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BRIEF REPORT

Autologous Stem Cell Transplantation Restores Immune Tolerance in Experimental Arthritis by Renewal and Modulation of the Teff Cell Compartment

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Objective. **Autologous stem cell transplantation (ASCT) induces long-term drug-free disease remission in patients with juvenile idiopathic arthritis. This study was undertaken to further unravel the immunologic mechanisms underlying ASCT by using a mouse model of proteoglycan-induced arthritis (PGIA).**

Methods. **For initiation of PGIA, BALB/c mice received 2 intraperitoneal injections of human PG in a synthetic adjuvant on days 0 and 21. Five weeks after the first immunization, the mice were exposed to total body irradiation (7.5 Gy) and received (un)manipulated bone marrow (BM) grafts from mice with PGIA. Clinical scores, T cell reconstitution, (antigen-specific) T cell cytokine production, and intracellular cytokine expression were determined following autologous BM transplantation (ABMT).**

Results. **ABMT resulted in amelioration and stabilization of arthritis scores. BM grafts containing T cells and T cell–depleted grafts provided the same clinical benefit, with similar reductions in PG-induced T cell proliferation and the number of PG-specific autoantibodies. In vivo reexposure to PG did not exacerbate disease. Following ABMT, basal levels of disease-associated proinflammatory cytokines (interferon-γ [IFNγ], interleukin-17 [IL-17], and tumor necrosis factor α [TNFα]) were reduced. In addition, restimulation of T cells with PG induced a strong reduction in disease-associated proinflammatory cytokine production. Finally, although the remaining host T cells displayed a proinflammatory phenotype following ABMT, IFNγ, IL-17, and TNFα production by the newly reconstituted donor-derived T cells was significantly lower.**

Conclusion. **Taken together, our data suggest that ABMT restores immune tolerance by renewal and modulation of the Teff cell compartment, leading to a strong reduction in proinflammatory (self antigen–specific) T cell cytokine production.**

Juvenile idiopathic arthritis (JIA) and rheumatoid arthritis are autoimmune diseases that often lead to major disability. The introduction of biologic agents such as anti–tumor necrosis factor α (anti-TNFα) has been a major step forward in controlling disease symptoms. In general, however, these treatments cannot induce drug-free disease remission. Furthermore, some patients fail to respond to conventional treatment or become unresponsive to treatment over time. For severely ill patients with JIA, autologous stem cell transplantation (ASCT) has proven to be an effective last resort (1,2). ASCT induces drug-free disease remission in a majority of patients during followup of up to 80 months posttransplantation (3).

The drug-free disease remission achieved by the majority of patients undergoing ASCT suggests that ASCT can, at least temporarily, restore immune tolerance in JIA. However, the underlying mechanisms are

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largely unknown. Data from our previous study of patients with JIA suggested that both renewal of the Treg cell compartment and re-programming of Teff cells may play a role (4). Unfortunately, T cell reconstitution after ASCT in humans cannot be monitored, because residual T cells and autologous graft–derived T cells are not distinguishable. A better understanding of the mechanisms would greatly favor the development of new treatment strategies that aim at not only immune suppression but also restoring immune tolerance.

Although it remains to be elucidated what underlies the impressive success of ASCT, immune reconstitution after profound depletion appears to favor development of tolerance over pathogenic immunity. Immediately after reinfusion with ASCT, the lymphopenic environment induces selective expansion and activation of the few T cell clones present. These T cells either survived the conditioning regimen (5) or may have been re-transferred with the graft and are potentially autoreactive. Therefore, lymphopenia-induced proliferation and activation of T cells may pose a risk of loss of self-tolerance early after ASCT. During this period, the presence of Treg cells may be essential to control T cell reconstitution and activation. At a later stage, the CD4+ T cell pool is further reconstituted by naive recent thymic emigrants, which is crucial for diversification of the T cell repertoire following ASCT (6) and may also play a role in the reestablishment of immune tolerance. Thus, the antigen specificity, differentiation, and function of the reconstituting T cells appear to be decisive for the efficacy of ASCT and warrant further investigation.

To elucidate this process, we developed an experimental model for ASCT in a mouse model of proteoglycan-induced arthritis (PGIA). PGIA is studied extensively, is clinically, immunologically, and histopathologically similar to human arthritis, and has a chronic relapsing–remitting course (7,8). Using this model, we previously demonstrated a crucial role for Treg cells in the recovery phase after autologous bone marrow transplantation (ABMT) (9). Here, we explore the influence of ABMT on the Teff cell compartment and demonstrate that ABMT-induced renewal of the T cell compartment leads to a strong reduction in proinflammatory (self antigen–specific) T cell responses.

MATERIALS AND METHODS

Mice. Female retired breeder BALB/c mice were obtained from Charles River Deutschland. CBy.PL(B6)-Thy1a.ScrJ mice, ages 7–10 weeks, were obtained from The Jackson Laboratory and served as donors for ABMT when indicated. The mice were maintained under standard conditions. After ABMT, recipient mice were housed under sterile conditions. All experiments were approved by the Animal Experiment Committee of the Faculty of Veterinary Medicine at Utrecht University.

Induction and assessment of arthritis. Arthritis was induced in BALB/c mice by 2 intraperitoneal injections of PG (400 μg) in 2 mg dimethyldioctadecylammonium bromide (DDA; Sigma Aldrich) 2 weeks and 5 weeks before bone marrow transplantation. PG was purified as described previously (7,8). The onset and severity of arthritis were assessed 3 times each week using a visual scoring system, as described previously (9). Briefly, the degree of joint swelling, redness, and deformation of each paw was scored on a scale of 0–4 to determine a total arthritis score (maximum possible score of 16 per mouse).

Treatment protocols. Two weeks after the second injection of PG in DDA, the recipient mice in which arthritis developed received a lethal dose (7.5 Gy) of total body irradiation. Next, ABMT was performed by intravenous injection of 2×10^6 BM cells obtained from syngeneic donor mice. Bone marrow was harvested by flushing the tibia and femur with Iscove's modified Dulbecco's medium including penicillin and streptomycin. BM cells (2×10^6) were resuspended in 200 μl 0.2% bovine serum albumin before intravenous injection into the tail vein. The mean percentage of T cells in BM grafts was 3.2%. For specific experiments, T cells were depleted from the BM graft using anti-mouse CD4 and anti-mouse CD8 MACS MicroBeads (Miltenyi Biotec). The T cell–depleted BM cells contained 0.67% (CD3+) T cells (data not shown).

Approximately 4–5 weeks after ABMT was performed, untreated arthritic mice and those that received unmanipulated grafts were given a booster dose of PG (400 μg administered intraperitoneally). The clinical response in these mice was compared with that in untreated arthritic mice and those that received unmanipulated grafts but were given an intraperitoneal injection of phosphate buffered saline (PBS) instead of PG.

In vitro assays. *T cell proliferation.* Seven weeks following ABMT, spleens and axial lymph nodes were harvested. Cells (2×10^5/well) were cultured in Iscove's modified Dulbecco's medium for 120 hours in the absence or presence of 10 μg/ml PG. During the last 16–18 hours of culture, 1 μCi ^{3}H-thymidine (Amersham) was added to each well. Proliferative responses were calculated as the median ^{3}H-thymidine incorporation (counts per minute) of triplicate wells.

Cytokine production. One, three, and seven weeks after ABMT, spleen cells (2×10^5) were cultured in culture medium for 96 hours in the presence of 10 μg/ml PG or 1 μg/ml soluble anti-CD3 (clone 145-2C11; BD PharMingen). Cytokine profiles were measured using a Mouse Cytokine Multiplex Kit (Bio-Rad) according to the manufacturer's instruction.

Flow cytometry and IgG1 enzyme-linked immunosorbent assay. For flow cytometric analysis, spleen, lymph node, and synovial fluid cells were stained for T cell receptor β (TCRβ), CD90.1, Ki-67 (BD PharMingen), CD4, CD90.2, FoxP3 (eBioscience), CD45RB, and CD44 (BioLegend). For IgG1 enzyme-linked immunosorbent assay, plates were coated with PG (0.5 μg/well) and blocked with 1.5% milk in PBS. Sera were added at a 1:100,000 dilution, and PG-specific antibodies were determined using peroxidase-conjugated rat anti-mouse IgG1

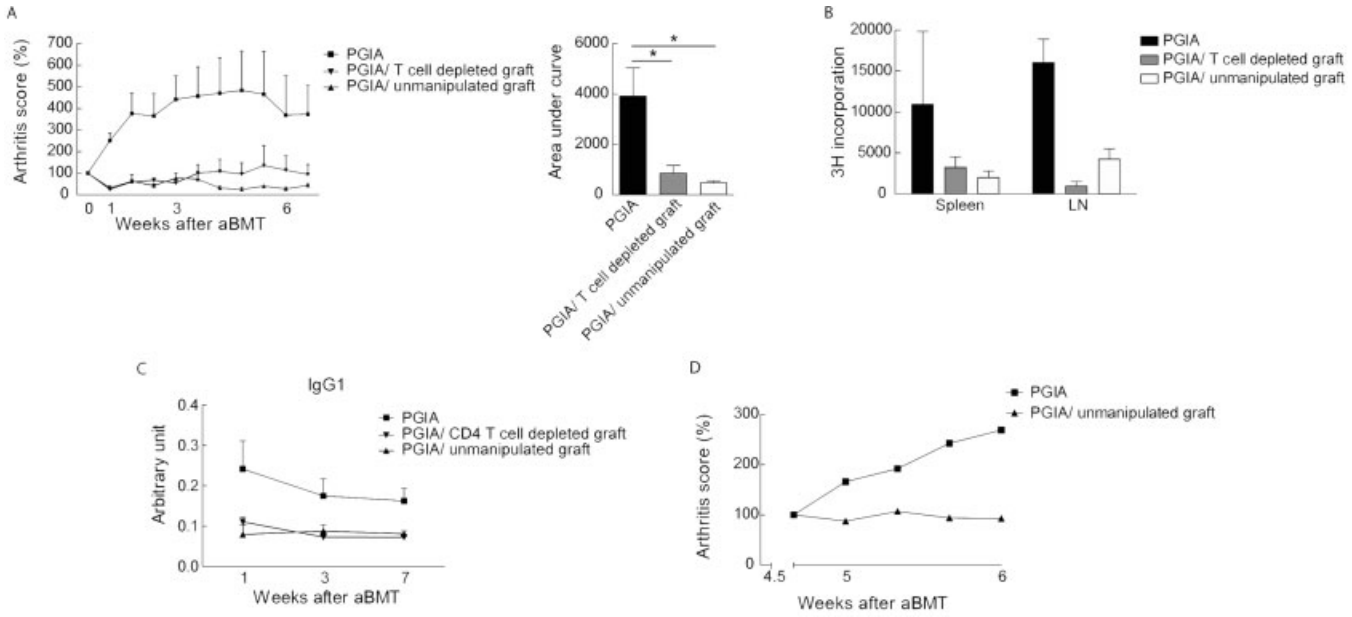

Figure 1. Reduction and stabilization of proteoglycan-induced arthritis (PGIA) after autologous bone marrow transplantation (ABMT). **A,** Left, Arthritis scores after ABMT. Arthritis scores were set to 100%, and the subsequent clinical effect was expressed as a percentage of the score at the time of transplantation. Results are representative of 2 separate experiments. Right, Area under the arthritis score curve. Values are the mean ± SEM (8 PGIA [control], 5 PGIA/T cell–depleted grafts, and 5 PGIA/unmanipulated grafts). $* = P < 0.05$ by Mann-Whitney U test. **B,** T cell proliferation after PG stimulation, as determined by ^{3}H-thymidine incorporation. Values are the mean ± SEM (2 PGIA, 3 PGIA/T cell–depleted grafts, and 4 PGIA/unmanipulated grafts). **C,** Levels of PG-specific IgG1 antibodies after ABMT, as determined by enzyme-linked immunosorbent assay. Values are the mean ± SEM (n = 5–8 PGIA, 4 PGIA/CD4+ T cell–depleted grafts, and 2–5 PGIA/unmanipulated grafts). **D,** Arthritis scores after administration of a booster dose of PG 4–5 weeks following ABMT. Arthritis scores were set to 100%, and values are the percent increase from the day after administration of the boost (n = 3 PGIA and 3 PGIA/unmanipulated grafts). LN = lymph node.

monoclonal antibody (clone X56; 1:1000). Serum antibody levels were calculated relative to mouse serum immunoglobulin fractions in the pooled sera of control mice.

Intracellular cytokine production. Seven weeks after ABMT, spleen and lymph node cells (5×10^5) were cultured with phorbol myristate acetate (25 ng/ml) and ionomycin (500 ng/ml; Calbiochem) for 5–6 hours. After 1 hour, GolgiStop (BD Biosciences) was added to the cultures. Cells were stained for anti-mouse TCRβ, CD4, CD90.1, TNFα, interleukin-17 (IL-17) (all from BD PharMingen), and interferon-γ (IFNγ) antibodies (eBioscience).

Statistical analysis. The Mann-Whitney U test was used to determine differences between untreated and ABMT-treated mice with PGIA. To achieve normal distribution for cytokine data, logarithmic transformation was performed before applying the Mann-Whitney U test. Wilcoxon's matched pairs signed rank test was performed to determine significant differences between host cells and donor cells. All data are presented as the mean ± SEM. *P* values less than 0.05 were considered significant.

RESULTS

Decreased disease activity using T cell–depleted or unmanipulated BM grafts for ABMT. Because the results of clinical studies have suggested that the presence of low numbers of potentially self-reactive memory T cells in an infused stem cell graft may pose a risk of disease relapse, we performed ABMT using T cell–depleted BM grafts and unmanipulated BM grafts. Both treatments resulted in a decrease in arthritis scores, which remained low compared with the scores of untreated mice with PGIA until the end of the observation period (Figure 1A). The area under the arthritis score curve was also significantly lower in both BMT treatment groups (T cell–depleted and unmanipulated grafts) compared with untreated mice with PGIA (Figure 1A).

To determine whether T cell depletion of the graft led to a difference in PG-specific responses, spleen and lymph node cells were restimulated with PG. After in vitro exposure to PG, proliferation was reduced in both treatment groups compared with the untreated control group (Figure 1B). In addition, the reductions in PG-specific IgG1 antibody levels following ABMT were similar in mice that received unmanipulated grafts and those that received CD4+ T cell–depleted grafts (Figure

1C), suggesting that the presence of low numbers of T cells in the graft does not influence treatment outcome.

Next, to determine persistence of tolerance to the disease antigen, a booster dose of PG was administered intraperitoneally to ABMT-treated and control mice with PGIA, 10 weeks after the first injection of PG in DDA. In control mice with PGIA, in vivo reexposure to PG resulted in increased arthritis scores, whereas arthritis scores in ABMT-treated mice remained stable (Figure 1D), indicating that ABMT induces in vivo tolerance.

Effect of ABMT on basal levels of antigen-specific T cell production of IFNγ, IL-17, and TNFα. Following ABMT, the number of CD4+ T cells was clearly reduced, and cells with a memory phenotype predominated (Figure 2A). The antigen specificity, differentiation, and function of reconstituting T cells are likely decisive for the efficacy of ABMT in autoimmune diseases. In untreated control mice, basal levels of IFNγ, IL-17, and TNFα were observed after culturing spleen cells without any stimulus (Figure 2B). After stimulation with PG, the production of IFNγ, IL-17, and TNFα almost doubled in the cell cultures of untreated mice (Figure 2C). In contrast, splenocytes from mice that underwent ABMT did not show IFNγ, IL-17, and TNFα production when cultured in medium alone. In addition, no production of IFNγ, IL-17, or TNFα could be measured following culture with PG. This absence of PG-induced production of proinflammatory cytokines was observed 1, 3, and 7 weeks following ABMT, suggesting long-term suppression of PG-specific T cell responses. The lack of cytokine production by splenocytes derived from mice treated with ABMT was not the result of a general impairment in cytokine production by these T cells, because nonspecific anti-CD3 stimulation induced similar levels of IFNγ, IL-17, and TNFα in the control and ABMT groups (Figure 2D). Taken together, these results show that ABMT leads to a less inflammatory environment and a strong reduction in self antigen–induced cytokine production.

Characteristics of the donor-derived CD4+ T cell compartment and the host CD4+ T cell compartment. As shown in Figure 2, the (antigen-specific) production of proinflammatory cytokines was reduced after ABMT. This could be attributable to a different environment created by conditioning but also by the renewal of the Teff cell population. By using unmanipulated BM with a congenic T cell marker (CD90.1), we were able to investigate the effect of ABMT on changes in the CD4+ T cell compartment. Seven weeks after ABMT, the majority of CD4+ T cells present in spleen and lymph nodes were donor derived, and these donor cells showed increased proliferation compared with the remaining host cells (Figure 3A). Importantly, donor-

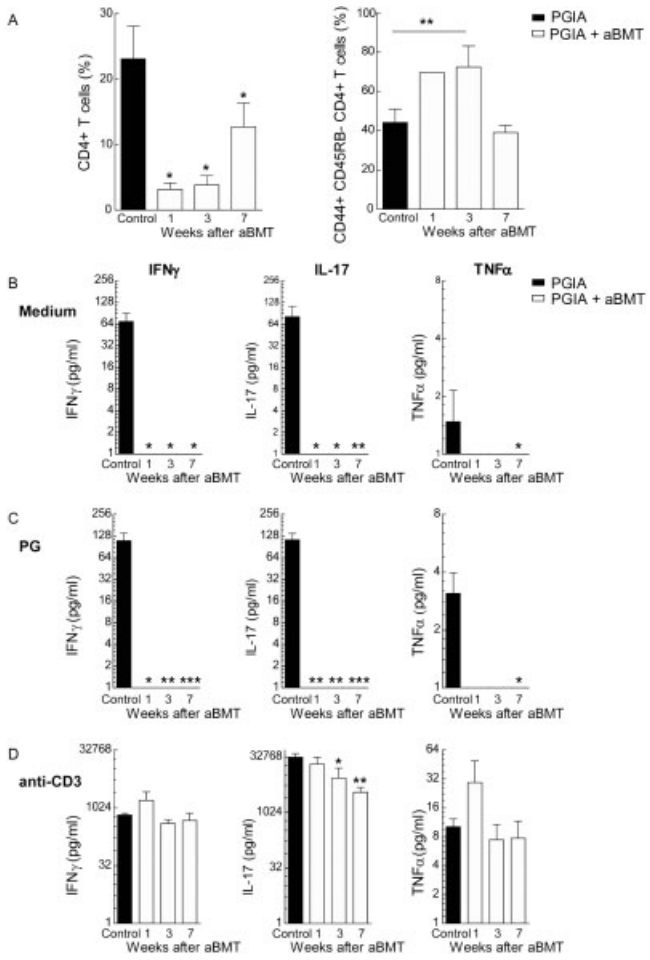

Figure 2. Reduced antigen-specific T cell production of proinflammatory cytokines following ABMT. **A,** Left, Percentages of CD4+ T cells following ABMT. Values are the mean ± SEM (control, n = 11; week 1, n = 5; week 3, n = 7; week 7, n = 9). Right, Percentages of memory CD4+ T cells following ABMT. Values are the mean ± SEM (control, n = 11; week 1, n = 1; week 3, n = 5; week 7, n = 6). **B–D,** T cell–specific cytokine production. Spleen cells were harvested 1, 3, and 7 weeks after ABMT with unmanipulated BM. The cells were cultured in medium alone (**B**) or in the presence of PG (10 μg/ml) (**C**) or anti-CD3 (1 μg/ml) (**D**) for 96 hours. The supernatants were collected and analyzed for interferon-γ (IFNγ), interleukin-17 (IL-17), and tumor necrosis factor α (TNFα) production. Mice with PGIA (control) were killed at different time points, and the data for these mice were pooled (n = 17–19). For mice with PGIA that underwent ABMT, n = 3 at week 1; n = 4 at week 3; and n = 6 at week 7. Values are the mean ± SEM on a $\log_2$ scale and are representative of pooled data from 2 separate experiments. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ versus control, by Mann-Whitney U test. See Figure 1 for other definitions.

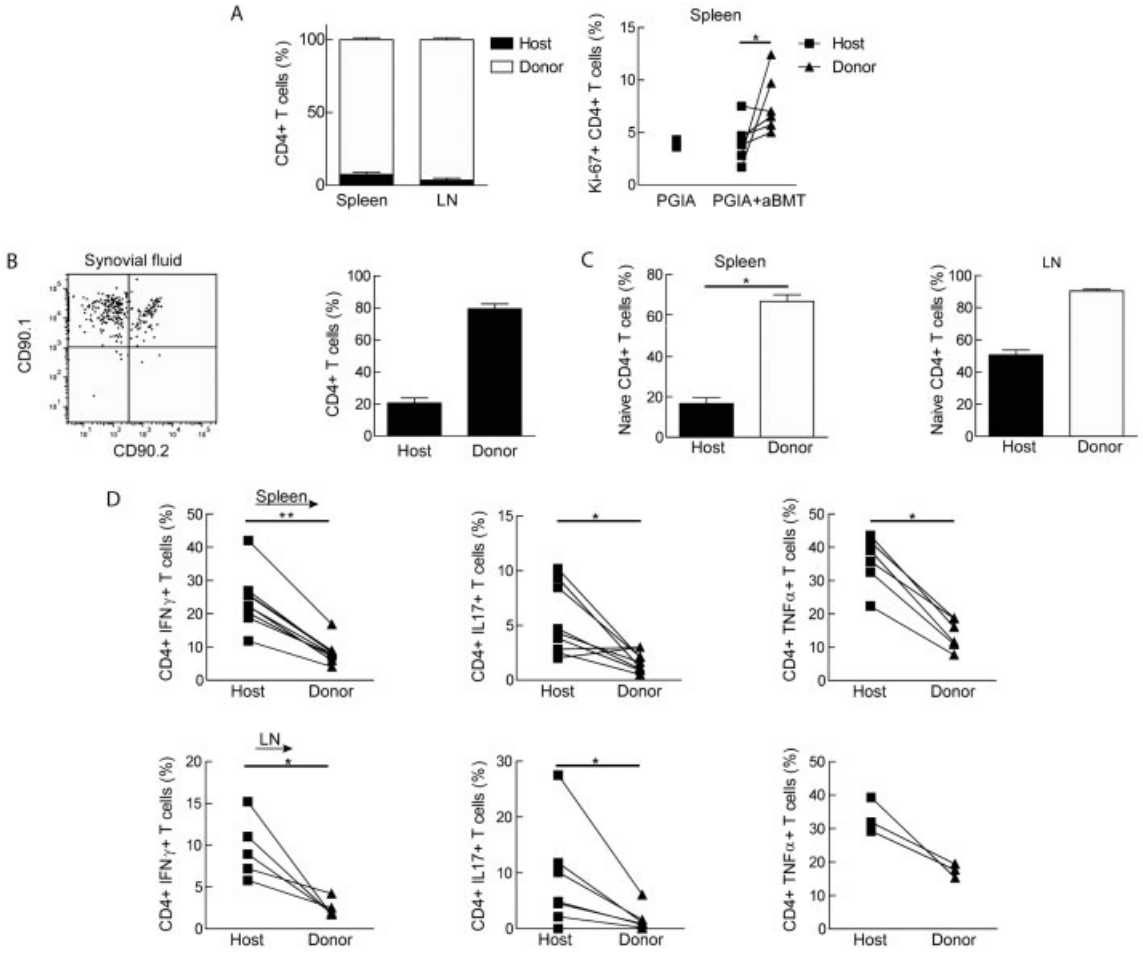

Figure 3. Disease-associated cytokines produced by naive donor-derived CD4+ T cells and residual host-derived T cells. **A,** Left, Percentages of T cell receptor β (TCRβ)+CD4+CD90.2+ (host) cells and TCRβ+CD4+CD90.1+ (donor) cells in spleen and LNs 7 weeks after ABMT (unmanipulated graft). Values are the mean ± SEM (n = 6). Right, Percentages of control, host, and donor Ki-67+TCRβ+CD4+ T cells (control, n = 4; PGIA + ABMT, n = 6). **B,** Left, Fluorescence-activated cell sorting analysis with gating for TCRβ+CD4+ synovial fluid cells. Host T cells are CD90.1CD90.2 double-positive, donor T cells are CD90.1+. Right, Percentages of host and donor CD4+ T cells in synovial fluid. Values are the mean ± SEM (n = 2). **C,** Percentages of host and donor naive (CD45RB^{high}CD44^{low}FoxP3−) CD4+ T cells in spleen (n = 6) and LNs (n = 3). Values are the mean ± SEM (spleen, n = 6; LNs, n = 3). **D,** Intracellular cytokine staining for interferon-γ (IFNγ), interleukin-17 (IL-17), and tumor necrosis factor α (TNFα) in splenocytes (top; n = 6–9) and LN cells (bottom; n = 3–7). Pooled data from 2 separate experiments are shown. $* = P < 0.05$; $** = P < 0.01$, by Wilcoxon matched pairs signed rank test. See Figure 1 for other definitions.

derived CD4+ T cells were also observed locally, in the synovial fluid of ABMT-treated mice (Figure 3B). As expected, the majority of donor T cells were naive, whereas most host CD4+ T cells showed a memory phenotype (Figure 3C). The percentages of host CD4+ T cells producing IFNγ, IL-17, or TNFα were significantly higher compared with the percentages of donor T cells, in both spleen and lymph nodes, confirming the less-activated status of donor-derived T cells (Figure 3D). Similar results were obtained using CD4+ T cell–depleted grafts (results not shown).

Taken together, these data demonstrate that ABMT induces renewal of the CD4+ T cell compartment by BM graft–derived T cells that display a more naive and less inflammatory phenotype and also home to the site of inflammation.

DISCUSSION

Autologous stem cell transplantation induces stable remission in a substantial portion of patients with severe JIA (1). Understanding the working mechanisms

of ASCT in autoimmune diseases may help in the development of new therapies that result in the same outcome but with less toxic side effects.

One of the original hypotheses for the success of ASCT in autoimmune disease is that ASCT eradicates autoaggressive T cell populations. Although conditioning ablates the T cell repertoire to a large extent, elimination is never complete, and there is a reasonable risk that autoaggressive T cells will persist. In addition, memory T cells will also be infused with the autologous stem cell graft. In peripheral blood stem cell–mobilized grafts, the number of T cells is 10-fold higher than the number in conventional BM grafts, but the T cells have a more naive phenotype (10). Here, we show in a mouse model of PGIA that the use of unmanipulated BM gives the same clinical results as T cell–depleted BM; this suggests that T cells present in the BM graft have a minimal effect on the clinical course of PGIA following ABMT. Recently, Snowden et al reported that none of the European Group for Blood and Marrow Transplantation registry outcome analyses to date support ex vivo depletion strategies (11). Consistent with this observation, more intense T cell depletion has also been associated with a higher rate of tolerance failure and the development of secondary autoimmune disease following ASCT (12).

Autoreactive T cells that survive conditioning represent another risk factor for the loss of self-tolerance following ASCT, especially in the setting of autoimmunity. Our results using a PGIA model of syngeneic BMT now demonstrate that remaining host CD4+ T cells proliferate vigorously early after ABMT and display a proinflammatory phenotype characterized by the production of IFNγ, IL-17, and TNFα. Because the development of PGIA is dependent on both IFNγ and IL-17 production (13,14), these results suggest a relatively high risk of loss of tolerance and relapse of arthritis shortly after ABMT. Consistent with this, in our multicenter study of ASCT in patients with JIA, 90% of disease relapses occurred within 9 months after ASCT (4). However, our data also show that despite the presence of these proinflammatory host CD4+ T cells, basal proinflammatory cytokine production and self antigen–specific cytokine responses are strongly reduced immediately after ABMT. In addition, the risk of early relapses may also be controlled by the presence and expansion of Treg cells, as shown in a previous study (9). Taken together, these results show that shortly after ASCT, there is a very delicate balance between lymphopenia-induced immune cell expansion and activation versus immune suppression and immune regulation that will determine the clinical outcome and early relapses.

Following the early reconstitution period, proinflammatory host T cells are thought to be steadily replaced by T cells derived from the autologous graft. In patients with JIA who underwent ASCT, it was suggested that renewal of the T cell compartment induces autoreactive T cells with a more regulatory phenotype (4). In our mouse model of PGIA, the "donor" cells indeed displayed a more naive and less inflammatory phenotype compared with the "host" cells, despite the enormous expansion of the number of donor cells. Furthermore, autoaggressive T cell responses remained low during the 7-week followup period after BMT. Taken together, our data indicate that ABMT resets the immunologic clock by renewing the functional CD4+ T cell compartment.

Restoration of immune tolerance is still considered the Holy Grail for the treatment of autoimmune diseases. ASCT is the only treatment that can lead to a sustained restoration of the immune balance in patients with severe autoimmune disease. Understanding the immunologic mechanisms of ASCT will help us develop new therapies that have the same clinical outcome but with less toxic side effects.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. van Wijk had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Delemarre, Roord, Zonneveld-Huijssoon, Rozemuller, Martens, Wulffraat, Prakken, van Wijk.

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Analysis and interpretation of data. Delemarre, Roord, van den Broek, Rozemuller, Martens, Broere, Glant, van Wijk.

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