In vivo engineering of mobilized stem cell grafts with the immunomodulatory drug FTY720 for allogeneic transplantation

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The immunological attributes of stem cell grafts play an important role in the outcome of allogeneic stem cell transplants. Currently, ex vivo manipulation techniques such as bulk T-cell depletion or positive selection of CD34+ cells are utilized to improve the immunological attributes of grafts and minimize the potential for graft-versus-host disease (GVHD). Here, we demonstrate a novel graft engineering technique, which utilizes the immunomodulatory drug FTY720 for in vivo depletion of naïve T (T_N) cells from donor G-CSF-mobilized grafts without ex vivo manipulation. We show that treatment of donor mice with FTY720 during mobilization depletes grafts of T_N cells and prevents lethal GVHD following transplantation in a major mismatch setting. Importantly, both stem cells and NK cells are retained in the FTY720-treated grafts. FTY720 treatment does not negatively affect the engraftment potential of stem cells as demonstrated in our congenic transplants or the functionality of NK cells. In addition, potentially useful memory T cells may be retained in the graft. These findings suggest that FTY720 may be used to optimize the immunological attributes of G-CSF-mobilized grafts by removing potentially deleterious T_N cells which can contribute to GVHD, and by retaining useful cells which can promote immunity in the recipient.

Keywords: Allogeneic stem cell transplantation · Graft engineering · Graft-versus-host disease · Stem cell mobilization · T cells

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Introduction

Graft engineering has played a crucial role in improving allogeneic stem cell transplantation (ASCT) and also broadening its potential application [1]. This has been underpinned by our greater understanding of the different cells that constitute the graft and their contribution to alloresponses [2]. Naive T (T_N) cells from grafts are the major cells responsible for graft-versus-host disease (GvHD), whereas other cells, such as effector memory T (T_EM), central memory T (T_CM), and NK, have potential for promoting immunity and antitumor activity, respectively [1, 3–6]. In order to mitigate alloresponses by donor T_N cells, current graft-engineering techniques utilize ex vivo bulk T-cell depletion or positive selection of CD34^+ cells. These techniques, however, can leave the graft devoid of useful immune cells, potentially making the recipient more susceptible to infection and relapse [1, 2]. Techniques specifically targeting depletion of T_N cells from grafts could potentially help maximize the capacity of grafts for immune reconstitution, antitumor activity as well as prevention of GvHD in recipients [1, 3–6]. Bleakley et al. have recently described a promising protocol in which T_N cells are depleted from mobilized grafts, but this still requires ex vivo manipulation of grafts and complex selection strategies [7]. Simpler and effective graft engineering techniques are therefore warranted to improve the outcomes of ASCT.

Different T-cell subsets have distinct trafficking patterns in the body. T_N cells (CD3^+ CD44^+ CD62L^+) primarily traffic through secondary lymphoid organs in search of their cognate antigen [8, 9]. T_CM cells (CD3^+ CD44^+ CD62L^-) have a similar trafficking route, while T_EM cells (CD3^+ CD44^- CD62L^-) mainly surveil the periphery and sites of inflammation [8, 9]. The trafficking of T_N cells is highly dependent on the sphingolipid metabolite sphingosine-1-phosphate (SIP), which is present at high levels in blood and lymph, and its receptor S1P1, which is expressed on T_N cells and other lymphocytes [10]. SIP acts as an egress factor facilitating the exit of T_N cells from secondary lymphoid organs [10]. Disruption of the S1P/S1P1 axis leads to inhibition of this egress and accumulation of T_N cells in secondary lymphoid organs. Lymphocytes such as T_EM cells are less affected due to their different trafficking routes and NK cells are similarly less affected as they utilize a different S1P receptor to exit lymph nodes [8, 11–13]. This specific property of T_N cells has been exploited in relapsing-remitting multiple sclerosis with the use of the immunomodulatory agent FTY720 [14]. FTY720 disrupts the S1P/S1P1 axis by inducing internalization and degradation of the S1P1 receptor [14], which leads to rapid sequestration of T_N cells, as well as B cells into secondary lymphoid organs and their depletion from peripheral blood (PB) [11]. While the immunomodulatory properties of FTY720 have been studied in various inflammatory conditions as a way of impacting disease, its use in manipulating mobilized stem cell grafts has not previously been examined [11, 12, 15, 16].

Currently, the majority of hematopoietic stem cell transplants are performed using G-CSF-mobilized PB stem cell grafts [17]. We have previously examined the role of SIP in different forms of mobilization and found that G-CSF mobilization is not negatively affected by disruption of the S1P/S1P1 axis with FTY720 [18].

Due to its selective capacity to deplete T_N cells from PB during steady state, we hypothesized that FTY720 could potentially be used during G-CSF mobilization to selectively deplete these grafts of T_N cells. However, it has not been previously examined if the capacity of FTY720 to deplete T_N cells from PB is maintained during G-CSF mobilization. In addition, there is currently no data regarding the kinetics of this depletion. Neither the impact of FTY720 on the engraftment capacity of mobilized stem cell grafts nor the immunological properties of such a graft, including its alloreactive capacity and maintenance of other lymphocyte subsets such as T_EM, T_CM, and NK cells, have been previously examined. Here, we demonstrate the capacity of FTY720 to selectively deplete T_N cells from PB in vivo during G-CSF mobilization. We show that NK-cell functions are not affected by FTY720 treatment and that they are not depleted from the grafts following treatment. We also show that T_EM and T_CM are retained in the graft. Importantly, we demonstrate that the alloreactive capacity of these grafts is blunted following in vivo T_N cell depletion with 1-day FTY720 treatment resulting in prevention of GvHD in a major mismatch transplant model. Furthermore, we show that the intrinsic engraftment capacity of stem cells from G-CSF-mobilized grafts is not affected by FTY720 treatment. Our data represent a proof of concept that in vivo graft engineering of G-CSF-mobilized donor grafts is feasible with FTY720 and that immunological attributes of these grafts can be improved by this method.

Results

FTY720 depletes T_N cells from PB but retains T_EM and NK cells at steady state and G-CSF mobilization

To assess the impact of FTY720 on different cell subsets in PB during steady state, C57BL/6 mice were treated with FTY720 (1 mg/kg) once daily for 4 days. The next day, PB was assessed for the different lymphocyte subsets by flow cytometry. FTY720 treatment decreased the percentage of CD3^+ cells by 72% (p = 0.0056) compared to control (Fig. 1A and B). The CD3^+ CD62L^- T_N subset was the major population depleted, decreasing by 61% (p < 0.0001) following treatment (Fig. 1A and C). In contrast, the relative percentages of NK and T_EM (CD3^+ CD62L^-) cells were elevated nearly two-fold (p = 0.0066 and p < 0.0001, respectively) by FTY720 (Fig. 1A, C, and D), likely reflecting the depletion of T_N cells. Decrease in CD62L^- cell subsets was observed only among T cells and not NK cells (Fig. 1A, C, and D).

To determine the impact of FTY720 on lymphocyte subsets from G-CSF-mobilized PB, mice were treated with FTY720 prior to and concomitantly during mobilization (Fig. 2A). FTY720 significantly reduced the percentage of T cells from mobilized PB compared to untreated controls, with the greatest reduction (88.5%, p < 0.0001) obtained after 7 days of treatment (Fig. 2B). This was
Figure 1. Flow cytometric analysis of T-cell and NK-cell populations in the PB of mice treated with FTY720 or PBS. Mice were treated with PBS or FTY720 for 4 days prior to blood harvest. (A) Gating strategies for analysis of flow cytometric data for T cells and NK cells. Flow cytometric analyses of (B) bulk T cells and NK cells, (C) T<sub>N</sub> cells (CD3<sup>+</sup>CD62L<sup>+</sup>) and T<sub>EM</sub> cells (CD3<sup>+</sup>CD62L<sup>−</sup>) and CD62L<sup>+</sup> and CD62L<sup>−</sup> NK cells are shown. Symbols represent individual mice, horizontal bars the means, and error bars ±SEM. Data are representative of a single experiment (n = 6 mice for PBS and n = 8 mice for FTY720 group). Data were compared using two-tailed unpaired Student’s t-test. §p < 0.01; †p < 0.001.

Figure 2. Impact of FTY720 treatment on T-cell, NK-cell, and B-cell populations in G-CSF-mobilized PB. Mice were either untreated or treated with FTY720 at 0.25, 0.5, 1, 2, and 7 days prior to PB harvest. (A) Schematic representation of different FTY720 treatment schedules relative to G-CSF mobilization. Arrows indicate the day of initiation of daily i.p. FTY720 injections to donors prior to sacrifice (X). G denotes initiation of twice daily s.c. injections of G-CSF to donors. Results of flow cytometric analysis of (B) bulk T cells (CD3<sup>+</sup>), (C) NK cells, (D) B cells (CD19<sup>+</sup>), (E) T<sub>N</sub> cells (CD3<sup>+</sup>CD44<sup>−</sup>CD62L<sup>+</sup>), (F) T<sub>EM</sub> cells (CD3<sup>+</sup>CD44<sup>+</sup>CD62L<sup>−</sup>), and (G) T<sub>CM</sub> cells (CD3<sup>+</sup>CD44<sup>−</sup>CD62L<sup>−</sup>) cells from G-CSF-mobilized mice. (B–G) Bar graphs represent cell percentages and scatter plots represent absolute counts with each symbol representing an individual mouse, horizontal bars the means, and error bars ±SEM. Data shown are from a single experiment representative of two independent experiments performed, except for CD19<sup>+</sup>, T<sub>CM</sub>, T<sub>EM</sub>, T<sub>N</sub>, and T<sub>EM</sub> subsets as well as the absolute cell counts which are representative of a single experiment (n = 5 mice for each treatment arm). Data were compared using two-tailed unpaired Student’s t-test. *p < 0.05; §p < 0.01; †p < 0.001 for each group compared with the untreated control.
also reflected in a reduction in T-cell numbers, which decreased by up to 96% (p < 0.0001) following 7 days of treatment (Fig. 2B). B-cell percentages and numbers were also reduced by up to 78.2% (p < 0.0001) and 92.5%, (p < 0.0001) respectively, after 7-day treatment compared to untreated controls (Fig. 2D). In contrast, the percentage of NK cells was significantly elevated by 3.8-fold (p < 0.0001) compared with untreated mobilized controls (Fig. 2C). However, numbers of NK cells in mobilized PB were not significantly altered (Fig. 2D). To better assess the effect of FTY720 treatment on T<sub>n</sub>, T<sub>EM</sub> as well as T<sub>CM</sub> subsets in G-CSF-mobilized PB, we used CD44 and CD62L staining (Fig. 2E–G). T<sub>n</sub> (CD<sup>3</sup> CD44<sup>-</sup> CD62L<sup>+</sup>) were decreased as early as 6 h post-FTY720 treatment and the greatest reduction observed following 7 days of treatment (Fig. 2E) with percentage and numbers decreasing by up to 95% (p < 0.0001) and 99.8% (p < 0.0001), respectively. T<sub>EM</sub> (CD<sup>3</sup> CD44<sup>+</sup> CD62L<sup>-</sup>) became the predominant T-cell population in the mobilized blood post-treatment, increasing in percentage up to 2.6-fold (p < 0.0001) after 1 day of treatment and up to three-fold (p < 0.0001) following 7 days of treatment. The numbers of T<sub>EM</sub> cells, however, were reduced by 74.8% (p < 0.001) and 88.3% (p < 0.001) following 1 and 7 days of treatment, respectively (Fig. 2F). T<sub>CM</sub> (CD<sup>3</sup> CD44<sup>+</sup> CD62L<sup>-</sup>) initially increased in percentage 1.3-fold (p < 0.001) in the mobilized blood 6 h post-FTY720 treatment but were reduced by up to 36.4% (p < 0.001) following 7 days of treatment. In contrast, their numbers decreased by up to 90.5% (p < 0.0001) after 1 day of treatment and up to 97.3% (p < 0.0001) after 7 days of treatment (Fig. 2G). These results demonstrate that NK cells are maintained in the G-CSF-mobilized blood following FTY720 treatment and that some T<sub>EM</sub> and T<sub>CM</sub> cells may be retained in the graft after FTY720 treatment due to their different clearance kinetics compared with T<sub>n</sub> cells.

Differences in the clearance rates of T<sub>EM</sub> and T<sub>CM</sub> cells compared with T<sub>n</sub> cells suggest that memory T cells may be retained in FTY720-treated grafts. To investigate this, we assessed levels of OVA-specific CD8<sup>+</sup> T cells in G-CSF-mobilized grafts from OVA-immunized mice untreated or treated with FTY720 1 day prior to sacrifice. The percentage of pentamer<sup>+</sup> CD8<sup>+</sup> T cells tended to decrease in PB of controls following mobilization (Fig. 3), while the percentage of pentamer<sup>+</sup> CD8<sup>+</sup> T cells tended to increase in mice following mobilization and 1 day of FTY720 treatment (Fig. 3, not significant). The trend of increased amounts of antigen-specific T cells in mice treated with FTY720 may reflect the sequestration of certain cell populations such as T<sub>n</sub> cells from the mobilized PB to organ tissue.

Together, these data suggest that FTY720 treatment can effectively deplete T<sub>n</sub> cells from G-CSF-mobilized grafts and that antigen-specific memory T cells can be retained.

FTY720 treatment does not affect NK-cell functionality at steady state and after G-CSF mobilization

To examine the functionality of NK cells retained following FTY720 treatment, we used an in vivo NK killing assay with BALB/c-derived B cells in PB pre- and post-G-CSF mobilization and T cells in PB pre- and post-G-CSF mobilization from OVA-specific CD8<sup>+</sup> T cells in PB pre- and post-G-CSF mobilization from untreated or 1-day FTY720-treated donors preimmunized with OVA. Data are representative of a single experiment (n = 5 donors per group). Data were compared using two-tailed unpaired Student’s t-test.

The impact of FTY720 on the alloreactivity of NK cells was also examined in a transplant setting. BALB/c/<sup>Rag2<sup>−/−</sup></sup> X<sup>R<sup>−/−</sup></sup> recipient mice were transplanted with T-cell-depleted (TCD) splenocytes from FTY720-treated, G-CSF-mobilized C57BL/6 donor mice and inoculated with a luciferase<sup>+</sup> BALB/c-derived B cell lymphoma cell line A20, 1 day post-transplant. Bioluminescence imaging 14 days post-A20 inoculation showed that TCD splenocytes from FTY720/G-CSF-treated mice decreased tumor burden compared with A20-only control or transplanted mice treated with an NK-depleting Ab (Fig. 4B and C).

The functionality of purified human NK cells following 19-h FTY720 treatment in vitro was assessed against K562 targets. Neither FTY720 nor FTY720P (the active phosphorylated form), at any of the physiologically relevant doses tested, had negative effects on NK-cell degranulation or cytokine production (Fig. 4D). Together, these data demonstrate that FTY720 has no detrimental effects on NK-cell function under steady state or during G-CSF mobilization and suggests that they may contribute to alloresponses in the recipient under the appropriate transplant setting.

Selective depletion of T<sub>n</sub> cells from G-CSF-mobilized PB grafts prevents GvHD

To investigate the capacity of FTY720 to minimize the GvHD potential of G-CSF-mobilized grafts in a major mismatch setting, lethally irradiated BALB/c recipients were injected intravenously with mobilized PB from C57BL/6 donors treated with PBS or
Figure 4. Assessment of in vivo NK-cell functionality following FTY720 treatment. (A) Killing capacity of NK cells from PB of C57BL/6 mice treated with FTY720 or PBS. Treated recipients were injected with an equal mixture of syngeneic target splenocytes (MHC$^{-}$CFSE$^{\text{high}}$ versus MHC$^{+}$CFSE$^{\text{low}}$). The relative rejection of MHC$^{-}$ splenocytes 1 day post-injection was assessed by comparing the ratio of CFSE$^{\text{high}}$ and CFSE$^{\text{low}}$ cells (defined as killing ratio) from recipient blood with preinoculate by flow cytometry. C57BL/6 mice treated with an NK-cell-depleting Ab were used as control. Symbols in scatter plots represent individual mice, horizontal bars the means, and error bars ± SEM ($n = 8$ mice for PBS and FTY720 groups; $n = 4$ mice for NK-cell-depleted controls; combined data from two experiments). Data were compared using two-tailed unpaired Student’s $t$-test. $^\dagger p < 0.001$. (B) Bioluminescence imaging of BRG mice, transplanted with grafts from FTY720-treated G-CSF-mobilized donors, 14 days post-injection with the luciferase$^{+}$ BALB/c-derived B cell lymphoma cell line A20. (C) Graphical representation of bioluminescence intensity from (B). Symbols in scatter plots represent individual mice, horizontal bars the means, and error bars ± SEM from a single experiment. Data were compared using one-tailed unpaired Student’s $t$-test. $^* p < 0.05; ^\ddagger p < 0.01$. (D) Degranulation (CD107a) and cytokine production (IFN-γ and TNF-α) by purified human NK cells cultured in media or different concentrations (10 or 100 nM) of FTY720 or FTY720P for 19 h against K562 targets was assessed by flow cytometry. NK cells without targets were used as controls. Data are shown as mean ± SEM of $n = 5$ donors pooled from two experiments. Data were compared using two-tailed unpaired Student’s $t$-test.
Figure 5. Effect of FTY720 graft engineering on the outcome of allogeneic stem cell transplantation. Recipient BALB/c mice received G-CSF-mobilized PB from PBS- or FTY720-treated C57BL/6 donors. Recipients injected with TCD BM served as controls. (A–C) Donors were treated with FTY720 for 2 days prior to and concomitantly during 5 days of G-CSF mobilization. Recipients did not receive an NK-depleting Ab. (D–I) Donors were treated with FTY720 only on the last day of the 5-day G-CSF mobilization prior to sacrifice. (D–F) Recipients received one dose of anti-asialo GM1 3 days prior to transplant (n = 6 per group). (G–I) Recipients received anti-asialo GM1 5 and 1 day prior to transplant (n = 6 per group except TCD BM (n = 4)). (A, D, and G) Survival curves, (B, E, and H) clinical scores on sacrifice, and (C, F, and I) weight curves for the various recipient BALB/c mice are shown. (J) Percent of donor cells (CD45.2+ H-2Kb+) >100 days post-transplant in PB of BALB/c recipients treated with anti-asialo GM1 5 and 1 day prior to transplant. Symbols in scatter plots represent individual mice, horizontal bars the means, and error bars ±SEM of the indicated numbers of mice, from a single experiment. Survival times were compared using log-rank survival statistics. Reconstitution was compared using two-tailed unpaired Student’s t-test. §p < 0.01; †p < 0.001; n.s.: not significant.

FTY720. Blood from two donor mice was used per recipient. Initially, blood from 7-day FTY720-treated mobilized donors was used. FTY720 treatment prevented lethal GvHD even at a high irradiation dose of 9.5 Gy (Fig. 5A–C). However, no long-term donor reconstitution followed in either FTY720 grafts or TCD BM controls except from one animal (data not shown) suggesting that both grafts were rejected. Graft rejection, which can be mediated by recipient NK cells, is a complication of T-cell depletion [20]. In order to protect grafts from rejection, recipient mice were lethally irradiated and injected with an NK-depleting Ab (anti-asialo GM1), according to manufacturer’s recommendations, 3 days before transplant. Irradiation was reduced to 8 Gy to minimize potential conditioning-related problems with the delayed transplant. FTY720 administration was reduced to a single dose, 1 day prior to graft harvest. A single dose of FTY720 was effective in preventing lethal GvHD, since all recipients of FTY720-treated grafts survived up to 100 days without signs of GvHD (Fig. 5D–F). In contrast, all PBS controls succumbed to GvHD by
Figure 6. Histological assessment of skin, gastrointestinal tract, and liver of recipient mice transplanted with FTY720- or PBS-treated, G-CSF-mobilized donor grafts. Recipient mice were transplanted with PBS-, FTY720-treated mobilized grafts or TCD BM grafts. Recipient mice transplanted with mobilized grafts from PBS-treated donors and which succumbed to GvHD between day 8 and day 38 were sacrificed and tissue samples obtained for histology. Mice transplanted with mobilized grafts from FTY720-treated donors or TCD BM transplanted controls did not succumb to GvHD and were sacrificed after >100 days post-transplant. Representative histological sections of (A) skin, (C) large intestine, and (E) liver from recipients are shown. Pathology scores of (B) skin, (D) large intestine, and (F) liver sections from recipient mice transplanted with PBS-, FTY720-treated G-CSF-mobilized grafts, or TCD BM grafts. Donors were treated with FTY720 only on the last day of the 5-day G-CSF mobilization prior to sacrifice. Recipients either received one dose of anti-asialo GM1 3 days prior to transplant ($n=6$ per group) or anti-asialo GM1 at 5 and 1 days prior to transplant. Symbols in scatter plots represent individual mice, horizontal bars the means, and error bars ±SEM of $n=6$ per group except TCD BM, $n=4$, combined data from two experiments. Histopathological scores were compared using one-way ANOVA with Bonferroni correction. $^\dagger p < 0.001$. Scale bars, 200 μm.

day 37 (Fig. 5D–F). However, grafts from neither FTY720-treated donors nor TCD BM controls, were reconstituted. Therefore, we repeated the same experiment but with anti-asialo GM1 injections 5 days and 1 day prior to transplantation. Again, GvHD was averted in recipients of FTY720-treated grafts (Fig. 5G–I). In this experiment, recipients of FTY720-treated grafts and recipients of TCD BM both showed partial donor reconstitution after sacrifice >100 days post-transplant (Fig. 5J). The low reconstitution of both grafts suggested a general problem with graft rejection rather than a drug-specific effect on engraftment. Accordingly, at least $10^6$ TCD BM cells are required for successful donor engraftment in such a major mismatch setting with lower doses compromising donor reconstitution [21]. Figure 6 demonstrates the capacity of FTY720 to prevent GvHD in skin (A and B), gastrointestinal tract (C and D), and liver (E and F). Together, these data demonstrate that FTY720 treatment in donors during G-CSF mobilization can prevent GvHD in recipients up to 100 days post-transplantation.

FTY720 treatment does not affect donor cell reconstitution

To assess the impact of FTY720 treatment on grafts in a more permissive setting, we performed transplants in congenic (CD45.1) recipients, using mobilized donor PB from two C57BL/6 mice, either treated with PBS or FTY720 for 1 day, per recipient. The level of donor reconstitution was assessed 18 weeks...
post-transplant. Secondary transplants using BM from primary recipients were performed to assess long-term donor reconstitution capacity. FTY720-treated grafts and PBS controls displayed similar levels of donor cell reconstitution in recipients 18 weeks following primary and secondary transplantation (Fig. 7A and B). The reconstitution of T and B cells as well as granulocytes was also comparable between the two grafts (Fig. 7C and D).

Discussion

Here we demonstrate a novel graft engineering strategy, which exploits the normal trafficking properties of different immune cell populations and stem cells to derive a graft product with improved immune characteristics and reduced potential for inducing GvHD. By using FTY720 to disrupt the S1P/S1P1 axis during G-CSF mobilization, we were able to effectively deplete donor-mobilized grafts of T_N cells in vivo without the need for further ex vivo manipulation. The depletion was sufficient to prevent GvHD in a major mismatch model. To our knowledge, this is the first demonstration of the immunomodulatory capacity of FTY720 during G-CSF mobilization and how it can be utilized to optimize the immunological attributes of mobilized grafts to prevent GvHD. Others have previously examined the use of FTY720 to try and mitigate GvHD [15, 22]. In these models, however, FTY720 was used in recipients following ASCT [15, 22]. This required prolonged treatment of recipients with FTY720, which was also found to alter immune reconstitution [22]. This is avoided in our system as potentially alloreactive cells are depleted from the graft prior to transplant avoiding the need for prolonged FTY720 treatment of the recipient. Additionally, we demonstrate that FTY720 treatment does not negatively impact the engraftment capacity of stem cells. We also show that NK cells, which have potential for promoting immunity, antitumor and anti-GvHD effect are maintained in mobilized grafts.
following FTY720 treatment and that FTY720 does not impair their function. T_{EM} and T_{CM} cells, which were decreased in mobilized PB following treatment, although at a slower rate than T_{N} cells, may also be retained and therefore potentially contribute to promoting immunity in the recipient. It has previously been shown by Sawicka et al. that T_{REG} are preferentially enriched in PB following FTY720 treatment during steady state [23]. Although the presence of T_{REG} was not examined in this study, it is possible that they are also retained in the graft and may contribute to dampening of GvHD.

Different graft engineering strategies have been examined and developed in order to prevent GvHD in ASCT [1]. These strategies, however, rely on ex vivo manipulation of grafts. Traditionally, total T-cell depletion has been performed but more recently selective depletion of T_{N} cells has also been investigated following successful data from animal models [3, 7]. In support of this, our selective in vivo depletion of T_{N} cells with FTY720 also resulted in effective prevention of GvHD, without requiring ex vivo manipulation of grafts. This has important implications for the simplification and potentially, cost-effectiveness of future graft engineering protocols.

It has been suggested that >2-log reduction of bulk T cells is minimally required to avoid GvHD [24, 25]. Although we did not achieve such a high reduction in bulk T cells from the G-CSF-mobilized grafts, we did achieve at least a 2-log reduction in T_{N} cells, which was sufficient to prevent GvHD in our major mismatch transplant model. This suggests that the level of T_{N} cell depletion, rather than that of bulk T cells, is important for prevention of GvHD. Currently, the level of T_{N} cell depletion required to prevent GvHD is unknown but our results provide a starting point for investigations into further defining this threshold. It has also previously been shown that bulk T-cell depletion of grafts alone can lead to problems with post-transplant EBV-related lymphoproliferative disorders due to an imbalance between T- and B-cell numbers in infused products [1]. FTY720 administration can potentially address this problem as we have shown that it can also deplete B cells significantly from G-CSF-mobilized PB.

Harnessing the potential of NK cells in ASCT for the treatment of malignant diseases is currently under intense investigation [26, 27]. The capacity of FTY720 to maintain NK cells in G-CSF-mobilized grafts suggests they would be at least a 2-log reduction in T_{N} cells, is important for prevention of GvHD. Currently, the level of T_{N} cell depletion required to prevent GvHD is unknown but our results provide a starting point for investigations into further defining this threshold. It has also previously been shown that bulk T-cell depletion of grafts alone can lead to problems with post-transplant EBV-related lymphoproliferative disorders due to an imbalance between T- and B-cell numbers in infused products [1]. FTY720 administration can potentially address this problem as we have shown that it can also deplete B cells significantly from G-CSF-mobilized PB.

Donor lymphocyte infusions using antigen-specific T_{EM} are now being explored to combat immunosuppression-related complications following ASCT [1, 6]. Our data show that treatment with FTY720 for 1 day is sufficient to prevent GvHD but also retain some antigen-specific memory T cells. Thus, using this protocol it may be possible to retain antigen-specific memory T cells from donors with preexisting immunity to help promote recipient immunity without the need for ex vivo isolation, again potentially decreasing the complexity of these procedures. This also suggests that FTY720 may be useful when given to donors in order to deplete the PB of T_{N} and B cells while retaining NK and memory T cells for improving the immunological attributes of donor lymphocyte infusions aimed at combating infections.

We have previously demonstrated that mobilization of hematopoietic progenitor cells with G-CSF is not affected by FTY720 [18]. Similarly, we did not observe differences in the engraftment rate of G-CSF-mobilized grafts from FTY720-treated mice compared with PBS-treated controls in our congenic transplant model. This supports our previous findings that mobilization with G-CSF is not impaired by FTY720 and also demonstrates that FTY720 treatment does not impair the engraftment of these grafts. While the engraftment of FTY720-treated grafts was unaffected in our congenic transplant model, we observed much lower engraftment rates in our major mismatch model. It has been demonstrated that recipient NK-cell-mediated graft rejection is increased following FTY720 treatment during steady state [23]. Although this novel approach shows promise, several hurdles must be overcome for its clinical potential to be realized. Currently, good safety data are only available regarding the effect of each individual agent in healthy volunteers, but not in combination. Thus, safety of the G-CSF and FTY720 combination must be determined. In our study, no obvious detrimental effects accompanied administration of the combined agents to animals. Another major hurdle is prevention of graft rejection. Better conditioning strategies specifically targeting recipient NK cells will be necessary to ensure successful engraftment of such a graft and retention of the potentially useful donor NK cells. Combining this strategy with mesenchymal stem cell infusions may be a potential solution, which could also synergize with this protocol to promote greater anti-GvHD effects, provide graft protection, and improve engraftment and donor reconstitution [29].

In summary, our study is a proof of concept that demonstrates for the first time how differences in the trafficking properties of stem and immune cells can be utilized for donor in vivo graft engineering of G-CSF-mobilized stem cell grafts. It shows that FTY720 administered during G-CSF mobilization can improve the immunological attributes of these grafts and prevent GvHD in the absence of ex vivo manipulation. These results may be applicable to the development of future transplantation and immunotherapy protocols and strongly suggest that clinical investigation into the use of FTY720 for improvement of ASCT is warranted.

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**Materials and methods**

**Reagents**

FTY720 (fingolimod) was purchased from Cayman Chemical (Ann Arbor, MI) and G-CSF (filgrastim) from Amgen (Solna, Sweden). Luciferin was from Gold Biotechnology (St. Louis, MO). H-2K\(^{b}\) - SIINFEKL - Pentamer - R-PE was from ProImmune (Oxford, UK)
and OVA peptides were from GenScript (Piscataway, NJ). The Dynabeads Mouse Pan T (Thy1.2) kit and CountBright Absolute Counting Beads were from Invitrogen (Lidingö, Sweden), and the human NK-cell isolation kit was from Miltenyi Biotec (Lund, Sweden). The murine NK-depleting Ab anti-NK1.1 (clone PK136) was a kind gift from Dr Benedict Chambers and anti-asialo GM1 was from Wako (Neuss, Germany). PolyI:C was a kind gift from Dr Annette Sköld. The anti-murine Ab anti-H-2Kb FITC, anti-CD45.2 APC, CD45.1 biotin, anti-CD3 PerCP Cy5.5, CD3 APC-Cy7, anti-CD62L PE-Cy7, and anti-NK1.1 Pacific Blue were from Biolegend (San Diego, CA). Anti-CD19 PE, anti-Gr-1 PE-Cy7, anti-CD8 PE-Cy5, and anti-B220-APC were from e-Bioscience (San Diego, CA). Anti-CD19 APC and anti-CD44 BV510 and anti-human; anti-CD56 (clone NCAM 16.2), anti-CD107a (clone H4A3) and anti-IFN-γ (clone 25723.11) were from BD Biosciences (San Jose, CA). Anti-CD3 (clone UCHT1) was from Dako (Glostrup, Denmark). Anti-TNF-α (clone MP6-XT22) was from eBioscience. CFSE and Streptavidin-qDot 605 were obtained from Life Technologies (Carlsbad, CA).

### Animals

All mice (C57BL/6, B6.SJ-1Prprca Pep3b/BoyJ [CD45.1], B6.Kb−/− D2−/−, BALB/c, BALB/c Rag2−/− γ−/− [BRG]) were bred and maintained at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet. Mice were used at 8–12 weeks of age and sex-matched. All animal experiments were approved by the Animal Ethics Committee of Stockholm in Sweden and conducted in accordance with institutional guidelines for animal care and use.

### Mobilization, drug treatment, and absolute cell counts

Mice were mobilized by subcutaneous injection of G-CSF (125 μg/kg) twice daily for 5 days unless otherwise specified and sacrificed the following day. FTY720 (1 mg/kg) was administered intraperitoneally daily either alone or concurrently during mobilization as specified. Absolute cell counts were performed using CountBright Absolute Counting Beads according to the manufacturer’s instruction.

### OVA immunization and memory T-cell assays

Mice were vaccinated subcutaneously in both right and left flank with 2 × 10⁶ BM-derived dendritic cells pulsed with 1 μM OVA peptide (SIINFEKL) twice with 1 day in between. Six weeks later, PB was assessed for SIINFEKL-specific CD8 T cells by pentamer staining. Twenty days later, mice were mobilized with G-CSF and left untreated or treated with FTY720 as described above. Mice were sacrificed 1 day post-FTY720 treatment and PB collected by cardiac puncture and analyzed for the SIINFEKL specific CD8 T cells. Pentamer staining of PB CD8 T cells was performed according to the manufacturer’s instruction and in combination with CD8 and B220 monoclonal Ab.

### NK assays

An in vivo CFSE-based NK killing assay was performed as published [19]. Briefly, recipient C57BL/6 mice were treated with FTY720 or PBS for 5 days. The following day, splenocytes from MHC-deficient C57BL/6 (susceptible targets) and wild-type mice (resistant targets) were obtained and labeled 1:1 (15 × 10⁶ each) with 5 μM (CFSEhigh) and 0.5 μM (CFSElow) of CFSE dye (Molecular probes), respectively. Following washes, they were injected into the recipient mice. The ratio between the two populations before co-injection was determined by flow cytometry. NK-depleted (anti-NK1.1) recipient mice were used as negative controls. One day post-injection, mice were sacrificed and bled. The ratio of CFSEhigh and CFSElow cells present in PB was assessed by flow cytometry and compared with the pre-inoculation ratio. The killing ratio was calculated by dividing the ratio between targets 1 day postinjection by that of the preinoculate.

The in vivo killing capacity of NK cells from G-CSF-mobilized C57BL/6 mice treated for 1 day with FTY720 was assessed by transplanting 10 × 10⁶ TCD splenocytes intravenously into BRG recipient mice. Splenocytes were depleted of T cells according to manufacturer’s instructions (Dynabeads Mouse Pan T (Thy1.2) kit; Invitrogen). Recipients were also treated with 500 μg of polyI:C for NK-cell activation. Recipients receiving the same cells but also injected intravenously with 500 μg of anti-NK1.1 were used as controls. The following day, recipient mice were inoculated intravenously with 1 × 10⁶ luciferase+ A20 cells (BALB/c derived B cell lymphoma cell line provided by Prof. A. Grandien). A20 cells were maintained in complete medium (RPMI 1640 with 10% heat inactivated FCS and 2 mM l-glutamine; all from Invitrogen) supplemented with 0.05 mM 2-ME (Sigma-Aldrich). Mice injected with luciferase+ A20 cells alone also served as controls. Tumor burden was assessed by injecting mice with 150 mg/kg luciferin and assessing bioluminescence with the IVIS Spectrum CT (Caliper Life Sciences/Perkin Elmer, Hopkinton, MA).

Human NK cells were isolated from PB by negative selection according to manufacturer’s instructions (NK-cell isolation kit; Miltenyi Biotec). Approval was obtained from the Regional Ethics Review Board for the use of PB mononuclear cells from healthy donors. NK cells were cultured in complete medium alone or with FTY720 or FTY720P at the specified concentrations for 19 h. Degranulation and cytokine responses of NK cells against K562 (obtained from ATCC and maintained in complete medium) targets were then assessed [30].

### ASCT and GvHD

Mobilized grafts were transplanted into BALB/c recipients 3 days after lethal irradiation (8 Gy). Recipients were also treated either once (-3 days) or twice (-5 and -1 days) before transplant with...
anti-asialo GM-1 (according to manufacturer's specification) to deplete NK cells. Blood from two donor C57BL/6 mice (approximately 1.6 ml) was used per BALB/c recipient. On average 18.4 $\times$ $10^6$ cells (range 10–23.8 $\times$ $10^6$) from FTY720-treated mobilized blood and 22.5 $\times$ $10^6$ cells (11–33.6 $\times$ $10^4$) from PBS-treated mobilized blood was transplanted per recipient. Donor blood cells, after RBC lysis with CaCl$_2$, were washed in PBS and resuspended in 100 $\mu$L PBS prior to intravenous injection into the recipient. TCD BM cells were used as controls and 1 $\times$ $10^6$ cells were injected per recipient. Mice were assessed for disease every 2 days initially and daily following disease progression. Mice were examined for changes in weight, movement, posture, activity, skin integrity, piloerection, eye inflammation, appetite, respiration, bowel and urinary function. Mice not succumbing to disease after 40 days were monitored weekly until sacrificed after 100 days.

Assessment of GvHD pathology

Liver, large intestine, and skin from the abdominal region were fixed in 10% formalin for 24 h and embedded in paraffin. Five-micrometer sections were made from each organ, stained with H&E and slide mounted with DPX (Sigma-Aldrich). Sections were examined under 16 $\times$ magnification using a LEICA DM 400B microscope and scored blind. Skin sections were scored on the basis of the following criteria: epidermis (0, normal; 1, foci of interface damage in less than 20% of section with occasional necrotic keratinocytes; 2, widespread interface damage in more than 20% of section); dermis (0, normal; 1, slightly altered with mildly increased collagen density; 2, markedly increased collagen density); subcutaneous fat (0, normal; 1, reduced number of normal adipocytes; 2, serous fat atrophy); follicles (three to four fields) (0, normal number of hair follicles, approximately ten per field; 1, between five and ten follicles per field; 2, <5 follicles per field) and inflammation (0, none; 1, focal infiltrates; 2, widespread infiltrates). Livers were assessed for bile duct injury (manifested by nuclear hyperchromasia, lymphocytic infiltrate) and inflammation (infiltrating lymphocytes, neutrophils, and eosinophils). Disease scores of between 0 and 4 were assigned based on the number of tracts involved and severity of disease in each tract (0, none; 1, few tracts with mild disease; 2, numerous tracts involved but mild disease; 3, injury in most tracts; 4, severe involvement of most tracts). Intestinal GvHD was scored on the basis of inflammation (0, none; 1, mild; 2, moderate; 3, severe without ulceration; 4, severe with ulceration).

Congenic transplants

Lethally irradiated (11 Gy) congenic (CD45.1+) recipients were transplanted on the same day with PB from two donor C57BL/6 (CD45.2+) mice. PB was obtained from G-CSF-mobilized mice treated with PBS or FTY720 1 day prior to PB harvest. Donor blood was RBC lysed and resuspended in PBS prior to intravenous injection into recipient as described previously. The percentage of CD45.2+ cells in recipient PB was assessed via flow cytometry, 18 weeks post-transplant to determine donor reconstitution. Secondary transplants were performed in lethally irradiated CD45.1+ recipients using 5 $\times$ $10^6$ BM cells from primary recipient mice and donor reconstitution was assessed 18 weeks post-transplantation. The percentage of CD45.2+ cells in recipient PB along with CD3+, CD19+, and GR-1+ cells was assessed 18 weeks post-transplant to determine donor reconstitution.

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Abbreviations: ASCT: allogeneic stem cell transplantation · GvHD: graft-versus-host disease · PB: peripheral blood · S1P: sphingosine-1-phosphate · TCD: T-cell depleted · TCM: central memory T · TEM: effector memory T · TN: naive T

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