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Autologous stem cell transplantation restores immune tolerance in experimental arthritis by renewal and modulation of the T effector compartment

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Abstract

Objective—Autologous stem cell transplantation (aSCT) induces long-term drug free disease remission in patient with juvenile idiopathic arthritis. This study was undertaken to further unravel the immunological mechanism underlying aSCT by using a mouse model of proteoglycan (PG)-induced arthritis (PGIA).

Methods—PGIA was induced in BALB/c mice by two intraperitoneal injections of human proteoglycan in a synthetic adjuvant on days 0 and 21. Five weeks after the first immunization, mice received 7.5 Gy total body irradiation and (un)manipulated bone marrow grafts of PGIA mice. Clinical scores, T cell reconstitution, (antigen-specific) T cell cytokine production and intracellular cytokine expression were determined following autologous bone marrow transplantation.

Results—Autologous bone marrow transplantation (aBMT) induced amelioration and stabilisation of arthritis scores. Bone marrow containing T cells gave the same clinical benefit as T cell depleted grafts, with similar reduction in PG-induced T cell proliferation and PG-specific autoantibodies. In vivo re-exposure to PG did not result in disease exacerbation. Following aBMT, basal levels of disease associated pro-inflammatory cytokines (IFN γ , IL-17 and TNF α) were reduced. In addition, T cell re-stimulation with the disease antigen showed a strong reduction in disease-associated pro-inflammatory cytokine production. Finally, while remaining host T cells

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displayed a pro-inflammatory phenotype following aBMT, IFN γ , IL-17 and TNF α cytokine production by the newly reconstituted donor derived T cells was significantly lower.

Conclusion—Together our data suggest that aBMT restores immune tolerance by renewal and modulation of the T effector compartment, leading to a strong reduction in pro-inflammatory (self antigen-specific) T cell cytokine production.

Introduction

Juvenile Idiopathic Arthritis (JIA) and Rheumatoid Arthritis (RA) are autoimmune diseases, which often lead to major disability. The introduction of biological agents such as anti-TNF α has been a major step forward in controlling disease symptoms. However, in general these treatments cannot induce a drug free disease remission. Furthermore, some patients fail to respond to conventional treatment or become unresponsive over time. In JIA, for these severely ill patients, autologous stem cell transplantation (aSCT) has proven to be an effective last resort. It induces drug free disease remission in a majority of patients during a follow-up of up to 80 months post transplantation (1–3). The drug free disease remission achieved by aSCT in the majority of patients suggests that aSCT can, at least temporarily, restore immune tolerance in JIA. However, the underlying mechanisms are largely unknown. Data from the JIA cohort suggested that both renewal of the regulatory T cell compartment and reprogramming of effector T cells may play a role (4). Unfortunately, T cell reconstitution after aSCT in human subjects cannot be followed up because residual T cells and autologous graft-derived T cells are not distinguishable. A better understanding of the mechanisms would greatly favour the development of new treatment strategies that aim at not just immune suppression but restoring immune tolerance.

Although it remains to be elucidated what underlies the impressive success of aSCT, immune reconstitution after profound depletion appears to favor the development of tolerance over pathogenic immunity. Immediately after re-infusion with aSCT, the lymphopenic environment induces selective expansion and activation of the few T cell clones present. These T cells have survived the conditioning regimen (5) or may have been retransferred with the graft and are potentially auto-reactive. 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 regulatory T cells (Treg) may be essential to control T cell reconstitution and activation. In a later stage the CD4 T cell pool is further reconstituted by naïve recent thymic emigrants, which crucial for diversification of the T cell repertoire following aSCT (6) and may also play a role in the re-establishment of immune tolerance. Taken together, the antigen-specificity, differentiation and function of the reconstituting T cells appear decisive for the efficacy of aSCT and warrants further investigation.

To elucidate this process we developed an experimental model for autologous stem cell transplantation in proteoglycan (PG)-induced arthritis (PGIA). PGIA is extensively studied, has clinical, immunological and histopathological resemblance to human arthritis and has a chronic relapsing remitting course (7,8). In this model we have demonstrated a crucial role for Tregs in the recovery phase after autologous bone marrow transplantation (aBMT) (9).

We now explored the influence of aBMT on the effector T cell compartment and demonstrate that aBMT induced renewal of the T cell compartment leads to a strong reduction in pro-inflammatory (self antigen-specific) T cell responses.

Materials and Methods

Mice

Female retired breeder BALB/c mice were obtained from Charles River (Sulzfeld, Germany). CBy.PL(B6)-Thy1a.ScrJ mice of 7–10 weeks old were obtained from Jackson Laboratory, (Bar Harbor, USA) and served as donors for aBMT when indicated.

Mice were maintained under regular conditions. After aBMT, recipient mice were housed under sterile conditions. All experiments were approved by the local Animal Experiment Ethical Committee.

Induction and assessment of arthritis

Arthritis was induced in BALB/c mice by two intraperitoneal (i.p.) injections of 400 µg PG in 2 mg adjuvant dimethyl dioctadecyl ammonium bromide (DDA, Sigma Aldrich, Zwijndrecht, the Netherlands) two and five weeks before stem cell transplantation. PG was purified as described previously (7,8). Arthritis was assessed three times a week by a visual scoring system as described previously (9).

Treatment protocols

Autologous bone marrow transplantation—Two weeks after the second PG/DDA injection, recipient arthritic mice received a lethal dose of 7.5 Gy total body irradiation. Next, aBMT was performed by intravenous injection of 2×10^6 syngeneic bone marrow (BM) cells.

Bone marrow suspension—BM was harvested by flushing tibia and femur. 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 by magnetic depletion using anti-mouse CD4 and anti-mouse CD8 MACS micro beads (Miltenyi Biotec). The T cell depleted bone marrow cells contained 0.67% (CD3+) T cells (data not shown).

Re-boost with PG—4,5 weeks after aBMT, PGIA animals that received no treatment or aBMT with unmanipulated grafts, received a boost of 400µg PG i.p. The clinical response was compared to animals that received the same treatment, but received phosphate buffered saline (PBS) i.p.

In vitro assays

T cell proliferation—Seven weeks following aBMT, spleen and axial lymph nodes were harvested. 2×10^5 cells/well were cultured in supplemented IMDM culture medium for 120 hours in the absence or presence of 10 µg/ml PG. During the last 16–18 hours $1 \mu\text{Ci } ^3\text{H}$ (^3H -

TdR; Amersham, Buckinghamshire, U.K.) was added per well. Proliferative responses were calculated as the median ^3H incorporation (cpm) of triplicate wells.

Cytokine production—One, three and seven weeks after aBMT, spleen cells (2×10^5) were cultured in culture medium for 96 hrs in the presence of 10 $\mu\text{g/ml}$ PG or 1 $\mu\text{g/ml}$ soluble anti-CD3 (clone 145-2C11, BD Pharmingen, San Diego, CA). Cytokine profiles were measured using a mouse cytokine multiplex kit (Biorad, CA, USA) according to the manufacturer's instruction.

Flow cytometry—Spleen, LN and synovial fluid cells were stained for TCR- β , CD90.1, Ki-67 (BD Pharmingen), CD4, CD90.2, Foxp3 (eBioscience) CD45RB and CD44 (BD Biolegend).

IgG1 ELISA—Plates were coated with PG (0.5 $\mu\text{g/well}$) and blocked with 1.5% milk in PBS. Sera were added in a 1:100,000 dilution and PG specific antibodies (Ab) were determined using peroxidase conjugated IgG1 mouse monoclonal Ab (X56 rat IgG1, 1:1000). Serum Ab levels were calculated relative to mouse serum Ig fractions of pooled sera of control mice.

Intracellular cytokine production—Seven weeks post aBMT, spleen and LN cells were cultured (5×10^5) with phorbol myristate acetate (PMA, 25ng/ml) and Ionomycin (500ng/ml, Calbiochem) for 5–6 hours. After one hour, Golgistop (BD Biosciences) was added to the cultures. Cells were stained for anti-mouse TCR- β , CD4, CD90.1 and TNF α , IL-17 (all BD Pharmingen) and IFN γ antibodies (eBioscience).

Statistical analysis

To identify differences between aBMT-treated PGIA animals and untreated PGIA animals, the Mann-Whitney U test was used. To achieve normal distribution for cytokine data, logarithmic transformation was performed before applying Mann-Whitney U test. Significant differences between host versus donor cells was tested by using Wilcoxon matched-pairs signed rank test. All data are presented as the mean + SEM values (error bars). P values < 0.05 were considered significant.

Results

Autologous BMT decreases equally disease activity using T cell depleted or unmanipulated BM grafts

Since it has been suggested in clinical studies that the presence of low numbers of potentially self-reactive memory T cells in the infused stem cell graft may pose a risk to relapsing of the disease, aBMT was performed with T cell depleted bone marrow grafts and compared with unmanipulated bone marrow grafts. Both treatments gave a decrease of arthritis scores after aBMT and remained low compared to untreated PGIA animals till the end of the observation period (Figure 1A, left panel). The area under the arthritis curve was also significantly lower in both BMT treatment groups (T cell depleted and unmanipulated grafts) versus untreated PGIA mice (Figure 1A, right panel).

To determine if T cell depletion of the graft led to a difference in PG-specific responses, spleen and lymph node cells were re-stimulated with PG. Both treatment groups showed reduced proliferation after *in vitro* exposure to PG compared to PGIA control mice (Figure 1B). In addition, PG-specific IgG1 antibody levels following aBMT were similarly reduced in both animals treated with unmanipulated grafts or CD4⁺ T cell depleted grafts (Figure 1C), suggesting that the presence of low T cell numbers in the graft does not influence treatment outcome.

Next, to determine persistent tolerance for the disease antigen, 7 weeks after the first PG/DDA injection, aBMT-treated animals and PGIA control mice received an *i.p.* booster with PG. In PGIA control mice, *in vivo* re-exposure to PG resulted in increased arthritis scores whereas arthritis scores in aBMT treated animals remained stable (Figure 2D), indicating that aBMT treatment induces *in vivo* tolerance.

Decreased basal levels and suppression of antigen-specific T cell production of IFN γ and IL-17 following aBMT

Post aBMT, there was a clear reduction in CD4⁺ T cells (Figure 2A, left panel) with a predominance of a memory phenotype (Figure 2A, right panel). Antigen-specificity, differentiation and function of reconstituting T cells are likely decisive for efficacy of aBMT in autoimmune diseases. In untreated animals basal levels of IFN γ , IL-17 and TNF α were found after culture of spleen cells without any stimuli (Figure 2B). After PG stimulation, the amount IFN γ , IL-17 and TNF α production was doubled in cell cultures of untreated animals (Figure 2C). In contrast, splenocytes of transplanted animals did not show IFN γ , IL-17 and TNF α production when cultured in medium alone. In addition, upon culture with PG, still no IFN γ , IL-17 or TNF α production could be measured. This absence of PG induced pro-inflammatory cytokines was observed 1, 3 and 7 weeks following aBMT suggesting a long-term T cell effect of the transplantation. The lack of cytokine production by splenocytes derived from aBMT treated animals was not the result of a general impairment in cytokine production by these T cells as non-specific anti-CD3 stimulation induced similar levels of IFN γ , IL-17 and TNF α in the control and aBMT treated groups (Figure 2D).

Together these results show that aBMT leads to less inflammatory environment and a strong reduction in self-antigen induced cytokine production.

Donor derived CD4⁺ T cell compartment is more naive and less pro-inflammatory compared to host T cell compartment

As shown in Figure 2, the (antigen-specific) production of pro-inflammatory cytokines was reduced after aBMT treatment. This could be due to a different environment created by conditioning, but also by the renewal of the (effector) T 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. As shown in Figure 3A left panel, 7 weeks after aBMT, the majority CD4⁺ T cells present in spleen and LN were donor derived, and these donor cells showed increased proliferation compared to remaining host cells (Figure 3A, right panel). Importantly, donor derived CD4⁺ T cells were also found locally, in the synovial fluid of aBMT treated mice (Figure 3B). As expected the majority of

donor T cells were naïve whereas most host CD4⁺ T cells showed a memory phenotype (Figure 3C). The percentage of host CD4⁺ T cells producing IFN γ , IL-17 or TNF α was significantly higher compared to the percentage of donor T cells in both spleen in LN, confirming the less activated status of donor-derived T cells (Figure 3D). Similar results were obtained using CD4⁺ T depleted grafts (data not shown).

Together these data demonstrate that aBMT induces renewal of the CD4⁺ T cell compartment by BM graft-derived T cells that display a more naïve 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 for autoimmune diseases may help us to develop new therapeutic treatments with the same outcome, but with less toxic side effects.

One of the original hypotheses for the success of aSCT in autoimmune disease has been the eradication of auto-aggressive T cell populations. Although conditioning ablates the T cell repertoire to a large extent, elimination is never complete and there is a reasonable risk of persisting auto-aggressive T cells. 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 times higher but the T cells have a more naïve phenotype (10). Here, we show that in the PGIA aBMT mouse model use of unmanipulated BM gives the same clinical results as T cell depleted BM, suggesting a minimal effect of T cells present in the BM graft on the clinical course following aBMT. Recently, the European group for blood and marrow transplantation also stated that so far there is no evidence to support the use of *ex vivo* manipulated bone marrow cells (11). In line with this, 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).

Auto-reactive T cells that have survived the conditioning represent another risk factor for the loss of self-tolerance following aSCT, especially in an autoimmune setting. Our results in the PGIA syngeneic model now demonstrate that remaining host CD4⁺ T cells proliferate vigorously early after aBMT and display a pro-inflammatory phenotype characterized by the production of IFN- γ , IL-17 and TNF- α . Since arthritis development in PGIA is dependent on both IFN γ and IL-17 production (13,14) these results suggest that there is a relatively high risk of loss of tolerance and relapse of arthritis shortly after aBMT. In line with this, in the aSCT JIA multi-centre study, 90% of disease relapses occurred within 9 months after aSCT (4). However, our data also show that despite the presence of these pro-inflammatory host CD4⁺ T cells, basal pro-inflammatory 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 regulatory T cells (Treg) as we have shown in a previous paper (9). 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 re-constitution period, the pro-inflammatory host T cells are thought to be steadily replaced by T cells derived from the autologous graft. In JIA aSCT-treated patients, it was suggested that renewal of the T cell compartment induces auto-reactive T cells with a more regulatory phenotype (4). In the PGIA aBMT model the “donor” cells indeed displayed a more naïve and less inflammatory phenotype than the “host” cells despite their enormous expansion. Furthermore, auto-aggressive T cell responses remained low during the 7 week follow-up after BMT. Together our data indicate that aBMT resets the immunological 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. Autologous SCT is the only treatment that can lead to a sustained restoration of the immune balance in severe autoimmune disease. Understanding the immunological 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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References

1. Swart JF, Lindemans CA, van RA, Boelens JJ, Prakken BJ, Wulffraat N. Changing winds in refractory autoimmune disease in children: clearing the road for tolerance with cellular therapies. *Curr Opin Rheumatol.* 2012; 24:267–273. [PubMed: 22418744]
2. Brinkman DM, de K I, ten CR, van Rossum MA, Bekkering WP, Fasth A, et al. Autologous stem cell transplantation in children with severe progressive systemic or polyarticular juvenile idiopathic arthritis: long-term follow-up of a prospective clinical trial. *Arthritis Rheum.* 2007; 56:2410–2421. [PubMed: 17599770]
3. Wulffraat N, van RA, Bierings M, Vossen J, Kuis W. Autologous haemopoietic stem-cell transplantation in four patients with refractory juvenile chronic arthritis. *Lancet.* 1999; 353:550–553. [PubMed: 10028984]
4. de Kleer I, Vastert B, Klein M, Teklenburg G, Arkesteijn G, Yung GP, et al. Autologous stem cell transplantation for autoimmunity induces immunologic self-tolerance by reprogramming autoreactive T cells and restoring the CD4+CD25+ immune regulatory network. *Blood.* 2006; 107:1696–1702. [PubMed: 16263787]
5. Verburg RJ, Flierman R, Sont JK, Ponchel F, van DL, Levarht EW, et al. Outcome of intensive immunosuppression and autologous stem cell transplantation in patients with severe rheumatoid arthritis is associated with the composition of synovial T cell infiltration. *Ann Rheum Dis.* 2005; 64:1397–1405. [PubMed: 15829573]
6. Muraro PA, Douek DC, Packer A, Chung K, Guenaga FJ, Cassiani-Ingoni R, et al. Thymic output generates a new and diverse TCR repertoire after autologous stem cell transplantation in multiple sclerosis patients. *J Exp Med.* 2005; 201:805–816. [PubMed: 15738052]

7. Glant TT, Mikecz K. Proteoglycan aggrecan-induced arthritis: a murine autoimmune model of rheumatoid arthritis. *Methods Mol Med.* 2004; 102:313–338. [PubMed: 15286393]
8. Glant TT, Finnegan A, Mikecz K. Proteoglycan-induced arthritis: immune regulation, cellular mechanisms, and genetics. *Crit Rev Immunol.* 2003; 23:199–250. [PubMed: 14584879]
9. Roord ST, de JW, Boon L, Wulffraat N, Martens A, Prakken B, et al. Autologous bone marrow transplantation in autoimmune arthritis restores immune homeostasis through CD4+CD25+Foxp3+ regulatory T cells. *Blood.* 2008; 111:5233–5241. [PubMed: 18256318]
10. Ottinger HD, Beelen DW, Scheulen B, Schaefer UW, Grosse-Wilde H. Improved immune reconstitution after allotransplantation of peripheral blood stem cells instead of bone marrow. *Blood.* 1996; 88:2775–2779. [PubMed: 8839875]
11. Snowden JA, Saccardi R, Allez M, Ardizzone S, Arnold R, Cervera R, et al. Haematopoietic SCT in severe autoimmune diseases: updated guidelines of the European Group for Blood and Marrow Transplantation. *Bone Marrow Transplant.* 2012; 47:770–790. [PubMed: 22002489]
12. Daikeler T, Labopin M, Di GM, Abinun M, Alexander T, Miniati I, et al. Secondary autoimmune diseases occurring after HSCT for an autoimmune disease: a retrospective study of the EBMT Autoimmune Disease Working Party. *Blood.* 2011; 118:1693–1698. [PubMed: 21596847]
13. Doodes PD, Cao Y, Hamel KM, Wang Y, Rodeghero RL, Mikecz K, et al. IFN-gamma regulates the requirement for IL-17 in proteoglycan-induced arthritis. *J Immunol.* 2010; 184:1552–1559. [PubMed: 20028652]
14. Doodes PD, Cao Y, Hamel KM, Wang Y, Farkas B, Iwakura Y, et al. Development of proteoglycan-induced arthritis is independent of IL-17. *J Immunol.* 2008; 181:329–337. [PubMed: 18566398]

percentage increase from the day after the boost. * Indicates p-value <0.05 compared to the PGIA group calculated using Mann-Whitney U test.

Figure 2. Reduction of antigen-specific T cell pro-inflammatory cytokine production post aBMT
(A) Percentage of total and memory CD4⁺ T cells. Spleen cells were harvested post aBMT (unmanipulated graft) and stained for FACS analysis. Left panel. Control N=11, 1 week N=5, 3 weeks N=7, 7 weeks N=9. Right panel Control N=11, 1 week N=1, 3 weeks N=5, 7 weeks N=6. Shown are the mean + SEM bars. **B, C and D. T cell specific cytokine production.** Spleen cells were harvested one, three and seven weeks after aBMT with unmanipulated BM. The cells were cultured in medium alone (**B**) or in the presence of PG (10 μ g/ml) (**C**) or anti-CD3 (1 μ g/ml) (**D**) for 96 hours. The supernatants were collected and

analyzed using Multiplex Immuno Assay for IFN γ , IL-17 and TNF α production. Pooled data of two separate experiments are shown. PGIA animals without aBMT (Control) were sacrificed on different time points and data of these animals are pooled together in the graph N=17–19, PGIA+ aBMT N=3 for 1 week, N=4 for 3 weeks, N=6 for 7 weeks. Depicted are mean cytokine production + SEM values at a 2-log scale. * Indicates p-value <0.05, ** indicates p-value <0.01 and *** indicates p-value <0.001 compared to the control PGIA group calculated using Mann-Whitney U test.

Figure 3. Naive donor derived CD4⁺ T cell compartment produces less disease associated cytokines compared to residual host derived T cells

(A) Left panel, percentage of TCRβ⁺ CD4⁺ CD90.2⁺ (host) cells versus TCRβ⁺ CD4⁺ CD90.1⁺ (donor) cells in spleen and LN 7 weeks after aBMT (unmanipulated graft). Mean values + SEM values are depicted, N=6. Right panel, percentage of control PGIA, host, and donor Ki-67+TCRβ+CD4⁺ T cells. Control PGIA N=4, PGIA+aBMT N=6. (B) In this experiment host T cells are CD90.1CD90.2 double positive, donor T cells are CD90.1+. Left panel shows FACS dot plot gated for TCRβ+CD4+ synovial fluid cells. Right panel shows percentage host versus donor CD4⁺ T cells in synovial fluid. N=2. Mean values + SEM

values are depicted. **(C)** Percentage naive (CD45Rb high, CD44 low, Foxp3 negative) CD4⁺ T cells in spleen (left panel) and LN (right panel). Spleen N=6, LN N=3. Mean values + SEM values are depicted. **(D)** Intracellular cytokine staining for IFN γ , IL-17 and TNF α . Upper three graphs show splenocytes, N=6–9. Lower three graphs show LN cells, N=3–7. For both organs pooled data of two separate experiments are shown. * Indicates p-value <0.05, ** indicates p-value <0.01 between host and donor T cells measured with Wilcoxon matched-pairs signed rank test.