Cytokine Modulation of Liver Annexin 1 Expression during Experimental Endotoxemia

Catherine de Coupade,* Maureen N. Ajuebor,[†]
Françoise Russo-Marie,* Mauro Perretti,[†] and
Egle Solito*

From the Department of Cell Biology,* Institut Cochin de
Génétique Moléculaire, Paris, France; and the Department of
Biochemical Pharmacology,[†] The William Harvey Research
Institute, Pharmacology Division, St. Bartholomew's and the
Royal London School of Medicine and Dentistry, London,
United Kingdom

Annexin 1 (ANXA1) is a calcium-binding protein endowed with anti-inflammatory properties. Using an extra-hepatic system, we showed that interleukin (IL)-6 regulates ANXA1 expression at the transcriptional level. The purpose of this study was to determine whether ANXA1 synthesis was modulated by IL-6 during experimental inflammation. We have compared liver ANXA1 expression during systemic and localized inflammatory reaction, using lipopolysaccharide (LPS) and turpentine. LPS treatment strongly induced ANXA1 expression in the liver of wild-type (WT) animals (+600%) whereas a modest increase (+60%) was measured in IL-6 knockout (KO) animals. Turpentine treatment did not affect the expression of ANXA1 in either animal type. LPS enhanced serum corticosteroid levels equally in WT and IL-6 KO mice, whereas higher tumor necrosis factor (TNF)- α and IL-1 β levels were released in IL-6 KO animals. Injection of mouse recombinant IL-6 to IL-6 KO animals before LPS or TNF- α challenge, replenished ANXA1 liver synthesis to that of WT animals. Exogenous ANXA1 but not ANXA5, administered to IL-6 KO mice before LPS challenge inhibited TNF- α release. We propose that ANXA1 acts as a novel acute phase protein, which is controlled in the liver by TNF- α and IL-6, and which may contribute to the resolution of systemic endotoxemia through a negative feedback on TNF- α release. (*Am J Pathol* 2001, 159:1435–1443)

The synthesis of acute phase proteins (APPs) is a protective mechanism of the host during conditions of distress, inflammation, and systemic endotoxemia.¹ The synthesis and release of class 1 APP (eg, serum amyloid A) is initiated by the proinflammatory cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)- α via a mechanism that is IL-6 mediated. Class 2 APPs (eg, α 2-macroglob-

ulin) are induced by IL-6 alone and by cytokines related to the IL-6 family.¹ APPs provide a buffering mechanism that protects the organism from the damaging actions of proinflammatory mediators that are produced in large amounts during acute and chronic inflammatory diseases.^{2–4} The role of endogenous IL-6 in regulating class 2 rather than class 1 APPs has been also addressed using mice deficient in the IL-6 gene [IL-6 knockout (KO) mice].⁵ In these animals, the hepatic expression of α 2-macroglobulin is compromised after intramuscular injection of turpentine oil,⁶ whereas the acute-phase response induced by lipopolysaccharide (LPS) is unchanged. The contribution of other cytokines such as TNF- α , which is released in higher amounts in the IL-6 KO mice, has been implicated in this response.⁵

Annexin 1 (ANXA1) is a glucocorticoid-inducible protein endowed with profound anti-inflammatory activity. Its powerful action in experimental models of inflammation were attributed originally to its ability to inhibit the activity of phospholipase A₂ and, therefore, the production of eicosanoids.^{7,8} More recent studies have demonstrated ANXA1 ability to down-regulate the process of neutrophil⁹ or monocyte¹⁰ adhesion to the activated endothelium. Our previous studies using the lung adenocarcinoma A549 cell line showed that ANXA1 expression is up-regulated by IL-6 and corticosteroids.¹¹ The response to IL-6 was mediated by a C/EBP β transcriptional factor that binds to a specific region of 30 bp.¹² From the pattern of stimulation induced by IL-6 and dexamethasone we have proposed that ANXA1 may participate in host defense as a new APP.¹¹

ANXA1 is expressed in a tissue-specific manner in rodents and, for instance, the liver¹³ or primary hepatic cell¹⁴ show negligible expression of ANXA1 in basal conditions. However, transgenic mice that developed a hepatocarcinoma expressed ANXA1 (in the liver) in a strictly temporal manner, ie, before tumor development.

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Present address of C. d. C.: NIH Pain Center, University of California, 521 Parnassus Ave., San Francisco, CA 94143.

Present address of M. N. A.: Department of Pharmacology and Therapeutics, University of Calgary, 3330 Hospital Dr. NW, Calgary, AB, Canada T2N 4N1.

Address reprint requests to Egle Solito Department of Neuroendocrinology, Imperial College School of Medicine, Hammersmith Campus, Commonwealth Building, Du Cane Rd., London 12 ONN, London, UK. E-mail: e.solito@ic.ac.uk.

Similarly, up-regulation of an ANXA1 isoform phosphorylated on tyrosine 21 was detected during liver regeneration after partial hepatectomy.¹⁴

Glucocorticoid hormones modulate several facets of the host inflammatory response. During experimental endotoxemia, circulating glucocorticoid [corticosterone (CCS) in rodents] levels increase in a time-dependent manner.¹⁵ The role of this hormonal response is not to down-regulate cytokine production,¹⁶ but rather to favor the synthesis of APP in the liver.^{2,17} *In vitro*, glucocorticoids are required for optimal APP induction by IL-1 or IL-6.¹

The present study was performed to monitor ANXA1 expression in the liver of wild-type (WT) and IL-6 KO animals during experimental inflammation. ANXA1 expression was almost absent in the liver of naïve mice. LPS and turpentine were used as agents inducing systemic or local inflammation, respectively. ANXA1 blocks TNF- α release occurring during a systemic inflammatory reaction. We propose ANXA1 as a novel APP, which contributes to the resolution of inflammation.

Materials and Methods

Animals and Treatments

Male C57BL/6J IL6 KO¹⁸ (kindly provided by Transgenic Alliance-IFFA-CREDO, Lyon, France) and control mice (C57BL/6J) from the same genetic background (Center d'Élevage R. Janvier, Le Genest St. Isle, France) (28 to 32g body weight) were used for all experiments. Mice were maintained in standard conditions under a 12-hour light/dark cycle and fed *ad libitum* a chow diet of 6.5% fat, 53% carbohydrate, and 18.6% protein. The procedure followed in the care and killing of the study animals was in accordance with European Community standards on the care and use of laboratory animals (Ministère de l'Agriculture, France; authorization no. 1975).

LPS (from *Escherichia coli* serotype 055:B5) was purchased from Sigma Chemical Co. (St Louis, MO), resuspended in sterile pyrogen-free saline solution and injected intraperitoneally at a dose of 1 mg/kg body weight.¹⁹ A volume of 100 μ l of steam-distilled turpentine was injected intramuscularly.⁵ Turpentine oil (British Pharmacopoeia) was from Thornton and Ross (Huddersfield, England).

Mouse recombinant TNF- α or mouse recombinant IL-6 (R & D Systems, Oxon, UK) were administered intravenously at a dose of 1 μ g per mouse.^{17,20,21} Human recombinant ANXA1 and ANXA5 were administered intravenously at a dose of 10 μ g per animal 30 minutes before LPS treatment.²² Neutralizing rabbit anti-mouse IL-6 or anti-mouse TNF- α polyclonal IgG (R&D Systems, Oxon, UK) were administered intraperitoneally (5 μ g per mouse) 1 hour before LPS (1 mg/kg intraperitoneally).²³ Whereas the glucocorticoid antagonist RU-486 (or mifepristone; Roussel-Uclaf, Romainville, France) was co-injected with LPS, at the dose of 20 mg/kg intraperitoneally.²⁴

ANXA1 mRNA and Protein Level Analysis

At the reported times after stimulus administration, liver total RNA from control and IL-6 KO mice was extracted using the RNAeasy Qiagen Kit (TEBU, Paris, France), following the manufacturer's instructions. After hybridization with the ANXA1 cDNA-radiolabeled probe, filters were hybridized to a DNA fragment coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously reported.²⁵ RNA-DNA hybridization was quantified by densitometric computer analysis in a series 400 Phosphorimager from Molecular Dynamics (Sunnyvale, CA). For protein analysis, liver proteins from WT or KO animals treated or not were extracted in RIPA buffer²⁶ containing protease and phosphatase inhibitors [100 μ mol/L phenylmethyl sulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin (Boehringer Mannheim, Indianapolis, IN), 1 μ mol/L Na₃VO₄, 1 μ mol/L NaF (Boehringer)]. Protein aliquots (30 μ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis²⁷ and electroblotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Immunoreactive proteins were revealed after immunoblotting with a rabbit polyclonal ANXA1 antibody (1/1000).¹⁴ A mouse monoclonal α -tubulin antibody (1/1000; Amersham SA, France) was used as internal control for protein level standardization. Densitometric analysis was performed using an Ultrascan XL Laser densitometer (Agfa, Ridgefield Park, NJ).

Isolation and Culture of Primary Hepatocytes

Hepatocytes were isolated by *in situ* collagenase perfusion of IL-6 KO mice liver according to Decaux and colleagues.²⁸ Perfused liver was minced in M199 medium (Life Technologies, Inc., Gaithersburg, MD) and filtered through a 70- μ m-mesh filter. Viability of recovered cells exceeded 90% as determined by trypan blue exclusion test. Hepatocytes were then plated at a density of 1×10^6 in 60-mm diameter and kept in culture according to Decaux and colleagues,²⁸ in the presence of 1 μ mol/L of dexamethasone (Sigma Chemical Co., St. Louis, MO). After 3 hours, medium was removed and the hepatocytes were cultured under serum-free conditions at 37°C in 5% CO₂ atmosphere.

CCS and TNF- α Measurement

Blood CCS levels were measured by radioimmunoassay, according to the manufacturer's instructions (ICN Pharmaceuticals Ltd., Basingstoke, UK). An enzyme-linked immunosorbent assay kit purchased from R&D System (Abingdon, UK) was used to measure serum TNF- α levels.

Immunohistochemistry

Fresh resected liver and specimens were fixed in 4% paraformaldehyde and embedded in paraffin as described.¹⁴ Sections (5 μ m) were immunostained with a polyclonal antibody directed against the N-terminal do-

main of ANXA1 (1:5000 final dilution) and subsequently developed using a Vectastain ABC kit (Vector Laboratories, Burlingame, Ca). Preimmune serum was used for control staining.¹⁴

Statistical Analysis

All values in the figures and text are expressed as mean $\pm$ SEM of n observations, where n represents the number of animals studied. Data sets were examined by one- and two-way analysis of variance, and individual group means were compared with Student's unpaired t -test. A P value < 0.05 was considered significant.

Results

LPS Injection Induces ANXA1 Expression in IL-6 KO and WT Mice

A low degree of ANXA1 expression was detected in the livers of WT and IL-6 KO mice in basal conditions (Figure 1A). Treatment with LPS (1 mg/kg body weight, intraperitoneally) induced a time-dependent expression of the protein and the mRNA with a peak at 4 hours (approximately sixfold increase greater than basal expression; Figure 1, B and C, referred to the protein level whereas Figure 1D is referred to mRNA expression). ANXA1 protein expression was elevated in WT animals also at the 24-hour time point, whereas it had gone back to basal values by 48 hours after LPS. A modest increase in liver ANXA1 protein level was measured in IL-6 KO mice at all time points under study, with an approximate increase of 60% at 4 hours after LPS (Figure 1B). Similarly, the induction in ANXA1 expression did not last up to the 24-hour time point. Treatment of mice with turpentine did not modify ANXA1 expression in the liver of either WT or IL-6 KO animals (Figure 1C).

No differences in lung or thymus ANXA1 were detected between WT and IL-6 KO mice either in the basal condition or after LPS or turpentine treatment (data not shown). Therefore in the next series of experiments we investigated the *in vivo* mechanism(s) operating during endotoxemia to increase ANXA1 expression in the liver.

Plasma TNF- α , IL-1 β , and CCS Concentration Increases after LPS Administration to Normal and IL-6 KO Animals

To explore whether CCS could participate in ANXA1 induction after LPS administration, we measured serum CCS level both before and after treatment with the two inflammogens. Figure 2A shows that equivalent CCS values were measured in WT and IL-6 KO mice under basal conditions. A comparable increase in CCS plasma levels was seen after LPS treatment in both animal types, with a peak at 4 hours after LPS and values back to normal by the 24-hour time point. LPS treatment induced a significant IL-1 β release maximal at 4 hours in both animal types with a higher magnitude in IL-6 KO mice (~ 3 times

Figure 1. ANXA1 expression in liver of LPS- or turpentine-challenged animals. **A:** Constitutive ANXA1 protein expression (37 kDa) in the liver of WT and IL-6 KO mice as analyzed by Western blot (see Materials and Methods). The immunoblot was stripped and reprobed with anti- α -tubulin mAb (55 kDa). Two animals per group are shown, although data are representative of at least five experiments with similar results. **B:** Time course of ANXA1 protein expression in WT and IL-6 KO mice after LPS (1 mg/kg intraperitoneally) treatment. **C:** The same after turpentine (100 μ l, intramuscularly) treatment. Densitometric analysis was performed using an Ultrascan XL laser densitometer (Agfa). **D:** Northern blot analysis of the time course for ANXA1 mRNA expression in the liver of WT and IL-6 KO mice after LPS (1 mg/kg body intraperitoneally). mRNA is expressed as percent increase of naïve animals normalized to GAPDH mRNA expression. Data for the Western blot are expressed as arbitrary units normalized against tubulin, and are shown as mean values $\pm$ SEM, $n = 10$ mice, whereas for Northern blot analysis, histograms are from three animals per group. *, $P < 0.01$ versus WT.

more than WT mice; Figure 2B). A similar sharp increase in serum TNF- α concentration was measured after LPS injection: in IL-6 KO mice TNF- α levels were almost twice as high as those measured in WT mice. After the 90-minute time point, a time-dependent decrease was observed in both animal species, returning almost back to basal by the 4-hour time point (Figure 2C).

Immunolocalization of LPS-Induced ANXA1 in the Mouse Liver

To identify the cellular source of ANXA1 in the liver, immunohistological analysis was performed on tissues collected from WT and IL-6 KO animals, with or without treatment with LPS. Control liver tissues from WT and KO animals show no obvious staining for ANXA1 (Figure 3, a and b). The large majority of ANXA1 expression in the liver of endotoxic mice was observed in most hepatocytic

Figure 2. Effect of LPS and turpentine on serum CCS, IL-1 β , and TNF- α levels in WT and IL-6 KO mice. Mice received 1 mg/kg LPS or 10 ml/kg sterile saline at time 0. CCS levels (A), IL-1 β (B), and TNF- α (C) levels in the serum as measured by radioimmunoassay and ELISA, respectively. Data are shown as mean values $\pm$ SEM, $n = 6$. *, $P < 0.01$ versus appropriate value at time 0. \$, $P < 0.01$ versus correspondent WT value.

cells 4 hours after LPS injection into WT mice (Figure 3c), and with less expression in IL-6 KO animals (Figure 3d).

Recombinant IL-6 Restores Liver ANXA1 Levels in IL-6 KO Mice after LPS Injection

Injection of mouse recombinant IL-6 (1 μ g/mouse, 4 hours) to IL-6 KO mice augmented ANXA1 expression after LPS injection, bringing it back to the degree of

Figure 3. ANXA1 immunolocalization in liver of WT or IL-6 KO mice treated with LPS. **a** and **b**: WT and IL-6 KO liver sections showed no or scarce ANXA1 expression. **c** and **d**: Intense ANXA1 immunostaining is detected in both animal types 4 hours after LPS (1 mg/kg intraperitoneally) injection. All sections were counterstained with Mayer's hematoxylin. Scale bars, 450 μ m.

expression detected in WT mice (Figure 4). In this set of experiments an approximate eightfold increase in ANXA1 protein was measured in LPS-treated WT animals, and a value of 9 ± 0.5 -fold increase was attained in IL-6 KO mice treated with LPS + IL-6. Interestingly, IL-6 alone was not sufficient to promote ANXA1 expression (Figure 4).

Next, the effect of TNF- α , which levels varied between the two types of animals after LPS, was tested alone or together with IL-6. Injected at the dose of 1 μ g per mouse, TNF- α significantly induced liver ANXA1 expression in WT animals, at levels similar to those achieved with LPS. Quite surprisingly, a pronounced response was also measured in IL-6 KO mice (Figure 4). However, co-injection with IL-6 (1 μ g) produced maximal ANXA1 expression in the latter animals.

Figure 4. Modulation of hepatic ANXA1 protein expression by IL-6 and TNF- α . Western blot analysis of ANXA1 liver expression after treatment with mouse recombinant IL-6 (1 μ g i.v.) or TNF- α (1 μ g i.v.), alone or in combination with LPS (1 mg/kg intraperitoneally). Protein extracts were prepared as described in Material and Methods. Densitometric analysis was performed using an Ultrascan XL laser densitometer (Agfa). Values represent percent of ANXA1 expression relative to respective controls (WT or IL-6 KO mice). Data are shown as mean values $\pm$ SEM, $n = 6$. *, $P < 0.05$ and **, $P < 0.01$ versus relative control (LPS- or TNF- α -treated animals).

Figure 5. IL-6 and TNF- α up-regulate ANXA1 expression in isolated hepatocytes. Primary hepatocytes were obtained by liver perfusion from WT or IL-6 KO animals as described in Material and Methods. Cells were then treated with IL-6 (10 ng/ml) or TNF- α (2 ng/ml) for the reported time points. Proteins were extracted and Western blot performed according to Material and Methods. **A:** A representative Western blot is reported for cells taken from both WT and IL-6 KO animals. **B:** Data (normalized against α -tubulin expression) expressed as percent of untreated hepatocytes (control) are shown as mean values $\pm$ SEM, from six different WT or IL-6 KO-perfused liver preparations. *, $P < 0.05$ versus relative control (unstimulated cells).

ANXA1 Expression in Perfused Hepatocytes from WT and IL-6 KO Mice Stimulated in Vitro

To verify if IL-6 or TNF- α was able to target the ANXA1 expression directly on the hepatocyte, primary cultured hepatocytes prepared from perfused livers of WT or IL-6 KO animals were treated with the two different cytokines. In cells taken from WT mice, exogenous IL-6 induced an up-regulation of ANXA1 expression already at 3 hours (Figure 5A). In contrast IL-6 KO animal primary hepatocytes needed a prolonged (24 hours) incubation with IL-6 (10 ng/ml) to produce a significant response. A similar effect was seen with TNF- α (2 ng/ml), although the response to this cytokine was more rapid in primary hepatocytes from both WT and KO (Figure 5, A and B). There was no synergism or additive effect when the cytokines were co-incubated for 24 hours.

Effect of Neutralizing Antibodies against TNF- α or IL-6 on ANXA1 Expression

Between 50 and 60% inhibition of LPS-induced liver ANXA1 protein expression was measured in WT and IL-6 KO mice pretreated with an anti-TNF- α antibody (Figure 6). In this set of experiments, the effect of the glucocorticoid antagonist RU-486 was also tested. RU-486 (20

Figure 6. Inhibition of ANXA1 protein expression by neutralizing anti-IL-6 and anti-TNF- α antibodies. WT or IL-6 KO mice were treated by neutralizing antibodies (10 μ g intraperitoneally) raised against TNF- α or IL-6 1 hour before LPS (1 mg/kg intraperitoneally) administration. RU-486 (20 mg/kg intraperitoneally) was administered simultaneously with LPS. Total proteins were extracted from livers and ANXA1 expression was evaluated by Western blot. Histogram represents ANXA1 expression (arbitrary unit) in WT or KO animals, and are representative of three independent experiments each performed with four mice per group. *, $P < 0.01$.

mg/kg) was highly effective in WT mice (~60% reduction in the ANXA1 response), whereas it was significantly less active in IL-6 KO mice (Figure 6). A mutual exclusion and certainly not an additive effect were observed when the two treatments were given together.

The effect of an anti-IL-6 antibody was tested in WT mice: comparable inhibition (between 30 and 45%) in liver ANXA1 protein expression was measured, again without an additive effect with the glucocorticoid antagonist RU-486 (Figure 6).

Administration of Human Recombinant ANXA1 Reduces TNF- α and IL-1 β Release

In IL-6 KO mice, TNF- α release was twofold to threefold higher than in WT mice (see Figure 2). As suggested by Fattori and colleagues,⁵ this indicates that endogenous IL-6 controls TNF- α production during endotoxemia. Because ANXA1 is up-regulated by IL-6, and because IL-6 modulates TNF- α production, we next tested if ANXA1 participates in the modulation of TNF- α release. Figure 7 illustrates these data, with injection of mouse recombinant IL-6 to IL-6 KO mice being able to significantly reduce TNF- α (Figure 7A) but not IL-1 β (Figure 7B) plasma levels measured after LPS. We then tested the effect of recombinant ANXA1. In this series of experiments, LPS released >2 ng/ml of TNF- α as measured at 90 minutes after injection. A significant reduction (~25%) in TNF- α or (77%) IL-1 β was measured in the group of animals treated with ANXA1, whereas the structurally related protein ANXA5 was essentially inactive (Figure 7).

Discussion

This study stems from the investigation of Fattori and colleagues,⁵ in which the liver response to systemic and

Figure 7. Extracellular role of ANXA1 in endotoxemic IL-6 KO mice. IL-6 (1 μ g i.v.), human recombinant ANXA1 (10 μ g i.v.), or ANXA5 (10 μ g i.v.) were administered 1 hour before LPS (1 mg/kg intraperitoneally) challenge. **A:** Circulating TNF- α levels were measured by ELISA at the 90-minute time point. **B:** Circulating IL-1 β was measured by ELISA at 4-hour time point. Data are shown as mean values $\pm$ SEM, $n = 6$. *, $P < 0.01$ versus LPS alone.

local inflammation, produced with LPS and turpentine, respectively, was studied in the mouse. At variance from serum amyloid protein A,⁵ ANXA1 expression in the liver was not modified by intramuscular injection of turpentine oil in either WT or IL-6 KO mice. In contrast, marked liver ANXA1 gene and protein synthesis were induced by LPS, and this was attenuated in IL-6 KO mice. We therefore conclude that liver ANXA1 is not overexpressed as a result of local inflammation (eg, as the one induced by turpentine) but rather as the result of a systemic inflammatory condition (eg, experimentally reproduced with LPS). For this reason, turpentine-induced inflammation was no longer used in the subsequent experiments.

LPS injection to the experimental animal initiates a cascade of events that has been investigated in detail throughout the past two decades, characterized by a time-dependent and concerted release of proinflammatory cytokines.^{29–31} Monocyte/macrophage-derived TNF- α is the first cytokine release by LPS with plasma peak around 90 minutes,^{5,15,32} and high levels of TNF- α were also measured in our experimental conditions. This surge in TNF- α is a prerequisite to subsequent production of other mediators including IL-1 β , IL-8,^{33,34} and

Figure 8. Proposed mechanism for liver ANXA1 expression in experimental endotoxemia. LPS triggers the release of proinflammatory cytokines, including TNF- α , IL-6, and IL-1 β that promote expression of ANXA1 in the liver. CCS plays a permissive role. Higher ANXA1 expression, if released in the circulation, may exert a negative feedback on TNF- α and IL-1 β release.

IL-6.⁵ Cytokine therapy of human endotoxic shock, although promising, has failed to reduce mortality,³⁵ suggesting the need for other forms of therapies. In this study we focused on the anti-inflammatory protein ANXA1,³⁶ and we show that TNF- α and IL-6 modulate its liver expression during endotoxemia. It is tempting to propose that ANXA1 expression is part of an endogenous protective loop aiming to down-regulate the effects of endotoxemia.

The existence of a strict link between TNF- α and ANXA1 protein expression in the liver is supported by the fact that administration of an anti-TNF- α antibody to LPS-treated mice reduced, whereas TNF- α addition to WT animals increased, ANXA1 expression. The changes seen after LPS or TNF- α treatment were remarkably high (6- to 10-fold greater than basal expression), probably because of the modest protein expression detected in the liver of untreated mice. Low basal expression of ANXA1 has also been reported in the rat liver.^{13,37} These data complement recent *in vitro* observations with U937 cells in which ANXA1 cellular content was increased on TNF- α treatment and before entering into apoptosis.³⁸ However, probably without surprise, the scenario operating in experimental endotoxemia and leading to up-regulation of ANXA1 protein levels was more complex. A defective ANXA1 expression was measured in LPS-treated IL-6 KO mice, strongly indicating a functional role for endogenous IL-6 in the regulation of ANXA1 synthesis in the liver. In addition, anti-IL-6 antibody inhibited LPS-induced ANXA1 expression in WT mice. Finally, injection of mouse recombinant IL-6 restored LPS ability to increase ANXA1 protein content in the liver of IL-6 KO mice. An augmented TNF- α concentration was also measured in the plasma of IL-6 KO mice which is probably responsible for the significant ANXA1 induction observed in these mice, although less than that in WT mice.

Together these data indicate the existence of a complex interplay between endogenous IL-6 and TNF- α in

this model of endotoxemia. Figure 8 summarizes this finding showing that endogenous IL-6 is required to down-regulate, at least in part, LPS-induced TNF- α synthesis and release, and both cytokines cooperate to achieve optimal ANXA1 expression after LPS treatment. The situation *in vitro* is clearly simpler, as isolated hepatocytes collected from either WT or IL-6 KO mice responded to the addition of IL-6 or TNF- α with increased ANXA1 expression, although with a different optimal time point. It is tempting to propose, also in view of the higher TNF- α levels measured in IL-6 KO mice, that in these animals TNF- α may partially compensate for IL-6 absence. Nonetheless, *in vivo*, this is not sufficient because IL-6 KO have a remarkably reduced hepatic ANXA1 expression after LPS injection. Because these mice responded well to TNF- α treatment alone (more or less as good as WT animals) it is possible that other factors released during endotoxemia can complicate the scenario. Overall, we would like to propose a major role for TNF- α with a crucial permissive action of IL-6 in modulating ANXA1 liver expression in endotoxemic naïve mice.

The scenario is complicated further by the effect of the glucocorticoid antagonist RU-486. Glucocorticoid's interrelationship with cytokines occurs at multiple levels. With respect to liver function, CCS ability to up-regulate cytokine receptors^{39–41} and therefore facilitate cytokine effects on liver production of APPs^{42,43} has long been known. Similarly, cytokines such as TNF- α and IL-1 β modulate expression of the glucocorticoid receptor and its interaction with specific nuclear response elements.^{44,45} In our experimental set up endogenous CCS was released in WT and IL-6 KO animals to a similar extent, and therefore it is unlikely to account for the differences observed in liver ANXA1 expression during endotoxemia. Nonetheless, the glucocorticoid antagonist RU-486 diminished LPS-induced ANXA1 expression in the IL-6 KO mice, an expression in itself modest when compared to WT animals. Therefore, endogenous CCS does not modulate liver ANXA1 expression directly, but it may exert a permissive effect of the inducing activity of IL-6 and TNF- α . It is now accepted that endogenous glucocorticoid hormones released during endotoxemia do not acutely control cytokine generation,⁴⁶ but rather have the function to permit an optimal APP response.^{2,4} In addition, endogenous glucocorticoids exert an important effect on induction of tolerance, as seen after repeated exposure to LPS.^{15,47,48}

The mechanism(s) by which IL-6 and TNF- α , alone or together with CCS, operate to stimulate ANXA1 synthesis and protein expression during endotoxemia are at present obscure. *In vitro*, we showed that IL-6 (24 hours, but not another gp130 cytokine family member) trans-activated ANXA1 promoter expression through NFIL-6 transcription factor and produced *de novo* ANXA1 synthesis in A549 lung adenoma cell line.¹¹ These data are confirmed by the experiments with primary hepatocytes stimulated *in vitro* with IL-6. It is likely that a similar mechanism occurs *in vivo* in the liver. Data in the literature⁴⁹ showed a reduced expression of C/EBP- β (murine equivalent of NFIL-6) in nuclei extracts from the hepatocytes

taken from IL-6 KO compared to WT mice after LPS treatment. In preliminary experiments we could confirm this reduction and observed restoration of C/EBP- β localization after IL-6 treatment (Egle Solito, unpublished results). Finally, IL-6 and TNF- α interaction at the level of each cytokine receptor(s) has also been reported⁵⁰ but the involvement of this mechanism in the events observed in our study is at the moment a matter of speculation.

The inhibitory role of IL-6 on TNF- α production,⁵ which is confined to the peripheral system (macrophage cells and not microglia), is well known.⁵⁰ In contrast less is known about the role of ANXA1 on TNF- α release. In one report, the glucocorticoid inhibitory effect on TNF- α release from human monocytes *in vitro* was shown to be mediated by endogenous ANXA1 acting in an autocrine manner.⁵¹ We show here for the first time that ANXA1, but not the structurally related protein ANXA5, blocked the release of TNF- α after *in vivo* LPS challenge. This effect is similar to that seen with IL-6 itself, and in view of the inducing action displayed by the latter cytokine, we propose that ANXA1 may be a mediator of this action of IL-6. A different pattern of effect was seen with respect to IL-1 β release, with ANXA1 but not IL-6 treatment, being able to produce a highly significant inhibition.

Many results argue in favor of an extracellular role of ANXA1.^{52,53} Because ANXA1 is externalized on the cell membrane after either glucocorticoid⁵¹ or IL-6 treatment¹¹ we cannot exclude a possible mechanism in which ANXA1 can interact with a ligand^{10,54} on the plasma membrane. Cellular and systemic responses to endotoxin are produced by binding to receptors such as monocyte/macrophage CD14,^{55–57} leading to induction of inflammatory reaction. It could be envisaged that ANXA1 could block such pathway of signaling. On the other hand human recombinant ANXA1 binds to lipid A possibly modulating its activity and/or interaction with other endotoxin-binding proteins.⁵³ Finally, ANXA1 down-regulates macrophage/monocyte activation,^{51,58,59} and because cells of this lineage produce the majority of TNF- α and IL-1 β released after LPS injection⁵⁰ a mechanism of selective down-regulation of cell activation could explain the significant inhibitory effect displayed by human recombinant ANXA1 on circulating levels of these two cytokines. Because ANXA1 does not directly inhibit IL-1 β secretion from human monocytes,⁶⁰ at variance from TNF- α ,⁵¹ it is tempting to propose that ANXA1 inhibition of LPS-induced raise in serum IL-1 β could be secondary to TNF- α inhibition, or that IL-1 β is coming from other cell sources (eg, Kupffer cells) sensitive to ANXA1. Finally, it is interesting that ANXA1 and IL-6 may be complementary in their *in vivo* protective role during endotoxemia, with the former displaying stronger inhibition on IL-1 β , and the latter being much more effective on TNF- α . It is likely that with these data we are beginning to unravel a novel positive loop between IL-6 and ANXA1 that guarantees an effective inhibition on the synthesis of proinflammatory cytokines.

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