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(54) **Title:** PREVENTION OF THE INTERACTION BETWEEN TENASCIN-C AND MIR-155 TO TREAT SEPSIS

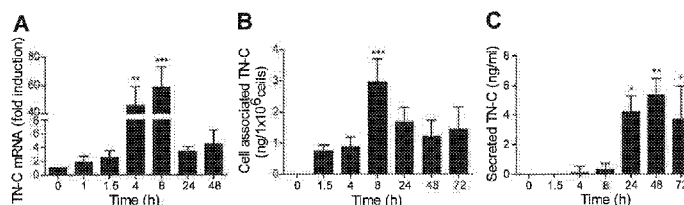


Figure 3

(57) **Abstract:** An agent which reduces the level and/or activity of tenascin-C and/or miR-155 for use in the treatment of pathogen induced inflammation, and methods of screening and determining the efficacy of agents for treating pathogen induced inflammation.

PREVENTION OF THE INTERACTION BETWEEN TENASCIN-C AND MIR-155 TO TREAT SEPSIS

The present invention relates to agents for use in the treatment of pathogen induced inflammation, in particular bacterial sepsis.

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Bacterial sepsis is a clinical term used to describe symptomatic bacteremia, with or without organ dysfunction. Bacterial sepsis is characterized by the presence of acute inflammation throughout the entire body, and is, therefore, frequently associated with fever or lower-than-average temperature, an elevated or low white blood cell count and elevated heart and respiratory rate. The modern concept of sepsis is that the host's immune response to the infection causes most of the symptoms of sepsis, resulting in hemodynamic consequences and damage to organs. Bacterial sepsis is triggered by release of toxic products from gram-negative or gram-positive bacteria (e.g. lipopolysaccharide (LPS) molecules from the outer wall of gram-negative bacteria or exotoxins produced by gram-positive bacteria) which activate the complex inflammatory pathways and cause disease.

Bacterial sepsis is a common consequence of uncontrolled bacterial infection and is potentially fatal. Approximately 20–35% of patients with severe bacterial sepsis and 40–60% of patients with septic shock die within 30 days. Many others die within the ensuing 6 months. Bacterial sepsis accounts for about 9% of overall mortality in the US.

Current treatment for sepsis comprises intra venous administration of broad spectrum antibiotics which may or may not be effective depending on the cause or scope of infection. Moreover, treatment of patients with antibiotics is not a cure for sepsis as often following administration of antibiotics there is a release of LPS from gram-negative bacteria. As such, antibiotic administration is often followed by blood transfusion, mechanical ventilation to aid respiration and/or dialysis to help with kidney function.

Furthermore, as antibiotic use has increased, many strains of bacteria have become resistant to antibiotics, making the treatment of sepsis more difficult. The incidence of *S. aureus* bacteraemia (SAB), particularly bacteraemia caused by methicillin-resistant *S. aureus* (MRSA) strains, has increased dramatically in recent years in the USA and

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in some European countries. Resistance of *S. aureus* strains to antibiotics has been increasing; thus, the ability of these pathogens to spread in both hospital and community settings has increased. Increased antibiotic resistance, in addition to the increased frequency of invasive surgery, increased use of intravascular devices, and
5 increased numbers of patients with immunocompromised status because of HIV infection or immunosuppression after transplantation or cancer treatment or a splenectomy, has led to sharp increases in the incidence of SAB and *S. aureus* infective endocarditis (SAIE) over the past 30 years.

10 There is therefore a need for further or alternative therapies for the treatment of bacterial sepsis.

According to a first aspect the invention provides an agent which reduces the level and/or activity of tenascin-C and/or miR-155 for use in the treatment of pathogen
15 induced inflammation.

Bacterial sepsis is an example of pathogen induced inflammation. Preferably the pathogen induced inflammation is caused by lipopolysaccharide (LPS), which preferably acts by inducing the activation of TLR4. Alternatively, or additionally,
20 pathogen induced inflammation may be caused by microbial structures, such as flagellin, which may act by inducing the activation of TLR5; or compounds such as peptidoglycan and lipoteichoic acids from gram-positive bacteria, which may act by inducing the activation of TLR2.

25 Furthermore, exotoxins produced by gram-positive bacteria, including the toxic shock syndrome toxin-1 (TSST-1) from *Staphylococcus aureus*, the streptococcal pyrogenic exotoxin A (SPEA) and the streptococcal mitogenic exotoxin Z (SMEZ), may induce bacterial sepsis.

30 The pathogen induced inflammation, and in particular septic shock, may be caused by one or more of the following bacteria *E. coli*, *Streptococcus pyogenes*, *staphylococcus aureus*, *Vibrio vulnificus*, *Staphylococcus saprophyticus*, *Pseudomonas aeruginosa*, *Viridans streptococci*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *P. gingivalis* and *Rickettsia rickettsiae*.

Other examples of pathogen induced inflammation include periodontal disease, meningococcal sepsis, meningitis, gastroenteritis, cystitis, and gonococcal arthritis. Periodontal disease is one of the most common inflammatory diseases and affects approximately 100 million people in the US. The disease is caused by an infection
5 with the gram-negative bacterium *P. gingivalis*, which acts by inducing the activation of TLR2 and TLR4 and leads to inflammatory bone destruction.

Meningococcal sepsis and meningitis are caused by infection with *Neisseria meningitidis*. The pathogen induced inflammation is caused by the meningococcal
10 lipopoly(oligo)saccharide (LOS), which acts by inducing the activation of TLR4. Gastroenteritis is an inflammation of the stomach and the intestine caused by infection with *E. coli* (or *Salmonella*, *Shigella* or *Campylobacter*). Cystitis is a urinary bladder inflammation caused by infection with *E. coli* (or *Staphylococcus saprophyticus*). Gonococcal arthritis is caused by infection with the gram-negative diplococcus
15 *Neisseria gonorrhoeae*. In the USA, gonococcal arthritis is the most common form of septic arthritis.

The agent of the invention is preferably not for the treatment of sterile tissue damage, that is tissue damage which is not caused directly by infection, such as rheumatoid
20 arthritis.

The agent may act directly to reduce the level and/or activity of tenascin-C. The agent may act indirectly to reduce the level and/or activity of tenascin-C.

25 The agent may act directly to reduce the level and/or activity of miR-155. The agent may act indirectly to reduce the level and/or activity of miR-155.

The agent may act on tenascin-C to reduce the level and/or activity of miR-155

30 The agent may act directly to reduce the level of tenascin-C below the threshold required to induce inflammation. The agent may act indirectly to reduce the level of tenascin-C below the threshold required to induce inflammation.

Preferably a reduction in the level of tenascin-C is a reduction in the circulating level of an active form of tenascin-C.

Preferably a reduction in miR-155 is a reduction in the circulating level and/or in the
5 cellular level of an active (mature) form of miR-155.

Preferably a reduction is defined as when a sample contains less than about 80%, less than 75%, less than about 70%, less than about 65%, less than about 60%, less than about 65%, less than about 50% the level or activity of tenascin-C and/or miR-155
10 observed in a reference sample.

Preferably the reference value, to which the determined levels or activities of tenascin-C and/or miR-155 are compared, is the level or activity of tenascin-C and/or miR-155 observed in a sample from one or more subjects that do not have any
15 detectable pathogen induced inflammation, or any clinical symptoms of a pathogen induced inflammation, and have so called "normal values" of tenascin-C and/or miR-155.

The level of tenascin-C and/or miR-155 may be determined in a sample of blood,
20 such as a whole blood sample, plasma or serum. In an alternative embodiment the sample may be urine. Preferably the sample is a serum sample.

Tenascin-C is a pro-inflammatory extracellular matrix glycoprotein (Midwood et al (2009) Nat. Med. 15: 774-780), that induces inflammatory cytokine synthesis in
25 primary human macrophages and synovial fibroblasts by activation of the pattern recognition receptor, TLR4, in sterile tissue induced inflammation. Tenascin-C is a large hexameric protein of approximately 1500kDa. High levels of tenascin-C have been found at sites of inflammation, for example in the synovial fluid of rheumatoid arthritis patients and in tumor stroma. The involvement of tenascin-C in pathogen
30 induced inflammation has to date not been known.

The level or activity of the tenascin-C or miR-155 present in a sample may be determined by any suitable assay, which may comprise the use of any of the group comprising immunoassays, spectrometry, western blot, ELISA, immunoprecipitation,
35 slot or dot blot assay, isoelectric focussing, SDS-PAGE and antibody microarray

immunohistological staining, radio immuno assay (RIA), fluoroimmunoassay, an immunoassay using an avidin-biotin or streptoavidin-biotin system, etc or combinations thereof. The level of tenascin-c mRNA or miR-155 present in a sample may be determined by any suitable assay, which may comprise the use of any of the group comprising RT-PCR, real time PCR, microarray assays, northern blot, nuclease protection assay (RPA), serial analysis of gene expression (SAGE) and amplified differential gene expression (ADGE). These methods are well known to persons skilled in the art.

10 Preferably it is the level of cell associated tenascin-C which is determined, that is intracellular and/or membrane bound tenascin-C. Preferably the level of soluble extracellular tenascin-C is not determined/considered in the invention.

miR-155 is a short non-coding RNA that is very well conserved throughout the animal kingdom.

Mouse miR-155 (MIMAT0000165) has the sequence;
UUAAUGCUAAUUGUGAUAGGGGU (Seq ID No: 1)

20 Human miR155 (MIMAT0000646) has the sequence;
UUAAUGCUAAUCGUGAUAGGGGU (Seq ID No: 2)

The miR-155 gene is about 1500 bases long located on the BiC area on chromosome 21 band q21.3. Transcription in this area releases a non-coding RNA product that upon processing finalization becomes miR-155. This particular chromosomal location shows strong sequence conservation in humans, mouse and chickens.

The agent may act on miR-155 or tenascin-C at a post-transcriptional level and may be a viral vector, including retroviruses/lentiviruses, adenoviruses, adeno-associated viruses, pox viruses, alphaviruses and herpes viruses, or a non-viral vector, including naked DNA, oligonucleotides, lipoplexes and polyplexes.

Preferably the agent to reduce levels of tenascin-C is an antagonist of tenascin-C

35 The agent to reduce levels of tenascin-C and/or miR-155 may be a small molecule, a protein, a peptide, a polymer, an antibody, a nucleic acid, an siRNA, an antisense oligonucleotide, an antagomer, an aptamer or a polysaccharide.

If the agent is an antibody it may be a human, humanized, mouse or chimeric antibody.

- 5 The agent may be for systemic and/or local administration

In one embodiment the agent acts to prevent, inhibit or reduce interaction between tenascin-C and miR-155.

- 10 The efficacy of an agent according to the invention may be measured by observing the level of TNF-alpha in a sample. Preferably a reduction in TNF-alpha will be seen if an agent according to the invention is working to reduce inflammation.

- 15 According a further aspect the invention provides the use of an agent that reduces the level of tenascin-C and/or miR-155 for the preparation of a medicament for the prevention or treatment of pathogen induced inflammation.

- 20 According to another aspect the invention provides a method of treating pathogen induced inflammation comprising administering to a subject in need thereof an effective amount of an agent according to the invention.

- 25 According to a still further aspect the invention provides a pharmaceutical composition comprising an agent according to the invention and a pharmaceutically acceptable excipient.

- Suitable acceptable excipients and carriers will be well known to those skilled in the art. These may include solid or liquid carriers. Suitable liquid carriers include water and saline. The proteins of the composition may be formulated into an emulsion or they may be formulated into biodegradable microspheres or liposomes. The composition may be formulated to protect the agent from degradation.
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- The composition may also comprise polymers or other agents to control the consistency of the composition, and/or to control the release of the antigen/secreted protein from the composition.
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The composition may also comprise other agents such as diluents, which may include water, saline, glycerol or other suitable alcohols etc; wetting or emulsifying agents; buffering agents; thickening agents for example cellulose or cellulose derivatives; preservatives; detergents, antimicrobial agents; and the like.

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Preferably the active ingredients in the composition are greater than 50% pure, usually greater than 80% pure, often greater than 90% pure and more preferably greater than 95%, 98% or 99% pure. With active ingredients approaching 100% pure, for example about 99.5% pure or about 99.9% pure, being used most often.

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According to a further aspect the invention provides a method for determining the efficacy of a treatment for pathogen induced inflammation comprising the steps of:

- i) determining the level and/or activity of tenascin-C and/or miR-155 in a biological sample from a subject;
- 15 ii) administering said treatment to the subject;
- iii) determining the level and/or activity of tenascin-C and/or miR-155 in a biological sample from said mammalian subject at a time following administration of said treatment

wherein a reduction in the level and/or activity of tenascin-C and/or miR-155
20 following administration of the treatment is indicative of said treatment being a therapy for pathogen induced inflammation.

According to a yet further aspect the invention provides a method of screening agents/compositions for efficacy in the treatment of pathogen induced inflammation.

25 In one aspect the invention provides an *in vivo* method of screening agents/compositions for efficacy in the treatment of pathogen induced inflammation comprising the steps of:

- i) determining the level and/or activity of tenascin-C and/or miR-155 in a biological sample from a subject, the subject may be known to be suffering
30 from pathogen induced inflammation;
- ii) administering said agent/composition to the subject;
- iii) determining the level and/or activity of tenascin-C and/or miR-155 in a biological sample taken from the subject at a time following administration of said agent/composition;

wherein a reduction in the level and/or activity of tenascin-C and/or miR-155 following administration of the agent/composition is indicative of said agent/composition being a therapy for pathogen induced inflammation.

5 In another aspect the invention provides an *in vitro* method of screening agents/compositions for efficacy in the treatment of pathogen induced inflammation comprising the steps of:

- i) providing a biological sample obtained from a subject;
- 10 ii) determining the level and/or activity of tenascin-C and/or miR-155 in the biological sample;
- iii) treating the biological sample with said agent/composition *in vitro*;
- iv) determining the level and/or activity of tenascin-C and/or miR-155 in the biological sample at a time following administration of said agent/composition;

15 wherein a reduction in the level and/or activity of tenascin-C and/or miR-155 following administration of the agent/composition is indicative of said agent/composition being a therapy for pathogen induced inflammation.

20 The biological sample in all aspects of the invention may be a sample of blood, such as a whole blood sample, plasma or serum. In an alternative embodiment the sample may be urine. Preferably the sample is a serum sample.

The invention also provides for agents/compositions identified by any method of the invention.

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The skilled man will appreciate that preferred features of any one embodiment and/or aspect of the invention may be applied to all other embodiments and/or aspects of the invention.

30 The present invention will be further described in more detail, by way of example only, with reference to the following figures in which:

Figure 1 – demonstrates that *Tnc*^{-/-} mice are less susceptible to LPS-induced sepsis (A and B) Body weight (A) and mobility (B) loss in *tnc*^{+/+} and *tnc*^{-/-} mice upon LPS injection, monitored over 8 h. Data in (A) are presented as

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percent loss relative to non-injected mice. (n=17 (A) or 10 (B) mice per group; mean $\pm$ SEM); ****= $P<0.0001$ and ***= $P<0.001$ compared to non-injected mice (A). Data are from three (A) or two (B) independent experiments. (C) Time-course analysis of TNF- α , IL-6 and HMGB1 in plasma from tnc+/+ and tnc-/- mice after LPS injection by ELISA (n=3 to 5 mice per group; mean $\pm$ SEM); ****= $P<0.0001$, ***= $P<0.001$ and **= $P<0.01$. Data are representative of three independent experiments. (D) Immunohistochemical analysis of neutrophil elastase+ cell infiltration in lungs from tnc+/+ and tnc-/- mice 0, 1.5, 4 and 8 h after LPS injection (n=4 LPS-injected mice per genotype and 3 non-injected mice per genotype); scale bars, 100 μ m. Data are representative of three independent experiments. (E and F) Immunoblot analysis of plasma tenascin-C in tnc+/+ (1-3) and tnc-/- (4-6) mice 8 h (E) and 0, 1.5, 4 and 8 h (F) after LPS injection. Results are representative of three independent experiments. (G) ELISA of plasma tenascin-C in tnc+/+ mice 0, 1.5, 4 and 8 h after LPS injection (n=7; mean $\pm$ SEM). Data are from two independent experiments.

Figure 2 – demonstrates that allogeneic bone marrow transplantation rescues LPS-induced circulating TNF- α in tnc-/- mice. (A) Quantification of male donor cell engraftment in female recipients 8 weeks after bone marrow transplantation by quantitative RT-PCR analysis of mouse testis-specific Y-encoded protein (TSPY) gene in erythrocyte-depleted peripheral blood cells. Data are shown as percentage of donor chimerism relative to that of male control mice. (B) Quantitative RT-PCR analysis of tenascin-C mRNA in erythrocyte-depleted peripheral blood cells of female recipients 1.5 h after LPS injection. Results are presented relative to those of tnc+/+ / +/+ chimeric mice. *= $P<0.05$ compared to tnc+/+ / +/+ chimeric mice. (C and D) ELISA of TNF- α in plasma from tnc+/+ / +/+ and tnc+/+ / -/- (C) and tnc-/- / -/- and tnc-/- / +/+ (D) chimeric mice 1.5 and 8 h after LPS injection. Results represent measurements of three mice per group (mean $\pm$ SD); ****= $P<0.0001$ and **= $P<0.01$.

Figure 3 demonstrates that tenascin-C expression is transiently induced by LPS. (A) Quantitative RT-PCR analysis of tenascin-C mRNA in BMDMs stimulated for 1, 1.5, 4, 8, 24 and 48 h with 100ng/ml LPS. Results are

presented relative to those of untreated BMDMs. Data are from seven independent experiments (mean $\pm$ SEM); ***= $P<0.001$ and **= $P<0.01$ compared to non-stimulated cells. (B) ELISA of cellular tenascin-C in BMDMs stimulated for 1.5, 4, 8, 24, 48 and 72 h with 100ng/ml LPS. Results are normalized to the cell number. Data are from six independent experiments (mean $\pm$ SEM); ***= $P<0.001$ compared to non-stimulated cells. (C) ELISA of secreted tenascin-C by BMDMs stimulated for 1.5, 4, 8, 24, 48 and 72 h with 100ng/ml LPS. Data are from six independent experiments (mean $\pm$ SEM); **= $P<0.01$ and *= $P<0.05$ compared to non-stimulated cells.

Figure 4 - Tenascin-C post-transcriptionally regulates the synthesis of specific cytokine subsets in BMDMs (A) ELISA of TNF α , IL-6, CXCL1, IL-10, IL-1 β , IL-12 and TGF β 1 secreted by tnc+/+ and tnc-/- BMDMs stimulated for 1.5, 4, 8, 24 and 48 h with 100ng/ml LPS. Data are from six to nine independent experiments (mean $\pm$ SEM); ****= $P<0.0001$, ***= $P<0.001$, **= $P<0.01$ and *= $P<0.05$. (B) Quantitative RT-PCR analysis of TNF α , IL-6, CXCL1 and IL-10 mRNA in tnc+/+ and tnc-/- BMDMs stimulated for 0.5, 1, 1.5, 4, 8, 24 and 48 h with 100ng/ml LPS. Results are presented relative to those of untreated BMDMs. Data are from four to five independent experiments (mean $\pm$ SEM).

Figure 5 – demonstrates the translation of pro-inflammatory cytokines is inhibited in the absence of tenascin-C. (A) ELISA of secreted and cellular TNF α , IL-6 and CXCL1 in tnc+/+ and tnc-/- BMDMs stimulated for 1.5, 4, 8, 24 and 48 h with 100ng/ml LPS. Results are normalized to the cell number. Data are from five to nine independent experiments (mean $\pm$ SEM). (B) Total amounts of TNF- α , IL-6 and CXCL1 in tnc+/+ and tnc-/- BMDMs stimulated for 1.5, 4, 8, 24 and 48 h with 100ng/ml LPS; results are shown as the sum of the amounts of secreted and cellular protein fractions normalized to the cell number. Data are from five to nine independent experiments (mean $\pm$ SEM); ***= $P<0.001$, **= $P<0.01$ and *= $P<0.05$. (C) ELISA of cellular TNF- α , IL-6 and CXCL1 in tnc+/+ and tnc-/- BMDMs stimulated for 24 h with 100ng/ml LPS in the presence of 1 μ g/ml brefeldin A. Results are normalized to the cell number. Data are from three independent experiments (mean $\pm$ SEM); ****= $P<0.0001$ and *= $P<0.05$.

Figure 6 – shows that tenascin-C is required for induction of miR-155 in activated BMDMs and in septic mice. (A) Microarray analysis of miRNA profile in *tnc*^{+/+} and *tnc*^{-/-} BMDMs stimulated for 8 h with 100ng/ml LPS. The heat-map shows suppression (green) and induction (red) of expression as fold change on a log2 scale, relative to non-stimulated BMDMs (n=3 mice per genotype). (B and C) Expression of TLR-induced miRNAs in *tnc*^{+/+} and *tnc*^{-/-} BMDMs (identified in A) classified as early (B) and late (C) response genes (n=3 per genotype; mean ± SEM); **= P<0.01, ns = not significant. (D) Time-course analysis of miR-155, pri-miR-155, miR-146a and miR-21 expression in *tnc*^{+/+} and *tnc*^{-/-} BMDMs stimulated for 4-72 h (miR-155), 0.5-48 h (pri-miR-155) or 4-96 h (miR-146a, miR-21) with 100ng/ml LPS. RNA used in A (n=3 mice per group) and RNA isolated from BMDMs derived from two (miR-146a, miR-21) and four (miR-155, pri-miR-155) additional mice per genotype was analyzed by quantitative RT-PCR (mean ± SEM); **= P<0.01 compared to *tnc*^{-/-} BMDMs at the same time point. (E) Correlation of miR-155 expression with TNF-α synthesis in *tnc*^{+/+} and *tnc*^{-/-} BMDMs stimulated with 100ng/ml LPS for 4, 8 and 24 h (n=5 mice per genotype; R=1.0, P<0.05). (F) Quantitative RT-PCR analysis of miR-155 expression in spleen from *tnc*^{+/+} and *tnc*^{-/-} mice 1.5 h after LPS injection (n=3 per genotype; mean ± SEM); *= P<0.05. Data are from two independent experiments.

Figure 7 – illustrates that miR-155 overexpression rescues TNF-α release in *tnc*^{-/-} BMDMs in response to LPS. (A) Quantitative RT-PCR analysis of miR-155 expression in *tnc*^{+/+} untransfected BMDMs and *tnc*^{-/-} untransfected, mock transfected and transfected with control precursor or miR-155 precursor BMDMs stimulated for 8 h with 100ng/ml LPS. Results are presented relative to those of non-stimulated BMDMs (n=3; mean ± SEM); *= P<0.05 and ns = not significant. Data are representative of four independent experiments each with cells obtained from three mice per genotype. (B) ELISA of TNF-α secreted by *tnc*^{+/+} untransfected BMDMs and *tnc*^{-/-} untransfected, mock transfected and transfected with control precursor or miR-155 precursor BMDMs stimulated for 8 h with 100ng/ml LPS (n=6; mean ± SEM); ***= P<0.001, **= P<0.01 and ns = not significant. Data are from two independent experiments each with cells obtained from three mice per genotype.

Figure 8 - shows *Tnc*^{-/-} mice develop less severe symptoms upon LPS injection. (A) Representative images of the eyes of *tnc*^{+/+} and *tnc*^{-/-} mice 0, 4 and 8 h after LPS injection. Data are representative of three independent experiments. (B) Fur ruffling in *tnc*^{+/+} and *tnc*^{-/-} mice upon LPS injection, monitored over 8 h. (n = 10 per genotype; mean ± SEM); ****= P<0.0001. Data are from two independent experiments. (C) Immunohistochemical analysis of neutrophil elastase⁺ cell infiltration in liver from *tnc*^{+/+} and *tnc*^{-/-} mice 0, 1.5, 4 and 8 h after LPS injection (n = 4 LPS-injected mice per genotype and 3 non-injected mice per genotype); scale bars, 100 µm. Results are representative of three independent experiments. (D) Neutrophil elastase⁺ cell count in lungs and liver from *tnc*^{+/+} and *tnc*^{-/-} mice 0, 1.5, 4 and 8 h after LPS injection. Values are expressed as mean ± SD (n). Data are representative of three independent experiments.

Figure 9 – demonstrates that BMDMs mature effectively in the absence of tenascin-C in vitro and in vivo. (A-D) FACS analysis of CD11b and F4/80 surface expression in *tnc*^{+/+} and *tnc*^{-/-} BMDMs. (A) One representative plot per genotype is shown; (B) percentage of CD11b⁺/F4/80⁺ BMDMs; (C,D) mean fluorescence intensity (MFI) of CD11b⁺ (C) and F4/80⁺ (D) BMDMs (n = 3; mean ± SEM). (E-G) FACS analysis of intracellular CD68 expression in *tnc*^{+/+} and *tnc*^{-/-} BMDMs. (E) One representative histogram per genotype is shown; percentage (F) and MFI (G) of CD68⁺ BMDMs (n = 4; mean ± SEM); ns = not significant. (H) Quantitative RT-PCR analysis of CD11b, F4/80 and CD68 mRNA in *tnc*^{+/+} and *tnc*^{-/-} BMDMs. Results are presented relative to those of *tnc*^{+/+} BMDMs (n = 4; mean ± SEM); ns = not significant. (I-L) FACS analysis of CD11b and F4/80 surface expression in thioglycollate-elicited peritoneal macrophages from *tnc*^{+/+} and *tnc*^{-/-} mice. (I) One representative plot per genotype is shown; (J) percentage of CD11b⁺/F4/80⁺ macrophages; (K,L) MFI of CD11b⁺ (K) and F4/80⁺ (L) macrophages (n = 3; mean ± SEM); ns = not significant. (M-O) FACS analysis of intracellular CD68 expression in *tnc*^{+/+} and *tnc*^{-/-} macrophages. (M) One representative histogram per genotype is shown; percentage (N) and MFI (O) of CD68⁺ macrophages (n = 3; mean ± SEM). Data are from three or four (E-H) independent experiments.

Figure 10 - shows Tenascin-C does not affect LPS-induced activation of BMDMs. (A-J) FACS analysis of macrophage activation marker expression in *tnc*^{+/+} and *tnc*^{-/-} BMDMs non-stimulated or stimulated for 24 h with 100ng/ml LPS. (A-D) Analysis of MHC II and CD40 surface expression in *tnc*^{+/+} and *tnc*^{-/-} BMDMs +/- LPS. (A) One representative plot per genotype +/- LPS is shown; (B) percentage of MHC II⁺/CD40⁺ BMDMs +/- LPS; (C,D) MFI of MHC II⁺ (C) and CD40⁺ (D) BMDMs +/- LPS (n= 3; mean $\pm$ SEM); ns = not significant. (E-G) Analysis of CD86 surface expression in *tnc*^{+/+} and *tnc*^{-/-} BMDMs +/- LPS. (E) One representative histogram from *tnc*^{+/+} and *tnc*^{-/-} BMDMs +/- LPS is shown; percentage (F) and MFI (G) of CD86⁺ BMDMs +/- LPS (n = 3; mean $\pm$ SEM); ns = not significant. (H-J) Analysis of TLR4/MD-2 surface expression in *tnc*^{+/+} and *tnc*^{-/-} BMDMs +/- LPS. (H) One representative histogram from *tnc*^{+/+} and *tnc*^{-/-} BMDMs +/- LPS is shown; percentage (I) and MFI (J) of TLR4/MD-2⁺ BMDMs +/- LPS (n= 3; mean $\pm$ SEM); ns = not significant. Data are from three independent experiments.

Figure 11 – demonstrates TNF- α production in *tnc*^{-/-} BMDMs is rescued by miR-155 overexpression but not soluble tenascin-C (A) Quantitative RT-PCR analysis of miR-155 expression in *tnc*^{+/+} untransfected BMDMs and *tnc*^{-/-} untransfected, mock transfected and transfected with control precursor or miR-155 precursor BMDMs, all in the absence of any stimulation with LPS (n = 3; mean $\pm$ SEM). Data are representative of four independent experiments each with cells obtained from three mice per genotype. (B) ELISA of TNF- α secreted by *tnc*^{+/+} untransfected BMDMs and *tnc*^{-/-} untransfected, mock transfected and transfected with control precursor or miR-155 precursor BMDMs (n = 6; mean $\pm$ SEM). Data are from two independent experiments each with cells obtained from three mice per genotype. (C) ELISA of TNF- α and IL-6 secreted by *tnc*^{+/+} and *tnc*^{-/-} BMDMs stimulated for 24 h with 100ng/ml LPS without (-) or with recombinant purified tenascin-C (TN-C) (n=3; mean $\pm$ SEM). ***= P<0.001 compared to *tnc*^{+/+} BMDMs stimulated with LPS. Data are representative of three independent experiments each with cells obtained from three mice per genotype. The same results were obtained when equivalent amounts of commercially purified human tenascin-C were added.

Figure 12 – details the amino acid sequence of human tenascin C (Seq ID No 6).

Tenascin-C expression is required for the proinflammatory response to LPS

5 Using an experimental model of LPS-induced systemic inflammation in *tnc*^{+/+} and *tnc*^{-/-} mice a rapid increase in circulating tenascin-C was observed in septic *tnc*^{+/+} mice. Maximal tenascin-C levels in plasma are detected 90 min after LPS injection, long before the induction of HMGB1 release, an established tissue injury marker in sepsis. This data supports specific tenascin-C upregulation by LPS-induced TLR4
10 signaling rather than tissue damage-driven release of tenascin-C. Indeed, the data demonstrates that tenascin-C is expressed at both mRNA and protein level in LPS-activated BMDMs, the major cellular players in sepsis. In response to bacterial endotoxins, macrophages promptly release pro-inflammatory cytokines, including TNF- α , which is both crucial for effective innate immunity and a key pathologic
15 contributor to sepsis. However, septic *tnc*^{-/-} mice had significantly less circulating TNF- α , a phenomenon that was reversed by allogeneic bone marrow transplantation. In *tnc*^{-/-} mice, transplantation of *tnc*^{+/+} bone marrow rescued LPS-induced circulating TNF- α and vice-versa, indicating that bone marrow-derived cells from *tnc*^{-/-} mice exhibit defects in TNF- α production. Consistent with low circulating TNF- α , less
20 plasma IL-6 and reduced neutrophil infiltration to lungs and liver were observed in *tnc*^{-/-} mice after LPS injection compared to *tnc*^{+/+} mice. Furthermore, *tnc*^{-/-} mice failed to induce release of HMGB1, a late mediator of sepsis that is released by activated macrophages partly through a TNF- α -dependent mechanism. These in vivo data suggest that tenascin-C is an early response gene that regulates LPS-induced
25 TLR4-mediated inflammation operating upstream of TNF- α and HMGB1. This is further supported by in vitro findings that *tnc*^{-/-} BMDMs secreted significantly less TNF- α , IL-6 and CXCL1, upon stimulation with LPS than *tnc*^{-/-} cells. This effect was specific, as it was not observed in other cytokine subsets, including IL-1 β , IL-12 or TGF β 1. Furthermore, in BMDMs, tenascin-C appears to specifically control the switch
30 from anti- to pro-inflammatory cytokine programs downstream of TLR4 activation. In the absence of tenascin-C, not only do BMDMs induce submaximal TNF- α levels, they also secreted more IL-10, an anti-inflammatory cytokine that is key to dampening the inflammatory response to infection induced by TLR signaling. Thus, transient tenascin-C expression promotes an initial pro-inflammatory response that is later
35 suppressed by IL-10 synthesis.

Results

To examine the role of tenascin-C in host defence against pathogenic infection, sepsis was induced with a sublethal dose of LPS in *tnc*^{+/+} and *tnc*^{-/-} mice. The major clinical symptoms of sepsis, including weight loss, reduced mobility, uveitis, ruffled fur and diarrhea, were evident in *tnc*^{+/+} mice 1.5 h after injection of LPS, in contrast to *tnc*^{-/-} mice, which showed no symptoms by 1.5 h. While symptoms became progressively more severe in *tnc*^{+/+} mice 4 and 8 h after injection, only a mild phenotype was observed in *tnc*^{-/-} mice, which exhibited significantly less weight loss ($47\% \pm 16.8P<0.0001$ at 4h; $38\% \pm 4.5P=0.0006$ at 8h), less mobility impairment ($76\% \pm 20P<0.0001$ at 4h; $57\% \pm 26.6P<0.0001$ at 8h) and less fur ruffling ($50\% \pm 0P<0.0001$ at 4h; $56\% \pm 19.2P<0.0001$ at 8h) than *tnc*^{+/+} mice, with mild uveitis and diarrhea (Figures 1A and 1B, and 8A and 8B).

On the molecular level, sepsis is driven by a 'cytokine storm' comprising elevated circulating levels of pro-inflammatory cytokines induced in response to LPS. Analysis of plasma levels of key inflammatory mediators of sepsis showed that *tnc*^{-/-} mice synthesize significantly less TNF- α ($70\% \pm 12.1P<0.0001$) and IL-6 ($50\% \pm 8P<0.0001$) compared to *tnc*^{+/+} mice 1.5 h after LPS injection, consistent with the lack of clinical symptoms in the absence of tenascin-C. By 4 h after LPS injection, there was $79\% (\pm 8.7)$ significantly less TNF- α ($P=0.001$) in *tnc*^{-/-} mice compared to *tnc*^{+/+} mice. Plasma TNF- α and IL-6 dropped by 8 h after LPS injection similarly in both *tnc*^{-/-} and *tnc*^{+/+} mice. Furthermore, *tnc*^{-/-} mice fail to induce release of HMGB1, a late mediator that is essential for organ damage in sepsis, which was significantly increased in *tnc*^{+/+} mice 8 h after LPS injection (Figure 1C).

Tissue damage following systemic LPS injection results from immune cell recruitment to susceptible sites; most notably neutrophil infiltration to lungs and liver occurs rapidly during sepsis. Immunohistochemical staining of neutrophil elastase in lungs and liver from *tnc*^{+/+} mice 1.5 h after LPS injection demonstrated substantial neutrophil infiltration, which was significantly reduced in *tnc*^{-/-} mice, an effect also evident at 4 and 8 h after injection (Figures 1D and 8C, 8D).

To determine whether tenascin-C expression was elevated in mice with LPS-induced sepsis plasma tenascin-C in *tnc*^{+/+} mice was quantified after LPS injection.

Immunoblot analysis and ELISA revealed increased tenascin-C in mice upon administration of LPS. Notably, the kinetics of tenascin-C expression was similar to the early pro-inflammatory cytokines TNF- α and IL-6 peaking 1.5 h after LPS injection and decreasing over time (Figures 1E-G). Together these results indicate that
 5 tenascin-C expression is required for the pro-inflammatory response to LPS in vivo.

Bone marrow engraftment rescues TNF- α synthesis in tnc-/- mice

TNF- α was the cytokine most affected by tenascin-C ablation. To determine if the reduced plasma levels of TNF- α observed in tnc-/- mice is mediated by defects in
 10 their hematopoietic cell population, allogeneic bone marrow transplantation was performed. Whole bone marrow cells isolated from tnc+/+ or tnc-/- male mice were injected into sublethally irradiated tnc+/+ or tnc-/- female recipient mice. Tnc+/+/+/+, tnc+/+/-/-, tnc-/-/-/- and tnc-/-+/+ chimeric mice were generated. All mice displayed equally high donor engraftment as shown by quantitative RT-PCR analysis of the
 15 mouse testis-specific Y-encoded protein (TSPY) gene in erythrocyte-depleted peripheral blood cells from recipient mice (Figure. 2A). These data indicate that the majority of bone marrow cells are from the donor.

Analysis of tenascin-C mRNA in erythrocyte-depleted peripheral blood cells from
 20 recipient tnc+/+/-/- mice 1.5 h after LPS injection showed significantly reduced expression of tenascin-C compared to tnc+/+/+/+ mice. The tenascin-C mRNA observed in tnc+/+/-/- mice is likely produced by residual bone marrow cells of the tnc+/+ recipient. Likewise, replenishment of tnc-/- mice with tnc+/+ bone marrow resulted in significantly more tenascin-C expression compared to at levels half of that
 25 expressed by tnc+/+/+/+ -/-/-/- mice (Figure. 2B).

TNF- α levels in plasma from tnc+/+/-/- chimeric mice were 71% ($\pm$ 15.5) significantly lower ($P=0.0033$) than those from tnc+/+/+/+ chimeric mice 1.5 h after LPS injection (Figure 2C). Accordingly, 75% ($\pm$ 5.9) significantly more TNF- α ($P<0.0001$) was
 30 found in plasma from tnc-/-+/+ chimeric mice compared to tnc-/-/-/- chimeric mice (Figure 2D). 8 h after LPS injection, TNF- α was reduced almost to basal levels in each group. Collectively, these data indicate that tenascin-C derived from bone marrow-derived cells drives TNF- α synthesis during LPS-induced sepsis and that bone marrow-derived cells from tnc-/- mice exhibit defects in TNF- α synthesis in vivo.

LPS-activated bone marrow-derived macrophages express tenascin-C

Experiments were also undertaken to investigate whether primary bone marrow-derived macrophages (BMDMs) obtained from *tnc*^{+/+} mice express tenascin-C in response to LPS. A kinetic analysis of tenascin-C expression was performed in LPS-stimulated BMDMs. Non-stimulated cells showed low basal levels of tenascin-C expression, which significantly increased upon stimulation with LPS. Tenascin-C mRNA started to increase at 1 h and peaked between 4 and 8 h and returned to basal levels by 24 h (Figure 3A), as observed in human myeloid cells. Increased tenascin-C mRNA correlated with tenascin-C protein synthesis. Non-stimulated BMDMs produced no detectable tenascin-C, however, cell associated tenascin-C significantly increased after LPS activation and peaked 8 h after stimulation (Figure 3B). Tenascin-C was also secreted into the medium where its levels significantly increased 24 h after stimulation (Figure 3C). These data demonstrate that tenascin-C expression in BMDMs is transiently induced by LPS.

15

To verify that tenascin-C ablation does not affect macrophage development in vitro and in vivo, *tnc*^{+/+} and *tnc*^{-/-} BMDMs or freshly isolated peritoneal macrophages were assayed for CD11b, F4/80 and CD68 expression. Each maturation marker was equally highly expressed in *tnc*^{+/+} and *tnc*^{-/-} cells (Figures 9A-O), indicating that tenascin-C has no effect on macrophage maturation.

20

Tenascin-C post-transcriptionally regulates LPS-mediated synthesis of specific cytokine subsets in BMDMs

To further examine the mechanism behind impaired pro-inflammatory cytokine synthesis in *tnc*^{-/-} mice upon LPS administration, the effects of tenascin-C on cytokine synthesis in BMDMs was studied. There was no significant difference in IL-1 β , IL-12 and TGF β 1 secretion between *tnc*^{+/+} and *tnc*^{-/-} BMDMs. However, BMDMs obtained from *tnc*^{-/-} mice secreted significantly less TNF- α , IL-6 and CXCL1, and significantly more IL-10, upon stimulation with LPS than did *tnc*^{+/+} cells (Figure 4A).

30

The expression of TNF- α , IL-6, CXCL1 and IL-10 mRNA was induced in *tnc*^{+/+} BMDMs by LPS stimulation and was not significantly affected by tenascin-C ablation (Figure 4B). No difference in IL-12 and TGF β 1 mRNA expression was observed in

tnc^{+/+} and tnc^{-/-} BMDMs (data not shown). These results suggest that regulation of cytokine synthesis by tenascin-C occurs at a post-transcriptional level.

To ensure that the effect of tenascin-C on cytokine synthesis is not due to abnormal macrophage activation in vitro or in vivo, the expression of activation markers in tnc^{+/+} and tnc^{-/-} BMDMs or freshly isolated peritoneal macrophages was analysed. Equal expression of MHC II, CD40 or CD86 and the LPS receptor TLR4/MD2 was observed in tnc^{+/+} and tnc^{-/-} BMDMs (Figures 10A-J) or peritoneal macrophages (data not shown). Thus, tenascin-C has no effect on macrophage activation.

Tenascin-C promotes translation of pro-inflammatory cytokines

Impaired pro-inflammatory cytokine production in the absence of any reduction in cytokine mRNA levels in tnc^{-/-} cells led us to investigate whether tnc^{-/-} BMDMs exhibit abnormal intracellular cytokine levels as a consequence of deficient secretion.

There was no significant difference in cellular TNF- α , IL-6 and CXCL1 levels in LPS-stimulated tnc^{+/+} and tnc^{-/-} BMDMs (Figure 5A). Cytokine levels in the cellular fraction did not account for the defective cytokine secretion by tnc^{-/-} BMDMs as shown by the sum of the amounts of cellular and secreted protein fractions (Figure 5B). These data demonstrate that pro-inflammatory cytokine cellular trafficking occurs normally in BMDMs in the absence of tenascin-C.

The effect of tenascin-C on pro-inflammatory cytokine translation was also analysed. Brefeldin A, an inhibitor of ER-to-Golgi protein transport, resulted in intracellular accumulation of TNF- α , IL-6 and CXCL1 in tnc^{+/+} cells at 24 h. In contrast, intracellular accumulation of TNF- α was abrogated in tnc^{-/-} BMDMs. Significantly less IL-6 and CXCL1 also accumulated within the Golgi in tnc^{-/-} BMDMs (Figure 5C). Together these results indicate that tenascin-C may be required for effective translation of LPS-induced pro-inflammatory cytokines in BMDMs.

Tenascin-C controls TNF-alpha production by driving miR-155 expression

The data presented herein demonstrates that tenascin-C mediates an initial pro-inflammatory response to infection by post-transcriptionally modulating the expression of the early TLR-induced microRNA miR-155. This miRNA is processed from the non-protein-coding transcript of the bic gene. By means of a miRNA screen and validation analysis, it was found that expression of both primary transcript pri-

miR155 and mature miR-155 was induced in *tnc*^{+/+} BMDMs immediately after LPS stimulation. However, the induction of mature miR-155 in *tnc*^{-/-} BMDMs was significantly inhibited. This is underscored by the significantly lower miR-155 expression detected in vivo in the spleen of septic *tnc*^{-/-} mice. Consistent with this a
5 positive correlation between miR-155 expression and TNF- α production was observed in *tnc*^{+/+} BMDMs upon LPS stimulation. Interestingly, in the absence of tenascin-C, impaired TNF- α synthesis was parallel to impaired miR-155 expression. This observation that tenascin-C functions as a tuner of miR-155 expression for an effective TNF- α production was demonstrated by overexpressing miR-155 in *tnc*^{-/-}
10 BMDMs, which fully regained the ability to release TNF- α in response to LPS. These data suggest that tenascin-C induced by LPS in BMDMs uses miR-155 to modulate TNF- α levels.

Results

15 miRNA expression in *tnc*^{+/+} and *tnc*^{-/-} BMDMs stimulated with LPS for 8 h was analyzed with an array containing 375 miRNAs. 35 miRNAs were found that were reproducibly and significantly upregulated or downregulated by LPS (Figure 6A). A number of miRNAs have emerged as important regulators of TLR signaling including those induced by LPS activation of TLR4. Among these, expression of early response
20 genes such as miR-155, miR-125b and let-7i (Figure 6B) was observed, as well as late response genes, including miR-146a, miR-132 and miR-21 (Figure 6C). In *tnc*^{+/+} BMDMs LPS strongly induced miR-155 expression, which was significantly inhibited in *tnc*^{-/-} cells (Figures 6A,B). Using quantitative RT-PCR, these results were confirmed and showed that miR-155 expression was rapidly induced by LPS, peaked
25 at 24 h and progressively declined in *tnc*^{+/+} BMDMs. Conversely, LPS-induced miR-155 expression in *tnc*^{-/-} BMDMs was inhibited and did not recover later (Figure 6D). Furthermore, analysis of the primary transcript pri-miR-155 in the same samples revealed no significant difference between *tnc*^{-/-} and *tnc*^{+/+} BMDMs, which suggests that tenascin-C post-transcriptionally regulates LPS-induced miR-155 expression.

30

Changes in TNF- α levels and miR-155 expression were shown to significantly correlate in LPS-stimulated *tnc*^{+/+} BMDMs. Accordingly, low TNF- α levels correlated with low miR-155 expression in *tnc*^{-/-} BMDMs (Figure 6E). This effect was specific, as IL-6, CXCL1, and IL-10 synthesis did not correlate with miR155
35 expression ($P=0.233$; data not shown). To determine whether these *in vitro* data are

relevant *in vivo*, the expression of LPS-induced miR-155 in the spleen of *tnc*^{+/+} and *tnc*^{-/-} mice upon LPS injection was examined. *Tnc*^{-/-} mice expressed significantly less miR-155 than *tnc*^{+/+} mice (Figure 6F). Taken together, these data suggest that tenascin-C is required for the induction of miR-155 by LPS in BMDMs and in septic mice.

Tenascin-C controls TNF- α release via the induction of miR-155

To determine whether reconstituting miR-155 expression rescues LPS-induced TNF- α production in the absence of tenascin-C, the mature 23-nucleotide miRNA was overexpressed in *tnc*^{-/-} BMDMs. Cells were transiently transfected with a negative control precursor or pre-miR-155 precursor, which mimics the endogenous precursor miR-155. Transfection of pre-miR-155 precursor, but not control precursor, resulted in increased expression of mature miR-155 with or without LPS stimulation. However, stimulation of transfected *tnc*^{-/-} BMDMs with LPS for 8 h further increased miR-155 expression, which was similar to that induced by LPS in control *tnc*^{+/+} BMDMs (Figures 7A and 11A). Thus, impaired miR-155 expression in *tnc*^{-/-} BMDMs was recovered. The effect of miR-155 overexpression on cytokine TNF- α release in *tnc*^{-/-} BMDMs upon LPS stimulation for 8 h was analysed. In the absence of LPS, very low basal levels of TNF- α cytokines were detected in untransfected and transfected cells. TNF- α production in *tnc*^{-/-} BMDMs transfected with pre-miR-155 precursor and stimulated with LPS for 8 h was significantly higher than in LPS-activated *tnc*^{-/-} cells transfected with control precursor and was equal to that of control *tnc*^{+/+} BMDMs (Figures 7B and 11B). Thus, miR-155 overexpression rescued TNF- α release in *tnc*^{-/-} BMDMs in response to LPS. Together, these data provide evidence that tenascin-C post-transcriptionally controls the production of TNF- α in response to LPS via miR-155.

The addition of soluble tenascin-C at a concentration comparable to that made by LPS stimulated *tnc*^{+/+} BMDMs (<10ng/ml) did not rescue defects in *tnc*^{-/-} BMDM cytokine synthesis. Moreover, high concentrations of soluble tenascin-C (up to 1 μ g/ml) inhibited cytokine synthesis (Figure 11C).

Experimental Procedures

Mice. Littermate *tnc*^{+/+} and *tnc*^{-/-} mice were from heterozygous breeding pairs on a 129/SvJ background (Saga et al., 1992). All mice were male aged between 8-12

weeks, except for bone marrow transplantation which were female aged 6-8 weeks. For the generation of BMDMs, bone marrow was flushed from tibias and femurs of *tnc*^{+/+} and *tnc*^{-/-} mice and red blood cells were lysed with Red Blood Cell Lysis Buffer (Sigma). The resulting cells were cultured in DMEM (PAA) supplemented with
5 20% FCS (GIBCO), 1% Antibiotic-Antimycotic solution (PAA) and 50 μ M β -mercaptoethanol (Invitrogen) containing 100ng/ml recombinant murine M-CSF (Peprotech). After 7 d, adherent cells were washed and replated, then stimulated with LPS alone (100ng/ml; Enzo Life Sciences) or with brefeldin A (1 μ g/ml; Sigma). To avoid cell toxicity, we treated BMDMs with brefeldin A for the last 8 h of incubation
10 with LPS. For the generation of thioglycollate-elicited peritoneal macrophages, *tnc*^{+/+} and *tnc*^{-/-} mice were injected intraperitoneally with 1 ml thioglycollate (3% wt/vol; Sigma). Peritoneal exudates were collected 5 d after with cold PBS and cells were analyzed by FACS. For analysis of LPS-induced sepsis, *tnc*^{+/+} and *tnc*^{-/-} mice were injected intraperitoneally with lipopolysaccharides from *E. coli* 055:B5 (8mg/kg;
15 Sigma). Mice were observed for 8 h. Body weight, mobility, uveitis, respiration, fur ruffling and diarrhoea were recorded at 0, 1.5, 4 and 8 h after LPS injection. Mobility impairment was graded using an arbitrary scale where 0 = none, 1 = mild (reduced and slower movement), 2 = moderate (movement upon stimulation) and 3 = severe (no movement at all). Mice were killed after 1.5, 4 or 8 h and plasma and tissues were
20 collected. All procedures were approved by the UK Home Office.

ELISA. ELISA for TNF- α , IL-6, IL-8 (R&D), IL-1 β (eBioscience), IL-12, TGF β 1, IL-10, (BD Biosciences), HMGB1 and TN-C (IBL) were used to quantify secreted and cell-associated pro-inflammatory mediators in plasma, cell supernatants and total protein extracts respectively. Extracts were prepared by lysing cells (1x10⁶) with
25 0.1ml RIPA buffer containing a protease inhibitor cocktail (Sigma).

Immunohistochemical analysis. Formalin-fixed tissues from *tnc*^{+/+} and *tnc*^{-/-} mice were paraffin-embedded, cut into 4 μ m sections and stained with neutrophil elastase primary antibody (MCA7771G; AbD Serotec). Immunostaining specificity was confirmed by omitting the primary antibody or using a non-immune rat IgG2a
30 (MCA1212, AbD Serotec). Neutrophil elastase+ cell number was determined by counting cells in five different areas of the total tissue section.

Immunoblot. Plasma samples from *tnc*^{+/+} and *tnc*^{-/-} mice were resolved by SDS-PAGE and analyzed by Western blotting using goat anti-tenascin-C polyclonal Ab (AF3358; R&D Systems).

Bone marrow transplantation. Each *tnc*^{-/-} and *tnc*^{+/+} female recipient mouse was exposed to a single dose of 9 Gy at 80 cGy/min radiation in a γ irradiator (Nordion) and, 3 h later, intravenously injected with whole bone marrow cells isolated from one *tnc*^{-/-} or *tnc*^{+/+} male mouse resuspended in 0.2 ml PBS. Transplanted mice received
 5 antibiotic orally for 11 weeks (2.5% Baytril; Bayer). The degree of donor engraftment was assessed as described previously (Wang et al., 2002). Genomic DNA was extracted from peripheral blood cells of female recipient and control mice using a QIAamp DNA Blood Mini kit (Qiagen). DNA samples were analyzed for the presence of the Y chromosome by two-standard curve method based on quantitative real-time
 10 PCR in a Corbett Rotor-gene 6000 machine (Corbett Research) with TaqMan primers and probe designed to detect the TSPY gene (fwd 5'-TCCTTGGGCTCTTCATTATTCTTAAC-3' (Seq ID No: 3); rev 5'-GAGAACCACGTTGGTTTGAGATG-3' (Seq ID No: 4); probe 6FAM-TCCTGGATCAGAGTGGCTTACCCAGG-TAMRA (Seq ID No: 5); Applied
 15 Biosystems). Standard curves were obtained by mixing male and female DNA from non-treated control mice. Samples were standardized against a mouse GAPDH genomic primer/probe set (Applied Biosystems). Samples were calibrated against DNA from male non treated control mouse.

RNA extraction and quantitative real-time PCR. Total RNA was extracted from
 20 cells (1×10^6) using a RNeasy Mini Kit (Qiagen). cDNA was synthesized from equivalent amounts of RNA with AffinityScript reverse transcriptase and oligo(dT) primer (Stratagene). This was followed by quantitative real-time PCR in a Corbett Rotor-gene 6000 machine (Corbett Research) with TaqMan primer sets for mouse tenascin-C (Mm00495662_m1), TNF- α (Mm99999068_m1), IL-6 (Mm00446190_m1),
 25 CXCL1 (Mm00433859_m1), IL-10 (Mm00439614_m1), CD11b (Mm00434455_m1), CD68 (Mm00839636_g1), F4/80 (Mm00802529_m1) and HPRT1 (Mm00446968_m1) (Applied Biosystems). For miRNA analysis, total RNA including small RNA was extracted from cells (2×10^6) using Trizol (Invitrogen). For mature miRNA detection, RNA was reverse transcribed using the Taqman miRNA reverse transcription kit,
 30 including the miRNA-specific primers, followed by real-time PCR with individual miRNA TaqMan assays for the endogenous reference RNA RNU6B, miR-155, miR-146a and miR-21 (Applied Biosystems). For pri-miRNA detection, total RNA was reverse transcribed with AffinityScript reverse transcriptase and random primers (Stratagene), followed by real-time PCR with TaqMan primer sets for mouse pri-
 35 miR155 (Mm03306395_pri) and HPRT1 (Applied Biosystems). Changes in expression

were calculated by the change-in-threshold ($\Delta\Delta CT$) method with HPRT1 and RNU6B as endogenous controls for gene-expression and miRNA analysis respectively and were normalized to results obtained with untreated cells.

miRNA array. The expression profile of 375 miRNAs was analyzed with TaqMan
5 Low Density Arrays in an ABI 7900HT (Applied Biosystems). Total RNA was extracted from cells with Trizol. RNA integrity was analyzed using an Agilent RNA 6000 Nano kit and an Agilent 2100 bioanalyzer (Agilent Technologies). cDNA was synthesized with a TaqMan microRNA reverse transcription kit and Megaplex RT primers and was followed by real-time PCR with a TaqMan universal PCR master mix
10 and TaqMan array rodent microRNA cards (Applied Biosystems). Data were analyzed with SDS Relative Quantitation software (Applied Biosystems).

Transient transfection. For transfection of miRNA, 2×10^6 tnc-/- BMDMs were transfected with negative control precursor or miR-155 precursors at a final concentration of 5nM with siPORT NeoFX transfection agent (Ambion) for 8 h. Cells
15 were allowed to recover for 24 h before treatment with LPS for 8 h.

Statistical analysis. Statistical analysis was performed using Student's T-test, one-way ANOVA with Dunnett's multiple comparison post-test or two-way ANOVA with Bonferroni post-test where appropriate; correlation was assessed with Spearman's rho test (Prism 5; GraphPad Software).

Clinical evaluation of septic mice. Mice were observed during a period of 8 h upon LPS injection. Pictures of mice were taken to record uveitis at 0, 4 and 8 h after LPS injection. Uveitis was considered mild when eyes were watery, moderate when one or two eyes displayed pus and severe when one or two eyes were closed. Fur ruffling was recorded at 0, 1.5, 4 and 8 h after LPS injection and was scored as 0, 1, 2 or 3 for
25 absent, mild, moderate or severe, respectively.

Flow cytometry. For surface staining, peritoneal macrophages and BMDMs were stained for 20 min at 4°C with anti-F4/80-PE (Caltag Medsystems), anti-CD11b-APC, anti-MHC II (I-A/I-E)-PE, anti-CD40-APC, anti-CD80-PE, anti-CD86-FITC, anti-TLR4/MD-2-APC and appropriate isotype controls (ebioscience). For intracellular
30 staining, cells were fixed with 4% paraformaldehyde in PBS for 20 min, were made permeable with PBS containing 2% (vol/vol) BSA, 2mM EDTA, 0.02% NaN₃ and 0.05% (vol/vol) saponin and were stained with anti-CD68-APC (AbD Serotec). Samples were analyzed on a FACSCanto II (BD Bioscience) and data were analyzed with FlowJo software (TreeStar).

Treatment of BMDMs with soluble tenascin-C. To assess whether addition of exogenous tenascin-C to *tnc*^{-/-} BMDMs can rescue defects in cytokine synthesis in response to LPS, recombinant human tenascin-C, was synthesized and purified as described previously (Midwood et al., 2009), or commercially purified human
5 tenascin-C (IBL, Japan) was used. 1×10^5 *tnc*^{+/+} and *tnc*^{-/-} BMDMs were stimulated for 24 h with 100ng/ml LPS without or with 1 ng/ml to 1µg/ml recombinant purified tenascin-C. Cell supernatants were analysed by ELISA to quantify secreted TNF-α and IL-6 (R&D). Viability of the cells throughout the experimental time period was examined by the MTT cell viability assay (Sigma). Cell viability was not affected by
10 treatment with tenascin-C. Addition of tenascin-C to *tnc*^{-/-} and *tnc*^{+/+} BMDMs in the absence of LPS stimulated cytokine synthesis (data not shown).

CLAIMS

1. An agent which reduces the level and/or activity of tenascin-C and/or miR-155 for use in the treatment of pathogen induced inflammation.
- 5 2. Use of an agent that reduces the level of tenascin-C and/or miR-155 for the preparation of a medicament for the prevention or treatment of pathogen induced inflammation.
- 10 3. A method of treating or preventing pathogen induced inflammation comprising administering to a subject in need thereof an effective amount of an agent according to claim 1.
- 15 4. The agent of claim 1, the use of claim 2 or the method of claim 3 wherein the pathogen induced inflammation is bacterial sepsis.
5. The agent, use or method of any preceding claim wherein the pathogen induced inflammation is caused by lipopolysaccharide (LPS).
- 20 6. The agent, use or method of any preceding claim wherein the agent acts directly to reduce the level and/or activity of tenascin-C.
7. The agent, use or method of any preceding claim wherein the agent acts directly to reduce the level and/or activity of miR-155.
- 25 8. The agent, use or method of any preceding claim wherein the agent acts on tenascin-C to reduce the level and/or activity of miR-155.
9. The agent, use or method of any preceding claim wherein the agent acts to
- 30 reduce the level or activity of cell associated tenascin-C.
10. The agent, use or method of any preceding claim wherein the agent acts to reduce the level of tenascin-C and/or miR-155 to less than about 80% the level or activity of tenascin-C and/or miR-155 observed in a reference sample.

11. The agent, use or method of any preceding claim wherein the agent acts to prevent, inhibit or reduce interaction between tenascin-C and miR-155.

12. A pharmaceutical composition comprising an agent according to any of claims 1 and 4 to 11 and a pharmaceutically acceptable excipient.

13. A method for determining the efficacy of a treatment for pathogen induced inflammation comprising the steps of:

- i) determining the level and/or activity of tenascin-C and/or miR-155 in a biological sample from a subject;
- ii) administering said treatment to the subject;
- iii) determining the level and/or activity of tenascin-C and/or miR-155 in a biological sample from said mammalian subject at a time following administration of said treatment

wherein a reduction in the level and/or activity of tenascin-C and/or miR-155 following administration of the treatment is indicative of said treatment being a therapy for pathogen induced inflammation.

14. An *in vivo* method of screening agents/compositions for efficacy in the treatment of pathogen induced inflammation comprising the steps of:

- i) determining the level and/or activity of tenascin-C and/or miR-155 in a biological sample from a subject, the subject may be known to be suffering from pathogen induced inflammation;
- ii) administering said agent/composition to the subject;
- iii) determining the level and/or activity of tenascin-C and/or miR-155 in a biological sample taken from the subject at a time following administration of said agent/composition;

wherein a reduction in the level and/or activity of tenascin-C and/or miR-155 following administration of the agent/composition is indicative of said agent/composition being a therapy for pathogen induced inflammation.

15. An *in vitro* method of screening agents/compositions for efficacy in the treatment of pathogen induced inflammation comprising the steps of:

- i) providing a biological sample obtained from a subject;

- ii) determining the level and/or activity of tenascin-C and/or miR-155 in the biological sample;
- iii) treating the biological sample with said agent/composition *in vitro*;
- iv) determining the level and/or activity of tenascin-C and/or miR-155 in the biological sample at a time following administration of said agent/composition;

wherein a reduction in the level and/or activity of tenascin-C and/or miR-155 following administration of the agent/composition is indicative of said agent/composition being a therapy for pathogen induced inflammation.

16. An agent/composition identified by the method of any of claims 13 to 15

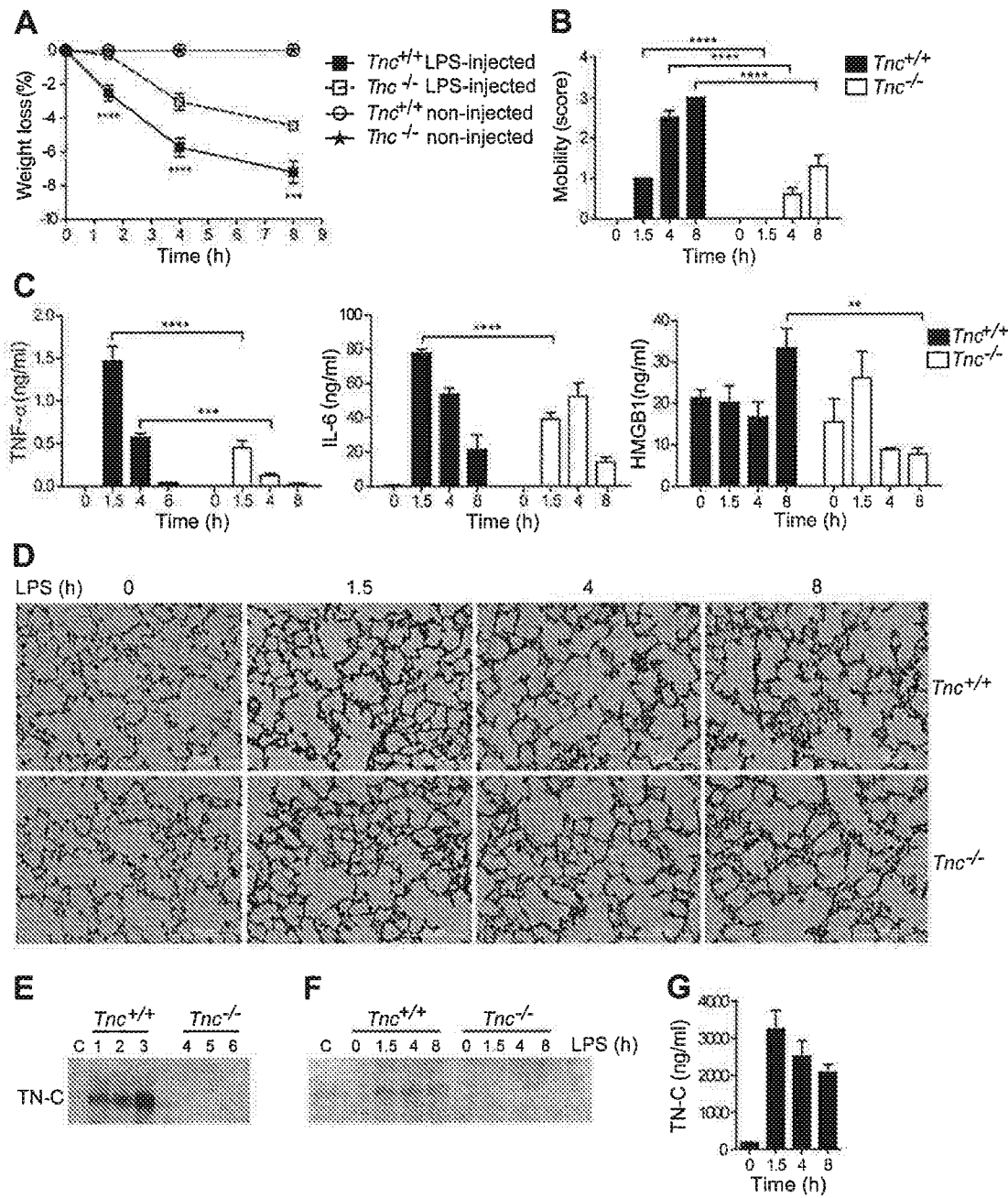


Figure 1

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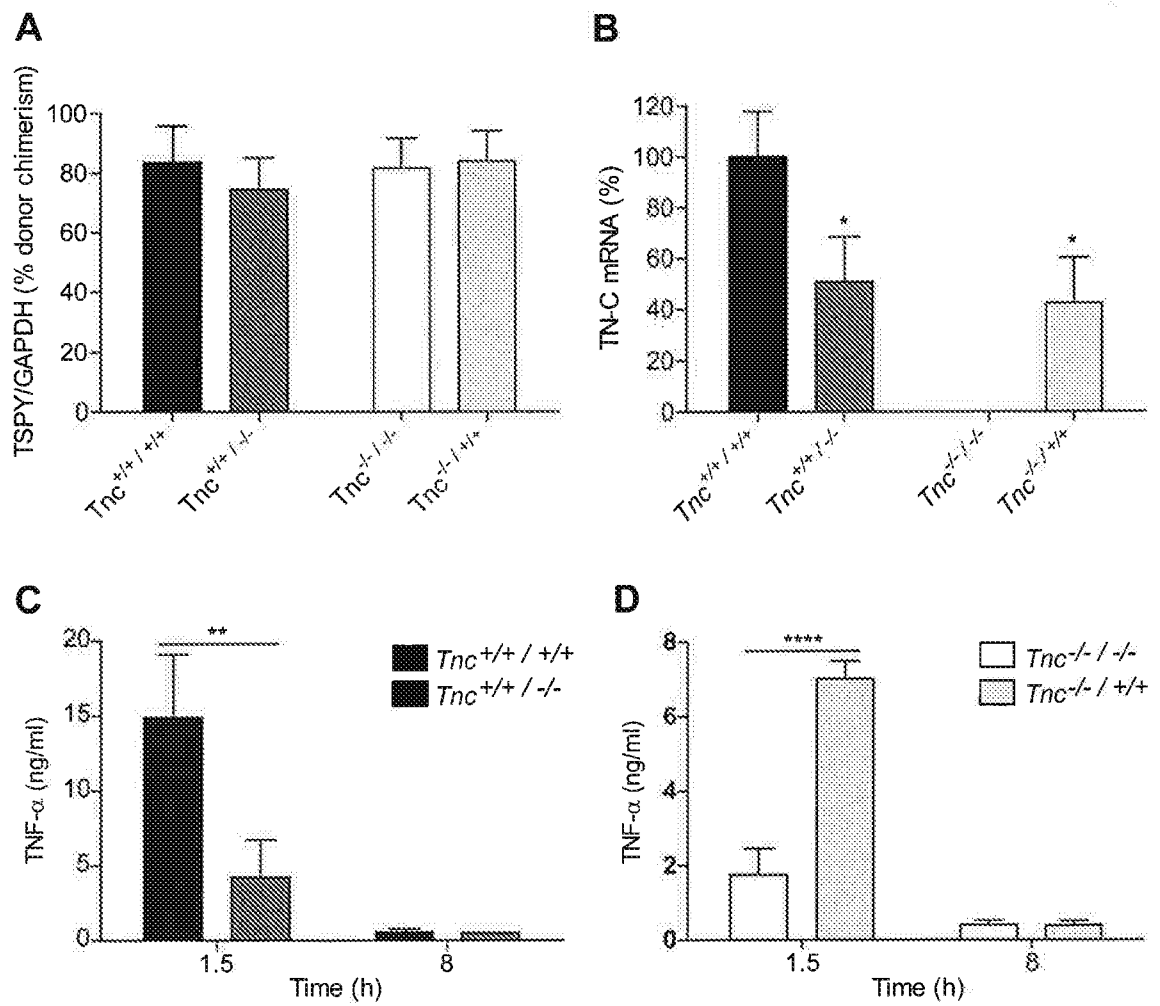


Figure 2

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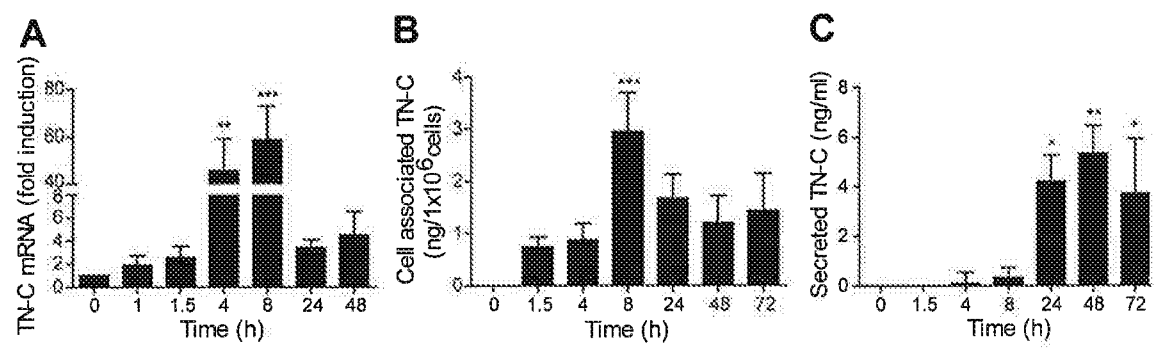
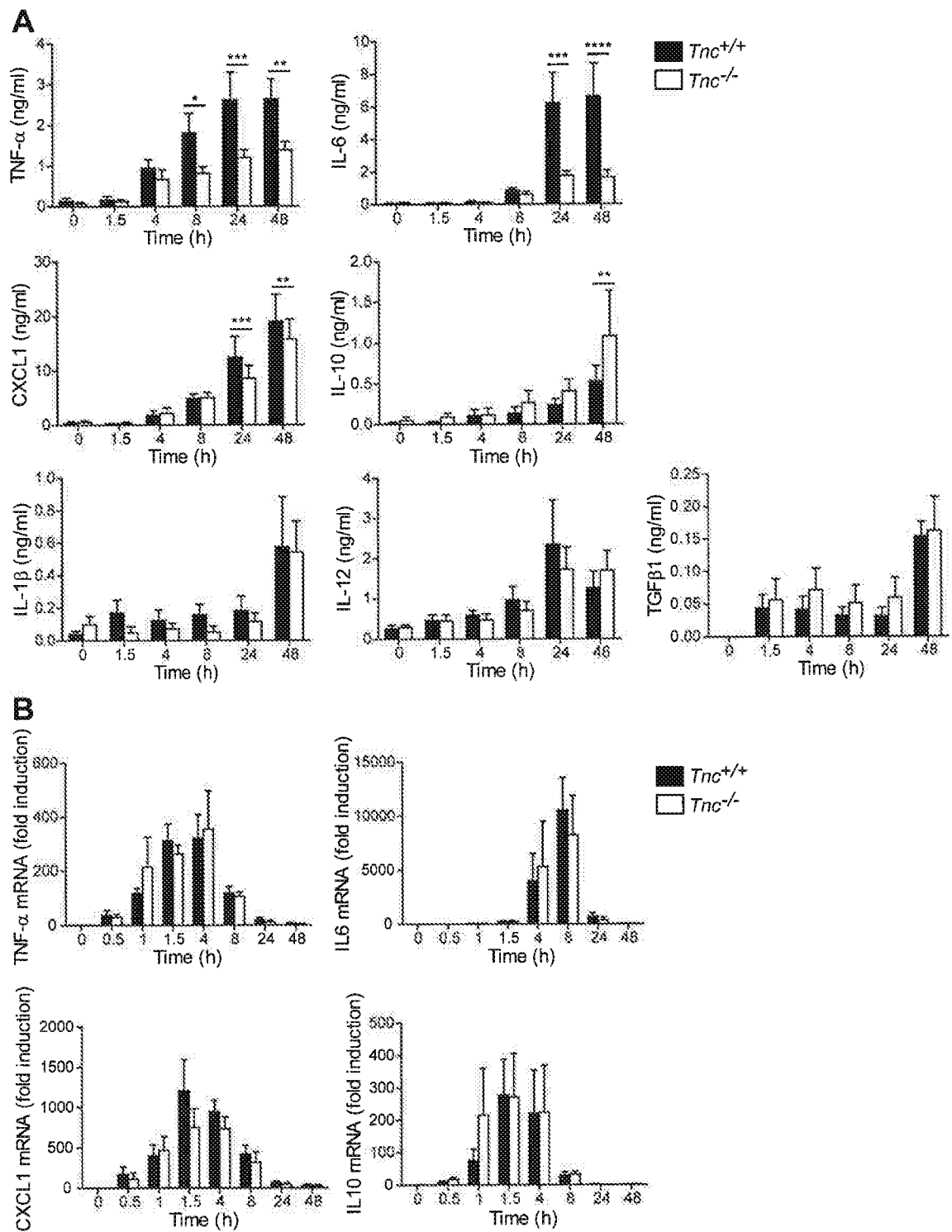


Figure 3

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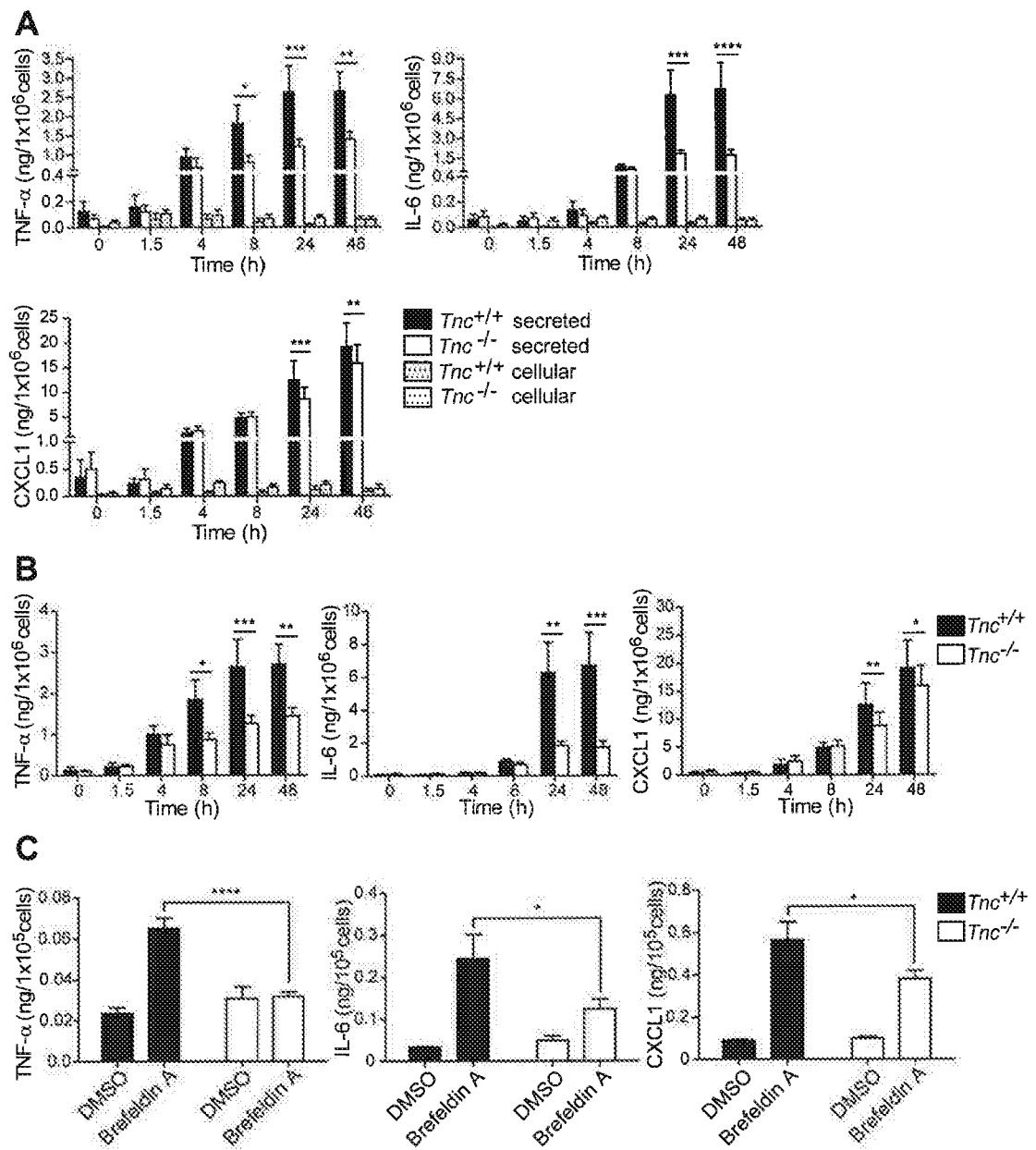


Figure 5

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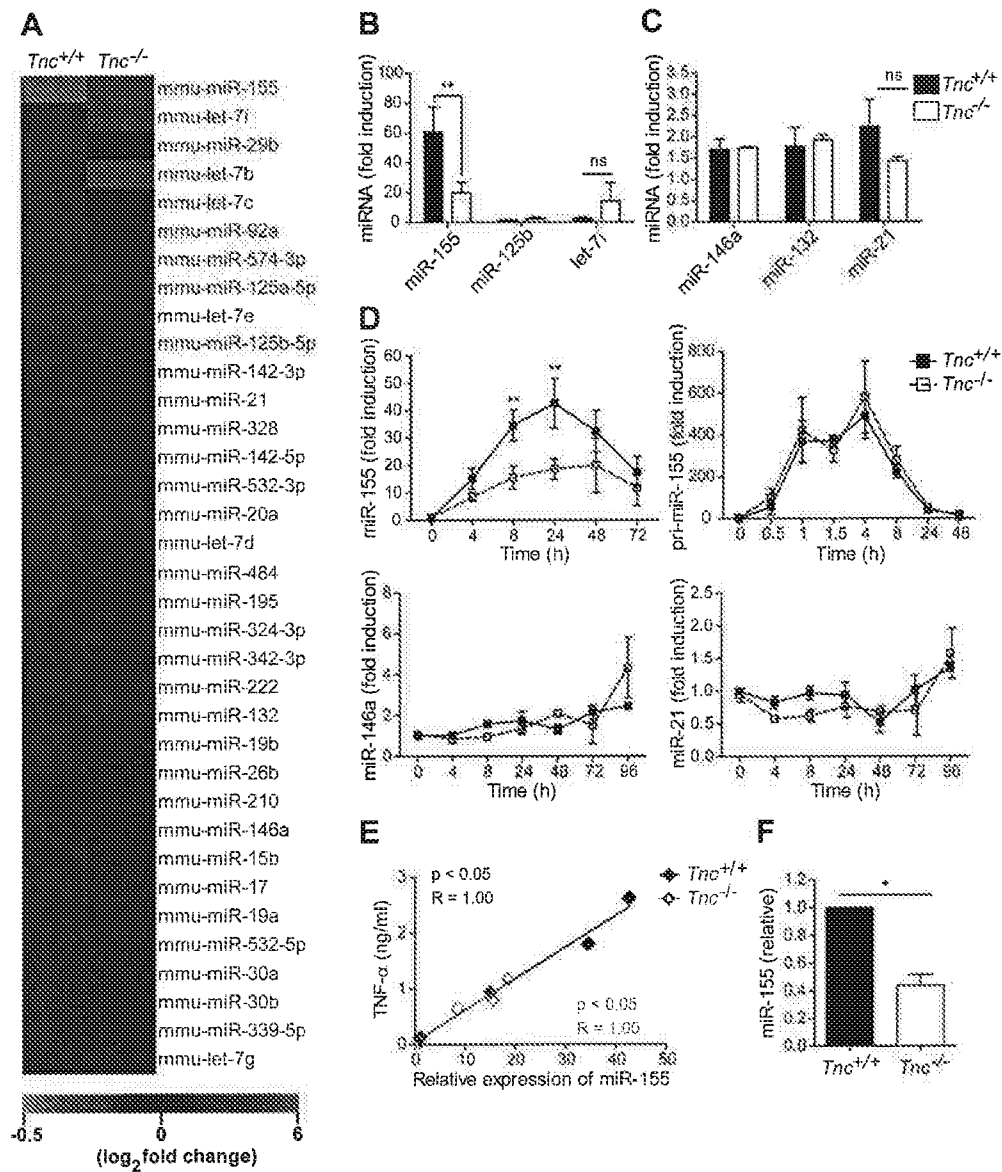


Figure 6

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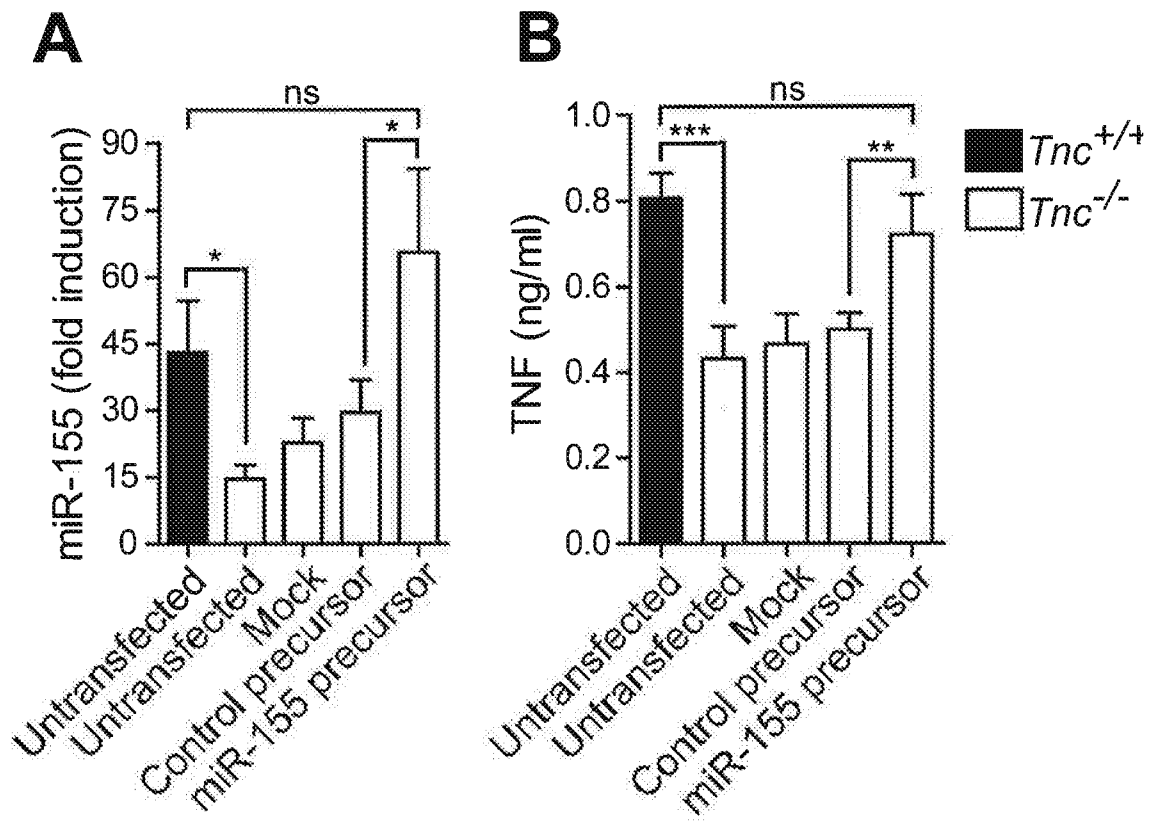


Figure 7

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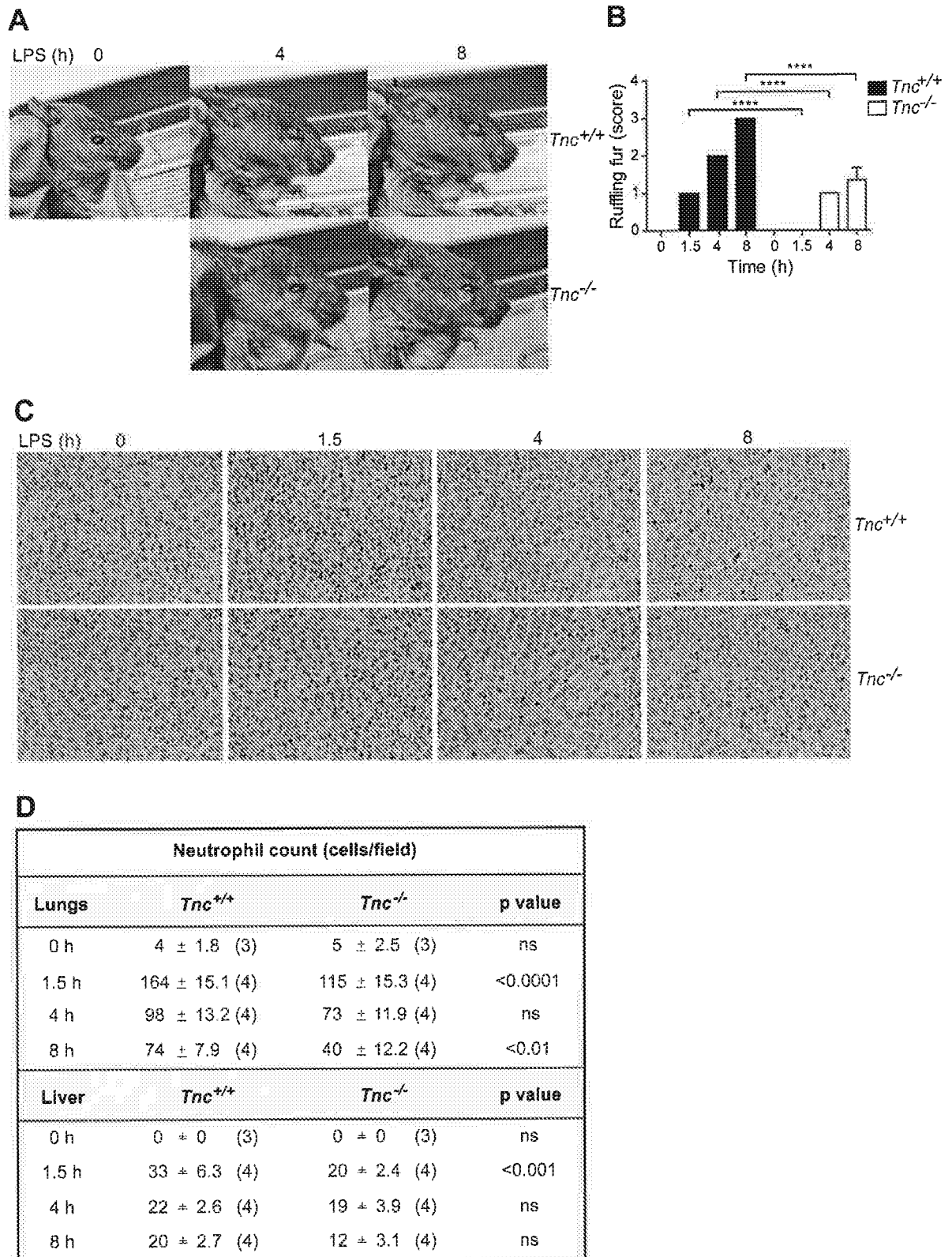


Figure 8

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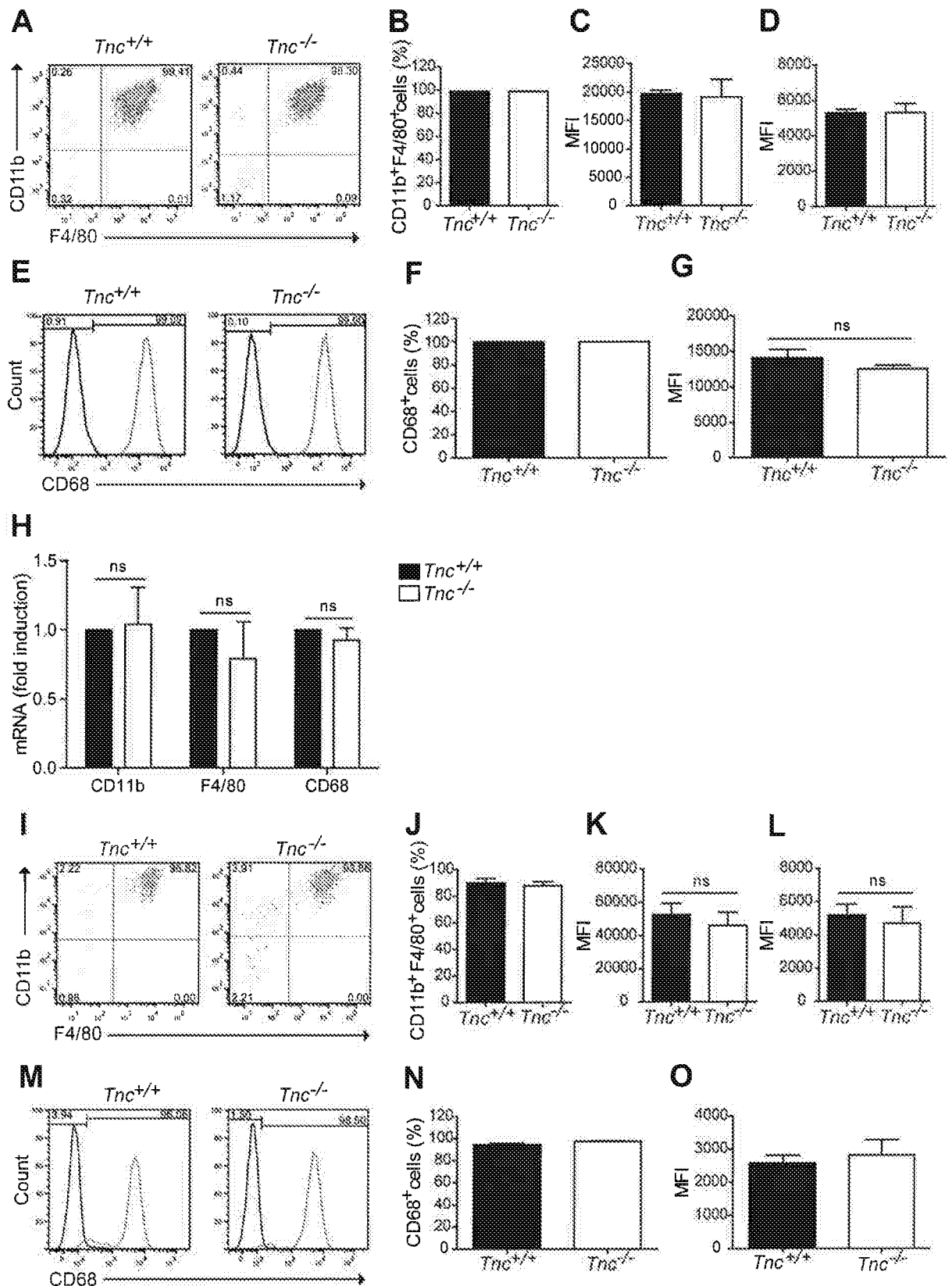


Figure 9

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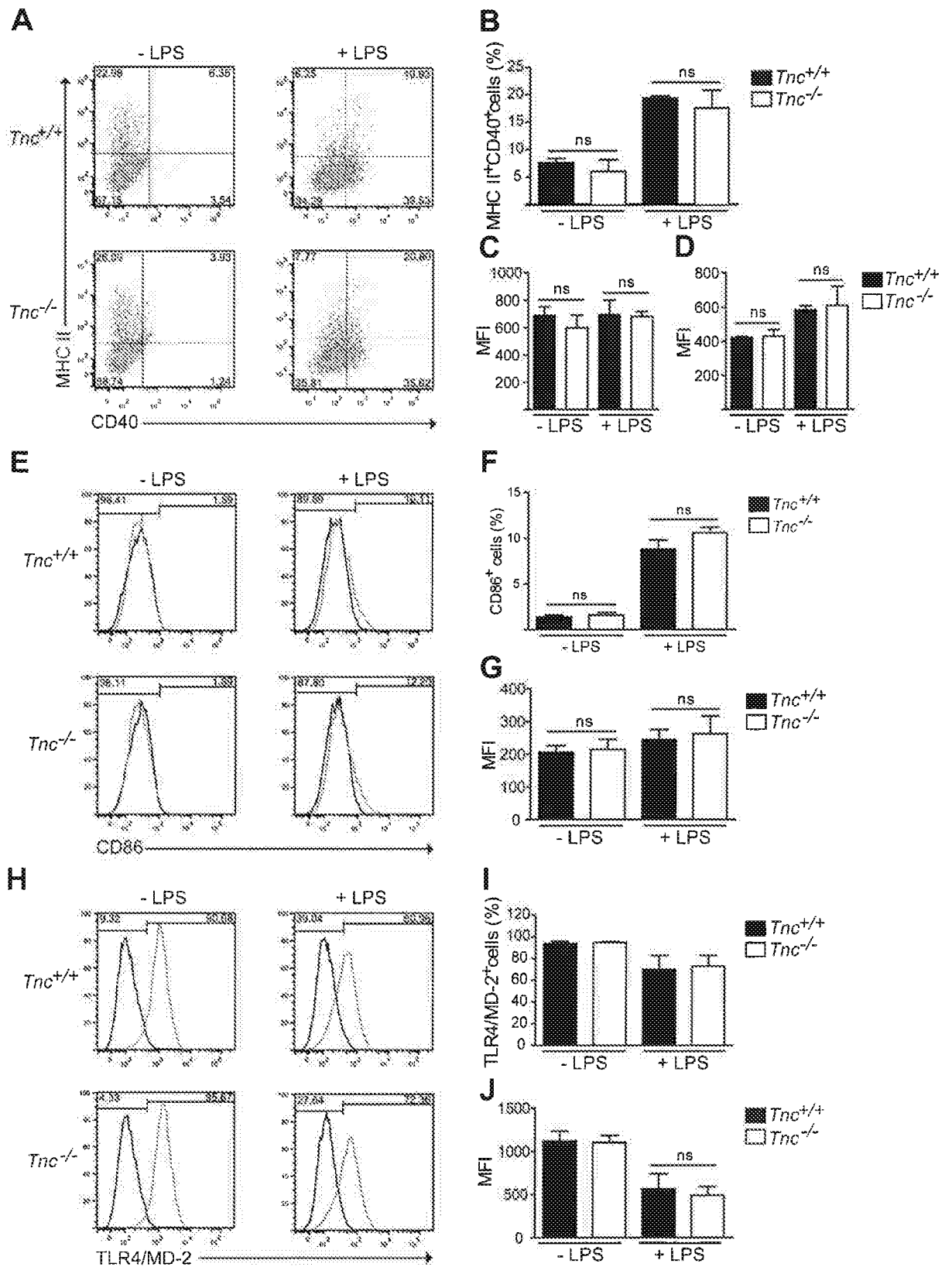


Figure 10

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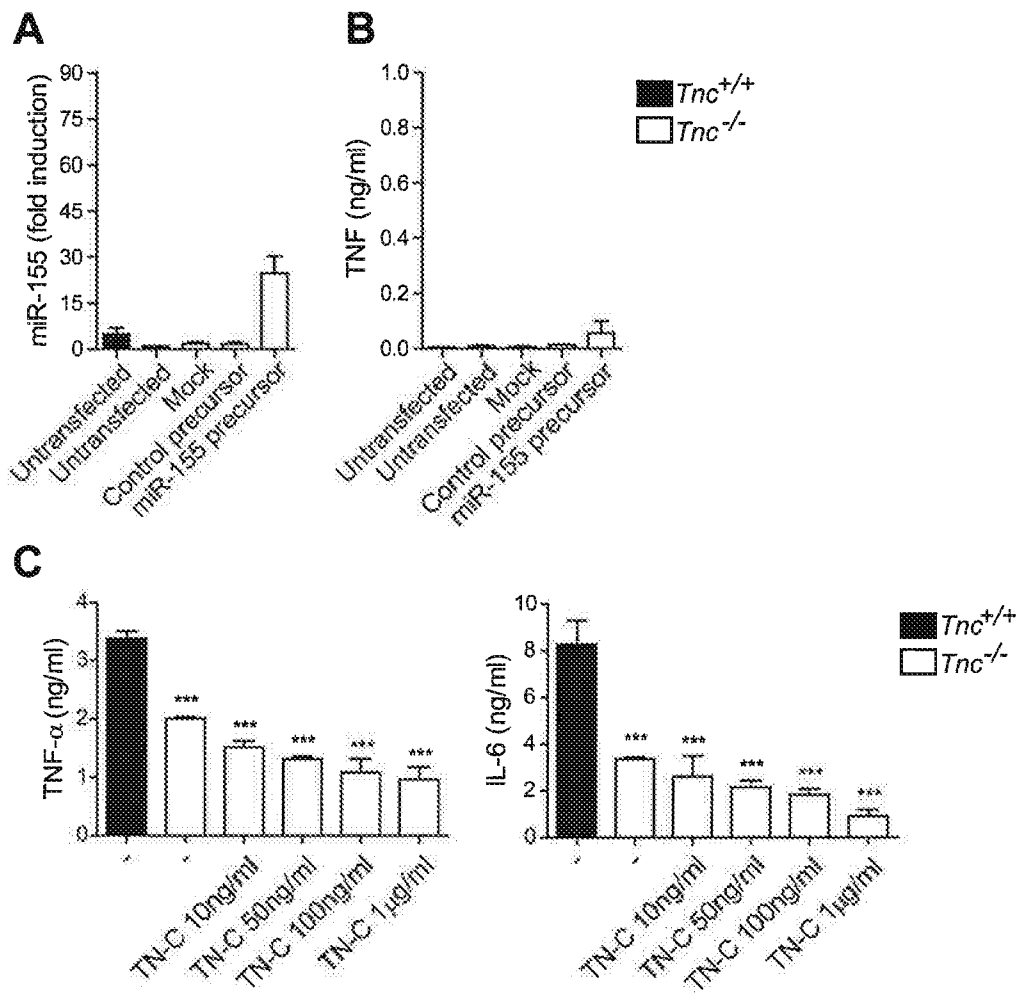


Figure 11

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10	20	30	40	50	60
MGAMTQLLAG	VFLAFLALAT	EGGVLKKVIR	HKRQSGVNAT	LPEENQPVVF	NHVYNIKLPV
70	80	90	100	110	120
GSQCSVDLES	ASGEKDLAPP	SEPSESFQEH	TVDGENQIVF	THRINIPRRA	CGCAAAPDVK
130	140	150	160	170	180
ELLSRLEELE	NLVSSLREQC	TAGAGCCLQP	ATGRLDTRPF	CSGRGNFSTE	GCGCVCEPGW
190	200	210	220	230	240
KGPNCSEPEC	PGNCHLRGRC	IDGQCICDDG	FTGEDCSQLA	CPSDCNDQ GK	CVNGVCICFE
250	260	270	280	290	300
GYAGADCSRE	ICPVPCSEEH	GTCVDGLCVC	HDGFAGDDCN	KPLCLNNCYN	RGRCVENECV
310	320	330	340	350	360
CDEGFTGEDC	SELICPND CF	DRGRCTINGTC	YCEEFTGED	CGKPTCPHAC	HTQGRCEE GQ
370	380	390	400	410	420
CVCDEGFAGV	DCSEKRCPAD	CHNRGR CVDG	RCECDDGFTG	ADCGELKCPN	GCSGHGR CVN
430	440	450	460	470	480
GQCVCDEGYT	GEDCSQLRCP	NDCHSRGR CV	EGKCVCEQGF	KGYDCSDMSC	PNDCHQHGR C
490	500	510	520	530	540
VNGMCVCDDG	YTGEDCRDRQ	CPRDCSNRGL	CVDGQCV CED	GFTGPDCAEL	SCPNDCHGQG
550	560	570	580	590	600
RCVNGQCVCH	EGFMGKDCKE	QRCPDCHGQ	GRCVDGQCIC	HEGFTGLDCG	QHSCPSDCNN
610	620	630	640	650	660
LGQCVSGRCI	CNEGYSGEDC	SEVSPPKDLV	VTEVTEETVN	LAWDNEMRVT	EYLVVYTP TH
670	680	690	700	710	720
EGGLEMQFRV	PGDQTSTIIQ	ELEPGVEYFI	RVFAILENKK	SIPVSARVAT	YLPAP EGLKF

Figure 12....

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730 740 750 760 770 780
 KSIKETSVEV EWDPLDIAFE TWEIIFRNMN KEDEGEITKS LRRPETSRYQ TGLAPGQEYE
 790 800 810 820 830 840
 ISLHIVKNNT RGPGLKRVTT TRLDAPSQIE VKDVTDTTAL ITWFKPLAEI DGIELTYGIK
 850 860 870 880 890 900
 DVPGDRTTID LTEDENQYSI GNLKPDTEYE VSLISRRGDM SSNPAKETFT TGLDAPRNLR
 910 920 930 940 950 960
 RVSQTDNSIT LEWRNGKAAI DSYRIKYAPI SGGDHAEDV PKSQQATTKT TLTGLRPGTE
 970 980 990 1000 1010 1020
 YGIGVSAVKE DKESNPATIN AATELDTPKD LQVSETAETS LTLLWKTPLA KFDRYRLNYS
 1030 1040 1050 1060 1070 1080
 LPTGQWVG VQ LPRNTTSYVL RGLEPGQEYN VLLTAEKGRH KSKPARVKAS TEQAPELENL
 1090 1100 1110 1120 1130 1140
 TVTEVGWDGL RLNWTAADQA YEHFIIQVQE ANKVEAARNL TVPGSLRAVD IPGLKAATPY
 1150 1160 1170 1180 1190 1200
 TVSIYGVIIQG YRTPVLSAEA STGETPNLGE VVVAEVLGWA LKLNWTAPEG AYEFFFIQVQ
 1210 1220 1230 1240 1250 1260
 EADTVEAAQN LTVPGLLRST DLPGLKAATH YTITIRGVTQ DFSTTPLSVE VLTEEVPMGM
 1270 1280 1290 1300 1310 1320
 NLTVTEVSWD ALRLNWTTTPD GTYDQFTIQV QEADQVEEAH NLTVPGSLRS MEIPGLRAGT
 1330 1340 1350 1360 1370 1380
 PYTVTLHGEV RGHSTRPLAV EVVTEDLPQL GD LAVSEVGW DGLRLNWTAA DNAYEHFVIQ
 1390 1400 1410 1420 1430 1440
 VQEVNKVEAA QNLTLPGLSLR AVDIPGLEAA TPYRVSIYGV IRGYRTPVLS AEASTAKEPE

Figure 12...continued

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1450 1460 1470 1480 1490 1500
IGNLNVSDIT PESFNLSWMA TDGIFETFTI EIIDSNRLLE TVEYNISGAE RTAHISGLPP

1510 1520 1530 1540 1550 1560
STDFIVYLSG LAPSIRTKTI SATATTEALP LLENLTISDI NPYGFTVSWM ASENAFDSFL

1570 1580 1590 1600 1610 1620
VTVVDSGKLL DPQEFTLSGT QRKLELRGLI TGIGYEVMS GFTQGHQTKP LRAEIVTEAE

1630 1640 1650 1660 1670 1680
PEVDNLLVSD ATPDGFRLSW TADEGVFDNF VLKIRDTKKQ SEPLEITLLA PERTRDITGL

1690 1700 1710 1720 1730 1740
REATEYEIEL YGISKGRSQ TVSAIATTAM GSPKEVIFSD ITENSATVSW RAPTAQVESF

1750 1760 1770 1780 1790 1800
RITYVPITGG TPSMVTVDGT KTQTRLVKLI PGVEYLVSI AMKGFEESEP VSGSFTTALD

1810 1820 1830 1840 1850 1860
GPSGLVTANI TDSEALARWQ PAIATVDSYV ISYTGEKVPE ITRTVSGNTV EYALTDLEPA

1870 1880 1890 1900 1910 1920
TEYTLRIFAE KGPQKSSTIT AKFTTDLDSP RDLTATEVQS ETALLTWRPP RASVTGYLLV

1930 1940 1950 1960 1970 1980
YESVDGTVKE VIVGPDTSY SLADLSPSTH YTAQIQALNG PLRSNMIQTI FTTIGLLYPF

1990 2000 2010 2020 2030 2040
PKDCSQAMLN GDTTSGLYTI YLNGDKAEAL EVFCDMTSDG GGWIVFLRRK NGRENFYQNW

2050 2060 2070 2080 2090 2100
KAYAAGFGDR REEFWLGLDN LNKITAQGQY ELRVDLRDHG ETAFAYDKF SVGDAKTRYK

2110 2120 2130 2140 2150 2160
LKVEGYSGTA GDSMAYHNGR SFSTFDKDTD SAITNCALSY KGAFWYRNCH RVNLMGRYGD

Figure 12....continued

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2170	2180	2190	2200
NNHSQGVNWF	HWKGHEHSIQ	FAEMKLRPSN	FRNLEGRRKR A

Figure 12....continued.

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/052276

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K45/00 A61P31/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TILI ESMERINA ET AL: "Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock", JOURNAL OF IMMUNOLOGY, vol. 179, no. 8, October 2007 (2007-10), pages 5082-5089, XP002716885, ISSN: 0022-1767 the whole document	1-10
X	US 2008/300211 A1 (BALTIMORE DAVID [US] ET AL) 4 December 2008 (2008-12-04) examples 3,16,17 ----- -/-	1-10



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 November 2013

Date of mailing of the international search report

11/12/2013

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Covone-van Hees, M

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2013/052276

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BERNDT A ET AL: "Reorganisation of the caecal extracellular matrix upon Salmonella infection-Relation between bacterial invasiveness and expression of virulence genes", VETERINARY MICROBIOLOGY, vol. 133, no. 1-2, 1 January 2009 (2009-01-01), pages 123-137, XP025655323, ELSEVIER BV, NL ISSN: 0378-1135, DOI: 10.1016/J.VETMIC.2008.06.025 [retrieved on 2008-07-04] the whole document	1-10
X	----- WO 2011/011775 A1 (UNIV CALIFORNIA [US]; SHEPPARD DEAN [US]; SU GEORGE [US]; ATAKILIT AMH) 27 January 2011 (2011-01-27) page 24, lines 1-3 - lines 18-20 claim 4 claims 12-15	1,3-10, 12-15
A	----- EP 2 395 019 A1 (UNIV TOKYO SCI EDUC FOUND [JP]) 14 December 2011 (2011-12-14) page 2, line 31 - line 32	1-15
X,P	----- PICCININI A M ET AL: "Endogenous control of immunity against infection: tenascin-C regulates TLR4-mediated inflammation via microRNA-155", CELL REPORTS, vol. 2, no. 4, 25 October 2012 (2012-10-25), pages 914-926, XP002696661, ELSEVIER INC, US ISSN: 2211-1247, DOI: 10.1016/J.CELREP.2012.09.005 [retrieved on 2012-10-19] the whole document	1-15
T	----- DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 2013, PICCININI ANNA M ET AL: "Cross-talk between endogenous danger signals and microRNAs: tenascin-C fine-tunes the immune response to infection via induction of miR-155", XP002716886, Database accession no. PREV201300707226 abstract -/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2013/052276

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	& INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE, vol. 32, no. Suppl. 1, 2013, page S9, 18TH WORLD CONGRESS ON ADVANCES IN ONCOLOGY / 16TH INTERNATIONAL SYMPOSIUM ON MOLECULAR MEDICINE; CRETE, GREECE; OCTOBER 10 -12, 2013 ISSN: 1107-3756(print) -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2013/052276

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2008300211	A1	04-12-2008	NONE

WO 2011011775	A1	27-01-2011	AU 2010275367 A1 02-02-2012
			CA 2767409 A1 27-01-2011
			CN 102481347 A 30-05-2012
			EP 2456460 A1 30-05-2012
			JP 2013500269 A 07-01-2013
			KR 20120052312 A 23-05-2012
			US 2012328604 A1 27-12-2012
			WO 2011011775 A1 27-01-2011

EP 2395019	A1	14-12-2011	EP 2395019 A1 14-12-2011
			US 2011294985 A1 01-12-2011
			US 2013071929 A1 21-03-2013
			WO 2010090272 A1 12-08-2010

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2013/052276

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 16
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 16

Present claim 16 encompasses compounds defined only by their desired function, contrary to the requirements of clarity of Article 6 PCT, because the result-to-be-achieved type of definition does not allow the scope of the claim to be ascertained. The fact that any compound could be screened does not overcome this objection, as the skilled person would not have knowledge beforehand as to whether it would fall within the scope claimed. In the absence of any example whatsoever of agents which reduces the level and/or the activity of tenascin-C and/or miR-155 and/or guidance in the application to structural limitations of the claimed agent/composition, undue experimentation would be required to screen compounds randomly.

The non-compliance with the substantive provisions is to such an extent that no meaningful search of claim 16 could be carried out at all (Article 17(2) PCT).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.