**Title Page:**

# A potent anti-inflammatory response in bat macrophages may be linked to extended longevity and viral tolerance.

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**Abstract**

Bats are unique among mammals given their ability to fly, apparent tolerance of deadly viruses and extraordinary longevity. We propose that these traits are linked and driven by adaptations of the innate immune system. To explore this hypothesis we challenged macrophages from the greater mouse-eared bat, *Myotis myotis* and the house mouse, *Mus musculus* with Toll Like Receptors (TLRs) ligands, lipopolysaccharides, LPS and polyinosinic-polycytidylic acid, Poly(I:C). Macrophages from both species presented a high level of mRNA induction of inferon *β* (*INF-β*), tumor necrosis factor (*TNF*) and interleukin-1*β* (*Il-1β*). However, in bat macrophages, this antiviral, proinflammatory response was balanced by a sustained high-level transcription of the anti-inflammatory cytokine *Il-10*, which was not observed in mouse, potentially resulting from adaptive regulation in bats. Additionally, phylogenomic selection tests across the basal divergences in mammals (*n* = 39) uncovered bat-specific adaptations in six genes involved in antiviral and proinflammatory signalling. Based on this pilot study, we put forward a hypothesis that bats may have evolved unique anti-inflammatory responses to neutralize proinflammatory stimuli resulting from flight. This in turn may drive their extraordinary longevity and viral tolerance by limiting inflammation driven ageing and infection-induced immunopathology. Further data from other individuals and bat species are required to advance this intriguing hypothesis.

**Keywords:** longevity, bat, viral response, phylogenetics, immune system evolution

**Introduction:**

Bats are the only mammals capable of powered flight, can tolerate deadly viruses and show exceptional longevity (Austad, 2010; Healy *et al.*, 2014; Brook and Dobson, 2015). Potentially these adaptations are linked, but the mechanisms behind this connection are currently unknown (O'Shea *et al.*, 2014). The demanding energetics of flight is linked with an increased risk of generating reactive oxygen species (Buffenstein *et al.*, 2008; O'Shea *et al.*, 2014). Resulting oxidative stress can cause inflammation by stimulating an inflammasome (Franceschi and Campisi, 2014) and has been associated with chronic immune disorders (Lood *et al.*, 2016), cancer (Sabharwal and Schumacker, 2014) and cellular ageing (Franceschi and Campisi, 2014). However, bats seem to defy ‘rate of living’ hypotheses, with

some species living up to 10 times longer than expected given their body size (Austad, 2010; Healy *et al.*, 2014; Munshi-South and Wilkinson, 2010).

During flight, bats exhibit a very high (15-16 fold) increase in metabolic rate (Speakman and Thomas, 2003). In mice, breeding directed at higher metabolic rates resulted in stronger immune responses to immune challenges (Książek and Konarzewski, 2012). Also it has been previously suggested that flight in bats evolved in tandem with adaptations of their innate immune system (Zhang *et al.*, 2013). Potentially, bats may have evolved the ability to prevent excessive activation of their immune responses as a protective mechanism against the consequences of their flight induced high metabolic rate, which in turn could lead to both their extended longevity and tolerance of viral infections. Inflammation is a driving factor of ageing process (Franceschi and Campisi, 2014) and the innate immune system is the first line of host defence during infection playing a crucial role in the early recognition and subsequent activation of a proinflammatory response to invading pathogens (Medzhitov and Janeway, 2000). The excessive or uncontrolled release of proinflammatory cytokines in response to infection, a 'cytokine storm', can lead to tissue damage (Tisoncik *et al.*, 2012). Therefore, the difference between surviving or dying from an acute infection depends on how quickly the host can dampen the initial pathogenic response and return to homeostasis before damage occurs (Rouse and Sehrawat, 2010). The mechanism underlying the shift in cytokine profile is currently unclear and is still under investigation.

Bats are considered the natural reservoir for multiple, highly pathogenic zoonotic viruses e.g. Ebola, SARS-CoV, MERS-CoV, rabies (O'Shea *et al.*, 2014; Brook and Dobson, 2015; Weyer *et al.*, 2015). A true natural reservoir can host the virus with little to no pathological effects (Weyer *et al.*, 2015). Indeed, a fruit bat, *Eidolon helvum*, which tested positive for Ebola antibodies was tracked after 13 months without any clinical signs of infection (Hayman *et al.*, 2010; Weyer *et al.*, 2015). Bats show also no documented pathology in response to a number of non-viral intracellular pathogens, including some bacteria (*Bartonella* spp.) (Kosoy *et al.*, 2010), fungi (*Histoplasma capsulatum*) (Greer and McMurray, 1981), and protozoa (*Plasmodium* spp.) (Schaer *et al.*, 2013). By contrast, pathogens that predominantly occupy the extracellular space (e.g. *Borellia* spp. or *Pseudogymnoascus destructans*) typically cause pathology in bats (reviewed Brook and Dobson, 2015). Protection against intracellular pathogens is primarily orchestrated by cell-mediated immune responses via cytokine networks (Nath, 2001). Therefore, bats' tolerance to these pathogens could be explained by their ability to finely tune the balance of cytokine

responses, which may have arisen as an adaptation to deal with the cellular damage induced by their flight driven high metabolic rate.

We hypothesize that bats may have evolved unique anti-inflammatory responses to deal with high levels of flight induced metabolic stress. This in turn would slow down inflammation and inflammation driven ageing, resulting in extraordinary longevity. This potent anti-inflammatory response, could also limit infection-induced immunopathology, by preventing excessive release of proinflammatory mediators, and create a niche for pathogen persistence explaining why bats are asymptomatic reservoirs for deadly viruses and other intracellular pathogens.

We tested this hypothesis using regulators of innate immune system, macrophages (Kelly and O'Neill, 2015). *Mus musculus* (mouse) and *Myotis myotis* (bat) macrophages were challenged with synthetic Toll-Like Receptor (TLR) ligands, lipopolysaccharides, LPS and polyinosinic-polycytidylic acid, Poly(I:C), and the levels of representative proinflammatory and anti-inflammatory cytokines mRNAs were assayed with qPCR. Broadly, LPS is a major outer surface membrane components present in almost all Gram-negative bacteria and mimics a bacterial infection by stimulating TLR4 receptor on the cell surface, and PolyI:C is structurally similar to double-stranded RNA and mimics a viral infection via intracellular, endosomal TLR3 stimulation. As expected, both bat and mouse macrophages mounted a rapid antiviral (*interferon β, INF-β*) and proinflammatory response (*interleukin-1β*, *Il-1β* and *tumour necrosis factor, TNF*). However, in *M. myotis* this response was linked with sustained high levels of transcription of immunoregulatory *interleukin-10 (Il-10)*, suggesting that during infection, bats can rapidly restore homeostasis and dampen inflammation through increased production of anti-inflammatory mediators. In line with this hypothesis, the production of high levels of nitric oxide (NO), which is typical for stimulated mouse macrophages and may contribute to oxidative tissue damage during immune response, has not been observed in bats. Our phylogenomic selection tests also detected evidence of positive selection acting on innate immune system genes in bats.

## Materials and Methods

### Macrophage growth condition and treatments:

An approximately 1 month old female *M. myotis* was euthanized at a bat rescue centre and subsequently used in the present study under permit. The bat was missing all fingers and plagiopatagium on the left wing, either given a birth defect or a healed trauma and therefore could never fly. Otherwise she was apparently healthy (forearm length=57.4 mm; weight =

17.3 g) having been feed by her mother. Its long bones (femurs and humerus) were kept at 4$^{o}$C in the culture medium (Dulbecco's MEM high glucose with stabilized glutamine, Biochrom/Merck, 20% FBS, Gibco) supplemented with 1 % antibiotics mix (Penicillin-Streptomycin-Fungizone, Lonza BioWhittaker™) and 50 µg/ml gentamicin (Lonza BioWhittaker™), and delivered to the lab within two days. Murine bone marrow derived macrophageswere isolated from C57BL/6 young female mice from Harlan UK. All experiments were carried out with prior ethical approval from Trinity College Dublin Animal Research Ethics Committee. Bone marrow from C57BL/6 mice ($n$ = 2) as well as from *M. myotis* (MMY2607) was differentiated into macrophages as previously described (Tannahill *et al.*, 2013). Briefly, the bones were cut in an aseptic environment at the ends and the bone marrow was flushed out with PBS using a 23 gauge needle. Compared to murine bone marrow, upon visual inspection, the *M. myotis* bone marrow had higher fat content, but was otherwise similar. The bone marrow was dissociated into single cells by pipetting and then centrifuged for 5 min at 1500 rpm. The cell pellet was resuspended in Red Blood Cell Lysing Buffer Hybri-Max (Sigma) and incubated for 5 min at room temperature. Dulbecco's Modified Eagle Medium (DMEM) was added and cells pelleted again. Finally the cells were resuspended in DMEM supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (P/S) and 30% L929 mouse fibroblast supernatant as a source of M-CSF. For the bat bone marrow, 1/8 was plated into one 10 cm non-tissue culture coated petri dish in 10 ml. After 3 days, an extra 10% L929 supernatant was added. Cells were harvested after 7 days by scraping in ice-cold PBS. Cells were then replated at a concentration of $5x10^5$ cells per ml and used for experiments the following day. During the first experimental repeat, cells were seeded in 24-well plate at a density of $2.5x10^5$ cells/well. During the second experimental repeat, due to limited amount of *M. myotis* bone marrow available, cells were seeded in 48-well plate at a density of $1.25x10^5$ cells/well. TLR ligands used for bat and mouse bone marrow derived macrophages *in vitro* studies were LPS from *Escherichia coli*, serotype EH100 (Alexis), used at concentration of 100 ng/ml, and LMW Poly (I:C) tlrl-picw (InvivoGen), used at a concentration 33 µg/ml.

**RT PCR:**

Total RNA was extracted from cells using Qiagen RNeasy extraction kit. Cells were lysed in RLT buffer at indicated time points and stored at -80$^{o}$C until total RNA extraction according to the manufacturer's instructions. The extracted RNA was treated with DNase to remove genomic DNA contamination (Ambion DNase kit), and cDNA was synthesized by the use of

ThermoScientific first-strand cDNA synthesis kit according to the manufacturer's instructions. Real-time PCR was carried out on Stratagene Mx3000p thermocycler using KAPA SYBR® FAST Universal master mix according to manufacturer's instructions. Reaction conditions were: 3 min 95ºC, 40 cycles: 5s at 95ºC, 30s 60ºC, melting curve analysis: 1 min at 95ºC, 30s 60ºC, 30s at 95ºC. Relative transcript levels were calculated by the ΔΔCt method (Livak and Schmittgen, 2001) using actin beta as an internal control to which all transcripts were normalized. *Myotis myotis* specific primers were designed based on cDNA sequences retrieved from blood transcriptome data (Huang *et al.*, 2016) and/or *M. myotis* genome assembly (Foley, N.M., G.M. Hughes, Z. Huang, M. Clarke, D. Jebb, C. V. Whelan, E. J. Petit, F. Touzalin, O. Farcy, G. Jones, R. D. Ransome, M. J. O'Connell, G. Kerth, H. Rebelo, L. Rodrigues, S. J. Puechmaille and E. C. Teeling; unpublished data). Primer3 plus was used for design of *Myotis myotis* and mouse qPCR primers unless otherwise stated (Untergasser *et al.*, 2012). Primer sequences are provided in Table S1.

**Analysis of available literature data for pig, human and mouse:**

Microarray studies employing primary bone marrow derived (pig, mouse) and monocyte derived (human) macrophages challenged with LPS for 24 hr were selected based on literature and GEO NCBI search. The ratios between fold induction of anti-inflammatory *Il-10* and proinflammatory *TNF* were calculated based on GSE datasets/ supplementary material provided by the authors. Details of the analysed studies are summarized in Table S2.

**Nitric oxide determination by Griess reaction:**

Nitric oxide (NO) concentration was measured in cell culture supernatants using Griess Reagent System (Promega) according to manufacturer's instructions. The Griess method measures nitrite, which is a stable breakdown product of NO. Briefly, 200 µl of culture supernatant was mixed with 1300 µl of distilled water, followed by addition of 250 µl of sulphanilamide. After 5 minutes, 250 µl of N-1-naphthylethylenediamine dihydrochloride was added. 10 min later, absorbance was measured at 540 nm in 1.5 ml cuvettes (UVmini-1240 spectrophotometer, Shimadzu) and concentration of NO in the supernatant was determined using a dilution curve prepared with sodium nitrite standard.

**Statistical analysis:**

For each treatment, LPS and Poly(I:C), data were collected and analysed by 2-way ANOVA with time and species as factors. Presented p-values indicate the significance of the species

effect. Statistical analyses were performed using the GraphPad PRISM software (GraphPad Software, lnc).

**Positive selection tests:**

Selection tests were performed on 12 immune genes involved in sensing viral infection and activation of the innate antiviral and proinflammatory signalling cascade. Annotated gene sequences for *CASP1, RIG-I, IFN-β, IL-10, IL-18, IL-1β, IRF3, NLRP3, ASC, TBK1, STING* and *TNF* were downloaded from GenBank (Benson *et al.*, 2015) for a range of mammalian species. These data represented 12 mammalian orders, with species from all four eutherian superorders, spanning more than 100 million years of mammalian evolution (derived from Meredith *et al.*, 2011; laurasiatherian topology based on Foley *et al.*, 2016; *Myotis* toplogy based on Ruedi *et al*., 2013) (Fig. S1). Coding sequences for each gene in the exceptionally long-lived bat *M. myotis* were mined from unpublished genome assembly (authors' unpublished data) using BLASTN (Altschul *et al.*, 1990) and the closely related *Myotis lucifugus* as a reference. A total of six bat species were included: *Myotis lucifugus, Myotis myotis, Myotis brandtii* and *Eptesicus fuscus* representing the echolocating Vespertilionidae and *Pteropus vampyrus* and *Pteropus alecto* representing the non-echolocating Pteropodidae.

Translated amino acid sequences for each gene set were aligned using MUSCLE (Edgar, 2004). Genes showing more than 30% gaps per alignment (potentially due to poor genome assembly, coverage or annotation) were excluded from further selection analysis. The number of taxa per alignment ranged from n =36–39 Codon alignments for each gene were generated with Pal2Nal (Suyama et al., 2006) (see Supplementary Information). Signatures of selection were investigated using the CODEML program as part of the PAML suite of software (Yang, 2007). As CODEML requires a user input tree, a mammalian species tree based on Meredith *et al*. (2011), Foley *et al.* (2016) and Ruedi *et al.* (2013) was generated for each alignment. CODEML is designed to calculate likelihood-derived dN/dS rates (ω), where dN is defined as a number of non-synonymous substitutions per non-synonymous sites and dS is a number of synonymous substitutions per synonymous sites. Specifically, site models M7 (null) versus M8 (alternative) and branch-site models model A1 (null) versus model A (alternative) were implemented in this study. Model M7 assumes $\omega \leq 1$ (neutral or purifying selection) per site compared to M8, which allows sites under positive selection ($\omega > 1$). Branch-site model A1 constrains foreground and background branches to $\omega \leq 1$, while the alternative model A allows positive selection in the foreground branch. For all alignments, the bat ancestral branch was designated as foreground branch. Likelihood ratio

tests (LRTs) were performed comparing null and alternative models using a chi-squared distribution. Significant sites under positive selection were predicted using Bayesian empirical Bayes (BEB) values (Yang *et al.*, 2005). Correction for multiple testing of related hypotheses on the same data set was performed as described in Kirwan *et al.* (2013). Briefly, tests have been grouped into families of related tests with one family defined for each of *CASP1, RIG-I, IFN-β, IL-10, IL-18, IL-1β, IRF3, NLRP3, ASC, TBK1, STING* and *TNF*. Then, the false-discovery rate procedure of Benjamini and Hochberg (1995) was applied to all the members of a given hypothesis family using the program R (R Core Team, 2016).

## Results

### *Myotis myotis* and *Mus musculus* macrophages respond differently to LPS and Poly(I:C) challenge

L929 mouse conditioned medium containing murine macrophage colony stimulating factor-1 (M-CSF) was successfully used to induce *M. myotis* macrophage growth. Even though a smaller proportion of *M. myotis* bone marrow cells were alive after 7 days of differentiation compared to mouse bone marrow, *M. myotis* bone marrow-derived cells were indistinguishable in terms of size and shape to their murine counterparts. Bone marrow derived macrophages of *M. myotis* and *M. musculus* were treated with synthetic TLRs ligands, LPS and Poly(I:C). RNA was isolated at 1, 4, 24 hr time points and changes of pro- and anti-inflammatory cytokine genes expression were measured using qPCR. Both mouse and *M. myotis* macrophages mounted a potent antiviral and proinflammatory response, as demonstrated by induction of interferon-β (*IFN-β*), interleukin-1β (*Il-1β*) and tumor necrosis factor (*TNF*) by both treatments (Fig. 1A,B,C). *Myotis myotis* macrophages also elicited a rapid anti-inflammatory response, showing significantly higher and persistent induction of interleukin-10 (*Il-10*) compared to mouse. Notably, the mouse macrophages elicited a higher induction of *Il-1β* , which is an acute-phase signalling proinflammatory cytokine (Guarda *et al.*, 2011) in response to potent TLR ligand LPS. The ratio between fold induction of anti-inflammatory *Il-10* and proinflammatory *TNF* or *Il-1β* was higher in *M. myotis* compared to mouse, especially after 24 hr of the LPS or Poly(I:C) treatment (Fig. 1 E,F). *Il-10/TNF* mRNA induction ratios derived from previous literature studies of pig, human and mouse macrophages treated with LPS for 24 hr are also presented (Fig. 1G). Moreover, mouse, but not *M. myotis* macrophages, demonstrated increased production of NO, in response to LPS treatment (Fig. 1H). Consistently, qPCR analyses also showed strong induction of *iNOS* (inducible nitric oxide synthase) expression 24 hr after immune challenge only in the mouse

macrophages (ΔΔCt = -6.87±0.56 SEM for LPS; -3.93 ±3.99 SEM for Poly(I:C)), but not in *M. myotis* (ΔΔCt = -0.54±0.56 SEM for LPS; -0.28 ±0.61SEM for Poly(I:C)).

**Phylogenomic analyses reveal bat-specific adaptations of immune system**

We performed phylogenomic analyses and positive selection tests in 12 representative immune genes involved in sensing viral infection and activation of the innate antiviral and proinflammatory signalling cascade in 39 mammalian taxa spanning basal eutherian divergences (including six bat species; Fig. S1). The function of proteins encoded by tested genes is summarised in Table S3 and their position in the immune signalling pathway is highlighted in Fig. 2. Tests for adaptive evolution at codon sites across eutherian mammals revealed evidence of significant positive selection acting on all investigated genes except *Il-10* and *TBK1*. At least one BEB site was reported for all positively selected genes with exception of *TNF* (Table S4A). The Branch-Site test (A vs A1 model) revealed positive selection within the ancestral bat lineage acting on proinflammatory *Il-1β* (Table S4B). We also detected vespertilionid-specific, Myotis-specific or Pteropus-specific non-synonymous amino acid substitutions in *RIG-I*, *Il-1β*, *Il-18*, *NLRP3*, *STING* and *CASP1* in the positively selected mammalian BEB sites (Table S4C and Fig. 3).

**Discussion**

We hypothesize that bats may have evolved unique anti-inflammatory responses to neutralize the effects of flight-induced metabolic stress. Potent anti-inflammatory signalling could explain their long life spans, by reducing chronic inflammation and diminishing inflammation associated senescence. The increased anti-inflammatory responses could also explain how bats reduce, but not eliminate pathogen load and show no immunopathology during viral and other intracellular infections.

The genus *Myotis* holds the longevity record even among bats, with up to 10 species living beyond 20 years and two species exceeding 30 years despite their small body sizes (Munshi-South and Wilkinson, 2010). *Myotis myotis* is the largest species of the genus (weighting up to 45 g, which is comparable to a house mouse) with the record life span of 37.1 years (Gaisler *et al.*, 2003). Therefore, due to both its extraordinary longevity and relatively large size enabling obtaining sufficient numbers of bone marrow derived macrophages, *M. myoti*s is an excellent starting point for *in vitro* studies aimed at identification of the putative link between immune system and longevity in bats. However, sampling bone marrow from *M. myotis* requires sacrificing a wild animal and therefore this

study was limited to one individual euthanized due to an opportunistic event. This is typical of studies employing cell lines derived from wild bat species (Kuhl *et al.*, 2011 ; Miller *et al.*, 2016). *Myotis myotis* macrophages, of size and shape indistinguishable compared to their murine analogues, were successfully differentiated in medium containing mouse M-CSF, and were responsive to widely used TLR ligands, LPS and Poly(I:C) (Fig. 1). Activation of TLRs induces intracellular signalling pathways, leading to the production of specific sets of proinflammatory cytokines and chemokines, as well as antiviral type I interferons (Akira and Takeda, 2004). Poly(I:C) and LPS activate TLR3 and TLR4 receptors respectively (Matsumoto and Seya, 2008; Lu *et al.*, 2008). TLR3 which is a nucleic acid sensing receptor involved in response to viral pathogens, but also to sterile tissue damage (reviewed by Tatematsu *et al.*, 2014). TLR3 can be also upregulated by LPS via TLR4-dependent pathway (Ding *et al.*, 2017). TLR4 is stimulated by bacterial LPS (Lu *et al.*, 2008), but also during viral infections, for example by Ebola glycoproteins (Okumura *et al.*, 2010). Studying the effect of TLR3 and TLR4 activation is therefore highly relevant from the point of view of bat viral tolerance.

As anticipated, both mouse and *M. myotis* macrophages responded to immune treatment with significant induction of *Il-1β, TNF* and *IFN-β.* Interestingly, the anti-inflammatory cytokine *Il-10* shown higher and persistent induction in *M. myotis*, and ratio between fold induction of anti-inflammatory *Il-10* and proinflammatory *TNF* was one order of magnitude higher in *M. myotis* than in mouse (this experiment) and other mammals (pig, human, mouse: public datasets from comparable experimental settings, listed Table S2). Moreover, unlike mouse, *M. myotis* macrophages did not produce the proinflammatory and cytotoxic mediator, NO, in response to LPS, and presented no induction of inducible nitric oxide synthase. Therefore, in line with our hypothesis, in *M. myotis* the balance of immune response appears to be tipped towards anti-inflammation. These results suggest that the bat's unique immunity may be due to efficient maintenance of the careful balance between the proinflammatory and anti-inflammatory immune mediators. This could explain a recently reported decrease in acute inflammatory responses occurring despite the constitutive expression of *IFN-α* in bat cells and tissues (Zhou *et al.*, 2016*b*). Likewise, previous whole genome analyses revealed that all bats lack the PYHIN gene family involved in inflammasome formation in other mammals (Ahn *et al.*, 2016). Interestingly, wild caught *Molossus molossus* bats injected with LPS showed no leucocytosis or fever (Stockmaier *et al.*, 2015), which could be hypotheticaly explained by increased levels of antipyretic Il-10. However this observation might be species- or experimental set-up- specific, as LPS induced fever was observed in *Myotis vivesi*

(Otálora-Ardila *et al.*, 2016) and leukocytosis occurred in challenged *Carollia perspicillata* (Schneeberger *et al.*, 2013); therefore the LPS induced systemic responses in bats require further investigation. The proposed link between bat longevity, viral tolerance and anti-inflammatory response is certainly plausible also in the light of available evidence from other species. Number of anti-inflammatory CD33rSiglec family genes shown strong positive correlation with maximum life span in 14 mammalian species (Schwarz *et al.*, 2015) and longer life spans of laboratory rat strains have been linked with maintaining ability to synthesize not only proinflammatory TNF, but also anti-inflammatory Il-10 (Dimitrijević *et al.*, 2014). Interestingly, increased production of Il-10 promotes viral persistence (Brooks *et al.*, 2006) and higher levels of serum Il-10 are associated with asymptomatic infections in humans (Guiyedi *et al.*, 2015). Thus, it can be hypothesized that the upregulated production of Il-10, the key immunoregulator during infection, may make bats potent reservoir hosts for pathogens.

Positive selection may drive the functional divergence and specialization of proteins. We tested *Il-10* and other genes involved in sensing viral infection and activation of the innate antiviral and proinflammatory signalling cascade for positive selection across eutherian mammals. Our analyses revealed evidence of significant positive selection acting on all investigated genes except *Il-10* and *TBK1*. This suggests that the observed increase in *Il-10* gene induction in *M. myotis* macrophages does not result from adaptive modification of the protein but demonstrates a functional upregulation of the anti-inflammatory response. Bat-specific adaptation of immune system genes were also detected. The branch-site test identified positive selection within the ancestral bat lineage acting on *Il-1β*, and vespertilionid-specific, *Myotis*-specific and *Pteropus*-specific amino acid substitutions were revealed in *RIG-I, Il-1β, Il-18, NLRP3, STING* and *CASP1* (Fig. 3). Proteins encoded by these genes are involved in anti-viral and proinflammatory pathway (Fig. 2 and Table S3). RIG-I and STING mediate sensing cytoplasmic nucleic acids and subsequent activation of antiviral and proinflammatory signalling (Onoguchi *et al.*, 2010; Burdette and Vance, 2013; Wang *et al.*, 2016). Interleukin-1β is a gatekeeper of inflammation and mediates acute and chronic inflammatory diseases (Dinarello, 2011). Interleukin-18 is also a proinflammatory cytokine implicated in several autoimmune diseases (Dinarello *et al.*, 2013). NLRP3 is a component of inflammasome, essential for activation of caspase-1, which in turns activates both IL-1β and Il-18 by proteolytic cleavage (Guo *et al.*, 2015). These results highlight the presence of immune system adaptations unique to bats that may be driven by the acquisition of flight and, in concert with increased expression of anti-inflammatory mediators, contribute to the unique

immune response observed in bats and their exceptional longevity. Suppression of chronic inflammation was proposed an essential step towards further improvements in human healthy lifespans (Arai *et al.*, 2015). Therefore, future studies of bat-specific adaptations of immune system are of pivotal importance from the perspective of human medicine and potential therapeutic interventions.

## Conclusions

Bone marrow derived *M. myotis* macrophages were grown *in vitro* for the first time and their responsiveness to TLR ligands was demonstrated. We showed that bats can mount an effective antiviral, proinflammatory reaction, which is balanced by a sustained protective anti-inflammatory response. This may prevent the cytotoxic effects and tissue damage resulting from inflammation and inflammaging. Bat-specific adaptations of immune system genes were shown by phylogenomic selection tests. While only one *M. myotis* individual has been tested in this proof-of-concept study, recent technical advances in generating bat immune cells (Zhou *et al.*, 2016*a*) should, in the nearest future, enable cross-species experiments to ascertain if bat immune system is really rewired towards anti-inflammatory response and provide more mechanistic insights in the involved pathways.

## Supplementary information

All generated nucleotide, protein and codon alignments for 12 immune genes under investigation are available from: https://figshare.com/s/ae28c1bccd20507a02ae.

Supplementary Tables S1–S4, Fig. S1 and References. Table S1. Primers used in this study; Table S2. Literature studies used to calculate Il-10/TNF ratio for pig, mouse and human; Table S3. Summary of function of proteins involved in sensing of viral infection and activation of innate antiviral/proinflammatory signalling which were subjected to phylogenomic analyses; Table S4. Results of tests for adaptive evolution; Fig. S1. Mammalian species tree used in selection analysis; References. Supplementary Information is available exclusively on BioOne.

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**Figure legends**

**Fig .1. *M. myotis* and *M. musculus* macrophages present differential response to LPS and Poly(I:C) challenge.** Change in gene expression of A) *IFN-β*, B) *IL-1β*, C) *TNF*, D) *Il-10* in response to LPS and Poly I:C treatment at 1, 4 and 24 hr time points are presented as changes in delta Ct values normalized to *actin-β*. Ratios of the fold induction of anti-inflammatory *Il-10* to proinflammatory *TNF* (E) and *Il-1β* (F) are also presented. G) Literature derived *Il-10/TNF* ratios for other mammals. H) NO generated by stimulated *M. myotis* and mouse macrophages. Bars represent the mean of two experimental repeats ±SEM. Presented p-values indicate the significance of the difference between species from analysis of variance (ANOVA).

**Fig. 2. Signatures of adaptive evolution in genes involved in sensing of viral infection and activation of antiviral/proinflammatory signalling.** Genes under positive selection (site-test) in mammals are highlighted in green. Genes with sites under positive selection in bat lineages are indicated with an asterix.

**Fig. 3. Bat-specific sites under positive selection in the investigated immune genes.** Sites that had statistically significant evidence of positive selection acting across eutherian mammals were investigated for any amino acid substitutions unique to the ancestral bat

lineage, the vespertilionid lineage or the Pteropodid lineage. Bat lineages-specific sites are highlighted in yellow.

**Figure 1**

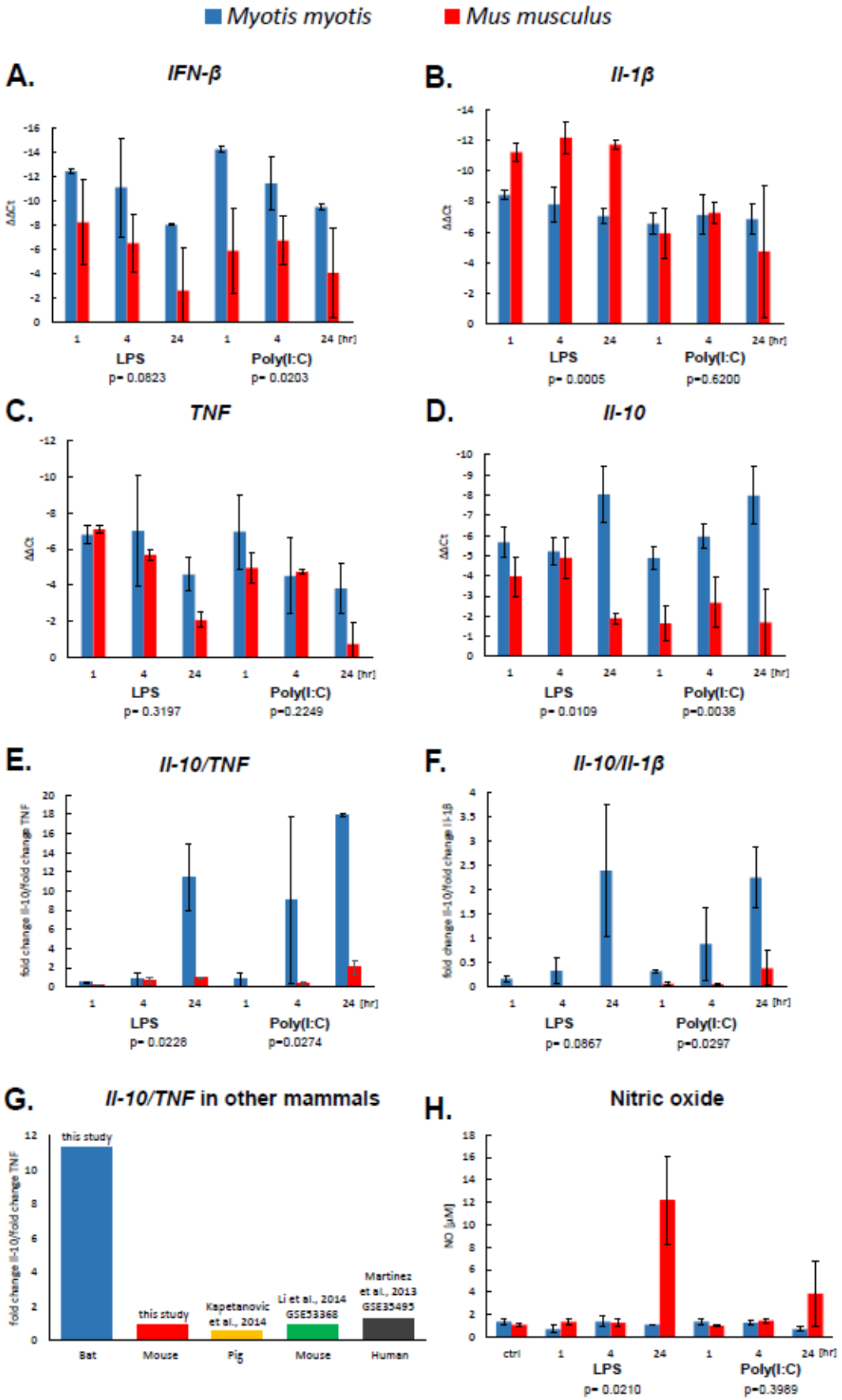

Myotis myotis
Mus musculus
A.
IFN-β
ΔΔCt
LPS
p= 0.0823
Poly(I:C)
p= 0.0203
B.
Il-1β
LPS
p= 0.0005
Poly(I:C)
p=0.6200
C.
TNF
LPS
p= 0.3197
Poly(I:C)
p=0.2249
D.
Il-10
LPS
p= 0.0109
Poly(I:C)
p=0.0038
E.
Il-10/TNF
fold change Il-10/fold change TNF
LPS
p= 0.0228
Poly(I:C)
p=0.0274
F.
Il-10/Il-1β
fold change Il-10/fold change Il-1β
LPS
p= 0.0867
Poly(I:C)
p=0.0297
G.
Il-10/TNF in other mammals
fold change Il-10/fold change TNF
this study
this study
Kapetanovic et al., 2014
Li et al., 2014 GSE53368
Martinez et al., 2013 GSE35495
Bat
Mouse
Pig
Mouse
Human
H.
Nitric oxide
NO [μM]
ctrl
LPS
p= 0.0210
Poly(I:C)
p=0.3989

**Figure 2**

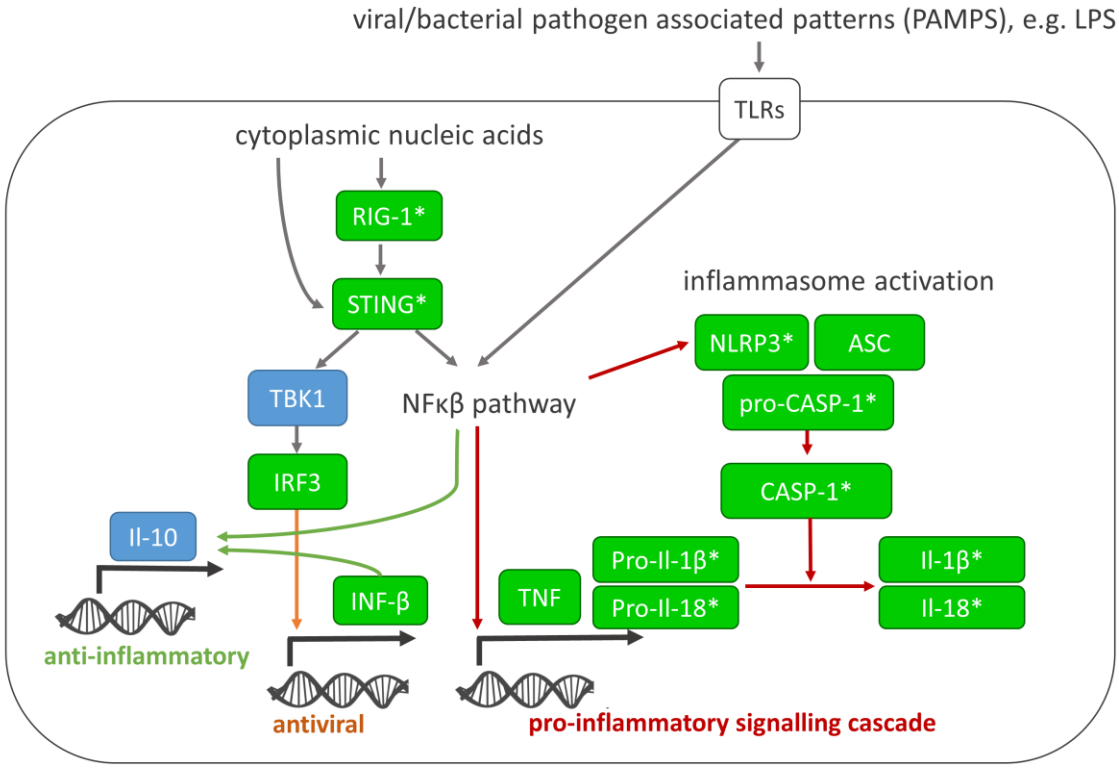
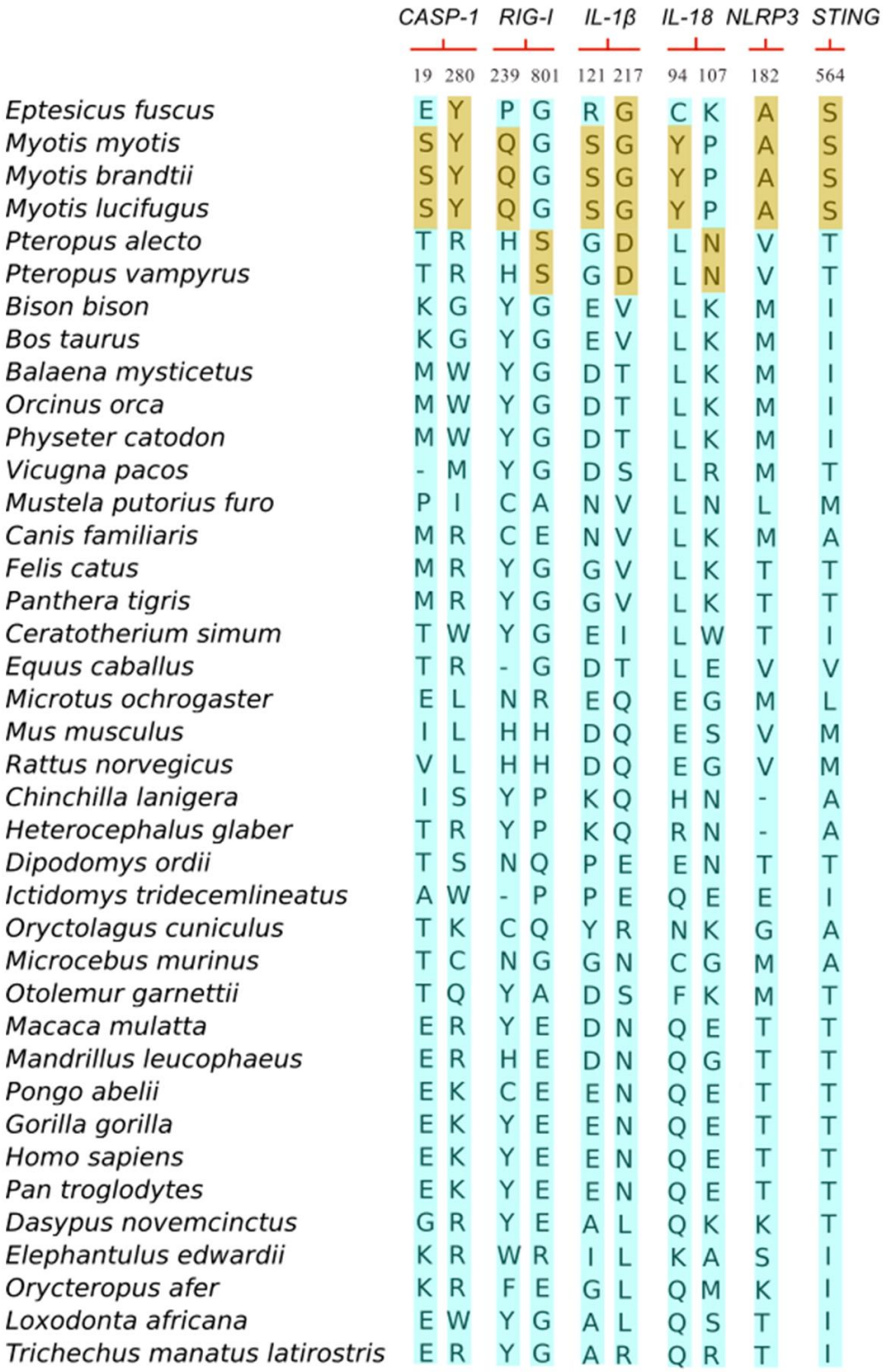

viral/bacterial pathogen associated patterns (PAMPS), e.g. LPS
TLRs
cytoplasmic nucleic acids
RIG-1*
STING*
TBK1
IRF3
NFκβ pathway
inflammasome activation
NLRP3*
ASC
pro-CASP-1*
CASP-1*
Il-10
INF-β
TNF
Pro-Il-1β*
Pro-Il-18*
Il-1β*
Il-18*
anti-inflammatory
antiviral
pro-inflammatory signalling cascade

**Figure 3**


CASP-1
RIG-I
IL-1β
IL-18
NLRP3
STING
19 280 239 801 121 217 94 107 182 564
Eptesicus fuscus E Y P G R G C K A S
Myotis myotis S Y Q G S G Y P A S
Myotis brandtii S Y Q G S G Y P A S
Myotis lucifugus S Y Q G S G Y P A S
Pteropus alecto T R H S G D L N V T
Pteropus vampyrus T R H S G D L N V T
Bison bison K G Y G E V L K M I
Bos taurus K G Y G E V L K M I
Balaena mysticetus M W Y G D T L K M I
Orcinus orca M W Y G D T L K M I
Physeter catodon M W Y G D T L K M I
Vicugna pacos - M Y G D S L R M T
Mustela putorius furo P I C A N V L N L M
Canis familiaris M R C E N V L K M A
Felis catus M R Y G G V L K T T
Panthera tigris M R Y G G V L K T T
Ceratotherium simum T W Y G E I L W T I
Equus caballus T R - G D T L E V V
Microtus ochrogaster E L N R E Q E G M L
Mus musculus I L H H D Q E S V M
Rattus norvegicus V L H H D Q E G V M
Chinchilla lanigera I S Y P K Q H N - A
Heterocephalus glaber T R Y P K Q R N - A
Dipodomys ordii T S N Q P E E N T T
Ictidomys tridecemlineatus A W - P P E Q E E I
Oryctolagus cuniculus T K C Q Y R N K G A
Microcebus murinus T C N G G N C G M A
Otolemur garnettii T Q Y A D S F K M T
Macaca mulatta E R Y E D N Q E T T
Mandrillus leucophaeus E R H E D N Q G T T
Pongo abelii E K C E E N Q E T T
Gorilla gorilla E K Y E E N Q E T T
Homo sapiens E K Y E E N Q E T T
Pan troglodytes E K Y E E N Q E T T
Dasypus novemcinctus G R Y E A L Q K K T
Elephantulus edwardii K R W R I L K A S I
Orycteropus afer K R F E G L Q M K I
Loxodonta africana E W Y G A L Q S T I
Trichechus manatus latirostris E R Y G A R Q R T I