Expression of Tolerance Associated Gene-1, a Mitochondrial Protein Inhibiting T Cell Activation, Can Be Used to Predict Response to Immune Modulating Therapies

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Immune modulating therapies or “biologics” are gaining more and more importance for treatment of autoimmune diseases and prevention of transplant rejection. However, so far biologics are not resonsably effective in all treated patients. The success rate varies from 20 to 80%. Therefore there exist a high demand for biomarkers predicting the response at a very early time point after onset of treatment. It also highlights that we still do not fully understand disease pathogenesis und the immunological mechanisms leading to a chronic inflammatory antself immune response.

Psoriasis is a T cell mediated disorder affecting ~2–3% of the Caucasian race. The disease manifests with marked infiltration of T lymphocytes, dendritic cells, and neutrophils in the skin and hyperplasia of the epidermis. An approved drug for treatment of psoriasis is Alefacept, a fully human protein composed of the extracellular portion of lymphocyte-function-associated Ag type 3 (LFA-3 or CD58) and the Fc domain of IgG1 (= LFA3TIP) (1–9). Its supposed action is based on inhibition of costimulation via blocking CD2 and granzyme-induced apoptosis due to FcyR III binding (9).

Alefacept is only effective in up to 50% treated psoriasis patients (10, 11). Moreover, biologics such as Alefacept are cost intensive and may cause side effects (3). Therefore, they are usually administered to patients who failed on conventional anti-inflammatory therapies (12). Severity of psoriasis is evaluated by the Psoriasis Area and Severity Index (PASI), Ref. 13, which in case of treatment with Alefacept, improves rather late during successful therapy. In a recent study, 55% of Alefacept-treated patients showed a remission of >50% of the psoriasis symptoms. Responding patients demonstrated a consistent reduction in quantity of infiltrating T cells, myeloid cells, dendritic cells, and of several key inflammatory molecules in psoriatic lesions (14). Thus, although changes in psoriatic plaque composition can be detected in parallel to improvements of disease severity, there is a demand for noninvasive biomarkers allowing an early discrimination between responding and nonresponding patients. An interesting study by Haider et al. (15) demonstrated that nonresponders express higher levels of T cell activation genes such as CD69 in pretreatment PBMCs.
To address the demand for noninvasive biomarkers monitoring treatment response in psoriasis patients, we analyzed peripheral transcription levels of CD3, CD69, Foxp3, PDL1, the tolerance associated gene-1 (TOAG-1), and the receptor for hyaluronic acid mediated migration (RHAMM) weekly by quantitative real-time PCR in a cohort of 20 psoriasis patients receiving Alefacept.

We observed that TOAG-1 mRNA expression is significantly up-regulated in PBMCs of psoriasis patients responding to Alefacept treatment, whereas RHAMM expression was decreased in responding patients. These differences already occurred between week 2 and 4 of Alefacept treatment and therefore much earlier than any significant improvement in disease severity (week 9).

We could also show that TOAG-1 protein is exclusively localized within mitochondria and enhances apoptosis upon overexpression in murine T cells.

Additionally, in vitro Alefacept treatment of human primary T cells resulted in inhibition of T cell activation, reduced conversion into activated CD137+ T cells, and thereby increased TOAG-1 expression. Alefacept-treated cultures are also characterized by enhanced apoptosis and diminished T cell proliferation due to an increase of PDL1 on APCs.

Materials and Methods

Design of psoriasis study

Twenty statistically matched patients regarding age, sex, and initial PASI (15 males, 5 females, aged between 28 and 70 years, median 50 years) with moderate to severe psoriasis (PASI: 7–36) were included in this study. Demographics are shown in supplementary Table A. They were treated with 15 mg Alefacept i.m. weekly. Peripheral blood was taken before first Alefacept application and then weekly until week five and thereafter every second week, until the end of treatment at week 13. PBMCs were isolated using Ficoll gradient centrifugation and frozen as pellets at −80°C until RNA isolation. At the same time points severity of disease and thereby possible reduction of symptoms was evaluated applying the PASI. Therapy was defined successful, if the PASI was reduced by at least 50% of the initial score, which was shown previously to be a clinically relevant end-point in the evaluation of psoriasis (16). Investigators analyzing the samples were blinded to the outcome of the study. The protocol concerning human subjects was approved by the ethics committee of the Charité University Medicine Berlin Campus Mitte, Germany.

Quantitative real-time PCR

The isolation of RNA and subsequent analysis of mRNA-expression profiles by Real-Time PCR (ABI Prism 7700 and 7500 Sequence Detection System Taqman; PE Applied Biosystems) has been described previously (17). In brief, mRNA was isolated using the Absolutely RNA Mini Prep Kit (Stratagene) following the manufacturer’s protocol and reversely transcribed into activated CD137+ T cells, and thereby increased TOAG-1 expression. Alefacept-treated cultures are also characterized by enhanced apoptosis and diminished T cell proliferation due to an increase of PDL1 on APCs.

For cytokine release assay, supernatants of murine T cell cultures were harvested at the same time points as the cells and stored at −80°C until measurement via cytometric bead array (CBA, TH1/TH2 from Bender MedSystems).

Psoriasis patients were monitored before and at the end of therapy for surface expression of CD3, CD4, CD8, CD2, and CD45RO (BD Pharmingen) in isolated PBMCs.

All measurements were performed on a FACSCalibur using CellQuest Software (BD Bioscience) within 1 h after staining.

In vitro experiments with human primary T cells

To investigate the impact of Alefacept on mRNA expression in T cells, one way MLR was performed. For these purposes, blood was taken from two different donors. One was referred to as responder and the other as donor. Donor cells were treated with a CD3 depletion mixture (RosetteSep, Stem Cell Technologies) to deplete donor T cells. Subsequently, PBMCs were isolated by Ficoll gradient centrifugation and 3 × 10^6 cells/ml of each preparation, responder and donor, were cocultured in 96-well plates. For some experiments responder PBMCs have also been separated into CD8+ T cells or memory and naive CD4+ T cells via indirect MACS (Miltenyi Biotec). For cell separation after in vitro culture, cells were stained with anti-human CD8-FITC and anti-human CD137-APC (both BD Pharmingen) and individual T cell subpopulations were sorted on a FACSaria (purity ≥ 95%).

We also studied the effect of Alefacept on polyclonal T cell activation. Therefore, human T cells were purified as just described above and seeded in 96-well plates coated with 10 μg/ml anti-human CD3 (OKT3) and -CD28 (CD28.2) Abs (eBioscience). Alefacept (50 μg/ml) was added either plate bound, soluble or soluble but cross-linked with 10 μg/ml anti-human IgG1 Ab (MH1715, Invitrogen) to study the effect of immobilization on efficiency in vitro. Additionally, the impact of T cell activation was compared when adding Alefacept during seeding of the cells or 3 days later.

PDL1 interaction was blocked in some experiments using anti-human PDL1 (MIHI) in concentration as indicated (eBioscience).

Proliferation was determined by [3H]thymidine (0.028 MBq) incorporation for 18 h at indicated time points of culture.

Localization of TOAG-1 protein

The coding sequence of murine TOAG-1 (RZPD clone ID: IRAKp961K0177Q2) was flanked with two different sequences containing a BglII or a BamHI restriction site. Thereby the stop codon was deleted. This fragment was inserted into pEGFP-N1 for creating a fusion-protein of TOAG-1 and EGFP. For localization analysis, NIH-3T3 cells were seeded on cover slides in 24-well plates and 24 h later (60% confluence) transfected with either TOAG-1-EGFP (fusion-protein) or pEGFP-N1 (control) plasmid DNA using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection cells were either stained with MitoTracker Red CMXRos for mitochondria or LysoTracker Red for lysosomes (both dyes were obtained from Invitrogen) and embedded in mounting medium containing 4',6-diamidino-2-phenylindol to visualize nuclear DNA. Thereafter, red (organelle staining), green (TOAG-1 or control), and blue (nucleus) fluorescence images were taken separately via laser fluorescence microscopy (Leica). Overlays were arranged with Adobe Photoshop 7.0.

Retroviral construct and packaging cells

The coding sequence of TOAG-1 (~1400 bp) was inserted into moloney murine leukemia virus-derived vector pLXSN in front of an internal ribosomal entry site follow by the reporter gene sequence of GFP using standard cloning techniques. Retrovirus packaging was achieved by co-transfection with two helper plasmids coding either for gagpol or ecotropic env genes from murine leukemia virus. In brief, 1.5 × 10^7 HEK-293 cells/ml were seeded into 60 mm dishes and 24 h later transfected using calcium/phosphate precipitation. After changing medium (DMEM containing 10% heat-inactivated FCS, 4 mM L-glutamine, and 100 μg/mI penicillin, 100 μg/ml streptomycin) on the next day and further incubation for 48 h, supernatant was filtered and frozen at −80°C as aliquots. The retroviral titer was determined by infecting NIH-3T3 fibroblasts with supernatant containing final concentration of 5 μg/ml polybrene. Transduction rate was assessed after 48 h incubation using flow cytometry by detecting GFP and ranged between 90 and 99%.

In vitro experiments with murine primary T cells

B cells were enriched (RosetteSep, Stem Cell Technologies) from spleens of male C57BL6 mice (H2b) and suspension was prepared using a 40-μm cell strainer (Nunc). BALB/c (H2d) CD4+ T cells were isolated via MACS (indirect CD4+), Miltenyi Biotec) from likewise prepared lymph node cells.
Equal amounts of B and CD4\(^+\) T cells (3 × 10^6 cells/ml) were seeded to 96-well plates. TOAG-1 mRNA expression on the first 4 days of in vitro culture was determined in untreated cells and cells either treated with 50 ng/ml cyclosporine A or 1 μg/ml anti CD4 mAb.

For retroviral transduction, B and CD4\(^+\) T cells (3 × 10^6 cells/ml, each) from C57/BL6 mice were seeded to 96-well plates coated with 6 μg/ml anti-mouse CD3 (145-2C11) and 4 μg/ml anti-mouse CD28 (37.51) Abs (both eBioscience). Eighteen hours later, supernatant was removed and saved. Retrovirial supernatant was diluted 1/2 with culture medium (RPMI 1640 containing 10% heat-inactivated FCS, 4 mM l-glutamine and 100 μM penicillin, 100 μg/ml streptomycin, 5 mM β-ME) containing final concentration of 5 μg/ml polybrein and added to the cells for 90 min incubation. Subsequently, viral supernatant was removed and retained medium was returned to the cells. After 5 days incubation, cells were either harvested for quantitative real-time PCR or for flow cytometry. Transduction rate was assessed at the same time point using flow cytometry detecting GFP and ranged between 7 and 12%.

**Evaluation of results and statistics**

The expression of each gene of interest has been normalized to the housekeeping gene HPRT. The expression results determined in PBMC samples obtained before therapy onset (week 0) was defined as baseline and set to 100%. The expression results determined in PBMC samples taken during the therapy were related to the baseline level.

Statistical analysis was done with SPSS 12.0. In general Mann-Whitney test was used to calculate p values. For pair-wise comparison of quantitative real-time PCR results in sorted CD137\(^+\) and CD137\(^+\) T cell subpopulation, a paired t test was used. A p-value of ≤0.05 was considered significant (*, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001).

**Results**

**Alefacept therapy in psoriasis patients**

Twenty psoriasis patients were treated with 15 mg Alefacept i.m. weekly over a period of 12 wk. Psoriasis symptoms were analyzed before (week 0) and during the course of treatment (weekly until week 5, thereafter every second week). Blood samples were taken at the same time points. A successful therapy was defined as PASI reduction of ≥50% which was previously shown to be a clinically relevant endpoint (16). In our study nine of twenty patients responded with ≥50% PASI reduction. Accordingly 11 patients were considered nonresponders. One patient discontinued therapy at week five due to lack of improvement in disease severity. A significant difference between responders and nonresponders concerning improvement of psoriasis symptoms was not detectable until week nine of Alefacept therapy. Because nonresponders also showed a modest improvement of disease severity without reaching 50% PASI reduction, responders were indistinguishable from nonresponders during the first weeks of therapy as shown in Fig. 1A. Demographic characteristics of all 20 patients are shown in supplementary Table A. Mann-Whitney U test did not reveal any relevance regarding age (p = 0.094), gender (p = 0.206), or primary PASI score (p = 0.569) between responders and nonresponders (data not shown).

**Effect of Alefacept therapy on peripheral T cell subsets**

Patients’ blood was monitored regarding T cell counts before therapy and at the end of Alefacept treatment. CD4\(^+\) and CD8\(^+\) T cells were investigated concerning CD2 and CD45RO surface expression using flow cytometry. Alefacept is postulated to act predominantly on CD2\(^{high}\) memory T cells and is known to deplete CD8\(^+\) T cells (15). Therefore, monitoring treated patients for T cell surface markers might also discriminate responding from nonresponding patients. Compared with baseline, CD4\(^+\) T cells were reduced in responders and nonresponders to a similar extent during treatment, as shown in Fig. 1B. There were no differences detectable in CD2 and CD45RO surface expression on CD4\(^+\) T cells. Interestingly, reduction of CD8\(^+\) T cells expressing high levels of either CD2 or CD45RO is more pronounced in responding than in nonresponding patients but only at the end of therapy. This was not

**Peripheral TOAG-1 and RHAMM expression can discriminate between responders and nonresponders during Alefacept therapy**

To address the demand for noninvasive biomarkers, which would enable identification of responders early during therapy, we analyzed peripheral transcription levels of several genes including CD3, CD69, Foxp3, PDL1, the TOAG-1, and the RHAMM weekly by quantitative real-time PCR in our cohort of 20 psoriasis patients receiving Alefacept.

Gene expression data are shown relative to pretreatment values for better representation of individual changes over time. CD69 and PDL1 were not differently expressed on mRNA level prior or during Alefacept therapy between responders and nonresponders (supplementary Figs. 1 and 2). Furthermore, we observed no significant difference between responders and nonresponders in Foxp3 gene expression throughout therapy, although Foxp3 slightly increased in responders at week 2 and 3 of treatment.

In contrast, as shown in Fig. 2A, TOAG-1 was significantly up-regulated in responders very early during Alefacept therapy,
Combinational analysis of gene markers

Because TOAG-1 and RHAMM showed opposite regulation in responders upon Alefacept therapy we further tested whether the ratio of these genes may allow even better discrimination of responders and nonresponders. The TOAG-1/RHAMM gene ratio was calculated from relative changes (compared with predose) in each gene. Over time, responders were characterized by an increase of the gene ratio of up to 30-fold (Fig. 2). In contrast, the expression levels of RHAMM were unchanged in nonresponding patients and a decrease in responders starting very early after therapy onset and lasting as long as Alefacept was administered (Fig. 2). At week 3 of treatment, nonresponders were characterized by a significant 5.5-fold increased RHAMM mRNA expression. Due to individual differences in gene expression levels we could not discriminate responders and nonresponders based on their absolute TOAG-1 and RHAMM mRNA expression before Alefacept therapy.

Alefacept significantly reduces proliferation of human PBMCs and alters mRNA expression patterns during T cell activation

Next, we investigated whether Alefacept can directly alter expression of allo-stimulated PBMCs dose-dependently. Next, we tested whether Alefacept also influences TOAG-1 and RHAMM expression of allo-stimulated PBMCs. Alefacept was added either at the time of stimulation or 3 days later to mimic the more clinically relevant situation of targeting already activated T cells. Indeed, we observed a significant TOAG-1 mRNA expression of allo-stimulated PBMCs dose-dependently. Next, we tested whether Alefacept also influences TOAG-1 and RHAMM expression of allo-stimulated PBMCs. Alefacept was added either at the time of stimulation or 3 days later to mimic the more clinically relevant situation of targeting already activated T cells. Indeed, we observed a significant TOAG-1 mRNA expression of allo-stimulated PBMCs dose-dependently.
Alefacept reduces the frequency of activated CD137+ T cells

Next, we studied in which T cell subpopulation Alefacept induces TOAG-1 up-regulation and RHAMM down-regulation upon Ag stimulation. Because we are lacking specific anti-TOAG-1 Abs, we could not perform intracellular TOAG-1 staining in different T cell subpopulations. Therefore, we have studied TOAG-1 and RHAMM transcription in enriched T cell subpopulations after activation in vitro. We purified activated and nonactivated T cells after stimulation with alloantigen. For our experiments, we have chosen the activation marker 4-1BB (CD137) as our earlier experiments revealed no significant differences in expression of other markers such as CD69 between untreated and Alefacept treated cultures (see above and supplementary Fig. 4). Furthermore, it has been shown that CD137 expression can be used to detect and isolate Ag specific activated T cells (18–20).

As shown in Fig. 4A, allogeneic stimulation of CD8+ T cells during mixed lymphocyte cultures resulted in an increased frequency of CD137+ T cells. Alefacept treatment dramatically abolished CD137+ expression on stimulated CD8+ T cells.

Next, we studied TOAG-1 and RHAMM transcription in purified CD8+ CD137+ and CD137+ cells of unstimulated PBMCs vs untreated and Alefacept-treated mixed lymphocyte cultures. TOAG-1 transcription was high in CD137+ T cells regardless whether they were obtained from unstimulated PBMCs or untreated and Alefacept-treated mixed lymphocyte cultures (Fig. 4B). TOAG-1 transcription was reduced up to 4-fold in activated CD137+ T cells, again no significant difference of TOAG-1 mRNA expression between CD137+ T cells of untreated and Alefacept-treated cultures could be observed.

Conversely, transcription of RHAMM was increased up to 20-fold in activated CD137+ T cells compared with CD137− T cells. However, we could also detect significant differences in RHAMM transcription of CD137− T cells between untreated and Alefacept treated cultures. RHAMM transcription in CD137− T cells increased up to 20-fold upon allogeneic stimulation. This could be at least partially inhibited by Alefacept treatment. Interestingly, Alefacept treatment also decreased RHAMM transcription in CD137− T cells down-regulated TOAG-1 expression upon transition to CD137+ activated T cells. In contrast, increase in RHAMM expression occurs already at an earlier stage during T cell activation and is inhibited by Alefacept treatment at both stages of T cell activation.
The differences in RHAMM transcription of CD137\(^{-}\)T cells between untreated and Alefacept-treated cultures prompted us to calculate again a ratio of TOAG-1 and RHAMM gene expression in CD137\(^{-}\) and CD137\(^{+}\)activated T cells. As shown in Fig. 4C, we detected significant lower ratio values in CD137\(^{-}\) and CD137\(^{+}\)T cells of Alefacept treated as compared with untreated cultures.

**TOAG-1 is a mitochondrial protein**

The function of TOAG-1 is so far not known. Furthermore, the amino acid sequence of TOAG-1 reveals no similarities to any known peptides or proteins. However, a sequence analysis predicted a mitochondrial targeting signal. To understand the consequences of enhanced TOAG-1 expression we performed overexpression studies. At first, subcellular localization of TOAG-1 protein was determined by transfecting NIH-3T3 cells with either EGFP vector alone or a fusion-protein of TOAG-1 and EGFP. As shown in Fig. 5A, TOAG-1-EGFP accumulated within organelle-like structures of the cells but not within membranes or the nucleus. Staining of the cells with the mitochondrial dye Mito Tracker Red revealed a similar distribution as the fusion-protein. In the merge of green and red fluorescence, mitochondrial localization of TOAG-1 was most evident, as yellow areas are indicating colocalization. Transfection with EGFP vector control alone led to a diffuse staining of the whole cell. To further prove an exclusive localization of TOAG-1 within mitochondria, transfected cells were also stained for lysosomes. Results are shown as merge only in Fig. 5B. The lysosomal staining revealed a completely different spotty red fluorescence around the nucleus compared with the green fluorescence of TOAG-1 EGFP fusion-protein. In the merge no colocalizing areas were visible.

_Retroviral overexpression of TOAG-1 in primary murine CD4\(^{+}\) T cells led to a higher susceptibility to apoptosis and reduced \(\Delta \Psi m\)_

Mitochondria regulate T cells apoptosis (21). Because Alefacept treatment resulted in enhanced T cell apoptosis in vivo and in vitro but also in enhanced TOAG-1 transcription, we investigated whether TOAG-1 as a mitochondrial protein can influence mitochondrial function and apoptosis during T cell activation. Therefore, primary murine CD4\(^{+}\) T cells were forced to over-express TOAG-1 by retroviral transfer as described in material and methods. As shown in Fig. 6, A and B, TOAG-1 over-expressing cells after 5 days culture were characterized by an increased amount of cells with reduced \(\Delta \Psi m\) measured by TMRM staining which was associated with an increased susceptibility to apoptosis, analyzed by annexin V-PE binding, shown in Fig. 6, A and B.

_Alefacept causes loss of \(\Delta \Psi m\) and increased susceptibility to apoptosis_

Because Alefacept treatment in vivo caused loss of CD4\(^{+}\) and even more pronounced of CD8\(^{+}\) T cells in psoriasis patients, we determined whether Alefacept also influenced mitochondrial function and apoptosis induction in T cells in vitro. Therefore, mitochondrial membrane potential (\(\Delta \Psi m\)) was measured by staining the cells with TMRM. TMRM accumulation depends on the integrity of the mitochondrial membrane. Cells with low \(\Delta \Psi m\) show a weak TMRM fluorescence. Those cells are likely to undergo apoptosis (22).

Therefore human MLRs were performed using CD4\(^{+}\)CD45RA\(^{-}\), CD4\(^{+}\)CD45RO\(^{-}\), or CD8\(^{+}\) T cells as responders. As demonstrated in Fig. 7, allogeneic stimulation in the presence of Alefacept resulted in a higher percentage of cells with reduced \(\Delta \Psi m\) in comparison to untreated cultures.
all three responder populations studied. Addition of Alefacept led also to a significant increase of apoptotic cells. The influence of Alefacept was especially apparent when CD8+ T cells were used as responders. Furthermore, we analyzed the effect of Alefacept on activation of caspases 3/7 and 8 in alloantigen-stimulated CD8+ T cells. As shown in Fig. 7C, allogeneic stimulation resulted in an enhanced activation of caspases 3/7 and 8 in CD8+ T cells on day 4 after onset of MLR compared with unstimulated CD8+ T cells. Interestingly, addition of Alefacept either immediately or on day 3 of MLR further enhanced the activation of both caspases (Fig. 7C).

Alefacept enhances PDL1 expression on APCs and CD4+ T cells

To investigate whether presence of APCs is necessary for our observed effects, we stimulated naive CD4+ T cells using plate bound anti-CD3 and anti CD28 Abs in the presence or absence of Alefacept. As shown in supplementary Fig. 5A, there was no effect of either plate bound or soluble Alefacept on proliferation of human primary naive CD4+ T cells compared with untreated cells. Immobilization of soluble Alefacept by cross linking with an anti-human IgG1 Ab did also not result in reduced proliferation compared with the untreated control (supplementary Fig. 5B). Consistent with these findings Alefacept could also not influence TOAG-1 and RHAMM mRNA expression after T cell activation via CD3- and CD28 stimulation in the absence of APCs (supplementary Fig. 5B) (15).

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PD1 binding to PD1 and 2 is known to inhibit T cell activation. PD1 is highly expressed on APCs. Because Alefacept diminished T cell activation in an APC-dependent manner, we investigated whether Alefacept influences the expression of the negative co-stimulatory molecule PDL1 on the surface of APCs. Therefore, we performed MLRs with human PBMCs in the presence or absence of Alefacept. PDL1 expression on APCs and CD4+ T cells was determined using flow cytometry. As shown in Fig. 8A, Alefacept treated cultures contained significantly more PDL1+ APCs compared with untreated control cultures. Interestingly, Alefacept treatment also increased the amount of PDL1 expressing CD4+ T cells compared with untreated control cultures.
Furthermore, blocking of PDL1 by adding a neutralizing anti-human PDL1 Ab to Alefacept treated cultures reverted partially the diminished proliferation as shown in Fig. 8B.

**Discussion**

To identify new biomarkers predicting the outcome of immune modulating therapies, we performed a small study with Alefacept in 20 psoriasis patients with moderate to severe psoriasis. Weekly application of 15 mg Alefacept i.m. for at least 12 wk has been shown to result in a significant improvement of psoriasis symptoms evaluated by PASI score in not more than 20–50% of the patients dependent on the chosen clinical endpoint (13). For our study, 50% reduction of the PASI score was chosen as clinical endpoint (16). Nine of our patients showed a clinically relevant reduction (Fig. 1A). We analyzed the mRNA expression of CD3, CD69, Foxp3, PDL1, TOAG-1, and RHAMM in peripheral blood samples of our psoriasis patient’s cohort receiving Alefacept therapy.

These gene markers were selected for the following reasons: CD3 as a control to follow potential changes in T cell composition at transcriptional level, CD69 as Haider et al. (15) showed that nonresponders express higher transcription levels of CD69 in pretreatment PBMCs, Foxp3 to indirectly study changes in regulatory T cell frequencies, PD-L1 to assess differences in gene expression of negatively regulating costimulatory molecules (23–28), and TOAG-1 (Acc. No: BE115945) and RHAMM as they have been recently described to be differentially expressed in the periphery during induction and maintenance of immunological tolerance in allogeneic kidney graft recipients (29). TOAG-1 has been shown to be highly expressed during induction and maintenance of graft acceptance, whereas RHAMM transcription increased during graft rejection. The opposite transcriptional regulation of both gene markers after allogeneic transplantation already indicated their high diagnostic potential.

Changes in peripheral TOAG-1 and RHAMM transcription were significantly different between responding and nonresponding patients, especially between week 2 and 4 of treatment (Fig. 2A). Whereas TOAG-1 was significantly up-regulated in responders, nonresponding patients showed a significant up-regulation of RHAMM on mRNA level. The difference in RHAMM mRNA expression was detectable at any time point studied. The quality concerning the discrimination throughout therapy was even enhanced by calculating a ratio of TOAG-1 and RHAMM expressions. Both markers have not yet been described of playing a major role during chronic T cell activation in psoriasis.

RHAMM as receptor for hyaluronan (HA)-mediated motility is required for the migration of receptor expression cells (30). HA is involved in the process of wound healing, keratinocyte proliferation (31, 32), and angiogenesis (33). In a recently performed study, elevated RHAMM expression correlated with inflammation after bleomycin injury (33). Moreover, it has been shown that RHAMM compensates for CD44 in CD44<sup>H11002</sup>/H11002<sup>H11002</sup> mice in the collagen-induced arthritis model when the response to collagen was even more aggressive (34). Patients with active psoriasis show higher HA levels in blister fluids than patients with inactive psoriasis or healthy people (35). A study by Elkayam et al. (36) revealed that serum...
HA levels correlate with skin involvement in patients with psoriatic arthritis. We detected no significant differences in Foxp3 expression between responders and nonresponders. A recent study compared Foxp3 expression in CD4+CD25+ cells between samples of healthy people and psoriatic patients (37). They detected comparable high levels of Foxp3 in CD4+CD25high cells in normal and psoriatic blood. However, they showed that psoriatic Tregs are functionally deficient in suppressing effector T cell responses in both alloantigen-specific and polyclonal TCR stimulation assays. In the study by Haider et al. (15) Foxp3 was up-regulated in responders compared with nonresponders within the first 6 h of Alefacept treatment. However, at week 2 and further, both groups could no longer be distinguished. This is in accordance with our results. Furthermore, they detected a significant CD69 up-regulation at baseline in PBMCs from nonresponders compared with responder PBMCs (15). In our patient cohort levels in CD69 transcription were not significantly different at baseline between responder and nonresponder and we actually detected an increment of CD69 mRNA expression in all patients at week 2 and 3 of therapy compared with baseline level. At this point of therapy TOAG-1 and RHAMM mRNA expression distinguished responding from nonresponding patients. The differences Haider et al. (15) detected are restricted to the first hours after therapy onset but dilute within days or weeks. Therefore, there is only a short time window allowing discrimination between responders and nonresponders. However, our calculated ratio of TOAG-1 and RHAMM transcription levels could distinguish responding patients from nonresponding patients throughout therapy. Haider et al. (15) also found that CD8+ T cells are decreased in numbers rather rapidly after Alefacept administration to psoriasis patients. We obtained similar results in our study. Alefacept reduced all T cells in responders and nonresponders but CD8+ T cell counts decreased to a higher extent than CD4+ T cells (Fig. 1B), compared with baseline levels. In contrast to CD4+ T cell counts, CD8+ T cells were decreased more dramatically in responding than in nonresponding patients. However, this was not significant. This might be also due to the small number of patients included. CD2high expressing CD4+ and CD8+ T cells were significantly decreased in all patients, even though CD8+ T cells were more affected. This is likely, but not only due to the fact that FACS Abs used to stain CD2+ cells, compete dose dependently with Alefacept for CD2 binding (data not shown).

TOAG-1 was previously shown to be highly expressed in the graft and in whole blood of tolerance developing recipients after allogeneic kidney transplantation (29). In this study, we could show that TOAG-1 is localized within mitochondria (Fig. 5A). Both, mitochondrial localization and changes in mRNA expression levels of TOAG-1 during T cell activation suggests a possible role in regulation of T cell activation or apoptosis (38–46). Indeed, TOAG-1 over-expressing primary murine CD4+ T cells were characterized by lower ΔΨm (Fig. 6A) compared with control-transduced cells. ΔΨm plays decisive roles in driving ATP synthesis and regulating reactive oxygen species, which might lead to cell apoptosis (44, 47, 48). Because T cell activation is dependent on ATP, TOAG-1 might have a suppressive effect on this process (49). In contrast, loss of ΔΨm is known to be one of the earliest indices for an upcoming apoptosis (43). Indeed, TOAG-1 transduced cells showed a higher susceptibility to undergo apoptosis compared with GFP virus transduced cells as measured by annexin V (Fig. 6B) and 7-AAD staining (data not shown). Thus, TOAG-1 could influence T cell activation and apoptosis by diminishing the mitochondrial membrane potential (ΔΨm).

As TOAG-1 over-expression enhances T cell apoptosis, cells expressing high levels of TOAG-1 should be predominately depleted in vivo during Alefacept treatment. Indeed, when following the kinetics of TOAG-1 transcription in vivo, we detected only a transient increase of TOAG-1 gene expression in responders (Fig. 2) until week 4 which was followed by TOAG-1 down-regulation upon week 5. Thus, a successful Alefacept treatment might result in an increased frequency of cells expressing high levels of TOAG-1, which are subsequently deleted.

We also investigated the impact of Alefacept in vitro. Cells treated with Alefacept showed a significant reduction of proliferation in a dose-dependent manner on day 4 of MLR (Fig. 3A). In our in vitro experiments Alefacept was added at two different time points: at the beginning of T cell activation and 3 days later to mimic the more clinically relevant situation targeting preactivated T cells (Fig. 3B). We could show that TOAG-1 is significantly up- and RHAMM significantly down-regulated when adding Alefacept at the day of T cell activation. Delayed addition of Alefacept resulted in the same trend but was not significant. Furthermore, we performed the same experiments with different T cell subsets (Fig. 3, C and D). Alefacept had comparable effects on TOAG-1 and RHAMM mRNA expression levels of memory or naive CD4+ as well as CD8+ T cells. This is a very interesting result, because Alefacept is so far postulated to be more effective in memory T cells due to high expression of CD2 of the surface (9). Although
Alefacept decreased CD69 transcription in memory or naive CD4+ as well as in CD8+ T cells this was not significant.

Having detected differences in TOAG-1 and RHAMM mRNA expression between untreated and Alefacept treated mixed lymphocyte reactions, we investigated whether these changes occur in an activated T cell subpopulation upon stimulation with Ag in vitro. Allogeneic stimulation resulted in an enhanced frequency of CD137+ T cells in comparison to unstimulated control cells (Fig. 4, A and B). Whereas Alefacept-treated cultures were characterized by significantly reduced frequency of CD137+ T cells upon stimulation with alloantigen in vitro.

Interestingly, mRNA expression analysis in CD137+ and CD137− T cells revealed highly opposite patterns for TOAG-1 and RHAMM expression after cell activation. The expression of TOAG-1 is down-regulated in allogeneic stimulated CD137+ T cells but not CD137− T cells. In contrast, RHAMM is up-regulated preferentially in activated CD137+ T cells, although we also observed an increase in RHAMM expression in CD137− cells upon allogeneic stimulation. Alefacept could significantly block RHAMM induction in CD137− and CD137+ T cells.

Therefore, our results indicate that the differences in TOAG-1 and RHAMM gene expression between untreated and Alefacept treated mixed lymphocyte cultures are not only a result of reduced conversion into highly activated CD137+ T cells but also a result of an earlier inhibition of T cell activation. Thus, a ratio of TOAG-1 and RHAMM expression might reflect the balance between less and highly activated T cells and therefore be appropriate for the effectiveness of Alefacept treatment in vitro. Alefacept can inhibit T cell activation and enhance T cell apoptosis by up-regulating TOAG-1 and PDL1 expression, providing further insights into the immunosuppressive mechanisms of Alefacept. Furthermore, analysis of changes in TOAG-1 and RHAMM expression in peripheral blood allowed the discrimination between responders and nonresponders early after onset of Alefacept treatment in psoriasis patients and before clinical response parameters.

Further to our previous findings in transplantation models and patients, these data suggest that monitoring of TOAG1 and RHAMM gene expression in peripheral blood may be useful for the early prediction of the effectiveness of immunomodulatory therapies with biologics.

Our data indicate that biologics such as Alefacept (LFA3-Ig, psoriasis) or nondepleting anti-CD4 Abs (transplantation) inhibit/modulate T cell activation and thereby lead to a modified expression of key molecules that regulate T cell activation.

Acknowledgments
We are grateful to Biogen-IDEC for their permission to perform the study and for providing Alefacept.

Disclosures
The authors have no financial conflict of interest.

References
Supplementary material legends:

Material 1:

**CD69 mRNA expression during Alefacept therapy in vivo.**

CD69 mRNA expression in PBMC’s of all treated patients prior to therapy and at week 2 and 3 during Alefacept treatment. In order to be able to compare individual changes, the initial mRNA expression level of each gene and patient was also set 100 % and the following values were referred to that initial score. Displayed here are mean mRNA expression levels normalized to HPRT + SD in 9 responders (grey line) and 11 nonresponders (black line).

Material 2:

**PDL1 mRNA expression during Alefacept therapy in vivo.**

PDL1 mRNA expression in PBMC’s of all treated patients prior to therapy and at week 2 and 3 during Alefacept treatment. In order to be able to compare individual changes, the initial mRNA expression level of each gene and patient was also set 100 % and the following values were referred to that initial score. Displayed here are mean mRNA expression levels normalized to HPRT + SD in 9 responders (grey line) and 11 nonresponders (black line).

Material 3:

**Predictive power of TOAG-1, RHAMM and the ratio of TOAG-1/RHAMM expression during Alefacept therapy.**

Predictive power of TOAG-1, RHAMM and the ratio TOAG-1/RHAMM expression during Alefacept therapy. Receiver operating characteristic curves (ROC curves) were created with SPSS 12.0 for each gene and for the ratio.

Material 4:

**Influence of Alefacept on CD69 mRNA expression in vitro.**

CD69 mRNA expression on day 4 of MLR with either CD4+ naïve (white bars) or memory (grey bars) T cells or CD8+ T cells (black bars) normalized to HPRT expression and referred to 100 % expression level of untreated cells (dotted black line). Isolation of T cell subset was done prior to allogeneic stimulation via MACS. MLR’s were either treated with alefacept on day 0 or 3 after allogeneic stimulation; n=4

Material 5:

**Crosslinking to APC’s is essential for effectiveness of alefacept in vitro**

A) Proliferation measured by 3H thymidine incorporation assay on day 2 after T cell activation of CD4+CD45RA+ T cells with anti-CD3/CD28 antibodies in the presence (soluble
or plate bound) or absence of alefacept. Isolation of naïve CD4⁺ T cells was done prior to allogeneic stimulation via MACS. Results are shown as mean ± SD. n=3

B) Proliferation measured by 3H thymidine incorporation assay on day 2 after T cell activation of cells either treated with soluble alefacept cross-linked via anti-human-IgG1 antibody or without alefacept but with anti-human-IgG1 antibody. Isolation of naïve CD4⁺ T cells was done prior to allogeneic stimulation via MACS. Results are shown as mean ± SD. n=3

C) TOAG-1 and Rhamm mRNA expression level on day 2 after T cell activation via anti human CD3 and anti human CD28 antibodies (10µg/ml each) in the absence of allogeneic APCs in the presence or absence of alefacept. Results are shown as mean ± SD; n=3

Material 6:
Oligonucleotides used to quantify mRNA expression by quantitative Real Time PCR.

Material 7:
Demographics, PASI score at the beginning and maximal PASI reduction of all 20 participating patients, ratio in week 3 of alefacept treatment
Supplementary Figure 1

CD69 mRNA expression [% of week 0]

- Week 2
- Week 3

CD69 mRNA expression (units /HPRT)

- Non-responder
- Responder

Time of treatment

Week 0, Week 2, Week 3
Supplementary Figure 2

A graph showing PDL-1 mRNA expression levels over time for non-responders and responders. The y-axis represents PDL-1 mRNA expression units relative to HPRT, with three time points: week 0, week 2, and week 3. The graph indicates a decrease in expression levels over time for both groups, with responders showing a slightly lower expression compared to non-responders.
Predictive power of TOAG-1, RHAMM and the ratio TOAG-1/RHAMM
Receiver operating characteristic curves (ROC curves) were created with SPSS 12.0 for each gene and for the ratio

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Supplementary Figure 4

CD69 mRNA expression units / HPRT

leukocyte subpopulations

- CD4^+ CD45RA^+
- CD4^+ CD45RO^+
- CD8^+

no
Alefacept
## Supplementary table 1

Oligonucleotides used to quantify mRNA expression by quantitative Real Time PCR.

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Supplementary table 2

Demographics, PASI score at the beginning and maximal PASI reduction of all 20 participating patients, ratio in week 3 of alefacept treatment

* = Patient discontinued treatment at week 5.

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