High dose chemotherapy and autologous hematopoietic stem cell transplantation for rheumatoid arthritis

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CHAPTER 6

THE OUTCOME OF INTENSIVE IMMUNOSUPPRESSION AND AUTOLOGOUS STEMCELL TRANSPLANTATION IN PATIENTS WITH SEVERE RHEUMATOID ARTHRITIS IS ASSOCIATED WITH CHANGES IN THE COMPOSITION OF SYNOVIAL T CELL INFILTRATION.

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Abstract.
Objective. High dose chemotherapy (HDC) followed by autologous stem cell transplantation (ASCT) is an experimental treatment modality for patients with severe autoimmune diseases including refractory rheumatoid arthritis (RA). It is aimed at immunoablation to allow regeneration of a non-autoaggressive immune system from reinfused stem cells. This study was undertaken to determine clinical and immunological correlates of HDC + ASCT in patients with severe rheumatoid arthritis (RA), refractory to conventional therapy.
Methods. Seven RA patients treated with HDC and autologous peripheral blood grafts enriched for CD34+ cells underwent serial sampling of peripheral blood and synovial tissue specimens. Disease activity was assessed with disease activity scores (DAS), serum concentrations of C-reactive protein (CRP) , and human immunoglobulin (HIG)-scans, while the extent of immunoablation was determined by immunophenotyping of peripheral blood mononuclear cells and immunohistochemistry and double immunofluorescence of synovium.
Results. Clinical responders (n=5) differed from nonresponders (n=2), having stronger baseline expression of CD3, CD4, CD27, CD45RA, CD45RB, and CD45RO in synovium ($p<0.05$), higher activity on HIG-scans ($p= 0.08$) and a trend towards higher concentrations of CRP in serum. Subsequent remissions and relapses in responders paralleled reduction and re-expresssion respectively of T cell markers. A relative increased expression of CD45RB and CD45RO on synovial CD3+ T cells was observed after HDC + ASCT. No correlations were found between DAS and changes in B cells or macrophages infiltration or synoviocytes.
Conclusions. HDC + ASCT results in profound but incomplete immunoablation of both the memory and naive T cell compartment which is associated with long lasting clinical responses in the majority of patients. Our findings provide strong circumstantial evidence for a role of T cells in established rheumatoid arthritis, and demonstrate a role for the synovium in post-transplant T cell reconstitution.

Introduction.

High dose chemotherapy (HDC) followed by autologous stem cell transplantation (ASCT) is an experimental therapy for severe autoimmune diseases including refractory rheumatoid arthritis (RA). A number of clinical studies have demonstrated longterm responses in RA patients previously refractory to disease modifying anti-rheumatic drugs (DMARD) [1-9]. The rationale of this strategy is based on the concept of immunoablation by intense immunosuppression with subsequent regeneration of naive T lymphocytes derived from reinfused hematopoietic progenitor cells [10]. The mechanisms by which HDC+ASCT exerts its anti-rheumatic effects have not yet been defined. It has been postulated that intensive immunosuppressive therapy followed by ASCT may be effective for the control of RA because the conditioning regimen deletes the relevant autoreactive lymphocyte population and the reinfused stem cells develop into a lymphocyte population that acquires self tolerance. Experimental studies of autoimmune disease in rodents have lent support to this concept but no comprehensive studies have been done in humans. We examined serially taken samples from synovial tissue and blood from 7 RA patients treated with HDC + ASCT in an attempt to unravel pathogenetic mechanisms in RA. We used lineage specific markers to analyze cellular infiltrates in the synovium, as well as activation and inflammatory markers to assess disease activity at the tissue level.

Patients and methods.

Seven RA patients (mean age 49 years, range 35-55, disease duration 12 years, range 7-20) were treated at Leiden University Medical Center (LUMC) with HDC + ASCT as part of a multicenter phase I/II trial [8]. The protocol was approved by the LUMC Medical Ethical Committee and the patients gave written informed consent to undergo arthroscopic procedures as described below. All patients had an established diagnosis of rheumatoid arthritis according to ACR-criteria [11]. Eligibility criteria were destructive disease, failure to respond to ≥ 4 anti-rheumatic drugs including maximal tolerable dose of methotrexate and combination therapy, and a clinically inflamed knee. Four patients had also failed TNF-blockade. Parameters of disease activity and concurrent DMARDs are summarized in Table 1. All patients had a high disease activity score (DAS) at baseline as defined by EULAR criteria [12]. Autologous hematopoietic stem cells were mobilized using a single infusion of cyclophosphamide (CyC) 4 g/m^2 followed by filgrastim (G-CSF) 10 μg/kg/day subcutaneously until leukapheresis. Immunomagnetic selection of CD34$^+$ cells from the leukapheresis product was performed using the Clinimacs Device (Miltenyi Biotec, Munich, Germany). Disease-modifying antirheumatic drugs (DMARDs) were discontinued before mobilization and not reinstituted until at least three months post-transplantation if deemed necessary on the basis of recurrence or persistence of disease activity. Corticosteroids were tapered when possible. NSAIDs were continued in the lowest

dosage needed to control pain and morning stiffness. The conditioning regimen consisted of i.v. CyC 200 mg/kg followed by reinfusion of the CD34+ enriched graft.

Clinical assessment.

Clinical assessment was performed on the day synovial biopsies were obtained using the disease activity score (DAS) [13]. DAS = (0.54 x √ Ritchie articular index (tender joint count)) + (0.065 x number of swollen joints) + (0.33 x Ln ESR) + (0.0072 x patient disease activity visual analogue scale).

HIG-scan.

Human immunoglobulin scintigrams (HIG-scans) were performed at baseline and at 3 months post-transplant according to standard operating procedures [14]. Disease activity was assessed by scoring total uptake in joints by 2 independent observers (RV and MW) on a 4-point scale (0 = no, 1 = light, 2 = moderate, 3 = strong uptake).

Synovial tissue.

Patients underwent serial arthroscopy to obtain synovial tissue specimens. The baseline arthroscopy was performed prior to mobilization (n=7). The first arthroscopy after transplantation was performed at a mean interval of 86 days (range: 60-127 days) after transplantation (n=7). The second arthroscopy was performed with a mean interval of 1.4 years (range 405-577 days) after transplantation (n=5). The arthroscopy procedure was performed in the inflamed knee with a small-bore, 2.7-mm arthroscope (Storz, Tuttlingen, Germany) under local anesthesia (lidocaine 1%) using an infrapatellar skin portal for macroscopic examination of the synovium and a second suprapatellar portal for the biopsy procedure. Five mm biopsies of synovial tissue were taken using a grasping forceps (Storz) from the suprapatellar pouch, the medial gutter and cartilage-pannus junction. A minimum of 10 biopsy samples were obtained and analyzed per patient per time point. The tissue samples from each biopsy procedure were snapfrozen in Tissue-Tek OCT (Miles Inc. Diagnostic Division, Elkhart, IN) by immersion in 2-methylbutane at -70°C. Frozen blocks were stored in liquid nitrogen until sectioning. Five μm sections were cut, mounted on glass slides and stored at -70°C.

		Before HDC + ASCT, at the time of the first arthroscopy		3 months after HDC + ASCT, at the time of the second arthroscopy		1 year after HDC + ASCT, at the time of the third arthroscopy	
	Response	**Medication**	**DAS**	**Medication**	**DAS**	**Medication**	**DAS**
1	**good**	Cyclosporin 300 mg/day, Predinsone 10 mg/day Ketoprofen 400 mg/day	5.88	Prednisone 7.5 mg/day Ketoprofen 400 mg/day	2.11	Methotrexate 7.5 mg/week Ketoprofen 200 mg/day	2.78
2	**good**	Prednisone 10 mg/day Piroxicam 20 mg/day	4.31	None	0.89	None	2.62
3	**good**	Hydroxychloroquine 200 mg/day Cyclosporin 100 mg/day Ibuprofen 1200 mg/day	4.99	None	2.28	Ibuprofen 1200 mg/day	2.47
4	**moderate**	Methotrexate 17.5 mg/week Cyclosporin 150 mg/day Prednisone 10 mg/day Naproxen 1000 mg/day	6.61	Prednisone 7.5 mg/day Naproxen 1000 mg/day	3.85	Leflunomide 20 mg Prednisone 10 mg/day Naproxen 1000 mg/day	4.60
5	**good**	Methotrexate 15 mg/week Diclofenac 150 mg/day	5.58	Diclofenac 150 mg/day	2.05	Methotrexate 15 mg/week Ibuprofen 800 mg/day	2.78
6	**no**	Prednisone 10 mg/day Ibuprofen 1600 mg/day	4.71	Prednisone 5 mg/day Ibuprofen 1600 mg/day	5.16	Methotrexate 17.5 mg/week Prednisone 7.5 mg/week Ibuprofen 1600 mg/day Tramadol 150 mg/day	4.67
7	**good**	Methotrexate 20 mg/week Cyclosporin 100 mg/day Diclofenac 150 mg/day	5.88	Diclofenac 150 mg/day	2.11	Methotrexate 10 mg/week Diclofenac 150 mg/day	3.26

Table 1. Medication used at the time of arthroscopy. Four variable Disease Activity Score (DAS) = (0.54 x √ Ritchie articular index) + (0.065 x number of swollen joints) + (0.33 x Ln ESR) + (0.0072 x patient disease activity VAS).

Immunohistochemistry.

Sections of biopsies taken at baseline and three months post-transplant were stained with the following monoclonal antibodies: mouse anti-human CD3, CD4, CD8, CD27, CD45RA, CD45RB, CD45RO, CD55, CD56, CD68, CD25, CD62E, CD62L, CD69, HLA-DR, CD19, CD38, IL-1β, IL-12 and IFN-γ; rabbit anti-human TNF-α;rat anti-human IL-4 and IL-10 (Table 2A). The following markers were investigated on the one year post-transplant samples: CD3, CD4, CD8, CD27, CD45RA, CD45RB, CD45RO, CD55, CD68. Immunohistochemical staining procedures were performed as follows. Slides were warmed up to room temperature, fixed in acetone (Merck) at room temperature for 10 minutes, and air-dried. After each step, the sections were washed with phosphate-buffered saline (PBS, Apotheek LUMC, Leiden, The Netherlands). All incubations were carried out at room temperature. Endogenous peroxidase activity was inhibited using 0.1% sodium azide (Merck) and 1% hydrogen peroxide (Merck) in PBS. The monoclonal antibodies were diluted in PBS with 1% bovine serum albumin (BSA, ICN Biomedicals Inc., Aurora, OH), and incubated for 60 minutes. IL-1β, IL-4, IL-10, IL-12 and IFN-γ were incubated for 18 hours. For control sections, the IgG1 isotype control (anti-KLH, Pharmingen) or PBS were applied. The detection of the monoclonal antibodies was performed using affinity-purified and horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies (Dako), rabbit anti-rat-HRP (Dako) and goat anti-rabbit-HRP (BD Pharmingen), the biotinyl tyramide/streptavidin-HRP amplification system (NEN Life Science Products Inc., Boston, MA), and aminoethylcarbazole (AEC, Sigma, St. Louis, MO). The HRP-conjugated antibodies were diluted in PBS/BSA (1%) with 10 % normal human serum (NHS, Bloedbank LUMC, Leiden, The Netherlands) as blocking serum, and incubated for 30 minutes. A biotinyl tyramide/streptavidin-HRP amplification system (NEN Life Science Products Inc., Boston, MA), and aminoethylcarbazole (AEC, Sigma, St. Louis, MO) was used to enhance staining. A biotinyl tyramide solution was added and slides were incubated for 30 minutes, followed by subsequent incubation with streptavidin-HRP in PBS/BSA (1%) for 30 minutes. HRP-activity was detected using hydrogen peroxide as substrate, and AEC as dye. After washing with distilled water, the sections were counterstained with Mayer's Hämalaunlösung (Merck), and mounted with Kaiser's glycerol gelatine (Merck).

Clone	Specificity	Host	Source
Primary antibodies (unconjugated)			
UCHT-1	CD3	Mouse	Becton-Dickinson, San Jose, USA
MT-310	CD4	Mouse	Dako, Glostrup, Denmark
DK25	CD8	Mouse	Dako
CLB-CD27/1, 9F4	CD27	Mouse	CLB, Amsterdam, The Netherlands)
4KB5	CD45RA	Mouse	Dako
PD7/26	CD45RB	Mouse	Dako
OPD4	CD45RO	Mouse	Dako
BRIC110	CD55	Mouse	CLB
M0718	CD68	Mouse	Dako
MACT-1	CD25	Mouse	Dako
ENA-1	CD62E	Mouse	Sanbio, Uden, The Netherlands
Greg 56	CD62L	Mouse	Pharmingen, Woerden, The Netherlands
FN50	CD69	Mouse	Dako
M704	HLA-DR	Mouse	Dako
HD37	CD19	Mouse	Dako
HB7	CD38	Mouse	Becton-Dickinson
AS10	IL-1β	Mouse	Becton- Dickinson
24910.1	IL-12	Mouse	R&D Systems, Abingdon, United Kingdom
MAB285	IFN-γ	Mouse	Genzyme, Cambridge, USA
IP-300	TNF-α	Rabbit	Genzyme
JES 3-19F1	IL-10	Rat	Pharmingen
Secondary antibodies			
Goat anti-mouse-HRP	Mouse Ig	Goat	Dako
Goat anti-rabbit-HRP	Rabbit Ig	Goat	BD Pharmingen
Rabbit anti-rat-HRP	Rat Ig	Rabbit	Dako

Table 2A. Primary and secondary antibodies used for immunohistochemistry studies.

Immunofluorescence double staining.

In order to characterize subsets of CD3 positive cells in the five responders, double staining procedures were performed with CD45RA, CD45RB, CD45RO and CD27 (Table 2B). The following combinations of markers were used in order to identify different cell types: naive T cells (CD45RA and CD27), memory T cells (CD45RO) and early versus more mature T cells (CD45RB) in combinations described in Table 2B. Antibodies were diluted in PBS with 1% BSA. Visualization of CD45RA, RB and RO antibodies was performed by a second incubation period with rabbit anti-mouse antibody (Dako, 30 minutes at 4 °C) conjugated to TRITC diluted in PBS with 1% BSA. Remaining free binding sites of the rabbit anti-mouse Ig polyclonal antibody were blocked by incubation with 20% normal mouse serum (NMS) in PBS for 20 minutes at 4 °C. To detect CD3 positive cells slides were then incubated with a mouse anti-human CD3 antibody conjugated to FITC in PBS with 5% NMS for 30 minutes at 4 °C. Visualization of CD27 with CD3 was performed with mouse anti-human CD3 followed by incubation period with rabbit anti-mouse antibody (Dako, 30 minutes at 4 °C) conjugated to TRITC. Free binding sites of the sheep anti-mouse Ig polyclonal antibody were blocked by incubation with 20% normal mouse serum in PBS for 20 minutes at 4 °C. To detect CD27 positive cells slides were then incubated with a mouse anti-human CD27 antibody conjugated to FITC in PBS with 5% NMS for 30 minutes at 4 °C. Between all incubation periods, slides were washed with cold (4 °C) PBS. Primary, secondary and tertiary reagents were titrated to obtain optimal results.

Microscopic analysis of immunohistochemical stained slides.

Sections were coded and randomly analyzed. All areas of each biopsy section were examined and histologic features were scored semi-quantitatively by two observers (RJV and RF or RJV and LD), who were blinded to clinical data. The expression of CD3, CD4, CD8, CD27, CD45RA, CD45RB, CD45RO, CD55, CD56, CD68, CD25, CD62E, CD62L, CD69, HLA-DR, CD19, CD38, IL-1β, IL-4, IL-12, IFN-γ, TNF-α, and IL-10 was scored on a five-point scale (0-4). A score of 0 was given to those sections with minimal infiltration and/or low expression, while a score of 4 represented large infiltration by numerous lymphocytes, macrophages or a strong expression of a certain cell surface marker. For the evaluation of CD4+ cells, only cells with lymphocyte morphology were included, since CD4 can be expressed on monocytes. The scoring is calibrated for each marker, and has been developed previously by examining $\geq$ 5 biopsies of rheumatoid synovial tissues [15]. Interobserver readings were identical or differed by only one point, and all differences that did occur were resolved by mutual agreement.

Microscopic analysis of Hematoxylin/eosin-stained slides.

Hematoxylin/eosin-stained sections were scored for the degree of infiltration with lymphocytes, plasma cells, and polymorphonuclear cells (PMNs) on a five-point scale (0-4) by two observers (RJV and PMK), who were blind to clinical data. These quantitative

scores corresponded with the numbers of cells per high-power field (HPF, 787.5*x*), as described earlier [16]. A score of 0 was given to those sections with minimal infiltration, while a score of 4 represented infiltration by numerous cells, as follows: score for lymphocytes 0 was 0-50, 1 was 51-200, 2 was 201-400, 3 was 401-600, and 4 was more than 600 lymphocytes per 4 HPFs; score for plasma cells 0 was 0-3, 1 was 3-25, 2 was 26-85, 3 was 86-150, and 4 was more than 150 plasma cells per 4 HPFs; and score for PMNs 0 was 0-3, 1 was 3-10, 2 was 11-22, 3 was 23-85, and 4 was more than 85 PMNs per 4 HPFs. In addition, each tissue was scored for synovial lining hyperplasia on a four-point scale (0-3; where 0 was 1-2, 1 was 3-4, 2 was 5-6, and 3 was more than 6 cell layers). A composite inflammation score was calculated by summing the scores for the four components: synovial lining hyperplasia, and infiltration with lymphocytes, plasma cells and PMNs (range 0-15).

Microscopic analysis of immunofluorescence stained slides.

Scoring of immunofluorescence double staining in the five responders was done by counting at least 100 of single or double positive cells.

Peripheral blood T-cell reconstitution analysis.

Immunophenotyping studies were done on peripheral blood mononuclear cells obtained at baseline, and at 1, 3, 6, 9 and 12 months after transplantation. The following combinations of markers were used in order to identify different cell types: naive CD4 and CD8 T cells, memory CD4 and CD8 T cells and early versus more mature CD4 and CD8 T cells (see Table 2C for antibodies used).

Statistical analysis.

Non-parametric techniques (Wilcoxon signed-rank tests) were used to test whether parameters of disease activity and immunological parameters after transplantation differed significantly from baseline. Differences between responders and non-responders were assessed using the Mann-Whitney U test. A Pearson correlation coefficient was calculated to assess the relationship between serum concentrations of CRP and scores on HIG-scans.

Clone	Specificity	Host	Source
Primary antibodies (unconjugated)			
4KB5	CD45RA	Mouse	Dako, Glostrup, Denmark
PD7/26	CD45RB	Mouse	Dako
OPD4	CD45RO	Mouse	Dako
UCHT-1	CD3	Mouse	BD
Primary antibodies (conjugated)			
UCHT-1	CD3-FITC	Mouse	BD Pharmingen , San Diego, USA
M-T271	CD27-FITC	Mouse	BD
Secondary antibodies			
R0270 rabbit anti-mouse TRITC	Mouse IgG	Rabbit	Dako

Table 2B. Primary and secondary antibodies used for immunofluorescence double staining.

Clone	Specificity	Host	Source
Primary antibodies (conjugated)			
RPA-T4	CD4-PE	Mouse	BD
MT310	CD4-PE	Mouse	Dako
RPA-T8	CD8-PE	Mouse	BD
3B5	CD8-PE	Mouse	Serotec
L48	CD45RA-FITC	Mouse	BD
PD7/26	CD45RB-FITC	Mouse	Dako
UCHL1	CD45RO-FITC	Mouse	Dako

Table 2C. Antibodies used for flow cytometric analysis of peripheral blood T lymphocytes.

Results.

Clinical efficacy.

The 7 patients displayed a dichotomous clinical response to HDC + ASCT with 5/7 patients attaining a good response based on the EULAR response criteria for disease activity (mean DAS from 5.33 to 1.89; p=0.04), at 3 months post-transplant. Of the two remaining patients (referred to as 'nonresponders') one had a moderate response initially (DAS from 6.61 at baseline to 3.85 at 3 months), but then progressed, while the other failed to respond at all (DAS from 4.71 at baseline to 5.16 at 3 months). Four patients had also failed TNF-blockade. Two of these four patients responded favourably to HDC and ASCT. At the time of the second arthroscopy none of the 7 patients was on DMARDs, however, these were reinstituted during the first year post-transplant in the 2 nonresponders and 3/5 responders because of flares. One year after transplantation the 5 responders underwent a third arthroscopy. The mean DAS in these patients at the time of third biopsy was 2.78 (range 2.47-3.26, p=0.04 versus baseline)(Table 1).

HIG-scan.

Baseline HIG-scores and CRP-concentrations were highly correlated (r=0.91, p<0.01), as were the changes after HDC + ASCT (r=0.91, p=0.01). The 5 clinical responders versus 2 nonresponders had increased baseline scores on HIG-scan (mean 2.4 versus 0.5, p= 0.08) and serum concentrations of C-reactive protein (mean 54.2 mg/L versus 34 mg/L, p=0.44).

Hematoxylin/eosin-stained slides.

The mean inflammation score in the whole group decreased from 6.17 at baseline to 2.33 at 3 months after transplantation (p=0.06), and from 5.75 to 1.75 in the responders (p= 0.14). A >50% reduction in the inflammation score was observed in 4/7 patients post-transplant (Figure 1).

	Responders (n=5)		Non-responders (n=2)	
	Baseline	**Three months**	**Baseline**	**Three months**
CD3	3.00	1.60	0	0.50
CD4	2.60	1.20	0	0.50
CD8	2.00	2.00	0	0.50
CD25	2.00	0.60	0	0
CD27	3.00	1.00	0	0.50
CD45RA	2.60	0.60	0	0.50
CD45RB	3.20	2.00	0	1.00
CD45RO	3.40	1.80	0	1.00
CD19	1.60	0.60	2.00	0
CD38	3.00	2.20	2.00	2.00
CD68-lining	1.80	2.40	0.50	1.50
CD55-lining	2.60	3.80	1.50	1.50
CD-68-sublining	2.20	2.00	0.50	0.50
HLA-DR	2.60	2.60	0	1.50
CD62L	0.60	1.20	0	0.50
CD62E	0.40	0.20	1.00	0
CD56	2.20	1.80	1.50	3.00
IL-1β	1.00	0.60	0	4.0
TNF-α	2.1	1.8	1.00	3.25
IFN-γ	2.00	2.60	1.00	2.00
IL-12	2.00	1.20	2.00	3.50
IL-10	1.20	2.30	1.50	3.50

Table 3. Immunohistochemical scores at baseline and three montsh after HDC + ASCT for responders and non-responders.

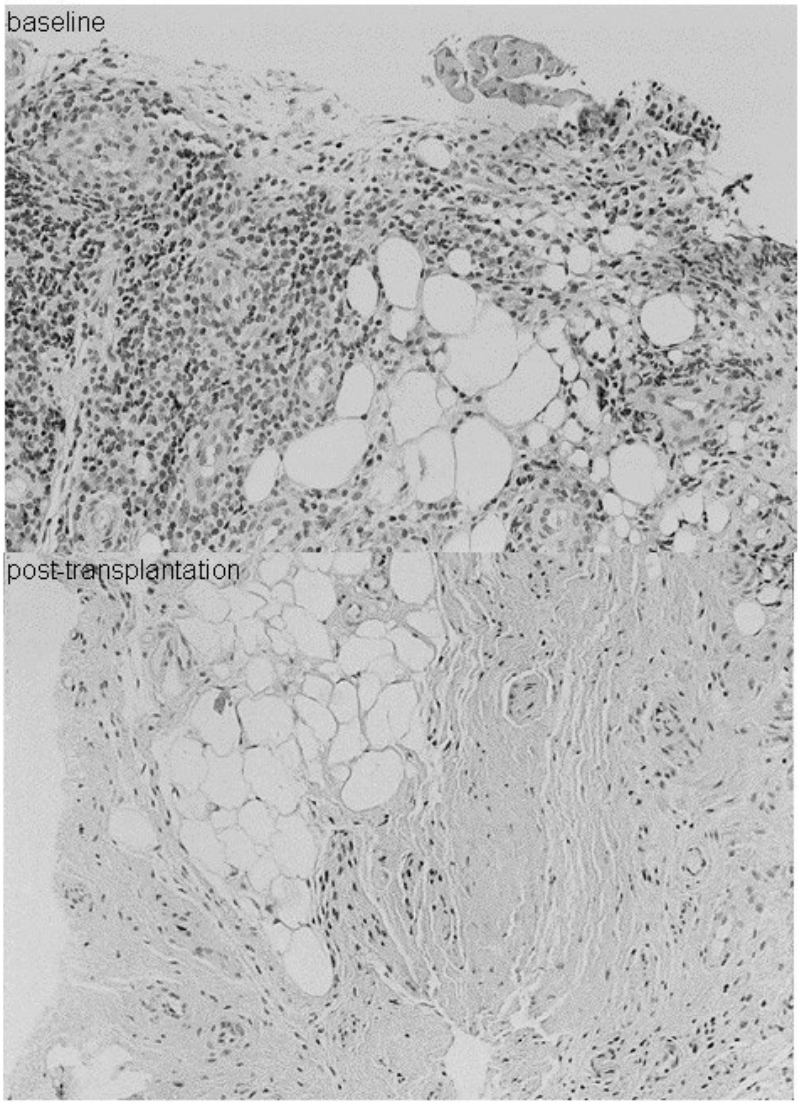


Figure 1. Synovial tissue taken from TX2 before and 3 months after HDC + ASCT. Infiltration with numerous lymphocytes and plasma cells before SCT, which were almost absent after SCT.

Immunohistochemistry.

Clinical responders had a high expression at baseline of CD3, CD4, CD27, CD45RA, CD45RB, CD45RO in synovium while the nonresponders lacked a significant synovial T cell infiltrate (Table 3 and Figure 2). The expression of these markers decreased at 3 months post-transplant in the responders, which was statistically significant for CD45RA and CD27 (p=0.04). When the changes between responders and nonresponders were compared, statistical significant differences were found for CD45RA and CD27 (p=0.05)(Mann-Whitney U test) (Figure 2,3). Changes in other surface markers and cytokines were found but these were not statistically significant except IL-10 which was significantly higher in the whole group at 3 months (p=0.04) and IL-1 which was significantly higher in non-responders at 3 months post-transplant (4.0 vs 0.60; p=0.02) (Table 3). Expression of IL-4 and TGF-β was considered too low to allow meaningful analyses. At one year after transplantation expression of CD3, CD4, CD45RA, CD45RO, but not CD45RB, had returned to baseline levels in the responders (Figure 3).

Immunofluorescence double staining.

Immunofluorescence double staining was performed in the five responders to investigate co-expression of CD27 and CD45R isoforms on synovial CD3 T cells. At baseline 72% of CD27+ cells were CD3+ (range 56-86%) and 88% of CD45RO+ cells were CD3+ (range 57-99%) (Figure 4). Vice versa, of CD3+ cells 68% were CD45RO+ (range 55-93), 22% CD45RA+ (range 5.5-32.7), and 34% CD45RB+ (range 16.5-86). Three months after transplantation CD45RA+ was expressed only on 9% of CD3+ cells but CD45RO+ on 90% (Figure 4). A high proportion of CD3+ T cells co-expressed CD45RB (66%, range 48.9-72.4, p=0.04) at 3 months post-transplant. One year after transplantation the distribution of CD45R-isoforms on CD3+ T cells had returned to baseline levels (Figure 6).

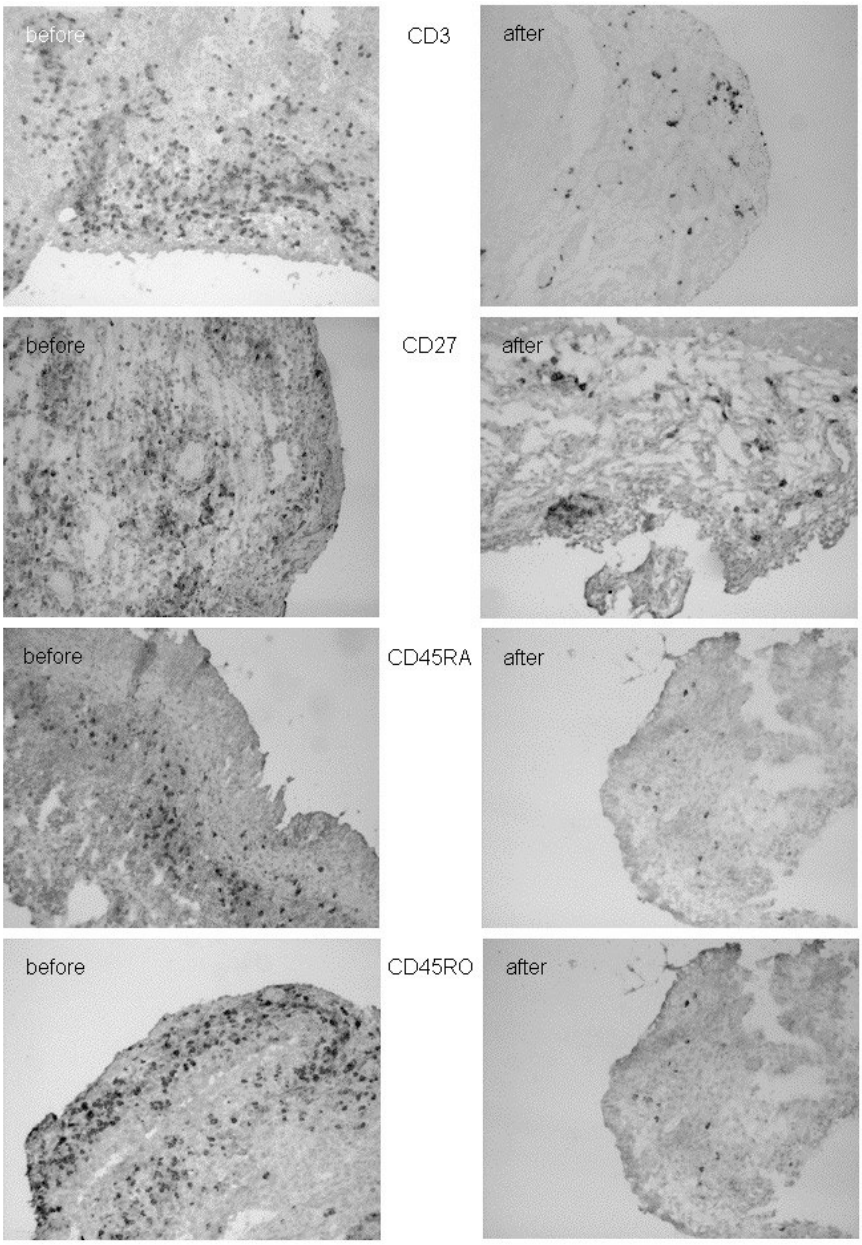


Figure 2. Immunohistochemical stained synovial tissue in a responder with CD3, CD27, CD45RA and CD45RO, before (left) and 3 months after HDC + ASCT (right).

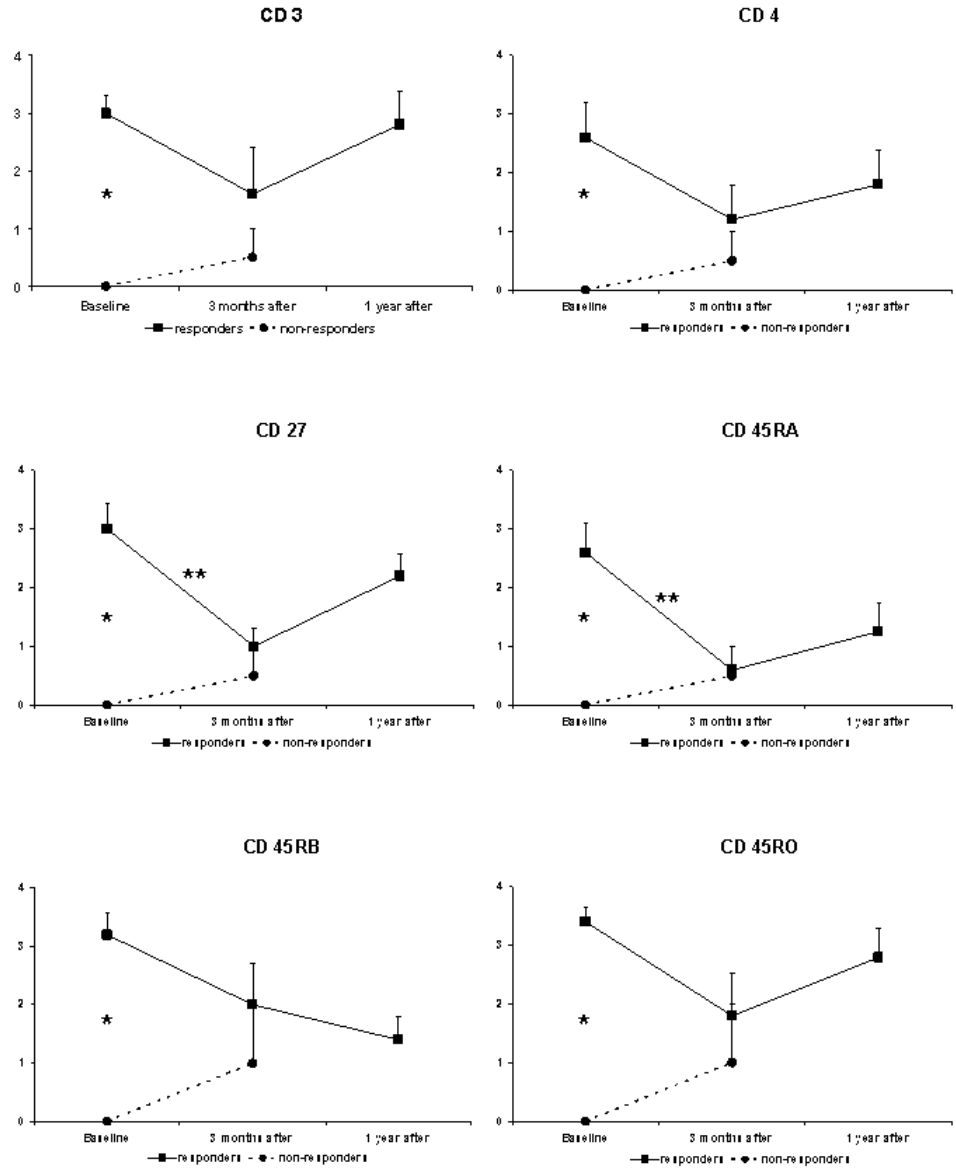


Figure 3. Semiquantitative infiltration scores before, 3 months after and 1 year after high dose chemotherapy and autologous stem cell transplantation of responders and non-responders for CD3, CD4, CD27, CD45RA, CD45RB, CD45RO. * $P < 0.05$. Statistically significant baseline difference between responders and non-responders. ** $P < 0.05$ Statistically significant decrease at 3 months post-transplant in responders CD45RA and CD27.

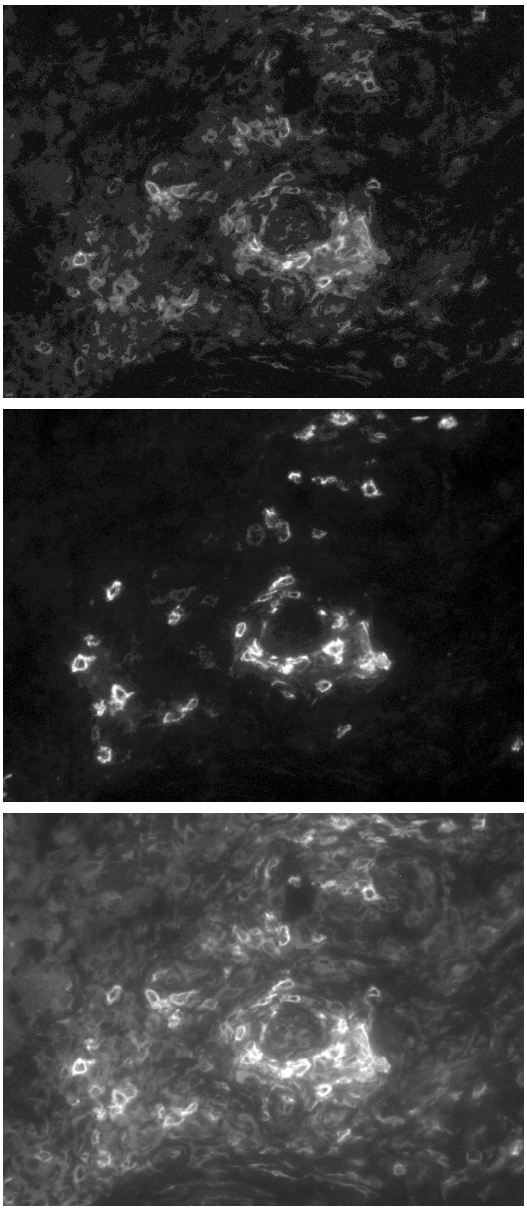


Figure 4. Co-expression of CD3 and CD45RO in RA synovial tissue before transplantation in a responder. CD3 (FITC = upper) and CD45RO (TRITC = middle) were detected using immunofluorescence techniques. A. Rheumatoid synovial tissue showing CD3+ cell infiltrate. B. Rheumatoid synovial tissue showing CD45RO+ cell infiltrate. C. Double positive cells, showing numerous double positive and only few CD45RO single positive cells (red).

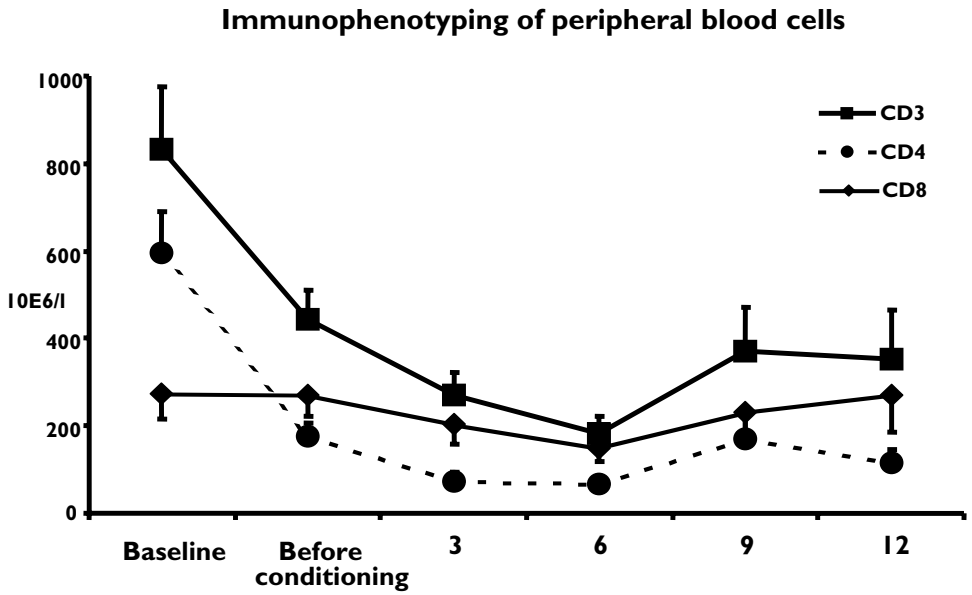
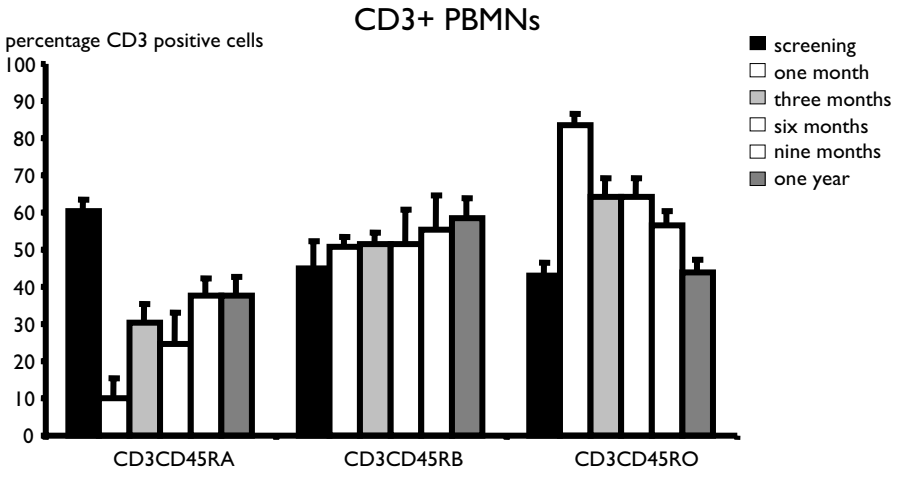


Figure 5. Absolute cellcount in peripheral blood mononuclear cells in the five responders for CD3, CD4 and CD8. Absolute cellnumbers were calculated by multiplying absolute lymphocyte count (10^6/l ± SEM) by the percentage of each subset determined by flow-cytometry after isolation peripheral blood mononuclear cells by density gradient centrifugation.



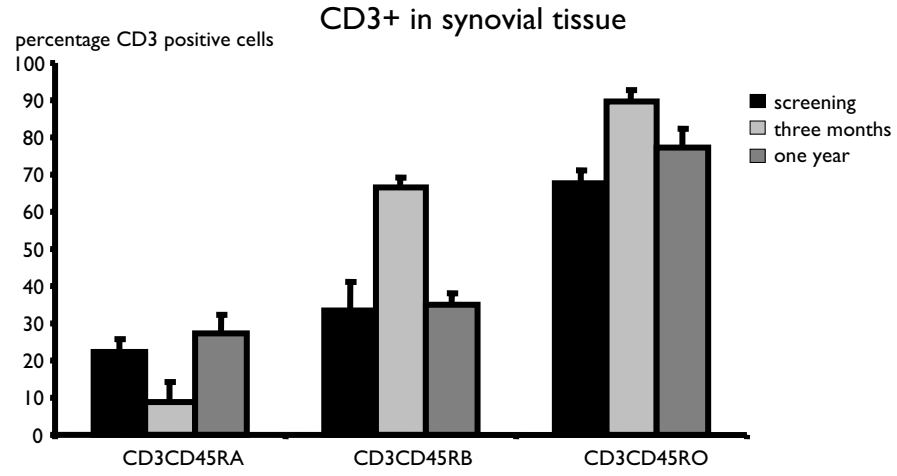


Figure 6. The mean number of CD 45RA, RB and RO expressed as a percentage of $CD3^+$cells.
A. Immunofluorescence double staining of peripheral blood mononuclear cells with CD3 plus CD45RA, RB or RO in the 5 responders at screening and at 1, 3, 6, 9 and 12 months after HDC + ASCT. Results expressed as % of CD3 cells expressing CD45R-isoform.
B. Immunofluorescence double staining of synovial cells with CD3 plus CD45RA, RB or RO in the 5 responders at screening and 3 and 12 months after HDC + ASCT. Results expressed as % of CD3 cells expressing CD45R-isoform.

Cell surface antigens in peripheral blood.
In order to compare the reconstitution results of T cell subsets in the synovial tissue in the five responders, flow cytometric analyses of peripheral blood mononuclear cells in the five responders were performed focussing on CD45RA, RB and RO expression on CD4+ and CD8+ cells. Absolute cell counts in peripheral blood after HDC + ASCT were characterized by prolonged lymphopenia of CD4+ T cells and transient expansion of CD8+ T cells (Figure 5). Numbers of B-cells, monocytes and NK cells decreased transiently but had returned to baseline numbers by 3-6 months (not shown). We then focussed on the relative reconstitution of T cells in blood versus synovium. As can be seen in Figure 6, a greater proportion of CD45RA+ T cells and a lower proportion of CD45RO+ T cells were present in peripheral blood when compared to synovial tissue at baseline. In the first months post-transplant the percentage of CD45RA decreased and remained lower than baseline for the duration of follow-up. A relative increase in CD45RO+ T cells was observed, which was statistically significant up to 6 months after transplantation ($p=0.03$). This was mostly due to the transient peripheral expansion of CD8+ memory T cells (not shown). The percentage of CD45RB+ cells remained relatively constant over time in peripheral blood, contrasting to the findings in synovial tissue as decribed above.

Discussion.
The present study was undertaken to advance our understanding of the immunological effects of high dose chemotherapy and autologous stem cell transplantation (HDC+ASCT) in rheumatoid arthritis (RA). This experimental treatment modality has been employed in recent years as a means to profoundly perturb the immune system of patients suffering from severe autoimmune disease including RA. Substantial improvements of disease activity have been reported in a number of studies, although treatment failures and relapses were observed as well [reviewed in 10]. Data on immunological correlates of the clinical responses in RA are scarce, but the persistence of serum autoantibodies in many transplanted patients suggested that eradication of autoreactive B cells was not achieved, possibly contributing to the failure to induce longterm remissions in these patients. Of all parameters investigated in this study those involving the T cell compartment were most pronounced, particularly in the patients with high inflammatory activity as measured by serum levels of C-reactive protein and uptake on HIG-scans. Failure to respond to TNF blocking therapy or any other clinical or laboratory parameter was not predictive of clinical response. The induction of (partial) remission was, however, associated with strong baseline expression and subsequent reduction of CD3, CD4, and the differentiation markers CD27 and the CD45-isoforms (RA,RB,RO) in synovium, while expression of these markers had returned to baseline levels at the 1-year biopsy at a time disease had relapsed to varying extents in most patients. Although changes in other cell subsets

occurred, no association was found with respect to disease activity and the number of macrophages (CD68+ cells), B cells (CD19+ cells), plasma cells (CD38+ cells) or fibroblast-like synoviocytes (CD55+ cells). Marked expression of pro-inflammatory cytokines remained detectable after transplant, notably of IL-1 in the two nonresponders. Of interest was the high proportion of CD45RB+ CD3+ T cells at 3 months post-transplant, and the gradual increase of this subset among peripheral blood T cells. This subset has recently been reported to be increased in peripheral blood of RA patients versus healthy controls, reflecting accelerated differentiation of naive CD45RA T cells under the influence of inflammation [17]. Co-expression of high levels of CD45RB and CD45RO and loss of CD45RA on T cells has been shown to reflect a phenotype typical of recently activated T cells [18]. The overrepresentation of T cells with a similar phenotype in the synovium at 3 months suggests either the selective migration of newly developed T-cells expressing high levels of CD45RB from blood, and/or local differentiation and/or expansion in the synovium, probably under the influence of homeostatic pressures or a local inflammatory drive as suggested by the results on cytokine expression. Any of these possibilities could contribute to the prolonged depletion of T cells from peripheral blood, which is a feature of RA patients following lymphocytotoxic therapies [19-23].

Our data, though for obvious reasons snapshot in nature, provide strong circumstantial evidence for an active role of T cells in perpetuation of disease activity [24]. Whether attracted to or expanding in the synovium specifically or nonspecifically, interaction of T cells with residual lymphoid or myeloid cells or resident cells such as synoviocytes could turn a subclinical into a clinically manifest synovitis [25, 26]. The findings from our study on the central role of CD4+ T cell infiltration add to the accumulating evidence from earlier case reports of patients with RA and juvenile idiopathic arthritis treated with HDC+ASCT and patients with psoriatic arthritis treated with T cell depleting monoclonal antibody therapy [27-29].
Our study raises the important question as to whether more intense immunosuppression to the extent of myeloablation or post-transplant immunosuppression is necessary to attain durable remissions. Preclinical studies and a recent registry analysis of transplants in human autoimmune disease have lent support to this concept [30, and submitted]. In none of our patients was complete ablation of the synovial T cell compartment achieved, at least not at the timepoints evaluated. Also, autoantibodies (rheumatoid factor and anti-CCP) remained detectable in serum taken at monthly intervals for 2 years (not shown). Given the complex immune dysregulation in RA, notably in late stages, it is conceivable that a more comprehensive treatment may indeed be needed. The key goal from a T cell centered perspective would be to induce sufficient immunoablation to allow regeneration of regulatory elements. HDC+ASCT could provide a window of opportunity to establish tolerance against selected autoantigens. Our data suggest such a strategy would not be

effective in patients with a low synovial T cell load. Whether different pathogenetic mechanisms are involved in such patients remains to be determined, but a recent study does point in this direction [31]. A baseline synovial biopsy seems useful to discrimate the two categories.
To our knowledge, this is the first comprehensive study to examine clinical and immunologic correlates of HDC+ASCT in RA. Although the number of patients investigated was small, and technical issues precluded the use of in situ multiparameter staining of the synovial T cell population, the data reveal interesting aspects of pathogenetic mechanisms operative in rheumatoid arthritis. The association of clinical responses with T cell debulking in the joint, of recurrence of disease activity with re-emergence of T cell infiltration of synovium, and the lack of a relation between disease activity and other cell types in blood or synovium lend support to the concept that T cells play a role in established disease. The changes in relative expression of the different CD45-isoforms in synovial tissue versus blood suggest that T cell repopulation in the joint is dictated by local homeostatic forces, selective homing or antigenic stimulation. More complete eradication of the synovial T cell compartment or post-transplant immunosuppression may be needed to induce more robust remissions, but whether the risks of these steps outweigh the risks of more intense immunosuppression remains to be determined.

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