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Neoantigen and tumor antigen-specific immunity transferred from immunized donors is detectable early after allogeneic transplantation in myeloma patients

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Abstract

To enhance the therapeutic index of allogeneic hematopoietic stem cell transplantation (HSCT), we immunized ten HLA-matched sibling donors prior to stem cell collection with recipient-derived clonal myeloma immunoglobulin, idiotype (Id), as a tumor antigen, conjugated with keyhole limpet hemocyanin (KLH). Vaccinations were safe in donors and recipients. Donor-derived KLH- and Id-specific humoral and central and effector memory T cell responses were detectable by day 30 after HSCT and were boosted by post-transplant vaccinations at 3 months in most recipients. One patient died prior to booster vaccinations. Specifically, after completing

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CONFLICTS OF INTEREST

The authors declare that there are no competing financial interests.

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treatment 8/9 myeloma recipients had persistent Id-specific immune responses and 5/9 had improvement in disease status. Although regulatory T cells increased after vaccination, they did not impact immune responses. At a median potential follow-up period of 74 months, six patients are alive, the 10 patients have a median progression-free survival of 28.5 months, and median overall survival has not been reached. Our results provide proof of principle that neoantigen and tumor antigen-specific humoral and cellular immunity could be safely induced in HSCT donors and passively transferred to recipients. This general strategy may be used to reduce relapse of malignancies and augment protection against infections after allogeneic HSCT.

Keywords

myeloma; transplantation; allogeneic; donor vaccination; idiotype

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) can eradicate hematologic malignancies through a combination of cytotoxic therapy and non-specific, immune-mediated effects of the allograft (1–3). However, relapse remains a significant cause of treatment failure (4) and novel strategies are necessary to enhance the graft-versus-tumor (GVT) effect. Here, we immunized HSCT donors with a patient-derived tumor antigen with the goal of inducing antitumor immunity in the donors prior to HSC collection and passively transferring the immunity to recipients by HSCT. Since most tumor antigens are self-antigens and vaccination with self-antigens may potentially induce autoimmunity, the use of a tumor-specific antigen is necessary for this approach in order to safely administer the vaccine to donors.

The clonal immunoglobulin produced by multiple myeloma (MM) cells has unique amino acid sequences within the variable regions termed, idiotype (Id), that are distinct from normal immunoglobulins and therefore can serve as tumor-specific antigen for therapeutic vaccination (5),(6). Active immunization with tumor-derived Id conjugated to a carrier, keyhole limpet hemocyanin (KLH) and administered together with granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjuvant was found to be highly immunogenic (7, 8) and, was recently shown to improve clinical outcome in follicular lymphoma patients in a randomized phase III trial (9). However, the immunogenicity of Id vaccines in MM patients was disappointing possibly due to self-tolerance and/or immunosuppressive state of the patients resulting from therapy or the disease (10–12). Vaccination of HSCT donors with a healthy immune system may potentially circumvent the barriers for active immunotherapy against tumors in recipients (13,14). In a limited number of MM patients undergoing allogeneic or syngeneic bone marrow transplantation, immunization of their respective donors with MM Id was safe and induced Id-specific humoral and cellular immunity (15–17).

The current study is distinct from our prior study by virtue of the following: (1) our prior study did not permit demonstration of transfer of vaccine-induced immunity to either KLH or Id, because the prior protocol design included *pre*-transplant vaccination of the recipients

(as well as the donors (15–18); (2) Peripheral blood stem cell grafts may contain up to 10 times more lymphocytes than bone marrow grafts and may result in more rapid donor lymphoid engraftment (19–21) and enhance the transfer of humoral and cellular immunity. Thus, the current study uses blood stem cells as the transfer element (instead of marrow); and (3) the current study uses a reduced intensity cytotoxic regimen (instead of myeloablation) (22). The scientific hypotheses tested in the current study of 10 donor-recipient pairs were: (1) that cellular immunity to a tumor antigen could be transferred directly from donor to recipient, and (2) that donor-derived immunity could be boosted by MM recipient vaccination post-transplantation.

MATERIALS AND METHODS

Subjects

Patients were ≥18 years of age, had IgG or IgA MM, a sibling that matched at 6/6 or 5/6 HLA antigens, adequate organ functions, and an M-protein concentration in plasma that was at least 70% of the total immunoglobulin of the corresponding isotype at study entry. After plasmapheresis for vaccine generation, patients were required to achieve at least a partial remission following conventional chemotherapy. The protocol was approved by the National Cancer Institute Institutional Review Board, and informed written consent was obtained from all patients and donors.

Treatment

While recipients were receiving conventional chemotherapy with etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, and fludarabine (EPOCH-F), a novel salvage regimen for MM before reduced-intensity allogeneic HSCT (22), donors were immunized with three subcutaneous injections of Id-KLH+GM-CSF vaccine at 10, 8, and 4 weeks before hematopoietic stem cell donation (Fig. 1). Approximately 3–4 weeks after the final immunization, donors received filgrastim (10 μg/kg/day) subcutaneously for hematopoietic stem cell mobilization and starting on day 5 donors underwent daily aphereses until a minimum of 3×10^6 $CD34^+$ cells/kg-recipient weight were obtained and cryopreserved.

MM patients received a reduced-intensity conditioning regimen consisting of fludarabine and cyclophosphamide as previously described (22). GVHD prophylaxis consisted of cyclosporine for 180 days plus methotrexate (5 mg/m^2) on days +1, +3, +6, and +11 post-transplant. MM patients were vaccinated with three subcutaneous injections of Id-KLH +GM-CSF at 3, 4, and 6 months post-transplant. Responses were evaluated using the European Group for Blood and Marrow Transplantation criteria available at the time this study was performed (23).

Vaccine formulation and Immune assays

Details on these are provided in Supplementary Methods.

Statistical analysis

A paired t-test or Wilcoxon signed-rank test was used to evaluate differences in immune responses or $Foxp3^+$ T cells between different time points. Overall survival and progression-free survival were determined by the Kaplan-Meier method. All *P* values are two-tailed.

RESULTS

Safety in donors

Ten MM patients and their respective HLA-matched sibling donors were enrolled in this study (Table 1; Supplementary Table 1). All donors completed their scheduled vaccinations. Common adverse effects (AEs) included grade 1–2 injection site reactions, arthralgia, myalgia, or bone pain with vaccination. One donor experienced grade 3 lymphopenia; another donor experienced grade 3 thrombocytopenia, hypophosphatemia, and hypokalemia. All AEs resolved within 4 weeks after completing vaccinations and no long-term AEs were noted after a minimum of 12 months follow-up.

Recipient characteristics and clinical outcome

All 10 recipients engrafted; median donor T-cell ($CD3^+$) chimerism at 28 days post-transplant was 100% (range, 97–100%). Grade II-IV acute GVHD was noted in 4/10 recipients. All nine evaluable patients developed chronic GVHD (limited=5; extensive=4). Nine recipients completed their post-transplant vaccinations (R2–R10). One recipient died 69 days post-transplant and did not receive vaccinations (Table 1). Transient grade 1–2 toxicities observed with vaccinations in recipients included injection site reactions, arthralgia, and elevated liver function tests. Transient grade 3 toxicities, including rigors, hypotension, dyspnea, and/or elevated liver function tests, were noted in 5 patients. Five of 9 patients who were evaluable at day 100 had improvement in their disease status post-transplant (Table 1). Three patients died of transplant-related complications. Six recipients were alive after a median potential follow-up of 74.3 months (for all 10, potential range: 57–117 months). Median progression-free survival is 28.5 months. Median overall survival has not been reached. Two recipients remain in complete remission, 60 and 57 months post-transplant, respectively, without further therapy (Table 1).

Induction and transfer of antibody responses

Antibodies to KLH were detected in all donors (Supplementary Table 1). Antibody responses were of both IgM and IgG isotypes in all 10 donors (Figs. 2 A,B; Supplementary Figs. 1 A,B). In the recipients, anti-KLH antibody responses were detected as early as 30 days post-transplant in all 9 patients assessed. Similar to the donors, the anti-KLH antibody responses in the recipients were of both IgM and IgG isotypes and increased significantly after post-transplant immunizations (Figs. 2 C,D; Supplementary Figs. 1 C,D).

Anti-Id antibody responses were induced in 7/10 donors (D2, D4, D5, D6, D7, D8, and D9) assessed (Fig. 2E; Supplementary Fig. 1E; Supplementary Table 1). Low anti-Id antibody titers were detectable in 6/9 recipients in the immediate post-transplant period but were amplified significantly in only three (R2, R6 and R8) after post-transplant immunizations (Fig. 2F; Supplementary Fig. 1F). Anti-Id antibodies in donors and recipients specifically

bound to the vaccinated Id protein but not to isotype-matched irrelevant Id protein with the exception of recipient 2 who had a polyreactive anti-Id antibody response (Figs. 2 G,H; Supplementary Fig. 1F). Together, these results suggest that humoral immunity can be induced against neoantigen in all donors, against tumor antigen in most, but not all donors, and both passively transferred to the recipients. Furthermore, antibody responses can be boosted by post-transplant immunizations in the recipients.

Induction and transfer of cellular responses

Postvaccine PBMC from all donors responded to KLH by producing substantial amounts of T_H1-like cytokines, IL-2, TNF-α, GM-CSF, and IFN-γ, compared with prevaccine PBMC (Fig. 3A; Supplementary Table 1). Interestingly, we also observed production of T_H2-like cytokines, IL-5, IL-10, and IL-13 (Fig. 3A; Supplementary Table 1). Anti-KLH T_H1 and T_H2 responses were detected as early as 30 days post-transplant in all 9 recipients assessed and were boosted by post-transplant immunizations in 8/8 recipients assessed (Fig. 3B). Remarkably, the cytokine production profile in the recipients was comparable with the respective donors before and after post-transplant immunizations. For example, donor 4 and recipient 4 did not produce GM-CSF, donor 10 and recipient 10 did not produce IL-10, and IL-4 was not produced by any of the donor or recipient PBMC (Figs. 3 A,B).

Anti-Id T_H1 and T_H2 cytokine responses were observed in 5 and 6 of the 10 donors, respectively. Altogether 7/10 donors had Id-specific cellular immune responses (Fig. 4A and Supplementary Table 1). Anti-Id T_H1 or T_H2 cellular responses were observed in all 8 recipients assessed (R1 and R9 were not evaluable) (Figs. 4B–D) and were enhanced by post-transplant immunizations in 5 recipients (R2, R3, R5, R6, and R10) (Figs. 4 C,D). More importantly, 4 of 5 patients that had improvement in their disease post-HSCT (Table 1) had evidence of transfer of Id-specific immunity (R2, R3, R8, and R10; R9 was not evaluable). Together, these results suggest that similar to humoral immune responses, anti-KLH and anti-Id cellular immune responses were induced by vaccination in the donors and were passively transferred by HSCT to all 8 recipients that were assessed. Furthermore, the transferred cellular immune responses were boosted by post-transplant immunizations in the recipients.

Effector and central memory T cells were induced by vaccination

Using intracellular cytokine assay, we confirmed and further characterized the T-cell origin of the cytokine responses above. We confirmed that KLH-specific $CD4^+$ T cells were significantly higher in frequency in postvaccine PBMC compared with prevaccine PBMC in 9/9 donors assessed ($P<0.05$; Figs. 5 A,B; Supplementary Fig. 2A). KLH-specific $CD4^+$ T cells could be detected 90 days post-transplant in 8/8 recipients assessed and could be transferred from the donor to the recipient (Supplementary Fig. 2B). Moreover, they increased further after post-transplant immunizations in 6 recipients (Figs. 5 C,D; Supplementary Fig. 2A). The KLH-specific $CD4^+$ T cells were of both effector ($CD27^{+/-}CD62L^-$) and central ($CD27^+CD62L^+$) memory phenotype in both donor-recipient pairs analyzed (Supplementary Fig. 2C).

Regulatory T cells in donors and recipients

To determine whether regulatory T cells (Tregs) were induced by vaccination, we assessed the number of $Foxp3^+$ T cells by flow cytometry and Epitest assay (24). The absolute number of Tregs increased significantly in 9/10 donors after first vaccination as compared to prevaccine levels (D2–D10: $P<0.01$, paired *t*-test; Fig. 6A). However, Tregs declined to baseline levels after second and third vaccinations (data not shown). Tregs also increased significantly in 7/8 recipients after the three post-transplant immunizations as compared to determination at 90–100 days post-transplant ($P<0.05$, paired *t*-test; Fig. 6B). The change in Treg numbers showed similar trend when their numbers were estimated using two different techniques, flow cytometry and methylation status of Foxp3 gene (24). Since Foxp3 may be induced in activated T cells, we determined the cytokine production profile of Tregs. $CD4^+Foxp3^+$ T cells did not produce TNF-α and IL-2 and cytokine-producing KLH-specific $CD4^+$ T cells did not express Foxp3, suggesting that $Foxp3^+$ cells are not activated T cells (Fig. 6C). Although Tregs are expected to be immunosuppressive, they did not correlate with KLH- or Id-specific humoral or cellular immune responses in donors or recipients (data not shown).

DISCUSSION

Here, we demonstrated that humoral and cellular immunity could be safely induced against a candidate tumor antigen (Id) in HSCT donors and can be passively transferred to all MM patients. Furthermore, initially weak immune responses against tumor antigen could be boosted by additional post-transplant vaccinations in the recipients (Figs. 2C,D,F,H, 3B, 4C,D, and Supplementary Figs. 1C,D,F). These findings, taken together with chimerism studies showing that the T cells were 100% donor origin by day 30 and strong evidence of concurrent immunity to KLH, a neoantigen to which the transplant recipients had no prior exposure, strongly suggest that vaccine-induced tumor antigen immunity can be passively transferred to the recipients and is demonstrable very early after HSCT. Remarkably, the immune responses against both KLH and Id were observed while patients were on immunosuppressive GVHD prophylaxis. Finally, the detection of both antigen-specific effector and central memory T cells suggests that vaccinating donors might result in both immediate and long-lasting immunity in recipients.

We noted improvement in disease status in 5/9 patients who were evaluable at 100 days after HSCT (Table 1). Because of limited sample size, it is not possible to draw definitive conclusions about correlation between immune responses and clinical outcomes. Nevertheless, we found that improvement in disease post-HSCT correlated with transfer of Id-specific immunity in all 4 patients that were assessable. Evidence of autoimmunity was not detected in the donors or recipients. Long-term survival lasting beyond 57 months was observed in 6/7 patients who did not die of transplant-related complications (Table 1). Future randomized studies are warranted to determine clinical efficacy of donor immunization.

Nonetheless, our results may have implications for improving the therapeutic index of allogeneic HSCT. First, donor immunization may be used as a general strategy to enhance the GVT effect in MM and other malignancies for which defined tumor-specific antigens

exist. Second, vaccinating donors against infectious pathogens may be a potential strategy for prophylaxis against complications of early and late infections in the recipients. Indeed, enhanced antibody titers against infectious disease antigens were demonstrated in transplant recipients receiving hematopoietic grafts from donors immunized with infectious disease vaccines (25–30). However, these studies did not formally demonstrate transfer of vaccine-induced immunity, because they used recall antigens, and cellular immunity was not determined. Furthermore, passive transfer of preexisting virus-specific T-cell immunity from unimmunized HSCT donors to recipients was shown to be inefficient (31, 32). Compared with adoptive transfer of donor-derived *ex vivo* expanded viral antigen-specific T cells (33, 34), active immunization of the donors may be more appealing and potentially more beneficial because of the ease of administering the vaccine and induction of both humoral and cellular immunity.

The KLH in our vaccine formulation also served as an internal control to assess the immunocompetency of donors and recipients. Heterogeneity was observed for both KLH- and Id-specific immune responses in donors and recipients. Overall, though, the magnitude of the humoral and cellular immune responses to KLH was substantially higher than Id-specific immune responses in both donors and recipients, as might be expected for an exogenous neoantigen (Figs. 2–4 and Supplementary Fig. 1). These results also suggest that although myeloma Id is considered to be a tumor-specific antigen, its immunogenicity in healthy donors might be limited by mechanisms of self-tolerance due to presence of shared epitopes between the tumor Id and host immunoglobulins. Indeed, polyreactive immune responses against Id were reported in Id vaccine studies suggesting presence of shared epitopes (35–37). Finally, it should be noted that evaluation of immune responses in cryopreserved, compared with fresh PBMC, may have underestimated the immunogenicity of the vaccine in donors and recipients, and may explain why post-transplant anti-Id immune responses were observed in R3 (Fig. 4C) even though they were not detected in D3 (Supplementary Table 1). Nevertheless, following protocol treatment, all 8 recipients that were assessable had detectable antibody or cellular responses, or both, to Id.

The increase in Tregs after vaccination in donors and recipients (Figs. 6A,B) is also rather intriguing and may provide a possible explanation for the lower immunogenicity of Id. Although the increased Tregs in recipients may be part of normal immune reconstitution after HSCT, induction of Tregs by the Fc region of Id (38) or by shared epitopes (39) following vaccination may have contributed to increased Tregs in both donors and recipients. Although Tregs did not negatively correlate with induction of immune responses, they may still suppress antitumor immunity in the tumor microenvironment. Therefore, future studies using alternative vaccine constructs, including those formulated with only the variable regions of tumor immunoglobulin (35, 37) or combination with strategies to deplete Tregs may be desirable.

While we demonstrated that Id-specific immunity could be passively transferred in the early post-transplant setting, it remains unclear as to whether the infusion of lymphocytes from vaccinated donors at this time point results in optimal antitumor activity. Lymphocytes infused in the early post-transplant period are exposed to immunosuppressive agents such as calineurin inhibitors (e.g. cyclosporine) and anti-proliferative agents (e.g. methotrexate,

mycophenolate mofetil), which are necessary to prevent GVHD. These agents may either blunt or eliminate antigen-specific lymphocytes (40). As such, it may be necessary to infuse additional cells at later time points when the risk of GVHD is less or boost immune responses through post-transplant vaccinations. Indeed, based on recent studies that suggest presence of high-levels of homeostatic cytokines (e.g. IL-7, IL-15) after immune depletion (41), it could be hypothesized that vaccinations earlier in the post-transplant setting may enhance the frequency of Id- and KLH-specific responses more than what was observed in our study.

In summary, our results provide proof of principle that cellular immunity induced against a model neoantigen (Fig. 3B) and tumor-specific antigen (Fig. 4 B–D) in the HSCT donors can be transferred to MM patients. The conclusion is important, because prior studies, including donor-derived Ig allotypes in recipients, largely investigated transfer of humoral immunity from donors to recipients (25, 26). Second, donor-derived immunity could be boosted by recipient vaccination. The most definitive data was for boosting of humoral immunity (Fig. 2C,D, H) while further increases of T cells was more variable. This variability may have been due to the fact that detection of antigen-specific T cells may be more sensitive to variables of compartmentalization (blood vs. trafficking to tissues) and the timing of sample acquisition for immune response analysis (generally 4 weeks, rather than 2 weeks, after vaccination) may have not been optimal for detecting peak cytotoxic T-cell responses. A third, unexpected, conclusion was that this transferred donor-derived immunity can be detected relatively early after allotransplant, within the first 30 days, despite iatrogenic immunosuppression (GvHD prophylaxis), suggesting that for future trials it may be feasible to administer recipient booster vaccinations earlier than day 100. Finally, donor vaccination was associated with only transient, acceptable toxicity. These conclusions have been used to design and activate a subsequent randomized Phase II trial of donor vaccination in MM with a primary clinical endpoint.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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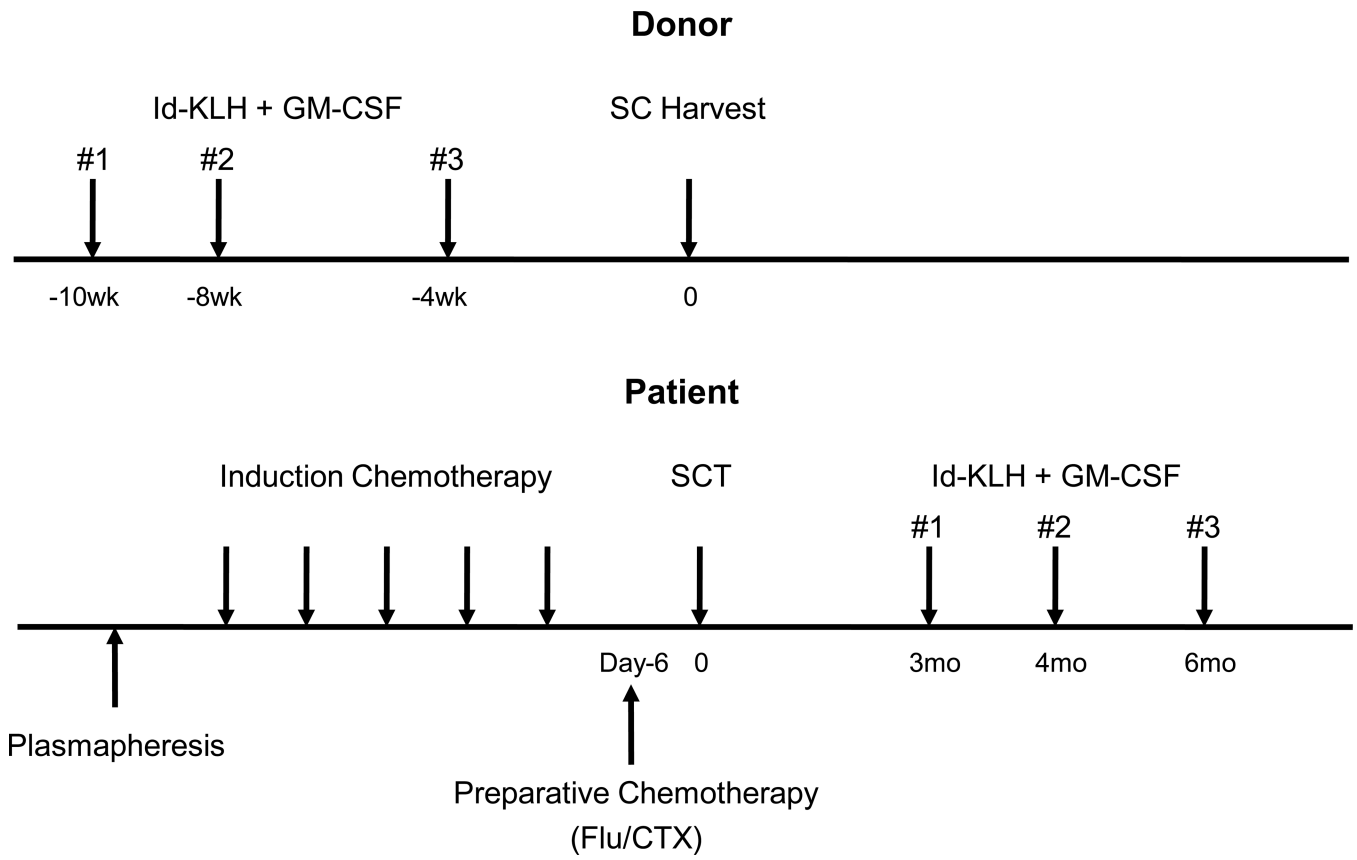


Figure 1. Clinical trial schema

Multiple myeloma (MM) patients underwent plasmapheresis after enrollment to isolate myeloma idiotype (Id) from plasma and were treated with three to five cycles of etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, and fludarabine (EPOCH-F) induction chemotherapy every 21 days. While MM patients were receiving EPOCH-F, their HLA-matched sibling donors were immunized with Id-KLH+GM-CSF vaccine three times at the indicated time points and peripheral blood stem cells were harvested 3–4 weeks after third vaccination. Patients received reduced-intensity preparative chemotherapy with fludarabine and cyclophosphamide (Flu/CTX) prior to stem cell transfer. MM Patients received three post-transplant immunizations with Id-KLH+GM-CSF vaccine at the indicated time points.

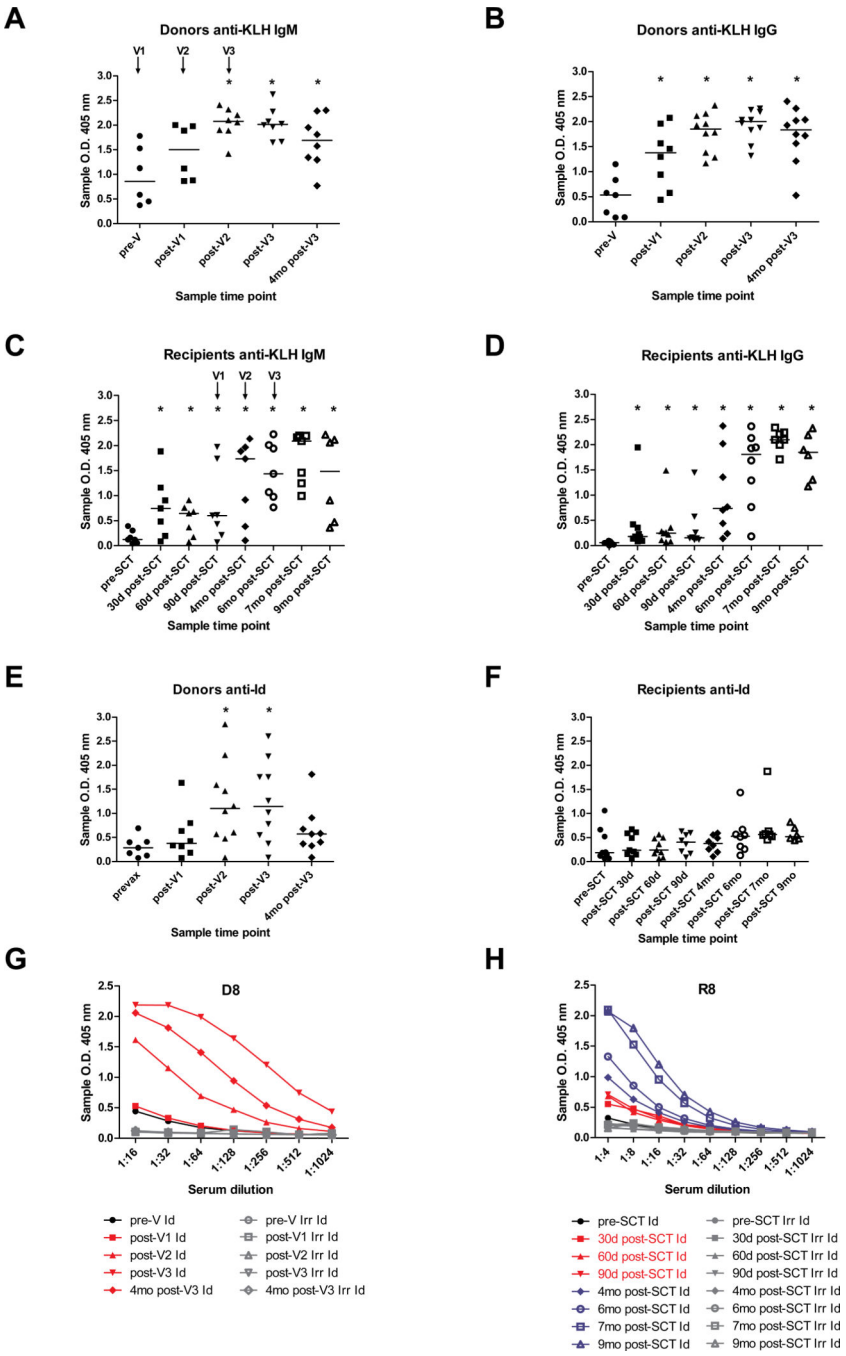
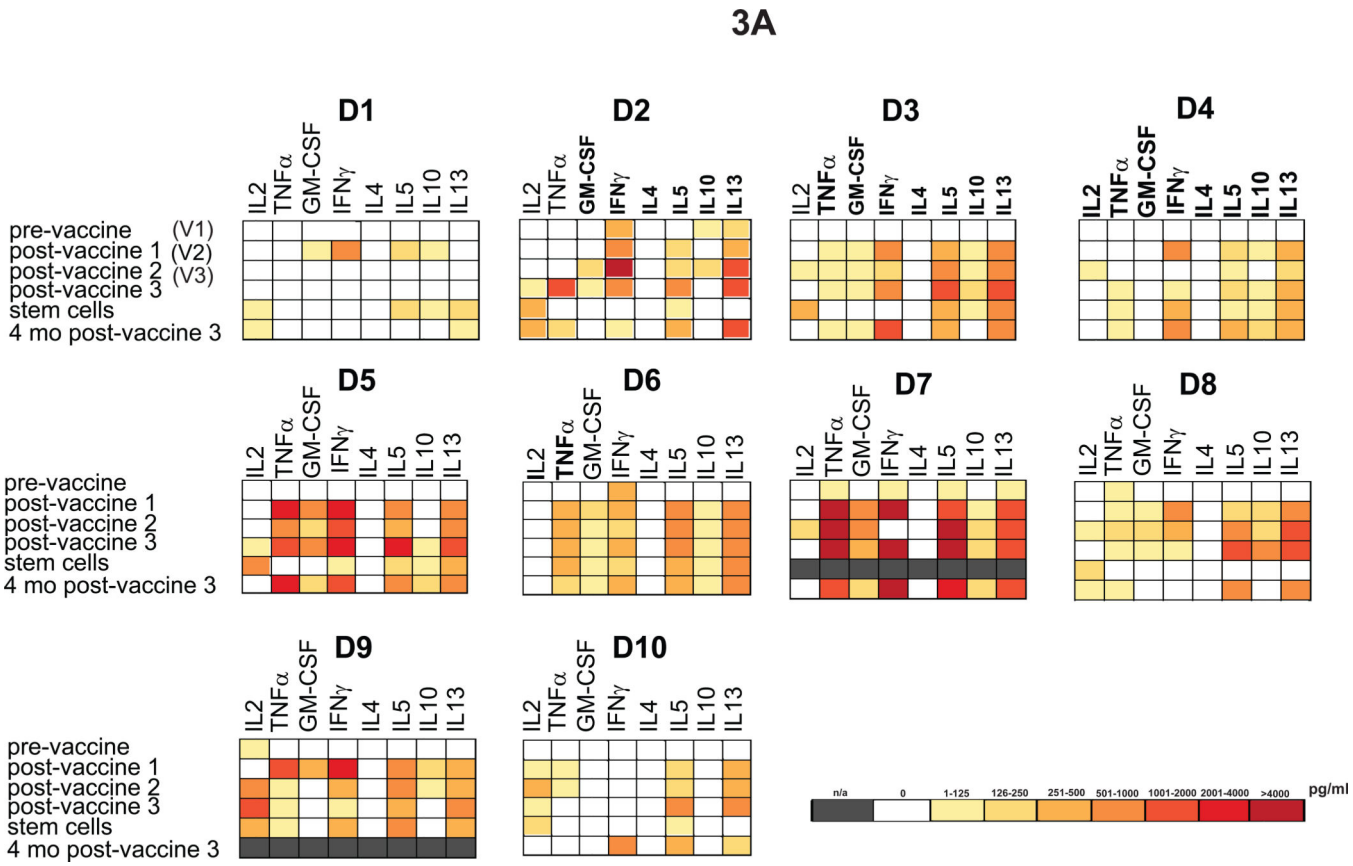


Figure 2. KLH and Id-specific antibody responses were induced in the donors and transferred to recipients

Prevaccine (pre-V) or pre-hematopoietic stem cell transplant (pre-SCT) and postvaccine (post-V) or post-SCT serum samples from the indicated time points in the donors (A, B, E, G) and recipients (C, D, F, H) were tested in parallel for KLH (A–D) and Id- (E–H) specific antibody responses by ELISA as described in the Materials and Methods. Post-SCT samples at 4 mo, 6 mo, 7 mo and 9 mo were obtained one month after the 1st, two months after the 2nd, and one and three months after the 3rd post-SCT vaccination, respectively. Vaccination time points are indicated by arrows as V1, V2, and V3 in the donors (**A**) and recipients (**C**).

(**A**–**F**) Sample optical density (OD) measurements at a serum dilution of 1:32 are shown. Horizontal bars indicate median for each group. Significant increase ($P < 0.05$) in antibody titers in postvaccine or post-SCT groups compared with prevaccine or pre-SCT groups respectively is indicated by an asterisk. Significant anti-Id antibody responses in individual patients are indicated by #. *P* values were calculated by Wilcoxon signed-rank test. Antibody responses against KLH were either IgM (A, C) or IgG (B, D) subtype. (**G, H**) Representative anti-Id antibody titration curves in donor 8 and recipient 8 are shown. Serum samples from the indicated time points were tested at various dilutions for reactivity against vaccinated Id or isotype-matched Id proteins of irrelevant specificity (Irr Id).



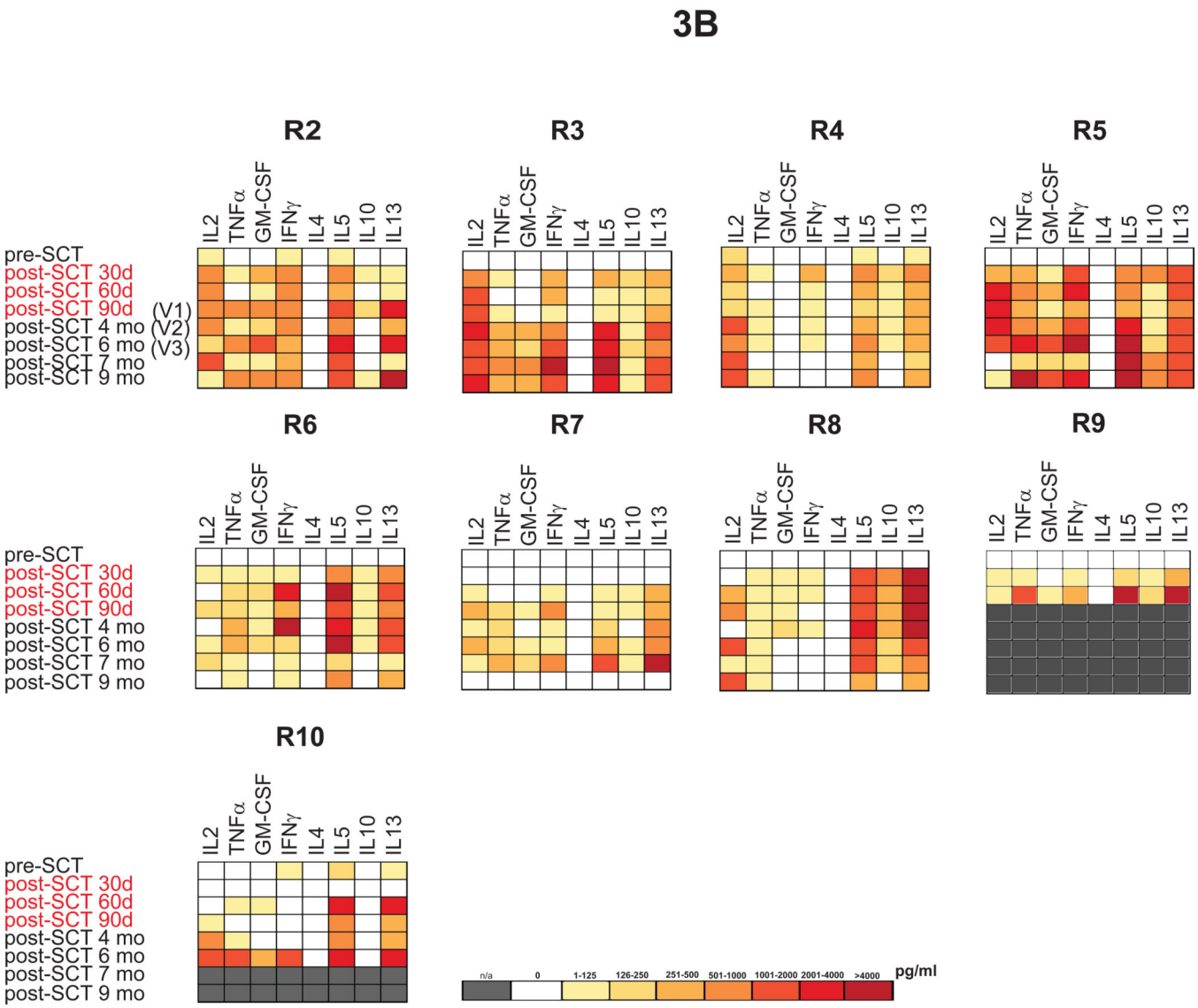
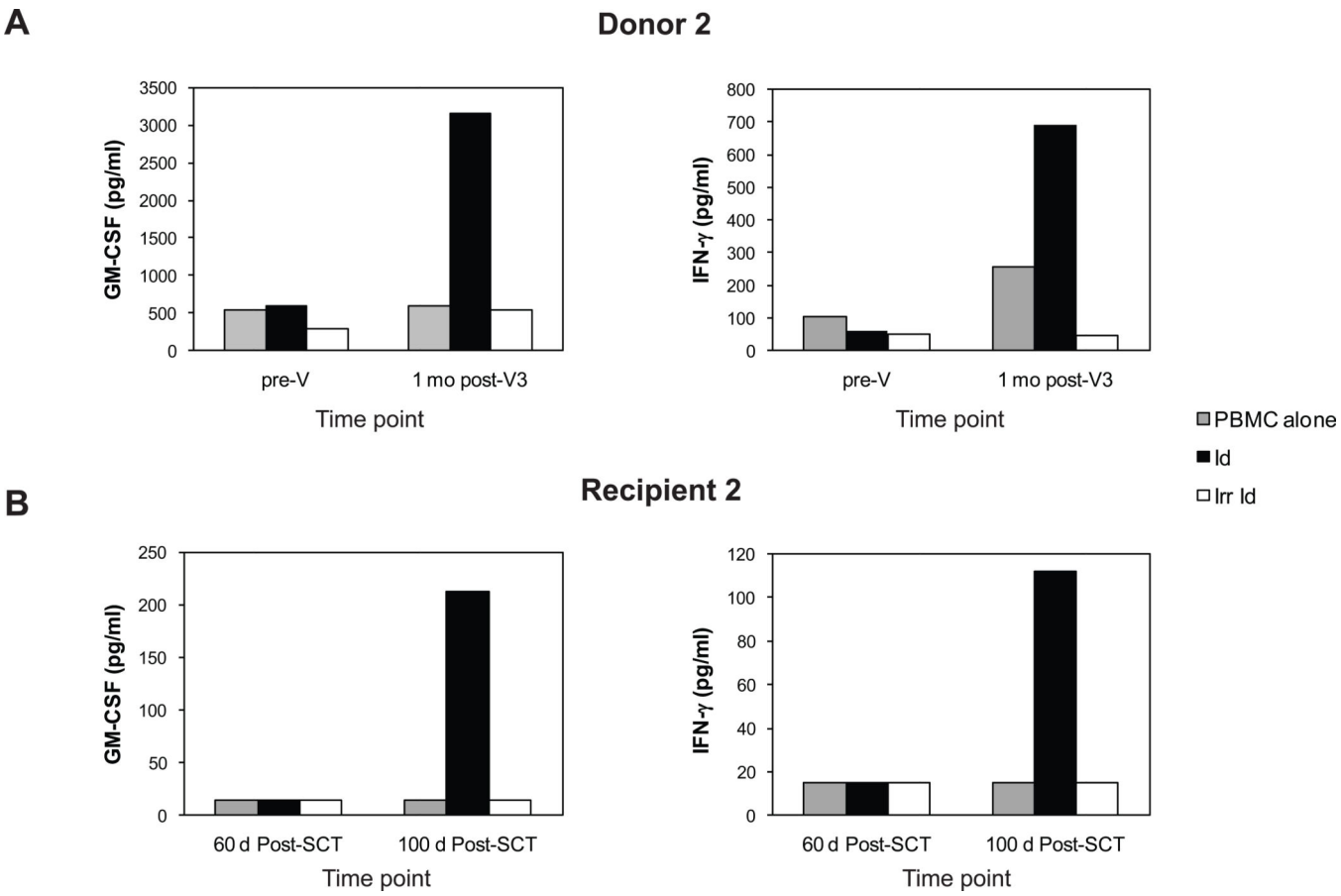


Figure 3. Cellular responses against KLH

Cryopreserved pre and postvaccine or pre and post-hematopoietic stem cell transplant (pre- and post-SCT) PBMC samples from the indicated time points in the donors (D1–D10) (**A**) and recipients (R2–R10) (**B**) were tested in parallel for reactivity against KLH in a cytokine induction assay as described in the Materials and Methods. Post-SCT samples at 4 mo, 6 mo, 7 mo and 9 mo were obtained one month after the 1st, two months after the 2nd, and one and three months after the 3rd post-SCT vaccinations, respectively. Vaccination time points are indicated as V1, V2, and V3 in the donors (**A**) and recipients (**B**). KLH-specific cytokine production was calculated by subtracting cytokines produced by PBMC in the absence of antigen from that in the presence of KLH at each time point. KLH-specific cytokine production is presented as a heat map according to the scale shown.



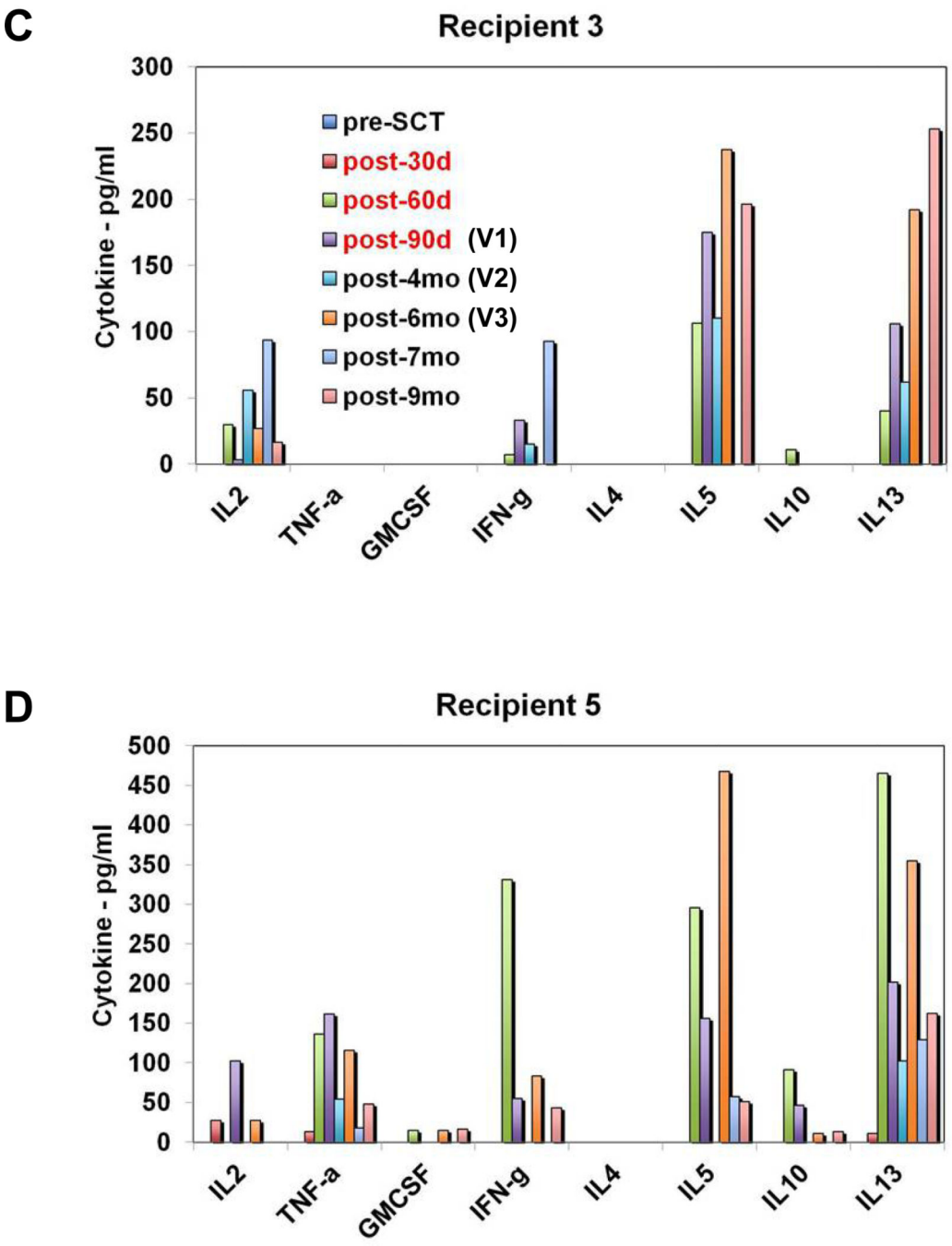


Figure 4. Cellular responses against Id

Fresh (**A, B**) or cryopreserved (**C, D**) pre and postvaccine or pre and post-hematopoietic stem cell transplant (pre- and post-SCT) PBMC samples from the indicated time points in Donor 2 (**A**) and recipients R2, R3, and R5) (**B–D**) were tested for reactivity against Id or irrelevant Id (Irrel. Id) in a cytokine induction assay as described in the Materials and Methods. Post-SCT samples at 4 mo, 6 mo, 7 mo and 9 mo were obtained one month after the 1st, two months after the 2nd, and one and three months after the 3rd post-SCT vaccinations, respectively. Vaccination time points for the recipient are indicated as V1, V2,

and V3 (**C**). Cytokine production in each well is shown (**A, B**). Id-specific cytokine production was calculated by subtracting cytokines produced by PBMC in the absence of antigen from that in the presence of Id at each time point (**C, D**).

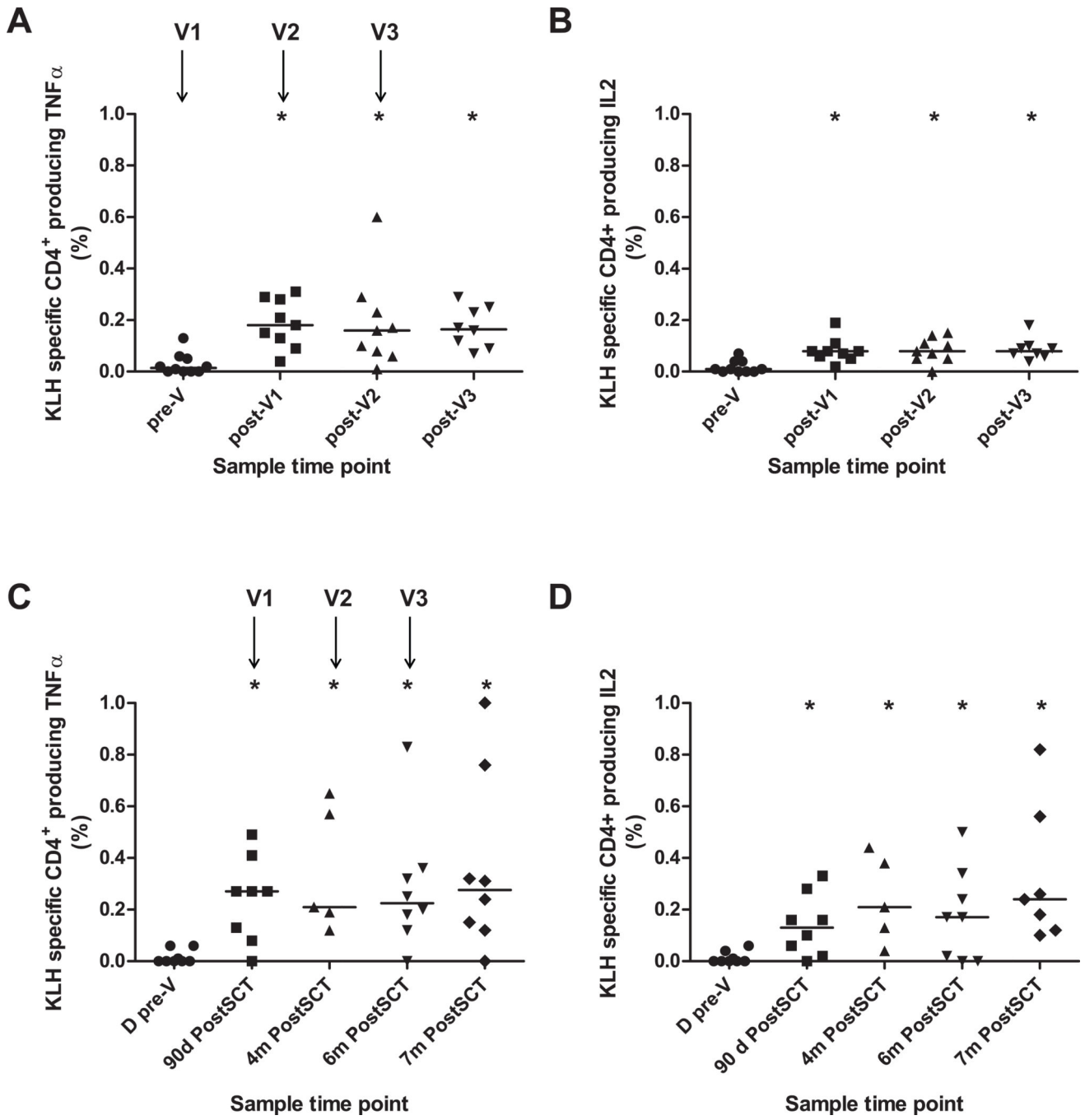


Figure 5. Frequency of KLH-specific T cells in donors and recipients

Cryopreserved PBMC samples from various time points in donors (**A, B**) and recipients (**C, D**) were cultured in medium alone, KLH, or bovine serum albumin (BSA) for 24 h with Brefeldin A added for the last 14 h. Production of TNF-α (**A, C**) and IL-2 (**B, D**) was assessed by intracellular cytokine staining as described in Materials and Methods. Vaccination time points are indicated by arrows as V1, V2, and V3 in the donors (**A**) and recipients (**C**). Frequency of KLH-specific $CD4^+$ T cells was calculated by subtracting cytokine-producing $CD4^+$ T cells in the absence of KLH from that in the presence of KLH

at each time point. Significant increase ($P < 0.05$) in KLH-specific $CD4^+$ T cells in donor postvaccine or recipient post-SCT groups compared with donor prevaccine group is indicated by an asterisk. *P* values were calculated by Wilcoxon signed-rank test. Post-HSCT samples at 4 mo, 6 mo, and 7 mo were obtained one month after the 1st, two months after the 2nd, and one month after the 3rd post-SCT vaccination, respectively. KLH-specific $CD4^+$ T cells producing TNF-α or IL-2 were detected in 7 of 8 evaluable recipients at 90 days post-SCT and in 8 of 8 following post-transplant immunizations.

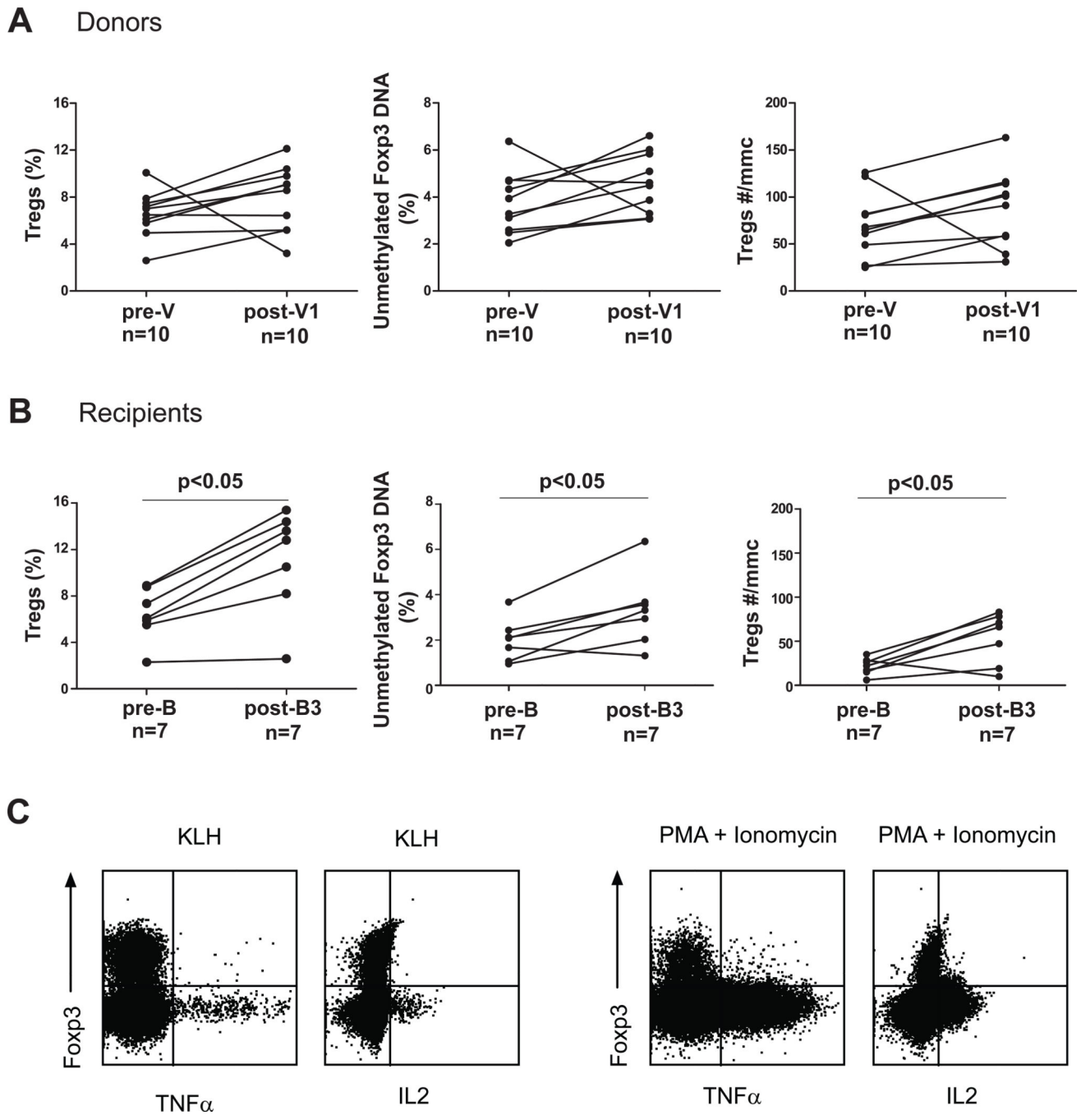


Figure 6. Regulatory T cells (Tregs) in donors and recipients

(**A, B**) The percentage of Foxp3$^+$ T cells in the peripheral blood CD4$^+$ T cells and PBMC was determined by flow cytometry and methylation status of the Foxp3 gene, respectively in donors (A) and recipients (B). The absolute number of Foxp3$^+$ T cells in the peripheral blood was calculated as described in Materials and Methods. *P* values were calculated by paired *t*-test for donors D2–D10. A significant increase in the percentage and absolute number of Foxp3$^+$ cells was observed at postvaccine 1 time point in 8 of 10 donors as compared with prevaccine time point (D2–D10: p<0.01, paired T-test). (**C**). Cryopreserved

PBMC from 7 mo post-HSCT time point from two recipients were cultured for 24 h in medium alone, KLH, or phorbol-12-myristate-13-acetate (PMA) and ionomycin. Intracellular staining was performed to determine the production of TNF-α and IL-2 in $CD4^{+}Foxp3^{+}$ and $CD4^{+}Foxp3^{-}$ T cells. Representative data from recipient 7 is shown.

Table 1

Recipients' characteristics and clinical outcome

Recipient	Age/ Sex	Prior therapies	Status pre-HSCT	Best response post-HSCT	Acute GVHD	Chronic GVHD	TTP	Current status (Cause of death)
1	60/M	VAD	PR	PR (d28)	+	NE	NE	Died d67 (Grade IV GVHD)
2	54/M	Thal; High Dose Dex; Melphalan/ Auto SCT	PR	VGPR (d28)	−	+	28.5 mo	PD 94+ mo
3	53/F	VAD; EDAP; Mel/Auto SCT; Dex	PR	VGPR (6 mo)	−	+	25 mo	PD 89+ mo
4	54/M	Pulse Dex/Radiation; VAD; Bus/ CTX/Auto SCT; Thal; Arsenic; Dex	PR	PR (12 mo)	−	+	NE	Died 13 mo (bacterial pneumonia)
5	51/F	Pulse Dex/ Radiation	PR	PR (d100)	−	+	15 mo	PR 77+ mo
6	55/M	VAD; DT-PACE	PR	PR (6 mo)	+	+	NE	Died 10 mo (bacterial pneumonia; CHF)
7	47/F	VAD; Thal/Dex	PR	PR (d28)	+	+	41 mo	VGPR* 71+ mo
8	56/M	Thal/Dex; Doxil; Bortezomib	PR	VGPR (d28)	−	+	9 mo	Died 59 mo (PD)
9	55/M	Thal/Dex	PR	CR (d100)	+	+	N/A	CR 60+ mo
10	44/M	VAD, DT-PACE	PR	CR (12 mo)	−	+	N/A	CR 57+ mo

Status pre-HSCT, disease status immediately prior to allogeneic hematopoietic stem cell transplantation (HSCT); GVHD, graftversus-host disease; Ab, antibody response; Th1, T helper 1 immune response; Th2, T helper 2 immune response; M, male; F, female; PR, partial response; SD, stable disease; VGPR, very good partial response; CR, complete response; d, days; mo, months; NE, not evaluable (date of progression was censored at the date of death); p, polyspecific; N/A, not applicable; CHF, congestive heart failure; *, patient achieved VGPR on salvage therapy with lenalidomide; VAD, vincristine, doxorubicin, and dexamethasone; Thal, thalidomide; Dex, dexamethasone; EDAP, etoposide, dexamethasone, cytarabine, and cisplatin; Mel, melphalan; Auto SCT, autologous stem cell transplantation; Bus, busulfan; CTX, cyclophosphamide; DT-PACE, dexamethasone, thalidomide, cisplatin, doxorubicin, cyclophosphamide, and etoposide.