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ORIGINAL ARTICLE

Molecular signature of mice T lymphocytes following tolerance induction by allogeneic BMT and CD40-CD40L costimulation blockade

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Summary

Tolerance induction by mixed chimerism and costimulation blockade is a promising approach to avoid immunosuppression, but the molecular basis of tolerant T lymphocytes remains elusive. We investigated the genome-wide gene expression profile of murine T lymphocytes after tolerance induction by allogeneic bone marrow transplantation (BMT) and costimulatory blockade using the anti-CD40L antibody MR1. Molecular functions, biological processes, cellular locations, and coregulation of identified genes were determined. A total of 113 unique genes exhibited a significant differential expression between the lymphocytes of MR1-treated Tolerance (TOL) and untreated recipients Control (CTRL). The majority of genes upregulated in the TOL group are involved in several signal transduction cascades such as members of the MAPKKK cascade (IL6, Tob2, Stk39, and Dusp24). Other genes involved in lymphocyte differentiation and highly expressed in the TOL group are lymphotactin, the estrogen receptors (ERs) and the suppressor of cytokine signaling 7. Common transcription factors such as ER 1 alpha, GATA-binding protein 1, insulin promoter factor 1, and paired-related homeobox 2 could be identified in the promoter regions of upregulated genes in the TOL group. These data suggest that T lymphoctes of tolerant mice exhibit a distinct molecular expression profile, which needs to be evaluated in other experimental tolerance models to determine whether it is a universal signature of tolerance.

Introduction

Transplantation tolerance through mixed chimerism is an attractive strategy, whose clinical translation is hampered by the remaining toxicities associated with the required bone marrow transplantation (BMT) conditioning. The use of costimulation blockade (CTLA4Ig and/or anti-CD40L [CD154]) as part of allogeneic bone marrow transplantation (BMT) protocols allowed the induction of mixed chimerism with markedly milder regimens than had previously been possible [1]. Murine protocols without global T-cell depletion [2,3] or even without any cytoreductive conditioning at all [3–5] became feasible for the first time. While the use of CTLA4Ig can be avoided in some situations [6,7], anti-CD40L is an indispensable component of all these mixed chimerism models.

Anti-CD40L treatment is of remarkable potency in numerous murine models of autoimmune disease and transplantation. Nevertheless, its mechanisms of action are still not fully understood. Induction of anergy, regulation, and deletion by anti-CD40L has all been proposed, and has to some extent been demonstrated, in one model or another [8]. Furthermore, a signal through CD40L or the requirement of complement and Fc-mediated effector mechanisms have been observed in some studies [6,9–12], whereas in others the absence of CD40L could substitute

for anti-CD40L antibody treatment [13,14]. Even though several anti-CD40L mAbs triggered unacceptable toxicites in (pre)clinical trials and their clinical development seems unrealistic, an anti-CD40 mAb has been shown to be similarly effective, and thus clinical blockade of the CD40-CD40L pathway is possibly attainable.

Models of allogeneic BMT differ markedly from other models with respect to the mechanisms contributing to tolerance induction [8]. When combined with allogeneic BMT, anti-CD40L treatment is unique in triggering a progressive extrathymic clonal deletion of donor-reactive T cells [2,3,15], thus allowing bone marrow engraftment without global depletion of the recipient's T-cell repertoire. Activation-induced cell death and apoptosis via the mitochondrial death pathway seem to be involved in this process [16,17]. Notably, no other mAb has been described to have the capability of triggering this form of deletion after nonmyeloablative BMT. Tolerance is observed in such BMT recipients before peripheral deletion is complete. Recent evidence reveals that during this phase immediately following BMT, CD4 T-cell-mediated regulatory mechanisms play a critical role [18–20]. Once peripheral deletion is complete and the bone marrow has engrafted, tolerance is maintained through central clonal deletion, without an essential role for regulation.

Previously, researchers tried to identify molecular signatures of regulatory lymphocytes in animal models of tolerance using SAGE (serial analysis of gene expression) [21]. The authors investigated 29 SAGE libraries of T cells and antigen-presenting cells focusing on distinct expression patterns in $CD4^+$ $CD25^+$ regulatory T cells and interleukin 10 (IL10)-producing regulatory T cells. The lymphocyte populations clearly separated on the basis of their expression profiles and were no more related to each other than they are to Th1- or Th2 cells.

Various groups used microarrays to reveal differential gene expression between Th1- and Th2 lymphocytes [22,23] as well as between Tc1- and Tc2 cells [24]. Matsui *et al.* [25] studied gene expression profiles in tolerizing murine cardiac allografts after costimulation blockade, but the present study is the first to evaluate whole genome expression profiles of lymphocytes after costimulation blockade. Besides, the experimental protocol used relies on the mixed chimerism approach, which relies on different tolerance mechanisms, and whose feasibility was demonstrated in nonhuman primate studies [26] and a small clinical pilot series of renal transplant recipients [27].

Using whole genome cDNA microarrays, we previously showed in human donor kidney biopsies that unique molecular signatures can be derived to separate grafts with immediate graft function from those with subsequent development of post-transplant acute renal failure [28]. The bioinformatics workup of these expression data including promoter analysis revealed that some of the abundantly expressed genes are coregulated by a combination of transcription factors suggesting the choreographed regulation [29].

The aim of the present study was to identify the unique molecular signature in T lymphocytes of mice made tolerant by BMT and CD40L blockade. This signature may then be evaluated in other experimental tolerance protocols and could potentially be used to identify candidates for immunosuppression withdrawal trials in human solid organ transplantation.

Methods

Mouse model of tolerance induction

Six- to 12-week-old mice were purchased from Charles River (Sulzfeld, Germany) and were kept under specific pathogen-free conditions. Groups of B6 mice ($n = 4$/group) received nonmyeloablative total body irradiation (3 Gy) one day before being transplanted with a conventional dose of fully allogeneic unseparated Balb/c bone marrow cells harvested from the tibiae, femurs, and humeri (approximately 16×10^6/mouse). Recipients were either left untreated (group Control (CTRL)), which invariably leads to BMC rejection without chimerism or tolerance, or received treatment with anti-CD40L (group Tolerance (TOL); 1 mg MR1 i.p. day 0), which leads to peripheral deletion, regulation, chimerism, and tolerance [2,19,30]. An MR1 is a hamster antimouse-CD40L mAb and was purchased from Bioexpress Inc. (West Lebanon, NH, USA). Mice were considered chimeric if they showed at least 10% donor cells within the myeloid lineage. Because only chimeric animals were included in the TOL group (to ensure that only splenocytes from tolerant animals were analyzed in this group), only three mice could be analyzed. Furthermore, we focused on quick and equal processing of splenocytes of both groups before analysis.

All experiments were approved by the local review board of the University of Vienna, and were performed in accordance with national and international guidelines of laboratory animal care.

Sample Preparation

Three weeks after BMT, mice were splenectomized and spleens were crushed for 1 min in Red Blood Cell Lysing Buffer (Sigma, Vienna, Austria) with the plunger of a syringe. The cells were filtered through a 70-μm nylon cell strainer. CD4 cells were enriched by incubation in Nylon Wool Fiber Type 'R' (Kisker Biotech, Steinfurt, Germany) for 1 h at 37 °C. Total RNA of T lymphocytes was isolated by using the TRIZOL® method (Invitrogene Corp., Carlsbad, CA, USA). Quality of the isolated total RNA

was checked by gel electrophoresis and with Agilent Bioanalyzer and RNA6000 LabChip® kit (Agilent, Palo Alto, CA, USA). Universal mouse RNA was used as reference RNA (Stratagene, La Jolla, CA, USA). Sample and reference mRNA were labeled in a two-step procedure with CyScribe cDNA post-labeling kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Sample preparation and hybridization were performed in random order. Detailed protocols, webfigures and webtables can be found on our website http://www.meduniwien.ac.at/nephrogene (see data – Molecular signature of tolerant T lymphocytes). The investigator performing the array experiments was blinded for the treatment group.

Microarray Hybridization and Scanning

Mouse cDNA microarrays holding 38 806 cDNA features were obtained from the Stanford University Functional Genomics core facility (Stanford University, CA, USA). The experiment procedure was performed as described previously [28,31]. Labeled samples were hybridized in Corning hybridization chambers (Corning Inc., Corning, NY, USA) overnight in a water bath (14 h, 65 °C). Arrays were scanned with a GenePix 4100A scanner (Axon Instruments, Union City, CA, USA). Image griding and calculation of spot intensity were performed using GENEPIXPRO 4.1 software.

Image-, grid- and result files are stored at the Stanford microarray database (http://genome-www5.stanford.edu/MicroArray/SMD/) [32]. Information on samples, material, experimental set-up, and procedures follow MIAME guidelines to be found at http://www.mged.org [33].

Data processing, statistical analysis, and functional annotation

The dataset consisted of 37 509 cDNA features exhibiting the spot intensities of 1.2-fold over background in either channel 1 or -2. Of those, 26 536 held a UniGene Cluster ID, and 10 973 were expressed sequence tags (ESTs) not assigned to a UniGene Cluster yet. In a first preprocessing step, a quality filter was applied on the dataset by considering only genes and ESTs with at least 80% of valid entries in all array experiments yielding 37 198 cDNA features. 1390 data points (0.62% of all experiments) remained unavailable. No correction for a putative batch bias was necessary because only one array batch was used in the whole analysis for all arrays.

An unsupervised hierarchical clustering algorithm was used for graphical representation of the differentially expressed genes [34]. Euclidean distance was used as distance measure in the dendrogram, and complete linkage was used as linkage rule in the cluster algorithm. Cluster analysis was performed with the MultiExperiment Viewer (MeV) developed at The Institute of Genomic Research (TIGR) [35].

An unpaired *t*-statistic of log-transformed expression values was used to evaluate differences between lymphocytes of TOL- and CTRL-mice. Genes with a fold change of two or higher and a *P*-value <0.001 were considered statistically significant regulated between the two groups. No adjustment for multiple testing was performed.

The resulting genes were analyzed with regard to their molecular functions, biological processes, and cellular locations using gene ontology terms (GO-Terms) from the Gene Ontology Consortium [36]. The SOURCE tool from the Stanford Genomics Facility was used to associate GO-Terms to the genes of interest [37]. Functional grouping of genes was based on GO-Terms, PANTHER ontologies, and information derived from iHOP, a protein information extraction tool [38,39]. Molecular pathways holding a significant number of genes were identified using the Gene Expression Data Analysis Tools of the PANTHER Classification System. Biological processes of genes were compared with the PANTHER reference dataset holding all 23 520 annotated genes. The expected to the observed frequency of genes to certain ontology categories were compared by using the chi-squared test.

Transcription factor-binding site (TFBS) analysis

The two gene sets of differentially regulated genes between group TOL and group CTRL were used in order to find transcription factors with an over-represented number of binding sites in the promoter regions of the genes under study.

The CONFAC (conserved transcription factor-binding site finder) tool was used in order to find significantly enriched TFBS in the datasets [40]. Promoter sequences ranging from 3000 bp upstream to 500 bp downstream with respect to the transcription start site (TSS) were scanned for putative TFBS. Transcription factor-binding sites were retrieved from the TRANSFAC database and the matrix and core similarities were set to 0.85 and 0.95, respectively [41]. Only TFBS present in the mouse as well as in the orthologous human promoter sequence were further analyzed. A Wilcoxon rank-sum test was calculated in order to identify the transcription factors with a significantly higher number of binding sites in respect to a reference dataset of 200 randomly picked genes provided by the CONFAC tool.

Results

Genes abundantly expressed (fold change >3) in both groups compared with reference RNA

Mean sector and printing plate ANOVA R^2-values were between 5×10^{-3} and 6×10^{-2} in the microarray experi-

ments suggesting no dependence of results on spatial location or printing plate. A total of 418 cDNA clones were identified with a mean log R/G expression of three or higher in both groups, TOL and CTRL, respectively. Gene symbols could be assigned to 273 cDNA clones representing 168 unique genes (webtable 1). Among those are various reported to be expressed in lymphocytes with known biological roles in inflammatory processes and immune response, e.g. granzymes A and B, interleukin receptors 1, 2, 7, and 10, chemokine ligands 5 and 6 or the signal transducer and activator of transcription 1 (STAT1). The PANTHER Classification System was used to identify sets of genes with similar molecular functions involved in same biological processes. Biological processes like 'immunity and defense', 'signal transduction', 'cytokine and chemokine mediated signaling pathway', or 'T-cell-mediated immunity' are among the top of the list as depicted in webtable 2.

Metabolic and signal transduction pathways with a significant number of genes involved as reported by the PANTHER Classification System are depicted in webtable 3. Ten genes belong to the 'inflammation mediated by chemokine and cytokine signaling pathway' according to PANTHER (webfigure 1). Four genes are assigned to the 'Apoptosis signaling pathway' and three genes each are assigned to the pathways 'Interleukin signaling' and 'T-cell activation', respectively.

Forty of the 168 genes are assigned to the biological process 'immunity and defense' and 39 genes belong to the group 'signal transduction' group strengthening the experimental data obtained by the microarray experiments.

Genes differentially expressed between TOL and CTRL lymphocytes

A total of 113 unique genes could be identified showing significant differential expression between the TOL and the CTRL group. Sixty-two genes were higher expressed in the TOL group as depicted in Table 1, whereas 51 genes showed lower expression (Table 2). A cluster diagram of the two gene sets along with a graphical representation of the expression values is given in Fig. 1.

The PANTHER ontology categories with significantly enriched sets of genes are listed in Table 3. The majority of genes upregulated in the TOL group are involved in various signal transduction cascades. Four genes are members of the MAPKKK cascade, namely IL6, Tob2, Stk39, and Dusp24. Other genes involved in lymphocyte differentiation are the suppressor of cytokine signaling 7 (SOCS7), which is activated by IL6, lymphotactin (Xcl1), and the two ERs 1 alpha (Esr1) and 2 beta (Esr2).

Genes involved in immunity and defense in the CTRL group are the lymphocyte antigen 6 complex (Ly6a), immunoglobulin heavy chain 4 (Igh-4), or the histocompatibility 2, class II, locus DMa (H2-DMa).

Transcription factor analysis

The search for common regulatory mechanisms in the 62 genes upregulated in the TOL group resulted in 4 transcription factors with an enriched number of binding sites in the upstream regions of the specified genes (webfigure 2). The 4 transcription factors are estrogen receptor 1 alpha (Esr1), GATA-binding protein 1 (GATA1), insulin promoter factor 1 (Ipf1), and paired-related homeobox 2 (Prrx2). It is of note that Esr1 alpha along with ER 2 beta were among the 62 upregulated genes in group TOL.

No transcription factors with significantly higher numbers of binding sites in comparison with the reference dataset could be obtained when analyzing the 51 genes upregulated in the CTRL group.

Discussion

In the present paper, we showed that lymphocytes of tolerant mice exhibit a distinct gene expression signature compared with the controls. Specifically, genes with biological functions of signal transduction, cell communication and immunity, and defense were abundantly regulated in the TOL versus the CTRL group. The biological relevance of our findings can be appreciated by the fact that TFBSs of four transcription factors could be identified in the majority of upregulated genes. Additionally, in one of those factors, the ER alpha was also found experimentally to be overexpressed in the TOL group. Several other groups investigated the role of ERs in immune response and lymphocyte differentiation [42–46]. One main finding in these papers was that the suppressive effects of estrogen on immune response and inflammation are mediated via these receptors.

Another element of the four transcription factors with enriched-binding sites in our dataset is GATA1. GATA1 is a member of the GATA family of transcription factors and is known to be involved in erythroid lineage development [47]. Another member of the family, GATA3, sharing a similar-binding profile is necessary for Th2 development. GATA1, GATA2, GATA3, and GATA4 all enhance interleukin 4 (IL4) and interleukin 5 (IL5) production and inhibit interferon γ (IFNγ) production [48]. No connection between the other two transcription factors Ipf1 and Prrx2 and lymphocyte differentiation or activation has been reported yet.

The role of cytokines in the induction of tolerance with costimulation blockade is complex, and remains incompletely understood [8]. Evidence that costimulation blockade affects Th1 and Th2 responses differently comes from

Table 1. Representation of the 62 genes upregulated in the TOL group compared with the CTRL group.

Accession number	Gene name	Gene symbol	Mean expression TOL (+MR1)	Mean expression CTRL (−MR1)
Cell cycle, cell growth				
AV006366	Transducer of ERBB2, 2	Tob2	1.10	−0.21
U81451	Estrogen receptor (ER) 2 (beta)	Esr2	0.56	−0.66
AI327095	Neuroblastoma, suppression of tumorigenicity 1	Nbl1	−2.97	−4.13
AV069067	Cell growth regulator with EF hand domain 1	Cgref1	−0.07	−1.18
NM_007956	ER 1 (alpha)	Esr1	0.55	−0.53
AI836990	Pituitary tumor-transforming 1	Pttg1	0.75	−0.25
Immune response				
J03783	Interleukin 6	Il6	0.14	−1.18
AV033738	CD59a antigen	Cd59a	1.23	−0.06
BB033108	Guanylate nucleotide-binding protein 1	Gbp1	−0.28	−1.56
AA023592	Tumor-associated calcium signal transducer 2	Tacstd2	1.39	0.24
AV041212	Immunoglobulin superfamily, member 3	Igsf3	1.21	0.07
AV015435	Chemokine (C motif) ligand 1	Xcl1	3.50	2.44
Ion binding				
AV088650	Phospholipase A2, group IB, pancreas	Pla2g1b	1.26	−0.41
BG068505	Sciellin	Scel	0.72	−0.29
Membrane				
AA030070	Transmembrane 4 superfamily member 9	Tm4sf9	1.63	0.31
AV078126	ELOVL family member 7, elongation of long chain fatty acids (yeast)	Elovl7	0.71	−0.46
AV048217	Receptor (calcitonin) activity-modifying protein 1	Ramp1	1.01	−0.13
AA023493	Transmembrane protein with EGF-like and two follistatin-like domains 1	Tmeff1	−2.04	−3.13
AV043458	DnaJ (Hsp40) homolog, subfamily C, member 5 beta	Dnajc5b	1.01	−0.02
Metabolism				
BG069483	Adiponectin receptor 1	Adipor1	2.93	0.92
BG071664	Serine/threonine kinase 24 (STE20 homolog, yeast)	Stk24	1.64	0.11
BG068078	Cysteine dioxygenase 1, cytosolic	Cdo1	1.07	−0.14
AV086000	Aldehyde dehydrogenase family 3, subfamily A1	Aldh3a1	−1.96	−3.10
AV094499	Myo-inositol 1-phosphate synthase A1	Isyna1	3.09	1.99
AV035504	Homogentisate 1, 2-dioxygenase	Hgd	0.90	−0.11
AV036103	Glutathione peroxidase 7	Gpx7	0.57	−0.44
Nucleus				
AI840760	RNA-binding motif protein 9	Rbm9	−1.57	−3.22
AV114032	Breast carcinoma amplified sequence 3	Bcas3	1.54	0.51
Protein modification				
BG063607	Serine (or cysteine) proteinase inhibitor, clade B, member 9b	Serpinb9b	0.86	−0.62
AA152808	Serine/threonine kinase 39, STE20/SPS1 homolog (yeast)	Stk39	1.10	−0.26
AV032732	Ubiquitin carboxy-terminal hydrolase L1	Uchl1	0.61	−0.72
AW548360	F-box and leucine-rich repeat protein 7	Fbxl7	0.58	−0.62
AV041151	DnaJ (Hsp40) homolog, subfamily B, member 8	Dnajb8	1.41	0.31
Signal transduction				
BG069262	Amyloid beta (A4) precursor protein-binding, family B, member 2	Apbb2	0.02	−1.28
BG067838	Suppressor of cytokine signaling 7	SOCS7	0.59	−0.68
AV040149	Dual specificity phosphatase 24 (putative)	Dusp24	0.93	−0.25
AV061251	Islet amyloid polypeptide	Iapp	0.60	−0.52
Structural				
BG066108	Mitochondrial ribosomal protein L49	Mrpl49	1.32	−1.31
AV012366	Procollagen, type VI, alpha 3	Col6a3	−2.84	−5.35
AV037622	Fibrinogen, alpha polypeptide	Fga	1.85	0.64
AV006132	Procollagen, type XV	Col15a1	0.73	−0.37
BG067360	Activity-regulated cytoskeletal-associated protein	Arc	0.55	−0.46
Transcription, translation				
BG074279	Ankyrin repeat domain 10	Ankrd10	2.89	0.85
NM_008601	Microphthalmia-associated transcription factor	Mitf	2.25	0.49
AV134878	Inhibitor of growth family, member 1-like	Ing1l	0.80	−0.62

Table 1. (contd)

Accession number	Gene name	Gene symbol	Mean expression TOL (+MR1)	Mean expression CTRL (−MR1)
AI839271	Heat shock factor-binding protein 1	Hsbp1	1.05	−0.24
AV006196	Eukaryotic translation elongation factor 1 alpha 2	Eef1a2	1.03	−0.15
AV078332	LIM domain binding 2	Ldb2	0.48	−0.68
AU015927	General transcription factor III C 1	Gtf3c1	−2.71	−3.86
BG063389	Processing of precursor 1, ribonuclease P/MRP family, (*Saccharomyces cerevisiae*)	Pop1	−0.25	−1.29
Transport				
BG068601	Clathrin, light polypeptide (Lca)	Clta	1.35	−1.03
AI838973	Cellular retinoic acid-binding protein II	Crabp2	0.70	−0.63
AV253834	Potassium voltage-gated channel, shaker-related subfamily, beta member 2	Kcnab2	2.36	1.11
AV001270	Solute carrier family 34 (sodium phosphate), member 1	Slc34a1	0.57	−0.62
BE292615	Vesicle-associated membrane protein 1	Vamp1	0.92	−0.19
BG072581	Glycosylphosphatidylinositol-specific phospholipase D1	Gpld1	1.04	0.02
Others				
AV006110	Williams–Beuren syndrome chromosome region 16 homolog (human)	Wbscr16	0.51	−1.58
BG069724	Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 13	Als2cr13	0.86	−0.46
AU045206	Cordon-bleu	Cobl	−0.05	−1.21
AV046974	Stimulated by retinoic acid gene 8	Stra8	1.92	0.77
AV006161	START domain containing 8	Stard8	0.96	−0.19
AV036018	Pregnancy-specific glycoprotein pseudogene 1	Psg-ps1	4.82	3.71

Genes are categorized based on the GO Terms and PANTHER classifications and are ranked by difference of mean expression between the two groups studied. Numbers are log2 of expression values.

experiments using STAT4−/− and STAT6−/− recipients, which revealed that anti-CD40L is more effective at suppressing an alloresponse in the absence of normal Th1 responses (i.e. in predominantly Th2, STAT4−/− mice) [49]. This 'deficit' could be overcome, however, for instance by administering very high doses of the anti-CD40L mAb, indicating that Th1 cells are not inherently resistant to CD40L blockade. However, in another study, IFNγ, a Th1 cytokine, was shown to be critical for the effect of anti-CD40L (given together with CTLA4Ig) in skin and heart graft models [50]. These results were derived from models not involving BMT, which are known to be different mechanistically [8]. Less is known about the role of cytokines in BMT tolerance systems. One particular protocol involving depletion of CD8 cells and anti-CD40L tolerized both Th1 and Th2 responses, and did not require IFNγ as demonstrated by using IFNγ-deficient strains both as recipient and donor [15]. Whether a certain cytokine milieu favors tolerance induction after BMT with costimulation blockade remains currently unknown.

Other genes involved in lymphocyte differentiation that were upregulated in the TOL group are lymphotactin (Xcl1), ERs as described above and the SOCS7, which is activated by IL6. Lymphotactin (Xcl1) is reported to inhibit $CD4^+$ T-cell proliferation through a decreased production of Th1 (IL2, IFNγ) but not Th2 (IL4, IL13) lymphokines [51]. Another study demonstrated that lymphotactin costimulates death of $CD4^+$ T cells via apoptotic pathways dependent on Fas-FasL signaling [52]. Xcl1 along with guanylate nucleotide-binding protein 1 (Gbp1) was both reported by Matsui *et al.* [25] to be highly upregulated in tolerizing cardiac allografts compared with syngeneic isografts after costimulation blockade. Gbp1 is induced by IFNγ, interleukin 1 alpha and beta and tumor necrosis factor alpha (TNFA). Interferon gamma and interleukin 1 beta are highly expressed in our lymphocyte populations. SOCS7 along with Interleukin 6 (IL6) is involved in the JAK-STAT signaling pathway repressing the signaling cascade in conjunction with other members of the SOCS family [53].

Cobbold *et al.* [21] used SAGE technology to study gene expression of 29 libraries of T cells and antigen-presenting cells. The authors focused on the identification of a distinct expression patterns in $CD4^+$ $CD25^+$ regulatory T cells and IL10-producing regulatory T cells. Most of the SAGE tags mapped to yet unknown genes in the two populations of regulatory T cells. Upregulated genes in $CD4^+$ $CD25^+$ cells are the transcriptional regulator SIN3A and interleukin 17 (IL17). Genes highly expressed in IL10-producing regulatory T cells are the tumor necrosis factor receptor superfamily, members 18 and 9, kallikrein 8 (Klk8), lymphocyte antigen 6 complex locus A (Ly6a), or the pituitary tumor-transforming 1 (Pttg1). Interestingly, Pttg1 was also upregulated in the TOL group in our study

Table 2. Representation of the 51 genes downregulated in the TOL group compared with the CTRL group. Genes are categorized based on the GO Terms and PANTHER classifications and are ranked by difference of mean expression between the two groups studied. Numbers are log2 of expression values.

Accession number	Gene name	Gene symbol	Mean expression TOL (+MR1)	Mean expression CTRL (−MR1)
Cell cycle, cell growth				
AV057778	Dual specificity phosphatase 22	Dusp22	−2.34	−0.89
DNA repair				
AV089058	RAD23b homolog (*S. cerevisiae*)	Rad23b	−1.51	−0.34
AV113813	Retinoblastoma-binding protein 4	Rbbp4	−1.87	−0.80
Hormone				
AV223385	Prolactin-like protein B	Prlpb	−2.38	−0.96
AV003292	Cholecystokinin	Cck	−0.42	0.75
Immune response				
AA166427	Immunoglobulin heavy chain 4 (serum IgG1)	Igh-4	−0.35	1.57
AV058058	Histocompatibility 2, class II, locus DMa	H2-DMa	0.52	1.76
AV084245	Synaptophysin-like protein	Sypl	−1.68	−0.50
BG075512	Membrane metallo endopeptidase	Mme	−1.58	−0.49
AV038265	Lymphocyte antigen 6 complex, locus A	Ly6a	0.06	1.06
BG065454	N-myc downstream regulated gene 1	Ndrg1	−4.58	−3.58
Ion binding				
BG070042	Sorbitol dehydrogenase 1	Sdh1	−2.92	−1.74
Membrane				
BG073453	Neuropilin 1	Nrp1	−2.36	−0.95
AV140547	Transmembrane 4 superfamily member 8	Tm4sf8	−2.83	−1.69
AV016927	Integrin beta 1 (fibronectin receptor beta)	Itgb1	−2.21	−1.15
Metabolism				
AV058563	Protein phosphatase 1, regulatory (inhibitor) subunit 2	Ppp1r2	−1.86	−0.30
AV088048	2,4-dienoyl CoA reductase 1, mitochondrial	Decr1	−0.75	0.40
AV006379	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7	Hsd3b7	−2.50	−1.39
BG064242	Pyruvate dehydrogenase complex, component X	Pdhx	−1.16	−0.11
AA175957	Heparanase	Hpse	−1.06	−0.02
Nucleus				
BG071765	Microrchidia	Morc	−4.87	−1.06
AV030488	High mobility group nucleosomal-binding domain 3	Hmgn3	−2.23	−0.87
AV140192	Nucleoporin 54	Nup54	−0.78	0.47
AV037082	Small nuclear ribonucleoprotein D1	Snrpd1	−2.00	−0.90
Protein modification				
AV028546	Protein-l-isoaspartate (d-aspartate) *O*-methyltransferase 1	Pcmt1	−1.00	0.98
AV090021	F-box only protein 31	Fbxo31	−1.73	0.13
BG065250	Cathepsin H	Ctsh	3.74	5.07
AV003067	Anterior pharynx defective 1c homolog (*Caenorhabditis elegans*)	Aph1c	−1.36	−0.34
Signal transduction				
AV268070	Synaptojanin 2	Synj2	−0.27	0.85
AV024420	Inositol polyphosphate phosphatase-like 1	Inppl1	−2.22	−1.12
BG076225	Dual specificity phosphatase 16	Dusp16	−0.96	0.11
Structural				
AV145175	Tubulin, beta 5	Tubb5	−2.41	−1.39
Transcription, translation				
AV053996	Recombining-binding protein suppressor of hairless-like (*Drosophila*)	Rbpsuhl	−0.96	0.28
AV147450	Zinc finger protein 148	Zfp148	−1.56	−0.33
AV083890	Myocyte enhancer factor 2C	Mef2c	−1.66	−0.48
AV084534	Eukaryotic translation initiation factor 4E-binding protein 2	Eif4ebp2	−0.42	0.75
AV067123	Myelocytomatosis oncogene	Myc	−1.51	−0.45
AV051741	ELL-associated factor 2	Eaf2	−0.31	0.74
Transport				
BG074144	Solute carrier family 40 (iron-regulated transporter), member 1	Slc40a1	0.73	2.46
AV048889	Trafficking protein particle complex 2	Trappc2	−1.04	0.18

Table 2. (contd)

Accession number	Gene name	Gene symbol	Mean expression TOL (+MR1)	Mean expression CTRL (−MR1)
AV052841	Solute carrier family 33 (acetyl-CoA transporter), member 1	Slc33a1	−1.82	−0.62
AV006077	RAB6, member RAS oncogene family	Rab6	−1.11	−0.01
BG063927	Chloride intracellular channel 4 (mitochondrial)	Clic4	−1.86	−0.77
AV052493	Solute carrier family 31, member 2	Slc31a2	−0.79	0.27
AV006147	Succinate dehydrogenase complex, subunit D, integral membrane protein	Sdhd	−1.16	−0.13
AV085180	Cytochrome c oxidase, subunit Va	Cox5a	−1.37	−0.35
Others				
AV087712	PDLIM1-interacting kinase 1 like	Pdik1l	−1.64	0.36
AV083984	Fer-1-like 3, myoferlin (*C. elegans*)	Fer1l3	−4.32	−2.96
BG064140	IBR domain containing 3	Ibrdc3	−0.34	0.71
AV149935	4-nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1 (*C. elegans*)	Nipsnap1	−0.95	0.09
BG075495	Echinoderm microtubule-associated protein like 4	Eml4	−0.79	0.24

whereas the lymphocyte antigen 6 complex locus A was upregulated in the CTRL group.

A group of investigators lead by Hamalainen *et al.*[22] used oligonucleotide arrays to identify distinct gene expression patterns between Th1- and Th2 lymphocytes. They identified 34 genes with differential expression between the two populations under study. Interferon gamma, TNFA, along with interleukin 8 (IL8), colony-stimulating factor 2 (CSF2), and chemokine ligands 3 (CCL3), 4 (CCL4), and 5 (CCL5) are preferentially expressed in Th1 cells compared with Th2 cells. Transcripts with higher expression in Th2 cells on the other hand are interleukins 4, 5 and 13 (IL4, IL5, and IL13) along with the macrophage migration inhibitory factor (MIF). These findings were confirmed by another study conducted by Chtanova *et al.* The authors also used oligonucleotide arrays to reveal distinct gene expression profiles in mouse Th1 and Th2 as well as Tc1 and Tc2 cells. In addition to the genes found by Hamalainen *et al.*, they also identified differentially expressed genes involved in transcriptional regulation like GATA-binding protein 3 (GATA3), signal transducer and activator of transcription (STAT4), avian musculoaponeurotic fibrosarcoma AS42 oncogene homolog (Maf), or T-box 21 (Tbx21).

Flechner *et al.* [54] used Affymetrix gene chips to study gene expression profiles of peripheral blood lymphocytes (PBLs) in kidney transplant patients. They identified distinct patterns in PBMs as well as in kidney biopsies between patients with well-functioning transplants without rejection and transplants undergoing acute rejection. However, both groups of patients received conventional immunosuppressive therapy and thus the expression profiles of the PBL cannot be compared with our data on costimulation blockade.

A limitation of our study is that only a single time point has been analyzed. Even though previous work indicates that the chosen time point of 3 weeks post-BMT is in a critical phase for tolerance induction [2,18], we cannot formally exclude that different gene expression profiles would be found at other time points. The use of wild-type donor–recipient strain combinations entails the lack of appropriate methods for tracking donor-reactive T cells. Thus, by analyzing a pool of lymphocytes which contains T cells of all specificities, some genes differently regulated in alloreactive T cells might have remained undetected because of the absolute low yield. Furthermore, we used nylon wool passage to enrich T cells despite the limitations of this technique, because magnetic cell sorting (MACS) separation and subsequent fluorescence activated cell sorting (FACS) sorting of T cells resulted in inconsistent array readings because of low yield and manipulation during the sorting procedure. It is unlikely that donor T cells were already present in the TOL group, because they only develop slowly over time [55]. We did not include a nonirradiated control group in our study, because our primary interest was to detect differences in the gene expression profile between tolerant and rejection lymphocytes and because such a control is not necessary, as both groups underwent the same regimen of total body irradiation (and bone marrow infusion), any observed difference in gene expression between the two study groups is thus unrelated to irradiation. Moreover, naíve murine lymphocytes exhibit a distinctly different molecular signature than both of our groups (GEO. GDS544, GDS237). Thus, the observed differences in gene expression between the two study groups are most likely because of the presence of tolerance.

In summary, we showed that a distinct genome-wide gene expression profile in T lymphocytes is associated

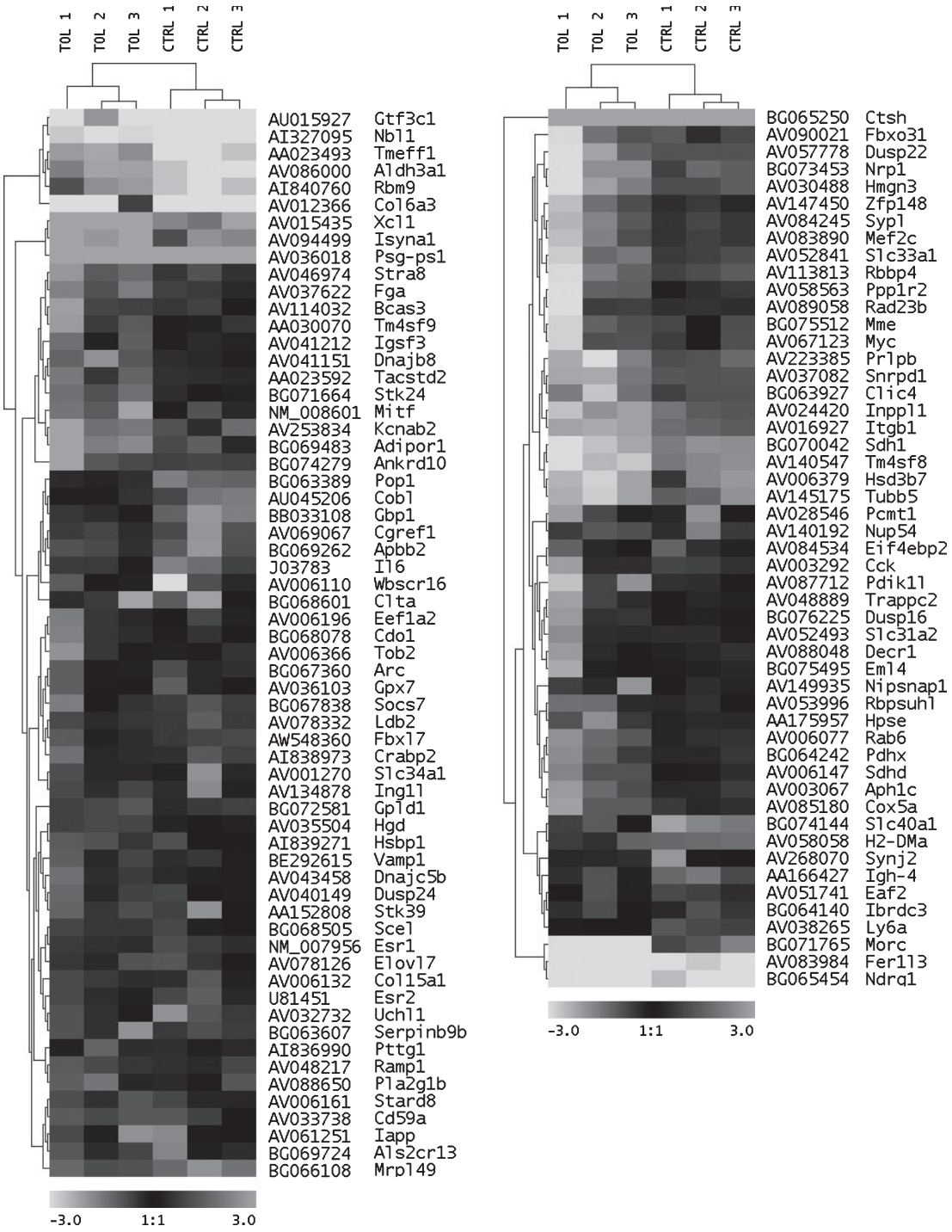

Figure 1 Expression profiles of differentially regulated genes between the TOL group and the CTRL group. Euclidean distance was used as distance measure and complete linkage as the linkage rule. Red spots indicate abundantly expressed transcripts whereas green spots indicate low expressed transcripts in comparison with the reference RNA. A clear separation between the two groups is detected. GenBank accession numbers and gene symbols are used for labeling the cDNA clones. Genes upregulated in the TOL group are depicted on the left whereas genes downregulated in the TOL group when compared with the CTRL group are shown on the right.

Table 3. Biological processes statistically enriched with differentially expressed genes between the TOL and the CTRL group. The reference dataset consisted of all 23 520 genes with an assigned biological process. Categories are sorted in descending order by the total number of genes in each group.

Biological process	Nr. of genes	Ref. dataset	*P*-value
Genes upregulated in the TOL group compared with the CTRL group ($n = 62$)			
Signal transduction	20	3666	0.001
Cell communication	9	1288	0.006
Immunity and defense	8	1303	0.021
Intracellular-signaling cascade	7	869	0.008
Cell proliferation and differentiation	6	753	0.014
Cell cycle control	5	389	0.004
Oncogenesis	5	597	0.021
Lipid, fatty acid and steroid metabolism	5	694	0.036
MAPKKK cascade	4	215	0.003
Steroid hormone-mediated signaling	3	31	0.000
Amino acid catabolism	2	44	0.006
JNK cascade	2	51	0.008
Oogenesis	2	61	0.012
Detoxification	2	76	0.017
JAK-STAT cascade	2	87	0.022
Tumor suppressor	2	107	0.033
Other oncogenesis	2	110	0.034
Phospholipid metabolism	2	113	0.036
Genes downregulated in the TOL group compared with the CTRL group ($n = 51$)			
Intracellular protein traffic	5	902	0.045
Carbohydrate metabolism	4	511	0.025
Cell motility	3	290	0.025
Exocytosis	2	161	0.048
Other immune and defense	2	88	0.016
Regulated exocytosis	2	63	0.008
Tricarboxylic acid pathway	2	27	0.002

with tolerance after BMT and MR1 costimulation blockade. Several genes involved in lymphocyte differentiation and suppression were identified to be overexpressed in the TOL group of BMT mice. Among these are the suppressor of cytokine signaling 7, the two ERs 1 alpha and 2 beta, pttg1, or lymphotactin known to costimulate apoptotic mechanisms in $CD4^+$ T cells. This molecular signature may be evaluated for its prospective validity of monitoring clinical tolerance.

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