Platelet gene therapy induces robust immune tolerance even in a primed model via peripheral clonal deletion of antigen-specific T cells

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While platelet-specific gene therapy is effective in inducing immune tolerance to a targeted protein, how the reactivity of pre-existing immunity affects the efficacy, and whether CD8 T cells were involved in tolerization, is unclear. In this study, ovalbumin (OVA) was used as a surrogate protein. Platelet-OVA expression was introduced by 2bOVA lentivirus transduction of Sca-1⁺ cells from either wild-type (WT)/CD45.2 or OT-II/ CD45.2 donors followed by transplantation into OVA-primed WT/CD45.1 recipients preconditioned with 6.6 Gy of irradiation. Sustained platelet-OVA expression was achieved in >85% of OVA-primed recipients but abolished in animals with high-reactive pre-existing immunity. As confirmed by OVA rechallenge and skin graft transplantation, immune tolerance was achieved in 2bOVA-transduced recipients. We found that there is a negative correlation between platelet-OVA expression and the percentage of OVA-specific CD4 T cells and a positive correlation with the OVA-specific regulatory T (Treg) cells. Using the OT-I/WT model, we showed that antigen-specific CD8 T cells were partially deleted in recipients after platelet-targeted gene transfer. Taken together, our studies demonstrate that robust antigen-specific immune tolerance can be achieved through platelet-specific gene therapy via peripheral clonal deletion of antigen-specific CD4 and CD8 T effector cells and induction of antigen-specific Treg cells. There is an antagonistic dynamic process between immune responses and immune tolerance after platelet-targeted gene therapy.

INTRODUCTION

Gene therapy has recently shown great promise for the treatment of genetic diseases such as hemophilia A and hemophilia B.[1–10] However, immune responses to the transgene product or viral proteins are still a significant concern in gene therapy.[4,9,11,12] Undesired immune responses can be an obstacle in gene therapy and in protein replacement therapy, as they may result in a failure of the treatment.[13] Also, undesired immune responses are a problem in graft-versus-host diseases and autoimmune diseases.[14–17] Thus, developing a gene therapy protocol that can promote antigen-specific immune tolerance may provide a new intervention to prevent conditions or treat diseases caused by undesired immune responses.

We have developed a gene therapy protocol in which transgene expression is specifically targeted to platelets under control of the platelet-specific αIIb promoter via lentivirus (LV)-mediated hematopoietic stem cell (HSC) transduction followed by transplantation.[18,19] Using a FVIII-deficient hemophilia A model, we have demonstrated that platelet-targeted FVIII expression (2bF8) triggers neither primary nor secondary anti-FVIII immune responses.[20] In the model with pre-existing anti-FVIII immunity, we found that anti-FVIII inhibitory antibodies (inhibitors) decline with time after 2bF8 gene therapy.[21,22] However, the intensity of the preconditioning regimen required for gene therapy of the inhibitor model is more stringent than that for the non-inhibitor model.[22] Further studies showed that 2bF8 lentiviral gene delivery to HSCs can restore hemostasis and induce immune tolerance in hemophilia A mice.[23,24] When a similar gene therapy protocol is applied to a hemophilia B model, in which FIX expression is targeted to platelets, sustained platelet-FIX expression can also be obtained in treated animals, resulting in phenotypic correction as well as FIX-specific immune tolerance induction.[25]

To investigate whether our platelet-targeted gene therapy protocol can be applied to induce immune tolerance to a non-coagulation protein, we used the ovalbumin (OVA) model.[26] We found that targeting OVA expression to platelets (2bOVA) in mice without pre-existing anti-OVA immunity (the unprimed model) can promote immune tolerance to OVA via peripheral clonal deletion of antigen-specific CD4 T cells and expansion of antigen-specific regulatory T (Treg) cells. However, it is unclear how the reactivity of pre-existing immunity affects the efficacy of immune tolerance induction and the potential mechanisms in platelet-targeted gene therapy.
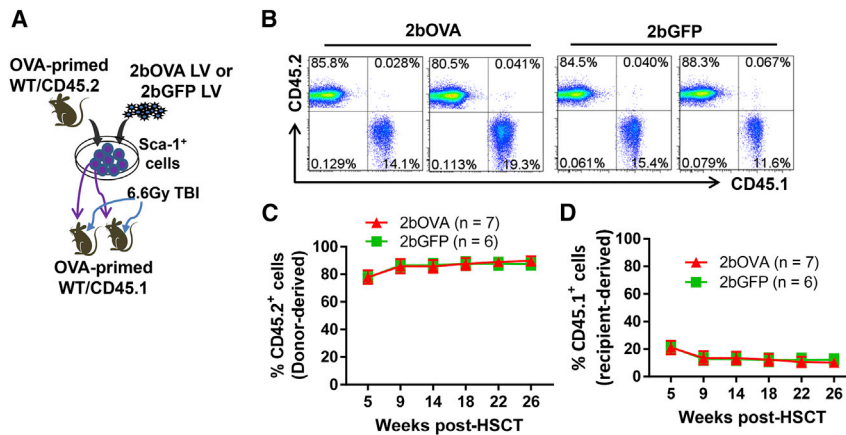
Figure 1. Cell phenotype in the OVA-primed wild-type (WT) CD45.1[+] recipients that received 2bOVA-transduced WT Sca-1^{+} cell transplantation (the WT/WT model)

Blood samples were collected by retro-orbital bleeds from recipients that received 2bOVA- or 2bGFP (a control vector)-transduced WT/CD45.2[+] Sca-1^{+} cells, and 1:10 of 3.8% sodium citrate was used as an anti-coagulant. Whole blood counts were performed using the Vet ABC hematology analyzer. Leukocytes were isolated, stained with CD45.1 and CD45.2 antibodies, and analyzed by flow cytometry to determine the engraftment in transduced recipients. (A) Schematic diagram of experimental design for the primed WT/WT model. (B) Representative dot plots from flow cytometry analysis of chimerism 14 weeks after HSC transplantation (HSCT). (C) Percentage of donor-derived leukocytes in transduced recipients over time after HSCT. (D) Percentage of recipient-derived leukocytes in transduced recipients over time after HSCT. These results demonstrate that high-level engraftment is achieved in recipients that received 2bOVA- or 2bGFP-transduced HSCs.

In the current study, we used the OVA model to investigate the impact of the intensity of pre-existing immunity on the efficacy of platelet-targeted gene transfer in antigen-specific immune tolerance induction in the primed model. We explored the impact of pre-existing immunity on 2bOVA genetically manipulated engraftment, neoprotein expression in platelets, and antigen-specific immune tolerance induction after platelet-targeted gene therapy using the wild-type (WT), OVA-specific CD4 T cell receptor (TCR) transgenic (OT-II), and OVA-specific CD8 TCR transgenic (OT-I) mouse models.

RESULTS

Sustained platelet-OVA expression can be obtained after 2bOVA lentiviral gene delivery to HSCs in an OVA-primed model

To investigate the efficacy of immune tolerance induction in platelet-targeted gene therapy in the primed model, we first used C57BL/6 WT mouse models. Both donors and recipients were WT mice and immunized with OVA to induce anti-OVA antibody development. OVA expression was introduced by 2bOVA transduction of HSCs from OVA-primed WT/CD45.2 donors followed by transplantation into OVA-primed WT/CD45.1 recipients preconditioned with 6.6 Gy total body irradiation (TBI) (Figure 1A). 2bGFP was used as a control vector in parallel. After bone marrow (BM) reconstitution, the chimerism in the 2bOVA group was similar to that in the 2bGFP control group as determined by flow cytometry (Figures 1B–1D). At 14 weeks after transplantation, the engraftments in the 2bOVA and 2bGFP groups were 85.7% ± 4.6% (n = 7) and 86.6% ± 4.5% (n = 6) (Figure 1C), respectively. In addition, we monitored whole blood counts in recipients. There were no statistically significant differences in whole blood counts between 2bOVA- or 2bGFP-transduced recipients versus untransduced controls (Figures S1A–S1C), demonstrating that ectopic expression of neoprotein in platelets does not alter blood counts and that pre-existing anti-OVA immunity does not diminish 2bOVA-transduced platelets.

To confirm the viability of 2bOVA-transduced hematopoietic cells in 2bOVA-transduced recipients, we used PCR to detect proviral DNA in peripheral leukocytes. As shown in Figure 2A, the OVA expression cassette was detected in all recipients that received 2bOVA-transduced Sca-1^{+} cells, but not in 2bGFP-transduced or untransduced recipients. To determine platelet-OVA protein expression in transduced recipients, we used a platelet lysate OVA ELISA assay. We found that six of seven 2bOVA-transduced recipients had sustained platelet-OVA expression with an average expression level of 5.4 ± 0.9 ng/10[8] platelets to 8.5 ± 2.3 ng/10[8] platelets during the study period (Figure 2B). The other recipient, which was the animal with the highest anti-OVA total immunoglobulin G (IgG) titer in that group (see the next section), had barely detectable levels of platelet-OVA. The average platelet-OVA expression in 2bOVA-transduced recipients was 5.9 ± 2.9 ng/10[8] platelets (n = 7). No OVA was detected in 2bGFP-transduced recipients (n = 6) or untransduced transplanted controls (n = 4), as expected (Figure 2C). These data demonstrate that sustained neoprotein expression is attainable after platelet-targeted gene transfer in a primed model, but the intensity of immune responses might affect the neoprotein expression.

Antigen-specific immune tolerance is induced after platelet-targeted gene therapy in the primed model

To investigate the immune responses after 2bOVA gene transfer in OVA-primed mice, we monitored anti-OVA total IgG titers. As shown in Figure 3A, the titers of anti-OVA total IgG in the untransduced transplanted control, 2bGFP, and 2bOVA groups were 1,370 ± 1,791 (n = 4), 810 ± 695 (n = 6), and 1,194 ± 1,392 (n = 7) before HSC transplantation (HSCT), and 24,340 ± 30,587, 2,097 ± 1,666, and 14,014 ± 16,824, respectively, 5 weeks after transplantation, with no statistically significant differences among the groups. To compare the changes of anti-OVA total IgG titers at various time points in different groups during the study period, the titers before HSCT were defined as 1 (Figure 3B). As transduced cells were transplanted into OVA-primed mice 1 week after the last OVA immunization,
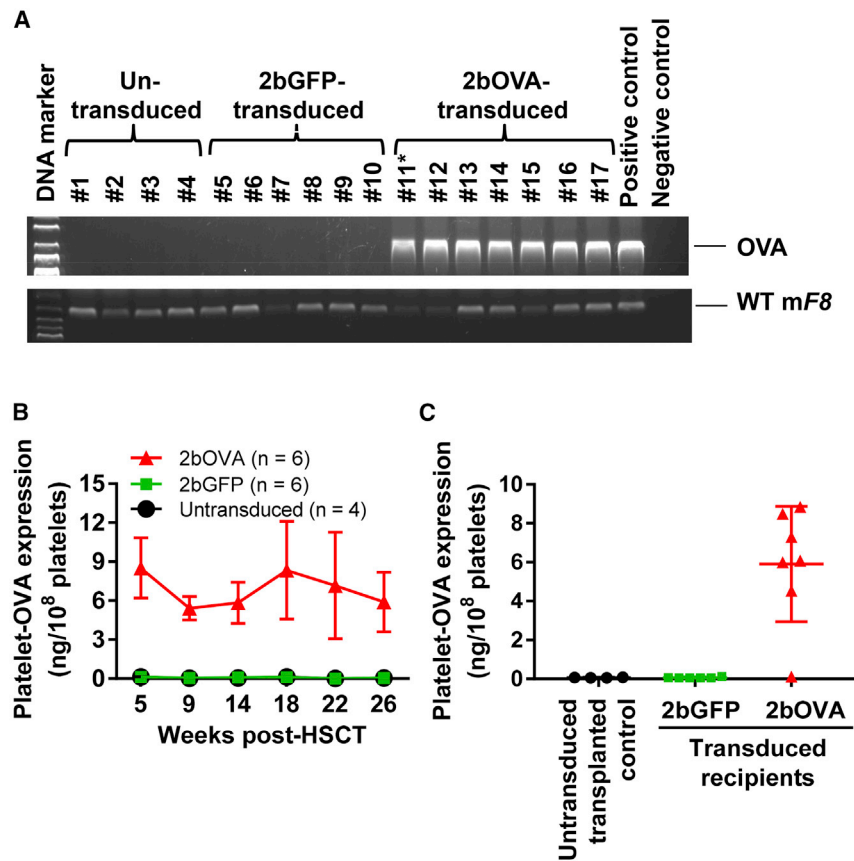
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Figure 2. OVA expression in 2bOVA-transduced OVA-primed recipients (the WT/WT model)

Blood samples were collected starting at week 5 after transplantation. Leukocytes and platelets were isolated. DNA was purified from leukocytes, and the 2bOVA expression cassette was amplified by PCR analysis. Isolated platelets were lysed in 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate] (CHAPS), and platelet lysates were used for ELISA to determine OVA expression levels. (A) PCR analysis of the OVA transgene in transduced recipients. PCR of WT mouse F8 (mF8) was used as an internal control to measure the integrity of DNA. pWPT2bOVA plasmid DNA was used as a positive control. (B) OVA protein expression in transduced primed recipients over time after transplantation. The levels of OVA transgene protein expression in platelet lysates from transduced recipients were determined by ELISA. OVA protein was detected in six of seven 2bOVA-transduced recipients. One of the 2bOVA-transduced recipients (#11 in A), which had the highest anti-OVA titer before transplantation, failed to achieve sustained platelet-OVA expression. (C) Average OVA expression in 2bOVA-transduced OVA-primed recipients. Each data point represents the average data from each recipient over time during the study period.

transplantation. Of note, full-thickness tail skin grafts from actin-mOVA transgenic mice were successfully engrafted onto 2bOVA-transduced OVA-primed recipients (including the one with high reactive pre-existing anti-OVA immune responses and barely detectable platelet-OVA) and survived throughout the remaining lifetimes (>8 months) of the animals (Figures 3E and 3F). In contrast, skin grafts were completely rejected within 60 days after transplantation in control 2bGFP-transduced and untransduced transplanted recipients (Figure 3F). Taken together, these data demonstrate that strong antigen-specific immune tolerance is induced after 2bOVA lentiviral gene delivery to HSCs even in a primed model.

Immune tolerance is established even in the OVA-specific OT-II model after platelet-targeted OVA gene transfer

To explore how pre-existing immunity impacts immune tolerance establishment after platelet-specific gene transfer in the OVA-primed model, we transduced HSCs from OVA-specific CD4 TCR transgenic mice (OT-II/CD45.2) with 2bOVA LV and transplanted into OVA-primed WT/CD45.1 recipients preconditioned with 6.6 Gy TBI (Figure 4A). 2bGFP-transduced and untransduced transplanted mice were used as controls. After BM reconstitution, the chimerism was comparable between the 2bOVA and control groups (Figure 4B). The average OVA protein level was 18.6 ± 12.3 ng/10^8 platelets (n = 10) in the 2bOVA-transduced group and no platelet-OVA was detected in 2bGFP and untransduced transplanted groups (Figure 4C). One of 10 2bOVA-transduced recipients, that is, the animal that had the highest level of anti-OVA total IgG before transplantation, had barely detectable platelet-OVA expression (Figures 4C

anti-OVA total IgG titers in all recipients peaked at 5 weeks after transplantation and then declined with time in all groups. When the titers in the 2bOVA group dropped 6 months after transplantation, recipients were rechallenged with OVA. All of the anti-OVA titers in 2bGFP-transduced recipients increased dramatically, while only one in the 2bOVA group slightly increased. To compare the changes of anti-OVA total IgG titers upon OVA rechallenge between groups, the titers before OVA rechallenge were normalized to 100%. Normalized anti-OVA antibody titers in the 2bOVA group were significantly lower than those in the 2bGFP and untransduced transplanted groups after OVA rechallenge (Figure 3C).

To ensure that the immune system was not inactive in 2bOVA-transduced recipients, animals were immunized intravenously with unrelated antigen recombinant human FVIII (rhF8) at 200 U/kg/week for 4 weeks, an immunization protocol known to induce anti-FVIII immune responses even in WT animals.[27,28] As shown in Figure 3D, all 2bOVA-transduced recipients developed anti-FVIII inhibitors after rhF8 immunization with no significant differences between the 2bOVA group and the 2bGFP or untransduced transplanted control.

To investigate whether cellular immune tolerance is established in primed animals after 2bOVA gene transfer, we performed skin graft
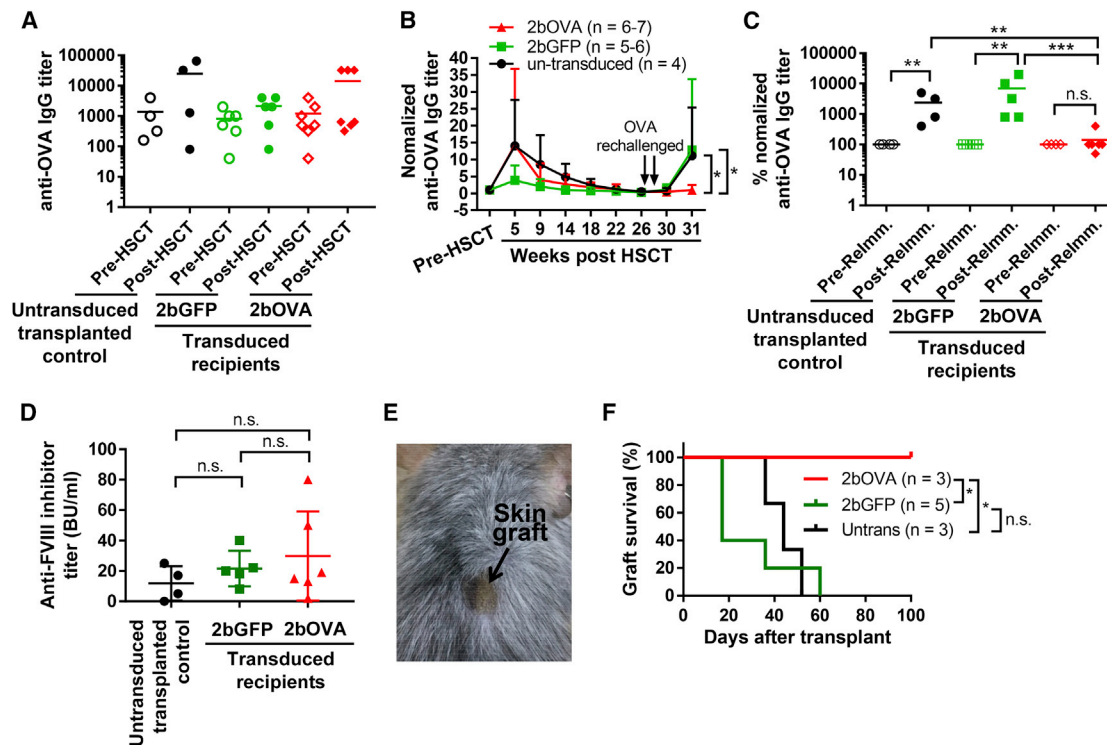
Figure 3. The development of anti-OVA immune tolerance in 2bOVA-transduced OVA-primed mice (the WT/WT model)

To investigate how the primed immune system responds after platelet-targeted gene transfer, we monitored anti-OVA total IgG titers in treated animals by ELISA. (A) Anti-OVA titers in recipients before and 5 weeks after HSCT. (B) Anti-OVA titers during the study course. The titer at the pre-HSCT time point was normalized to 1. (C) Changes in anti-OVA titers after OVA rechallenge. To investigate whether immune tolerance was induced in 2bOVA-transduced recipients, recipients were immunized with OVA (20 μg/mouse by i.p. injection) twice. One week after each rechallenge, plasmas were collected for ELISA to determine anti-OVA total IgG titers. The titer at the pre-HSCT time point was normalized to 100%. (D) Titers of anti-FVIII antibodies. To investigate whether the immune tolerance developed in 2bOVA-transduced recipients is OVA-specific, mice were further challenged with the unrelated antigen recombinant human FVIII (rhF8) at 200 U/kg/week intravenously (i.v.) (four times), and anti-FVIII inhibitory antibodies (inhibitors) were determined by a Bethesda assay. (E and F) Skin graft transplantation. To further confirm the antigen-specific immune tolerization developed in 2bOVA-transduced OVA-primed recipients, tail skin grafts from Act-mOVA transgenic mice were transplanted onto recipients. Complete graft rejection was recorded. 2bGFP-transduced and untransduced transplanted controls were performed in parallel. Representative skin graft on the 2bOVA-transduced recipient (8 months after skin transplantation) is shown in (E). Skin graft survival rate is shown in (F). These data demonstrate that antigen-specific immune tolerance is achieved after platelet-specific gene therapy in a primed model. $^*p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$. n.s., not significant (for difference between the two groups).

and 4D). Similar to the WT model, there were no statistically significant differences in the titers of anti-OVA antibodies between the 2bOVA, 2bGFP, and untransduced transplanted control groups before and 5 weeks after HSCT (Figure 4D). Anti-OVA antibody titers in all groups declined with time (data not shown). After at least 15 weeks after HSCT, when animals were rechallenged with OVA, anti-OVA titers in all recipients in the 2bGFP group (n = 5) and five of six in the untransduced transplanted group increased, while none of the titers in the 2bOVA group increased (Figure 4E). When the titers of anti-OVA total IgG before rechallenge were normalized to 100%, normalized anti-OVA antibody titers after rechallenge in the 2bOVA group (30 ± 15) were significantly lower than those in the untransduced and 2bGFP groups (4,530 ± 10,033 and 5,015 ± 8,386, respectively) (Figure 4E). To ensure that platelet-OVA expression was sustained, sequential BM transplantation was carried out by transplanting BM cells from primary recipients that received 2bOVA-transduced Sca-1^{+} cells into secondary

recipients. As shown in Figure 4F, platelet-OVA levels in secondary recipients were comparable to those in primary recipients. These results demonstrate that platelet-specific OVA gene delivery to HSCs can still induce immune tolerance in the OVA-primed WT mice even though the donors are OVA-specific TCR transgenic mice and platelet-OVA expression is sustained.

Antigen-specific CD4 T cells were deleted in peripheral lymphoid organs in the OVA-primed model after platelet-targeted OVA gene transfer

To explore how platelet-targeted gene transfer affects antigen-specific CD4 T cells in peripheral blood in the primed model, we monitored CD4 T cells in peripheral blood monthly by flow cytometry analysis. After BM reconstitution, the leukocyte engraftments in the 2bOVA group were similar to those in the control groups, but donor-derived OVA-specific CD45.2^{+}CD4^{+} (OT-II/CD4) T cells in the 2bOVA group were consistently significantly lower than in the untransduced
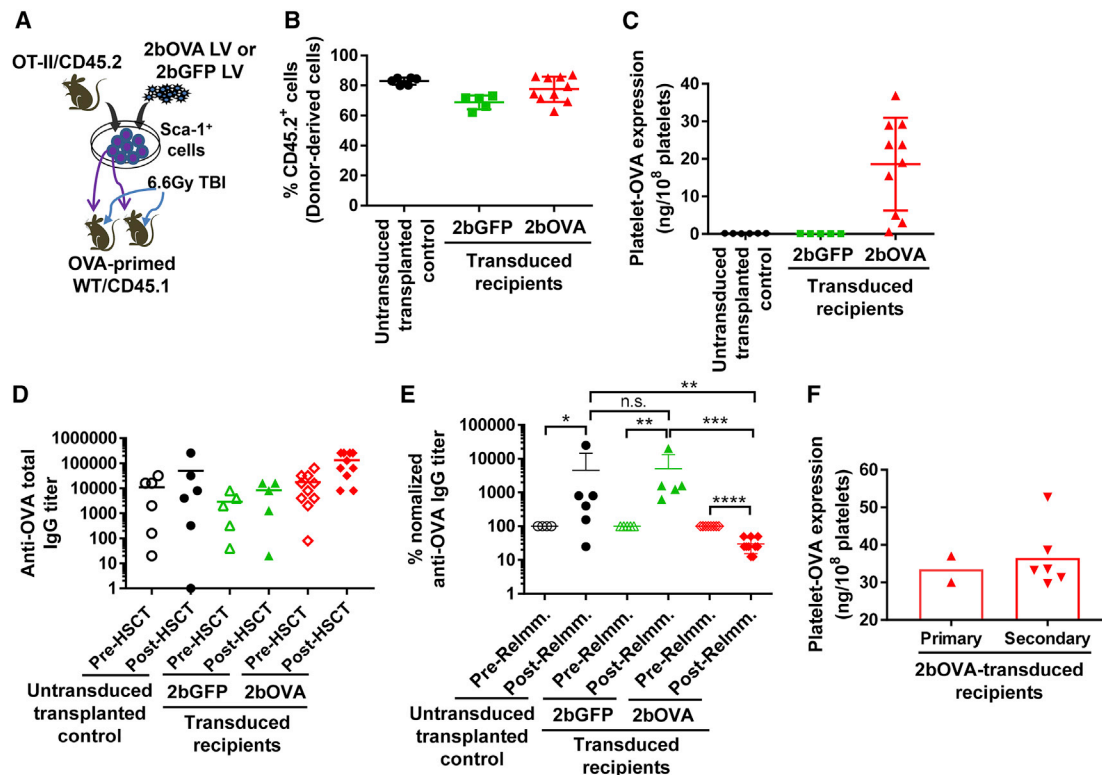
Figure 4. Assessment of OVA expression and immune responses in the OVA-primed model with OVA-specific CD4 T cells (the OT-II/WT model)

In this model, recipients (WT/CD45.1) were preimmunized with OVA to induce anti-OVA antibody development and then were preconditioned with 6.6 Gy followed by transplantation of 2bOVA-transduced Sca-1+ cells from OVA-specific OT-II/CD45.2 TCR transgenic mice. Animals were analyzed by flow cytometry for engraftment and ELISA for OVA expression and for anti-OVA total IgG titers. (A) Schematic diagram of experimental design for the primed OT-II/WT model. (B) Engraftments in recipients after bone marrow (BM) fully reconstituted (at week 14 after HSCT). (C) Average OVA expression levels in transduced recipients. Data presented are the average platelet-OVA expression from multiple time points during the study period as determined by ELISA. (D) Anti-OVA total IgG before and 5 weeks after transplantation as determined by ELISA. (E) Changes in anti-OVA titers after OVA rechallenge in OVA-primed recipients that received 2bOVA-transduced OT-II HSCs. Animals were reimmunized with OVA at a dose of 20 μg/mouse/week twice by i.p. injection after at least 15 weeks after HSCT. One week after the second rechallenge, plasmas were collected for ELISA to determine the titers of anti-OVA total IgG. The titer in each animal before reimmunization was defined as 100%. 2bGFP-transduced and untransduced transplanted OVA-primed recipients were used as controls in parallel. These data demonstrate that immune tolerance can be achieved in the primed model, even using the OVA-specific TCR transgenic donors. (F) Platelet-OVA expression in the primary and secondary 2bOVA-transduced recipients. BM cells were collected from two of the 2bOVA-transduced primary recipients and transplanted into six secondary recipients (CD45.1/WT) preconditioned with 6.6 Gy TBI. After BM was reconstituted, platelets were collected from recipients, and OVA expression levels were determined by ELISA. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. n.s., not significant (for difference between the two groups).

and 2bGFP groups in peripheral blood during the entire study period (Figures 5A and 5B; Figures S2A and S2B). In 2bOVA-transduced recipients, 8 of 10 had less than 2% CD45.2+CD4+ T cells, but CD45.2+CD4+ cells in the other two recipients were similar to those obtained in the 2bGFP and untranduced transplanted controls. Furthermore, both the percentage and the total number of antigen-specific Treg cells (CD45.2+CD4+CD25+Foxp3+) in the 2bOVA group were significantly higher than in the 2bGFP or untransduced transplanted control groups (Figures 5C and 5D) in peripheral blood. The average level of platelet-OVA expression through the study period in each recipient was negatively associated with the percentage of donor-derived OVA-specific CD4 T cells (CD45.2+CD4+) (Figure 5E) and positively associated with OVA-specific Treg cells (Figure 5F) in peripheral blood in OVA-primed mice after platelet-specific OVA gene transfer.

To investigate the OVA-specific CD4 T cells in lymphoid organs in the OVA-primed model after platelet-targeted OVA gene transfer, 1 month after recipients were rechallenged with OVA protein by intraperitoneal (i.p.) injection, animals were euthanized for analysis. Cells isolated from lymph nodes, spleen, and thymus were stained and analyzed by flow cytometry. The percentage and total number of OVA-specific (donor-derived CD45.2+CD4+) T cells in both superficial lymph nodes and mesenteric lymph nodes were significantly lower in the 2bOVA group compared to the 2bGFP and untransduced groups (Figures 6A–6D). The percentage, but not the total number, of OVA-specific CD4 T cells in the spleen was significantly lower in the 2bOVA group compared to the 2bGFP and untransduced control groups (Figures 6E and 6F). There were no differences in either the percentage or total cell number of CD45.2+CD4+ T cells in the thymus among the three groups (Figures 6G and 6H), indicating that central
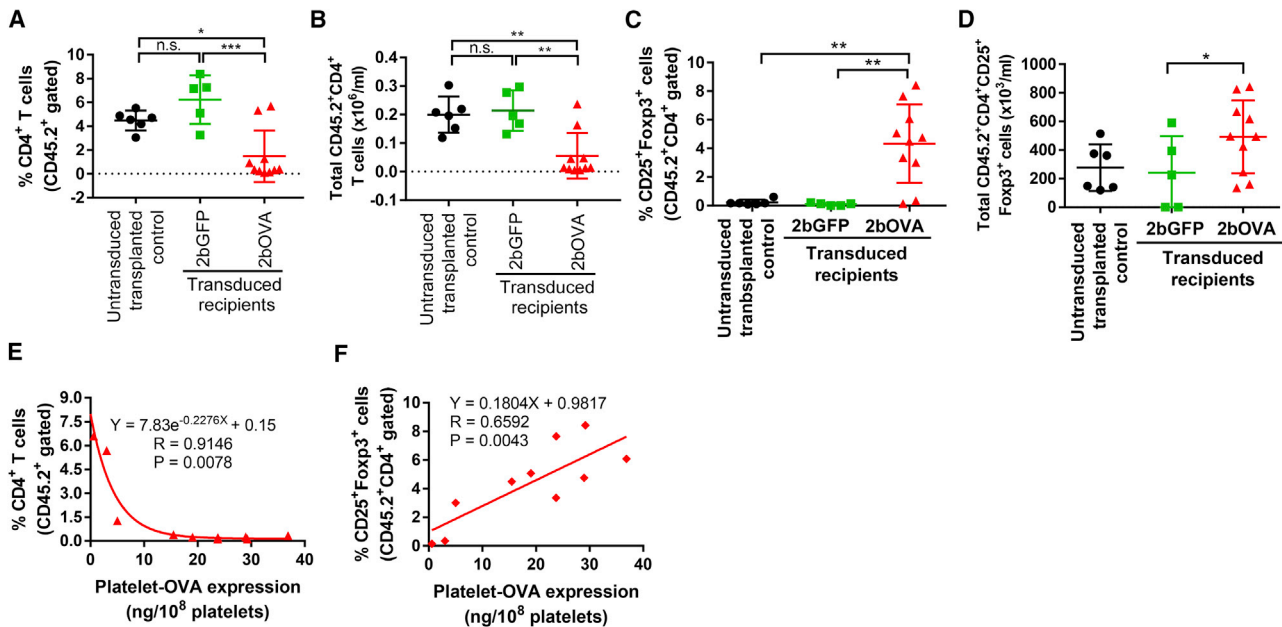
Figure 5. Flow cytometry analysis of OVA-specific CD4$^+$ (CD45.2$^+$CD4$^+$) T cells and OVA-specific regulatory T (Treg) cells (CD45.2$^+$CD4$^+$CD25$^+$Foxp3$^+$) in peripheral blood of OVA-primed recipients that received 2bOVA-transduced OT-II HSCs (the OT-II/WT model)

Blood samples were collected monthly after HSCT by retro-orbital bleeds, and leukocytes were isolated. Cells were stained for cell markers CD45.1, CD45.2, CD4, CD25, and Foxp3. Samples were run on a BD LSR II flow cytometer, and data were analyzed using FlowJo software. Cells were gated on CD45.2$^+$ (OT-II transgenic donor-derived) and analyzed for CD4$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ (Treg) cells. (A) Percentage of OVA-specific CD4 T cells at 14 weeks after HSCT. (B) Total number of OVA-specific CD4 T cells in peripheral blood at week 14 after HSCT. (C) Percentage of OVA-specific Treg cells at 14 weeks after HSCT. (D) Total number of OVA-specific Treg cells at 14 weeks after HSCT. (E) Correlation between the level of platelet-OVA expression and the percentage of OVA-specific CD4 T cells at 10 weeks after HSCT in 2bOVA-transduced OVA-primed recipients. (F) Correlation between the level of platelet-OVA expression and the percentage of OVA-specific Treg cells at 14 weeks after HSCT in 2bOVA-transduced OVA-primed recipients. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant (for difference between the two groups).

tolerance may not play a role in the immune tolerance induced after platelet-targeted gene therapy. Meanwhile, the frequency and the total number of endogenous CD4$^+$ T cells were similar in the three groups (data not shown). Of note, the percentage and total number of donor-derived Treg cells in the 2bOVA group were significantly higher than those in the 2bGFP group in the spleen (Figures 7A and 7B). In lymph nodes, the percentage of donor-derived Treg cells in the 2bOVA group was significantly higher than that in the 2bGFP and untransduced groups, but there were no significant differences in the total numbers of donor-derived Treg cells among the three groups (Figures 7C–7F). There were no differences in thymus among groups in either the percentage or total number (Figures 7G and 7H).

Taken together, these data demonstrate that platelet-specific gene therapy can induce immune tolerance in a primed system through two distinct pathways, that is, peripheral clonal deletion of antigen-specific CD4$^+$ T cells and expansion of antigen-specific Treg cells.

Antigen-specific CD8 T cells can be deleted after platelet-targeted OVA gene transfer

To investigate how platelet-specific gene therapy impacts antigen-specific CD8 T cells, we used the OT-I/WT model, in which all

donor-derived CD8 T cells are OVA-specific. Sca-1$^+$ cells were isolated from OVA-specific CD8 TCR transgenic OT-I/CD45.2 mice, transduced with 2bOVA LV, and transplanted into WT/CD45.1 recipients (Figure 8A). 2bGFP LV was used as a control vector in parallel. After transplantation and BM reconstitution, the percentage of donor-derived leukocytes (CD45.2$^+$) in the 2bOVA group was significantly lower than that in the 2bGFP control group (Figure 8B). Low levels of platelet-OVA expression were obtained in 2bOVA-transduced recipients at week 5 (0.72 ± 0.65 ng/10^8 platelets, n = 8) and dropped to barely detectable levels at week 16 (0.17 ± 0.38 ng/10^8 platelets) after transplantation (Figure 8C). To determine the transduction efficiency, we used flow cytometry to analyze 2bGFP LV-transduced platelets. There were 33.9% ± 12.9% platelets that were positive with GFP in 2bGFP LV-transduced recipients (Figure 8D), demonstrating that high HSC transduction efficiency and successful transplantation were achieved. Of note, the percentage of donor-derived OVA-specific CD8 T cells in the 2bOVA group was 48% lower than in the 2bGFP control group (11.5% ± 4.2% and 11.7% ± 7.5% in the 2bOVA group and 21.6% ± 3.4% and 21.9% ± 4.2% in the 2bGFP group, respectively, at weeks 8 and 12 after HSCT) (Figure 8E). Interestingly, we found that the percentage of the total OVA-specific CD8$^+$Foxp3$^+$ cells in the 2bOVA group (0.19% ± 0.14% and 0.15% ± 0.18%, respectively) was significantly higher
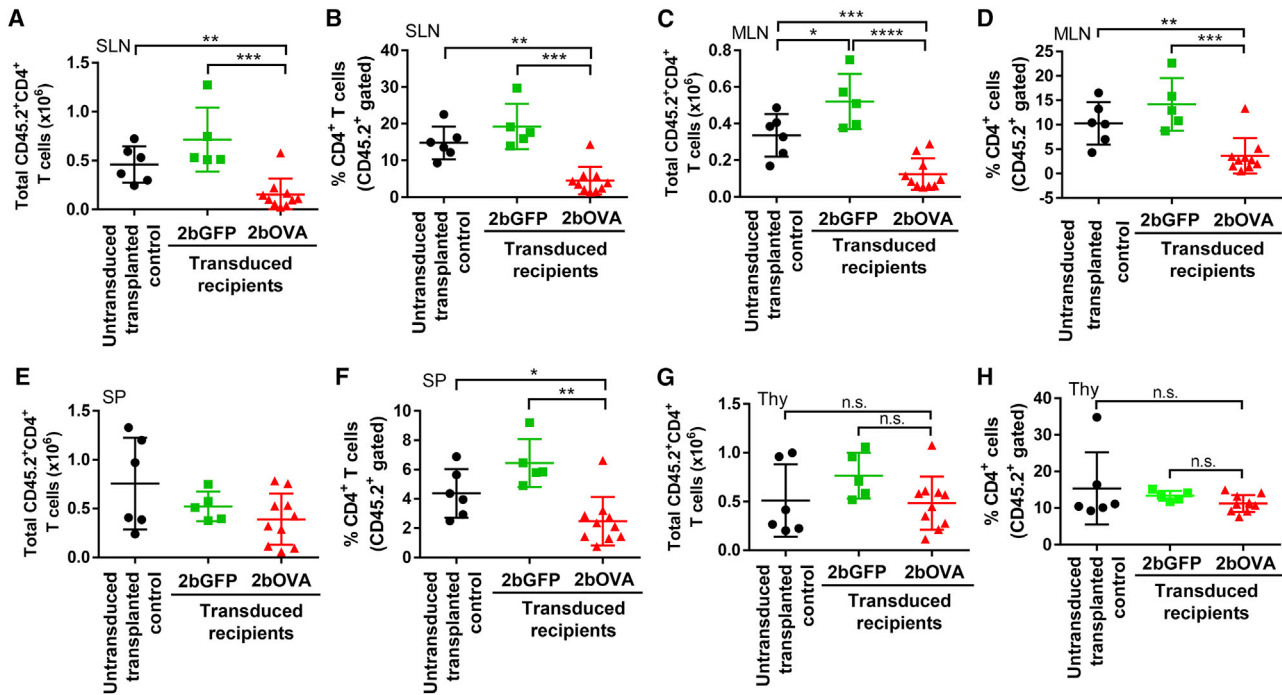
Figure 6. Flow cytometry analysis of OVA-specific CD4 T cells in the spleen, lymph nodes, and thymus of 2bOVA-transduced OVA-primed recipients (the OT-II/WT model)

Three weeks after OVA reimmunizations, recipients were euthanized and spleen (SP), superficial lymph nodes (SLNs), mesenteric lymph nodes (MLNs), and thymus (Thy) were isolated. Cells from lymphoid organs were stained with anti-mouse CD45.1, CD45.2, CD4, and CD8 antibodies. Samples from 2bGFP-transduced and untransduced transplanted recipients were used as controls in parallel. Samples were run on a BD LSR II flow cytometer, and data were analyzed using FlowJo software. Cells were gated on $CD45.2^+$ (OT-II transgenic donor-derived) and analyzed for $CD4^+$ T cells. (A) Total OVA-specific CD4 T cells in SLNs. (B) Percentage of OVA-specific CD4 T cells in SLNs. (C) Total OVA-specific CD4 T cells in MLNs. (D) Percentage of OVA-specific CD4 T cells in MLNs. (E) Total OVA-specific CD4 T cells in SP. (F) Percentage of OVA-specific CD4 T cells in SP. (G) Total OVA-specific CD4 T cells in the thymus. (H) Percentage of OVA-specific CD4 T cells in the thymus. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. n.s., not significant (for difference between the two groups).

than that in the 2bGFP group ($0.05\% \pm 0.01\%$ and $0.03\% \pm 0.01\%$, respectively) (Figures 8F and 8G). Taken together, our data from these studies showed that antigen-specific CD8 T cells were partially deleted, although platelet-OVA expression was not sustained in the OT-I/WT model. Our data indicate that there is an antagonistic dynamic process between immune reaction and immune tolerance after platelet-targeted gene therapy.

DISCUSSION

Immune tolerance induction is desired in gene therapy as well as protein infusion. Inducing immune tolerance in the primed model is much more challenging than in the unprimed model, as the immune system is already sensitized by immunogen and the mechanisms of reestablishing immune tolerance could be different.[29–31] In the current study, we showed that platelet-specific gene therapy can promote antigen-specific immune tolerance through peripheral clonal deletion of antigen-specific CD4 and CD8 T cells and the expansion of antigen-specific Treg cells even when the immune system is already activated. We found that the intensity of the pre-existing immunity affects the efficacy of platelet-targeted gene therapy, indicating that there is an antagonistic dynamic process between immune responses

and immune tolerance after platelet-targeted gene therapy in a primed model.

Our previous studies have demonstrated that using sufficient preconditioning is essential for engrafting genetically manipulated HSCs for platelet-specific gene therapy in hemophilia A mice with pre-existing immunity. The intensity of preconditioning required for sustained platelet-derived neoprotein expression in the primed model is much stronger than that for the non-primed model.[21,22] In the current study, we used a non-myeloablative preconditioning regimen of 6.6 Gy TBI, one of the optimal preconditioning regimens for immune tolerance induction in platelet gene therapy,[24] to investigate how the immune system responded to platelet-targeted gene transfer when recipients were already primed and had already developed various titers of antibodies before gene therapy. Animals with various levels of immune responses that received HSCT from the same pool of transduced cells under the same preconditioning allowed us to investigate how the intensity of immune responses impacts the efficacy in neoprotein expression and antigen-specific immune tolerance after platelet-specific gene therapy. Our studies show that effective engraftment was achieved in all of the recipients regardless of the intensity of
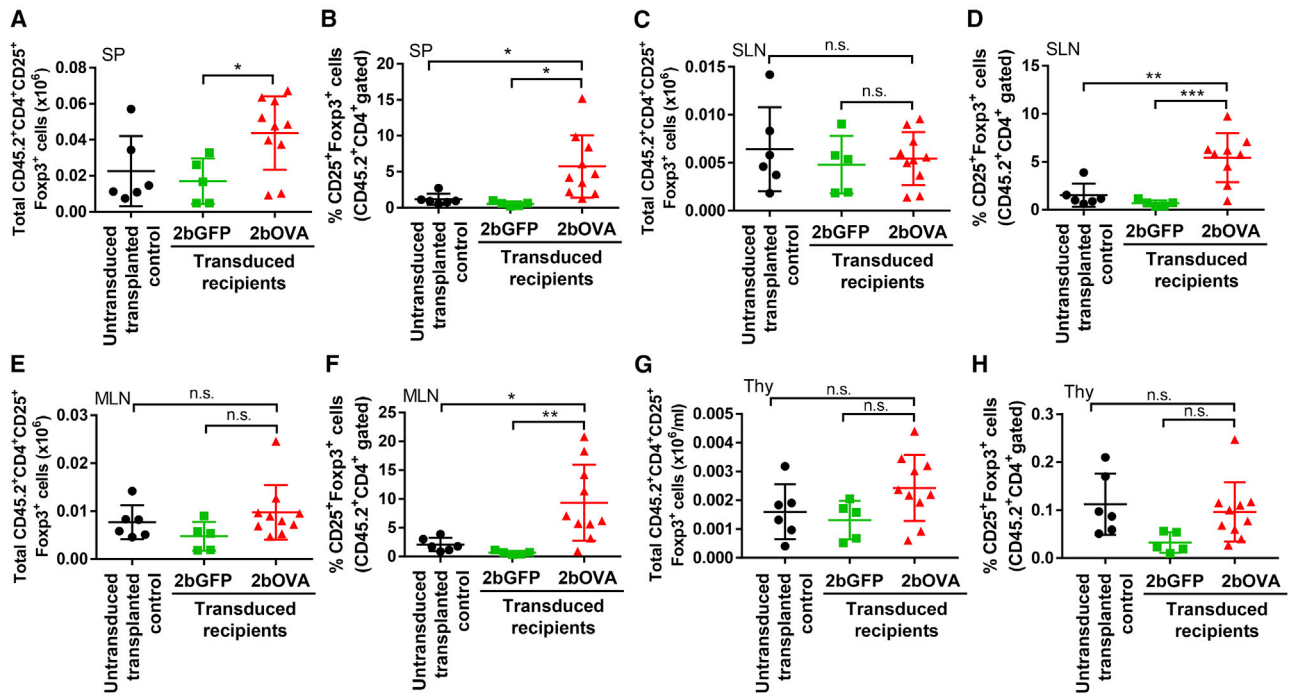
Figure 7. Flow cytometry analysis of OVA-specific Treg cells in the SP and LNs of 2bOVA-transduced OVA-primed recipients (the OT-II/WT model)

Three weeks after OVA reimmunizations, recipients were euthanized and SP, SLNs, and MLNs were isolated. Cells from lymphoid organs were stained with anti-mouse CD45.1, CD45.2, CD4, CD25, and Foxp3 antibodies. Samples from 2bGFP-transduced and untransduced transplanted recipients were used as controls in parallel. Samples were run on a BD LSR II flow cytometer, and data were analyzed using FlowJo software. Cells were gated on $CD45.2^+$ (OT-II transgenic donor-derived) and analyzed for Treg cells ($CD4^+CD25^+Foxp3^+$). (A) Total OVA-specific Treg cells in SP. (B) Percentage of OVA-specific Treg cells in SP. (C) Total OVA-specific Treg cells in SLNs. (D) Percentage of OVA-specific Treg cells in SLNs. (E) Total OVA-specific Treg cells in MLNs. (F) Percentage of OVA-specific Treg cells in MLNs. (G) Total OVA-specific Treg cells in the thymus. (H) Percentage of OVA-specific Treg cells in the thymus. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant (for difference between the two groups).

pre-existing anti-OVA immunity, but platelet-OVA expression was limited in 10%–15% of recipients that had high titers of anti-OVA antibodies before transplantation in both the WT and OT-II models.

The titer of anti-OVA total IgG peaked 5 weeks after transplantation in all three groups and then declined with time thereafter in all recipients, both transduced and untransduced. Although our animals underwent 6.6 Gy TBI followed by HSCT, the peak time of anti-OVA humoral immune responses in our model is similar to that reported previously in which OVA was immunized into naive animals without any preconditioning.[32] Furthermore, a memory anti-OVA immune response can be elicited, and CAG-OVA skin graft was rejected in control mice from the 2bGFP-transduced and untransduced transplanted groups. These results suggest that 6.6 Gy TBI preconditioning followed by HSCT does not impair the function of the immune system in terms of both humoral and cellular immune responses.

While animals were continuously exposed to platelet-OVA after receiving 2bOVA-transduced HSCs, anti-OVA immune responses in 2bOVA-transduced recipients were not boosted. Instead, the titers declined with time even after the rechallenge with exogenous OVA protein, and the skin grafts from CAG-OVA transgenic mice were successfully engrafted and maintained through the remaining life-

times of the animals. More strikingly, immune tolerance was also induced in the recipients that had a high titer of anti-OVA total IgG with a barely detectable level of platelet-OVA expression after 2bOVA gene transfer in both the WT and OT-II models. Of note, platelet-targeted gene transfer does not cause global immune suppression, as all of the animals developed anti-FVIII inhibitors after being challenged with a high dose of rhF8, and the titers were comparable to those in the 2bGFP and untransduced control groups. Thus, results from our current study demonstrate that strong antigen-specific immune tolerance can be reestablished in the primed model after platelet-specific gene delivery to HSCs.

It is still unclear why platelet-OVA expression was diminished when animals had high immune reactivity before gene transfer. The platelet counts in the recipients with high-titer anti-OVA IgG were similar to those in other recipients (data not shown), indicating that it might not be due to the destruction of platelets containing OVA. We speculate that there were some high-reactive OVA-specific immune cells that persisted even after 6.6 Gy TBI preconditioning in the animals with high pre-existing immunity and that early stage megakaryocyte progenitors were killed. In these progenitors, the IIb promoter is activated, but the cells do not have storage granules to store neoprotein OVA. Thus, the progenitors that produced neoprotein OVA might
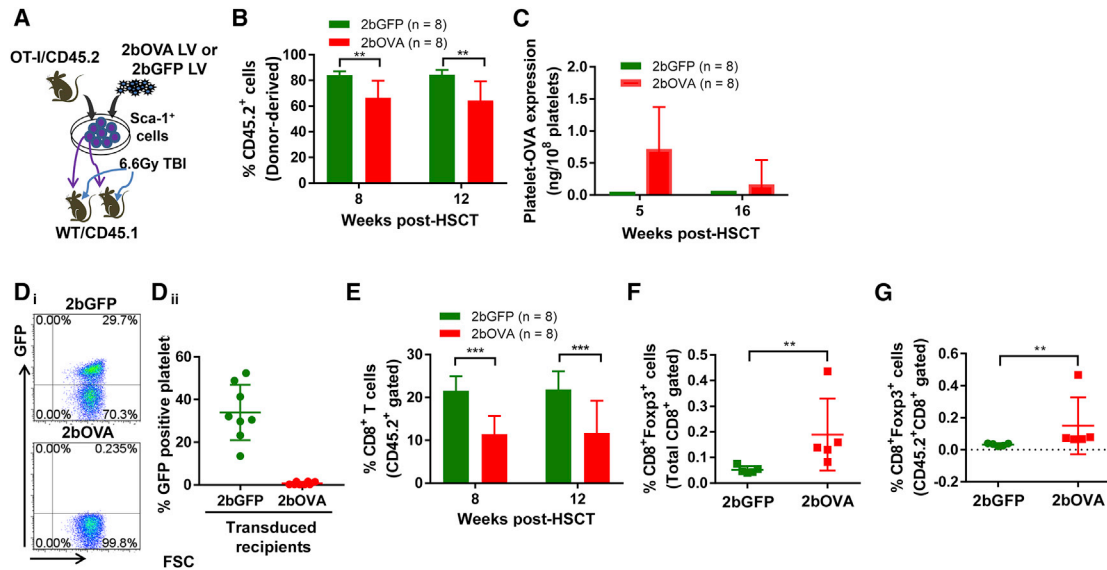
Figure 8. Assessment of OVA expression and antigen-specific CD8 T cell responses in the OT-I/WT model

In this model, recipients (WT/CD45.1) were preconditioned with 6.6 Gy followed by transplantation of 2bOVA-transduced Sca-1^{+} cells from OVA-specific CD8 TCR transgenic (OT-I/CD45.2) mice. Animals were analyzed by flow cytometry for engraftment and immune cells, and ELISA for OVA expression. (A) Schematic diagram of experimental design for the primed OT-I/WT model. (B) Engraftments in recipients after BM reconstituted at weeks 8 and 12 after HSCT. (C) OVA expression levels in transduced recipients determined by ELISA on platelet lysates. (D) Flow cytometry analysis of platelets for GFP expression. Blood samples were collected at week 12 after HSCT and stained for platelet surface markers GPIb (CD42) and GPIIb (CD41). Cells were gated on CD41^{+}CD42^{+} and analyzed for GFP expression. Representative dot plots are shown in (Di), and summarized data are shown in (Dii). (E) Percentage of OVA-specific CD8 T cells (CD45.2^{+}CD8^{+}) in recipients. Cells were gated on CD45.2^{+} (OT-I transgenic donor-derived) cells and analyzed for CD8^{+} T cells. (F) Percentage of total CD8^{+}Foxp3^{+} Treg cells in recipients. Cells were gated on CD8^{+} and analyzed for Foxp3^{+} cells. (G) Percentage of OVA-specific CD8^{+}Foxp3^{+} Treg cells in recipients. Cells were gated on CD45.2^{+}CD8^{+} and analyzed for Foxp3^{+} cells. **p < 0.01, ***p < 0.001.

be eliminated by high-reactive immune cells that remained after preconditioning with sublethal 6.6 Gy TBI, resulting in limited levels of platelet-OVA in these mice. While 6.6 Gy TBI preconditioning is the optimal preconditioning regimen for platelet-targeted gene therapy of the non-primed model,[24,26] our current study indicates that it may be suboptimal for the primed model if the immune system is highly reactive, which may require much more intense preconditioning. This is in agreement with our previous findings in platelet-targeted FVIII expression in hemophilia A mice with pre-existing anti-FVIII immunity, in which busulfan preconditioning alone was insufficient for platelet-FVIII gene therapy, but busulfan plus anti-thymocyte globulin or a low dose of TBI was sufficient.[22] Further studies are warranted to elucidate which population of cells gets killed, how cells get killed, and who is responsible as the killers in platelet gene therapy for the primed model with high-reactive immune responses.

It has been shown that TBI can not only create space in BM for engraftment but also synchronize thymocyte development progression.[33] Our data from both the unprimed (previous study[26]) and primed (the current study) models demonstrate that immune tolerance induced after platelet-specific gene therapy is peripheral, not central, tolerance. Using the OVA-specific TCR transgenic model, we confirmed that after 2bOVA gene transfer, antigen-specific CD4 T cells were deleted in peripheral blood, lymph nodes, and spleen in OVA-primed mice. The antigen-specific Treg cells were expanded.

Our results show that percentages of antigen-specific CD4 T and Treg cells in peripheral blood correlate with the levels of platelet-OVA expression. The animal that had the highest anti-OVA total IgG titer expressed the lowest platelet-OVA (0.58 mU/10[8] platelets) and had the highest percentage (8.2%) of OVA-specific CD4 T cells at the early time point (week 6) after transplantation. The OVA-specific CD4 T cells declined with time to 1.9% by the time the animal was euthanized at week 26 for terminal experiments. Importantly, the animal was tolerized to the OVA protein rechallenge.

The antagonistic process between immune responses to the neoprotein and clonal deletion of antigen-specific CD4 T cells and the expansion of antigen-specific Treg cells determines whether immune tolerance can be achieved after gene transfer. If a preconditioning regimen does not sufficiently quench down the highly reactive immune system, early stage megakaryocyte progenitors may be eliminated. Alternatively, if certain levels of platelets containing OVA are achieved, this may gradually eliminate the antigen-specific CD4 T cells and induce antigen-specific Treg cells to expand and reestablish antigen-specific immune tolerance. Our results from the OT-I/WT model support the antagonistic process between immune responses to neoprotein and clonal deletion of antigen-specific T cells. Platelet-targeted gene transfer might induce clonal deletion of antigen-specific CD8 T cells, resulting in antigen-specific cellular tolerance. However, an excessive number of antigen-specific CD8

T cells might destroy 2bOVA-transduced cells if those cells were not effectively eliminated by platelet-derived OVA after platelet-targeted gene transfer. What threshold of antigen-specific CD8 T cells could be fully tolerized by platelet-targeted gene transfer, and how platelet gene therapy induces clonal deletion of antigen-specific CD4 and CD8 T cells are subjects under investigation by our group.

In summary, our studies demonstrate that robust antigen-specific immune tolerance can be achieved after platelet-specific gene therapy via peripheral clonal deletion of antigen-specific CD4 and CD8 effector T cells and expansion of antigen-specific Treg cells. Although modulating existing immune responses and reestablishing tolerance is more challenging than preventing a primary immune response,[29] our studies strongly suggest that platelet-targeted gene therapy is a promising approach to induce antigen-specific immune tolerance for the treatment of diseases with undesired immune responses, such as hemophiliacs and autoimmune diseases.

MATERIALS AND METHODS

Antibodies and reagents

Fluorophore directly conjugated anti-CD45.1, anti-CD45.2, anti-CD4, anti-CD8, anti-CD25, anti-Foxp3, and anti-B220 monoclonal antibodies (mAbs) were purchased from eBioscience (San Diego, CA, USA). Anti-mouse CD41 mAb directly conjugated with phycoerythrin (PE) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse CD42b mAb conjugated with DyLight 649 was purchased from Emfret Analytics (Eibelstadt, Germany). A Foxp3 transcription factor staining buffer set was purchased from eBioscience. Mouse BD Fc Block (purified rat anti-mouse CD16/CD32) was purchased from BD Pharmingen (Franklin Lakes, NJ, USA). The EasySep mouse SCA1 positive selection kit was purchased from STEMCELL Technologies (Cambridge, MA, USA). X-VIVO 10 media were purchased from Lonza (Walkersville, MD, USA). The QIAamp DNA blood mini kit was purchased from QIAGEN (Germantown, MD, USA). GoTaq green master mix was purchased from Promega (Madison, WI, USA). OVA protein was purchased from Sigma (St. Louis, MO, USA; catalog no. A2512, grade VI). 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; a zwitterionic detergent) was purchased from MP Biomedicals (Solon, OH, USA). A chicken egg OVA ELISA kit was purchased from Alpha Diagnostic International (San Antonio, TX, USA). Recombinant human full-length FVIII (rhfF8; Kogenate FS) was from Bayer Healthcare (Whippany, NJ, USA).

Mice

WT/CD45.1, WT/CD45.2, OT-II/CD45.2 (in which 98% of CD4$^+$ T cells express major histocompatibility complex class II-restricted OVA$_{323-339}$-specific Vα2Vβ5 TCR[34]), OT-I/CD45.2 (in which 98% of CD8$^+$ T cells express OVA$_{257-264}$-specific Vα2Vβ5 TCR),[35] and Act-mOVA (in which OVA is expressed under control of the chicken β-actin promoter and detected in all tissues[36]) mice used in this study were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in our animal facility. All animals used in this study were of a C57BL/6 genetic background. Isoflurane or xyla-

zine/ketamine were used for anesthesia. All mice were kept in pathogen-free micro-isolator cages in the animal facilities operated by the Medical College of Wisconsin. Animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

OVA-primed mouse model

All WT/CD45.1 and WT/CD45.2 mice were immunized with full-length OVA protein by i.p. injection at a dose of 100 μg/mouse and boosted with 20 μg/mouse 2 weeks later to induce anti-OVA antibody development to establish the OVA-primed model. One week after the second immunization, 100 μL of blood was collected from a retro-orbital bleed and plasma was isolated to determine the titers of anti-OVA total IgG using ELISA as previously described.[25,26] The animals that developed anti-OVA total IgG were defined as OVA-primed mice and used for the following research.

HSC transduction and transplantation

2bOVA LVs were produced as described in our previous study.[26] Sca-1$^+$ cells were isolated from either OVA-primed WT/CD45.2, OT-II/CD45.2, or OT-I TCR transgenic donor mice using the EasySep mouse SCA1 positive selection kit following the protocol provided by the manufacturer. Sca-1$^+$ cells were transduced with 2bOVA or 2bGFP LV as previously described.[26,37] Untransduced Sca-1$^+$ cells were cultured in parallel as a control for the primed model studies. After transduction, approximately 1×10^6 cells in 200 μL of X-VIVO 10 media were transplanted via retro-orbital injection into each OVA-primed WT/CD45.1 recipient that was preconditioned with a sub-lethal 6.6 Gy of TBI. Age- and sex-matched animals with comparable levels of anti-OVA IgG were randomly assigned to the three groups receiving the transplantation of either 2bOVA- or 2bGFP-transduced cells or untransduced Sca-1$^+$ cells. Animals were analyzed starting at 4 weeks after transplantation. Blood samples were collected by retro-orbital bleeds using 1:10 of 3.8% sodium citrate anticoagulant, and whole blood counts were performed using the Vet ABC hematology analyzer (Scil Animal Care Company, Gurnee, IL, USA). Plasma, leukocytes, and platelets were isolated as previously described[21] for assays.

OVA expression and immune response studies

To determine OVA expression in 2bOVA-transduced recipients, two assays, that is, (1) PCR detection of proviral OVA expression in peripheral blood cell-derived DNA and (2) ELISA determination of OVA protein expression levels in platelets, were performed following the procedures as previously described.[26] Samples from 2bGFP-transduced and untransduced transplanted recipients were used as controls in parallel.

To investigate immune responses in recipients with pre-existing anti-OVA immunity after platelet-specific OVA gene transfer, anti-OVA total IgG titers were monitored. After BM was fully reconstituted, OVA expression and anti-OVA immune responses were assessed, and recipients from both the WT/WT and OT-II/WT models were rechallenged with OVA at a dose of 20 μg/mouse/week via i.p.

administration twice. One week after OVA reimmunization, plasmas were collected for ELISA to determine the titers of anti-OVA total IgG.

Some recipients from the WT/WT model subsequently received skin graft transplantation from Act-mOVA transgenic mice as previously described[26] to investigate the impact of platelet-targeted gene therapy on T cell immune responses in the primed model after platelet-targeted gene transfer. Rejection was recorded as the day when the graft was no longer attached to the recipient.

To investigate whether platelet-targeted gene transfer affects immune responses to an unrelated protein, some recipients from the WT/WT model were immunized with rhF8 at a dose of 200 U/kg/week for 4 weeks by intravenous injection. One week after the last rhF8 immunization, plasmas were collected and anti-FVIII inhibitor titers were determined by a modified Bethesda assay.[38] Recipients from the 2bGFP-transduced and untransduced transplanted controls were used in parallel.

Flow cytometry analysis

Flow cytometry analysis was used to determine chimerism, T cell populations, and 2bGFP transduction efficiency in transplantation recipients. Leukocytes from peripheral blood, lymph nodes, spleen, and thymus were stained for cell markers CD45.1, CD45.2, CD4, CD8, B220, CD25, and Foxp3 as described in our previous study.[26] To determine the 2bGFP LV transduction efficiency, platelets were stained for CD41 and CD42 as described in our previous study[39] and analyzed for GFP expression. All samples were run on a BD LSR II flow cytometer (BD Biosciences, Sparks, MD, USA) and analyzed using FlowJo software (FlowJo, Ashland, OR, USA). Samples from WT/CD45.1 and WT/CD45.2, OT-II/CD45.2, or OT-I/CD45.2 mice were used as controls.

Statistical analysis

All data are presented as the mean ± SD, and statistical comparisons of two experimental groups were evaluated by the unpaired two-tailed Student's t test or Mann-Whitney test when data failed in the normality test. The one- or two-way analysis of variance followed by the Tukey test was used to determine whether there were statistically significant differences among the means of three or more groups. The Kruskal-Wallis test followed by Dunn's test was used to compare the groups when data failed in the normality test. A value of $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.12.026.

AUTHOR CONTRIBUTIONS

J.L. designed the study, performed experiments, and analyzed data; J.C. performed experiments and analyzed data; J.A.S. performed experiments and provided comments on the manuscript; J.H. provided administrative support for J.L.; C.B.W. helped to design research; and Q.S. designed research, analyzed data, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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