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Chronic psychosocial stress promotes systemic immune activation and the development of inflammatory Th cell responses

Dominic Schmidt [a,1], Stefan O. Reber [b,1], Catherine Botteron [a], Thomas Barth [a], Daniel Peterlik [a], Nicole Uschold [b], Daniela N. Männel [a], Anja Lechner [a,*]

[a] Institute of Immunology, University of Regensburg, 93053 Regensburg, Germany
[b] Institute of Zoology, University of Regensburg, 93053 Regensburg, Germany

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ABSTRACT

Recent studies indicate that chronic psychosocial stress favors the development of generalized immune dysfunction. During stressor exposure neuroendocrine factors affect numbers and functionality of leukocytes. However, the exact mechanisms leading to systemic changes in immune functions during stress are still not clear. During chronic subordinate colony housing, a model of chronic psychosocial stress, mice developed spontaneous colonic inflammation. Decreased glucocorticoid signaling, induced by a combination of adrenal insufficiency and glucocorticoid resistance, was thought to prevent tempering of local immune cells, and to promote tissue inflammation. In this study we investigated changes in the systemic immune status after chronic subordinate colony housing and analyzed potential mechanisms underlying those alterations.

Analysis of T helper cell subsets in peripheral lymph nodes revealed a reduction of regulatory T cells, accompanied by increased T cell effector functions. Generalized activation of T cells was shown by elevated cytokine production upon stimulation. In addition, we observed no apparent shift towards T helper type 2 responses. It is likely, that the previously reported hypocorticism in this stress model led to a steady production of inflammatory Th1, Th2, and Th17 cytokines and obstructed the shift towards an anti-inflammatory response.

In conclusion, we established chronic subordinate colony housing as a model to investigate the outcome of stress on the systemic immune status. We also provide evidence that distinct T helper cell subtypes react differentially to the suppressive effect of glucocorticoids.

1. Introduction

Chronic psychosocial stress is a burden of modern societies and has long been recognized as a risk factor for numerous affective and somatic disorders (Agid et al., 1999; Cohen et al., 1991; Dimsdale, 2008; Kiecolt-Glaser et al., 2002; Levenstein, 1998). Over the past decades stress-related pathologies have been linked to a decreased overall glucocorticoid (GC) signaling, either induced by a break-down of hypothalamo–pituitary–adrenal (HPA) axis function or the development of GC-resistance (Cutolo et al., 2003; Davis et al., 2008; Raison and Miller, 2001). Due to the lack of clinically relevant animal models a causal link between chronic stress-induced reduction in GC signaling and disease development is still elusive. Therefore, there is an urgent need for appropriate animal models mimicking those conditions.

Chronic subordinate colony housing (CSC) has recently been established as a clinically relevant model of chronic psychosocial stress in male mice. CSC causes symptoms such as development of increased state anxiety, reduced weight gain, increased adrenal mass, an insufficiency to release GC upon challenge with corticotropin *in vitro*, elevated plasma norepinephrine (NE) levels, reduced thymus weight, and the development of spontaneous colitis during exposure to CSC (Reber et al., 2007; Reber and Neumann, 2008). Also, as a consequence, CSC aggravated dextran sodium sulfate (DSS)-induced colitis in mice as indicated by an increased histological damage score and enhanced secretion of IFN-γ, TNF, and IL-10 by *in vitro* stimulated mesenteric lymph node cells (Reber et al., 2008). Thus, CSC can be used as an animal model to investigate mechanisms involved in stress-induced local immune alterations. In particular, the lack of immune suppressive GC due to adrenal insufficiency and GC-resistance of splenocytes indicates CSC as a useful model studying stress-related immune

* Corresponding author. Address: Institute of Immunology, University of Regensburg, Franz-Joseph-Strauss-Allee 11, 93053 Regensburg, Germany. Fax: +49 941 944 5462.
E-mail address: anja.lechner@klinik.uni-regensburg.de (A. Lechner).
[1] Both authors contributed equally to this article.

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pathologies also observed in humans. However, the consequence of CSC on the systemic immune status has not been addressed so far.

During ongoing immune responses either T helper (Th) 1 or Th2 cells emerge, which act by coordinating effector functions of either macrophages and cytotoxic T cells or B cells, respectively (Mosmann and Coffman, 1989). This model has recently been expanded by the identification of a third Th cell subtype, namely Th17 cells (Mangan et al., 2006; Veldhoen et al., 2006). Th17 cells promote granulocyte development and recruitment during homeostasis and infection, but are also associated with tissue inflammation and chronic inflammatory disorders (Kramer and Gaffen, 2007). To prevent chronic inflammations, regulatory T (Treg) cells inhibit effector T cell activity (Sakaguchi et al., 2006). Interestingly, although functionally opposed, Th17 and Treg cells share common developmental pathways. On the one hand TGF-β-induced up-regulation of the transcription factor Foxp3 is necessary for Treg cell function, on the other hand, in combination with IL-6, TGF-β favors differentiation of Th17 cells (Bettelli et al., 2006; Huber and Schramm, 2006).

To elucidate whether the local gut inflammation induced by CSC is also accompanied by a systemic immune activation, we monitored the activation status of peripheral T cells. Furthermore, we were interested in the impact of reduced GC-responsiveness on systemic immune parameters in the CSC model. We observed that CSC enhanced T cell functionality; cytokine secretion by T cells was increased after re-stimulation *in vitro* and the relative number of Treg cells was reduced in peripheral lymph nodes. In addition, T cell activation was accompanied by enhanced B cell activity as indicated by increased numbers of germinal centers in the spleen.

2. Methods

2.1. Animals

Male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19–22 g (experimental mice) or 30–35 g (dominant mice) were individually housed in standard cages for one week before the experimental procedure started. All mice were kept under standard laboratory conditions (12-h light/dark cycle, lights on at 06:00 h, 22 °C, 60% humidity) and had free access to tap water and standard mouse diet. All experimental protocols were approved by the Committee on Animal Health and Care of the local government, and performed according to international guidelines on the ethical use of animals.

2.2. Chronic subordinate colony housing

CSC was conducted as previously described (Reber et al., 2007). In brief, four experimental subordinate mice were housed together with a larger dominant male in a polycarbonate observation cage ($38 \times 22 \times 35$ cm) for 19 consecutive days. Before starting the CSC procedure, the future dominant males were tested for their aggressive behavior. Males that started to injure their opponents by harmful bites were not used. On days 8 and 15 the dominant males were replaced to avoid habituation. In all colonies, the larger male mouse established a dominant status by chasing and attacking all four experimental mice. In parallel, single housed control (SHC) mice remained undisturbed in their home cages except for change of bedding once a week.

2.3. Antibodies and reagents

The following antibodies were purchased from eBioscience (Frankfurt, Germany): rat anti-mouse CD4 (clone RM4-5), rat anti-mouse CD8α (clone 53-6.7), rat anti-mouse CD25 (clone PC61), rat anti-mouse/rat Foxp3 (clone FJK-16s), rat anti-mouse IFN-γ (clone XMG1.2), rat anti-mouse IL-17 (clone TC11-18H10.1), hamster anti-mouse CD3ε (clone 145-2C11), rat anti-mouse CD45R (clone RA3-6B2). Peanut agglutinin (PNA) was obtained at Vector Laboratories (Burlingame, USA). Alexa Fluor 546 conjugated streptavidin (Invitrogen, Karlsruhe, Germany) and goat anti-rat Alexa Fluor 488 (Invitrogen) were used as secondary reagents. Rat anti-mouse CD62L (clone MEL-14) and FITC, PE, and APC labeled isotype control IgG were purchased from BD Biosciences (Heidelberg, Germany). Hamster anti-mouse CD3ε (clone 145-2C11) was purified from hybridoma cell culture supernatant using protein-G-Sepharose columns (GE Healthcare, München, Germany) and used for stimulation experiments.

2.4. Isolation of lymph node cells and staining of Treg cells

Inguinal, brachial, and axillary lymph nodes were harvested 19 days after induction of CSC. A single cell suspension was prepared in PBS supplemented with 2% FCS and cell numbers were determined. Cell surface staining was performed as previously described (Potzl et al., 2008) using anti-CD4, anti-CD25, and anti-CD62L antibodies. For detection of intracellular Foxp3, the Treg staining kit (eBioscience) was applied according to the manufacturer's instructions.

2.5. Stimulation of lymph node cells and intracellular cytokine staining

Lymph node cells were incubated in cell culture medium (RPMI, 10% FCS, penicillin (100 U/ml) and streptomycin (100 U/ml)). All cell culture reagents were purchased from PAN Biotech (Aidenbach, Germany). Cells were stimulated with plate-bound anti-CD3ε (5 μg/ml). Dexamethasone (DEX; Fortecortin, Merck Pharma GmbH, Darmstadt, Germany) was used at the following concentrations: 10, 100, and 1000 ng/ml. After 16 h of stimulation ionomycin (1 μM; Sigma–Aldrich) and phorbol-12-myristat-13-acetat (PMA, 50 ng/ml; Sigma–Aldrich) in the presence of brefeldin A (BFA, Sigma–Aldrich) were applied for 4 h. Intracellular staining was conducted using the IC fixation buffer in combination with the permeabilization buffer (eBioscience) according to the manufacturer's instructions.

2.6. Flow cytometric analysis

Data was collected on the LSR II flow cytometer (BD Biosciences) and analyzed using the DIVA software (BD Biosciences). All staining profiles were based on live-gated cells, as determined by forward and sideward scatter properties. Exclusion of doublet cells was performed by SSC area – SSC width gating.

2.7. Determination of cytokine production

Cells were stimulated with plate-bound anti-CD3ε (5 μg/ml) for 48 h *in vitro* with or without DEX (10 ng/ml) and cell culture supernatants were collected for ELISA. IFN-γ protein quantification was performed using the DUO-ELISA kit (R&D Systems) following the manufacturer's instructions. The detection limit for IFN-γ was 20 pg/ml. For determination of all other cytokines Th1/Th2 FlowCytomix Multiplex Kit (Bender MedSystems, Vienna, Austria) was used according to the manufacturer's instructions. The DEX-mediated reduction in cytokine secretion was calculated as follows: For each culture, cytokine levels were normalized to the mean of anti-CD3ε-treated control cells (media) of either SHC or CSC mice set to 100%.