MUB40 Binds to Lactoferrin and Stands as a Specific Neutrophil Marker

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Abstract:
Neutrophils are the most abundant immune cells recruited to inflamed tissues. Due to the lack of dedicated tools, their detection and study has been hampered. Here, we show that MUB40 binds to lactoferrin, the most abundant protein stored in neutrophil specific and tertiary. Lactoferrin is specifically expressed and secreted by neutrophils among other leukocytes, making MUB40 a specific neutrophil marker. Naïve mammal neutrophils were efficiently labelled with fluorescent MUB40-conjugates (-Cy5, Dylight405). A peptidase-resistant retro-inverso MUB40 peptide (RI-MUB40) was synthesized and its lactoferrin-binding property was validated. Neutrophil lactoferrin secretion was assessed in vitro by live microscopy on infected neutrophils in the presence of RI-MUB40-Cy5. Systemically administered RI-MUB40-Cy5 accumulated at
sites of inflammation in vivo in a mouse arthritis model and therefore proposes itself as a potential novel tool. Improving neutrophils' detection with the universal and specific MUB40 marker will promote their study in inflammatory diseases.

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### Opposed Reviewers:

- No specific reviewers listed.
Dear Dr. Ciancetta,

We thank you for the opportunity to resubmit a revised version of our manuscript entitled: «The MUB40 peptide binds to lactoferrin and stands as a specific neutrophil marker» by Anderson and colleagues. We have addressed all the reviewer comments and critics and we performed requested experiments.

We have included additional results in this new version of our manuscript (mainly Figures 2, 3 and S2). Importantly, based on a reviewer comment, we have now demonstrated that MUB40 can be used as a neutrophil marker using flow cytometry. We thank the reviewer suggestion.

We are looking forward to hearing from you, do not hesitate to contact me for further missing informations.

Sincerely,

Benoit Marteyn, PhD
Senior scientist, CR1 Inserm (U1202)
Institut Pasteur, Paris - Unité de Pathogénie Microbienne Moléculaire
Gustave Roussy Cancer Campus, Villejuif – Laboratoire de Thérapie Cellulaire
France
REVIEWERS' COMMENTS
We thank the reviewers for their time and thoughtful comments. Please find below our answers and addings.

Reviewer #1: The authors provide evidence that MUB40 allows for reliable detection of lactoferrin and thereby might serve as strategy to visualize neutrophils in vivo. The authors describe the chemical synthesis, structure and binding properties of MUB40 and a retro-inverso peptidase resistant version of MUB40. They move on and demonstrate that MUB40 labels human and other mammalian granulocytes. They provide evidence that lactoferrin is stored in specific and tertiary granules. Of utmost interest they demonstrate that their strategy allows for detection of lactoferrin degranulation in vitro using live cell imaging by using a peptidase-resistant retro-inverso MUB40 peptide.

This is an exciting report on a new strategy to assess inflammation by using a specific ligand for an antimicrobial molecules. This strategy might pave the way to new tools for visualization of inflammatory processes in general and therefore is of broad interest.

My only concern is related to the issue whether lactoferrin is granulocyte specific. I acknowledge that the authors tested whether Lactoferrin is expressed in CD14+ human monocytes, Cd19+ B cells and CD3+ T cells. However, according to Immgen database (https://www.immgen.org/) lactoferrin (lactotransferrin, Ltf) is expressed in BM-macrophages, Ly6C+ classical monocytes; some expression also in B cell precursors and hematopoetic stem cells in the mouse (Immgen database; immgen.org). Since the authors claim that Lactoferrin might serve as a marker for granulocytes I feel that a analysis of these cell types is warranted as well (e.g. isolation of the cells ex vivo (e.g. by FACS sorting) and staining with the retro-inverso MUB40 as done in Fig. 5.)

We thanks the reviewer for this comment and we agree that the potential use of MUB40 in flow cytometry was not described in our original manuscript. In the revised version, we included new results showing that in human and mouse blood samples, neutrophils are specifically labeled with MUB40 fluorescent conjugates. None of the other mature leucocytes (including Ly6C+ classical monocytes) are labeled with these markers. Since we previously demonstrated that human CD34+ HSC were not labeled with MUB40 fluorescent conjugates (Figure 2B), we confirm here the specificity of the neutrophil labeling (page 9, lines 7-18). To our knowledge, lactoferrin is only detected at high concentrations in neutrophil granules and in secreted fluids (including milk). In the Immgen database mentioned by the reviewer, we did not find any original manuscript describing the expression of lactoferrin in monocytes.

Minor:
- Labelling of Fig. 4 C. What does "FT" stand for?
FT corresponds to the flow-through fraction (unbound). This comment has been included in the revised figure legend (page 30
- The authors might want to consider elaborating in more detail on the synthesis of the MUB40 and retro-inverso peptide.
We thank the reviewer for noticing these missing information which have not been included, by mistake, in the submitted manuscript. We re-introduced this section in the supplementary informations (form page 2 line 1 to page 3 line 8) and referred in the main text (page 6 line 3).

Reviewer #2:

This paper outlines an impressive piece of work in which the authors synthesize and validate a new neutrophil marker using an extensive range on in vivo and in vitro models.
The paper appears technically sound, with each experiment well reasoned and explained. The paper displays a logical progression from synthesis through the various complexities of models. The authors outline potential follow-up studies and for the most-part the information given would allow re-use.
We thank the reviewer for these positive and encouraging statements.

Below I have outlined my few points that could benefit from further explanation:

1. Differentiation between Mub40 and Mub70. In the discussion the authors note that both MUb70 and Mub40 labeled neutrophil granules by interacting with a lactoferrin glycosylation moiety. What therefore is the advantage of Mub40 over Mub70?
The main advantages of MUB40 compared to MUB70 are associated to its shorter length making its synthesis quicker, easier and cost-effective. It allowed the design and production of various forms of MUB40 with different conjugated molecules (various fluorophores, biotin, etc..) and a non-cleavable retro-inverso version.
The lonely functional difference between MUB40 and MUB70 is its ability to label goblet cell granules, probably due to its smaller size (Figure 1B and main text, page 6 lines 6-9).

2. Could the authors further define the transient nature of the Mub40 labeling in live cells? What is the concentration co-efficient of the labeling vs lactoferrin concentration? It appears the transient nature is more pronounced in cell culture than in vivo.
We thank the reviewer for raising this important question. Unfortunately, the lactoferrin concentration in neutrophil granules vs supernatant is difficult to assess. MUB40 concentration in labeling solution is high enough to allow the detection of high lactoferrin concentration in animal/human inflammatory sites (Fig. 5B/C and Fig. 6)
The transient RIMUB40 labeling of neutrophils infected with
Shigella has not yet been minded. As we can rule out the possibility of RIMUB40 degradation, we think that it may be due to the solubilization of secreted lactoferrin in the culture medium. Although labeled with RIMUB40, the resulting fluorescent signal might not be high enough to be detected with a fluorescent microscope. Conversely, at the site of degranulation on the cell surface lactoferrin is more concentrated and detected with RIMUB40 (main text, page 10 lines 22-24). In vivo, we hypothesize that lactoferrin may be accumulating within inflammatory foci, (figs. 5B and 5C) (main text page 11 line 5 and page 11 lines 16-20). This point was further outlined in page 6 line 18 «(inflammatory sites) are hypothesized to contain a high concentrated level of lactoferrin »

3. If both Mub40 and Mub70 label colonic mucosa, why is there no apparent labeling on the colonic mucosa in Figure 3c? Is the Mub40 signal in 3C only extracellular?

We acknowledge that the mouse colonic mucus is not labeled with MUB40 in Fig. 3C, which is consistent with the inefficient labeling of mouse colonic mucus with MUB70, compared to human, guinea pig, rabbit colonic mucus, as described in Coic et al, 2012, J. Biol. Chem. This observation remains unexplained.

4. Is any specific permeation step needed to enable MuB40 labeling in fixed cells?

After fixation, neutrophils are labeled with MUB40 in the presence of a detergent, either Saponin (0.1%) or Triton (0.1%). The potential use of Triton as been further outlined in the revised version of the manuscrismet (main text p20, line 1)

5. How many mice were included in each of the 2 experiments in the 5C? Can the authors comment on the apparent fluorescent signal from the animals spine?

In the experiment shown in 5C I originally started with 5 ctrl mice + 5 arthritic mice, however 1 of the arthritic mice died during the imaging preparation, so in this group are only 4 mice (as indicated in the Figure Legend) which results in 10 and 8 imaged hind ankles.

In the first experiment, we included 3 ctrl and 5 arthritic mice - but I recall that the imaging settings were quite different... Finally, in the latest experiment we have 4 controls and 5 K/BxN mice (which is probably the better repetition in the end than the first one).

The signal that appears to result from the spinal cord is in fact due to a superficial injury of the skin that occurred during the shaving/depilation of the animal. Superficial signals appear indeed very strong with this imaging technique as they are not quenched by their passage through tissues as the ones from the
ankle joints. As a consequence, small skin lesions due to injury or mouse bites appear indeed very strong.
**MUB** binds to l... and stands as a specific neutrophil marker

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Abstract

Neutrophils are the most abundant immune cells recruited to inflamed tissues. Due to the lack of dedicated tools, their detection and study has been hampered. Here, we show that MUB40, binds to lactoferrin, the most abundant protein stored in neutrophil specific and tertiary. Lactoferrin is specifically expressed and secreted by neutrophils among other leucocytes, making MUB40 a specific neutrophil marker. Naïve mammal neutrophils were efficiently labelled with fluorescent MUB40-conjugates (-Cy5, Dylight405). A peptidase-resistant retro-inverso MUB40 peptide (RI-MUB40) was synthesized and its lactoferrin-binding property was validated. Neutrophil lactoferrin secretion was assessed in vitro by live microscopy on infected neutrophils in the presence of RI-MUB40-Cy5. Systemically administered RI-MUB40-Cy5 accumulated at sites of inflammation in vivo in a mouse arthritis model and therefore proposes itself as a potential novel tool. Improving neutrophils’ detection with the universal and specific MUB40 marker will promote their study in inflammatory diseases.
Introduction

Polymorphonuclear neutrophils are the most abundant immune cell population recruited to inflammatory tissues. Once recruited, neutrophils release proteins and cytokines contributing to the overall innate immune response. Proteins secreted by neutrophils upon their stimulation, are stored in pre-formed granules. Neutrophils contain four distinct granule populations named azurophil granules (α), specific granules (β1), tertiary granules (β2) and secretory vesicles (γ), which are sequentially formed during granulopoiesis. When activated, neutrophils sequentially release the protein content of stored granules. Granules formed during the later stage of granulopoiesis are more prone to exocytosis. Neutrophil granule protein content has been first investigated by Borregaard and colleagues and since then extensively studied in vitro and in vivo during inflammation and immune response (Cowland and Borregaard, 2016; Kjeldsen et al., 1999; Sengelov et al., 1995) (Borregaard et al., 2007) (Faurschou and Borregaard, 2003) (Soehnlein et al., 2009). Increased seric concentrations of neutrophil secreted proteins were reported in various inflammatory diseases such as inflammatory bowel disease (IBD) (Gouni-Berthold et al., 1999) or colorectal cancer (Ho et al., 2014). Inflammatory states were also correlated with high concentration of neutrophil secreted proteins in faeces (IBD (Däbritz et al., 2014) (Lehmann et al., 2015)) or in sputum (cystic fibrosis (Sagel et al., 2012)).

Among neutrophil secreted proteins, lactoferrin is the most abundant and suitable neutrophil-derived faecal marker of inflammation (Sugi et al., 1996) (Martins et al., 1995) including IBD (Sipponen, 2013) (Stragier and Van Assche, 2013). However, to date, no inflammation-imaging method based on lactoferrin detection is described, although specifically stored in neutrophil granules, and not expressed by other white
blood cells. Lactoferrin is an 80 kDa glycoprotein produced by neutrophils and exocrine glands located in respiratory and gastrointestinal tracts (Peen et al., 1996). Lactoferrin antimicrobial activity is associated with its iron sequestration property, limiting pathogens’ (bacteria, viruses, fungi) survival and spreading (Orsi, 2004). Neutrophil lactoferrin is locally secreted by neutrophils at bacterial infection sites (Masson et al., 1969).

Here we characterize MUB40, a novel marker of neutrophils, which binds to neutrophil lactoferrin, stored in β1 or β2 granules and released in the extracellular compartment, upon neutrophil activation. Here, MUB40 has been validated as a new inflammation marker in an infectious inflammatory model in tissues infected with the pathogenic enterobacteria Shigella flexneri, but also in sterile inflammatory models.

**Results**

**MUB40-peptides chemical synthesis, structure, and binding properties**

MUB40 is a 40-amino acid peptide, derived from the MUB70 marker, originally characterized for its ability to bind the human colonic mucus (Coïc et al., 2012). Four overlapping 40-amino acid peptides (named MUB40#1, MUB40#2, MUB40#3, and MUB40#4) covering the MUB70 sequence were designed (Figure 1A), chemically synthesized (see Methods), and conjugated to fluorophore or biotin via the N-ter added cysteine when required for further study (see Supplementary Methods). The MUB40 peptide synthesis strategy was set up based on MUB70 synthesis (Coïc et al., 2012), incorporating secondary amino acid surrogates (Dmb and pseudoproline...
dipeptides (Figure S1A). As a result, lowering of aggregation propensity and aspartimide formation produced the MUB\textsubscript{40} peptide with a satisfactory yield (see Supplemental Methods for detailed information).

MUB\textsubscript{40}\#1 (corresponding to MUB\textsubscript{70} C-terminal part), conjugated to Cy5 (MUB\textsubscript{40}\#1-Cy5) was the only peptide, which conserved the ability to bind to and fluorescently label the human colonic mucus (Figure 1B and S1B). In contrast with MUB\textsubscript{70}-Cy5, MUB\textsubscript{40}\#1-Cy5 allowed in addition the labelling of goblet cell granules on fixed slides, most likely due to its reduced size and the resulting improved accessibility to granule content (Figure 1C). Similarly to MUB\textsubscript{70} (Coïc et al., 2012), we show here by Analytical Gel Filtration (see Supplemental Methods) that MUB\textsubscript{40}\#1 (theoretical MW 4.9 kDa), combined as a trimer (experimental Mr 15.9 kDa), unlike the three others overlapping peptides which rather organized as tetramers (MUB\textsubscript{40}\#2 theo MW 4.8 kDa; exp: Mr 22.0 kDa, MUB\textsubscript{40}\#3 theo MW 4.9 kDa; exp Mr 20.5, and MUB\textsubscript{40}\#4 theo MW 4.8 kDa; exp Mr 20.4 kDa) (Figure 1D). The structure of MUB\textsubscript{40} peptides was analyzed by Circular Dichroism (CD). The four resulting signals were almost similarly shaped and could be assigned to unstructured peptide chains or polyproline II-like scaffolds (Fig 1E). In regards to the thermal transition curves (data not shown), no obvious conformational changes were identified and we assumed a global unordered structure for all peptides. Nevertheless, slight differences were observed between the CD spectra, mostly in the negative band within the 200 nm range: MUB\textsubscript{40}\#1 showed a lower intensity signal, significantly 1 to 1.5 nm red-shifted, as compared to other MUB\textsubscript{40} peptides (#2, #3 and #4) minima. Even if no structural element was evidenced from the CD profiles, this singularity could reflect a specific feature of MUB\textsubscript{40}\#1. As a conclusion, MUB\textsubscript{40}\#1, hereafter-named MUB\textsubscript{40}, highlighted a similar
oligomerization state and mucus-binding properties compared to MUB\textsubscript{70} and was further characterized in this study.

**MUB\textsubscript{40} labels human and other mammalian granulocytes**

In addition to its colonic mucus binding property, confirmed on fixed human colon explants infected with *Shigella flexneri* (*S. flexneri*) (Figure 1F, see Methods), MUB\textsubscript{40}-Cy5 labelled a specific cell population in the colonic mucosa, which were hypothesized to be polymorphonuclear neutrophils (neutrophils), the most abundant immune cell population recruited upon *Shigella* invasion (Sansonetti et al., 1999). To confirm this hypothesis, human neutrophils were purified from healthy volunteers (see Supplementary Methods). When incubating fixed purified human neutrophils with MUB\textsubscript{40}-Cy5, a fluorescent labelling was confirmed and appeared to be granular (Figure 1G), suggesting that MUB\textsubscript{40} target was stored in neutrophil granules.

We further confirmed that regardless of the fluorophore conjugated to MUB\textsubscript{40} (MUB\textsubscript{40}-Cy5 or MUB\textsubscript{40}-Dylight405, see Supplementary Methods), human neutrophil granules were specifically labeled (Figure 2A). Neutrophils differentiate from pluripotent haematopoietic stem cells (HSC, CD34+) in the bone marrow during granulopoiesis, characterized by the formation of promyelocytes, myelocytes, metamyelocytes, band cells, segmented neutrophilic cells, and mature neutrophils. To assess the specificity of mature neutrophil labelling with MUB\textsubscript{40}-Cy5, hematopoietic stem cells were purified from cytapheresis product and differentiated to neutrophils (see Supplementary Methods). We confirmed that MUB\textsubscript{40}-Cy5 did not label human hematopoietic stem cells until their differentiation into mature
neutrophils *in vitro* (Figure 2B). In order to anticipate its use in the presence of living cells, a non-cleavable retro-inverso version of MUB₄₀, named RI-MUB₄₀, was synthesized with non-natural D-amino acids. RI-MUB₄₀ was resistant to trypsin proteolysis, whereas MUB₄₀ was rapidly degraded (Figure 2C-D). The neutrophil granule binding property of RI-MUB₄₀ conjugated to Cy5 (RI-MUB₄₀-Cy5) was similar to MUB₄₀-Cy5 (Figure 2A).

The specificity of neutrophil labeling with MUB₄₀ conjugated peptides was further assessed on human and mouse blood samples. We demonstrated by flow cytometry that human neutrophils were efficiently labeled with MUB₄₀-Alexa405 after cell fixation and permeabilization; naïve cells were not labeled (Figure 3A). This result was consistent with the localization of MUB₄₀-Alexa405 target in neutrophil granules and the inability of MUB₄₀-Alexa405 to cross plasma membrane. Following the same procedure, peripheral blood mononuclear cells (PBMCs) were not labeled with MUB₄₀-Alexa405 (Figure 3B), as confirmed by immunofluorescent labeling (Figure S2A). Similarly, among mouse circulating leucocytes, neutrophils were specifically labeled with MUB₄₀-Alexa405, after fixation and permeabilization, not PBMCs (including B-lymphocytes, T-lymphocytes, Ly6C-/+ monocytes, Figure 3C and S2B-C).

We demonstrate that all mammalian neutrophils tested were efficiently labeled with MUB₄₀-Cy5, as illustrated with mouse, but also guinea pig circulating neutrophils (Figure 3D). In animal models of shigellosis (see Supplementary Methods), neutrophils recruited to the intestinal mucosa infected with *Shigella* were specifically labeled with MUB₄₀-Cy5 or MUB₄₀-Dylight405, as illustrated in guinea pig colon (*Shigella flexneri*; MUB₄₀-Cy5 or MUB₄₀-Dylight405, Figure 3E), mouse colon (*Shigella sonnei*, MUB₄₀-Cy5, Figure 3F, described in (Anderson et al., 2017)), and
rabbit ileum (Shigella flexneri, MUB<sub>40</sub>-Dylight405, Figure S3).

These results confirm the potential use of MUB<sub>40</sub> peptide as a specific neutrophil marker in physiological and pathophysiological conditions, in humans and in various animal models. We next aimed at identifying the specific MUB<sub>40</sub> target in neutrophil granules.

**MUB<sub>40</sub> binds to lactoferrin stored in specific and tertiary granules**

The four classes of neutrophil granules (α, β<sub>1</sub>, β<sub>2</sub>, and γ) were fractionated on a three-layer Percoll gradient (see Methods), as previously described (Kjeldsen et al., 1999). To confirm the appropriateness of the approach we subjected the different fractions to mass spectrometry and could identify the most abundant proteins stored in each granule population (see Supplementary Methods): cathepsin G, neutrophil elastase, and myeloblastin in azurophil granules; lactoferrin, NGAL, cathelicidin C, and lysozyme C in specific granules; and lactoferrin, MMP-9, NGAL, cathelicidin C, and protein S100-A9 in tertiary granules, as previously reported (Figure 4A, S4 and Table S1).

The MUB<sub>40</sub> target was mainly stored in specific (β<sub>1</sub>) and tertiary (β<sub>2</sub>) granules, as revealed by western blot using a biotinylated version of RI-MUB<sub>40</sub> (RI-MUB<sub>40</sub>-biotin) (see Supplementary Methods) when granule contents were analyzed on SDS Page gel (Figure 4A) or on Ag-Page gel (allowing the separation of high molecular-weight complexes, see Supplementary Methods) (Figure 4B). Both approaches allowed the detection of a signal in β<sub>1</sub>/β<sub>2</sub> fractions with RI-MUB<sub>40</sub>-Biotin suggested that MUB<sub>40</sub> target were present in these samples. A stronger signal was observed when separating samples on Ag-Page, suggesting that MUB<sub>40</sub> target may form or be associated with high molecular complexes or aggregates. The detection of a signal
on Ag-Page in the γ fraction with RI-MUB$_{40}$-Biotin might be due to an incomplete fractionation of the complexes with this standard procedure (Figure 4B). The propensity of lactoferrin, specifically stored in β1/β2 fractions, to polymerize in the presence of cations such as Ca$^{2+}$ or Fe$^{3+}$ (Bennett et al., 1981) (Mantel et al., 1994) is hypothesized to be responsible of this phenomenon. The granule fractionation stringency was assessed by immunodetecting lactoferrin exclusively in specific (β1) and tertiary (β2) granules (Figure 4A).

Lactoferrin was identified as a target of MUB$_{40}$ in neutrophil granules, by a pulldown assay with RI-MUB$_{40}$-Biotin (Figure 4C). This result was confirmed by immunofluorescence experiments on fixed human neutrophils, showing a similar localization of the RI-MUB$_{40}$-Cy5 and α-lactoferrin fluorescent signals (Figure 4D).

The MUB$_{40}$ lactoferrin-binding property was finally demonstrated with human purified lactoferrin incubated in a RMPI 1640 medium supplemented with 10 mM Hepes and 3 mM glucose, which allowed the formation of lactoferrin oligomers, as previously performed in other medium (Bennett et al., 1981) (Mantel et al., 1994). Again, in this experimental model, a similar localization of the RI-MUB$_{40}$-Cy5 and α-lactoferrin fluorescent signals was observed (Figure 4E). N-deglycosylation of lactoferrin with PNGase abolished MUB$_{40}$-Cy5 labeling, suggesting that lactoferrin glycosylation moiety was essential for MUB$_{40}$ binding to lactoferrin (Figure 4E-F).

As a conclusion, MUB$_{40}$ is a marker of lactoferrin, which is the most abundant protein stored in neutrophil specific and tertiary granules. The potential use of MUB$_{40}$-Cy5 as a marker of lactoferrin secretion was further assessed during *Shigella flexneri* infection *in vitro* and in *in vivo* models of inflammation.
Detection of lactoferrin degranulation with MUB\textsubscript{40}-Cy5 \textit{in vitro} and \textit{in vivo}

Neutrophil granule inducible exocytosis (or degranulation) occurs in the presence of an inflammatory stimulus, such as bacterial infection. Here, for the first time, the degranulation process could be assessed \textit{in vitro} on living neutrophils infected with \textit{Shigella flexneri} by live fluorescence microscopy in the presence of RI-MUB\textsubscript{40}-Cy5: transient, dot-shaped fluorescent signals were detected on the cells’ surface (Figure 5A and Movie S1), strongly suggesting that exocytosed lactoferrin was bound extracellularly to RI-MUB\textsubscript{40}-Cy5. Since RI-MUB\textsubscript{40}-Cy5 was not degraded by proteases (Figure 2C-D), the fact that transient lactoferrin labeling might be due to its solubilization in the culture medium leading to the dilution of the fluorescent signal.

Lactoferrin detection with RI-MUB\textsubscript{40}-Cy5 was strictly extracellular, confirming that this marker does not cross plasma membrane of live cells, similarly to MUB\textsubscript{70} (Coïc et al., 2012). \textit{In vivo}, neutrophil lactoferrin secretion was successfully revealed on fixed tissues with MUB\textsubscript{40}-Cy5 in the guinea pig colonic mucosa infected by \textit{Shigella flexneri}; revealing lactoferrin accumulation within the bacteria foci vicinity (Figure 5B), consistent with previous studies (Masson et al., 1969). The assessment of neutrophil recruitment during \textit{Shigella flexneri} invasion could not be investigated in the guinea pig model of shigellosis, since the targeted organ deep localization is not compatible with fluorescence imaging techniques on living animals.

Further validation of RI-MUB\textsubscript{40} in living animals suffering of shigellosis was not possible due to the localization of the infected organ, the colon, deep into the abdomen, making the assessment of fluorescent signals difficult. Alternatively, the capacity of RI-MUB\textsubscript{40} to specifically label inflammatory tissues was evaluated in an arthritis mouse model (sterile inflammation), using the K/BxN serum transfer model (Bruhns et al., 2003). Indeed, following systemic administration (i.v. injection) of RI-
MUB$_{40}$-Cy5 into arthritic mice, but not their naïve controls, a fluorescent signal accumulated at inflammatory sites, localized in joints, which are known to contain a large population of activated neutrophils and are hypothesized to contain a high concentrated level of lactoferrin (Figure 5C). The inflammation in articulations was confirmed by the detection of luminol (a MPO substrate, i.v. injection) bioluminescence (Figure 5C). Notably, epifluorescent (RI-MUB$_{40}$-Cy5) and bioluminescent (luminol) signals were significantly higher in arthritic mice compared to controls (Figure 5C, $p \leq 0.0001$, T-test) and correlated (Figure 5D, $p \leq 0.0001$, T-test).

In conclusion, we demonstrated here that MUB$_{40}$ and RI-MUB$_{40}$ peptides allowed the assessment of neutrophil lactoferrin detection in vitro or in vivo. Therefore, the potential use of RI-MUB$_{40}$ as an inflammatory disease marker, associated with neutrophil recruitment and activation, was further evaluated on human inflamed tissues.

**Neutrophil labeling with MUB$_{40}$ on human inflamed tissues**

Our results indicated that neutrophil lactoferrin could be labeled with MUB$_{40}$ or RI-MUB$_{40}$ peptides intracellularly on fixed samples (cells and tissues) or extracellularly upon its secretion by living neutrophils upon activation. Neutrophil recruitment and activation was further successfully assessed on various human biopsies from patients diagnosed with sterile or infectious inflammatory diseases with MUB$_{40}$ and illustrated here with a malignant fibrous histiocytoma (Figure 6A) and a streptococcal skin abscess (Figure 6B), respectively. In both cases, recruited neutrophils and secreted lactoferrin were labeled with MUB$_{40}$-Cy5 and a $\alpha$-lactoferrin antibody: corresponding fluorescent signals were co-localized (Figure 6A-B, $p \leq 0.001$, T-test).
In conclusion we demonstrated that MUB$_{40}$ peptides allow detection of human lactoferrin and therefore offers a new tool for the assessment of neutrophil recruitment and activation in human inflammatory tissues.

**Discussion**

Here we designed, synthesized and validated MUB$_{40}$ and RI-MUB$_{40}$ peptides as novel markers of neutrophil lactoferrin, which may be considered as universal markers of mammalian neutrophils and will facilitate neutrophil detection and study in animal models of inflammation, including mouse, rabbit, or guinea pig (Figures 3 and S3), as illustrated by our recent report in guinea pigs (Arena et al., 2016).

As a novel lactoferrin marker, MUB$_{40}$ will contribute to a better understanding of lactoferrin modulatory and antimicrobial functions *in vitro* and *in vivo*. To date, most studies have focused on the importance of mucus lactoferrin in the protection of the respiratory tract from inflammation or infection *in vivo* (Valenti et al., 2011)-(Sagel et al., 2009)-(Dubin et al., 2004) or in lung epithelial cell culture models (Calu3 (Babu et al., 2004)). Lactoferrin abundance and function in the intestinal tract has been less investigated, although confirmed in humans (Peen et al., 1996); its protective role was confirmed in a mouse model of colitis (Ye et al., 2014). The resistance of lactoferrin to bacteria secreted serine proteases (SPATE) proteolysis strongly support its importance in preserving the epithelial lineage from bacterial aggression (Gutierrez-Jimenez et al., 2008). The use of MUB$_{40}$ will promote lactoferrin studies in intestinal inflammation and infection.

We previously characterized MUB$_{70}$ colonic mucus-binding property mediated by its ability to interact with a Mucin 2 glycosylation moiety (Coïc et al., 2012). Here we
have confirmed that its shorter derivative, MUB$_{40}$-Cy5, similarly labeled colonic mucus and goblet cells’ granules (Figure 1B, C and F). We demonstrated that MUB$_{70}$ (data not shown) and MUB$_{40}$ peptides labeled neutrophil granules (Figures 1F and 2A) by interacting with a lactoferrin glycosylation moiety (Figure 4E-F). Taken together, these results raised the question of the specificity of MUB$_{70}$ and MUB$_{40}$ targets in mucus samples and in neutrophil granules. Muc2 and lactoferrin are both present in the colonic mucus, and both play a key role in the protection of the colonic mucosa from colitis (Ye et al., 2014)-(Faure et al., 2004). For technical reasons, it was not possible to isolate and purify either Muc2 or lactoferrin from the mucus matrix, which is a complex and dense hydrogel. Considering that mucins are not expressed by neutrophils, lactoferrin can be considered a specific target of MUB$_{40}$ in neutrophil granules. This assumption is supported here by the demonstration of the interaction between purified human lactoferrin and MUB$_{40}$ (Figure 4C and F). However, we cannot rule out the possibility that MUB$_{40}$ may label lactoferrin present in colonic mucus together with Muc2. Although difficult, further investigations will be required to address the MUB$_{40}$ target specificity in colonic mucus.

Using RI-MUB$_{40}$-Cy5, we could reveal neutrophil degranulation for the first time in inflammatory tissues in vivo with a non-invasive method (Figure 5C-D). Notably the intensity of fluorescent signal correlated with disease severity, suggesting that RI-MUB$_{40}$-Cy5 is not only a marker for inflammation in vivo, but also allows the appreciation of inflammation intensity.

We demonstrated that MUB$_{40}$ is a specific neutrophil marker, which may be used, in a broad range of in vitro assays. Further investigations will be required to validate RI-MUB$_{40}$ as a potentially new inflammation marker in vivo, including a pre-clinical study (toxicity, bioavailability, specificity and sensitivity of its lactoferrin-binding property).
Labeling RI-MUB40 with radioactive elements may be envisaged for inflammation site localization with non-invasive inflammation-imaging methods such as scintigraphy, PET or SPECT (Wu et al., 2013) (Zhang et al., 2010) (Zhang et al., 2007) (Locke et al., 2009).

Significance

Neutrophils are major players of the innate immune response during inflammation and infection, although their detection remains difficult in animal models and humans. Here we describe MUB40 as a specific marker of neutrophils, binding to lactoferrin, stored in specific and tertiary granules or released upon neutrophil activation. MUB40 and its retro-inverso derivative RI-MUB40 allow the imaging of neutrophils in vitro and in vivo in inflammation animal models; these markers will open new doors in neutrophil study, non-invasive live imaging of inflammation and diagnostic of inflammatory diseases.

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Material and Methods

MUB40 peptides series synthesis

The synthesis were carried out on a 100µmoles scale on an ABI 433 synthesizer (Applied Biosystems, Foster City, CA) from a polystyrene AM-RAM resin and using conventional FMOC chemistry. N-terminal acetylation was achieved by treating the peptide resins at the end of the synthesis with acetic anhydride for 30 minutes. As a result, all peptides were N-terminal amide and C-terminal acetylated. For the purpose of structural analysis (CD and gel filtration), MUB40 peptides were submitted to a N-ethyl maleimide treatment in order to prevent covalent dimer formation. Fluorophore labeling and biotin derivatization were performed through the conjugation of their maleimide derivatives to the free sulfhydryle peptides. All purification steps and HPLC analysis were done by C18 Reverse Phase columns. Final characterization by electro-spray mass analysis were consistent with the expected masses (between brackets) : Cy5-labeled MUB40#1: 5549.740 (5550.115); Cy5-labeled MUB40#2: 5447.472 (5447.933); Cy5-labeled MUB40#3: 5588.273 (5589.170); Cy5-labeled MUB40#4: 5501.778 (5502.093); Cy5-labeled RI-MUB40#1 (RI-MUB40): 5551.265 (5550.115); Dylight 405-labeled MUB40#1 (MUB40-D405): 5519.849 (5518.9.. – restricted proprietary information); RI-MUB40#1-Biotin (RI-MUB40-Biotin): 5296.994 (5296.792). Detailed synthesis and derivatization steps and the biophysical analysis of MUB40 peptides are described in Supplementary Methods.

Neutrophil granules purification and fractionation.

Neutrophil granules were collected from purified polymorphonuclear neutrophils,
following the procedure described previously (Kjeldsen et al., 1999). Neutrophils were resuspended in PBS (2.7. 10⁶ cell/mL) with 0.5 μL/mL DFP (Sigma-Aldrich) and incubated on ice for 15 min prior centrifugation (1300 rpm; 10 min). Cells were resuspended (20.10⁶ cell/mL) in a relaxation buffer (KCl 100 mM, NaCl 3 mM, MgCl₂ 3.5 mM, PIPES 10 mM, adjusted at pH 6.8) with a cocktail of protease inhibitors (400 mM leupeptin, 400 mM pepstatin, 3 mM PMSF, 1 mM orthovanadate) and supplemented with 1 mM ATP, 1 mM EDTA, and 1.25 mM EGTA. Cells were lysed by nitrogen cavitation (350 psi, 20 min). Cell lysates were centrifuged at 3000 rpm for 15 min to remove remaining cells and nuclei. For total granule recovery, lysates were centrifuged at 16000 rpm for 45 min; granules were resuspended in protease inhibitor-containing relaxation buffer (described above) and stored at -80°C. For granule fractionation, lysates were centrifuged onto a 3-layers Percoll gradient (densities 1.120 g/mL - 1.090 g/mL - 1.050 g/mL) at 37,000 x g for 30 min at 4°C, as described in (Kjeldsen et al., 1999). From the top to the bottom, γ (secretory vesicles), β2 (tertiary granules), β1 (specific granules), and α (azurophil granules) fractions were collected. Remaining Percoll solution was removed by ultracentrifugation (100,000 x g for 90 min at 4°C); purified granules were collected in inhibitor-containing relaxation buffer and stored at -80°C.

**Human and mouse models of inflammation**

**Colon explant surgical collection.** In summary, human colon segments (ascending, descending, and sigmoid colon) were obtained from fully informed patients undergoing surgery for colon carcinoma and were analyzed anonymously. Patient written consent was obtained, according to the French bioethics law. None of the
patients had undergone radiotherapy or chemotherapy. According to the pathologist’s examination rules for the longitudinally bisected colon, a healthy segment of tissue, which was distant from the tumor region and devoid of metastatic cells, was removed. Tissues were processed according to the French Government guidelines for research on human tissues and the French Bioethics Act with the authorization n°RBM 2009-50.

*Human inflammatory tissues.* Human biopsies from patients diagnosed for malignant fibrous histiocytoma and for a streptococcal skin abscess were collected and processed at the Kremlin Bicêtre Hospital, Anatomy and Pathological Histology Department, headed by Dr. Thierry Larzure. Tissue samples were fixed in formaldehyde and further embedded in paraffin. 10 μm sections were obtained using a microtome (Leica Biosystem). Tissues were labeled with a mouse anti-lactoferrin primary monoclonal antibody (Hycult biotech, clone 265-1K1, 1:50 dilution), MUB40-Cy5 (1 μg/mL), and Dapi (1:1000 dilution) as described below.

*Ex vivo infection of human colonic tissue.* Human colon explants were infected with *S. flexneri* 5a (M90T) pGFP, as described in (Nothelfer et al., 2014) and adapted from (Coron et al., 2009). Briefly, colonic tissues were cut into ~5-cm² segments and pinned flat, with the submucosa facing down, onto a 4% agarose layer in tissue culture Petri dishes containing DMEM/F12 culture medium (Invitrogen) supplemented with 10% FBS, glutamine, and 2.1 g/L NaHCO₃ (Sigma-Aldrich). *S. flexneri* (M90T) 5a pGFP was added at ~2 × 10⁸ bacteria per cm² of tissue. Bacteria were allowed to settle for 15 min at room temperature before incubation at 37°C, 5% CO₂ for 6 h on a slowly rocking tray. Tissue was fixed by overnight incubation with 4% PFA (Euromedex) and 0.1 M Hepes (Gibco) in PBS. For whole-mount staining,
tissues were fixed on a 40 × 11-mm tissue culture dish (TPP) with Histoacryl tissue glue (Braun). To obtain 150-µm-thick sections, the tissue was embedded in low-melting agarose according to (Snippert et al., 2011) and cut with a vibratome (VT1000E; Leica).

**Mouse arthritis model.** 6-7 weeks old female C57BL/6J mice were purchased from Charles River France, housed under specific pathogen-free (SPF) conditions and handled in accordance with French and European directives. Mouse protocols were approved by the Animal Ethics committee CETEA number 89 (Institut Pasteur, Paris, France) and registered under #2013-0103, and by the French Ministry of Research under agreement #00513.02. Arthritis was induced by i.v. injection of 120 μL K/BxN serum and arthritis scored as described previously (Bruhns et al., 2003). Mice injected with physiological saline were used as controls. On day 6 after serum transfer, mice were anesthetized, shaved, depilated and injected i.p. with luminol (10 mg/mouse) and i.v. with 5 μg/mouse MUB-40 Cy5. Epifluorescence, bioluminescence and CT images were acquired 10-90 min after injection using an IVIS SpectrumCT (Perkin Elmer).

**Flow cytometry**

**Human.** Neutrophils and PBMCs were separated as described in Supplementary Methods. Naïve cells were resuspended in PBS + EDTA 2mM. Fixed and permeabilized were were obtained by incubation in PFA 3.2% for 30 min and resuspension in PBS + Triton 0.1% for 30 min. Naïve and fixed/permeabilized cells were incubated with CD45-FITC and MUB_{40}-Alexa405 peptide (1 μL/mL) for 15 min at room temperature. Cells were analyzed with a FACSCANTO II (BD) and data were analyzed using Flowjo software.
Mouse. Blood leucocytes were purified by dextran, washed in PBS EDTA and kept on ice. For hematopoietic populations staining, 2 to 3 million cells were resuspended in PBS EDTA, blocked with 16/32 for 15 minutes and stained with antibodies for 30 minutes at 4°C (CD45 PE-CF594, CD3e FITC, B220 PE-CY7, NKP46 FITC, CD11b PE from BD biosciences, and Ly6G APC-H7, Ly6C APC from Biolegend). Cells were washed with PBS EDTA and fixed in PFA 3.3% at room temperature during 15 minutes. Cells were washed and resuspended in PBS-0.1% Triton for 5 minutes. Cells were then incubated with MUB40-Alexa405 peptide (1 μL/mL) for 15 minutes at RT and washed. Not fixed cells incubated with peptide or fixed cells not incubated with peptide were used as negative control. Cell fluorescence was determined using a Fortessa (BD biosciences) and analyzed with Kaluza Software (Beckman Coulter). The fluorescence intensity was quantified.

**Fluorescent markers and cell labeling**

**Fixation and staining procedures.** For microscopy studies, purified or cultured cells and polymerized lactoferrin were resuspended onto 24-well plates containing 12mm coverslips in RMPI 1640 + 10 mM Hepes (when cultured in the autologous plasma) and centrifuged at 300 x g for 10 min. Culture media were removed and cells were fixed in 4% Paraformaldehyde (PFA) for two hours. Fixed cells were washed three times in PBS + 0.1% saponin (Sigma-Aldrich), prior immunolabeling with primary antibodies in the same buffer for 1 hour (Triton 0.1% can alternatively be used for cell permeabilization). After three additionnal washes in PBS + 0.1% saponin, secondary antibodies and fluorescent markers were incubated for one hour.
Coverslips were washed three times in PBS + 0.1% saponin, three times in PBS, and three times in deionized H$_2$O and mounted with prolong gold mounting media.

**Antibodies, fluorescent markers.** For immunofluorescence assay, lactoferrin was detected with a mouse anti-lactoferrin primary monoclonal antibody (Hycult biotech, clone 265-1K1, 1:50 dilution) and an anti-mouse-FITC conjugated secondary antibody (1:1000). Nuclei were detected with DAPI (Life Technologies), Lyophilized MUB$_{40}$ peptides (MUB$_{40}$#1-Cy5, MUB$_{40}$#2-Cy5, MUB$_{40}$#3-Cy5, MUB$_{40}$#4-Cy5, MUB$_{40}$#1-Dylight, RI-MUB$_{40}$-Cy5, and RI-MUB$_{40}$-Biotin) were solubilized in a phosphate buffer pH8 at a 1 mg/mL concentration and used at a 1:1000 dilution. For PBMC staining CD19-APC (B lymphocytes), CD3-APC (T lymphocytes), and CD14-PercP (monocytes, macrophages) (BD Bioscience) were used at a 1:1000 dilution together with Dapi (1:1000) and MUB$_{40}$-Cy5 (1 mg/mL). Actin was stained with Phalloidin-Rhodamine red X (RRX) (Jackson Immunoresearch Antibodies) used at a 1:1000 final dilution.

**Biochemistry**

**Electrophororesis.** Proteins were separated by either SDS Page (4-12% gradient, at 150 volts for 90 min) or Ag-Page (0.7 % (w/v) agarose gels in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA pH 8), containing 0.1% (w/v) SDS at 65 volts for 3 hours).

**Transfer.** Protein samples separated by SDS Page were transferred to nitrocellulose membrane performing electrophoretic protein transfer. Protein samples separated by Ag-Page were transferred to nitrocellulose membrane using a vacuum blotter (GE HealthCare); 40 mbar pressure in 4 x SSC (0.6 M sodium chloride, 60 mM tri-sodium citrate).
RI-MUB_40-Biotin immunoblotting. Transferred nitrocellulose membranes were blotted in PBS with RI-MUB_40-Biotin (1 μg/mL) for 3 hours, washed three times (15 min) in PBS, incubated with HRP-conjugated Streptavidin (Thermo Scientific ref. N100, 1:1000 dilution) for one hour, and washed three times (15 min) in PBS. RI-MUB_40-Biotin binding was detected with chemiluminescence (ECL kit, GE Healthcare) using an imaging system (B:Box, Syngene).

RI-MUB_40-Biotin Pulldown assay. Lyophilized, biotinylated retro-inverso MUB_40 (RI-MUB_40-Biotin) was solubilized in 1 mL (185 μg/ml final concentration) of binding buffer (20 mM NaH_2PO_4, 0.15 M NaCl, pH 7.5). 150 μL (277 ng) of solubilized RI-MUB_40-Biotin was loaded onto 200 μL of washed/packed Streptavidin Sepharose High Performance beads (GE HealthCare). The loaded beads were incubated with gentle rocking for 1 hour at room temperature with 1 mg of purified neutrophil granule fractions. Pulldown fractions were transferred to columns and washed with 10 mL fresh binding buffer. Bound RI-MUB_40-Biotin and co-purified proteins were eluted with 500 μL 8M Guanidine-HCl, pH 1.5. Eluted proteins were mixed 1:1 with 2x Laemmli buffer (4% SDS, 20% Glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris HCl, pH 6.8). Samples were run on 4-20% SDS-Page gels and stained with InstantBlue™ (Expeodeon, ref. ISB1L) for 1 hour before destaining in deionized H_2O. Stained gels were imaged and ≈80 kDa co-purified band was cut out and sent for mass spectrometry identification.

Lactoferrin polymerization. Human purified lactoferrin (ref : L1294, Sigma-Aldrich) was resuspended in deionized H_2O (35 μg/mL). Lactoferrin was incubated in RPMI + 10 mM Hepes supplemented with 10 mM FeCl_3, at indicated concentrations (0.35 or 3.5 μg/mL) onto 24-well plates containing 12mm coverslips overnight at 37°C,
adapted from similar lactoferrin polymerization experiments performed in iron saturated phosphate buffer (Mantel et al., 1994).

**Lactoferrin deglycosylation.** About 10 µg of human purified lactoferrin (ref : L1294, Sigma-Aldrich) was treated by Peptide-N-Glycosidase F (PNGaseF, New England Biolabs, Ipswich MA, USA) for N-deglycosylation. Protein was treated exactly as described in manufacturer protocol. Twelve unit of PNGase F were added for 4 h at 37°C for the removal of N-glycosylation.

**Lactoferrin polymers interaction with MUB$_{40}$-Cy5.** Lactoferrin polymers were centrifuged at 2000 rpm for 10 min and fixed in Paraformaldehyde (PFA) 4% for one hour. Lactoferrin polymers were immunolabeled in PBS + 0.1% saponin (Sigma-Aldrich) with a mouse anti-lactoferrin primary monoclonal antibody (Hycult biotech, clone 265-1K1, 1:50 dilution) and an anti-mouse-FITC conjugated secondary antibody (1:1000), together with MUB$_{40}$-Cy5 (1µg/mL). Slides were washed three times in PBS and three times in deionized H$_2$O and mounted with prolong gold™ mounting media.

**Lactoferrin polymers interaction with RI-MUB$_{40}$-Biotin.** 5 µg lactoferrin polymers performed in RPMI 1640 +10 mM Hepes (see above) were separated by Ag-Page, transferred to nitrocellulose membrane and blotted with RI-MUB$_{40}$-Biotin (1µg/mL) in PBS, as described above.

**Author contributions**

YMC and FB synthesized MUB$_{40}$ peptides. BB performed biophysical analyses. MA, JS, LI, CR, DT and TCB performed biochemical and mass spectrometry analyses. NL and VL performed HSC experiments. MA, PV, ETA, JYT, GN, KN, FJ and BSM
validated MUB\textsubscript{40} peptides in indicated animal models. TL validated MUB\textsubscript{40} peptides in human biopsies. BSM designed the experiments and wrote the manuscript together with FB, FJ and MM.

**Competing financial interests**

The authors declare no competing financial interests

**Materials & Correspondence**

Correspondence and material request (including MUB\textsubscript{40} peptide derivatives) should be addressed to Dr. Benoit Marteyn

**Bibliography**


Figure legends

Figure 1. MUB_{40}, derived from MUB_{70}, binds to the human colonic mucus and neutrophil granules

(A) Schematic representation of the four 40-amino acid peptides named MUB_{40}\#1, MUB_{40}\#2, MUB_{40}\#3, MUB_{40}\#4, overlapping and covering the MUB_{70} sequence (Coïc et al., 2012) (from C-terminal to N-terminal, see Figure S1A). (B) Human colonic mucus layer was labelled with the MUB_{40}\#1-Cy5 fluorescent conjugate (1 \, \mu g/mL, magenta). Actin (red); bar, 10 \, \mu m; not MUB_{40}\#2-\#4 peptides conjugated to Cy5 (shown in Figure S1B). (C) Colonic goblet cell granules were labelled with MUB_{40}\#1-Cy5 (1 \, \mu g/mL, magenta), which was not the case for MUB_{70}-Cy5 (Coïc et al., 2012) or MUB_{40}\#2-\#4 peptides (data not shown). Actin (red); bar, 20 \, \mu m. (D) Elution profiles of MUB_{40} peptides (#1 - #4) were obtained by analytical gel filtration (absorbance at 280 nm). Samples were prepared at 10\mu g/ml in the elution buffer (20 mM phosphate buffer, 150 mM NaCl, pH7.4). MUB_{40}\#1 assembled as a trimer, similarly to MUB_{70} (Coïc et al., 2012), not MUB_{40}\#2, MUB_{40}\#3, and MUB_{40}\#4. The relative masses (15.9 ; 22.0 ; 20.5 and 20.4 respectively) were estimated from standard proteins’ elution volume (as indicated). (E) Far-UV Circular Dichroism spectra of the four MUB_{40} peptides at 60 \mu M in 20 mM sodium phosphate buffer (pH 7.4) in the presence of 50 mM NaCl, showing a specific structural signal of MUB_{40}\#1, compared to others peptides. (F) A human colonic explant was infected with _Shigella flexneri_ pGFP (green) and labelled with MUB_{40}\#1-Cy5 (1 \, \mu g/mL, magenta) and Dapi (blue) after fixation and permeabilization. The colonic mucus layer and infiltrated...
neutrophils (see (G)), were labelled with MUB₄₀#1-Cy5, as imaged by two-photon microscopy. Bar, 50 μm (G) The labelling of polymorphonuclear neutrophils with MUB₄₀#1-Cy5 (1 μg/mL, magenta) was confirmed on human purified neutrophils, showing a granular staining. Nucleus was stained with Dapi (blue). Bar, 5 μm.

**Figure 2. Mature myeloid cells were specifically labelled with MUB₄₀#1-Cy5 (hereafter named MUB₄₀-Cy5)**

(A) Human polymorphonuclear neutrophils were similarly labelled with MUB₄₀ conjugated with Cy5 (magenta) or Alexa405 (blue) and with a retro-inverso (RI) MUB₄₀ peptide, designed with non-natural D-amino acids, conjugated with Cy5 (magenta). Nuclei were stained with Dapi (blue or white). Bars, 20 μm. (B) Human hematopoietic stem cells (CD34+) were not labelled by MUB₄₀-Cy5 during their proliferation, when a positive staining (magenta) was obtained upon their differentiation in polymorphonuclear neutrophils in the presence of G-CSF, IL-3, and IL-6 (2 weeks). Nuclei were stained with Dapi (blue). Bars, 50 μm. (C-D) RI-MUB₄₀ peptide was not degraded by trypsin. (C) MUB₄₀ and RI-MUB₄₀ final peptide concentration was 0.25 mg/mL and trypsin to protein ratio was 1:20 (w/w). HPLC profiles of purified MUB₄₀ and RI-MUB₄₀ peptides incubated with Trypsin during the 0, 1, 3, and 24h at 37°C. The percentage of peptide stability over the time are shown in (D). Results are expressed with Mean ± S.D. (n=3).
Figure 3. Specific labelling of neutrophils (human, mouse, guinea pig) with MUB₄₀ conjugated fluorescent peptides using flow cytometry or fluorescent imaging.

(A) Naïve (blue) and fixed/permeabilized human neutrophils (red) were incubated with MUB₄₀-Alexa405 (1 µg/mL) and analyzed by flow cytometry. The efficient labelling of fixed/permeabilized neutrophils with MUB₄₀-Alexa405 confirms its intracellular target. MFI were calculated with three independent samples, error bars show S.D., *** indicates p < 0.001. (B) Human neutrophils were separated on a Percoll gradient (see Supplementary Methods) and subsequently fixed and permeabilized. MUB₄₀-Alexa405 (1 µg/mL) specifically labels neutrophils, not PBMCs. MFI were calculated with three independent samples, error bars show S.D., *** indicates p < 0.001. (C) MUB₄₀-Alexa405 labelling of mouse leucocytes was assessed by flow cytometry. The gating strategy and negative control are described in Fig. S2B-C. MUB₄₀-Alexa405 (1 µg/mL) specifically labels neutrophils, not mononuclear cells, including B and T lymphocytes and Ly6C- or Ly6C+ monocytes. MFI were calculated with three independent samples, error bars show S.D., ** indicates p < 0.01. Consistent results were obtained by immunofluorescent microscopy shown in Fig. S2A. (D) Guinea pig and mouse neutrophils were fixed and labelled with MUB₄₀-Cy5 (magenta). Nuclei were stained with Dapi (blue). Bars, 10 µm. (E) Upon *Shigella flexneri* 5a pGFP (green) infection of the guinea pig colonic mucosa, infiltrated neutrophils were labelled with MUB₄₀-Cy5 (magenta) or MUB₄₀-Alexa405 (blue). Bars, 100 µm. (F) Upon *Shigella sonnei* pMW211 (pDsRed) (red) oral challenge of mice, a local colonization of the colonic mucosa was observed,
associated with a recruitment of neutrophils labelled with MUB40-Cy5 (magenta). Actin was stained with Phalloidin-FITC (green), bar,100 μm.

Figure 4. MUB40 binds specifically to lactoferrin, stored in neutrophil specific (β1) and tertiary granules (β2)

(A) Human neutrophil granules were purified and fractionated on a 3-layers Percoll gradient (Kjeldsen et al., 1999). Total granule, α (azurophil granules), β1 (specific granules), β2 (tertiary granules), and γ (secretory vesicles) fractions were separated on a 10% SDS Page gel and stained with Coomassie. Most abundant proteins in each fraction was identified by mass spectrometry (right panel) (raw data presented in Figure S4 and Table S1). The preferential labelling of the β1 and β2 fractions with RI-MUB40-Biotin (1 μg/mL) together with a α-lactoferrin antibody was observed by western blot using Streptavidin-HRP (bottom). (B) Granule fractions (α, β1, β2 and γ) were additionally separated on an Ag-Page gel (allowing the identification of high molecular-weight complexes) prior to transferring proteins onto a nitrocellulose membrane. The preferential labelling of the β1 and β2 fractions with RI-MUB40-Biotin (1 μg/mL) was confirmed by western blot using Streptavidin-HRP. (C) Purified neutrophil granules were incubated with RI-MUB40-Biotin to identify its target. The most abundant protein present in the output was identified by mass spectrometry as lactoferrin (78 kDa). FT corresponds to the flow-through fraction (D) The labelling of lactoferrin by MUB40 was confirmed by immunofluorescence on fixed human neutrophils, showing a co-localization of the fluorescent signals using RI-MUB40-Cy5 (magenta) and a α-lactoferrin antibody (green). DNA was stained with Dapi, bar, 20
µm. (E) In order to confirm the labelling of human lactoferrin by MUB40, commercial purified lactoferrin was allowed to polymerize in RPMI 1640 culture medium (0.35 µg/mL and 3.5 µg/mL) at 37°C (adapted from (Bennett et al., 1981); (Mantel et al., 1994)). Polymerized lactoferrin was fixed and immunolabeled with MUB40-Cy5 (magenta) and an α-lactoferrin antibody (green). bar, 20 µm. Deglycosylated lactoferrin (PNGase treatment) was no longer labelled by MUB40-Cy5. (F) Lactoferrin (naïve and deglycosylated) polymers (5 µg) were separated on an Ag-Page gel prior to transfer onto a nitrocellulose membrane. The interaction between lactoferrin and MUB40 was confirmed by western blot with RI-MUB40-Biotin (1 µg/mL) and streptavidin-HRP.

Figure 5. Neutrophil lactoferrin degranulation assessment with RI-MUB40-Cy5 in vitro and in vivo in infectious and sterile inflammation models

(A) The kinetics of the lactoferrin degranulation (cell surface exposure, white arrows) was assessed by live microscopy on neutrophils infected by Shigella flexneri pGFP (green) (at MOI 20) in the presence of RI-MUB40-Cy5 (1µg/mL, magenta) (RPMI 1640 medium supplemented with 10 mM Hepes at 37°C). Images were acquired every 60s for 9 min (see Movie S1), bar 10 µm. RI-MUB40-Cy5 did not cross the plasma membrane of living cells (data not shown), similarly to MUB70 (Coïc et al., 2012). (B) Neutrophil lactoferrin degranulation is detected in Shigella flexneri 5a pGFP (green) foci of infection in the guinea pig mucosa with MUB40-Cy5 (1µg/mL, magenta) (white arrow) DNA was stained with Dapi (blue). Bar, 20 µm. (C-D) RI-MUB40-Cy5 accumulates at sites of inflammation in vivo. C57Bl/6J mice were
injected i.v. with serum from K/BxN mice (K/BxN, n=4) or saline (saline, n=5) (day 0).

**C** Photon emission corresponding to luminol degradation by myeloperoxidase activity present in the joints was measured 6 days post arthritis induction (top panels). Bioluminescence in regions of interest (red circles) is expressed as average radiance (p/s/cm²/sr; left scatter plot). Accumulation of RI-MUB₄₀-Cy5 fluorescent signal (640nm/700nm) in the joints of arthritic mice but not in control mice (middle panels and lower panels as merged picture with CT). Epifluorescence in regions of interest (red circles) is expressed as average radiant efficiency (p/s/cm²/sr/[uW/cm²]; right scatter plot). **D** Correlation of bioluminescent signal and RI-MUB₄₀-Cy5 fluorescent signal in indicated regions of interest (hind ankle joints). Data in (C) are representative of two independent experiments. Error bars correspond to the SEM, **** indicates p ≤ 0.0001 (unpaired t-test).

**Figure 6. Inflammatory tissues labelling with MUB₄₀-Cy5**

Tissue inflammation is characterized by neutrophil recruitment and potentially associated with lactoferrin degranulation. The validation of MUB₄₀ peptides (here MUB₄₀-Cy5) as markers of inflammation was confirmed on human biopsies, here **(A)** a malignant fibrous hystiocytoma (sterile inflammation) and **(B)** a streptococcal skin abscess (infectious inflammation). Lactoferrin was stained with an anti-lactoferrin antibody (green) and MUB₄₀-Cy5 (1µg/mL, magenta), DNA was stained with Dapi (blue). Bars, 150 µm. Surface plots and correlation of fluorescent signals were obtained with the Fiji software.
Anderson et al., Fig. 1

A

MUB\textsubscript{70}
MUB\textsubscript{40} #1
MUB\textsubscript{40} #2
MUB\textsubscript{40} #3
MUB\textsubscript{40} #4

B

Human colonic mucus

Actin
MUB\textsubscript{40}-Cy5 #1
Merge

C

Human colon goblet cells

Actin
MUB\textsubscript{40}-Cy5 #1
Merge

D

\textbf{E}

F

Human colon infected with \textit{Shigella flexneri}

G

Human neutrophil

MUB\textsubscript{40}-Cy5/DIC/DAPI/Merge

Shigella
MUB\textsubscript{40}-Cy5#1
Dapi
**Figure 4**

**A**

**Human neutrophil granule fractions**

<table>
<thead>
<tr>
<th>MW (kDa)</th>
<th>Total</th>
<th>α</th>
<th>β1</th>
<th>β2</th>
<th>γ</th>
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<tbody>
<tr>
<td>100 -</td>
<td>SDS-Page</td>
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<td>10 -</td>
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</tbody>
</table>

**MS analysis**

1. Lactoferrin
2. Lactoferrin
3. Gelatinase (MMP-9)
4. Cathespin G
5. Elastase
6. Myeloblastin
7. NGAL
8. Cathelicidin (LL-37)
9. Lysozyme C
10. Protein S100-A9

**B**

**neutrophil granule fractions**

<table>
<thead>
<tr>
<th>α</th>
<th>β1</th>
<th>β2</th>
<th>γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>WB</td>
<td>RI-MUBα-Biotin</td>
<td>Ag-Page</td>
<td></td>
</tr>
</tbody>
</table>

**C**

**Neutrophil granules**

**Pulldown RI-MUBα-Biotin; negative control**

**Input**

**Lactoferrin** (78 kDa)

**Output**

**RI-MUBα-Biotin**

**D**

**Human neutrophils**

**Dai**

**Lactoferrin**

**RI-MUBα-Cy5**

**Merge**

**E**

**Oligomerized human Lactoferrin**

0.35 μg/mL

**Lactoferrin**

**MUBα-Cy5**

**Merge**

3.5 μg/mL

**Lactoferrin**

**MUBα-Cy5**

**Merge**

**F**

**Purified human Lactoferrin**

PNGase -

**WB RI-MUBα-Biotin**

PNGase +

**Ag-Page**
Anderson et al., Fig. 5

A

Neutrophils infected with *Shigella flexneri* pGFP + RI-MUB<sub>40</sub>-Cy5

00:00 01:00 03:00 05:00 07:00 09:00

DIC

S. flexneri pGFP

RI-MUB<sub>40</sub>-Cy5

Merge

B

Guinea pig colon infected with *Shigella flexneri* (foci of infection)

Dapi

Shigella

MUB<sub>40</sub>-Cy5 (Lactoferrin)

Merge

C

mock injected K/BxN injected K/BxN injected

Luminol

Bichrom fluorescence

RI-MUB<sub>40</sub>-Cy5

Epifluorescence

Merge CT + RI-MUB<sub>40</sub>-Cy5

D

Correlation Luminol/RI-MUB<sub>40</sub>

Pearson r: 0.84

R<sup>2</sup>: 0.70

P value <0.0001
Figure 6

Andersen et al., Fig. 6

**A**
Malignant fibrous histiocytoma

![Images of Dapi, Lactoferrin, MUB_{40}-Cy5, and Merge]

**Correlation**
Lactoferrin/MUB_{40}-Cy5
Pearson r: 0.82
R^2: 0.65
P value <0.001

**Surface plot analysis**

- Dapi
- Lactoferrin
- MUB_{40}-Cy5

**B**
Streptococcal skin abscess

![Images of Dapi, Lactoferrin, MUB_{40}-Cy5, and Merge]

**Correlation**
Lactoferrin/MUB_{40}-Cy5
Pearson r: 0.87
R^2: 0.74
P value <0.001

**Surface plot analysis**

- Dapi
- Lactoferrin
- MUB_{40}-Cy5
Anderson et al, Supplementary informations

Supplementary Figure legends

Supplementary Figure 1. MUB\textsubscript{40} peptides sequence and colonic mucus binding property

(a) Operational sequences for the syntheses of MUB\textsubscript{40}#1, MUB\textsubscript{40}#2, MUB\textsubscript{40}#3, MUB\textsubscript{40}#4, where the secondary amino acid substitutes are indicated in red (dipeptides pseudoproline) or green (dipeptides Dmb). The proline residues are indicated in blue. (b) As described in Fig. 1b with MUB\textsubscript{40}#1-Cy5, Human colonic mucus layer was labelled with the MUB\textsubscript{40}#2-Cy5, MUB\textsubscript{40}#3-Cy5, and MUB\textsubscript{40}#4-Cy5 fluorescent conjugate (1 \(\mu\)g/mL, magenta). Actin (red); bar, 10 \(\mu\)m.

Supplementary Figure 2. Human or mouse peripheral blood mononuclear cells (PBMC) are not labeled by MUB\textsubscript{40}-Cy5 or MUB\textsubscript{40}-Alexa405

(A) Human PBMCs were purified from blood samples and fixed in PFA 3%. CD3\(^+\) (T lymphocyte), CD14\(^+\) (monocytes, macrophage), and CD19\(^+\) (B lymphocyte) cells (red) were not stained with MUB\textsubscript{40}-Cy5 (magenta), using the same protocol as in Fig. 2D (neutrophils). Bars are 10 \(\mu\)m. (B) Gating strategy for identification of mouse hematopoietic cell populations in peripheral blood by flow cytometry. From total cells, CD45\(^+\) and singlets were selected. In a “Singlets” gate, neutrophils (Ly6G\(^+\)) were selected. After exclusion of neutrophils, lymphoid cells were identified in a “Not neutro” gate, B lymphocytes (B220\(^+\)), T lymphocytes (CD3\(^+\)) and NK cells (NKP46\(^+\)). After exclusion of lymphoid cells in a “not neutrophil not lymphoid” gate, monocytes were gated as CD11b\(^+\) cells and divided in two subsets, Ly6C\(^+\) classical and Ly6C\(^-\) patrolling monocytes. (C) Fluorescence intensity of MUB\textsubscript{40}-Alexa405 peptide in...
distinct hematopoietic cell populations from peripheral blood was acquired and Mean fluorescence intensity (MFI) was calculated from three independent samples. Error bars correspond to the SD, * indicates p ≤ 0.05 (unpaired t-test).

Supplementary Figure 3. Infiltrated neutrophil detection in the rabbit intestinal mucosa

Immunofluorescence detection of neutrophils (MMP-9, green) in a rabbit ileum section. Actin was stained with RRX-phalloidin (red), and neutrophils were labeled with MUB_{40}-Dylight405 (blue) at a final concentration of 1 µg/mL and a anti-gelatinase (MMP-9) antibody (green). Bar is 30 µm.

Supplementary Figure 4. Neutrophil granule fractionation and protein content separation by electrophoresis

Neutrophil granules were purified as described in Methods and fractionated on a three-layer Percoll gradient, as described previously. ≈ 10 µg of each sample (α, β1, β2 and γ granule fractions) were separated on a 12% SDS-PAGE gel and stained with InstantBlue Protein Stain (Sigma-Aldrich). Indicated bands (a-g) were cut and further analysed by Mass Spectrometry for protein identification (see Supplementary Table 1).

Supplementary Table 1. Fractionated neutrophil granule protein by Mass Spectrometry

Proteins from gel bands described in Fig. 4 and Supplementary Fig. 4 were further identified by LC-MSMS. The top 3 proteins are herein reported. Proteins selection was done according to 2 criteria: their theoretical mass range must be in accordance
with their migration on SDS gel and their iBAQ (Intensity based Absolute Quantification) metrics².

Supplementary Movie01. Dynamics of lactoferrin secretion by neutrophils infected with *Shigella flexneri* pGFP, in the presence of RI-MUB₄₀-Cy5.

Time-lapse imaging of living purified human neutrophils in the presence of RI-MUB₄₀-Cy5 (magenta, 1 µg/mL) and *S. flexneri* pGFP (MOI 20). Image were acquired every 30s using a widefield epifluorescent microscope (Zeiss Definite Focus) at 37°C in the presence of 5% CO₂. Individual images are shown in Fig. 5a.
Supplementary Methods

Synthesis and cleavage. The synthesis were carried out on a 100µmoles scale on an ABI 433 synthesizer (Applied Biosystems, Foster City, CA) equipped with a conductivity flow cell to monitor Fmoc deprotection from a polystyrene AM-RAM resin (capacity 0.41 mmol/g for MUB₄₀ peptides and 0.62 mmol/g for retro-inverso RI-MUB₄₀#1, Rapp Polymere GmbH, Tuebingen, Germany). Standard Fmoc amino acids, Dmb, and pseudoproline dipeptides were activated with HCTU (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate) / DIPEA (N,N-diisopropylethylamine). Fmoc-D-amino acids and Hmb dipeptide were activated with HATU (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide) / DIPEA. All Fmoc-AAs and surrogates were single-coupled with eight-fold molar excess regarding the resin. Both coupling reagents, N-methyl pyrrolidone (NMP) and standard Fmoc amino acids were obtained from Applied Biosystems. Fmoc D-amino acids were obtained from Eurogentec (Eurogentec, Seraing, Belgium). Fmoc L and D-amino acids were side-protected as follows: tBu for aspartic acid, glutamic acid, serine, threonine, and tyrosine; Trt for cysteine and histidine; Boc for lysine; and Pbf (2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl) for arginine. Fmoc-Asp(OtBu)-(Dmb)Gly-OH dipeptides and pseudoproline (oxazolidine) dipeptides were purchased from Merck-Novabiochem. Fmoc-D-Asp(OtBu)-(Hmb)Gly-OH was purchased from Bachem (Bubendorf, Switzerland). Piperidine was purchased from Sigma-Aldrich (St Louis, MO, USA).

N-terminal acetylation was achieved by treating the peptide resin at the end of the synthesis with acetic anhydride for 30 minutes. As a result, all peptides were N-terminal amide and C-terminal acetylated.
Cleavage from the solid support and deprotection of the amino acid side chains were accomplished in one step by 3 h treatment at room temperature with a mixture of TFA / ethanedithiol / triisopropylsilane / water (92.5 / 2.5 / 2.5 / 2.5) for MUB$_{40}$ peptides or TFA / ethanedithiol / triisopropylsilane / water / phenol (92 / 2.5 / 2 / 2.5 / 1) for 4 h for RI-MUB$_{40}$. After filtration of the resin, the cleavage mixtures were poured into ice-cold diethyl ether. The precipitates were recovered by centrifugation, washed three times with cold diethyl ether, dried, resuspended in a mixture of water and acetonitrile, and freeze dried.

**HPLC analysis of synthesized peptides.** Analysis of crude mixtures and purity control of the final peptides were performed by RP-HPLC on an Agilent (Santa Clara, CA, USA) 1100 Series liquid chromatograph and monitored with a photodiode array detector by absorbance at 230 nm, according to the following methods. A linear gradient from 15% to 40% of acetonitrile in aqueous solvent A (50mM ammonium acetate, pH 6.5) over 20 min was applied at a 0.35 ml/min flow rate on a Symmetry 300 C18 3.5 µm 2.1 x 100 mm column (Waters, Manchester, UK). To check the purity of RI-MUB$_{40}$ derivatives, supplementary analyses were done in 0.08% aqueous TFA by applying a 25%-50% acetonitrile gradient on an Aeris Peptide 3.6µ XB-C18 column.

**Peptides purification.** The free sulfhydryl crude peptides MUB$_{40}$#1, #2, & #3 were solubilized at a final concentration of 20 mg/ml in a mixture of solvent A and acetonitrile, 8:2 v/v. Crude MUB$_{40}$#4 was solubilized at the same concentration in water with aqueous ammoniac (pH8) and 10 equivalent of DTT (1,4-Dithio-DL-threitol). Crude RI-MUB$_{40}$ was solubilized at a final concentration of 3.5 mg/ml in solvent A. Those materials were purified by RP-MPLC (AP-100/200 flash, Armen Instrument, Saint Ave, France) on a preparative column (26 x 313 mm) packed with
100 Å 20 µm C18 Nucleoprep packing (Macherey & Nagel GmbH & Co, Düren, Germany), by applying a linear gradient of 15–70% (MUB\textsubscript{40} peptides) or 15-50% (RI-MUB\textsubscript{40}) solvent B (mixture of acetonitrile and solvent A, 8/2 v/v) in solvent A over 60 min at a 20 ml/min flow rate. The purification was monitored at 214 nm (UV detector K2501, Knauer, Berlin, Germany). The suitable fractions were pooled and freeze dried. The overall isolated yields (from 20% to 30%) were in concordance with the observed synthesis yields deduced from the crude’s HPLC analysis.

**MUB\textsubscript{40} peptides conjugation.** Cy5 and Dylight 405 (Thermofisher Scientific) conjugations were operated in a 0.1 M Phosphate buffer pH=6 (MUB\textsubscript{40} peptides) or pH=7.2 (RI-MUB\textsubscript{40}), using 1.2 equivalent of the correspondent maleimide derivative (InvitroGen) in the presence of 1.5 equivalent of TCEP (Tris (2-carboxyethyl)phosphine) per mole of cysteine residue. Repeating one time, this 30 minute coupling protocol was necessary to achieve completion of RI-MUB\textsubscript{40} labeling. The labeled peptides were purified by RP-HPLC using a linear gradient of 15–40% acetonitrile over 20 min at a 6 ml/min flow rate, either on a Nucleosil 5µm C18 300 Å semi-preparative column equilibrated in solvent A (MUB\textsubscript{40} peptides) or on a Kromasil 5µm C18 300 Å semi-preparative column (AIT, Houilles) equilibrated in 50mM Triethyl ammonium acetate (RI-MUB\textsubscript{40}). The purity was checked according to the formerly-described HPLC analytical method. The exact concentration was determined by quantitative amino acid analysis (Hitachi, L-8800 analyzer), giving a 50% to 60% conjugation isolated yield.

Biotinylated RI-MUB\textsubscript{40} was obtained by adding the free sulfhydrlie peptide to 5 equivalents of the maleimide derivative (EZ-linked maleimide-PEG2-biotin, Thermoscience) in 0.1M phosphate buffer (pH6). The biotinylated peptide was purified by HPLC in 50mM ammonium acetate on a Kromasil 5µm C18 300 Å semi-
preparative column, using a linear gradient of 15–40% acetonitrile over 20 min at a 6 ml/min flow rate. A double peak was observed in the analytical HPLC profile, which was attributed to the resolution of the two isomers resulting from the addition of the sulfhydryl peptide on the maleimide double bond.

_Electrospray ionisation mass spectrometry._ Mass spectrometry was carried out on a quadrupole-TOF Micro mass spectrometer (Waters) equipped with a Z-spray API source. Capillary, sample cone, and extraction cone voltages were set at 3kV, 40V, and 10V, respectively. Source and desolvation temperatures were set at 80 and 250°C, respectively. Data were acquired by scanning over the m/z range 150–2000 at a scan rate of 1 s and an interscan delay of 0.1 s. Peptides were dissolved in a mixture of water/methanol/acetic acid (49.5/49.5/1, v/v/v) at a concentration of 1 µg/µl and analyzed in positive-ion mode by infusion at a flow rate of 5 µl/min. Around fifty spectra were combined and the resultant raw multi-charged spectra were processed using the MaxEnt1 deconvolution algorithm embedded in the Masslynx software. Given the deconvolution process of MaxEnt1, applied to the charged molecules (the Cy5 moiety is positively charged), final characterization was consistent with the expected masses (between brackets): Cy5-labeled MUB₄₀#1: 5549.740 (5550.115); Cy5-labeled MUB₄₀#2: 5447.472 (5447.933); Cy5-labeled MUB₄₀#3: 5588.273 (5589.170); Cy5-labeled MUB₄₀#4: 5501.778 (5502.093); Cy5-labeled RI-MUB₄₀#1 (RI-MUB₄₀): 5551.265 (5550.115); Dylight 405-labeled MUB₄₀#1 (MUB₄₀-D405): 5519,849 (5518.9.. – restricted proprietary information); RI-MUB₄₀#1-Biotin (RI-MUB₄₀-Biotin): 5296.994 (5296.792).

**MUB₄₀ peptides biochemical and biophysical characterization**

_Size Exclusion Chromatography._ Gel filtration was performed using an Agilent 1100
system (Agilent Technologies; Palo Alto, CA, USA) and monitored by absorbance at 280 nm. MUB₄₀ peptides were solubilized in the gel filtration buffer (20 mM phosphate buffer, 150 mM NaCl, pH7.4) at a concentration of 10µg/ml, 100µg/ml, or 1mg/ml), separately injected on a Yarra™ 3μ SEC-2000 300 x 7,8 mm column (Phenomenex, Le Pecq, France), and eluted at a 0,5 ml/min flow rate. The column was calibrated with a mixture of standards proteins (ribonuclease A, 13.7 kDa; carbonic anhydrase 29 kDa; ovalbumin 44kDa; GE Healthcare) complemented with a custom synthetic peptide of our own library (peptide x, 5.1 kDa; Institut Pasteur). The logarithm of the molecular weights were plotted versus the corresponding partition coefficients (Kav = (Ve - Vo)/(Vc -Vo); Ve, elution volume; Vo, void volume; Vc, geometric column volume), giving log(Mr) = 2,3108 – 2,2361Kav as a calibration curve equation.

Circular dichroism. Far-UV Circular Dichroism (CD) spectra were recorded on an Aviv215 spectropolarimeter (Aviv Biomedical) between 190 and 260 nm using a cylindrical cell with a 0.02 cm path length and an averaging time of 1 s per step. Prior analysis, MUB₄₀ pepides were solubilized at a 60 µM final concentration in 20 mM sodium phosphate buffer (pH 7,4) in the presence of 50 mM NaCl. Scans were repeated consecutively three times and merged to produce an averaged spectrum. Results were corrected using buffer baselines measured under the same conditions and normalized to the molar peptide bond concentration and path length as mean molar differential coefficient per residue. MUB₄₀ Peptides were solubilized at a 60 µM final concentration in 20 mM sodium phosphate buffer (pH 7,4) in the presence of 50 mM NaCl.

Trypsin proteolysis. MUB₄₀-Cy5 and RI-MUB₄₀-Cy5 were dissolved in 50mM ammonium bicarbonate, pH 8. Prior to digestion and owing to the propensity of
maleimide derivatives to undergo ring-opening in a basic environment (1), we incubated both peptides at 37°C overnight. Ring-opening completion was checked before trypsin addition. Doing so, we avoided overlapping of N-terminal digest fragments resulting from co-existing succinimidyl thioether and succinamic acid thioether peptides. Along this treatment, minor foot-peaks appeared besides the major peak, which was linked with the well-known side reaction of deamidation and concomitant isomerization, leading to aspartyl and isoaspartyl forms of the peptides (2). Lyophilized trypsin (Thermo scientific) was reconstituted using 50mM acetic acid, diluted with 50mM ammonium bicarbonate and added to the peptide solution so as to obtain a final peptide concentration of 0,25 mg/mL and a protease to protein ratio of 1:20 (w/w). Samples were directly incubated on the injection sampler thermostated at 37°C. HPLC and LC-MS analyses were performed as previously described in Supplementary Information, applying a 15% to 40% linear gradient of acetonitrile in 10mM ammonium acetate over 20 minutes. An Aeris Peptide 3.6µ XB-C18 column was employed for LC-MS analysis of the L-peptide digest fragments, which were identified in positive electrospray ionization mode (data not shown).

Cell biology and neutrophil fractionation

Ethics. All participants gave written informed consent in accordance with the Declaration of Helsinki principles. Peripheral Human blood was collected from healthy patients at the ICAReB service of the Pasteur Institut (authorization DC No.2008-68). Hematopoietic Stem Cells were purified from cytapheresis products collected from healthy patients stimulated 5 days with G-CSF at the Gustave Roussy Cancer Campus (Villejuif, France). Human blood was collected from the antecubital vein into tubes containing sodium citrate (3,8% final) as anticoagulant molecules.
Polymorphonuclear neutrophils purification. Human polymorphonuclear neutrophils were purified as described previously. Briefly, plasma was removed by centrifugation (450 x g, 15 min); blood cells were resuspended in 0.9% NaCl solution supplemented with 0.72% Dextran. After red blood cells sedimentation, white blood cells were pelleted and further separated on a two layer Percoll (GE Healthcare) (51% - 42%) by centrifugation (at 240 x g, 20 min). PBMC (top layer) were isolated from polymorphonuclear neutrophils (bottom layer). Red blood cells were removed from the latter fraction using CD235a (glycophorin) microbeads (negative selection) (Miltenyi Biotec). PBMCs and polymorphonuclear neutrophils were resuspended in the autologous plasma. Guinea pig and mouse polymorphonuclear neutrophils were purified with the same procedure.

Hematopoietic Stem cells (CD34+) purification, proliferation and differentiation. HSC were purified from cytapheresis products with a CD34 Microbead Kit Ultrapure, according to the manufacturer procedure (Miltenyi). Lin−/CD34+ HSC purity yield was >98%. CD34+ HSC were cultured in StemSpan SFEM II supplemented with SCF (100 ng/ml), IL-3 (10 ng/ml), and IL-6 (100 ng/ml) at 37°C with 5% CO₂. Neutrophil differentiation was induced in StemSpan SFEM II containing G-CSF (10 ng/ml), SCF (100 ng/ml), and IL-3 (10 ng/ml) for 13 days at 37°C with 5% CO₂.

Bacterial strains and growth conditions. Shigella flexneri 5a pGFP (M90T) strain was grown in GCTS broth or on TCS agar plates supplemented with 0.01% Congo Red (Sigma-Aldrich) and Ampicillin (100 µg/ml). Shigella sonnei was acquired from the Institut Pasteur strain collection (CIP 106347) and is a clinical isolate from a 1999 Paris infection. The strain was grown in GCTS supplemented with Ampicillin (100 µg/ml) to maintain the pMW211 plasmid.
**Neutrophil infection.** Human purified neutrophils were cultured in glass-bottom dishes (Mattek) for live fluorescent microscopy in a RMPI 1640 (Life Technologies) culture medium supplemented with 10% of heat inactivated Human Serum (Sigma-Aldrich). Neutrophils (5.10^5 cell/mL in 2 mL) were infected with exponentially-grown *Shigella flexneri* 5a pGFP at a Multiplicity Of Infection (MOI) 20 in RMPI 1640 (Life Technologies) culture medium supplemented with 10 mM Hepes (Life Technologies) at 37°C. Infected neutrophils were centrifuged at 2000 rpm for 10 min prior imaging.

**Animal models of shigellosis**

*Guinea pig.* The experimental protocol was approved by the french Ethic Committee Paris (n°20140069, 2014). Young guinea pigs (Hartley, <150g, Charles River) were anaesthetized and infected intrarectally with 10^9 CFU exponentially grown *Shigella flexneri* 5a (M90T) pGFP as previously described. Infection occurred during 8 hours before animals were sacrificed and infected colons collected and fixed in 4% Paraformaldehyde (PFA) for two hours. For immunohistochemical staining, infected guinea pig colon samples were washed in PBS and incubated at 4°C in PBS containing 16% sucrose for 4 hours, followed by incubation in PBS with 30% sucrose overnight. Samples were frozen in OCT (VWR 361603E) on dry ice. Frozen blocks were stored at -80 until sectioning. 10 to 30 µm sections were obtained using a cryostat CM-3050S (Leica). Tissue slices were labeled in PBS + 0.1% saponin (Sigma-Aldrich) with MUB₄₀-Cy5 or MUB₄₀-Dylight (1µg/ml) to localize recruited neutrophils. Slides were washed three time in PBS + 0.1% saponin, three times in PBS, and three times in deionized H₂O and mounted with prolong gold™ mounting media.

*Mouse.* The experimental protocol was approved by the french Ethic Committee
Female 6 week-old BALB/cJRi mice from Charles River were orally gavaged with streptomycin (100 µL of 200 mg/ml solution) 24 hours prior to *Shigella sonnei* infection. Mice were orally gavaged with $10^{10}$ CFUs *S. sonnei* carrying pMW211 expressing DsRed and monitored for 24 hours. At the end of the experiment, animals were sacrificed and tissue sections from the colon were extracted. Colon sections were placed in 4% paraformaldehyde (PFA) solution for 2 hours. PFA fixed tissue sections were passaged for 16 hours in 16% sucrose followed by 4 hours in 30% sucrose solutions. Fixed colon slices were embedded in OCT (VWR 361603E) and flash frozen in dry ice chilled 2-methylbutane. Frozen blocks were stored at -80°C until sectioning. A Leica CM3050S cryostat was used to cut 30 µM thick colon slices, which were absorbed to glass microscope slides. Tissue slices were prepared for fluorescence microscopy by incubation in 0.1% saponin for 1 hour followed by incubation with fluorescent markers specific for actin (phalloidin-FITC, Life Technology) and MUB$_{40}$-Cy5 at a final 1µg/ml concentration. Slides were washed in deionized H$_2$O and mounted with prolong gold<sup>TM</sup> mounting media.

**Rabbit.** The experimental protocol was approved by the French Ethic Committee Paris 1 (number 20070004, December 9th 2007). New Zealand White rabbits weighting 2.5-3 kg (Charles River) were used for experimental infections. For each animal, up to 12 intestinal ligated loops, each 5 cm in length, were prepared as described previously<sup>5-7</sup> and infected with $10^5$ CFU *S. flexneri* pGFP per loop. After 16h, animals were sacrificed and collected tissue were fixed in 4% Paraformaldehyde (PFA) for two hours. For immunohistochemical staining, infected rabbit ileum samples were washed in PBS, incubated at 4°C PBS containing 12% sucrose for 90 min, then in PBS with 18% sucrose overnight, and frozen in OCT (Sakura) on dry ice. 7 µm sections were obtained using a cryostat CM-3050 (Leica). Fluorescent staining was
performed in PBS + 0.1% saponin using Phalloidin-RRX (1:1000 dilution) to stain Actin, MUB$_{40}$-Cy5 (1µg/mL) and a mouse anti-lactoferrin primary monoclonal antibody (Hycult biotech, clone 265-1K1, 1:50 dilution) and an anti-mouse-FITC conjugated secondary antibody (1:1000) to stain infiltrated neutrophils. Slides were washed three times in PBS + 0.1% saponin, three times in PBS, and three times in deionized H$_2$O and mounted with prolong gold$^{tm}$ mounting media.

**Mass spectrometry analyses**

*Digestion of proteins.* Coomassie-stained bands detected on gel were cut and rinsed three times in a 50/50 mix of water/acetonitrile (ACN). Proteins were reduced (10 mM Dithiothreitol (DTT)) and further alkylated (50 mM Iodoacetamide) in-gel. In-gel tryptic digestion was performed by adding 400 ng sequencing grade modified trypsin (Promega France, Charbonnières, France) in 50 mM NH$_4$HCO$_3$ for 18 h at 37° C. Tryptic peptides were recovered by washing the gel pieces twice in 0.5% FA-50% ACN and once in 100% acetonitrile, and all supernatants were collected in the same tube and evaporated to almost dryness.

*LC-MS/MS of tryptic digest.* Digested peptides were analyzed by nano LC-MS/MS using an EASY-nLC 1000 (Thermo Fisher Scientific) coupled to an Orbitrap Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen). Half of each sample was loaded and separated at 250 nL.min$^{-1}$ on a home-made C$_{18}$ 30 cm capillary column picotip silica emitter tip (75 µm diameter filled with 1.9 µm Reprosil-Pur Basic C$_{18}$-HD resin, (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany)) equilibrated in solvent A (0.1 % FA). Peptides were eluted using a gradient of solvent B (ACN ,0.1 % FA) from 2% to 5% in 5 min, 5% to 35% in 30 min, 30% to 50% in 5 min at 250 nL/min flow rate (total length of the chromatographic run was 50 min
including high ACN level steps and column regeneration). Mass spectra were acquired in data-dependent acquisition mode with the XCalibur 2.2 software (Thermo Fisher Scientific, Bremen) with automatic switching between MS and MS/MS scans using a top-15 method. MS spectra were acquired at a resolution of 60000 with a target value of $3 \times 10^6$ ions. The scan range was limited from 300 to 1700 $m/z$. Peptide fragmentation was performed via higher-energy collision dissociation (HCD) with the energy set at 28 NCE. Intensity threshold for ions selection was set at $1 \times 10^5$ ions with charge exclusion of $z = 1$ and $z > 6$. The MS/MS spectra were acquired at a resolution of 17500 (at $m/z$ 400). Isolation window was set at 2 Th. Dynamic exclusion was employed within 30s.

Data were searched using MaxQuant\textsuperscript{1} (version 1.5.3.8) (with the Andromeda search engine) against a human database (20202 entries, downloaded from Uniprot the 2016.05.26).

The following search parameters were applied: carbamidomethylation of cysteines was set as a fixed modification, and oxidation of methionine and protein N-terminal acetylation were set as variable modifications. The mass tolerances in MS and MS/MS were set to 5 ppm and 20 ppm, respectively. Maximum peptide charge was set to 7, and 5 amino acids were required as minimum peptide length. Results were filtered by a 0.01 false discovery rate at both protein and peptide levels.

**Microscopy and Image analysis**

*Confocal microscopy.* Fixed cells (polymorphonuclear neutrophils, PBMC, haematopoietic stem cells), guinea pig colon and rabbit ileum infected with *S. flexneri* pGFP were imaged on a laser-scanning TCS SP5 confocal microscope (Leica). Z-stack images were taken with 1 $\mu$M step-size increments. Obtained Z-stack images
were processed with Fiji software

*Spinning disk microscope.* Mouse colons infected with *S. sonnei* pDsRed were imaged on a Bruker Opterra fluorescence microscope using a Zeiss Plan-Apochromat 63x/1.40 oil immersion lens and Prairie View software version 5.3. Z-stack images were taken with 0.5 µM step-size increments. Obtained Z-stack images were stitched with Fiji software version 2.0.0-rc-30/1.49u and visualized using IMARIS software version 8.3.1.

*Live epifluorescence microscopy.* Viable neutrophils infected with *S. flexneri* pGFP were imaged on a Definite focus live microscope (Zeiss) equipped with a temperature control chamber (37°C) and a 63x oil immersion objective. Images were acquired every 60s. Z-stack images were taken with 2 µM step-size increments. Obtained Z-stack images were processed with Fiji software to generate Movie 01 and the corresponding extracted images shown in Fig. 5a.

*Two-photons microscopy.* Human colonic tissue segments infected with *S. flexneri* pGFP were imaged using a commercial laser-scanning microscope (LSM710, Meta, Zeiss, Germany) as described previously. MUB₄₀-Cy5 was detected using multiphoton excitation (MPE, magenta), Acquisitions were performed with Zen 2008 SP 1.1 software acquisition package developed by ZEISS. Imaris software (http://www.bitplane.com) was used to prepare final images.

**Bibliography**


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### b

![Actin, MUB<sub>40</sub>-Cy5 #2, Merge](image1)

![Actin, MUB<sub>40</sub>-Cy5 #3, Merge](image2)

![Actin, MUB<sub>40</sub>-Cy5 #4, Merge](image3)
Rabbit ileal loop infected with Shigella

Anderson et al., Fig. S3
Anderson et al., Fig. S4
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