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Fibronectin extra domain a limits liver dysfunction and protects mice during acute inflammation☆

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ABSTRACT

Background and aim: The primary transcript of fibronectin (FN) undergoes alternative splicing to generate different isoforms, including FN containing the Extra Domain A (FN_EDA+), whose expression is regulated spatially and temporarily during developmental and disease conditions including acute inflammation. The role of FN_EDA+ during sepsis, however, remains elusive.
Methods: Mice constitutively express the EDA domain of fibronectin ($EDA^{+/+}$); lacking the FN EDA domain ($EDA^{-/-}$) or with a conditional ablation of EDA + inclusion only in liver produced FN (alb-CRE^{+}EDA floxed mice) thus expressing normal plasma FN were used. Systemic inflammation and sepsis were induced by either LPS injection (70 mg/kg) or by cecal ligation and puncture (CLP) Neutrophils isolated from septic patients were tested for neutrophil binding ability.
Results: We observed that $EDA^{+/+}$ were protected toward sepsis as compared to $EDA^{-/-}$ mice. Also alb-CRE^{+}EDA floxed mice presented reduced survival, thus indicating a key role for EDA in protecting toward sepsis. This phenotype was associated with improved liver and spleen inflammatory profile. Ex vivo experiments showed that neutrophils bind to a larger extent to an FN_EDA + coated surface as compared to FN, thus potentially limiting their over-reactivity.
Conclusions: Our study demonstrates that the inclusion of the EDA domain in fibronectin dampens the nflammatoryi consequences of sepsis.
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1. Introduction

Sepsis is characterized by a dysregulated inflammatory response to an infection [1,2]. The incidence of sepsis is rising at a rate of 8.8% per year and the number of deaths increases continuously despite the advances in critical care medicine [3]. Although inflammatory cytokines produced by immune cells of both innate and adaptive systems are critical for the host to eradicate the invading pathogens [4,5], their uncontrolled release has been proposed to contribute to sepsis-related over-inflammation. At the same time, in septic patients, neutrophil and macrophage functions are largely impaired, and this can also contribute to bacterial growth [6].

To this aim, corticosteroids, interleukin (IL)-1R antagonists, toll-like receptor (TLR)-antagonists, and tumour necrosis factor (TNF) antagonists were all tested in clinical trials, as potential strategies to limit the overactivation of the inflammatory response during sepsis. However, none of these approaches was successful, suggesting that, beyond uncontrolled inflammation, other pathophysiological mechanisms could contribute to death following severe sepsis [7–12].

Among the acute phase proteins modulated during sepsis, changes in fibronectin (FN) levels were proposed as a prognostic marker of sepsis severity in humans and the injection of the FN fragment was shown to protect from sepsis [13–18]. Previous

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Abbreviations and acronyms

FN	Fibronectin
EDA	Extra domain A
CLP	Cecal Ligation and Puncture
IIICS	Type 3 connecting segment
$EDA^{+/+}$	Constitutive expressing FN + EDA
$EDA^{-/-}$	Constitutive lacking FN-EDA
$EDA^{wt/wt}$	Control animals
Alb-CRE	CRE-Recombinase driven under albumin promoter

reports have shown that FN or its fragments can potentially interact with host pathogens during normal and pathological conditions [19,20].

FN exists in different isoforms which are generated as a consequence of the alternative splicing of a single primary transcript, with the inclusion of the extra domain A (EDA) being one of the most relevant and resulting in the generation of EDA-containing FN (FN_EDA+) [21]. The liver is the major contributor of soluble plasma FN, a variant that does not contain the EDA domain [22]. Vice versa, FN produced in other tissues undergoes alternative splicing and represents a key component of the extracellular matrix (ECM) [23]. Interestingly, during sepsis, the balance between the different FN isoforms in ECM is altered with an increase in FN_EDA+ and a reduction in FN lacking EDA (FN_EDA-) [17,24]. Whether these changes reflect the adaptation of the ECM to regulate leukocyte *trans*-endothelial migration and tissue penetration and how this contributes to the regulation of infection is debated [25].

In this study, we sought to investigate the role and mechanism of action of FN_EDA+ in the pathogenesis of polymicrobial sepsis. We took advantage of mouse models constitutively expressing EDA ($EDA^{+/+}$), constitutively lacking EDA ($EDA^{-/-}$), or a conditional knock-out model, in which the liver produces FN_EDA-, whereas other tissues produce only FN_EDA+. The latter is an animal model with plasma FN levels similar to control mice but always presenting EDA inclusion in FN produced in peripheral tissues [26,27].

Our study shows that the presence of FN_EDA + protects from sepsis by interacting with neutrophils and limiting the uncontrolled inflammatory response.

2. Results

2.1. Constitutive inclusion of the EDA domain in fibronectin decreases mortality in both LPS and CLP models of septic shock

Following the intraperitoneal injection of a sub-lethal dose of LPS, $EDA^{+/+}$ mice showed an increased survival rate compared to $EDA^{wt/wt}$ and $EDA^{-/-}$ animals (Fig. 1A). Of note, mice with liver-specific deletion of the EDA exon (Alb-CRE + $EDA^{+/+}$ mice produce FN_EDA-only in the liver and therefore FN_EDA-is present in the circulation while FN_EDA + production is maintained in other tissues), had a profile similar to that of $EDA^{wt/wt}$ and $EDA^{-/-}$ mice (Fig. 1A). This finding suggests that liver-derived circulating FN_EDA + might protect from sepsis. To further address this hypothesis, the impact of FN_EDA+ was tested in a second model of sepsis (cecal ligation puncture (CLP)-induced polymicrobial sepsis). Under this experimental condition, $EDA^{+/+}$ mice presented a significant increase in survival compared to both $EDA^{wt/wt}$ and $EDA^{-/-}$ mice (Fig. 1B). The survival rate of Alb-CRE + $EDA^{+/+}$ mice was similar to that of $EDA^{wt/wt}$ and $EDA^{-/-}$ mice (Fig. 1B), again casting for a role of circulating FN_EDA+ in protecting from sepsis. To exclude the possibility that the increased survival observed in $EDA^{+/+}$ mice could be the consequence of lower circulating FN levels rather than the peculiar production of FN_EDA+, we compared the survival of EDA haplodeficient mice ($EDA^{+/wt}$) and $EDA^{-/wt}$ mice to CLP-induced polymicrobial sepsis. The two models differ in FN isoforms, with $EDA^{+/wt}$ presenting a certain amount of FN_EDA+ in plasma; of note $EDA^{+/wt}$ mice had an increased survival rate when compared to $EDA^{-/wt}$ (Fig. 1C).

2.2. Constitutive inclusion of the EDA domain in fibronectin reduces plasma levels of hepatic enzymes

Given that the presence of FN_EDA + significantly increased the survival rate of $EDA^{+/+}$ mice, we sought to understand the mechanism beyond this effect. First, we measured the levels of circulating markers of inflammation with a focus on liver enzymes [28]. We found that 24 h after the CLP challenge, plasma levels of creatinine, AST, and ALT were significantly lower in $EDA^{+/+}$ mice than in $EDA^{wt/wt}$ and $EDA^{-/-}$ mice (Fig. 2A-C). These data are in line with what was reported above and suggest that FN_EDA + could be associated with reduced organ dysfunction during sepsis. The latter observation prompted us to investigate whether the clearance of bacterial overload could be improved in $EDA^{+/+}$ mice.

2.3. Constitutive inclusion of the EDA domain in fibronectin promotes changes in circulating innate immune cell distribution

To address whether the improved outcome observed in $EDA^{+/+}$ mice was the consequence of a different immune response, we evaluated possible changes in the blood distribution of immune cells during acute inflammation (Supplemental Fig. 1). Following LPS injection, a general increase in myeloid CD11b + cells was observed in all genotypes, but only $EDA^{wt/wt}$ mice showed a significant increase in the prevalence of CD11b + cells compared to the baseline (Fig. 3A). The increased frequency of CD11b + cells in $EDA^{wt/wt}$ mice after 24h was mainly related to a relative increase in neutrophils (Fig. 3B), paralleled with a decreased proportion of monocytes (Fig. 3C). Within the monocyte population, a similar distribution of Ly6Chigh, intermediate and low subsets was observed (Fig. 3D-F). These findings are consistent with previous reports displaying an increase in neutrophils during LPS-mediated endotoxemia [29,30] and would support the hypothesis of an involvement of fibronectin in the immune response. Indeed, it has been proposed that fibronectin could stabilize the attachment of the pathogen to the endothelium under flow conditions [25,31,32] favouring leukocyte transmigration.

2.4. The inclusion of the EDA domain in fibronectin increases splenic markers of bacterial clearance

To address whether this could be the case in our experimental setting, we evaluated the role of fibronectin in splenic cellular clearance. First, we profiled changes in key inflammatory markers in the spleen and observed an increased expression of mediators involved in bacterial clearance such as CXCL1, INFgamma or IL4 in $EDA^{+/+}$ mice compared to $EDA^{wt/wt}$ and $EDA^{-/-}$ while other chemokines and cytokines were not different or reduced like IL6 (Fig. 4A-F). Of note, we did not find any difference in cytokines mainly contributed by macrophages (Supplementary Fig. 2) or eosinophils (Supplementary Fig. 3), thus limiting a role for these cells in promoting survival to polymicrobial sepsis in $EDA^{+/+}$ mice. Altogether, these data suggest that splenic acute responses do play a major role in mediating survival.

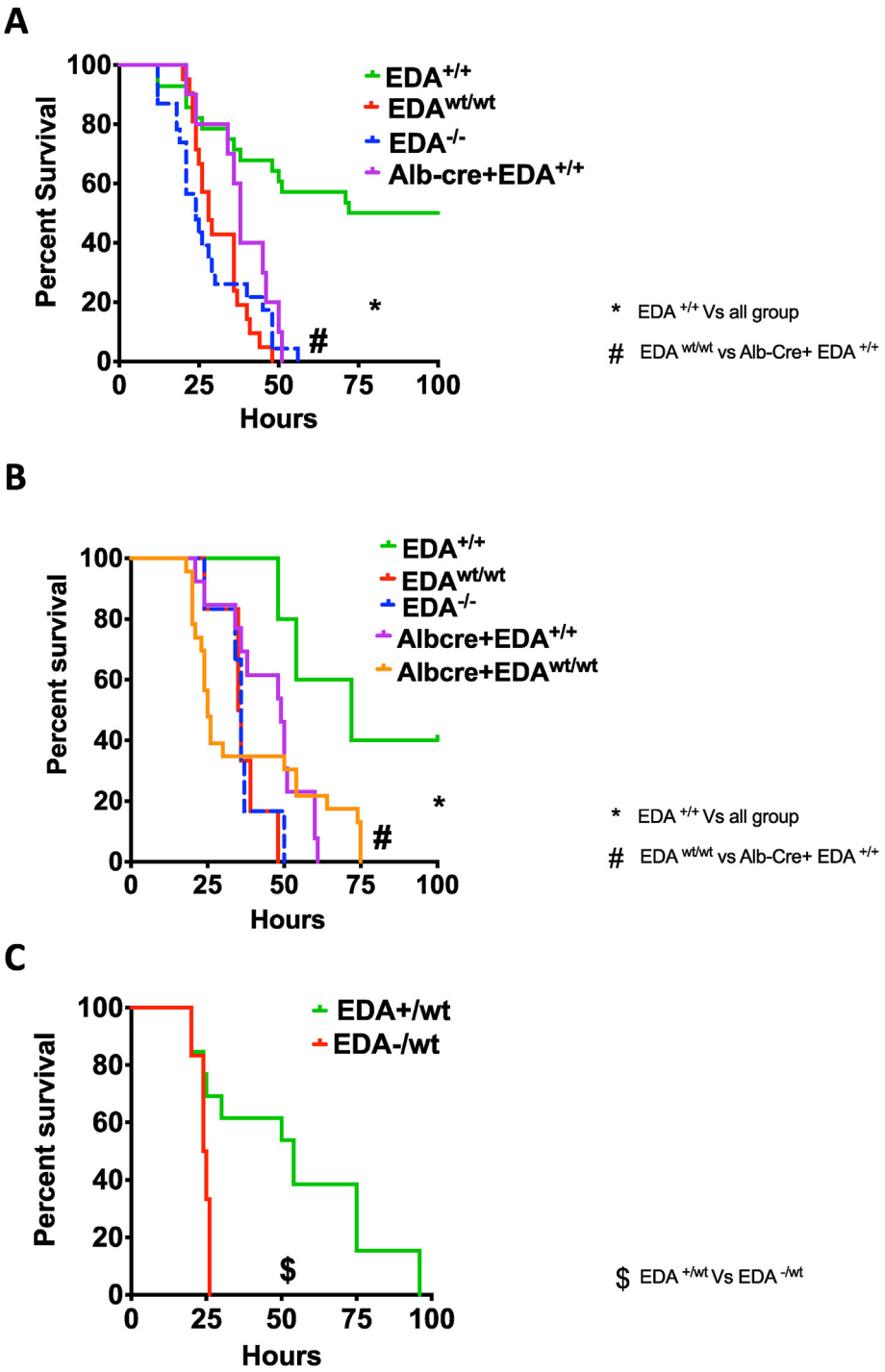

Fig. 1. Constitutive inclusion of the EDA domain in fibronectin increases the survival in murine models of endotoxemia and CLP. A) Survival curve of $EDA^{+/+}$, $EDA^{-/-}$, and $EDA^{wt/wt}$ mice (N = 6) towards CLP model of polymicrobial sepsis B) Survival curve of $EDA^{+/+}$, $EDA^{-/-}$, and $EDA^{wt/wt}$ mice (N = 6) towards LPS model of polymicrobial sepsis. C) Survival curve of $EDA^{+/wt}$, EDA-/wt and EDA ±mice (N = 6) in CLP model of polymicrobial sepsis D) Survival curve of $EDA^{+/+}$ albcre+, $EDA^{wt/wt}$albcre + animals (N = 6) in CLP model of polymicrobial sepsis. Data are expressed in Mean ± SEM.*P < 0.01, one-way ANOVA Comparing $EDA^{+/+}$ vs $EDA^{wt/wt}$ and $EDA^{-/-}$.

2.5. Fibronectin-EDA is affected in during metabolic diseases and increases neutrophil adherence under flow conditions

ScRNAseq data showed that Fibronectin (FN1) expression as well as FN_EDA + expression is increased during obesity in human liver (Fig. 5A to D) mainly in Cholangiocyte, Hepatocytes and Fibroblast (FN1) and Fibroblast and Endothelial cells (FN_EDA+) compared to controls. Furthermore, to evaluate the effect of the EDA domain inclusion in fibronectin on immune response, we used human activated neutrophils isolated from septic shock patients and tested their adherence to FN_EDA + - compared to FN_EDA- coated surfaces (Fig. 5E). An increased adherence to an FN_EDA + -coated surface was observed suggesting that the presence of the EDA domain might favour neutrophil rolling and adhesion.

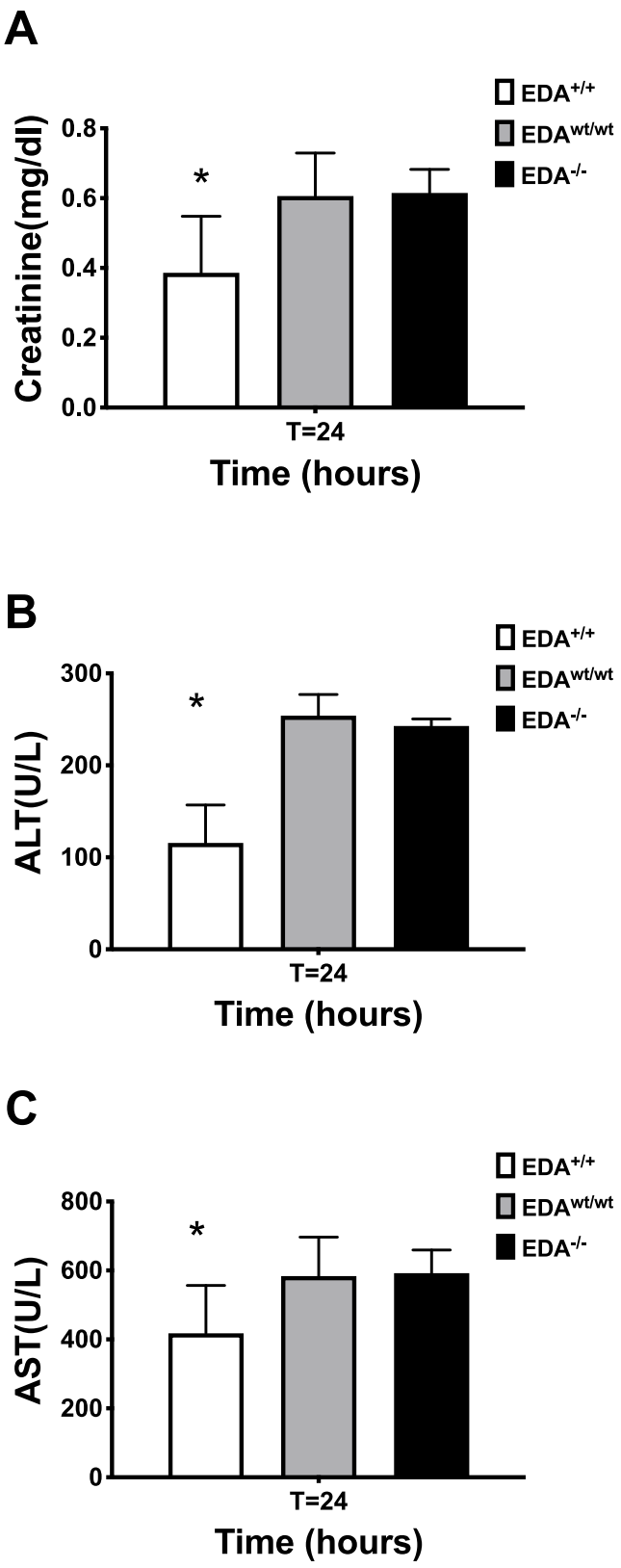

Fig. 2. Constitutive inclusion of the EDA domain in fibronectin reduces plasma levels of non-specific enzymes. A) Creatinine, B) alanine transaminase (ALT), and C) aspartate transaminase (AST) are shown in absolute values. Data are expressed in mean ± SEM of at least 6 animals per group. *P < 0.05, one-way ANOVA comparing $EDA^{+/+}$vs $EDA^{wt/wt}$ and $EDA^{-/-}$for time point 24 h (T = 24).

3. Discussion

Physiologically, plasma FN lacks the EDA domain and can be deposited in extracellular matrices [26]. Plasma FN levels change under different pathological conditions including atherosclerosis [33], obesity [34], diabetes [35] and sepsis [36]. During sepsis, for instance, a poorer outcome is observed among subjects with the most pronounced plasma FN level reduction [37–42]. These findings raised the question of whether fibronectin could play a role during acute inflammation thanks to its capability to act at the crossroad between the vasculature and the systemic response [18,43–45]. Few studies have shown that plasma fibronectin could be a potential biomarker and a possible opsonin in sepsis [17] however, a clear explanation is still lacking.

Our data indicate that the inclusion of the EDA domain in circulating fibronectin increases the survival in murine models of CLP and LPS-mediated endotoxemia. Liver-specific knock-out of EDA, which results only in circulating FN devoid of EDA, while EDA_FN+ is produced in peripheral tissues since birth, were not protected from sepsis, thus indicating that plasma EDA_FN + plays a key role in the observed protective effects. This finding is further supported by the observation in EDA ± mice, which present circulating levels of fibronectin similar to a WT mouse but have higher plasma levels of FN_EDA + than WT, are more protected from CLP and LPS-mediated endotoxemia and mortality.

In humans, although contradictory findings have been reported, a general reduction of FN plasma levels was observed during acute inflammation associated with sepsis [13] coupled with increased production of circulating FN containing EDA [46,47]. Despite this, the concentration of plasma FN_EDA+ in patients with sepsis who survived was significantly lower than that in non-survivors [13]. Whether this is indirectly marking the ability of FN_EDA + to opsonize bacteria and favour the elimination of the complex or increased deposition in tissues during acute and severe inflammation is unclear.

Interestingly we observed that neutrophils appeared to adhere more strongly to cellular FN (which contains EDA) as compared to plasma fibronectin (which does not contain EDA). This observation is in line with the available findings suggesting that the presence of EDA confers the ability to interact with different integrins and redirect leukocyte-matrix interaction [48].

Notably, FN_EDA+ was proposed to activate TLR-4 and to promote the expression of genes involved in the inflammatory response [49]. This response was shown to be critical in improving the response to infection and indeed inhibiting the TLR-4 response results in reduced bacterial clearance and augment sepsis [50]. Such increase in FN_EDA + -mediated responses could be beneficial under severe and acute infections.

Is this mechanism relevant for vascular disorders such as atherosclerosis? We have previously shown that FN_EDA+, although it did not impact on atherosclerotic plaque area in apolipoprotein E and LDL-receptor-deficient mice, resulted in a more stable atherosclerotic plaque phenotype. This highlights the relevance of FN_EDA + as an isoform at the crossroad between plaque burden and stability [27]. On the same line, the observation in the context of acute inflammation following sepsis in this work highlights the role of alternative splicing of FN as a mechanism finely tuned to balance physiopathological responses under different vascular acute and chronic inflammatory conditions.

In summary, our study suggests that the presence of FN_EDA+ in plasma increases survival during severe sepsis-related inflammation. The possibility that this phenotype could depend on the increased neutrophil retention and increased expression of splenic cell markers facilitating the clearance of bacterial load should be explored further.

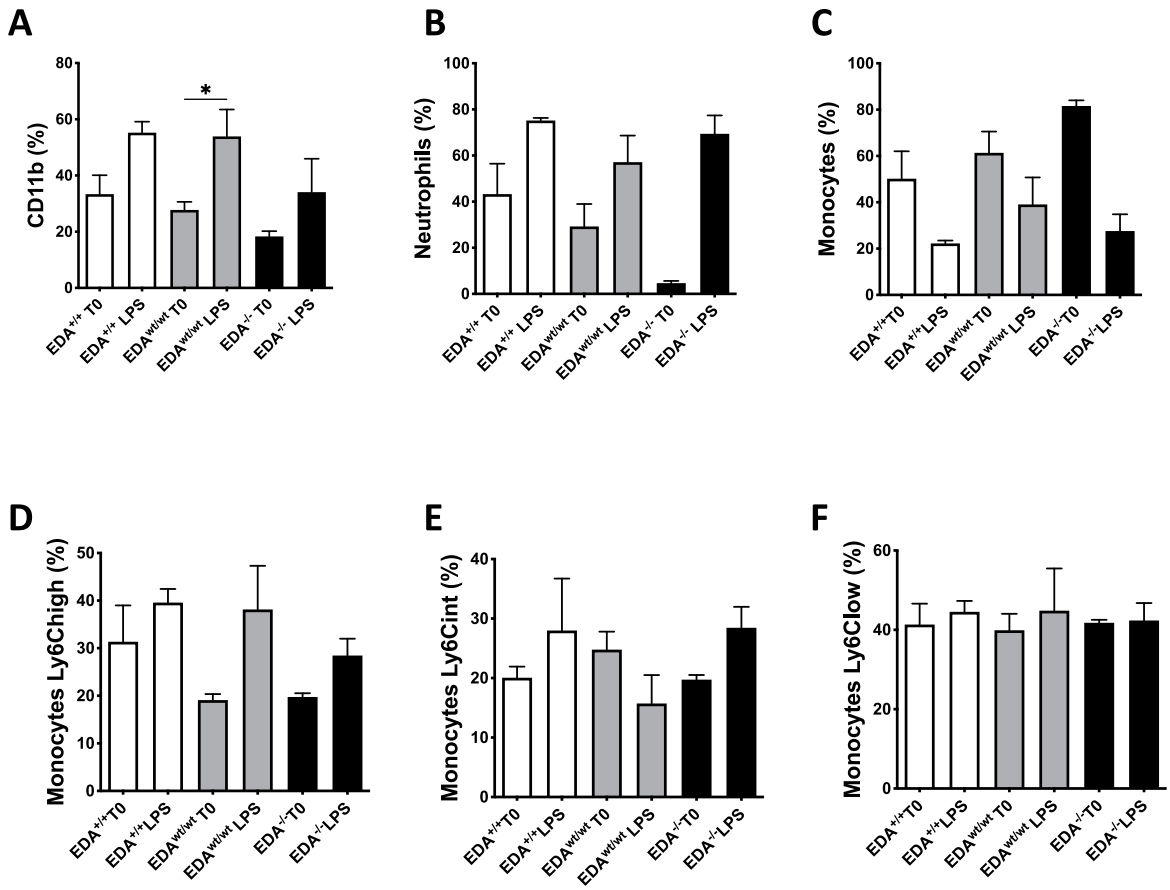

Fig. 3. Characterization of systemic inflammatory cells after LPS injection. A) Characterization of monocytes and neutrophils in $EDA^{+/+}$, $EDA^{wt/wt}$ and $EDA^{-/-}$ mice at time zero and after challenge with LPS. B) CD11b positive cells out of Leukocytes. C) Neutrophil percentage identified as LY6C + Ly6G + cells. D) Monocyte percentage identified as Ly6C + Ly6G-cells). E-G) Monocytes respectively low, intermediate and high based on the positivity to Ly6C. Data are presented as mean ± SEM, n = 2–7,*P < 0.05.

4. Materials and methods

4.1. Animal models and reagents

The mouse models were generated as described previously [51,52]. The liver-specific knock-out of fibronectin containing the EDA domain was generated as described [23]. $EDA^{wt/wt}$ mice were used as controls. All the animals were on a C57BL/6 background. Heterozygous mice $EDA^{+/wt}$, $EDA^{-/wt}$ and EDA ± were also used. The amount of total fibronectin and EDA domain-including fibronectin produced in these animal models has been described previously [23]. All experiments were performed using age-matched littermate controls. The investigation conforms to the European Commission Directive 2010/63/EU and was approved by the local authorities (Progetto di Ricerca Protocollo 2009/3 and 2012/2). Animal studies are reported in compliance with the ARRIVE guidelines [53,54]. Mice were housed in Tecniplast ventilated cage systems under specific pathogen-free conditions (standard 12 h light/dark cycle), with wood shaving-based bedding, free access to chow and autoclaved water, and housed with no more than five animals per cage. All mice were randomly allocated to cages designated for specific treatment groups by vivarium staff, upon transfer from the breeding barrier unit into the animal housing room. All mice purchased from vendors or transferred from our breeding barrier unit were acclimatized in the animal housing room for 7 days before commencing experiments.

4.2. Sepsis models

Two models of sepsis were used. Model 1: mice aged 8–10 weeks were injected intraperitoneally with LPS (70 mg/kg of body weight, Sigma Aldrich) to induce endotoxemia. Animals were monitored for up to 100 h. Model 2: Cecal ligation and puncture (CLP) procedure was used as a confirmatory model, as described previously [55]. Post-operative survival was monitored as described [55].

4.3. Plasma transaminase profiling

Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed with a colorimetric method using Cobas Mira Plus analyzer (Horiba®, ABX, France) 0 and 24 h after CLP.

4.4. Flow cytometric analysis

Red blood cells were lysed using Lysing Buffer (Becton Dickinson, Italy) [54]. Monocytes and neutrophils counts were assessed by flow cytometry with a NovoCyte cytometer (ACEA) using the combination of the following antibodies: antiCD11b-PE, antiLy6C-FITC, and antiLy6G-PerCP antibodies from eBioscience [56]. A representative panel of the gating strategy is presented in the supplemental section.

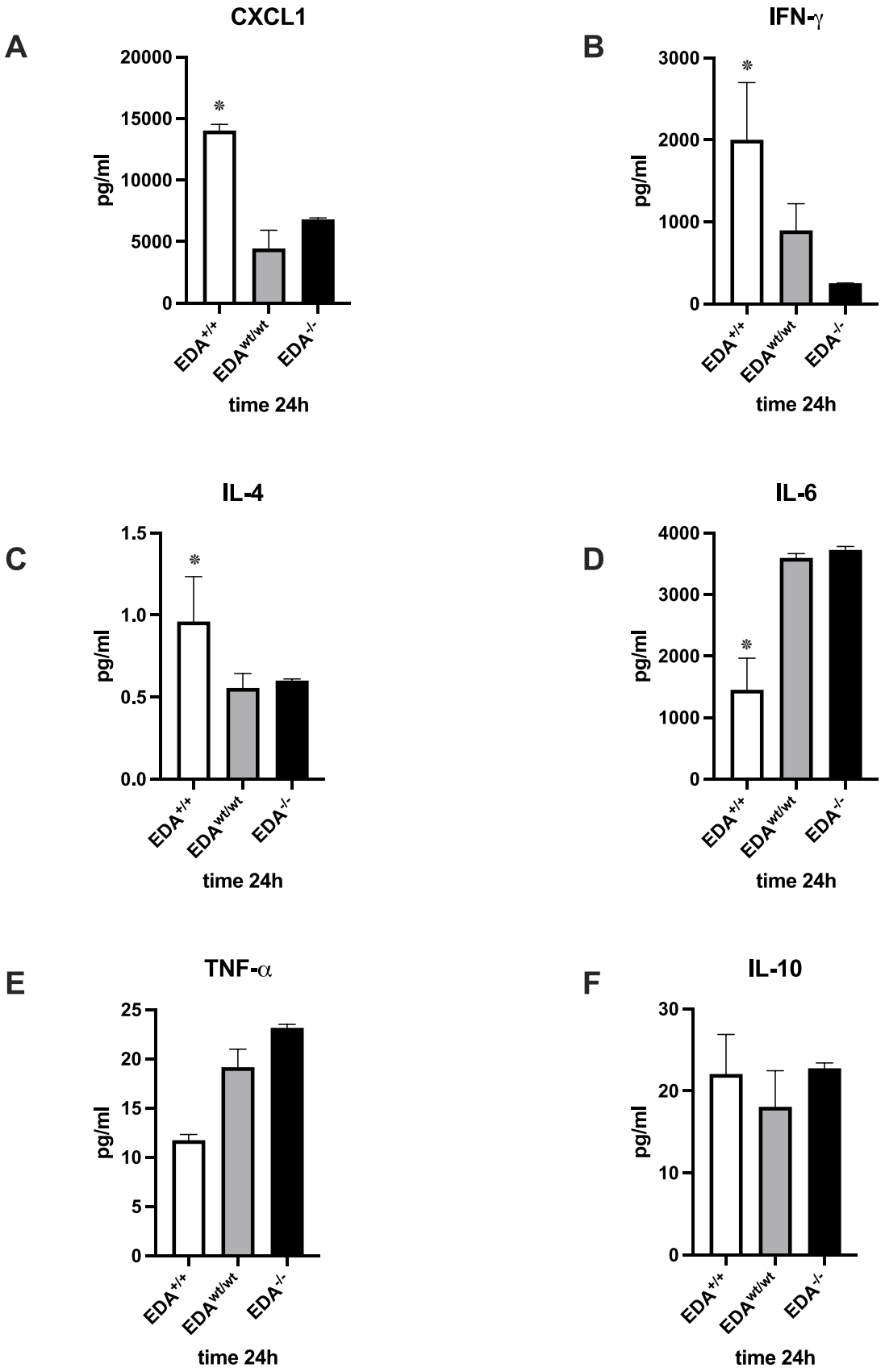

Fig. 4. Markers of bacterial clearance were upregulated in the spleen of EDA$^{+/+}$ mice. A) CXCL1, B) IFN-γ, C) IL-4, D) IL-6, E) TNF-α, and F) IL-10 levels were evaluated in all three genotypes. Splenic caspase 3 levels were lower in EDA$^{+/+}$and EDA$^{wt/wt}$ mice compared to EDA$^{-/-}$mice signifying an improved cell survival. *P < 0.05, Student T-test vs time point 24h of respective genotype.

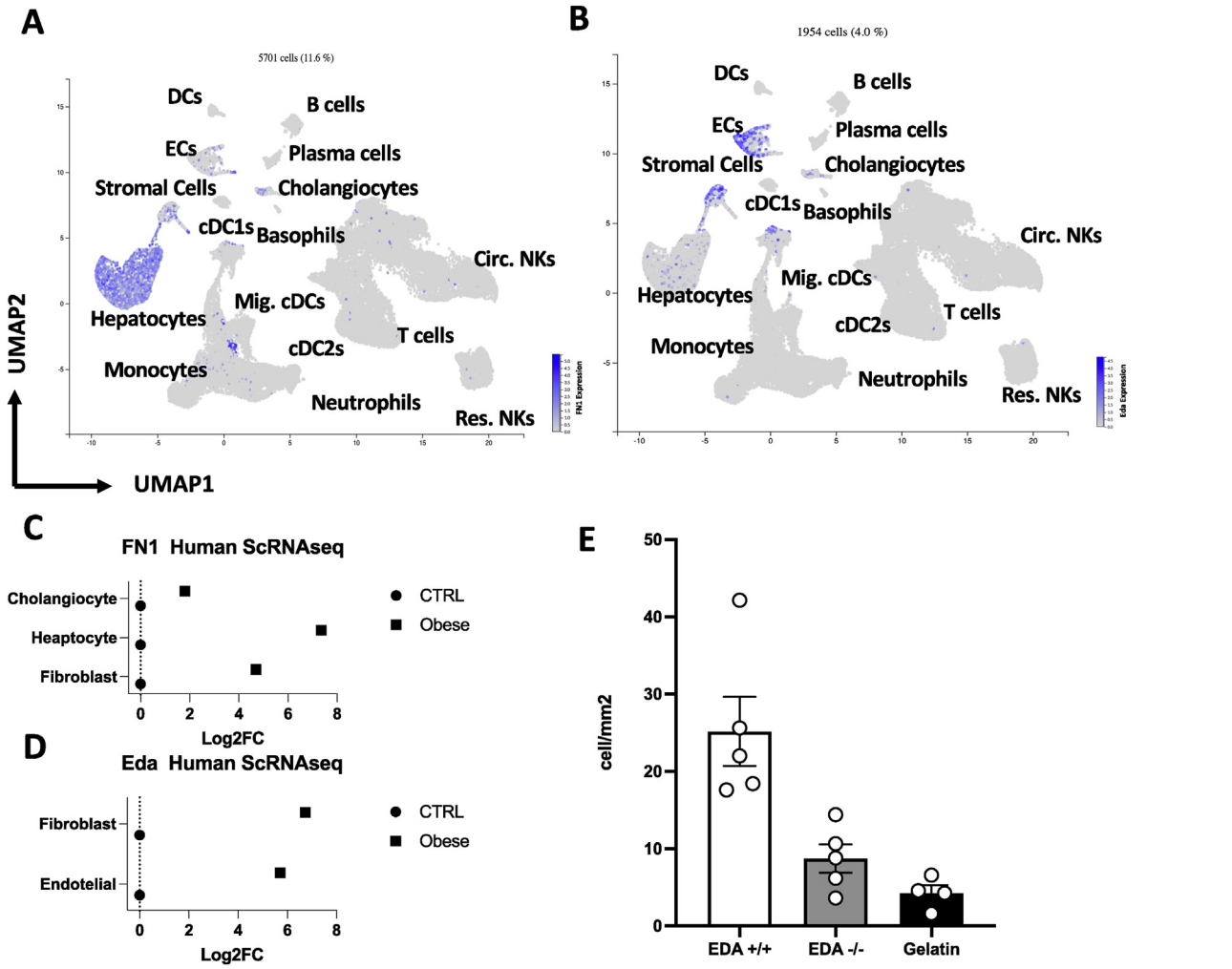

Fig. 5. Fibronectin expression is affected by obesity and when presenting EDA contribute to neutrophil adhesion. A-B) FN1 expression on hepatic subpopulation. C-D) Eda expression on hepatic subpopulation Data obtained from publicly available datasets at https://www.livercellatlas.org/download.php. E) Isolated neutrophils from human septic shock patients adhered to EDA + fibronectin more compared to EDA-fibronectin or Gelatin. *P < 0.05, One-way ANOVA comparing $EDA^{+/+}$ vs $EDA^{-/-}$and gelatin n = 4.

4.5. Tissue cytokine array

The spleen from the different experimental models was harvested 24 h after the LPS injection. Total protein was extracted by adding 50 μl of ice-cold lysis buffer (20 mm Tris-HCl, pH 7.5, 100 mm NaCl, 10 mm MgCl2, 1 mm EDTA, 1 mm EGTA, 0.5% Nonidet P-40, 2.5 mm sodium pyrophosphate, 1 mm β-glycerophosphate, 1 mm Na3VO4, 25 mm NaF, 1 μg/ml of leupeptin, 1 μg/ml of aprotinin, 1 mm PMSF, and 1 mm DTT) to each sample. The cell lysate was transferred to a 1.5-ml microcentrifuge tube, debris was pelleted by centrifugation (10,000 rpm for 10 min), then protein levels were quantified by the Lowry method. Protein concentration was normalized to 1 μg/μl 25 μl of the extract was diluted in 25 μl PBS and analyzed for the following cytokines: eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-alpha, and VEGF using Eve Technologies, (University of Calgary).

4.6. Neutrophil flow chamber experiment

Neutrophils were isolated from patients with sepsis as described before [57]. Patient score details and source of infection are reported in Supplemental Table 1. Neutrophils were allowed to run on a flow chamber coated with either fibronectin + EDA or fibronectin-EDA at 0.5 dyne/cm^2 as described previously [58]. Cell attachment was recorded and plotted for a flow of 0.5 dyne/cm^{2cri}.

4.7. Analyses of single cell transcriptome data

Publicly available datasets were analyzed in this study. These data retrieved from the GEO repository under accession numbers GSE192742. All data can be found here: https://www.livercellatlas.org/download.php. The data for DEGs are derived from public dataset [59].

4.8. Statistics

All data are represented as mean ± standard error (SEM) unless stated otherwise. Each experiment was performed at least a minimum of 4 times and statistical significance was assessed using One-way ANOVA (Graph Pad PRISM 7). For some experiments, additional tests such as Student paired T-test and Tukey Comparison among the groups were performed. P-values <0.05 were used where appropriate.

Author contribution

Participated in research design: VKPV, PU, GDN, AFM; performed experiments, data analysis: VKPV, PU, AM, LDD, FB, AD; wrote or contributed to the writing of the manuscript: VKPV, GDN, AFM.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.athplu.2023.05.002.

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