Zuliani-Alvarez, Lorena and Marzeda, Anna M. and Deligne, Claire and Schwenzer, Anja and McCann, Fiona E. and Marsden, Brian D. and Piccinini, Anna M. and Midwood, Kim S. (2017) Mapping tenascin-C interaction with toll-like receptor 4 reveals a new subset of endogenous inflammatory triggers. Nature Communications, 8 (1). ISSN 2041-1723

Access from the University of Nottingham repository: http://eprints.nottingham.ac.uk/48271/2/Zuliani%20et%20al%20Nat%20Commun%202017%20Suppl%20Mat.pdf

Copyright and reuse:

The Nottingham ePrints service makes this work by researchers of the University of Nottingham available open access under the following conditions.

This article is made available under the Creative Commons Attribution licence and may be reused according to the conditions of the licence. For more details see: http://creativecommons.org/licenses/by/2.5/

A note on versions:

The version presented here may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher’s version. Please see the repository url above for details on accessing the published version and note that access may require a subscription.

For more information, please contact eprints@nottingham.ac.uk
Supplementary Figure 1. Biophysical characterization of FBG-C, -R, -W, -X. 

a. Protein purity was verified by silver staining of 1 µg of FBG-C, -R, -W and -X.

b. Anti-His tag western blot of 1 µg of FBG-C, -R, -W and -X was used to confirm protein identity.

c. Circular dichroism (CD) spectra in the far UV region of FBG-C, -R, -W and -X was used to show similar folding profiles for each. The buffer control signal is shown as a black solid line and the FBG-C, -R, -W and -X negative peaks as black dashes.

d. Protein purity was verified by silver staining of 1 µg of recombinant human TLR4 from R&D systems.
Supplementary Figure 2. Cytokine induction by FBG-C, -R and –W is TLR4 dependent and not due to LPS contamination. a, b. Primary human macrophages were pre-incubated for 6 h with 3µM TLR4 inhibitor TAK242 (a) or for 30 min with 25 µg mL\(^{-1}\) of TLR4 polyclonal antibody (b) prior to stimulation with LPS (1 ng mL\(^{-1}\)) or FBG-C, -R and –W (1 µM), or no stimulation (-) for 24h. Data are shown as mean ± SEM from 3 independent donors. Paired t-test vs non-treated with TAK242 or TLR4 antibody, *p<0.05, **p<0.01, ***p<0.001. c. % of inhibition ± SEM of LPS, FBG-C, -R and -W activity by TLR4 inhibitor TAK242. d. % of inhibition ± SEM of LPS, FBG-C, -R and -W activity by TLR4 antibody. e, f. THP1 NF-κB cells (e) or primary human macrophages (f) were left unstimulated (-) or stimulated with LPS (1ng ml\(^{-1}\)) or FBG-C, -R, -W and –X (1 µM) incubated for 30 min with polymyxin B (PMB) or boiled for 15 min. NF-κB activation was measured after 24 h using QUANTI-Blue™. Cytokine synthesis was measured by ELISA Data shown as mean ± SEM, N=3 independent experiments. Paired t-test PMB or boiled vs non- treated, *p<0.05
Supplementary Figure 3. Peptides 5 and 6 can induce NF-κB and cytokine synthesis in a TLR4 dependent manner. a. Primary human macrophages were left unstimulated (-) or stimulated with LPS (1 ng mL⁻¹), FBG-C (1 µM) or 20 µM of peptides 1-9 for 24 h and cytokine synthesis was measured by ELISA. b. Primary human macrophages were pre-incubated for 6 h with 3 µM TAK242 or DMSO, prior to stimulation with 1 ng mL⁻¹ of LPS, 1 µM of FBG-C or 100 µM of peptide 5. Cytokine synthesis was measured after 24 h by ELISA. c. THP1 NF-κB cells were left unstimulated (-) or stimulated with 20, 50 or 100 µM of peptides or scrambled peptide controls for 24 h and NF-κB activation was measured using QUANTI-Blue™. d. THP1 NF-κB cells were left unstimulated (-) or stimulated with 0.5 ng mL⁻¹ of LPS or 100 µM of peptide 5 for 24 h. Samples were previously incubated for 30 min with 10 µg ml⁻¹ polymyxin B or boiled for 15 min. NF-κB activation was measured using QUANTI-Blue™. Data are shown as mean ± SEM, N=3 independent experiments or 3 independent donors. One-way ANOVA vs unstimulated cells or paired t-test vs non-treated, *p<0.05, **p<0.01, ***p<0.001. e. Increasing doses of TLR4 were pre-incubated with 200 µM of peptides before adding them to 96 well plates coated with FBG-C. Curves were fitted in GraphPad Prism using one binding site hyperbola equation. Data are shown as mean ± SEM, N=3.
Supplementary Figure 4. Modelling the structure of FBG-C, -R, -W and –X. a. The structures of FBG-C, -R, -W and –X were modelled based on the C-terminal fibrinogen γ chain or the FIBDC crystal structure (FIBDC not shown). Sub-domain protein organisation is highlighted: A-subdomain in blue, B-subdomain in red and P-subdomain in yellow. The location of peptides 5, 6 and 7 are indicated by the arrows in the structure of the FBG-C model. b. FBG-C, -R, -W and –X models were aligned and coloured using PyMOL Molecular Graphics System highlighting in yellow conserved amino acid sequences. Figure on right panel rotated 180° compared to figure on the left. c. FBG-C, -R, -W and –X models were aligned and coloured using PyMOL Molecular Graphics System highlighting in red conserved structure. Figure on right panel rotated 180° compared to figure on the left.
Supplementary Figure 5. Peptide variants of loop 5 can induce NF-kB activation. a. THP1 NF-kB cells were unstimulated (-) or stimulated with 20, 50 or 100 µM of different peptide versions of loop 5 for 24 h and NF-kB activation was measured using QUANTI-Blue™. Data shown as mean ± SEM, N=3 independent experiments. One-way ANOVA vs unstimulated cells. b. Primary human macrophages were left unstimulated or stimulated for 24h with 20, 50 and 100 µM of loop 5-4, loop 5-mutation 1, loop 5-mutation 2 and loop 5 mutation-3. IL-8 synthesis was measured by ELISA. Data shown as mean ± SEM. N=3 independent donors. Paired t-test vs loop 5-4, *p<0.05, **p<0.01, ***p<0.001. c. THP1 NF-kB cells were left unstimulated (-) or stimulated with 20, 50 and 100 µM of peptides loop 5-4, loop 5-deletion, loop 5-mutation 1, loop 5-mutation 2 and loop 5 mutation-3. NF-kB activation was measured after 24h using QUANTI-Blue™. Data shown as mean ± SEM. N=3 independent experiments. Paired t-test vs loop 5-4, *p<0.05, **p<0.01, ***p<0.001.
Supplementary Figure 6. The linear sequences of loops 5, 7 and 10 of FBG-C are not conserved in human FRePs. 

**a.** Multiple sequence alignment of the FBG domain of tenasin-C of different species.  
**b.** Multiple sequence alignment of human FBG domains. The amino acids involved in TLR4 activation and binding in loop 5, 7 and 10 are highlighted in a black box. The alignment was coloured according to the Clustal colour scheme. 

**Light blue:** hydrophobic. **Red:** positive charged. **Green:** Polar. **Pink:** conserved column of cysteine. **Violet:** negative charge. **Orange:** glycine. **Yellow:** proline. **Cyan:** aromatics.

Supplementary Figure 6. The linear sequences of loops 5, 7 and 10 of FBG-C are not conserved in human FRePs.
Supplementary Figure 7. Synthesis of and cytokine induction by FIB-G, FIC-1 and ALP-4. Protein purity was verified by silver staining of 1 µg of FIB-G (a), FIC-1 (b) and ALP-4 (c)(left panel). Anti-His tag western blot of 1 µg of each protein was used to confirm protein identity (middle panel). Circular dichroism (CD) spectra in the far UV region were used to show similar folding profiles for each. The buffer control signal is shown as a black solid line and the FIB-G, FIC-1 and ALP-4 negative peaks as black dashes (right panel). d. Primary human macrophages were stimulated with FBG-C, FIB-G, FIC-1 and ARP-4 (1 µM) incubated for 30 min with polymyxin B (PMB) or boiled for 30 min. IL-6 synthesis was measured by ELISA. Data shown as mean ± SEM from 3 independent donors. Paired t-test vs non-treated, ns, not significant.
Supplementary Table 1. Percentage of identity amongst the amino acid sequences of the FBG domains of human tenascin-C, -R, -W and -X (FBG-C, -R, -W, –X)

|       | FBG -C | FBG -X | FBG -R |
|-------|--------|--------|--------|
| FBG -X| 52.89  | -      | -      |
| FBG-R | 59.65  | 54.67  | -      |
| FBG-W | 53.02  | 49.56  | 55.74  |
Supplementary Table 2. Amino acid sequences of the peptides used in this study.

| Peptide | Sequence |
|---------|----------|
| P1      | TIGLLYPFKDCSQAMLNGDTTSGLYTIYL |
| P2      | YTIYLNGDKAEALEVFCDMTSDGGGWIVFL |
| P3      | WIVFLRRKNGRENFYQNWKAYAAGFGDRRE |
| P4      | GDRREEFWLGLDNLNKITAQGQYELRVD |
| P5      | ELRVDLRDHGGETAFAVYDKFSVGDAKTRYK |
| P6      | KTRYKLKVEGYSGTAGDSMAYHNGRSFST |
| P7      | RSFSTFDKTDTSATNCALSYKGAFWYRN |
| P8      | WYRNCHRVMGNGDMMNHSQGVNWFWHKG |
| P9      | FHWKGHEHSIQFAEMKLPSNRMLGRRKRA |
| P5S     | KGVFLTRYVTDARDVHFCKYGASRELEAKD |
| P6S     | YKGTSDHFRVGNSRYETSMGKAATLKY |
| P9S     | AFERHKMWKVRKLRAGHCPELHSNSRIFQF |
| Loop 5-1 | KTRYK |
| Loop 5-2 | GDAKTRYKLKVEG |
| Loop 5-3 | DKFSVGDAKTRYKLKVEGYS |
| Loop 5-4 | AFAVYDKFSVGDAAKTRYKLKVEGYSGTAD |
| Loop 5-deletion | GETAFAVYDKFSVGDAAVYDGYSGTADSMAY |
| Loop 5-mutant 1 | AFAVYDKFSVGDAAATAYKLKVEGYSGTAD |
| Loop 5-mutant 2 | AFAVYDKFSVGDAAKTRYALAVEGYSGTAD |
| Loop 5-mutant 3 | AFAVYDKFSVGDAAATAYALAVEGYSGTAD |

Nine peptides (P1-9) were used to map the FBG-C active site; together they span the whole sequence of FBG-C with each peptide containing a sequence that overlaps with the contiguous peptide. Scrambled versions of peptides 5, 6 and 9 (P5S, P6S, P9S) were made as controls. Peptides comprising loop 5 of FBG-C and adjacent amino acids were made including deletion and mutations in this amino acid sequence.
Supplementary Table 3. Peptides block TLR4-FBG-C binding.

| Protein        | KD (nM) ± SD | p value |
|----------------|-------------|---------|
| TLR4 only      | 68.85 ± 7.79|         |
| TLR4 + P1      | 78.75 ± 11.33| 0.280   |
| TLR4 + P2      | 83.29 ± 5.24| 0.056   |
| TLR4 + P3      | 64.45 ± 15.67| 0.672   |
| TLR4 + P4      | 42.63 ± 10.06| 0.023 * |
| TLR4 + P5      | 241.22 ± 89.15| 0.028 * |
| TLR4 + P6      | 370.82 ± 141.64| 0.021 * |
| TLR4 + P7      | 383.71 ± 113.31| 0.008 **|
| TLR4 + P8      | 31.87 ± 8.22| 0.048 * |
| TLR4 + P9      | 86.26 ± 16.57| 0.17    |

Increasing doses of TLR4 were pre-incubated with 200 µM of peptides before adding them to 96 well plates coated with FBG-C. The affinity of FBG-C binding to TLR4 was calculated using GraphPad Prism using one binding site hyperbola equation, N=3. Un-paired t-test vs TLR4 only. *p<0.05, ** p<0.01.
Supplementary Table 4. Affinity of TLR4 binding to FBG-C mutants.

**a.**

| Protein       | KD (nM) ± SD | p value |
|---------------|-------------|---------|
| FBG-C         | 58.21 ± 5.24|         |
| FBG-C mut 1   | 81.01 ± 15.45| 0.031 * |
| FBG-C mut 2   | 75.92 ± 15.58| 0.074   |
| FBG-C mut 3   | 121.10 ± 15.29| 0.0002 ***|

**b.**

| Protein       | KD (nM) ± SD | p value |
|---------------|-------------|---------|
| FBG-C         | 53.4 ± 7.93 |         |
| FBG-C mut 4   | 63.03 ± 8.92| 0.234   |
| FBG-C mut 5   | 336.4 ± 103.12| 0.009 **|

**c.**

| Protein       | KD (nM) ± SD | p value |
|---------------|-------------|---------|
| FBG-C         | 60.48 ± 11.43|         |
| FBG-C mut 6   | 86.69 ± 21.25| 0.135   |
| FBG-C mut 7   | 123.78 ± 28.33| 0.023 **|

96 well plates were coated with FBG-C or with FBG-C mutants, and TLR4 was added in a dose dependent manner. The affinity of each FBG variant for TLR4 was calculated using GraphPad Prism using one binding site hyperbola equation. N=4 for FBG-C, FBG-C mutant 1, 2 and 3 (a). N=3 for FBG-C, FBG-C mutant 4, 5, (b) 6 and 7 (c). Un-paired t-test vs FBG-C, *p<0.05, **p<0.01, ***p<0.001.
### Supplementary Table 5. Affinity of TLR4 binding to FBG-X mutants.

| Protein                | KD (nM) ± SD   | p value |
|------------------------|---------------|---------|
| FBG-C                  | 59.77 ± 7.08  |         |
| FBG-X                  | ND            |         |
| FBG-X mutant 1         | ND            |         |
| FBG-X mutant 2         | 173.09 ± 56.66| 0.007** |
| FBG-X mutant 3         | 154.96 ± 46.74| 0.006** |
| FBG-X mutant 4         | 78.33 ± 18.41 | 0.109   |

96 well plates were coated with FBG-C, FBG-X, FBG-X mutants 1, 2, 3 or 4, and TLR4 was added in a dose dependent manner. The affinity of FBG-C or FBG-X mutants to TLR4 was calculated using GraphPad Prism using one binding site hyperbola equation. N=4. Un-paired t-test vs FBG-C. **p<0.01.
Supplementary Table 6. FBG domain crystal structures

| Protein            | PDB ID |
|--------------------|--------|
| Angiopoietin 1     | 4EPU   |
| Angiopoietin 2     | 1Z3U   |
| FIBCD-1            | 4M7H   |
| Fibrinogen α chain | 1FZD   |
| Fibrinogen β chain | 1FZA   |
| Fibrinogen γ chain | 1FID   |
| Ficolin-1          | 2JHM   |
| Ficolin-2          | 4R9J   |
| Ficolin-3          | 2J5Z   |
Supplementary Table 7. The name and accession numbers of the 24 human FRePs

| Protein                                | Accession no. |
|----------------------------------------|---------------|
| Angiopoietin 1                         | Q15389        |
| Angiopoietin 2                         | O15123        |
| Angiopoietin 4                         | Q9Y264        |
| Angiopoietin-like protein 1            | O95841        |
| Angiopoietin-like protein 2            | Q9UKU9        |
| Angiopoietin-like protein 3            | Q9Y5C1        |
| Angiopoietin-like protein 4            | Q9BY76        |
| Angiopoietin-like protein 5            | Q86XS5        |
| Angiopoietin-like protein 6            | Q8NI99        |
| Angiopoietin-like protein 7            | O43827        |
| Fibrinogen α chain                    | P02671        |
| Fibrinogen β chain                    | P02675        |
| Fibrinogen γ chain                    | P02679        |
| Fibrinogen-like protein 1             | Q08830        |
| Fibroleukin                            | Q14314        |
| FIBCD-1                                | Q8N539        |
| Ficolin-1 (M)                          | O00602        |
| Ficolin-2 (L)                          | Q15485        |
| Ficolin-3 (H)                          | O75636        |
| MFAP4                                  | P55083        |
| Tenascin-C                             | P24821        |
| Tenascin-R                             | Q92752        |
| Tenascin-W                             | Q9UQP3        |
| Tenascin-X                             | P22105        |
Supplementary Table 8. The accession numbers of tenascin-C from different species

| Protein          | Accession number |
|------------------|------------------|
| *S. scrofa*      | Q29116           |
| *M. musculus*    | Q80YX1           |
| *R. norvegicus*  | B2LYI9           |
| *B. taurus*      | A0JN60           |
| *G. gallus*      | P10039           |
| *D. rerio*       | Q4FAI8           |
| *T. rubripes*    | Q7SZG1           |
Supplementary Table 9. Forward and reverse primer sequences for tenasin-R, -X and –W FBG domains.

| Gene | Accession number | Basepair number of FBG | Domain boundaries | Primer Sequence | Size of PCR product |
|------|------------------|------------------------|-------------------|----------------|-------------------|
| TNR  | NM_003285.2      | 696                    | 3937-4632         | GCC CAT ATG CAT CAT CAT CAT CAT CAT CAT CAT CAT GGA GGC CGG GTG TTC CCT CAT TNR GCC CTC GAG TTA GAA FBG CTG TAA GGA CTG CCG Reverse TTT TCT | 735 |
| TNX  | NM_019105.6      | 693                    | 12239-12931       | GCC CAT ATG CAT CAT CAT CAT CAT CAT CAT CAT CAT GGA GGC CAT CAT CAT ACCTCTTTCACCACGGGT TNR GCC CTC GAG TTA GCC FBG TCC CCC CGC TGG GGA G Reverse TTT TCT | 732 |
| TNW  | NM_022093.1      | 723                    | 3291-4013         | GCC CAT ATG CAT CAT CAT CAT CAT CAT CAT CAT CAT CAT CGT TTC CCA TNR GCC CTC GAG TTA GAA FBG CGT TCG CAG CCT TCC Reverse TCT CAG | 762 |

Each sequence has a His-tag (underlined) and a restriction site in the forward primer for Ndel (bold) and in the reverse primer for XhoI (bold).
**Supplementary Table 10. Forward and reverse primer sequences FBG domains from the fibrinogen chain γ, ficolin-1 and angiopoietin-like protein 4.**

| Gene     | Accession number | Basepair number of FBG | Domain boundaries | Primer          | Sequence                          | Size of PCR product |
|----------|------------------|------------------------|-------------------|----------------|-----------------------------------|---------------------|
| FGG      | BC021674.1       | 807                    | 556-1362          | FGG FBG Forward | CAC CCA TGG CAC ATC ATC ATC ATC ATC ATC AAA TCC AGC TCA CTG G | 845                 |
|          |                  |                        |                   | FGG FBG Reverse | GTT CTC GAG TTA AAC GTC TCC AGC CTG TTT GGC TCC C |                    |
| FCN1     | NM_002003.2      | 658                    | 362-1019          | FCN1 FBG Forward | CAC CAT ATG CAT CAT CAT CAT CAT CAT GTC TGT GCG ACA GGC CCA C | 694                 |
|          |                  |                        |                   | FCN1 FBG Reverse | GCT CTC GAG TTA GGC GGG CCG CAC CTT CAT CTC TG |                    |
| ANGPTL4  | NM_139314.1      | 687                    | 730-1416          | ANGPTL4 FBG Forward | AAA CAT ATG CAT CAT CAT CAT CAT CAT AGC CGC TGT CAC CGG CTG C | 723                 |
|          |                  |                        |                   | ANGPTL4 FBG Reverse | ACT CTC GAG TTA GGA GGC TGC CTC TGC TGC CAT G |                    |

Each sequence has a His-tag (underlined) and a restriction site in the forward primer for *Ncol* (fibrinogen chain γ, bold) or *Ndel* (ficolin-1 and angiopoietin-like protein 4, bold), and in the reverse primer for *Xhol* (bold).
Supplementary Table 11. Primer sequences used to insert specific mutations in FBG-C and FBG-X.

| Protein                  | Primer Forward | Primer Reverse               |
|--------------------------|----------------|------------------------------|
| FBG-C mutant 1           | Primer Forward | GTG GGA GAT GCC GCG ACT GCC TAC AAG CTG AAG GTG GAG |
|                          | Primer Reverse | CAC CTT CAG CTT GTA GGC AGT CGC GGC ATC TCC CAC GC |
| FBG-C mutant 2           | Primer Forward | GCC AAG ACT CGC TAC GCG CTG GCG GTG GAG GGG TAC |
|                          | Primer Reverse | GTA CCC CTC CAC CGC CAG CGC GTA GGC AGT CTT GG |
| FBG-C mutant 3           | Primer Forward | GTG GGA GAT GCC GCG ACT GCC TAC GCG CTG GCG GTG GAG GGG TAC |
|                          | Primer Reverse | GTA CCC CTC CAC CGC CAG CGC GTA GGC AGT CTC CAC AGC |
| FBG-C mutant 4 and 5     | Primer Forward | AGC AAC TTC AGA AAT CTT GAA GGC GGG GCC TAA CGG GC |
|                          | Primer Reverse | GC CGG TTA GCC CCC GCC TTC AAG ATT TCT GAA GTT GC |
| FBG-C mutant 6 and 7     | Primer Forward | GAC AAG GAC ACA CCT TCA GCC CTC ACC AGC TGT GCT CTG |
|                          | Primer Reverse | CAG AGC ACA GCT GGT GAG GGC TGA AGG TGT GTC CTT GTC |
| FBG-X mutant 1           | Primer Forward | CAC GTA GAC TCG GCT AAG GCG AAG TAC CGC CTC CAC TTG |
|                          | Primer Reverse | CAA GTG GAG GCG GTA CTT CGC CTT AGC CGA GTC TAC GTG |
| FBG-X mutant 2           | Primer Forward 1 | GAC TCC TTC CAC GTA GGC GAT GCT AAG GCG AAG TAC CGC |
|                          | Primer Reverse 1 | GCG GTA CTT CGC CTT AGC ATC GCC TAC GTG GAA GGA GTC |
|                          | Primer Forward 2 | GTA GGC GAT GCT AAG ACG AAG TAC CGC CTC AAG TTG GAG GGC |
|                          | Primer Reverse 2 | GCC CTC CAA CTT GAG GCG GTA CTT CGT CTT AGC ATC GCC TAC |
| FBG-X mutant 3           | Primer Forward 1 | TCC CCA GCG GGG AGA CGC AAA CTC GAG |
| FBG-X mutant 4 | CAC CAC CAC |
|----------------|------------|
| Primer Reverse 1 | GTG GTG GTG CTC GAG TTT GCG TCT CCC CGC TGG GG |
| Primer Forward 2 | CCA GCG GGG AGA CGC AAA CGC GCG TAG CAC CAC |
| Primer Reverse 2 | GTG GTG CTA GCG CGC TTT GCG TCT CCC CGC TGG |
| Primer Forward | CGT GAT CGG GAC GAC AAC AGC TTG ATC ATC AAC TGC GCT GTC TCC |
| Primer Reverse | GGA GAC AGC GCA GTT GAT GAT CAA GCT GTT GTC GTC CCG ATC ACG |