Learned immunosuppressive placebo responses in renal transplant patients

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Patients after organ transplantation or with chronic, inflammatory autoimmune diseases require lifelong treatment with immunosuppressive drugs, which have toxic adverse effects. Recent insight into the neurobiology of placebo responses shows that associative conditioning procedures can be employed as placebo-induced dose reduction strategies in an immunopharmacological regimen. However, it is unclear whether learned immune responses can be produced in patient populations already receiving an immunosuppressive regimen. Thus, 30 renal transplant patients underwent a taste-immune conditioning paradigm, in which immunosuppressive drugs (unconditioned stimulus) were paired with a gustatory stimulus [conditioned stimulus (CS)] during the learning phase. During evocation phase, after patients were reexposed to the CS, T cell proliferative capacity was significantly reduced in comparison with the baseline kinetics of T cell functions under routine drug intake (η_p^2 = 0.34). These data demonstrate, proof-of-concept, that learned immunosuppressive placebo responses can be used as a supportive, placebo-based, dose-reduction strategy to improve treatment efficacy in an ongoing immunopharmacological regimen.

conditioning | placebo | immunosuppression | T cells | transplantation

Our understanding of the mechanisms mediating placebo responses to medicines has grown substantially during the last 2 decades (1). A key strategic question is how to maximize placebo responses in clinical practice, with the goal of improving treatment outcomes and patient benefit (2). Placebo responses are generally defined as positive treatment outcomes that are caused by nonspecific treatment effects, such as the quality of patient–doctor communication, patients' expectations toward the benefit of a treatment, or associative learning procedures such as conditioning (3–5). Classical conditioning has been shown to not only mediate placebo-analgesia (6–8) but also modulate autonomous responses such as neuroendocrine variables and immunological functions (9, 10). The experimental evidence in rodents and humans demonstrating behavioral conditioned immunopharmacological effects suggests that associative learning procedures may be an effective and ethical unproblematic supportive strategy for pharmacological regimens in clinical situations. The aim would be to use the learned immunopharmacological response to substitute for a portion of the pharmacological agent, thereby reducing drug-induced unwanted adverse effects for the patient benefit (11–17).

Based on studies in experimental animals, we previously established a taste-immune conditioning paradigm in healthy human subjects (9), in which the calcineurin-inhibitor cyclosporine A (CsA) as an unconditioned stimulus is paired with a novel-tasting drink, the conditioned stimulus (CS). After several unconditioned stimulus–CS pairings during the acquisition (training) phase, the CS (drink) is presented together with placebo pills during the evocation. Reexposure to the CS induced a learned immunosuppressive effect, reflected by reduced cytokine (interleukin-2, IFN-γ) production and cytokine mRNA expression in peripheral blood lymphocytes (18). This conditioned immunosuppressive effect can be reproduced (19), and extinction of the learned immunosuppression can be inhibited, by combining reexposures to the CS with low- or subtherapeutic doses of the drug (12). These phase 1, proof-of-concept studies in healthy subjects demonstrate that the intense, bidirectional communication between the brain and the peripheral immune system can be exploited to induce learned placebo responses in immune functions (9, 20).

The intake of immunosuppressive drugs has serious adverse effects such as increased cardiovascular morbidity, nephrotoxicity, neurotoxicity, and hypertension (21–24). Reducing medication dosage without diminishing medication efficacy could improve clinical care for postorgan transplantation and chronic inflammatory autoimmune diseases. In clinical routine, the majority of these patients will already be receiving immunosuppressive treatment before possibly participating in an immunosuppressive learning paradigm as a supportive therapy. It is not clear, however, whether such patients could safely benefit form dose reduction through placebo-conditioning paradigms.

Thus, in this study, we combined a taste-immune conditioning paradigm in renal transplanted patients, who were already receiving an immunosuppressive drug regimen with calcineurin-inhibitors (CsA or tacrolimus). After analyzing baseline kinetic of immune functions and neuroendocrine parameters under

Significance

Akin to other physiological responses, immune functions can be modified in humans through associative conditioning procedures as part of a learned placebo response. However, it is unclear whether learned immune responses can be produced in patient populations already receiving an immunosuppressive regimen. In the present study, we demonstrate in renal transplant patients who were already receiving immunosuppressive treatment that learned immunosuppressive placebo responses increased efficacy of immunosuppressive medication. These data demonstrate that behavioral conditioning of drug responses may be a promising tool that could be used as a placebo-based dose-reduction strategy in an ongoing immunopharmacological regimen, the aim being to limit unwanted drug adverse effects and to improve treatment efficacy.

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Table 1. Sociodemographic/clinical characteristics of patients

Patients characteristics	Men	Women
Patients, *n*	24	6
Age, y	56.2 ± 12.6	52.8 ± 7.1
Body mass index	26.4 ± 3.8	30.1 ± 6.1
Calcineurin inhibitor, CsA/Tac	7/17	1/5
Time since transplantation, mo	79.7 ± 78.0	67.2 ± 40.8
Multiple transplantations, *n*	2	0

Data are shown as mean ± SD.

routine drug intake, patients combined their medication with a novel gustatory stimulus serving as CS twice a day for 3 consecutive days (acquisition). During evocation days, in addition to their morning and evening drug intake, patients received placebo pills at two additional points during the day, together with the gustatory CS. Blood samples were drawn and immunological and neuroendocrine parameters analyzed in comparison with the baseline condition of the pharmacological treatment only.

Results

Patients. Ninety-four renal transplanted patients were screened, of whom 30 met the entry criteria and were willing to participate in the study. Patients were completely informed about the scope and procedure of the study (see *Patient Information*) and gave written informed consent. The demographic characteristics are shown in Table 1.

Immunological and Neuroendocrine Outcome. The proliferation of $CD4^+$ T cells during the baseline measures without any drug-cue association significantly increased 6 and 10 h after the morning intake of the immunosuppressive medication. Exposure to the gustatory stimuli during the second evocation day induced a significant, conditioned reduction in the proliferative capacity of T cells (ANOVA, $F_{2,\ 56} = 14.36$; $P < 0.001$; $\eta_p^2 = 0.34$) 6 and 10 h after the morning drug intake ($t = 4.61$, $P < 0.001$/$t = 5.27$, $P < 0.001$, respectively), indicating that the learned immunosuppressive placebo response increased drug efficacy without changes in drug dosage (Fig. 1*A* and Tables S6 and S7). In parallel, there was a significant interaction effect in the γ-IFN mRNA expression in T cells (ANOVA, $F_{1.24,\ 34.77} = 3.83$; $P = 0.05$; $\eta_p^2 = 0.12$), reflected by a trend in conditioned inhibition of γ-IFN mRNA expression 10 h after the morning medication intake ($t = 1.88$; $P = 0.07$; Fig. 1*C*). The conditioned inhibition in T cell activity was not associated with alterations in IL-2 mRNA expression (ANOVA, $F_{1.60,\ 46.33} = 1.41$; n.s.; Fig. 1*B*) or with the circulation of T cell numbers, as cell counts of $CD3^+/CD4^+$ and $CD3^+/CD4^-$ did not differ between baseline and second evocation day (Tables S3, S7, and S8). In addition, the reduced T cell proliferation cannot be attributed to changes in the levels of immunosuppressive drugs, as cyclosporine A as well as tacrolimus levels did not significantly differ between conditions (Table S4).

Analyses of catecholamine plasma levels did not show significant differences between the baseline and second evocation conditions, neither for noradrenaline nor for adrenaline (Fig. 2). Cortisol plasma level showed a significant circadian decline and a significant interaction (ANOVA, $F_{2,\ 58} = 4.94$; $P < 0.01$; $\eta_p^2 = 0.15$), with a trend toward slightly lower cortisol levels 6 h after the morning drug intake during the second evocation day ($t = 1.95$; $P = 0.06$).

Additional analyses did not show significant differences between male and female patients, neither in the immunological nor in the endocrine parameters analyzed here.

Behavioral and Cardiovascular Measures. Patients trait anxiety and depression characteristic, analyzed with the Hospital Anxiety and Depression Scale and the State-Trait-Anxiety-Depression Inventory, were within the normal range. When patients were asked to rate the quality of the taste of the drink employed as the CS, there were no significant differences between the acquisition and evocation phases. In addition, heart rate and blood pressure, analyzed in parallel with the blood samples 2, 6, and 10 h during baseline and second evocation, did not differ between conditions (Table S5).

Discussion

After organ transplantation or chronic inflammatory autoimmune diseases, patients depend on immunosuppressive medication for the rest of their lives, which can cause severe adverse effects. In this study with kidney transplant patients, long-term treated with immunosuppressive drugs cyclosporine A or tacrolimus, the introduction of a taste-immune conditioning procedure induced a significant learned inhibition of T cell proliferative capacity. The conditioning paradigm increased the effectiveness of the medications without any increase in medication dose. These data provide a proof of concept that learned placebo responses in immune functions can be employed as additional therapy during immunosuppressive treatment to maximize treatment efficacy.

The phenomenon of learned immune responses is based on two phenomena: On the one hand, it depends on the intense bidirectional communication between the CNS and the immune system (20, 25), and on the other hand, it relies on the ability of an organism to "learn" a physiological reflex via associative learning processes (classical or behavioral conditioning) (26, 27). Experimental evidence in rodents and healthy human subjects has demonstrated behaviorally conditioned immunosuppressive effects, both in humoral and cellular immune functions (4, 28). Employing the calcineurin-inhibitor CsA as an unconditioned stimulus in a taste-immune learning paradigm, we explored this phenomenon of learned immunosuppression as part of a conditioning-induced placebo response in healthy male subjects. These studies demonstrate that the learned immune response, reflected by inhibition of cytokine production, can be reproduced during a second, unreinforced reexposure to the CS (19); that the learned

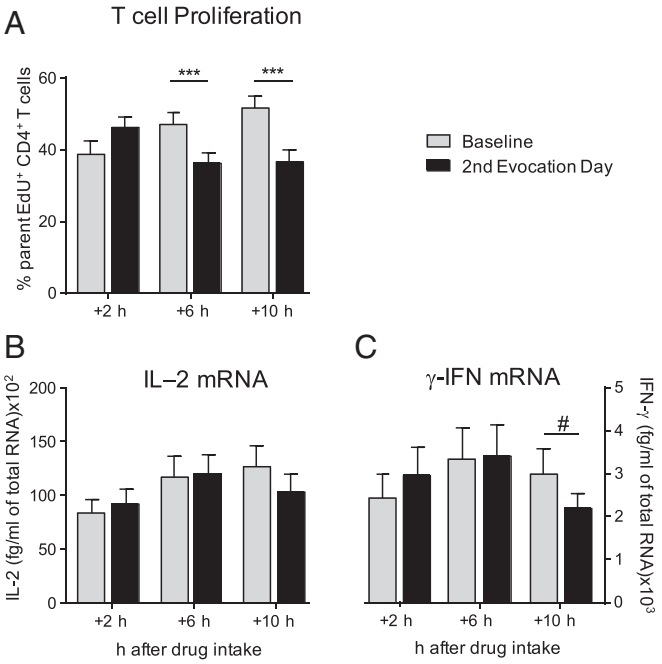

Fig. 1. Proliferation of $CD4^+$ T cells (*A*), IL-2 (*B*), and γ-IFN mRNA (*C*) expression in $CD3^+$ cells 2, 6, and 10 h after morning drug intake at baseline and during the second evocation day (data are shown as means ± STE). ***$P < 0.001$; #$P = 0.07$.

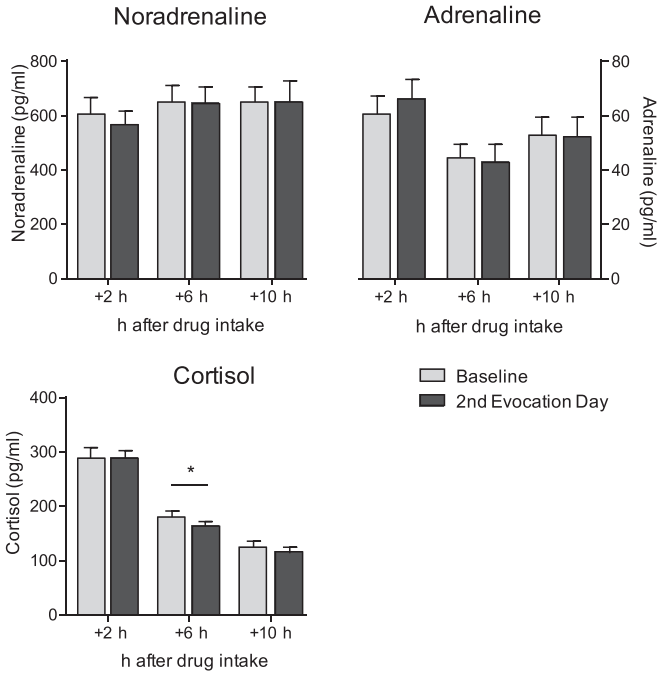

Fig. 2. Noradrenaline, adrenaline, and cortisol plasma levels (picograms per milliliter) 2, 6, and 10 h after morning drug intake at baseline and during the second evocation day (data are shown as means ± STE). *$P = 0.06$.

immune response can be induced by associative learning solely, and not via patient expectations (8, 29); and that extinction of the learned immunosuppressive response can be inhibited when subtherapeutic dosages of the immunosuppressant drug are applied together with the CS during prolonged evocation (12, 15).

Previous experimental data in rodents and healthy subjects supported the notion that classical conditioning of immunosuppressive drug responses is a promising tool that could be used to improve treatment outcomes. For example, evidence in rodents indicated that pretreatment with immunosuppressive drugs seems not to affect conditioned immune functions (30). However, the question arose of whether and to what extent patients already receiving long-term immunosuppressive therapy with calcineurin inhibitors such as CsA of tacrolimus (Tac) could still develop a learned immunosuppression. The data provided here demonstrate that implementing a taste-immune learning protocol into an unchanged drug regimen in long-term immunosuppressed renal transplant patients resulted in a learned immunosuppression reflected by an inhibition of T cell activity.

The potential clinical applicability of learned immunosuppressive responses has been convincingly demonstrated in rodents, where conditioned immune responses significantly reduced mortality in animals with inflammatory autoimmune disease and significantly reduced allergic responses or prolonged the survival time of transplanted vascularized organs (26, 31–34). These learned immune responses in rodents, as well as in healthy human subjects, were induced in the absence of any drug application or significantly reduced drug dosages administered during the evocation trials (4). These data suggest that learned immune responses may have a role as supportive therapy into an immunosuppressive regimen to maximize treatment efficacy and significantly decrease drug dosages, with the aim of reducing unwanted drug adverse effects (1). Furthermore, the toxic adverse effects of immunosuppressive substances such as calcineurin inhibitors (cardiovascular morbidity, nephrotoxicity, neurotoxicity, and hypertension) are predominantly caused by the pharmacological substance itself (21, 24). The behavioral conditioned immunosuppression exploits the brain–immune communication with significantly reduced drug dosages. We do not have yet direct evidence for potential reduction of long-term toxic drug adverse effects. However, we do know that the learned immunosuppression with CsA is diminishing the calcineurin activity, and that this effect seemed to be mediated via adrenoreceptors and distinct intracellular mechanisms compared with the drug effect (35, 36).

The precise mechanisms steering these learned placebo responses in immune functions in immunosuppressed patients are unclear. The learned immunosuppressive changes were neither associated with significant changes in cytokine mRNA expression, T lymphocyte numbers, plasma levels of cortisol, or catecholamines, nor associated with changes in cardiovascular or behavioral measures. In rodents, the conditioned immunosuppressive effects using CsA as an unconditioned stimulus are centrally mediated in the CNS via the insular cortex and the amygdala (37). In the periphery, the learned inhibition of calcineurin activity in lymphocytes seemed to be mainly mediated via noradrenergic innervation of lymphatic organs such as the spleen and β-adrenoreceptor-dependent mechanisms (9, 35, 36, 38). In addition, noradrenaline plasma levels were significantly correlated with the learned suppression of interleukin-2 levels in healthy volunteers (39). However, we could not identify any correlation between the learned T cell responses and sympathetic adrenal activity in the patient data presented here. In addition, patients receiving β-adrenoreceptor antagonists ($n = 20$) did not significantly differ in their learned T cell response from patients taking no β-blockers ($n = 10$). Further studies have to analyze the mechanisms mediating the learned immune responses in immunosuppressed patients.

The present study demonstrates the proof of principle that learned immunosuppressive placebo responses can be introduced into an ongoing immunosuppressive pharmacological regimen in renal transplant patients. There are a number of limitations, however, regarding possible clinical applicability of such learning strategies in clinical routine. The results of this study need to be replicated with a larger number of transplant patients over a longer period of time. Future studies have to examine whether these results also apply to patients with different requirements of long-term immunosuppressive therapy, such as chronic, inflammatory autoimmune diseases. In this context, it needs to be determined whether and to what extent the taste cues are essential in this learning paradigm to induce learned immunosuppressive effects. In healthy humans, extinction of the learned immunosuppressive response could be prevented by administering low therapeutic dosages of the drug together with the CS, inducing a reconsolidation process in the immune response (12, 15). In addition, inhibition of T cell proliferation or cytokine mRNA expression in lymphocyte are indicators of an immunosuppressive response; whether and to what extent this is also a corresponding marker for disease activity or the rejection process of transplanted organs has to be determined.

In general, analyzing long-term drug intake from the perspective of associative learning processes, behavioral conditioning of drug responses may be a promising tool that could be used as a placebo-based dose reduction strategy to limit unwanted drug adverse effects and to improve treatment efficacy (11, 14, 16, 17). For immunosuppressive regimens in particular, there is a need to understand which properties of which immunopharmacological agents can be behaviorally conditioned and which reinforcement schedules should be used to achieve optimal effects and prevent habituation or extinction of the learned immunopharmacological responses.

In summary, the clinical exploitation of learned placebo responses in immune functions is still in a very early stage. The data presented here provide experimental evidence that learned immunopharmacological responses can be implemented into ongoing immunosuppressive therapy in renal transplant patients.

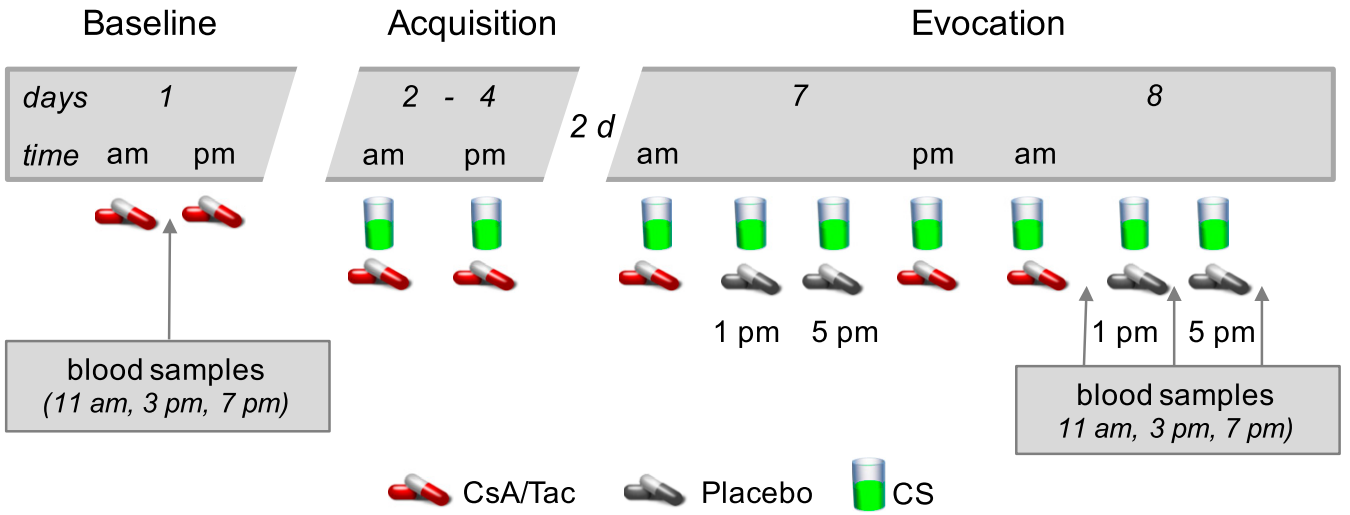

Fig. 3. During baseline measures (day 1; without any drug-cue association), blood samples were taken at three different times: 2, 6, and 10 h after morning (9 AM) drug intake. During the acquisition days 2–4, the immunosuppressive drug intake (CsA or Tac) at 9 AM and 9 PM was combined with the CS (green-colored, new-tasting drink). During evocation days (study days 7 and 8), the morning and evening drug intake was again combined with the CS. In addition, patients received placebo pills 4 h (at 1 PM), as well as 8 h (at 5 PM), together with the CS. At the second evocation day, blood samples were taken at three times: 2, 6, and 10 h after morning drug intake (9 AM).

Methods

Patients. Thirty renal transplanted patients (24 male, 6 female) with stable kidney function for at least 6 mo participated in the study (sociodemographic variables are listed in Table 1). Indication for transplantation was diverse, and time after transplantation was 77.2 (±71.6) months (Table S1). All patients received calcineurin-inhibitors as immunosuppressive regimen; n = 24 patients were treated with Tac (Prograf; Axicorp Pharma BV), n = 6 patients received CsA (Sandimmun optoral; Novartis Pharma GmbH). All patients took their immunosuppressive medication twice a day in a 12-h cycle (9 AM/9 PM). Patients with acute or chronic infections, increased C-reactive protein plasma concentration, rejection episodes within the last 6 mo, mental illness, tumor diseases, or allergies were excluded from the study. Patients received financial compensation for participating in the study. The study was approved by the Ethics Committee of the University Hospital Essen (13-5572-BO) and registered in the German Clinical Trial Register (DRKS00007693).

Study Design. Patients were recruited from the Outpatient Nephrology Clinic of the University Hospital Essen. All patients gave written informed consent and were provided full transparency about the background and procedure of the study. The conditioning paradigm was fully explained, and patients understood that the placebos that accompanied the CS drink during evocation were inactive/inert. On day 1 (baseline), patients were invited to the clinic, and blood was drawn at 2, 6 and 10 h after drug intake (9 AM) to analyze immunological and neuroendocrine parameters (Fig. 3). Days 2, 3, and 4 constituted acquisition, during which patients were asked to combine their immunosuppressive drug intake (CsA or Tac) at 9 AM and 9 PM, together with 50 mL of a green-colored novel gustatory stimulus consisting of colored strawberry milk, lavender oil, and green food coloring, employed as the CS in this taste-immune conditioning paradigm. After a 2-d break, during which the drug intake was not combined with the CS (drink), the evocation phase was performed on study days 7 and 8, where morning and evening drug intake was again combined with the CS (drink). In addition, patients received placebo pills 4 h (at 1 PM) as well as 8 h (at 5 PM) together with the CS to increase the salience of the CS as a medical intervention and to closely mimic the situation of taking the CS always together with a medication. The second evocation day (study day 8) was performed in the clinic after the identical procedure as the previous evocation day (drug intake + CS at 9 AM; placebo pill intake + CS at 1 AM and 5 PM). In addition, blood samples were taken at three points, 2, 6, and 10 h after morning drug intake (9 AM), to compare the neuroendocrine and immunological parameters with the baseline measure. Importantly, all patients did not change their medication schedules during the 8 d of the study: neither the immunosuppressive medication nor the accompanying medication.

CsA and Tac Blood Concentration. CsA and Tac blood concentrations in the whole blood were analyzed by the Central Laboratory of the University Hospital Essen. To determine the CsA level, an affinity chromatography-mediated immunoassay was used according to manufacturer's instructions. Levels were analyzed with the DIMENSION Xpand (Siemens Health Care GmbH). Tac levels were analyzed using a one-step immunoassay according to manufacturer's instructions. Afterward, the levels were analyzed via chemiluminescence with the Architect i1000SR (Abbott).

Cell Isolation. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare) and adjusted to 2.5×10^6 cells/mL in cell culture medium (RPMI 1640 supplemented with GlutaMAX-I, 25 mM Hepes, 10% FBS, 50 μg/mL gentamicin; Life Technologies).

T Cell Proliferation. To assess $CD4^+$ T cell proliferation, 2.5×10^5 PBMCs were stimulated with 2.5 μg/mL soluble anti-human CD3 antibody (clone: HIT3a; BD Pharmingen) in a 96-well flat-bottom tissue culture plate. After incubation (24 h, 37 °C, 5% CO_2), the Click-iT-EdU cell proliferation assay (Molecular Probes; Thermo Fisher Scientific) was used as previously described (40, 41). To identify $CD4^+$ T cells, cells were stained with APC-conjugated anti-human CD4 antibody (clone RPA-T4; BD Pharmingen) and anti-human CD3 monoclonal antibody (clone: HIT3a; BD Pharmingen). The percentage of proliferating $CD4^+$ T cells was determined on a FACS Canto II flow cytometer using FACS Diva software (BD Biosciences). T cell proliferation data observed in each patient in each condition are included in Table S6. FACS plots of one representative patient displaying the number of proliferating $CD4^+$ T cells at the different points (2, 6, and 10 h) during baseline and during the second evocation are shown in Fig. S1.

IFN-γ and IL-2 mRNA Expression. Next, 5×10^6 PBMCs were stimulated with 40 ng/mL soluble anti-human CD3 antibody (clone: HIT3a; BD Pharmingen) for 4 h (37 °C, 5% CO_2). After incubation, cells were lysed and total RNA was extracted using the RNeasy Micro Kit (QIAGEN) according to manufacturer's recommendations. Single-stranded cDNA was synthesized, using the High Capacity Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems), using Takyon Low Rox Probe MasterMix (Eurogentec) and the following cycling conditions: 5 min at 95 °C, followed by 40 cycles of 3 s at 95 °C, 20 s at 60 °C, and 26 s at 72 °C. Primers and probes for IL-2 (forward: 5′-CCAGGATGCTCACATTTAAGTTTTAC-3′; reverse: 5′-GAGGTTTGAGTTCTTCTTC TAGACACTGA-3′; probe: 5′-6-FAM-TGCCCAAGAAGGCCACAGAACTGAA-BHQ1-3′) and IFN-γ (forward: 5′-TCAGCTCTGCATCGTTTTGG-3′, reverse 5′-GTTCCAT TATCCGCTACATCTGAA-3′; probe: 5′-6-FAM-TTGGCTGTTACTGCCAGGACCCA TATGT-BHQ1-3′) were designed using Primer Express 3.0 software (Applied Biosystems) and were purchased from Microsynth. For quantification of

IL-2 and IFN-γ mRNA expression, serially diluted cDNA samples generated from purified specific PCR products (High Pure PCR Product Purification Kit; Roche Diagnostics) were used as external standards in each run. Results are expressed as fg/μg total RNA. Cytokine mRNA expression observed in each patient in each condition is included in Table S6.

T Cell Subpopulations. PBMC suspensions (2.5×10^6 cells/mL) were incubated with the following fluorochrome-conjugated monoclonal antibodies for 45 min: anti-human CD3 PE-Cy7 (clone SK7; BD Pharmingen) and anti-human CD4 AlexaFluor 405 (clone RPA-T4; AbD Serotec). Total T cells were identified by CD3 staining and T helper cells by CD3/CD4 double-staining on a FACS Canto II flow cytometer using FACS Diva software (BD Immunocytometry Systems). Total cell counts were obtained with an automated cell counter (XP-300; Sysmex Deutschland GmbH).

Neuroendocrine Parameters. Plasma cortisol concentrations were determined using a commercial ELISA (Cortisol ELISA; IBL International). Intra- and interassay variances were 5.6% and 6.9%, respectively. Plasma noradrenaline and adrenaline levels were determined by HPLCy with electrochemical detection (ChromSystems Instruments and Chemicals, Gräfelfing, Germany), as previously described (42).

Behavioral Measures. Sociodemographic data were collected from all participants, together with anxiety and depression scores, using the Hospital Anxiety and Depression Scale (43). The State-Trait-Anxiety-Depression Inventory (44) was assessed to document possible differences in trait and present state negative emotions during baseline and evocation. In addition, participants were asked to report the subjective taste quality of the drink (CS), using a visual analog scale (0, "like very much"; 100, "dislike very much").

Statistical Analysis. The Kolmogorov–Smirnov test was used to determine whether the data met the assumption of normality. The proliferation data were normally distributed. All other data were nonnormally distributed, and logarithmic transformations were applied before data analysis. Immunological, endocrine, cardiovascular, and behavioral parameters were compared with univariate or repeated-measures ANOVA, followed by post hoc Bonferroni tests. If not otherwise reported, ANOVA interaction effects (condition × time) are shown. Data analysis was performed using SPSS software (SPSS 22.0; SPSS Inc.). Statistical power was calculated using G-power software (version 3.1.9.2, www.gpower.hhu.de/) (45). For ANOVA time and interaction effects, analysis revealed sufficient power ($1 - \beta > 0.84$) for medium-sized effects ($f = 0.25$), and excellent power for large effects ($f = 0.40$; $n = 30$ patients; $\alpha = 0.05$). All data are expressed as mean ± standard error (STE). The significance level was set at $P < 0.05$.

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