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Pavlovian conditioning of immune function: animal investigation and the challenge of human application

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Abstract

Pavlovian conditioning of immune functions provided early impetus to the rapidly expanding knowledge of bi-directional communication among the immune, endocrine, and central nervous systems. Since these early investigations, the phenomenology of this response has been well characterized. However the neural mechanisms and biological relevance of conditioned immunomodulation remain unclear. To this end, we present here data from our laboratories that have: (1) revealed some of the neural mechanisms and biological relevance of an animal model of conditioned immunomodulation; (2) demonstrated the conditionability and potential mechanisms of conditioned immune responses in healthy humans, and (3) investigated conditioned immunomodulation in a clinical sample. Together, these data demonstrate that animal models provide a basis for investigating mechanisms whereby conditioned changes in immune function may modulate health status in a clinical realm.

Keywords: Behavioral conditioning; Psychoneuroimmunology; Rat; Human; Sympathetic nervous system

1. Introduction

Extensive evidence currently exists demonstrating bi-directional communication among the nervous, endocrine and immune systems [4,6,52,54]. One primary motivator for the explosion of scientific interest in the field of psychoneuroimmunology was the demonstration that an antibody response to antigen could be re-enlisted in rats via a Pavlovian conditioning paradigm [1].

Since this hallmark discovery, the conditionability of both cellular and humoral immune responses in laboratory animals has been well documented. However, to date little advance has been made in the understanding of neuroendocrine mechanisms of conditioned immunomodulation. Furthermore, the biological relevance of conditioned alterations in immune function is not well understood. An investigation of the mechanisms and biological relevance of conditioned immune alterations is possible via a number of research approaches. Firstly, animal models provide the opportunity for investigating both peripheral and central nervous system mechanisms of conditioned immunomodulation. Additionally, the effect of conditioned immunomodulation on disease outcome can be readily examined. To gain a further appreciation of the utility of conditioned changes of immune function, the conditionability of immune functions in humans is required, together with examination of neuroendocrine pathways that modulate this response. However, to appreciate how conditioned immunomodulation may alter disease state in humans, the effect of conditioned

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alterations of immune functions, and resulting disease status, must be examined in clinical samples.

Therefore, in an attempt to advance the understanding of mechanisms and biological relevance of conditioned immunomodulation, we summarize studies from our laboratories that have investigated the:

1. peripheral neuroendocrine mechanisms of conditioned immunomodulation in the rat, and biological significance of this response by utilizing a heart transplantation model (Exton, Schedlowski and collaborators).
2. Conditionability of immune changes in humans, and the potential neuroendocrine mediators (von Auer, Buske-Kirschbaum and collaborators).
3. Conditioned changes of immune function in a clinical sample undergoing cancer chemotherapy (Stockhorst, Göbel and collaborators).

2. Conditioned immunomodulation in the rat: mechanisms and biological relevance

Since the pioneering study completed by Ader and Cohen [1], numerous experiments have examined the conditionability of immune parameters in laboratory animals. The conditioning paradigm employs the pairing of a novel stimulus (conditioned stimulus; CS) with an immunomodulating drug (unconditioned stimulus; UCS). Upon re-exposure of the CS alone, immune functioning is altered in a similar manner to that which occurs following actual drug administration. Whilst a number of different stimuli have been implemented as the CS, the most commonly utilized is a novel tasting saccharin solution. The sweet drinking fluid is paired with administration of an immunomodifying agent. At a subsequent time point, re-exposure of the saccharin results in the animals avoiding consumption of the CS, which is termed conditioned taste aversion (CTA). Concomitantly, the animals demonstrate a modification of immune functioning that commonly mimics the actual drug effect.

Conditioned effects have been demonstrated both in humoral and cellular immunity [3]. Despite the establishment of a number of robust models, only a limited number of studies have attempted to examine the clinical relevance of conditioned changes in immune function. Specifically, the morbidity and mortality of animals with autoimmune disease is abated via conditioning using cyclophosphamide or cyclosporin A (CSA) as the UCS [2,35]. Additionally, the survival of heterotopic heart allografts can be extended using a conditioning paradigm that pairs saccharin as the CS with CSA as the UCS [25].

Furthermore, the mechanisms of conditioned changes in immune function and disease progression are poorly understood. However, there is some evidence suggesting that endocrine mediators, such as opioids and catecholamines modulate conditioned changes in immune function [38,39,48].

Therefore, as the data until now is inconclusive, we have developed a model to address the questions of mechanisms and biological relevance of conditioned immunomodulation in the rat.

2.1. Conditioned immunomodulation in the rat

We have developed a conditioning model to re-enlist specific immune changes produced by administration of CsA. CsA is a potent immunosuppressant extracted from *Tolypocladium inflatum Gams* [31]. This drug has become a cornerstone of organ transplant immunosuppressive therapy, due mainly to its specific inhibition of T lymphocyte interleukin-2 (IL-2) production [14,55]. Thus, cyclosporine diminishes levels of cytokine used by T cells to up-regulate a cellular immune response, thus reducing the ability of the immune system to reject a transplanted graft.

To produce a CTA paradigm male Dark Agouti (DA) rats were placed on a water deprivation regime for 5 days, allowing them 15 min of drinking at 0700 and again at 1700 each day (Fig. 1A). Our model implements a three learning (CS-UCS pairing) trial paradigm, with each learning trial separated by 72 h. On the fifth day, animals receive the first CS-UCS pairing. Conditioned animals receive a 0.2% saccharin solution (Sac) as the CS, paired with i.p. 20 mg/kg CsA as the UCS (Fig. 1B). In the afternoon session they are administered water (Wat) paired with i.p. saline injection. Sham conditioned rats are given Wat paired with CsA in the morning of the training days, and Sac in combination with saline in the afternoon. Three days following the final pairing, the CS alone is presented during each drinking session. This was repeated for the subsequent 2 days. Two extra control groups are implemented. CsA-treated animals were treated similarly to sham conditioned rats, however these animals received an additional CsA injection (20 mg/kg) following each of the first three CS re-exposures. This allows a comparison of the conditioned response with the actual drug effect. Additionally, an untreated group is utilized which is not manipulated during the entire conditioning procedure.

One hour following the third CS re-exposure animals are sacrificed, with blood, spleen, and lymph nodes removed for immunological analysis. Since CsA reduces T lymphocyte proliferation via inhibition of IL-2 synthesis, the effectiveness of the conditioning paradigm was initially investigated by analyzing IL-2 production following mitogen- (Concanavalin A, Con A) induced splenocyte proliferation. Fig. 2 presents representative data from a number of replications [16]. Conditioned rats display no significant suppression of lymphocyte

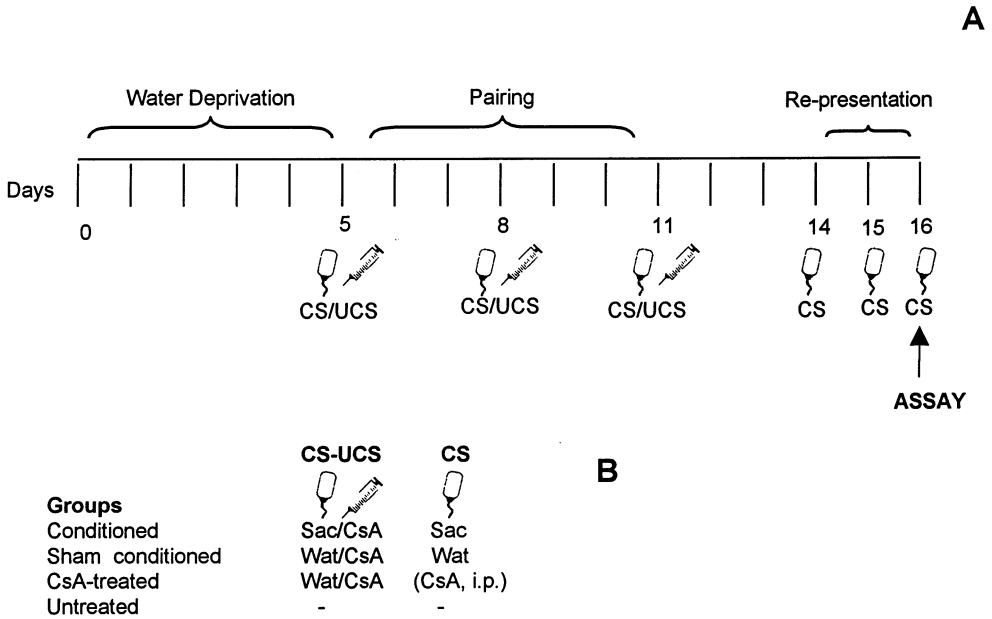

Fig. 1. Conditioned immunomodulation in the rat: conditioning paradigm and experimental groups. Animals received three CS-UCS pairings, followed by three CS reexposures. Animals were sacrificed one hour following the third CS reexposure, with blood and organs removed for immunological analysis.

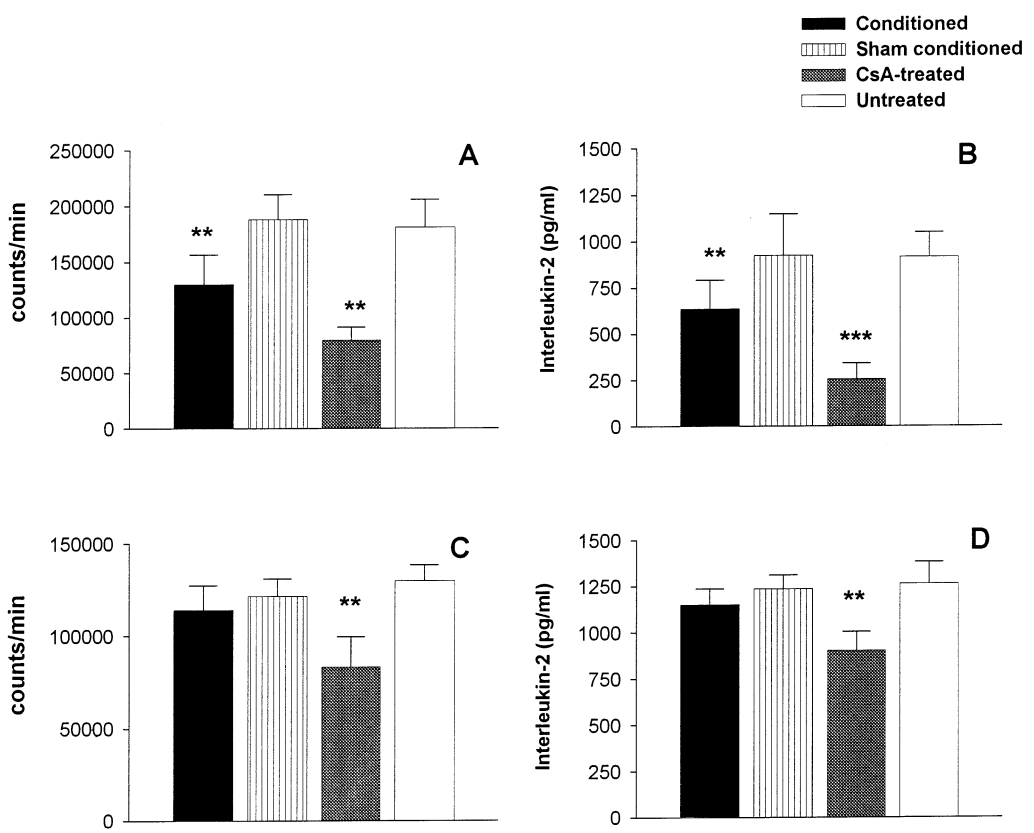

Fig. 2. Conditioning produces a reduction in splenocyte proliferation (A) and IL-2 production (B) which approaches that effected by cyclosporin administration. Surgical denervation of the spleen blocks the reduced proliferation and cytokine production in conditioned animals, but not in cyclosporin treated rats (C and D). ** $P < 0.01$, *** $P < 0.001$.

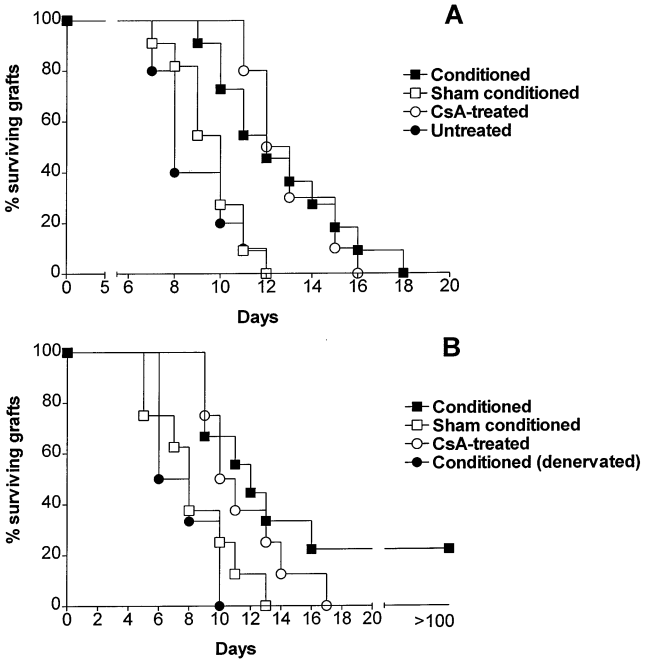

Fig. 3. (A) Conditioning prolongs the survival time of heart allografts in the rat to a similar magnitude as that achieved by short-course CsA treatment. (B) The addition of sub-therapeutic CsA regime to each group further improves graft survival in conditioned animals only. Importantly, the combination of conditioning and sub-therapeutic CsA therapy produced long-term survival (> 100 days) in 20% of animals. Furthermore, the conditioned prolongation of graft survival was completely blocked by prior surgical denervation of the spleen.

proliferation in the mesenteric lymph nodes (data not shown). However, significant reductions in mitogen-induced lymphocyte proliferation in the spleen is revealed (Fig. 2A). Similarly, conditioned rats display a reduction of IL-2 production compared to sham-conditioned animals (Fig. 2B) [16]. The conditioned reduction of IL-2 production and splenocyte proliferation approaches the magnitude of the immunosuppressive effect in CsA-treated animals. Importantly, the inhibition of splenocyte proliferation and IL-2 production in conditioned and CsA-treated rats is not due to altered leukocyte numbers or subset composition in the spleen [16].

2.2. Neuroendocrine mechanisms of conditioned immunomodulation

It has been suggested that paradigms investigating conditioned changes in immune function may produce 'stress effects', thus altering immune function via activation of the hypothalamus-pituitary-adrenal axis. However, when we analyze serum corticosterone levels at the time of IL-2 and proliferation assay, conditioned animals do not differ from control groups [16,17].

An alternative mechanism is that the CNS may communicate with the immune system during conditioning via the neural innervation of lymphoid organs such as the spleen [18,41]. This may be effected by paracrine means via the release of mediators from nerves situated in close proximity to lymphocytes [18,41]. In the spleen, noradrenaline is the primary transmitter released, and functionally operates as a neurotransmitter between the nerve and lymphocyte/macrophage [62]. In addition, the splenic parenchyma allows easy diffusion of noradrenaline [41]. Furthermore, sympathetic noradrenaline secretion mediates immunosuppression in the spleen after stress [15,56,67], hypothalamic lesioning or stimulation [33,46] and intracerebroventricular cytokine infusion [64].

Since we observed conditioned suppression of lymphocyte proliferation and cytokine production in the spleen but not mesenteric lymph nodes, we proposed that the contrary effects may have been due to a differential influence of the sympathetic nervous system on these organs. Therefore, we examined whether the conditioned effects would occur in animals with a denervated spleen [16]. Two weeks prior to conditioning, the splenic nerve was cut using standard methods [16]. Surgical denervation of the spleen significantly reduced the splenic noradrenaline content but did not affect CTA, indicating acquisition of the CS-UCS association [16]. However, splenic denervation completely abrogated the conditioned reduction in splenocyte proliferation, the efferent phase of the response (Fig. 2C). In addition, reduced splenocyte IL-2 production in conditioned rats was blocked by splenic sympathetic denervation (Fig. 2D) [16].

2.3. Biological relevance of conditioned immunomodulation

We subsequently wanted to establish that conditioned alterations in splenocyte proliferation and IL-2 secretion are functionally relevant. As the specific inhibition of T cell IL-2 production by CsA has resulted in the drug becoming a primary immunosuppressive agent used in transplantation medicine, we examined whether the conditioned immunosuppression would prolong the survival time of a transplanted heart allograft. Thus, conditioned DA rats received a heterotopic heart allograft from a Lewis (LEW) rat donor following the third conditioned stimulus re-exposures using standard techniques [47]. The CS was presented every day thereafter, and the survival time was determined. Conditioned animals displayed a longer latency to rejection of the graft and a significant increase in allograft survival time compared to sham conditioned and untreated animals (Fig. 3A). Furthermore, the magnitude of conditioned allograft prolongation was similar to the effect produced in CsA-treated rats [16].

Despite such biologically relevant findings, a common criticism of the results from conditioning experiments is that the effects are relatively small in comparison to

actual drug administration, and thus it is doubtful that conditioning has any clinical relevance as a stand alone therapy. Nevertheless, the ability of a suboptimal, albeit therapeutic dose of cyclophosphamide to inhibit the development of systemic lupus erythematosus in mice is enhanced by behavioral conditioning [2]. Therefore, we extended these data by examining whether a combination of conditioning and subtherapeutic CsA treatment can prolong heterotopic heart allograft survival in rats [17]. Specifically, the original conditioning plan was implemented, however on the first CS re-exposure day, conditioned, sham conditioned, and CsA treated groups were injected i.p with a 2 mg/kg (subtherapeutic) dose of CsA. This procedure was repeated on six further CS re-exposure days, with each subtherapeutic CsA administration separated by 48 h. Thus these groups received a subtherapeutic regime of seven 2 mg/kg CsA injections. Untreated rats were neither conditioned nor administered subtherapeutic CsA. Similar to the basic conditioning paradigm, CsA-treated animals received an additional CsA injection (20 mg/kg) following each of the first three CS re-exposures.

Animals which received subtherapeutic CsA and were behaviorally conditioned displayed a significant increase in survival time compared to rats which received subtherapeutic CsA and were only sham conditioned. Furthermore, even a short course of therapeutic CsA treatment (3×20 mg/kg) together with the subtherapeutic regime (7×2 mg/kg CsA) produced an increase in the mean survival time which was significantly lower than that achieved by the combination of subtherapeutic CsA and behavioral conditioning. The most striking effect observed by combining conditioning and subtherapeutic CsA was that 20% of these animals displayed long-term surviving grafts (>100 days). Moreover, splenic denervation completely abrogated the increased survival time induced by the combination of subtherapeutic CsA and behavioral conditioning (Fig. 3B) [17].

2.4. Conclusions

These studies have demonstrated that suppression of specific immune parameters by CsA can be re-enlisted via Pavlovian conditioning. Additionally, these data show that conditioned alterations of splenocyte function can be effected via direct neural innervation of the spleen. Furthermore, the conditioned changes of immune function are of biological relevance, as they are of sufficient magnitude to prolong the survival time of a functional heart allograft.

Thus, Pavlovian conditioned alterations of immune function in the spleen can be induced via sympathetic nervous system regulation. Sympathetic innervation in the spleen incorporates extremely close appositions between nerve terminals, and both lymphocytes and macrophages [41]. Indeed, noradrenaline functionally operates in the spleen as a neurotransmitter between the nerve and lymphocyte/macrophage [62], which bear functional adrenoceptors [4,20,30]. In addition, the splenic parenchyma allows easy diffusion of noradrenaline [41]. Therefore, the CNS can communicate with peripheral lymphoid organs via peripheral sympathetic nerves.

This communication produces functionally relevant alterations in immune function, as observed in the conditioned prolongation of heart allograft survival. Although conditioning paradigms have been shown to produce reliable alterations in immune function and to influence the course of a disease model in laboratory animals, the effects are typically small. Thus, it is likely that for this interesting phenomenon to have any practical relevance, it must be combined with drug therapy, with the aim of reducing the dose of medication required, and thus possibly limiting unwanted drug side effects. Therefore, this data showed that a dose of CsA that is previously ineffective in prolonging graft survival transforms to an effectual drug regime when coupled with behavioral conditioning. Thus, behavioral models can be utilized as a supplement to immunomodulatory drug regimes.

Animal models contribute to the ultimate goal of complementing pharmacotherapy by controlled behavioral paradigms in a clinical setting. However, to achieve this goal, conditioning principles must be applied in human models, examining the effectiveness of conditioned immunomodulation in healthy humans. Although this field is fraught with a number of technical difficulties that are not observed in basic animal research, significant advances have been made.

3. Conditioned immunomodulation in healthy humans: phenomenon and neuroendocrine mediators

Although the heuristic and biological relevance of classically conditioned immunomodulation has often been shown in animal models, there is currently little research investigating conditioned immunomodulation in humans. Early clinical observations suggest that immune related disorders such as allergic rhinitis or allergic asthma might be sensitive to associative learning processes. Thus, in early case studies it has been reported that an allergic attack can be provoked by exposure to an artificial rose or to the picture of a hay field in sensitive patients [27,40].

The first experimental approach to the possibility of a conditioned immunomodulation in humans was completed by Strutsovskaya [63]. He reported that after having received injections of gamma-globulin on four consecutive days, children with scarlet fever showed increased phagocytosis when treated with an injection of saline on day 5. These data provided first experimen-

Table 1
Experimental protocol for the conditioning of NK cell activity in healthy humans[a]

Group	Treatment					Expected alteration of NK cell activity
	Day 1	Day 2	Day 3	Day 4	Day 5	
Conditioned	CS+E	CS+E	CS+E	CS+E	CS+Sal	↑
Saline control	CS+Sal	CS+Sal	CS+Sal	CS+Sal	CS+Sal	–
Epinephrine control	CS+E	CS+E	CS+E	CS+E	CS+E	↑
Unpaired control	CS*+E	CS+E	CS+E	CS+E	CS+E	–

[a] CS, conditioned stimulus (sherbet sweet); E, epinephrine; Sal, saline; CS*, presentation of CS and UCS in a non-contingent manner.

tal evidence that the human immune system may be sensitive to classical conditioning. Nevertheless, definitive conclusions from these data cannot be made, as the early studies suffered from several methodological shortcomings such as small sample sizes or inadequate control groups.

Therefore, the specific goal of a series of studies conducted in our group was to examine whether immune function could be classically conditioned in humans. In an attempt to underline the clinical significance of the experiments, conditioned alteration of the number and activity of natural killer (NK) cells was examined. NK cells are large granular lymphocytes and are considered to play a major role in the immune surveillance against virally infected and neoplastic cells [26,69].

3.1. Conditioned increase of NK cell activity in blood

In the initial experiment, healthy volunteers were subjected to a basic classical conditioning protocol using epinephrine as a UCS [13]. In numerous studies it has been shown that epinephrine injection or infusion leads to a marked increase in NK cell number and activity in healthy subjects without significant side effects [32,52,53,65]. Due to its stimulating effect, epinephrine was considered to be an appropriate UCS in our protocol to induce a significant alteration of NK cell number and activity.

Healthy subjects were randomly distributed to four experimental groups (Table 1). Conditioned subjects were exposed to a sherbet sweet (CS), immediately followed by a subcutaneous injection of epinephrine (UCS) for four consecutive days. On day 5, the subjects were reexposed to the CS which was followed by an injection of saline on this day. Control groups were given either (a) the CS and saline injection (saline control) or (b) the CS and epinephrine injection, but in a non-associated manner (unpaired control). On the test day (day 5) both control groups were treated as the experimental group, i.e. received the sherbet sweet combined with a saline injection. An additional control group (epinephrine control) received the CS and the UCS on all experimental days.

To determine NK cell activity, blood samples were drawn five min before and 20 min after CS presentation. It was hypothesized that due to the close association between the sherbet sweet and the immunomodulatory effects of an epinephrine injection, reexposure of the subjects to the CS would result in conditioned elevation of NK cell activity.

Indeed, a significant increase of NK cell activity in the conditioned group was observed 20 min following CS reexposure (Fig. 4). Although treated identically as the conditioned group on this day, no alteration of NK cell activity was found in either the saline control group nor the unpaired control group. These findings support the position that firstly, the experimental treatment per se did not affect NK cell function (saline control) and further, that a strong association between the CS and the UCS during acquisition appears to be a prerequisite to obtain a conditioned elevation of NK cell activity (unpaired control group).

Thus, these data clearly demonstrate that immune function can also be modulated in humans via a classi-

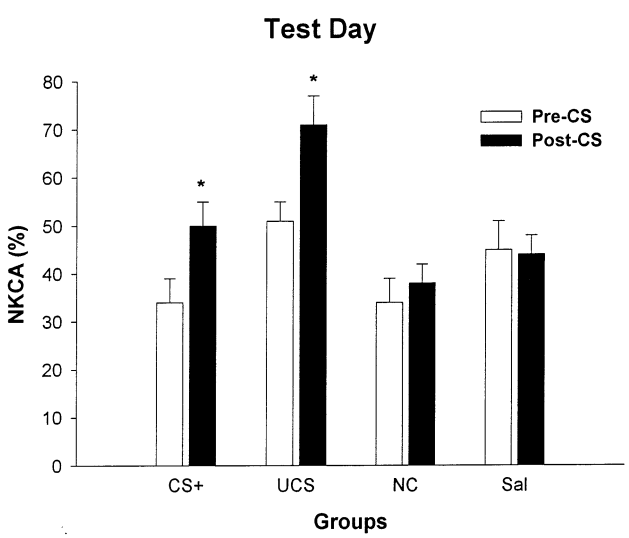

Fig. 4. Modulation of NK cell activity (NKCA, in % lysis; effector:target ratio = 50:1) in the conditioned group (CS +), UCS control group (UCS), unpaired control group (NC) and saline control group (Sal) five min before and 20 min following CS reexposure (* $P <$ 0.05).

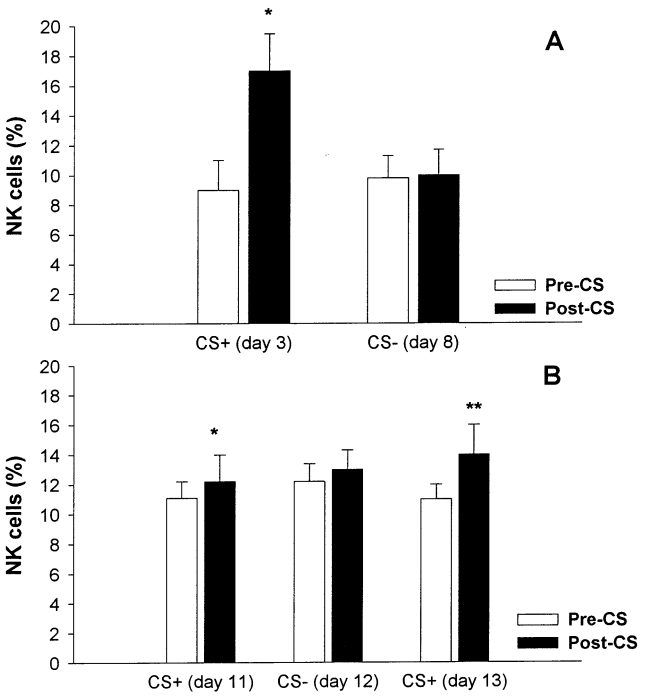

Fig. 5. Modulation of the percentage of NK cells following presentation of the CS+ and CS− on acquisition day 3 and 8 (A) and on test days 11, 12 and 13 (B). * $P < 0.05$; ** $P < 0.01$.

cal learning protocol. By pairing a neutral gustatory stimulus with an immunostimulating stimulus subjects showed conditioned enhancement of NK cell activity when reexposed to the taste alone.

3.2. Conditioned increase of NK cell numbers in blood

In order to clarify the mechanisms of a conditioned elevation of NK cell activity, we subsequently investigated whether NK cell numbers in the peripheral blood may be sensitive to associative learning processes [12]. In this study a discriminative conditioning protocol was used with each subject serving as his or her own control. In a discriminative learning protocol, two conditioned stimuli are introduced. The stimulus followed by the UCS is referred to as the CS^+ while the stimulus presented without the UCS is referred to as the CS^- [7]. With respect to the principles of classical conditioning, a conditioned response only after reexposure of the CS^+ was expected.

Healthy subjects were provided with ten acquisition trials. Five trials included presentation of CS^+ followed by the UCS, while in the other five experimental trials subjects were exposed to the CS^- alone. The order of stimulus presentation was randomized and identical for all subjects. We implemented a compound stimulus consisting of a sherbet sweet and white noise as the CS^+. This stimulus was followed by a subcutaneous injection of epinephrine, the UCS. As previously described, a subcutaneous injection of epinephrine is not only followed by an increase in NK cell activity but also by increased NK cell numbers in blood. In the CS^- trials, subjects were also exposed to a compound stimulus, consisting of a sherbet sweet combined with a specific tone which, however, remained without immunological reinforcement. Test trials on day 11, 12 and 13 were identical to the acquisition trials, except for the absence of the UCS. In the first and third test trial, subjects were reexposed to the CS^+, while in the second test trial they received the CS^-. A conditioned alteration of NK cell number was expected in the first but not in the second test trial. The third test trial was introduced to investigate possible extinction phenomena.

Blood samples were taken before and 20 min after stimulus presentation and assayed for NK cell number on acquisition days 3 and 8 as well as on the test days 11, 12 and 13. As expected, analysis of NK cell number on acquisition day 3 revealed a significant increase of cell number after the CS^+ training, but not after the CS^- training (Fig. 5A). Furthermore, on day 11 a significant increase after CS^+ reexposure was observed, with an even more pronounced effect on day 13 (Fig. 5B). As predicted, no alteration of NK cell number was observed on day 12 following CS^- presentation.

To summarize, these data conform to a discriminative conditioning analysis, demonstrating that only the CS^+ previously paired with the immunoenhancing injection of epinephrine leads to conditioned immunomodulation, while a comparable CS without previous immunological reinforcement did not provoke this effect. Furthermore, the data demonstrate that beside NK cell activity, NK cell number can also be modulated in a conditioning protocol and that alteration of NK cell number remains following one extinction trial. The latter observation concurs with animal studies that demonstrate a maintained CR even following five or six unreinforced CS presentations [9,11]. However, it should be noted that in some studies conditioned animals showed extinction of conditioned immunomodulation after exposure to more than one extinction trial [24,34,50,68]. Thus, it may be possible that a more frequent reexposure to the unreinforced CS may also have resulted in extinction of the CR in our subjects.

Taken together, the two experimental studies provide evidence of classically conditioned modulation of NK cell activity and NK cell number in humans. The findings suggest that humans differentiate between two similar cues of different immunological relevance in responding with an anticipatory learned response.

3.3. Conditioned NK cell numbers and activity: neuroendocrine mechanisms

In a further examination we investigated the neuroendocrine mechanisms producing the conditioned response. Furthermore, we aimed to demonstrate specific phenomena of classically conditioned immunomodulation such as extinction, spontaneous recovery or latent inhibition. Healthy subjects were randomly distributed into five experimental groups. Conditioned subjects received a sherbet sweet (CS) immediately followed by a subcutaneous injection of epinephrine (UCS) on four consecutive days. On day five, conditioned subjects were again assigned to four groups and were either reexposed to the CS (CS^+ control); received a β-adrenergic antagonist (propranolol) an hour before CS presentation (CS^+ blockade); remained without treatment (CS^0); or provided with the antagonist without being reexposed to the CS (CS^0 blockade). Another control group received the CS followed by saline on all experimental days (saline control). To investigate specific phenomena such as extinction, latent inhibition and spontaneous recovery selected groups underwent a second treatment period.

A significant increase in NK cell activity and NK cell number was observed in the conditioned group, with a high correlation between both parameters. Thus, it is possible that a conditioned rise of NK cell number may have contributed to increased NK cell activity observed on the test day. Presentation of the CS without the immunomodulating UCS before and after the acquisition did not result in latent inhibition or extinction of the CR. Most interestingly, subjects which received the β-adrenergic antagonist propranolol an hour before CS reexposure failed to show a conditioned response, suggesting that adrenergic mechanisms may be involved in the conditioned alteration of NK cell function in our protocol (unpublished data).

Therefore, the data from our investigations provide strong evidence that immune processes in humans can be modulated by associative learning processes. Furthermore, these results support animal data indicating that adrenergic activation is responsible for the conditioned alteration of immune function. It appears that the sympathetic nervous system plays a major role in producing classically conditioned immunomodulation in both animals and humans.

However, the clinical significance of conditioned alterations of immune responses in humans is yet to be determined. It remains the challenge to reveal whether conditioned changes in immune function in humans can possibly play a functional role in altering disease progression. Nevertheless, some data hints at the possibility of modulating disease course via classical conditioning in humans. Conditioned suppression of leukocyte numbers in the blood of autoimmune patients has been shown using a novel taste CS and cyclophosphamide as the UCS [23]. Conditioning has been shown to be able to suppress the progression of delayed-type hypersensitivity (DTH) reaction [57]. In contrast, the nasal tryptase release following dust mite exposure can be conditioned using a novel taste CS [21]. However, well controlled studies have not been able to condition skin inflammation to allergens [8]. Thus, although there remains a paucity of data examining the biological relevance of conditioned immunomodulation in humans, there is enough evidence to warrant further investigation.

To summarize, there is growing evidence that immune function can be influenced by classical learning processes in humans. Importantly, immune functions known to play a pivotal role in diseases such as autoimmune disease, cancer or allergy are conditionable. However, future research is necessary to further clarify the underlying biological mechanisms of conditioned immunomodulation in humans and the relevance of a learned immune response in disease prevention or progression. An extensive study of the classical conditioning of clinically relevant immune functions in new and efficient conditioning protocols may help elucidate the regulation of the human immune system by the brain. It may further lead to new therapeutic regimens which may include a learned immune response in the therapy of selected immune-related disorders.

4. Conditioned immunomodulation in humans: clinical application

Thus far we have shown that classical conditioning can effectively modulate immune function in both laboratory rodents and healthy humans. However, perhaps the greatest challenge is to reveal whether classical conditioning alters immune functions in patients with immune-relevant disease, and whether these effects are of sufficient biological relevance to alter disease progression. Therefore, our group has conducted a series of studies investigating the effects of classical conditioning in cancer patients receiving cytostatic drugs during chemotherapy [58–61].

In terms of classical conditioning, the situation of a patient receiving cytostatic drugs during chemotherapy can be described as a conditioning trial. The drugs, or more precisely their detection by the central nervous system (CNS), function as the UCS [49]. Cytostatic drugs induce a number of effects that are regarded as the UCR with reduction of tumor growth as the intended effect. Nevertheless, treatment induces unintended side-effects such as post-treatment nausea and post-treatment vomiting, where CNS involvement is mainly documented for post-treatment vomiting. Vomiting is assumed to be coordinated by a distributed

medullary control system predominantly consisting of the chemoreceptive trigger zone in the area postrema and its projections to the nucleus of the solitary tract which receives input from the abdominal vagus nerve [45]. Cytostatic drugs reduce food consumption and induce weight loss [59]. In addition, cytostatic drugs are immunomodulatory, typically suppressing a number of immune functions.

Stimuli contingently paired with the UCS, such as sight of the hospital or smell of the ward, function as CSs and trigger conditioned responses (CR) when the patient is re-exposed to the CS prior to a subsequent infusion. Anticipatory nausea and vomiting (ANV), learned food aversion, and anticipatory immunomodulation have thus far been investigated as putative CRs. Investigation of these CRs have focused on the examination of ANV, demonstrating that ANV conforms to typical parameters of classical conditioning [58], and is susceptible to reduction by an overshadowing protocol [61].

Despite the clinical relevance of conditioned immune function in cancer patients undergoing cytostatic therapy, only four studies have investigated this phenomenon, with incongruent results [10,19,36,37]. While one study reported a decrease in immune functions in the hospital compared to home environment [10], others observed increases in immune parameters [36,37], or found no functional changes [19]. It is likely that a number of factors, such as the type of cancer, the chemotherapy regime implemented, and the age of the subjects contribute to the inconsistent results.

Therefore, we investigated conditioned changes in immune function in cancer patients and aimed to extend the current data by investigating conditioned immune function in pediatric cancer patients [60]. In this study we focused particularly on the conditioned modulation of NK cell activity [10,36], since NK cells belong to the unspecific defense system that are able to lyse neoplastic cells [26,69]. In addition we analyzed the plasma concentration of the cytokines interleukin (IL)-1β, IL-2, IL-10, IFN-γ, tumor necrosis factor (TNF)-α. These cytokines are well documented as participating in either the behavioral response to cancer (IL-1β, TNF-α) [5,43], mediating the immune response to cancer or directly mediating tumor growth (IL-2, IFN-γ, TNF-α) [42,51,66].

Table 2
Inclusion criteria for conditioning in pediatric cancer patients

Inclusion criteria
1. 4–18 years of age
2. Absence of CNS or gastrointestinal tumors (to exclude organic reasons for ANV)
3. Experience of at least two chemotherapy cycles (to guarantee prior CS-UCS exposition)
4. At least 7 days free of emetogenic drugs between two cycles (to exclude pharmacologic reasons for ANV)
Diagnoses
Acute lymphatic leukemia ($n=7$), Ewing sarcoma ($n=4$), osteosarcoma ($n=4$), peripheral neuroectodermal sarcoma ($n=3$), non-Hodgkin lymphoma ($n=1$)

Nineteen pediatric cancer patients from the Clinic of Pediatric Hematology and Oncology, Heinrich-Heine-University Düsseldorf participated in the study. Patients fulfilled the inclusion criteria shown in Table 2. All patients received intravenous (i.v.) polychemotherapy using the standardized protocols of the German Society of Pediatric Oncology and Hematology.

Patients were observed during two consecutive chemotherapeutic cycles (cycles A and B; Fig. 6). Recording of symptoms commenced at home 2 days prior to the infusion (day −2, day −1), employing symptom lists. On the morning of a chemotherapeutic cycle (day 0), patients moved from home to hospital where they were reexposed to the hospital CS prior to infusion onset. Thus, within the anticipatory measurement interval, the CS content of the environment increased with temporal proximity to infusion onset. The post-treatment measurement commenced following the completion of the last infusion of a cycle. The patient was again observed for 2 days (day +1, day +2). During cycle B, blood was drawn at day −2 in the home environment (low CS content) and at the same time on day 0 in the hospital (high CS content) prior to the start of the infusion.

Symptoms were recorded using self-report graphically illustrated symptom-lists measuring occurrence and intensity of 10 symptoms (headache, dizziness, heart beat, nausea, hot flashes, sweating, weakness, vomiting, heart burn, loss of appetite). Each symptom-list covered a 12 h interval. The four symptom lists prior to the first infusion of each cycle constituted the anticipatory measurement interval. The four 12 h intervals following completion of the last infusion of a cycle represented the post-treatment measurement interval. Blood samples were analyzed for NK cell activity by a standard chromium release assay. Cytokine concentrations (IL-1β, IL-2, IL-10, TNF-α, and IFN-γ) were analyzed in plasma using commercial ELISA.

A high level of ANV and post-treatment nausea and vomiting (PNV) was observed in cycle A (ANV in 6 patients; PNV in 14 patients) compared to cycle B (ANV in three patients; PNV in 11 patients). This difference related to the stronger emetogenity of the drug protocol in cycle A (protocol of high emetogenity in nine patients) compared to cycle B (high emetogenity in six patients). Furthermore, UCS intensity and CS-UCS contiguity determined the level of anticipatory symptoms in cycle A [60].

In comparison to the home environment, patients experienced increased NK cell activity and plasma IFN-

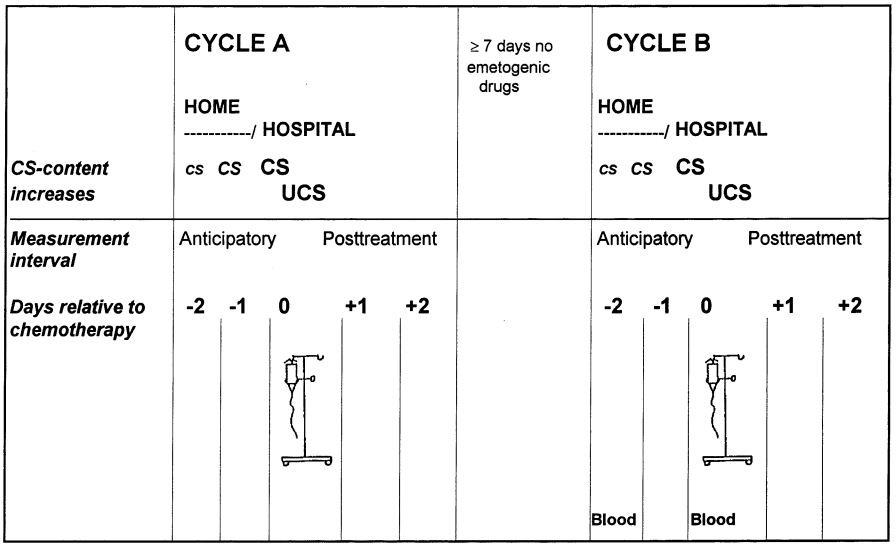

Fig. 6. Study paradigm. Cycle A and Cycle B represent two consecutive chemotherapeutic cycles. ANV was measured on 2 days (day −2, day −1) prior to the start of a chemotherapeutic cycle. The chemotherapy cycle commenced on day 0. PNV was monitored over 2 days (day +1, day +2) following completion of the cycle. Blood was drawn in cycle B at day −2 (home environment) and at day 0 (hospital) prior to infusion onset. CS, conditioned stimulus; UCS, unconditioned stimulus.

Table 3
NK cell activity (NKCA) and plasma cytokine (IFN-γ, IL-1β) concentrations in ANV+ and ANV− patients, measured in the home and in hospital

	NKCA (% cytolysis)		IFN-γ (pg/ml)		IL-1β (pg/ml)	
	Home, day −2	Hospital, day 0	Home, day −2	Hospital, day 0	Home, day −2	Hospital, day 0
ANV+	3.4 ± 0.8	7.9 ± 2.2**	3.7 ± 1.5	10.1 ± 3.9*	1.8 ± 1.4	2.8 ± 1.7
ANV−	5.2 ± 0.9	5.9 ± 1.2	5.8 ± 2.8	5.4 ± 2.1	1.4 ± 0.6	0.3 ± 0.2*

* $P<0.05$.
** $P<0.01$ Lam–Longnecker test for paired data.

γ concentrations in the hospital environment [60]. However, to address whether patients with ANV also develop anticipatory immunomodulation, we examined the conditioned immune parameters in the two patient subgroups. Patients were divided into those that developed ANV in at least one cycle (ANV+; $n=7$) or those that did not develop ANV, neither in cycle A nor in cycle B (ANV−; $n=11$). These groups did not differ in age, sex, or number of previous infusions and the absolute level of immune parameters.

However, ANV+ patients displayed increased NK cell activity and plasma IFN-γ levels from home (day −2) to the hospital (day 0) environment, in comparison to unchanged NK cell activity and cytokine concentrations in ANV− patients (Table 3). In contrast, ANV− patients demonstrated reduced levels of plasma IL-1β from home to hospital, whereas ANV+ patients showed no alteration in plasma levels of this cytokine (Table 3). No significant conditioned changes were observed in plasma levels of IL-2, TNF-α, or IL-10 in either group (data not shown).

In our sample, patients that reacted with an anticipatory change in nausea and/or vomiting to the CS-related hospital environment demonstrated parallel increases in NK cell activity and plasma concentrations of IFN-γ. These effects, known to form part of the UCR following chemotherapy [44], support previous data indicating a conditioned increase in NK cell activity in cancer patients [36].

The current effects appear to be unrelated to cortisol plasma levels (data not shown), thus suggesting that the HPA axis does not play a mediating role. However, as is indicated from the conditioning of immune parameters in animals (Section 2) and conditioned NK cell activity in humans (Section 3), it is possible that the sympathetic nervous system, in addition to endogenous opioids [29] may play an important role in producing the current conditioned effects.

Although the biological significance of the current effects are unknown, data from animal experiments suggest that classical conditioning has the potential to modify tumor progression. Classically conditioned in

creases in NK cell activity in mice [29] can prolong survival time following tumor inoculation [22]. Furthermore, active immunotherapy with allogeneic spleen cells in lymphoma-bearing animals is augmented by conditioning, thus resulting in a delay of tumor growth [28].

The current study thus demonstrates that classical conditioning in cancer patients produces changes in immune parameters relevant for tumor progression. These data provide preliminary evidence for the role of conditioning in a clinical realm. However, the data offer a number of interesting challenges. For example, the reproducibility over time of anticipatory immune responses needs to be investigated. Secondly, the neuroendocrine pathways producing such effects need to be analyzed, with an aim of possible manipulation of these pathways in mediating immune function in clinical samples. Finally, and the greatest challenge of all, is to examine conditioned changes in immune function in patients using an experimental design, to investigate whether conditioned immune alterations modulate disease progression.

5. Conclusions

Since the first controlled demonstration of conditioned changes in immune function more than 20 years ago [1], many models have been developed to demonstrate that numerous functions of the immune system are sensitive to classical conditioning. Nevertheless, there remains a paucity of knowledge concerning the neuroendocrine mechanisms driving these responses, as well as the possible clinical benefit to be derived from them. The current paper reviews studies from our laboratories that have made some progress towards understanding these questions.

Our data demonstrate that the sympathetic nervous system plays an integral role in effecting conditioned immunomodulation in both animals and healthy humans. Although it must be recognized that other neuroendocrine mechanisms are involved in the generation of CR, these data offer promise in the generation of beneficial conditioned effects, and the abrogation of detrimental ones.

The presented data also demonstrate that conditioned effects are biologically relevant. Classically conditioned immunosuppression modulates the course of disease in animal models. Additionally, conditioned changes of immune function are possible in healthy humans. Furthermore, classical conditioning alters disease-relevant immune parameters in clinical samples. These last data represent the great frontier to conditioned changes of immune function. It is the challenge of future research to cross the boundary of experimental manipulation toward a possible human application in clinical medicine.

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