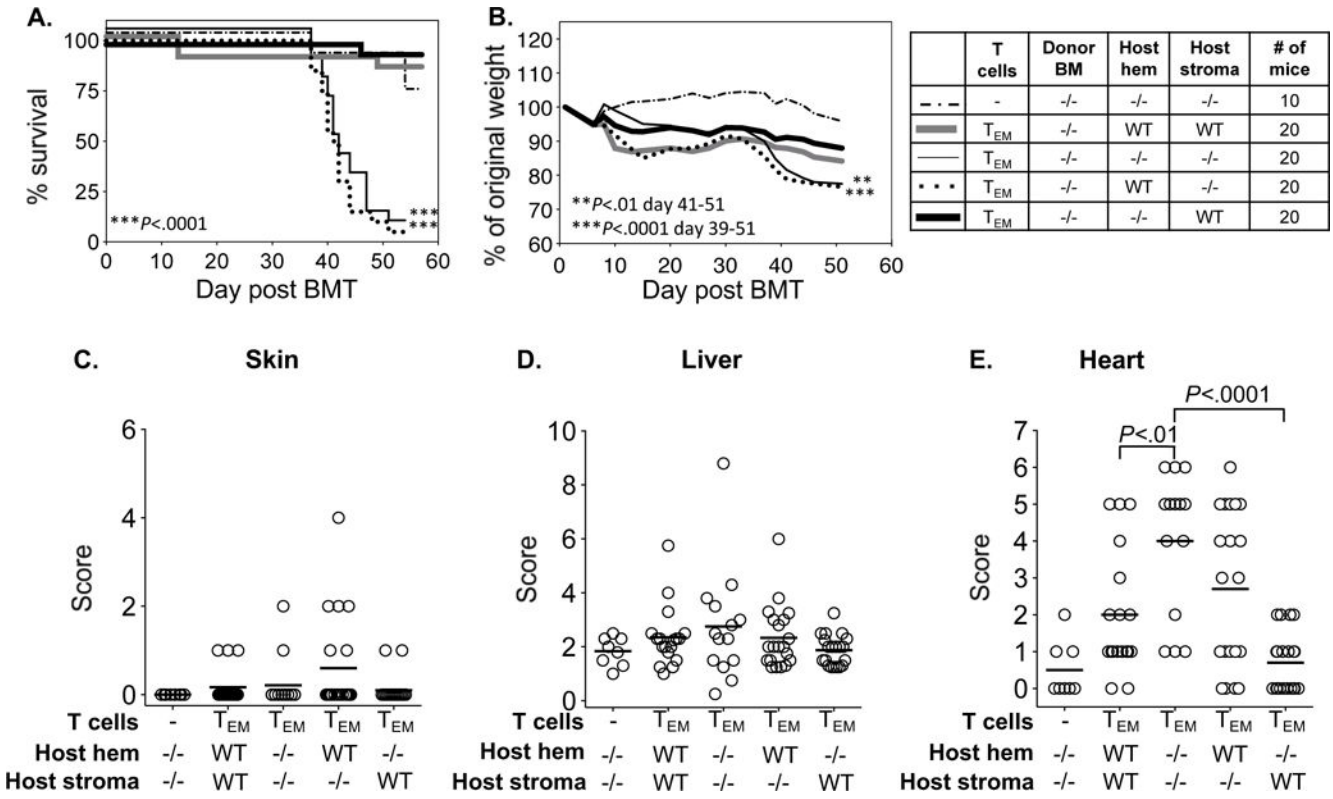


Figure 4. PD-1/2 expression on host stromal cells protects the heart from TS1 T_{EM}-induced disease

(A–E) The following BM chimeras were generated with WT HA104 and PD-L1/2$^{-/-}$ HA104 mice: (PD-L1/2$^{-/-}$ → WT), (WT → PD-L1/2$^{-/-}$), (PD-L1/2$^{-/-}$ → PD-L1/2$^{-/-}$), (WT → WT). 8 weeks later, chimeras were re-irradiated and transplanted with PD-L1/2$^{-/-}$ BM and 1000 TS1 T_{EM}. Shown are survival (A), weight loss (B) and pathology for the skin (C), liver (D) and heart (E). Data were combined from two independent experiments. For weight and survival curves, *P* values are relative to WT → WT recipients of PD-L1/2$^{-/-}$ BM and TS1 T_{EM}.

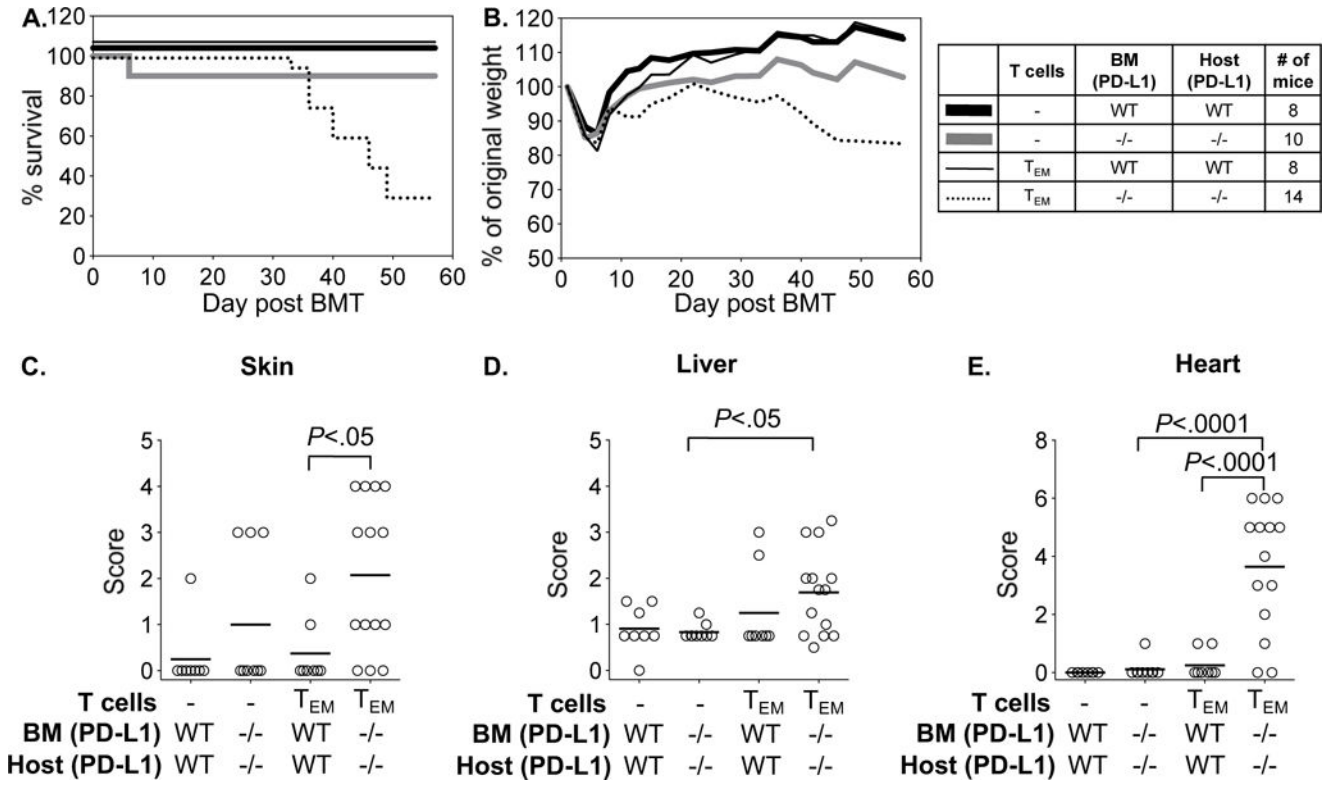


Figure 5. The absence of only PD-L1 is sufficient for the development of TS1 T_{EM}-induced heart disease

WT or PD-L1$^{-/-}$ BM alone or in combination with TS1 T_{EM} were transferred into irradiated HA104 WT or HA104 PD-L1$^{-/-}$ hosts, respectively. Shown are survival (A), weight loss (B) and pathology scores for the skin (C), liver (D) and heart (E). Data were combined from 2 independent experiments. *P* values for weight and survival curves are relative to HA104 recipients of WT BM and TS1 T_{EM}.

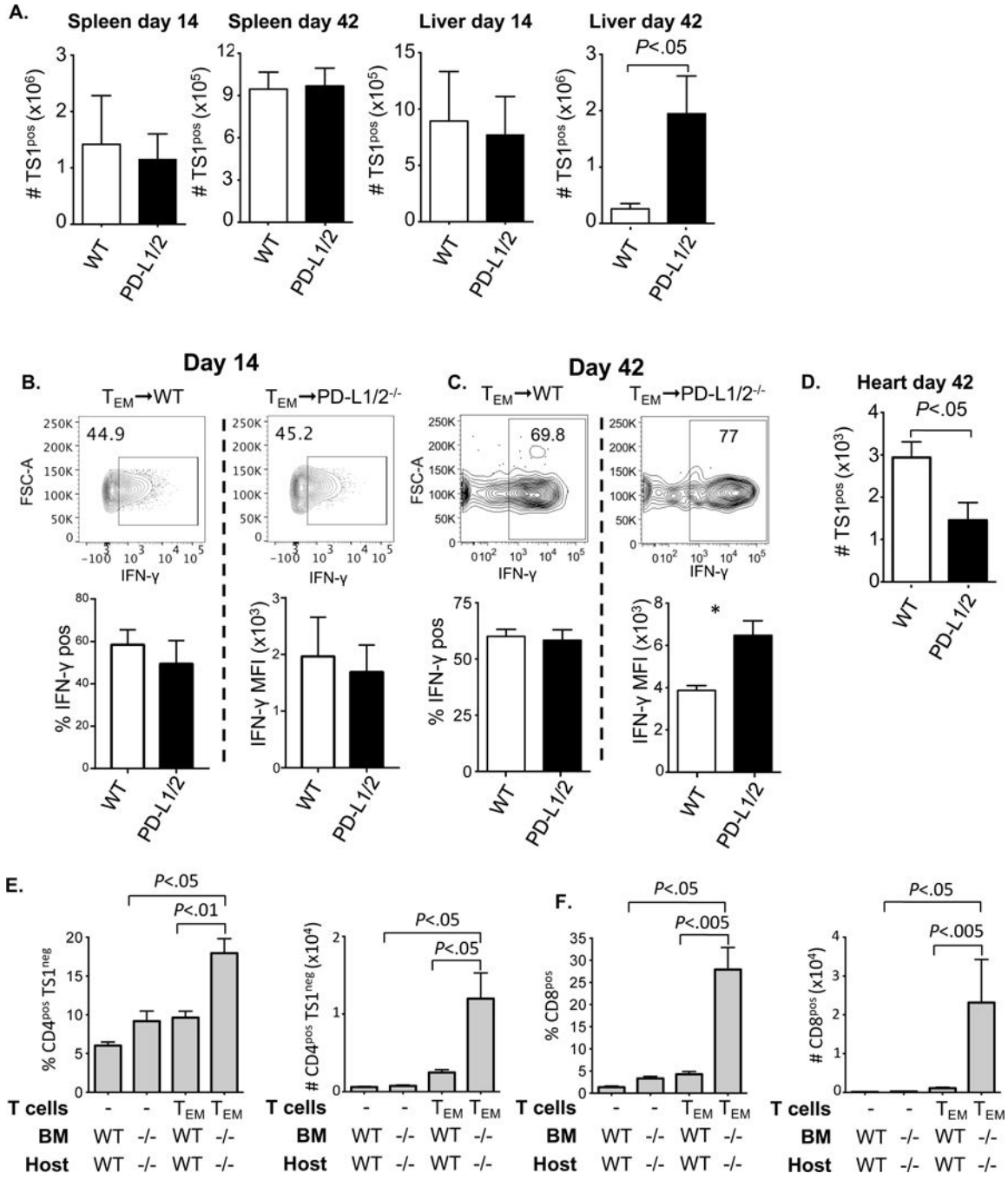
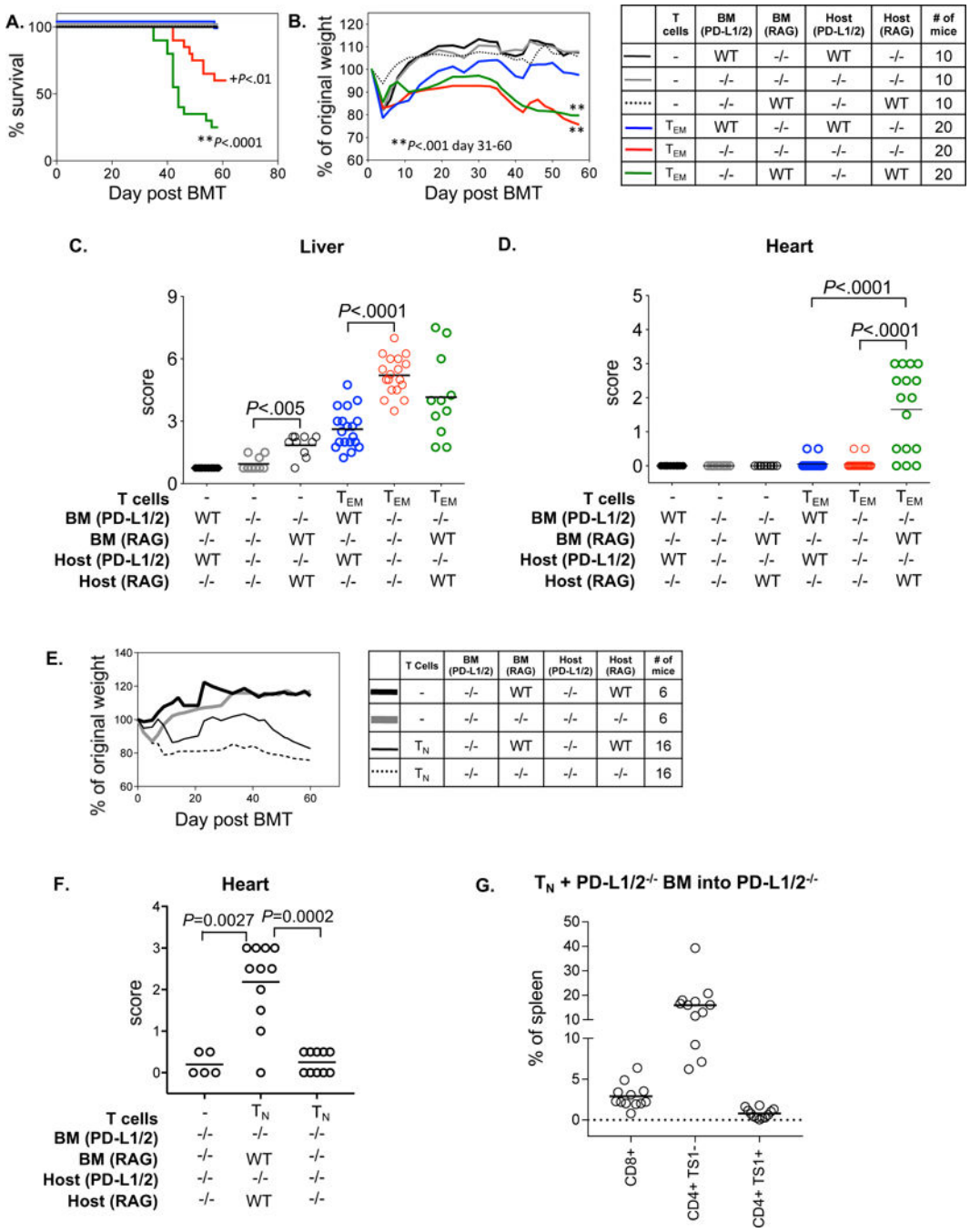


Figure 6. Characterization of TS1 and polyclonal T cells in the absence of PD-L1/2

(A–D) Irradiated WT or HA104 mice were reconstituted with WT or PD-L1/2$^{-/-}$ BM, respectively, with no T cells or with 1000 TS1 T_{EM}. (A) Number of TS1 cells in spleen and liver on days +14 and +42 and the numbers of TS1 from heart on day +42 (D). Data were combined from two independent experiments for a total of 8-12 mice per group. The percentages of splenic TS1 cells on days +14 and +42 that were IFN-γ^+ and the IFN-γ MFI are shown in (B) and (C). Data were combined from two independent experiments for a total of 19 mice per group for day +42 and from one experiment with 5 mice per group for day

+14. (E-F) The number of non-TS1 $CD4^+$ T cells and $CD8^+$ T cells per heart are shown. Data are representative of two independent experiments, each with 3-4 mice per group.



	T cells	BM (PD-L1/2)	BM (RAG)	Host (PD-L1/2)	Host (RAG)	# of mice
	-	WT	-/-	WT	-/-	10
	-	-/-	-/-	-/-	-/-	10
	-	-/-	WT	-/-	WT	10
	T_{EM}	WT	-/-	WT	-/-	20
	T_{EM}	-/-	-/-	-/-	-/-	20
	T_{EM}	-/-	WT	-/-	WT	20

	T Cells	BM (PD-L1/2)	BM (RAG)	Host (PD-L1/2)	Host (RAG)	# of mice
	-	-/-	WT	-/-	WT	6
	-	-/-	-/-	-/-	-/-	6
	T_N	-/-	WT	-/-	WT	16
	T_N	-/-	-/-	-/-	-/-	16

Figure 7. Polyclonal non-TS1 T cells are required for TS1-induced heart disease

(A–F) PD-L1/2$^{-/-}$ HA104 or PD-L1/2$^{-/-}$ RAG2$^{-/-}$ (triple KO; TKO) HA104 mice were transplanted with PD-L1/2$^{-/-}$ or TKO BM (respectively) with no T cells or with 1000 TS1 T_{EM} (A-D) or 300 TS1 T_N (E-F). The TS1 T_{EM} experiments had an additional group wherein HA104 RAG2$^{-/-}$ mice were transplanted with RAG2$^{-/-}$ BM with no T cells or with TS1 T_{EM}. Shown are survival (A), weight loss (B) and pathology scores for the liver (C), and heart (D) from the TS1 T_{EM} experiments. *P* values for weight and survival curves are relative to RAG2$^{-/-}$ HA104 recipients of TS1 T_{EM} and RAG2$^{-/-}$ BM and are significant

from day +31 onward. Data were combined from 2 independent experiments. Weight loss and heart pathology scores for the TS1 T_N experiment are shown in (E) and (F), respectively. Percentages of $CD8^+$, $CD4^+$ $TS1^-$ and $CD4^+$ $TS1^+$ cells in the spleen when mice were sacrificed for pathology are shown in (G). $P<0.05$ comparing weights of either TS1 group to its BM alone control from days 9-60 post BMT. TKO TS1 recipients had significantly more weight loss ($P<0.05$) than PD-L1/2$^{-/-}$ TS1 recipients on days 2, 7-9, 12-21, and 23-58. Data are from one experiment.

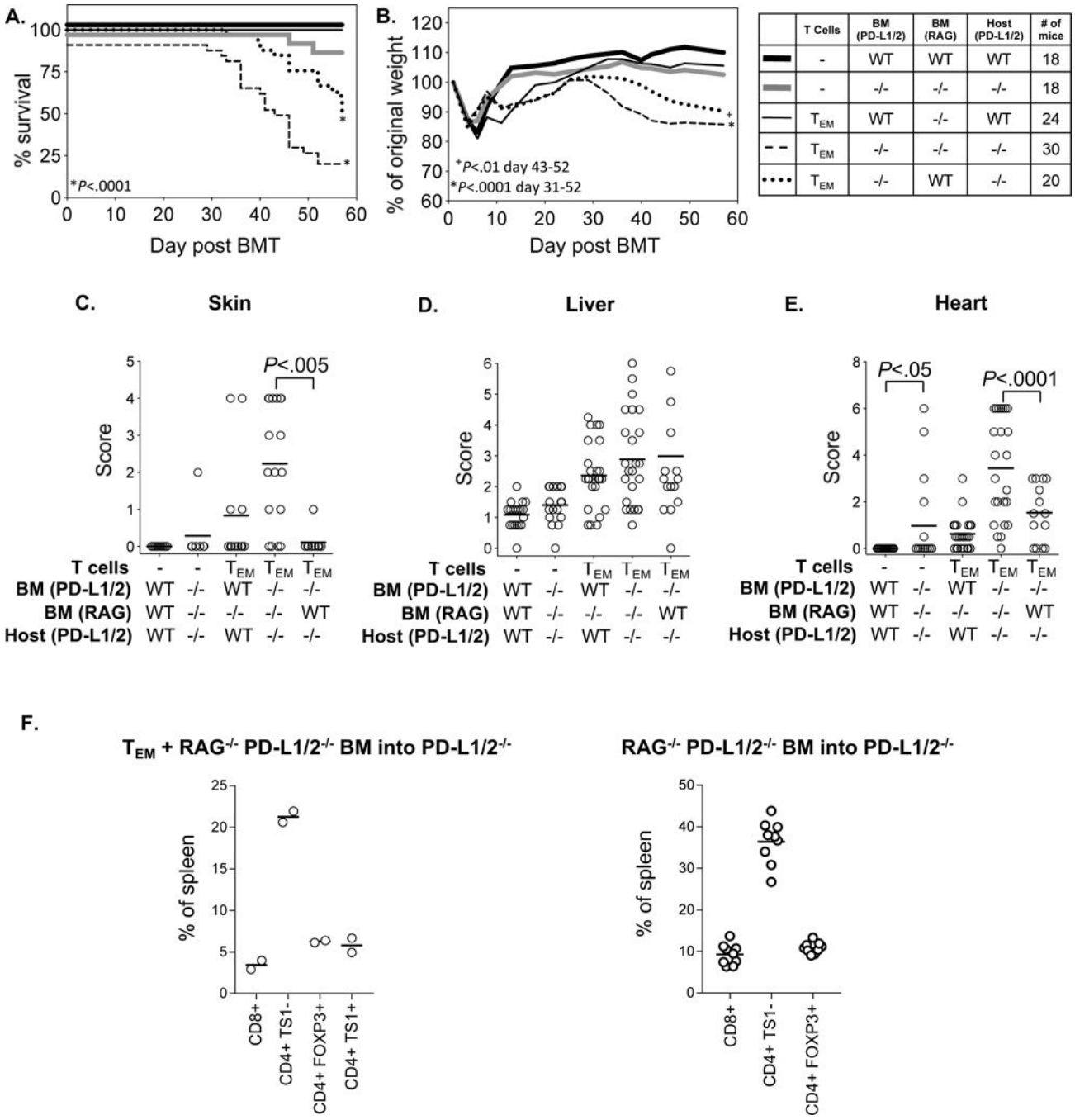


Figure 8. Polyclonal recipient T cells are sufficient for TS1 T_{EM} induced myocarditis

Irradiated PD-L1/L2$^{-/-}$ HA104 mice were reconstituted with 1000 TS1 T_{EM} and RAG2$^{-/-}$ PD-L1/2$^{-/-}$ BM or RAG2$^{+/+}$ PD-L1/2$^{-/-}$ BM. The following control groups were also transplanted: 1) HA104 mice reconstituted with BALB/c BM and no T cells; 2) PD-L1/2$^{-/-}$ HA104 mice reconstituted with PD-L1/2$^{-/-}$RAG2$^{-/-}$ BM and no T cells; and 3) WT HA104 mice reconstituted with RAG2$^{-/-}$ BM and 1000 TS1 T_{EM}. Shown are survival (A), weight loss (B) and pathology scores for the skin (C), liver (D) and heart (E). *P* values for weight and survival curves are relative to WT HA104 recipients of TS1 T_{EM} and RAG$^{-/-}$ BM.

Percentages of $CD8^+$, $CD4^+$ $TS1^-$ and $CD4^+$ $FOXP3^+$ $TS1^-$ and $CD4^+$ $TS1^+$ cells in the spleen at day 60 post-transplant for two PD-L1/2$^{-/-}$ recipients of TKO BM with and without TS1 T_{EM} are shown in (F). Data for (A-E) were combined from two independent experiments. Data in (F) were from one experiment.

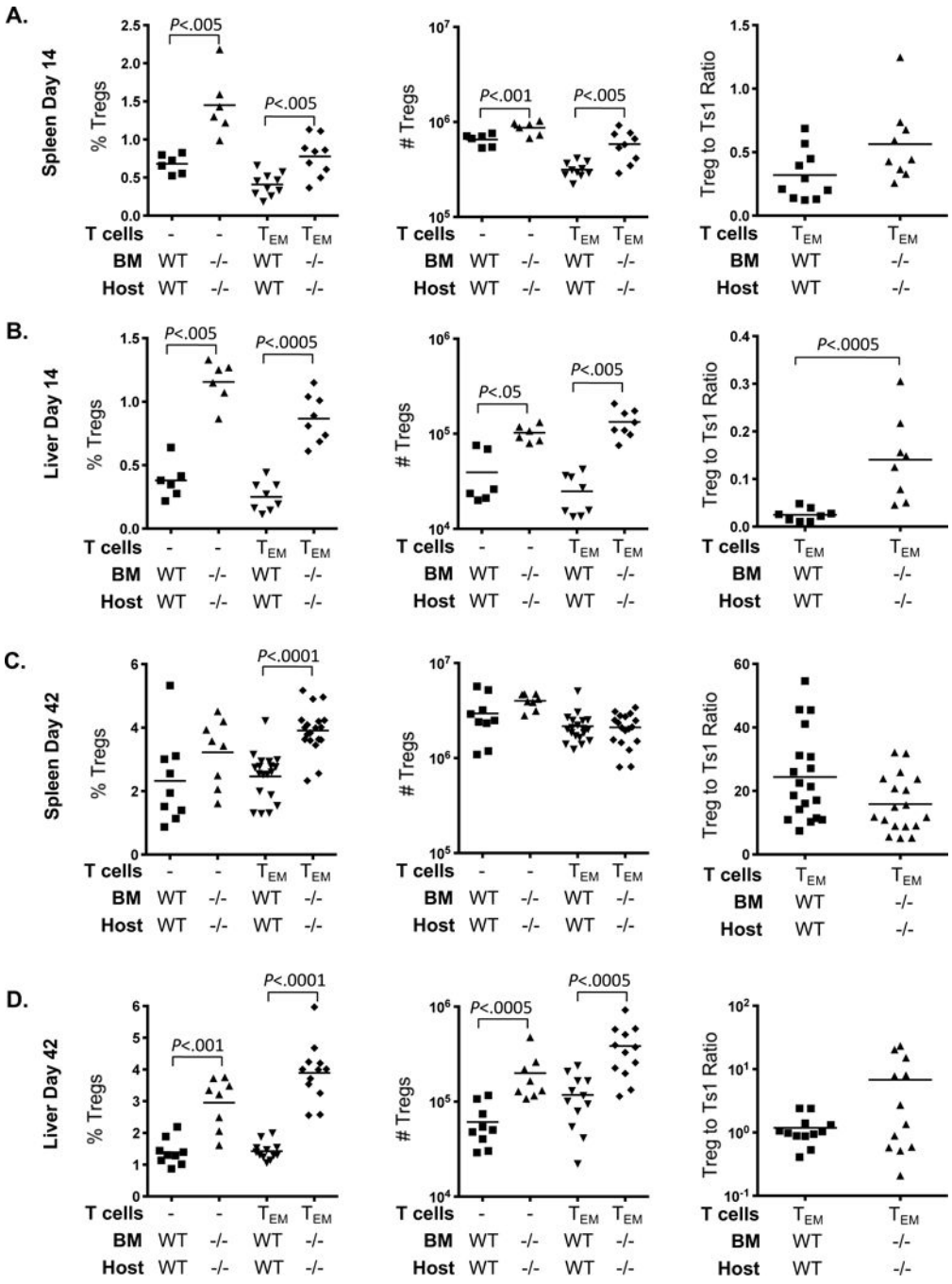
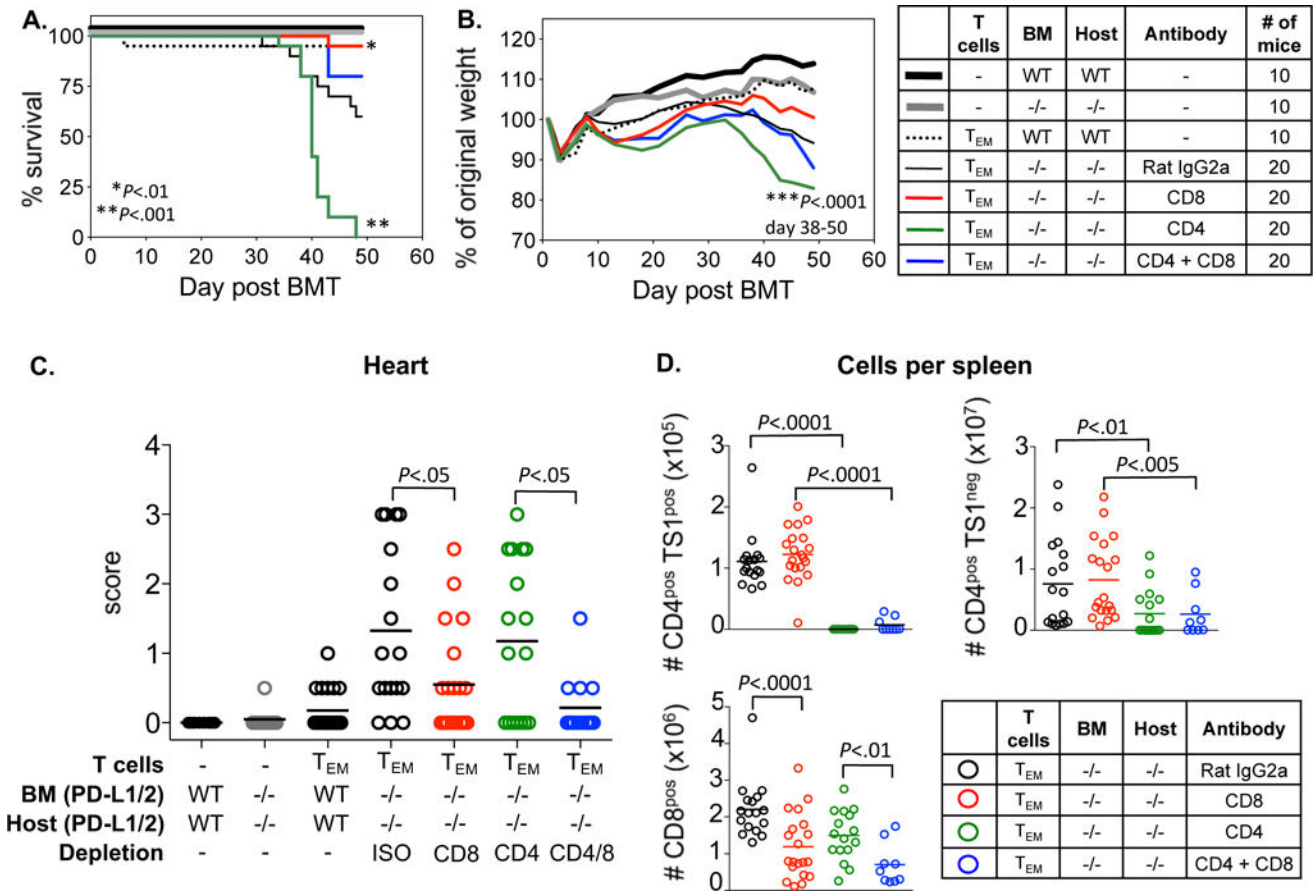


Figure 9. The absence of PD-ligands does not diminish Treg reconstitution

Irradiated WT or HA104 mice were reconstituted with WT or PD-L1/2$^{-/-}$ BM, respectively, with or without 1000 TS1 T_{EM}. Percentages and numbers of Tregs (CD4^{+} FOXP3^{+}) as well as Treg to TS1^{+} T cell ratios in the spleen and liver on day 14 and in the spleen and liver on day 42 are shown in A, B, C and D, respectively. Day 14 data were combined from two independent experiments and day 42 data were combined from 3 independent experiments. TS1 cell numbers from these experiments are shown in Figure 6A.



	T cells	BM	Host	Antibody	# of mice
	-	WT	WT	-	10
	-	-/-	-/-	-	10
	T_{EM}	WT	WT	-	10
	T_{EM}	-/-	-/-	Rat IgG2a	20
	T_{EM}	-/-	-/-	CD8	20
	T_{EM}	-/-	-/-	CD4	20
	T_{EM}	-/-	-/-	CD4 + CD8	20

	T cells	BM	Host	Antibody
	T_{EM}	-/-	-/-	Rat IgG2a
	T_{EM}	-/-	-/-	CD8
	T_{EM}	-/-	-/-	CD4
	T_{EM}	-/-	-/-	CD4 + CD8

Figure 10. In the absence of PD-L1/2, $CD8^+$ T cells are required for TS1-induced heart disease

WT or PD-$L1/2^{-/-}$ BM, with or without TS1 T_{EM}, were transferred into WT HA104 or PD-$L1/2^{-/-}$ HA104 hosts, respectively. PD-$L1/2^{-/-}$ TS1 recipients were given isotype control, anti-CD8, anti-CD4 or both anti-CD4 and anti-CD8 antibodies as follows: a) Isotype control and anti-CD8 were given on days −6, −4, 6, 13, 20, 27, 34 and 41; and b) anti-CD4 was given on days 27, 31, 35, 39, 42 and 46. Shown are survival (A), weight loss (B), and heart pathology (C). Number of splenocytes that were $CD4^+$ $TS1^-$, $TS1^+$, or $CD8^+$ are shown in (D). Data were combined from two independent experiments. For weight and survival curves *P* values are relative to isotype control recipients.