European Journal of Cell Biology 91 (2012) 576–581

Contents lists available at SciVerse ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.de/ejcb

Deletion of gp130 in myeloid cells modulates IL-6-release and is associated with more severe liver injury of Con A hepatitis

H.H. Lutz[a], S.D. Sackett[a], D.C. Kroy[a,c], N. Gassler[b], C. Trautwein[a,*]

[a] Department of Gastroenterology and Metabolic Disorders, RWTH University Hospital Aachen, University of Aachen (RWTH), Pauwelsstrasse 30, 52074 Aachen, Germany
[b] Institute of Pathology, RWTH University Hospital Aachen, Aachen, Germany
[c] Gastrointestinal Unit, Massachusetts General Hospital, Harvard Medical School, 55 Fruit Street, Boston, MA 02114, USA

ARTICLE INFO

Article history:
Received 23 June 2011
Received in revised form 9 August 2011
Accepted 8 September 2011

Keywords:
gp130
LysMCre
Concanavalin A
Hepatitis
IL-6
Macrophages

ABSTRACT

IL-6/gp130 dependent signaling plays an important role in modulating inflammation in acute and chronic diseases. The course of Concanavalin A- (Con A) induced hepatitis can be modulated by different immune-mediated mechanisms. IL-6/gp130-dependent signaling has been shown to be protective in hepatocytes. However, the role of this pathway in myeloid cells has not yet been studied. In our present study we used macrophage/neutrophil-specific gp130 knockout ($gp130^{\Delta Lys}$, KO) animals and analyzed its relevance in modulating Con A-induced hepatitis. Additionally, we performed *in vitro* studies with $gp130^{\Delta Lys}$-macrophages. We demonstrate that $gp130^{\Delta Lys}$ animals are more susceptible to Con A-induced hepatitis. This is reflected by higher transaminases, higher lethality and more severe liver injury as shown by histological staining. Using flow cytometry analysis we further could show that increased liver injury of $gp130^{\Delta Lys}$ animals is associated with a stronger infiltration of CD11b/F4/80 double-positive cells compared to wild-type ($gp130^{flox/flox}$, WT) controls. To further characterize our observations we studied thioglycolate-elicited peritoneal macrophages from $gp130^{\Delta Lys}$ animals. Interestingly, the LPS-dependent IL-6 release in $gp130^{\Delta Lys}$ macrophages is significantly reduced ($p < 0.05$) compared to WT macrophages. Additionally, IL-6 blood levels *in vivo* after Con A injection were significantly lower in $gp130^{\Delta Lys}$ animals compared to WT animals ($p < 0.05$). In summary, our results suggest that gp130-deletion in macrophages and granulocytes leads to diminished IL-6 release from these cells, which is associated with more severe Con A-induced hepatitis.

Introduction

Immune-mediated effects modulating the severity of acute and chronic liver diseases are of essential relevance, especially in viral or autoimmune hepatitis (Dienes and Drebber, 2010). Both etiologies are major global health problems and understanding the immunological mechanisms leading to disease progression is important in order to optimize therapeutic options. Immunological mechanisms are involved in triggering disease progression and thus are of critical importance in inducing liver fibrosis and its characteristic complications like portal hypertension, variceal bleeding and growth of hepatocellular carcinoma (Hernandez-Gea and Friedman, 2011).

Concanavalin A- (Con A) induced hepatitis is a well-established experimental mouse model which mimics autoimmune or viral hepatitis. In this model, T-cell activation leads to a specific cytokine production, the activation of immune cells such as macrophages and the concomitant development of severe hepatitis (Gantner et al., 1996; Tiegs et al., 1992). gp130 dependent pathways play major roles in various models of experimental liver disease. To date, its cell-specific role has mainly been studied and characterized in hepatocytes. Work from our own group has demonstrated a protective role for IL-6 in Con A-induced liver injury (Klein et al., 2005). IL-6 pretreatment of wild-type (WT) mice protects these animals from Con A-induced hepatitis, while hepatocyte-specific gp130 knockout animals show even more severe liver injury. Furthermore, IL-6 has also been shown to be protective in the chronic carbon tetrachloride (CCl4) mouse model using knockout animals with gp130 deletion in hepatocytes and non-parenchymal cells (Streetz et al., 2003).

The possible effects of gp130 dependent signaling in non-parenchymal cells such as neutrophils or macrophages in acute models of hepatitis have not yet been studied. Lipopolysaccharide (LPS) has no direct effect on gp130-dependent STAT-activation in hepatocytes. In contrast, LPS in macrophages is known to significantly influence IL-6-induced STAT3 and SOCS3-activation (Bode et al., 1999; Yasukawa et al., 2003). Driven by these data, we addressed the impact of gp130 in myeloid cells using neutrophil/macrophage

* Corresponding author. Tel.: +49 241 80 80866; fax: +49 241 80 82455.
E-mail address: ctrautwein@ukaachen.de (C. Trautwein).

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doi:10.1016/j.ejcb.2011.09.006

specific knockout ($gp130^{\Delta Lys}$) animals exposed to models of acute hepatitis. Thus, we investigated the impact of impaired gp130-signaling in macrophages using the Con A-induced liver injury model *in vivo* and further studied the impact of LPS stimulation on macrophages *in vitro*.

Material and methods

Animals

Specified pathogen-free (SPF) 8–12-week-old male C57BL6/J mice were obtained from the Animal Research Institute of the RWTH University Hospital Aachen. For each time point and group, 3–5 animals were treated in parallel. Macrophage/neutrophil-specific gp130 deficient animals were generated by crossbreeding $gp130^{flox/flox}$ animals with the lysozyme M-promotor Cre (LysM-Cre) tg mice ($gp130^{\Delta Lys}$) (Clausen et al., 1999). This animal strain carries loxP sites flanking exon 16 of the gp130 gene and has been previously described (Sander et al., 2008). LysMCre-negative ($gp130^{flox/flox}$) animals carry loxP sites flanking exon 16 of the gp130 gene without expressing Cre recombinase ($gp130^{flox/flox}$) and were used as controls (WT). Deletion efficiency was evaluated in peritoneal macrophages (for harvesting procedure see below) via PCR and was above 80%, consisting with previous publications on this Cre/loxP recombination system. All experiments were approved by the Institutional Animal Care and Use Committee of the RWTH University Hospital. The experiments were performed in agreement with the Guide for Care and Use of Laboratory Animals (National Institutes of Health publication no. 86-23, revised 1985).

Con A-induced hepatitis in B6 mice

Hepatitis was induced by intravenous (tail vein) injection of 20 mg/kg bodyweight of Con A. An equal volume of carrier solution was injected in control animals (saline). To evaluate severity of hepatitis, Alaninaminotransferase (ALT) levels were measured in serum. Blood samples were collected by retro-orbital puncture at specific time points (0, 24, 48 and 96 h after Con A injection).

Isolation of macrophages after thioglycolate-induced peritonitis, RNA isolation and RT-PCR

WT and $gp130^{\Delta Lys}$ animals were first treated with an i.p.-injection of 2 ml of 4% thioglycolate. After 72 h, animals were sacrificed and peritoneal cells were collected by peritoneal lavage, at this time point consisting mainly of macrophages. Next, cells were incubated in RPMI supplemented with 10% FCS and P/S for 2–4 h. Adherent cells were used and cultured in RPMI with p/s as described before (Hirohashi and Morrison, 1996). RNA was isolated at different time points after LPS stimulation, using RNeasy columns (Qiagen, Hilden, Germany) following the manufacturer's instructions. First-strand synthesis was performed with oligo(dT) primers and reverse transcription with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed using SYBR Green Reagent (Invitrogen, Carlsbad, CA, USA) in Applied Biosystems Prism 7300 real-time PCR system (Applied Bioscience, Foster City, CA, USA). Reactions were performed at least three times and GAPDH values were used to normalize gene expression. IL-6 mRNA levels were expressed in times vs. basal expression of IL-6 ("fold induction") in $gp130^{\Delta Lys}$ and WT macrophages, respectively. Basal expression of both cell types served each as control and did not differ significantly. Furthermore, basal expression was set to "1" for better comparability of mRNA levels.

Flow cytometry

Forty-eight hours after Con A injection, livers from control and $gp130^{\Delta Lys}$ animals were harvested. Tissue was minced into 1-mm pieces and digested in RPMI containing collagenase D (400 U/l) (Roche) and DNase I (0.01 mg/ml) (Boehringer, Mannheim, Germany) in a shaking water bath at 37° for 30 min. The remaining cellular suspension was filtered and washed twice in HBSS (Invitrogen, Carlsbad, CA, USA). This cell suspension was stained with fluorochrome-conjugated Antibodies (CD45 BD Biosciences, San Jose, CA, USA) and F4/80 (Serotec, Oxford, United Kingdom). Cell mixtures were then analyzed by flow cytometry Canto II (BD Biosciences, San Jose, CA, USA) and data were processed using FlowJo software (Tree Star, Ashland, OR, USA).

Blood samples

Blood samples were collected retro-orbitally at baseline and at different time points after treatment with Con A for both $gp130^{\Delta Lys}$ and WT mice as indicated. Measurement of ALT plasma enzyme activity was performed following a standard protocol in the Department of Laboratory Medicine of RWTH University Hospital Aachen, IL-6 blood levels were measured by ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol.

H&E staining and immunohistochemistry

For histologic and immunohistochemical evaluation, livers were harvested at different time points after Con A injection, as mentioned above. For histopathologic analyses, cryosections of 3 μm were stained with H&E using standard protocol. For immunofluorescent stainings, slides were first fixed with methanol and acetone for 5–10 min at room temperature and then stained. Macrophages and monocytes were stained with rat anti-mouse CD11b mAb (BD Biosciences, San Jose, CA, USA). As secondary antibody, Alexa Fluor 594 anti-rat antibody was used (Molecular Probes Invitrogen, Carlsbad, CA, USA). Nuclei were counterstained with DAPI.

Liver necrosis after Con A-induced hepatitis

The area of necrotic liver parenchyma was measured and quantified as mm^2 via light microscopy in representative cuts of the median lobe after H&E staining and compared as a percentage of total liver tissue area in those cuts as described before ($n = 3$) (Luedde et al., 2005). An Axioskop A microscope (Carl Zeiss MicroImaging, Göttingen, Germany) with Axiovision 3 software was used for this analysis.

Statistical analysis

The statistical analysis was conducted by the Student *t*-test for transaminase levels, necrotic areas in liver parenchyma, immunohistochemistry and flow cytometry. Comparison of the survival curves was performed using the Log-Rank (Mantel–Cox) test. Two-way ANOVA was used for evaluation of the time course of LPS-induced in IL-6 release *in vitro* and IL-6 blood levels *in vivo*. $p < 0.05$ was considered significant.

Results

Deletion of gp130 in myeloid cells leads to more severe Con A-induced hepatitis with higher morbidity and mortality compared to WT mice

To evaluate the role of gp130 dependent signaling in non-parenchymal cells for the development of an acute hepatitis,