ORIGINAL RESEARCH

Trametinib Attenuates Delayed Rejection and Preserves Thymic Function in Rat Lung Transplantation

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Abstract

Delayed immunological rejection after human lung transplantation causes chronic lung allograft dysfunction, which is associated with high mortality. Delayed rejection may be attributable to indirect alloantigen presentation by host antigen-presenting cells; however, its pathophysiology is not fully understood. The mitogen-activated protein kinase pathway is activated in T cells upon stimulation, and we previously showed that the MEK inhibitor, trametinib, suppresses graft-versus-host disease after murine bone marrow transplantation. We investigated whether trametinib suppresses graft rejection after two types of rat lung transplantation and analyzed its immunological mode of action. Major histocompatibility complex–mismatched transplantation from brown Norway rats into Lewis rats and minor histocompatibility antigen–mismatched transplantation from Fischer 344 rats into Lewis rats were performed. Cyclosporine (CsA) and/or trametinib were administered alone or consecutively. Acute and delayed rejection, lymphocyte infiltration, and pulmonary function were evaluated. Administration of trametinib after CsA suppressed delayed rejection, reduced inflammatory cell infiltration and fibrosis within the graft, and preserved pulmonary functions at Day 28. Trametinib suppressed functional differentiation of T and B cells in the periphery but preserved thymic T cell differentiation. Donor B cells within the graft disappeared by Day 14, indicating that delayed graft rejection at Day 28 was mainly due to indirect presentation by host antigen-presenting cells. Finally, trametinib administration without CsA preconditioning suppressed rejection after minor histocompatibility antigen–mismatched transplantation. Trametinib attenuates delayed rejection upon major histocompatibility complex–mismatched transplantation by suppressing indirect presentation and is a promising candidate to treat chronic lung allograft dysfunction in humans.

Keywords: MEK inhibitor; lung transplantation; delayed rejection; thymic function; chronic rejection

Clinical Relevance

Chronic lung allograft dysfunction (CLAD) causes high mortality in human lung transplantation. The MEK inhibitor trametinib attenuates delayed immunological rejection after major histocompatibility complex–mismatched rat lung transplantation, providing a promising candidate to treat CLAD.

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The number of lung transplantations has increased since this procedure was first successfully performed by Cooper in 1983 (1). However, the postoperative 5-year survival rate is only 54%, which is unsatisfactory compared with that of other types of solid organ transplantation (2). The primary causes of mortality are infection and immunological rejection, the latter of which is classified into acute and chronic types (2–4). Acute rejection is generally controlled by currently available immunosuppressants, such as calcineurin inhibitors and corticosteroids (5, 6). However, chronic rejection is often therapy resistant and irreversible, resulting in chronic lung allograft dysfunction (CLAD) (3).

CLAD causes fatal pulmonary dysfunction, and long-term use of multiple immunosuppressants increases the risks of serious opportunistic infections and secondary malignancies (7). Several immunological studies indicate that direct alloantigen presentation by donor antigen-presenting cells (APCs) to recipient T cells causes acute rejection (8, 9). On the other hand, chronic rejection and CLAD may be attributable to indirect alloantigen presentation by recipient APCs to recipient T cells after donor APCs disappear from the graft (10–12). Therefore, a novel immunomodulatory strategy that inhibits indirect alloantigen presentation without abrogating antimicrobial or antitumor immunity might suppress human CLAD. Unfortunately, there is no such candidate strategy.

The RAS/MEK/ERK pathway is activated in many cancers, and several MEK inhibitors, such as trametinib and cobimetinib, display clinical efficacy, and are approved for the clinical treatment of malignant melanoma in combination with BRAF inhibitors (13, 14). Notably, this pathway is downstream of T cell receptor signaling (15–18). We recently showed that pharmacological inhibition of MEK selectively suppresses graft-versus-host disease (GVHD) without abrogating antiviral or antitumor immunity after murine bone marrow transplantation (17, 18). MEK inhibition may also suppress graft rejection after solid organ transplantation; however, several topics should be investigated. In contrast with bone marrow transplantation, host immunity is not ablated and immunological tolerance is unlikely after lung transplantation (19). The thymus is not ablated, and it is unknown how MEK inhibition modulates thymic function upon solid organ transplantation.

We previously established an orthotopic left lung transplantation model in rats (20). Here, we used this model to investigate whether trametinib suppresses acute or delayed graft rejection. In addition, we analyzed the effects of trametinib on thymic function in a transplant setting. Although the roles of trametinib in lung transplantation differ from those in hematopoietic stem cell transplantation, we believe that trametinib is a novel and promising candidate to reduce chronic rejection and CLAD-related mortality in humans.

Methods

Animals

Lewis (LEW; $RT1^{l}$), brown Norway (BN; $RT1^{n}$), and Fischer 344 (F344; $RT1^{lv1}$) rats, aged 10–12 weeks, were purchased from Japan SLC. All rats were inbred, weighed 250–300 g, and were housed in a specific pathogen-free barrier facility. All the animal protocols were approved by the Institutional Animal Care and Use Committee of Kyoto University (Medkyo 16180).

Orthotopic Lung Transplantation

Recipients were LEW rats in all transplantations. The lungs of BN rats were implanted into LEW rats orthotopically for major histocompatibility complex (MHC)–mismatched transplantation, the lungs of F344 rats were implanted into LEW rats for minor histocompatibility antigen–mismatched transplantation, and the lungs of LEW rats were implanted into LEW rats for syngeneic transplantation. Orthotopic lung transplantation was performed as previously reported (20), and is described in the data supplement.

Treatment Protocols

Trametinib and cyclosporine (CsA) were purchased from MedChemExpress and Novartis Pharmaceuticals, respectively. Trametinib was reconstituted in DMSO (Wako Pure Chemical Industries), stored at −20°C, suspended in 0.5% methylcellulose (Sigma-Aldrich), and orally administered on 5 d/wk (17, 18, 21). Rats were subcutaneously administered 25 mg/kg CsA on 4 days in the first week and on 2 d/wk thereafter (20). After allogeneic transplantation, rats were treated with a single reagent for 28 days or switched to another reagent at Day 14. Specifically, rats were treated with DMSO (DMSO/DMSO), CsA (CsA/CsA), or 0.3 mg/kg trametinib (Tra0.3/Tra0.3) alone, or switched from CsA to DMSO (CsA/DMSO), 0.1 mg/kg trametinib (CsA/Tra0.1), or 0.3 mg/kg trametinib (CsA/Tra0.3). The doses of trametinib were determined based on previous studies that assessed its effects on murine bone marrow transplantation (17) and rat arthritis (22). These treatment protocols are summarized in Figure 1A. After minor antigen–mismatched transplantation from F344 rats into LEW rats, recipients were administered DMSO, CsA, or 0.3 mg/kg trametinib from Day 0 to Day 14 (minor-DMSO, minor-CsA, and minor-Tra0.3, respectively).

Histopathological Evaluation

The graft, spleen, and thymus were harvested, preserved with formalin, embedded in paraffin, and subjected to hematoxylin and eosin and Masson's trichrome staining. Histological evaluations were performed according to the criteria for acute lung rejection stipulated by the International Society for Heart and Lung Transplantation. The A grade is characterized by infiltration of perivascular mononuclear cells, whereas the B grade is characterized by lymphocytic bronchitis/bronchiolitis (23). Evaluations were performed by two pathologists and a chest surgeon in a blinded manner described in the data supplement.

Statistical Analysis

Continuous variables with a normal distribution were plotted as means (±SD). Other continuous variables were plotted as medians (ranges). Categorical variables were summarized as counts and proportions. Continuous variables were compared using the Student's *t* test or the Wilcoxon rank-sum test. Significant differences between multiple groups were determined by a one-way ANOVA. All *P* values are two-sided. A *P* value less than 0.05 was considered statistically significant. Statistical analyses were performed using JMP 12 software (SAS Institute) and GraphPad Prism 7 (GraphPad Software, Inc.).

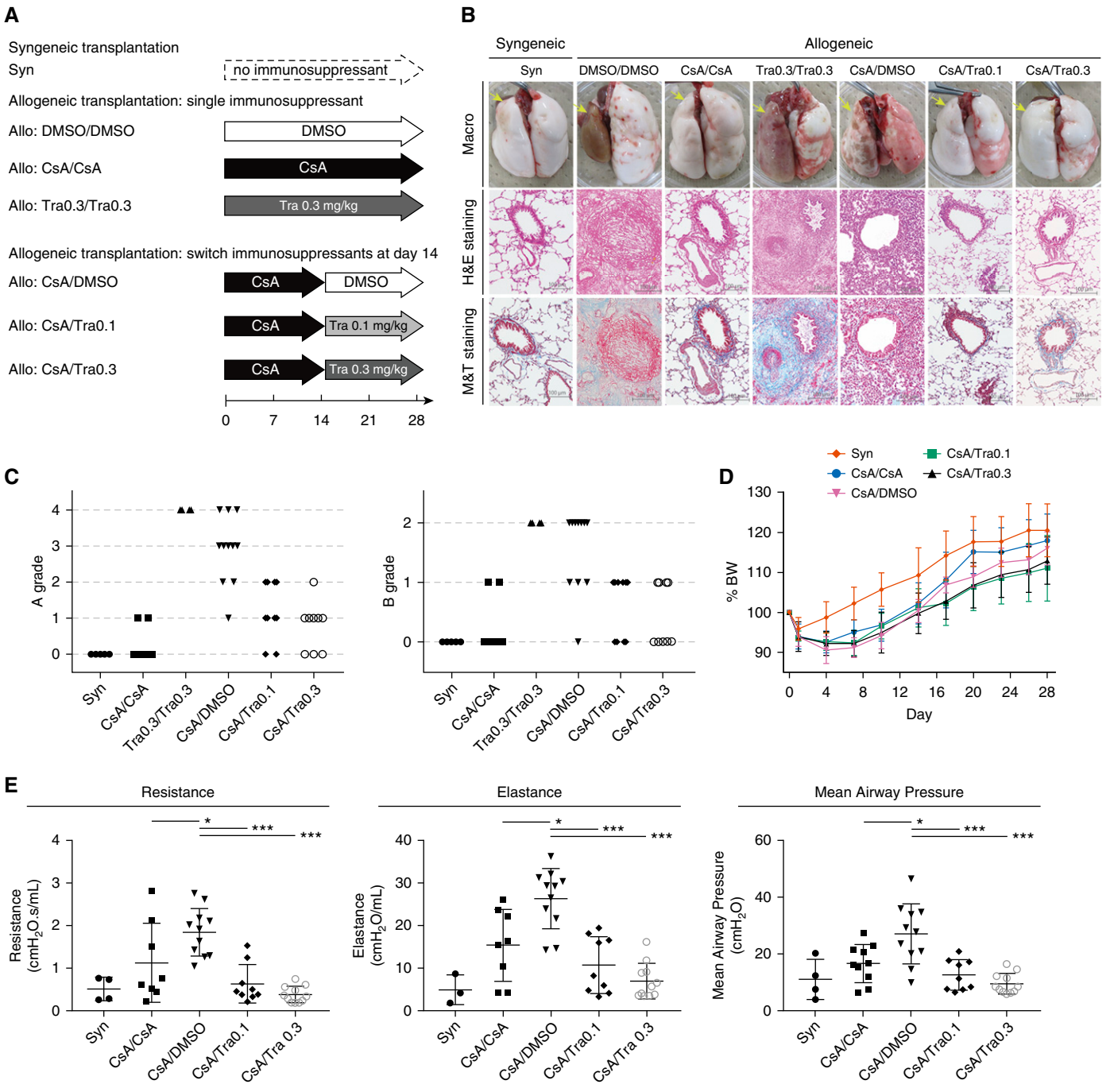

Figure 1. Trametinib attenuates delayed rejection after rat lung transplantation. Major histocompatibility complex (MHC)–mismatched orthotopic left lung transplantation was performed from brown Norway (BN) rats into Lewis (LEW) rats. Syngeneic lung transplantation from LEW rats into LEW rats was performed without immunosuppressants (syn; $n = 5$). (*A*) Treatment intervention protocols from Day 0 to Day 28 are summarized. In the single-immunosuppressant groups, DMSO, cyclosporine (CsA), or 0.3 mg/kg trametinib was administered from Day 0 to Day 28 (DMSO [DMSO/DMSO], $n = 3$; CsA [CsA/CsA], $n = 9$; and 0.3 mg/kg trametinib [Tra0.3/Tra0.3], $n = 5$). In the switch groups, CsA was administered from Day 0 to Day 14 and then 0.1 or 0.3 mg/kg trametinib or DMSO was administered from Day 15 to Day 28 (switched from CsA to DMSO [CsA/DMSO], $n = 5$; switched from CsA to trametinib [CsA/Tra0.1], $n = 10$; and switched from CsA to 0.3 mg/kg trametinib [CsA/Tra0.3], $n = 11$). (*B*) Macroscopic (arrows denote grafts) and microscopic (hematoxylin and eosin [H&E] and Masson's trichrome [M&T] staining) images of the lungs at Day 28. Scale bars: 100 μm. (*C*) Scores of A/B grades of acute rejection according to the criteria stipulated by the International Society for Heart and Lung Transplantation in each group. (*D* and *E*) Sequentially measured postoperative body weight (BW) as a percentage of preoperative BW (*D*) and pulmonary resistance, elastance, and mean airway pressure (*E*) (Wilcoxon chi-square test). $^{*}P < 0.05$ and $^{***}P < 0.001$. Allo = allogeneic; syn = syngeneic; tra = trametinib.

Results

Trametinib Attenuates Delayed Rejection after Rat Lung Transplantation

Syngeneic and allogeneic transplanted lungs treated with CsA/CsA for 28 days had a normal appearance without inflammatory cell infiltration (Figure 1B). However, allogeneic transplanted lungs treated with DMSO/DMSO or Tra0.3/Tra0.3 for 28 days had a reddish appearance, exhibited a decreased volume, and displayed inflammatory cell infiltration and fibrosis (Figure 1B). Next, recipients were initially treated with CsA and then switched to DMSO or 0.1 or 0.3 mg/kg trametinib on Day 14. CsA treatment for the first 14 days inhibited acute rejection at Day 14 (group c in Figures EA and EB in the data supplement); however, withdrawal of CsA resulted in rejection at Day 28 (CsA/DMSO in Figure 1B and Figures EA and EB), as indicated by bloody spots on the pleura, volume loss, and inflammatory cell infiltration. However, treatment with trametinib from Day 14 to Day 28 attenuated these effects in a dose-dependent manner (CsA/Tra0.1 and CsA/Tra0.3 in Figures 1B and 1C). Masson's trichrome staining indicated that fibrosis occurred around the bronchus and pleura in the DMSO/DMSO, Tra0.3/Tra0.3, and CsA/DMSO groups, but not in the CsA/CsA, CsA/Tra0.1, or CsA/Tra0.3 groups (Figure 1B). Histopathological scoring of rejection was performed. Recipients had lower scores of A and B rejection grades in the CsA/Tra0.1 and CsA/Tra0.3 groups than in the CsA/DMSO group (Figure 1C; Wilcoxon rank-sum test, both $P < 0.001$). Postoperative body weight (BW) as a percentage of preoperative BW was approximately equivalent in all the allogeneic transplantation groups (Figure 1D; one-way ANOVA, $P = 0.16$). Pulmonary function tests showed that resistance, elastance, and mean airway pressure were lower in the CsA/Tra0.1 and CsA/Tra0.3 groups than in the CsA/DMSO group (Figure 1E; one-way ANOVA, all $P < 0.001$). These parameters displayed marked inter-individual variation in the CsA/CsA group because some recipients developed pulmonary aspiration and pneumonia. Thus, trametinib does not suppress acute rejection after lung transplantation when delivered alone, but inhibits delayed rejection when administered after CsA.

Trametinib Suppresses Infiltration of T Cells into the Graft and Their Functional Differentiation in the Periphery

Infiltration of T cells into the spleen and graft was evaluated by immunohistochemistry. $CD3^+$ T cells were abundant in the spleen in each group (Figure 2A). These cells were scattered around vessels and bronchioles in the graft, and some infiltrated the alveolar space. This infiltration was obviously less extensive in the CsA/Tra0.3 and CsA/CsA groups than in the CsA/DMSO group (Figure 2A). The number of $Foxp3^+$ T cells was maintained in the trametinib-treated groups, but was slightly reduced in the CsA/CsA group (Figures 2B and 2C; one-way ANOVA, all $P < 0.001$). Flow cytometric analysis revealed that the CD4/8 ratio and the frequency of $CD62L^+$ T cells were lower in the graft than in the spleen and peripheral blood (Figures 2D–2F; one-way ANOVA, all $P < 0.001$). This implies that the spleen and peripheral blood are the sources of naive T cells and that $CD8^+$ effector memory T cells mainly infiltrate the graft. The frequencies of $CD4^+CD62L^+$ T cells in peripheral blood and the graft were higher in the CsA/Tra0.3 group than in the CsA/DMSO group (Figure 2F; one-way ANOVA, $P = 0.058$ [spleen], $P = 0.059$ [peripheral blood mononuclear cells (PBMCs)], $P = 0.001$ [graft]; Student's t test, $P = 0.031$ [PBMCs], $P < 0.001$ [graft]), indicating that trametinib suppresses functional differentiation and tissue infiltration of T cells in the periphery. Trametinib suppressed functional differentiation of $CD4^+$ T cells and infiltration of T cells into the graft, but preserved $Foxp3^+$ cells, which are potential regulatory T cells.

Trametinib Suppresses Activation of B Cells

Next, infiltration of B cells into the spleen and graft was evaluated. $CD79a^+$ cells were abundant in the spleen in each group (Figure 3A). The graft contained many $CD79a^+$ cells in the CsA/DMSO group, and this was suppressed by trametinib treatment (Figures 3A and 3B; one-way ANOVA, $P < 0.001$). Rat B cells can be detected as $CD45RA^+CD3^-$ cells (24, 25). Among them, IgM^+ cells are immature B cells, which reflect activated humoral immunity. Flow cytometric analyses detected a substantial B cell population in the graft in the CsA/DMSO group, but not in the CsA/Tra0.1 or CsA/Tra0.3 group (Figures 3C and 3D; one-way ANOVA, $P = 0.454$ [spleen], $P = 0.159$ [PBMCs], $P < 0.001$ [graft]). Furthermore, the percentages of IgM^+ cells among B cells in the peripheral blood and spleen were lower in the CsA/Tra0.1 and CsA/Tra0.3 groups than in the CsA/CsA group (Figures 3E and 3F; one-way ANOVA, $P < 0.001$ [spleen], $P = 0.013$ [PBMCs], $P = 0.648$ [graft], respectively).

Trametinib Preserves Differentiation of Thymic T Cells

Flow cytometric analyses of thymic lymphocytes at Day 28 revealed that $CD4^+$ cells were depleted in the CsA/CsA group, but preserved in the other groups (Figures 4A and 4B; one-way ANOVA, $P < 0.001$ [$CD4^+$], $P = 0.093$ [$CD8^+$]). In addition, most $CD8^+$ cells were $TCR\alpha/\beta^{low}$ in the CsA/CsA group (Figures 4C and 4D; one-way ANOVA, both $P < 0.001$). Thus, mature T cells in the thymus were depleted upon long-term administration of CsA, but preserved upon trametinib treatment (Figure 4E; one-way ANOVA, both $P < 0.001$). Next, the thymuses of LEW rats that had received lung transplants from BN rats were sequentially evaluated by histology. Treatment with CsA for 14 days resulted in atrophy of the thymic medulla and loss of $AIRE^+$ cells (Figure 4F, a and b). CsA treatment for a further 2 weeks resulted in complete loss of the thymic medulla and $AIRE^+$ cells (Figure 4F, c). This was reversed by withdrawal of CsA and treatment with trametinib (Figure 4F, d–f). Analyses of different subsets of cells in the thymus revealed that ERK1/2 expression was high in $CD4^-CD8^-$ double-negative cells as well as $CD4^+$ and $CD8^+$ single-positive cells, but low in $CD4^+CD8^+$ double-positive cells (Figures 4G and 4H; one-way ANOVA, $P = 0.004$). These results demonstrate that trametinib preserves thymic T cell differentiation from double-positive cells into $CD4^+$ and $CD8^+$ cells.

Donor B Cells Disappear from the Graft by Day 14 after Transplantation

Alloantigen presentation after transplantation begins with direct presentation by donor-derived APCs to

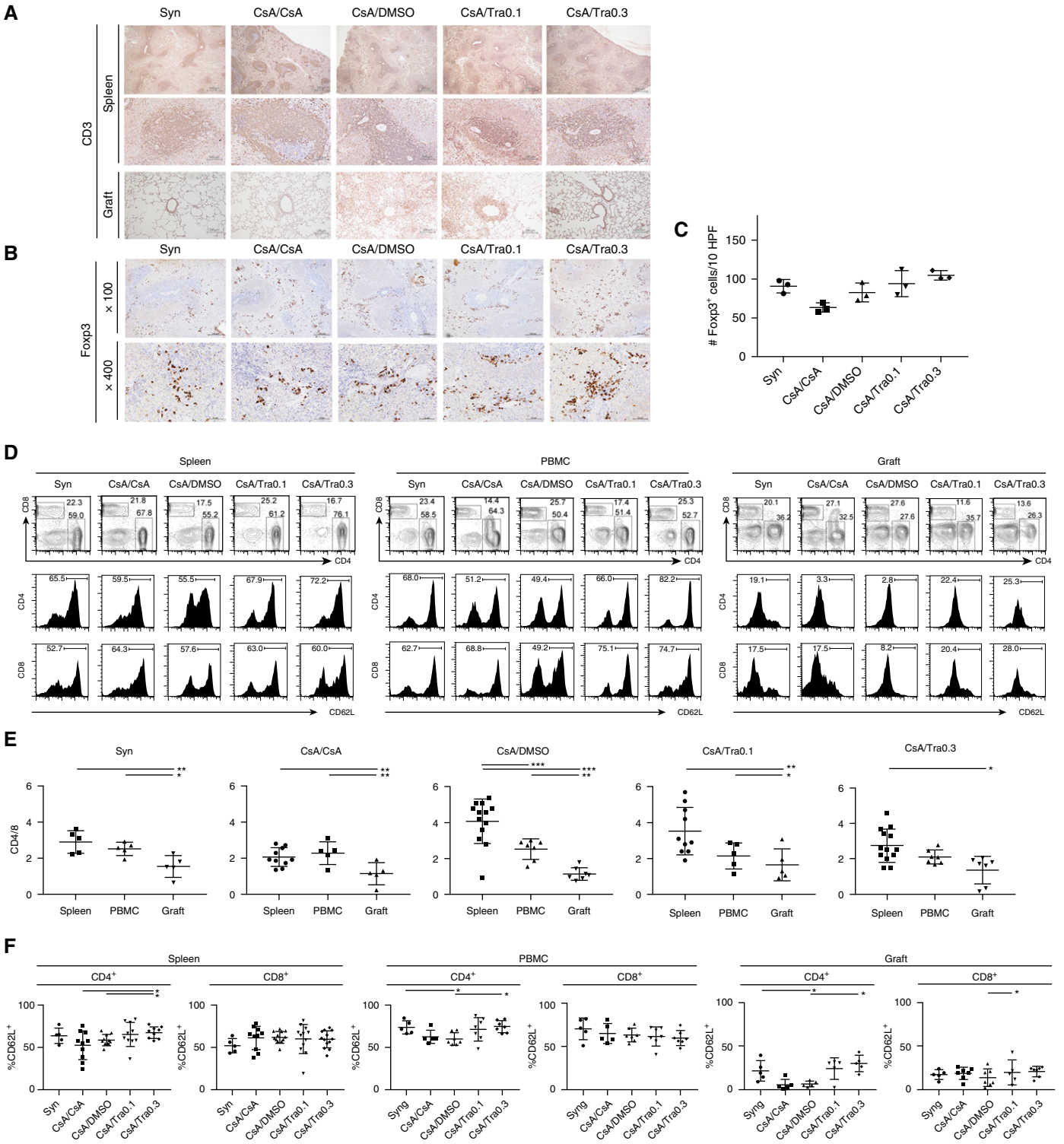

Figure 2. Trametinib suppresses functional differentiation of T cells in the periphery. (*A*) CD3 staining of the spleen and H&E staining of the graft at Day 28. (*B*) Foxp3 staining of the spleen at Day 28. (*A* and *B*) Images are representative of five independent experiments. Scale bars: 50 μm, 100 μm, 200 μm, and 500 μm. (*C*) Numbers of Foxp3$^+$ cells per 10 high-power fields (HPFs) are expressed as means ± SD (two-tailed Student's *t* test). (*D*) Flow cytometric analyses of the spleen, peripheral blood mononuclear cells (PBMCs), and the graft at Day 28. (*E*) The CD4/8 ratios in the spleen, peripheral blood, and the graft are expressed as means ± SD (two-tailed Student's *t* test). (*F*) The frequencies of CD62L$^+$ cells among CD4$^+$ and CD8$^+$ T cells are expressed as means ± SD (two-tailed Student's *t* test). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

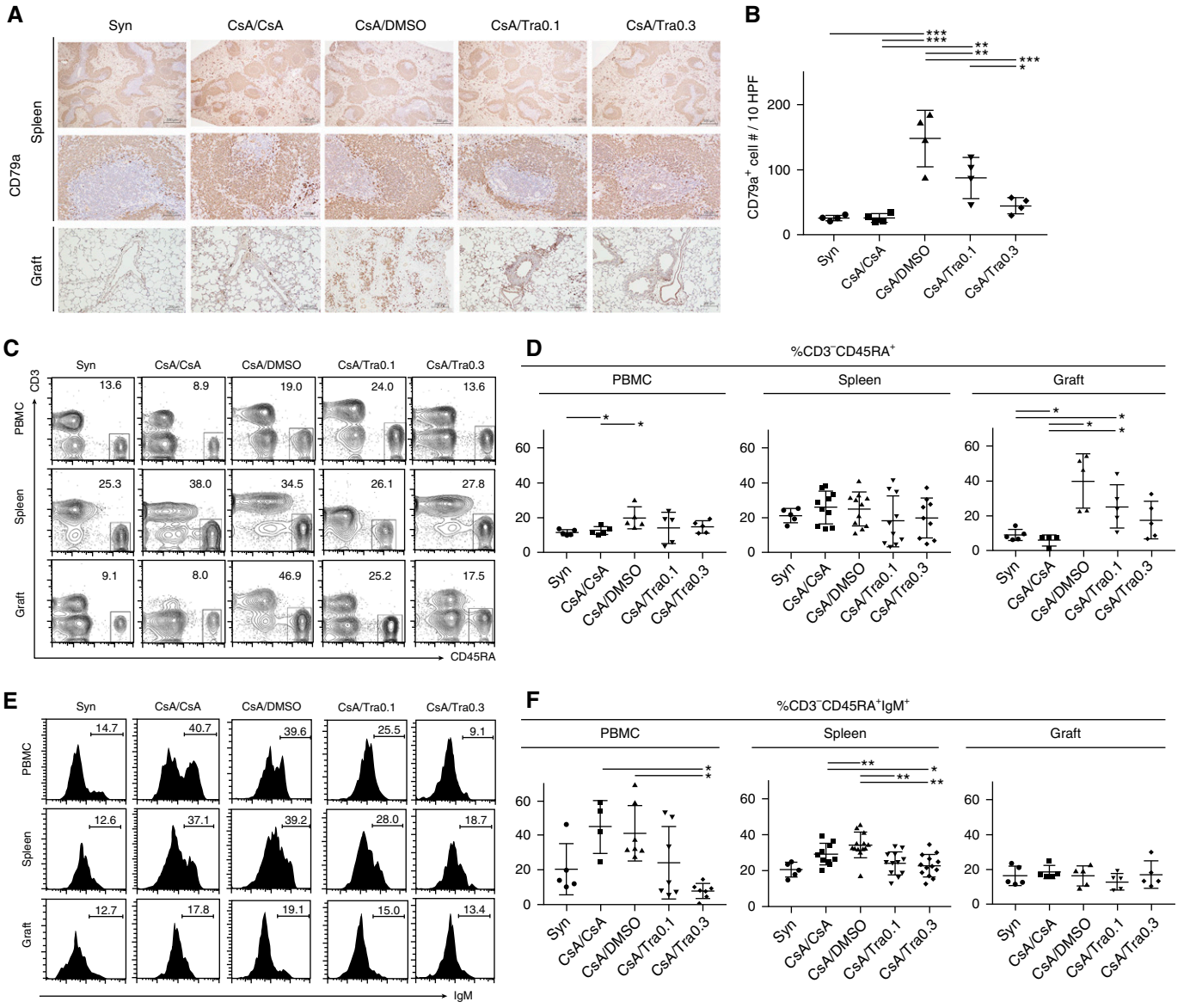

Figure 3. Trametinib suppresses B cell activation. (*A*) CD79 staining of the spleen (low and high power) and graft. Images are representative of five independent experiments. Scale bars: 100 μm, 200 μm, and 500 μm. (*B*) Numbers of $CD79^+$ cells per 10 HPFs are expressed as means ± SD (two-tailed Student's *t* test). (*C*) Flow cytometric analyses of the spleen, peripheral blood, and the graft at Day 28. The frequency of $CD45RA^+CD3^-$ cells among lymphocytes was determined to assess the level of B cells. (*D*) The frequencies of $CD45RA^+CD3^-$ cells among lymphocytes in peripheral blood, the spleen, and the graft at Day 28 are expressed as means ± SD (two-tailed Student's *t* test). (*E* and *F*) The frequency of IgM^+ cells among $CD45RA^+CD3^-$ cells was assessed (*E*). Aggregated data are expressed as means ± SD (two-tailed Student's *t* test) (*F*). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

recipient T cells (9, 12, 26). This is followed by indirect presentation by recipient-derived APCs as donor APCs are lost (9), although it is clinically difficult to differentiate between direct and indirect presentation. To investigate chimerism of B cells as important APCs in our model, $CD45RA^+CD3^-$ lymphocytes in the graft were sequentially analyzed by flow cytometry with an anti–MHC class II antibody that reacts with the MHC of LEW rats ($RT1^l$), but not with that of BN rats ($RT1^n$). This revealed that 5–10% of B cells were donor derived at Day 3 after transplantation, most of these cells disappeared by Day 14, and no donor B cells were detected at Day 28 (Figures 5A and 5B; one-way ANOVA, $P < 0.001$). This was observed in each treatment group, indicative of the sequential loss of passenger lymphocytes (i.e., donor lymphocytes in the graft [27]). Immunohistochemistry at Day 28 revealed that fewer MHC class II^+ cells infiltrated the graft in the CsA/Tra0.1 and CsA/Tra0.3 groups than in the CsA/DMSO group (Figure 5C). $CD79a^+$ cells colocalized with MHC class II^+ cells, implying that most MHC class II^+ cells were recipient-derived B cells (Figure 5D).

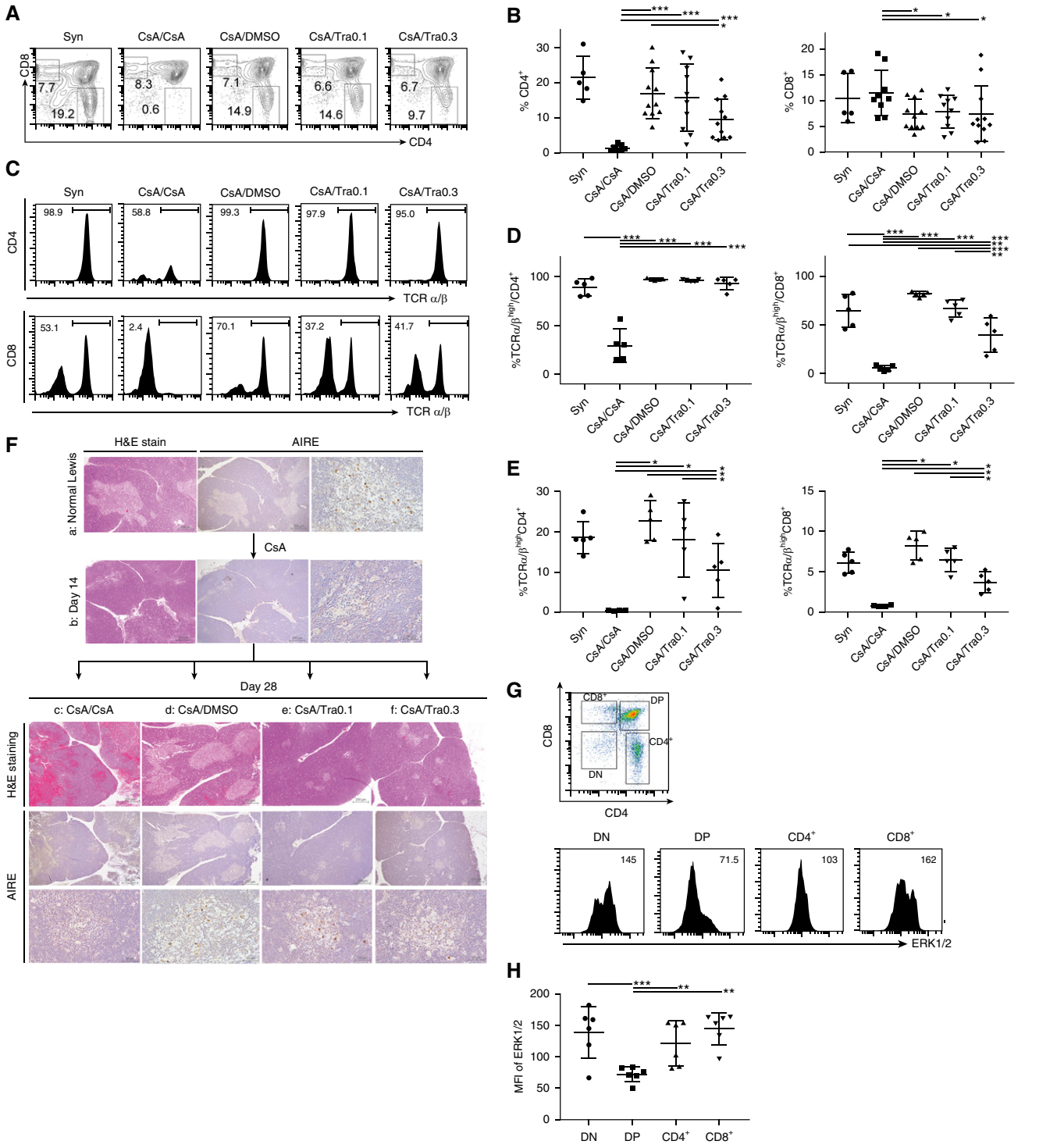

Figure 4. Trametinib preserves T cell differentiation in the thymus. (*A* and *B*) $CD4^+$ and $CD8^+$ T cells in the thymus at Day 28 after allogeneic transplantation from BN rats into LEW rats were identified by flow cytometry (*A*), and their frequencies are expressed as means ± SD (two-tailed Student's *t* test) (*B*). (*C* and *D*) $TCR\alpha/\beta^{high}$ cells among $CD4^+$ and $CD8^+$ T cells at Day 28 were detected (*C*), and their frequencies are expressed as means ± SD (two-tailed Student's *t* test) (*D*). (*E*) The frequencies of $TCR\alpha/\beta^{high}CD4^+$ and $TCR\alpha/\beta^{high}CD8^+$ T cells are expressed as means ± SD (two-tailed Student's *t* test). (*F*) LEW rats (*a*) underwent allogeneic lung transplantation from BN rats and were treated with CsA for 14 Days (*b*) and then with CsA (*c*), DMSO (*d*), 0.1 mg/kg trametinib (*e*), or 0.3 mg/kg trametinib (*f*) for another 14 days. H&E and AIRE staining of the thymus in each group is shown. Scale bars: 50 μm and 200 μm. (*G*) Flow cytometric analyses of $CD4^-CD8^-$ double-negative (DN) cells, $CD4^+CD8^+$ double-positive (DP) cells, $CD4^+$ cells, and $CD8^+$ cells in the thymus. The expression level of ERK1/2 and its mean fluorescence intensity (MFI) in each cell population are shown. (*H*) The MFIs of ERK1/2 in the four populations of T cells are expressed as means ± SD (two-tailed Student's *t* test). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. ERK1/2 = extracellular signal-regulated kinases 1/2.

Trametinib Suppresses Graft Rejection without CsA Preconditioning after Minor Antigen–Mismatched Transplantation

Rejection after minor histocompatibility antigen–mismatched transplantation is assumed to be associated with indirect alloantigen presentation by host APCs (10, 12, 28). We investigated whether trametinib suppresses this rejection without CsA preconditioning. Lungs of F344 ($RT1^{lv1}$) rats were transplanted into LEW rats (29). Recipients were administered DMSO, 25 mg/kg CsA, or 0.3 mg/kg trametinib from Day 0 to Day 14 (Figure 6A; minor-DMSO, minor-CsA, and minor-Tra0.3, respectively). The graft had a reddish appearance and a reduced volume with pleural congestion in the minor-DMSO group, whereas alveolar pneumatization and viable portions were maintained in the minor-Tra0.3 group (Figure 6B). Microscopic examination of the graft detected inflammatory cell infiltration and fibrotic changes in the minor-DMSO group, but not in the minor-Tra0.3 group (Figure 6B). Recipients showed lower scores of A and B grades of rejection in the minor-Tra0.3 group than in the minor-DMSO group (Figure 6C; Wilcoxon's rank-sum test, both $P < 0.001$). BW was recovered faster in the minor-Tra0.3 group than in the minor-DMSO and minor-CsA groups (Figure 6D; one-way ANOVA, $P = 0.021$). The frequencies of $CD4^+$ $TCR\alpha/\beta^{high}$ cells and $CD8^+$ $TCR\alpha/\beta^{high}$ cells in the thymus were higher in the minor-Tra0.3 and minor-DMSO groups than in the minor-CsA group (Figure 6E; one-way ANOVA, both $P < 0.001$). The frequencies of $CD4^+$ and $CD8^+$ T cells positive for pERK1/2 were lower in the minor-Tra0.3 group than in the minor-DMSO and minor-CsA groups (Figures 6F and 6G; one-way ANOVA, $P = 0.006$ and $P = 0.003$, respectively). Thus, trametinib suppresses rejection after minor histocompatibility antigen–mismatched transplantation without CsA preconditioning.

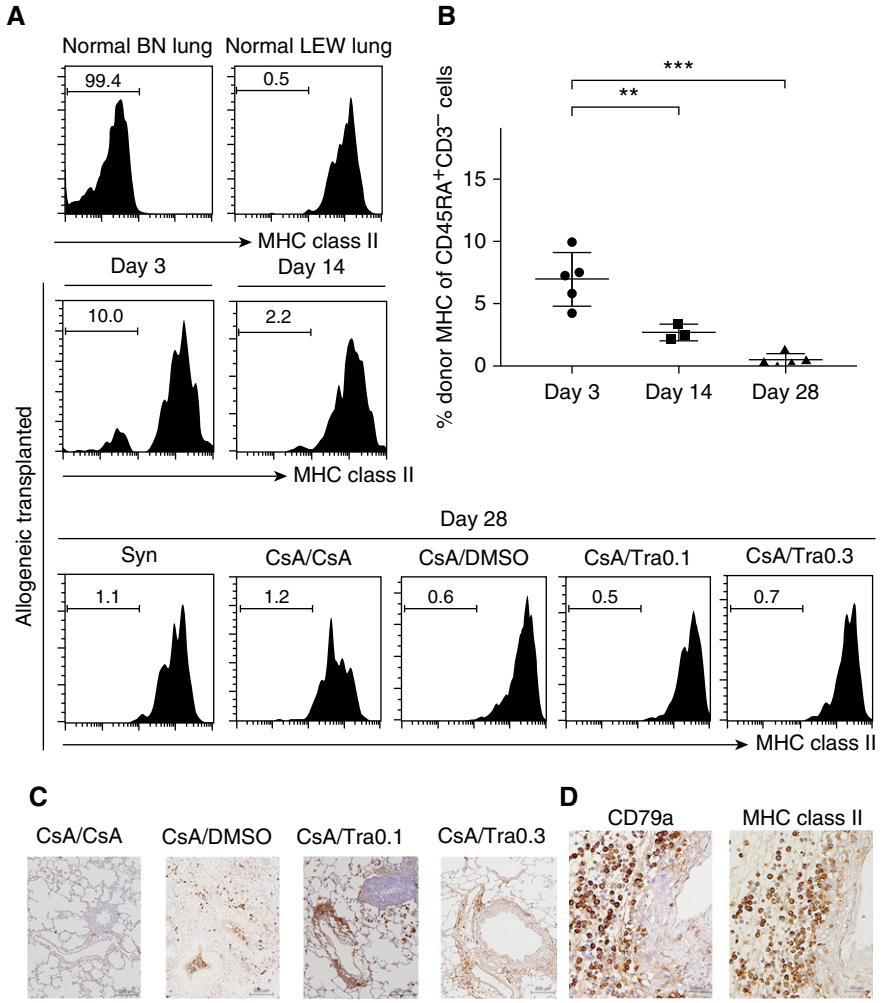

Figure 5. Donor B cells disappear from the graft by Day 14 after transplantation. (*A*) Flow cytometric analyses of lymphocytes in the left lungs of LEW rats, BN rats, grafts after 3 and 14 days of CsA treatment after lung transplantation from BN rats into LEW rats, and at Day 28 in each group. Expression of MHC class II of LEW rats (HIS19) in $CD45RA^+CD3^-$ cells was analyzed by gating $CD45RA^+CD3^-$ cells. (*B*) The frequencies of $HIS19^-$ cells among $CD45RA^+CD3^-$ cells are expressed as means ± SD (two-tailed Student's *t* test). (*C*) Staining of grafts in the allogeneic transplantation groups with the OX-3 antibody, which only reacts with the MHC class II of LEW rats. (*D*) Colocalization of $CD79a^+$ and MHC class II^+ ($OX\text{-}3^+$) cells in the graft in the CsA/Tra0.1 group. Scale bars: 200 μm and 50 μm. $^{**}P < 0.01$ and $^{***}P < 0.001$.

Discussion

Administration of the MEK inhibitor, trametinib, after CsA inhibited delayed rejection upon MHC-mismatched lung transplantation in rats. Trametinib suppressed differentiation of T and B cells in the periphery, but preserved thymic function and regulatory T cells. Given that trametinib suppresses GVHD without abrogating antitumor immunity upon bone marrow transplantation, it may ideally modulate immunity after solid organ transplantation. Trametinib is approved for clinical use in patients with malignant melanoma and is well tolerated. Based on our results, a clinical trial is warranted to investigate whether trametinib reduces the occurrence of or improves CLAD after human lung transplantation.

CsA treatment from Day 0 to Day 14 and 28 inhibited immunological rejection after MHC-mismatched transplantation (Figure 1B and Figure EB). On the other hand, trametinib treatment from Day 0 to Day 28 did not suppress rejection. However, trametinib treatment after CsA preconditioning for 14 days suppressed delayed rejection at Day 28 (Figure 1B). These results are in contrast with our previous finding that trametinib suppresses GVHD after bone marrow transplantation without calcineurin inhibitors (17, 18). This disparity may be due to differences between solid organ and bone marrow transplantations. GVHD is caused by alloreaction of donor lymphocytes against host tissues, whereas host lymphocytes

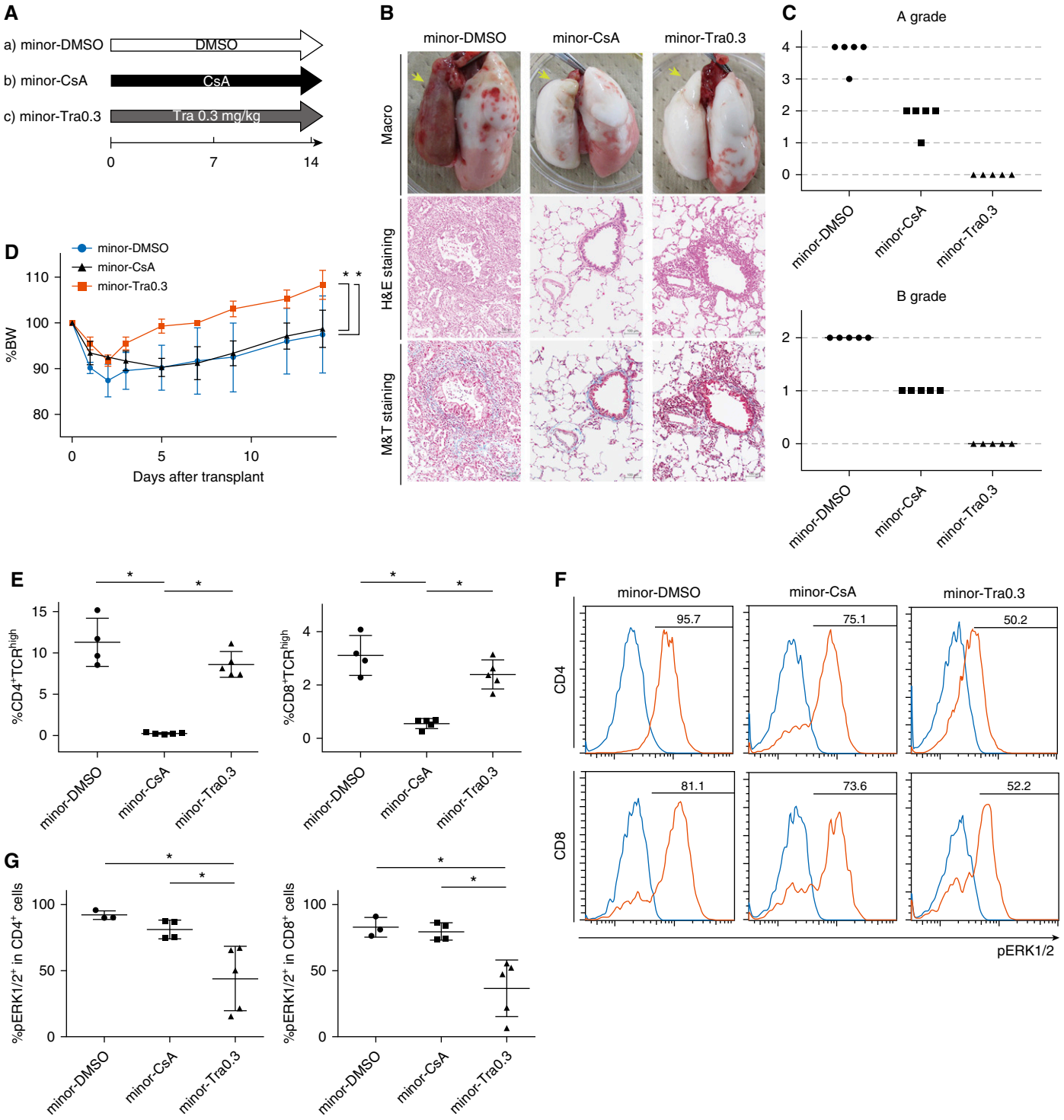

Figure 6. Trametinib attenuates acute rejection without CsA preconditioning after minor antigen–mismatched lung transplantation. (*A*) Lungs of Fischer 344 rats were transplanted into LEW rats. Recipients were administered DMSO, 25 mg/kg CsA, or 0.3 mg/kg trametinib five times per week (minor-DMSO, $n = 5$; minor-CsA, $n = 5$; and minor-Tra0.3, $n = 5$). (*B*) Macroscopic (upper section, yellow arrows denote grafts) and microscopic (middle section, H&E staining; lower section, M&T staining) images of the grafts at Day 14. Scale bars: 100 μm. (*C*) Scores of A/B grades of acute rejection according to the criteria stipulated by the International Society for Heart and Lung Transplantation. (*D*) Postoperative BW as a percentage of preoperative BW. (*E*) The frequencies of $CD4^+$ $TCR\alpha/\beta^{high}$ and $CD8^+$ $TCR\alpha/\beta^{high}$ cells at Day 14 are expressed as means ± SD (two-tailed Student's *t* test). (*F*) Flow cytometric analysis of ERK1/2 phosphorylation in peripheral blood $CD4^+$ and $CD8^+$ T cells at Day 14. Blue and red lines represent the results obtained without stimulation and upon stimulation with phorbol-12-myristate-13-acetate/ionomycin, respectively. (*G*) The frequencies of $pERK1/2^+$ cells among $CD4^+$ and $CD8^+$ cells are expressed as means ± SD ($n = 5$ per group). *$P < 0.05$. pERK1/2 = phospho-ERK 1/2.

damage the graft upon lung transplantation (30). High-dose chemotherapy and total-body irradiation ablate host immunity before bone marrow transplantation, and donor APCs present host tissue–derived antigens to donor T cells after transplantation (30, 31). By contrast, graft-resident donor APCs present donor-derived antigens to host T cells (direct presentation) immediately after lung transplantation. However, donor cells are gradually lost and, thereafter, host APCs present donor-derived antigens to host T cells (indirect presentation) (12, 32). Thus, alloantigen presentation changes from direct to indirect after lung transplantation.

Graft-resident donor B cells play important roles in direct presentation (8, 27, 33, 34). These cells disappeared by Day 14 in our model (Figure 5); therefore, delayed rejection at Day 28 after MHC-mismatched transplantation might be attributable to indirect presentation by host APCs. Indirect presentation is more important in rejection after minor histocompatibility antigen–mismatched transplantation (10, 28, 35), which was suppressed by trametinib without CsA preconditioning (Figure 6). Therefore, trametinib may selectively suppress indirect alloantigen presentation, although this requires verification. Graft-resident donor lymphocytes induce passenger lymphocyte syndrome in humans (36); however, these cells also disappear by Day 14 after transplantation (27, 37). CLAD is the most common cause of death after the first year after transplantation (2), and affects 50% of recipients within 5 years after lung transplantation (38). This disease may be strongly associated with chronic or delayed rejection. Collectively, trametinib may suppress immunological rejection leading to CLAD in humans.

The role of the mitogen-activated protein kinase pathway in thymic T cell differentiation has been mainly investigated in knockout mice (15, 39). Knockout of ERK1 reduces differentiation of $CD4^+CD8^+$ cells into $CD4^+$ and $CD8^+$ T cells (40–42), whereas pharmacological inhibition of MEK sustains differentiation toward $CD8^+$ T cells (15). However, thymic function has not been intensively investigated in transplant settings (43). Trametinib preserved some stages of T cell differentiation in the thymus, indicating that it maintains thymic function (Figure 4). $CD4^+CD8^+$ cells may have been unaffected by trametinib, because they demonstrate low expression of ERK1/2 (Figure 4G). CsA induces atrophy of the thymic medulla (44). We confirmed that it strongly abrogated thymic T cell differentiation into $CD4^+$ $TCR\alpha/\beta^{high}$ and $CD8^+$ $TCR\alpha/\beta^{high}$ T cells (Figures 4 and 6). Calcineurin inhibitors suppress thymic functions and increase the risk of infection (40, 44, 45), which is a serious problem after lung transplantation. Our data suggest that trametinib inhibits rejection and preserves antimicrobial and antitumor immunity by maintaining thymic function.

Trametinib suppresses activation and functional differentiation of T cells, and may also suppress activation of B cells (Figure 3). Several reports demonstrate that MEK inhibition suppresses humoral immunity (46, 47); however, its effects in transplant settings are unclear. Administration of trametinib results in rapid engraftment of B cells after murine bone marrow transplantation (17) and thereby reduces GVHD. Further study is required to investigate whether the effects of trametinib described in the current study are due to direct inhibition of B cell receptor downstream signaling by trametinib or indirect inhibition of humoral immunity by suppression of $CD4^+$ T cells. Detailed *in vitro* experiments must be performed to investigate the effects of MEK inhibition on B cells. Rejection after human lung transplantation is often associated with production of donor-specific antibodies (48, 49), and this might be inhibited by trametinib.

We have not confirmed our findings using MEK- or ERK-knockout animals, partly because such rats are unavailable. However, MEK- and ERK-knockout mice display fatal defects or radical abnormalities in hematopoiesis (50, 51), which may modulate transplant immunology. Our results are consistent with our previous findings that MEK inhibitors suppress T cell alloreactivity *in vitro* and GVHD after murine bone marrow transplantation (17, 18). This provides a strong rationale for clinical trials.

In summary, the MEK inhibitor, trametinib, suppressed delayed rejection after MHC-mismatched rat orthotopic left lung transplantation with CsA preconditioning. Trametinib suppressed functional differentiation of T and B cells in the periphery, but preserved thymic T cell differentiation. Graft-resident donor B cells disappeared by Day 14 after transplantation, indicating that indirect alloantigen presentation by host APCs contributes to this delayed rejection. Trametinib without CsA preconditioning suppressed rejection after minor histocompatibility antigen–mismatched transplantation, which is associated with indirect alloantigen presentation. Collectively, pharmacological inhibition of MEK might suppress CLAD or chronic rejection after human lung transplantation. Given that trametinib is approved for treatment of malignant melanoma and is well tolerated, our findings should be confirmed in clinical trials. ■

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