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Identification and functional characterization of the house finch interleukin-1 $\beta$

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# 1 Identification and functional characterization of the house finch interleukin-1 $\beta$

2

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15

## 16 Highlights

- 17 • Full-length house finch IL-1 $\beta$  was cloned, expressed, and its basic biological roles  
18 explored.
- 19 • House finch IL-1 $\beta$  modulates the expression of Th1/Th2 cytokines and nitric oxide  
20 production by activated immune cells.
- 21 • House finch IL-1 $\beta$  enhances the expression of acute phase protein and antimicrobial  
22 peptide by activated immune cells.

**23 Abstract**

24 Interleukin-1 $\beta$  (IL-1 $\beta$ ), an inflammatory cytokine of the IL-1 family, is primarily produced as a  
25 precursor protein by monocytes and macrophages, then matures and becomes activated through  
26 proteolytic catalysis. Although the biological characteristics of avian IL-1 $\beta$  are well known,  
27 little information is available about its biological role in songbird species such as house finches  
28 that are vulnerable to naturally-occurring inflammatory diseases. In this study, house finch IL-  
29 1 $\beta$  (HfIL-1 $\beta$ ) was cloned, expressed, and its biological function examined. Both precursor and  
30 mature forms of HfIL-1 $\beta$  consisting of 269 and 162 amino acids, respectively, were amplified  
31 from total RNA of spleen and cloned into expression vectors. HfIL-1 $\beta$  showed high sequential  
32 and tertiary structural similarity to chicken homologue that allowed detection of the expressed  
33 mature recombinant HfIL-1 $\beta$  (rHfIL-1 $\beta$ ) with anti-ChIL-1 $\beta$  antibody by immunoblot analysis.  
34 For further characterization, we used primary splenocytes and hepatocytes that are predominant  
35 sources of IL-1 $\beta$  upon stimulation, as well as suitable targets to stimulation by IL-1 $\beta$ . Isolated  
36 house finch splenocytes were stimulated with rHfIL-1 $\beta$  in the presence and absence of  
37 concanavalin A (Con A), RNA was extracted and transcript levels of Th1/Th2 cytokines and a  
38 chemokine were measured by qRT-PCR. The addition of rHfIL-1 $\beta$  induced significant  
39 enhancement of IL-2 transcript, a Th1 cytokine, while transcription of IL-1 $\beta$  and the Th2  
40 cytokine IL-10 was slightly enhanced by rHfIL-1 $\beta$  treatment. rHfIL-1 $\beta$  also led to elevated  
41 levels of the chemokine CXCL1 and nitric oxide production regardless of co-stimulation with  
42 Con A. In addition, the production of the acute phase protein serum amyloid A and the  
43 antimicrobial peptide LEAP2 was observed in HfIL-1 $\beta$ -stimulated hepatocytes. Taken together,  
44 these observations revealed the basic functions of HfIL-1 $\beta$  including the stimulatory effect on  
45 cell proliferation, production of Th1/Th2 cytokines and acute phase proteins by immune cells,

46 thus providing valuable insight into how HfIL-1 $\beta$  is involved in regulating inflammatory  
47 response.

48 **Keywords:** IL-1 $\beta$ ; house finch; cytokines; acute phase protein; avian

## 49 1. Introduction

50 Interleukin-1beta (IL-1 $\beta$ ) is the most studied prototypical pro-inflammatory cytokine  
51 because of its crucial role in the initiation of inflammation and regulation of innate and adaptive  
52 immune responses (Netea et al., 2015). IL-1 $\beta$  lacks a signal peptide and is primarily expressed  
53 by activated macrophages, monocytes, and dendritic cells as an inactive precursor form and  
54 remains in the cytosol, requiring proteolytic processing at its N-terminal region for optimal  
55 bioactivity (Black et al., 1988; Thornberry et al., 1992; Arend et al., 2008). Subsequently, it is  
56 cleaved by either an intracellular cysteine protease caspase-1 activated by inflammasome  
57 (Thornberry et al., 1992; Martinon et al., 2002) or by inflammasome-independent enzymatic  
58 processes such as neutrophil-derived serine proteases and pathogen-released enzymes (Netea et  
59 al., 2010). This cleaved IL-1 $\beta$  is secreted into the extracellular milieu, where it can induce its  
60 own transcription as mature and bioactive IL-1 $\beta$ . By binding to IL-1 type I receptor (IL-1R1),  
61 secreted IL-1 $\beta$  exerts its biological activities including T cell activation, B cell proliferation, and  
62 antigen recognition along with the induction of inflammatory genes, chemokines, and cell  
63 adhesion molecules (Burns et al., 2003; Dinarello, 2009). In mammals, IL-1 $\beta$  induces the  
64 development of Th17 cells in combination with IL-6 or TGF- $\beta$ , while the production of IL-23 is  
65 IL-1 $\beta$  dependent in monocytes which contributes to maintenance of Th17 cells (Weaver et al.,  
66 2007; Dong, 2008; van de Veerdonk et al., 2009). IL-1 $\beta$  also induces synthesis of  
67 cyclooxygenase type 2 (COX-2), type 2 phospholipase A, and inducible nitric oxide synthase  
68 (iNOS), leading to the production of prostaglandin-E2 (PGE2), platelet activating factor (PAF),  
69 and nitric oxide (NO) that causes fever, lower pain threshold, vasodilatation, and hypotension  
70 (Dinarello, 2009). Additionally, IL-1 $\beta$  is responsible for triggering the synthesis of the acute  
71 phase protein serum amyloid A (SAA), IL-6, neutrophil-selective CXC chemokines, and

72 macrophage inflammatory protein-2 (McColl et al., 2007). An abnormal increase of IL-1 $\beta$   
73 secretion is associated with the pathogenesis of auto-inflammatory diseases such as cryopyrin-  
74 associated periodic syndromes, which is related to an over-activation of caspase-1 (Campbell et  
75 al., 2016).

76 In avian species, chicken IL-1 $\beta$  (ChIL-1 $\beta$ ) was first identified and cloned from the  
77 chicken macrophage cell line HD11 stimulated with LPS (Weining et al., 1998). ChIL-1 $\beta$  has a  
78 similar gene structure to mammalian homologues (Giansanti et al., 2006) with 34% and 33%  
79 amino acid identity with the respective human and mouse orthologues; however, it lacks a  
80 conserved aspartic acid residue thus preventing the caspase-1 cleavage. Nonetheless, N-  
81 terminally truncated ChIL-1 $\beta$  lacking the predicted pro-domain exhibits significantly enhanced  
82 biological activity suggesting that precursor cleavage is critical for its maximal activity (Gyorfy  
83 et al., 2003). Another phylogenetically conserved aspartic acid residue was later discovered by  
84 cleavage of avian proIL-1 $\beta$  with either sea bass or human caspase-1, which is distinct from the  
85 cleavage site of mammalian homologues (Reis et al., 2012). Consistent with mammalian  
86 homologues, ChIL-1 $\beta$  expression is significantly enhanced following viral, bacterial, and  
87 protozoal infections. ChIL-1 $\beta$  mRNA expression was induced in the gut following *Eimeria*  
88 infection (Laurent et al., 2001; Hong et al., 2006a,b), enhanced mRNA level was also observed  
89 in macrophages from turkeys suffering from poult enteritis and mortality syndrome (PEMS), as  
90 well as in bursal cells from IBDV-infected chickens (Heggen et al., 2000; Eldaghayes et al.,  
91 2006). *Salmonella* spp. led to up-regulation of IL-1 $\beta$  mRNA in chicken cell lines and heterophils  
92 (Iqbal et al., 2005; Kogut et al., 2005). Macrophages exposed to either *Escherichia coli* or  
93 *Mycoplasma synoviae* increased IL-1 $\beta$  transcription (Lavric et al., 2008). These reports further  
94 highlight the important role of IL-1 $\beta$  in controlling the pathogenesis of many diseases.

95           The properties of IL-1 $\beta$  have been well studied in domestic poultry but not in wild birds,  
96           which are in close contact with domesticated animals and may act as natural reservoirs for many  
97           zoonotic pathogens. The house finch, *Haemorhous mexicanus*, is a small passerine songbird that  
98           originally inhabited western North America and later expanded to the eastern U.S. (Hill, 1993).  
99           House finches are relatively easy to capture and examine in captivity making them ideal  
100           organisms for studying the ecology of wildlife diseases, and they favored over domesticated  
101           birds to study the co-evolutionary relationship between host and pathogen during emergence of  
102           other diseases (Hurtado, 2012). Most recently, differential mRNA expression of IL-1 $\beta$  across  
103           populations following experimental *Mycoplasma gallisepticum* (MG) infection was documented  
104           (Adelman et al., 2013). However, the biological role of IL-1 $\beta$  in wild house finches still needs to  
105           be elucidated. To clarify this matter, we first cloned the precursor and mature forms of house  
106           finch IL-1 $\beta$  (HfIL-1 $\beta$ ), then investigated its basic function by measuring immune cell  
107           proliferation and differential mRNA expression of Th1/Th2 response elements, acute phase  
108           protein and antimicrobial peptide by activated immune cells.

109

## 110 **2. Materials and Methods**

### 111 2.1. Birds and tissue collection

112           House finches were captured in either July of 2012 or June-July of 2015 using cage traps  
113           and mist nets in Montgomery County, VA under permits from VDGIF (044569/2012 and  
114           050352/2015) and USFWS (MB158404-1). All finches were housed at constant day length and  
115           temperature, and were fed an *ad libitum* pelleted diet prior to and throughout experiments (Daily  
116           Maintenance Diet, Roudybush Inc. Woodland, CA). Following capture, adult individuals from  
117           both sexes were identified based on their plumage characteristics and tested for the exposure to



118 the pathogen as described in Park et al. (Data in Brief, submitted). After testing, only healthy  
119 birds that showed no clinical signs of disease and had no pathogen load (Grodio et al., 2008)  
120 were randomly selected for the subsequent experiments. All tissue samples, including brain,  
121 heart, liver, small intestines (duodenum, jejunum, ileum), spleen, thymus, bursa, lung,  
122 proventriculus and gizzard were collected from two individuals to assess HfIL-1 $\beta$  tissue  
123 distribution. Additionally, the primary cells were isolated from spleens and livers of 10  
124 randomly selected birds for further biological experiments.

125

## 126 2.2. Sequence and structural analyses

127 Nucleotide and amino acid sequences of HfIL-1 $\beta$  were aligned with other orthologous  
128 sequences obtained by BLAST search using Clustal Omega (Sievers and Higgins, 2014). The  
129 phylogenetic tree was constructed from the alignment using the neighbor joining (NJ) method  
130 within the MEGA4 program, with Poisson correction and complete deletion of gaps (Tamura et  
131 al., 2007). The stability of the branching order was confirmed by performing 1,000 bootstrap  
132 replicates. The theoretical molecular weight (MW) and isoelectric point (pI) were estimated  
133 using a Compute pI/MW tool from ExPASy (<http://www.expasy.org>). The three-dimensional  
134 structure of HfIL-1 $\beta$  was built by comparative modeling at the Robetta server  
135 (<http://rosetta.bakerlab.org>) (Kim et al., 2004). The model was superimposed with the X-ray  
136 structure of ChIL-1 $\beta$  using Discovery Studio 2.0 (Accelrys Inc., CA) and PyMOL (DeLano  
137 Scientific, CA).

138

## 139 2.3. Construction of recombinant HfIL-1 $\beta$ (rHfIL-1 $\beta$ ) expression plasmid

140 Both precursor and mature forms of HfIL-1 $\beta$  genes were amplified from total RNA  
141 extracted from house finch spleen using the primers designed based on partial genomic  
142 sequences of house finch (provided by D. Hawley) (Table 1). Using 1  $\mu$ g of total RNA, the first-  
143 strand cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, CA). The full-length  
144 HfIL-1 $\beta$  was amplified using the following conditions: initial denaturation at 92°C for 2 min, 35  
145 cycles of denaturation at 92°C for 15 sec, annealing at 54°C for 15 sec and extension at 72°C for  
146 30 sec, with a final extension at 72°C for 7 min. Synthesized precursor and mature forms of  
147 HfIL-1 $\beta$  were directly inserted into pCR2.1-TOPO vector (Invitrogen, CA) and transformed into  
148 *E. coli* TOP10 (Invitrogen). Transformants containing recombinant plasmid were selected by a  
149 combination of PCR screening and endonuclease digestion with *EcoR* I (New England Biolabs,  
150 MA), and confirmed by sequencing (Biocomplexity Institute at Virginia Tech, VA). For sub-  
151 cloning into a prokaryotic or eukaryotic expression vector, mature and precursor forms of HfIL-  
152 1 $\beta$  were digested with endonucleases *Bgl* II and *Xma* I (New England Biolabs) and ligated into  
153 pQE-30 (Novagen, CA) and pcDNA3.1 (Invitrogen), respectively. By colony PCR screening,  
154 positive clones including HfIL-1 $\beta$  were selected and verified by sequencing.

155

#### 156 2.4. Expression of rHfIL-1 $\beta$ and immunoblot analysis

157 HfIL-1 $\beta$  in pQE30 plasmid was introduced into *E. coli* BL21 (New England Biolabs) and  
158 cultured at 30°C overnight. The expression of HfIL-1 $\beta$  was induced by adding 1 mM IPTG  
159 (Gold Biotechnology, MO) and shaking incubation for 5 hr at 25°C. The cells were harvested by  
160 centrifugation and resuspended with 50 mM Tris (pH 7.5), 240 mM NaCl and 1 mg/ml lysozyme  
161 buffer. After cell lysis by sonication, soluble fraction containing HfIL-1 $\beta$  was collected by  
162 centrifugation, followed by purification using Ni<sup>+</sup>-resin (Biolone, MA). After endotoxin removal

163 using the ProteoSpin Endotoxin Removal Micro Kit (Norgenbiotek, ON, Canada), the purified  
164 rHfIL-1 $\beta$  was quantified using BCA protein assay and used in subsequent assays. To examine  
165 the binding reactivity of anti-ChIL-1 $\beta$  antibody, 1  $\mu$ g of the purified rHfIL-1 $\beta$ , and rChIL-1 $\beta$   
166 (Bio-Rad) as a positive control were loaded on SDS-PAGE gel under reducing conditions and  
167 transferred to PVDF membrane (Millipore, MA). The blot was incubated with anti-polyhistidine  
168 conjugated with HRP (Sigma, MO) or anti-ChIL-1 $\beta$  polyclonal antibody (Thermo Scientific, MA)  
169 in a 1: 1,000 dilution as the primary antibody and goat anti-rabbit IgG conjugated with HRP  
170 (Santa Cruz Biotechnology, CA) in a 1: 2,000 dilution as the secondary antibody. After washing,  
171 the blot was incubated with the SuperSignal West Pico chemiluminescent Substrate (Pierce, IL),  
172 and developed using a gel imaging system (Bio-Rad).

173

#### 174 2.5. HfIL-1 $\beta$ expression analysis in tissue

175 The expression of HfIL-1 $\beta$  in house finch tissues was determined by qRT-PCR and  
176 immunoblotting. In order to investigate HfIL-1 $\beta$  mRNA expression, various tissues were  
177 collected from two healthy house finches including brain, heart, liver, spleen, thymus, bursa,  
178 lung, proventriculus, gizzard and each small intestinal section. Total RNA was extracted using  
179 RNeasy Mini Kit (Qiagen, CA), followed by synthesis of the first-strand cDNA using High-  
180 Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA). Synthesized cDNA was  
181 mixed with 5  $\mu$ l of Fast SYBR Green Master Mix (Applied Biosystems) and 0.1  $\mu$ M primers in  
182 10  $\mu$ l final volume of qRT-PCR reaction. The following thermal cycling conditions were used:  
183 95 $^{\circ}$ C for 20 sec as initial denaturation, followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 3 sec,  
184 and annealing/extension at 57 $^{\circ}$ C for 30 sec. Transcription of HfIL-1 $\beta$  was normalized against  
185 the expression of GAPDH, followed by calibration using brain transcript level and  $2^{-\Delta\Delta CT}$  method

186 (Livak and Schmittgen, 2001). To examine HfIL-1 $\beta$  protein expression level, 50 mg of tissues  
187 were collected from same birds that we used for RNA extraction were homogenized and  
188 sonicated in RIPA buffer (Cayman Chemical, MI) supplemented with protease inhibitor cocktail  
189 (Sigma) and phosphatase inhibitors (1 mM NaF and 1 mM Na<sub>3</sub>VO<sub>4</sub>). After centrifugation at  
190 10,000 x g for 30 min, the supernatant was collected and protein concentration determined using  
191 BCA assay (Thermo Scientific), then a 20  $\mu$ g protein extract was resolved on SDS-PAGE gel  
192 under reducing conditions and analyzed by immunoblotting with anti-ChIL-1 $\beta$  antibody as  
193 previously described. In parallel, anti-GAPDH antibody (1:4,000; Millipore) was used as a  
194 reference for protein loading and for quantification of relative protein expression.

195

## 196 2.6. Isolation of splenocytes and hepatocytes

197 To isolate splenocytes, house finch spleens were excised and passed through a 0.22  $\mu$ m  
198 cell strainer (BD, CA). Cell debris was washed out of cell suspension with Hank's Salt Solution  
199 (HBSS; HyClone, UT), which was overlaid onto Histopaque-1077 (Sigma). After centrifugation  
200 at 400 x g for 30 min, mononuclear cells from the interphase were collected and mixed with PBS.  
201 By centrifugation, cells were collected and washed with RPMI-1640 (Mediatech, VA), and  
202 counted using a hemocytometer. Freshly isolated splenocytes were resuspended with RPMI-  
203 1640 containing 20% fetal calf serum (FCS; Atlanta Biologicals, GA) and 1%  
204 penicillin/streptomycin, and cultured in a 24-well plate at a cell density of  $1 \times 10^6$  cells/well  
205 overnight at 39°C with 5% CO<sub>2</sub> humidified air. For the isolation of hepatocytes, the livers were  
206 excised and cut into small pieces. After washing with HBSS, the pieces were incubated with  
207 0.25% trypsin in Dulbecco's Modified Eagle Medium (DMEM; Mediatech) for 18 hr at 4°C and  
208 then placed at 37°C for 30 min. The tissue pieces were passed through a 0.22  $\mu$ m cell strainer

209 and the collected cells were washed with DMEM. After determining cell viability and  
210 concentration by a hemocytometer, the cells were resuspended with DMEM supplemented with  
211 10% FCS and 1% penicillin/streptomycin and seeded at  $1 \times 10^6$  cells/well in a 24-well plate and  
212 then cultured overnight at  $39^\circ\text{C}$  with 5%  $\text{CO}_2$  humidified air.

213

## 214 2.7. Cell proliferation assay

215 The role of HfIL- $1\beta$  on cellular proliferation was investigated with either splenocytes or  
216 hepatocytes using CellTiter 96® Non-Radioactive Cell Proliferation Assay Kit (Promega, WI)  
217 according to manufacturer's protocol. Briefly,  $2 \times 10^5$  cells were seeded in a 96-well plate and  
218 incubated with medium alone, rHfIL- $1\beta$  (0.01 and 0.1  $\mu\text{g/ml}$ ) with or without Con A in the  
219 presence and absence of anti-ChIL- $1\beta$  antibody at  $39^\circ\text{C}$  with 5%  $\text{CO}_2$  for 12 hr. Incubated cells  
220 were treated with Dye Solution (15  $\mu\text{l}$ ) for 3 hr at  $39^\circ\text{C}$  with 5%  $\text{CO}_2$ , followed by addition of  
221 Solubilization Solution/Stop Mix. After 1 hr incubation at  $39^\circ\text{C}$ , the absorbance was measured  
222 at 570 nm and 630 nm using a microplate reader. The readings were corrected by subtracting the  
223 background value at 630 nm.

224

## 225 2.8. Cytokine transcripts analysis upon cell stimulation

226 Isolated splenocytes ( $1 \times 10^6$  cells/well) were cultured in a 24-well plates and treated with  
227 medium alone, Con A (10  $\mu\text{g/ml}$ ), rHfIL- $1\beta$  (0.1  $\mu\text{g/ml}$ ), or rHfIL- $1\beta$  (0.1  $\mu\text{g/ml}$ ) with Con A (10  
228  $\mu\text{g/ml}$ ) for 6 and 12 hr. Cell supernatants were collected for quantification of NO production and  
229 total RNA was extracted from the treated cells using RNeasy Mini Kit (Qiagen). Extracted RNA  
230 (1  $\mu\text{g}$ ) was reverse transcribed into cDNA using High-Capacity cDNA reverse transcript kit  
231 (Applied Biosystems). The transcript levels of Th1/Th2 cytokines (IFN- $\gamma$ , IL- $1\beta$ , IL-2, IL-10),

232 iNOS and a chemokine (CXCL1) were measured by qRT-PCR. The primers used for qRT-PCR  
233 analysis were designed within the conserved regions of the multiple sequence alignment of  
234 closely-related bird species including zebra finch, canary, and chicken. To measure hepatic gene  
235 expression by HfIL-1 $\beta$  treatment, isolated liver hepatic cells ( $1 \times 10^5$  cells/well) were cultured in  
236 24-well plates, followed by treatment with medium alone, Con A (10  $\mu$ g/ml), rHfIL-1 $\beta$  (0.01 and  
237 0.1  $\mu$ g/ml) or rHfIL-1 $\beta$  (0.01 and 0.1  $\mu$ g/ml) with Con A for 6 hr. After incubation, the cell  
238 supernatants were collected to measure NO production and total RNA was extracted as described  
239 earlier. The mRNA levels of the SAA, LEAP2, and IL-1 $\beta$  were analyzed. With the collected cell  
240 supernatants, NO production was measured using a Griess Reagent System (Promega).

241

## 242 2.9. Statistical analyses

243 All data were expressed as the means  $\pm$  SEM and analyzed by Student's t test or one-way  
244 ANOVA using JMP software (Ver 11). Differences between groups assessed by Tukey Kramer  
245 multiple comparison test were considered to be statistically significant at  $P < 0.05$  (\*),  $P < 0.01$   
246 (\*\*), or  $P < 0.001$  (\*\*\*).

247

## 248 3. Results

### 249 3.1. Sequence analyses of HfIL-1 $\beta$

250 The full-length HfIL-1 $\beta$  was predicted to encode a precursor form of 269 amino acids  
251 with a theoretical molecular weight of 30 kDa and isoelectric point of 6.74. Multiple sequence  
252 alignment of the deduced amino acid sequence with other orthologs revealed that the precursor  
253 form of HfIL-1 $\beta$  shares 76% and 94% similarity with chicken and zebra finch, respectively,  
254 while it has 28% and 27% similarity with human and mouse, respectively. Sequence comparison

255 revealed that HfIL-1 $\beta$  lacks the conserved aspartic acid (Asp/D) as the IL-1 $\beta$ -converting enzyme  
256 (ICE) cut site, and its mature form starts alanine at amino acid residue 108 producing a 162  
257 amino acid peptide with a predicted molecular weight of 18 kDa and isoelectric point of 8.52.  
258 This mature form has 32% and 34% similarity with the respective human and mouse sequences,  
259 and 84% and 97% similarity with the chicken and zebra finch, respectively. Phylogenetic  
260 analysis indicated that the IL-1 $\beta$  encoding region evolved into two distinct lineages among avian  
261 species and HfIL-1 $\beta$  being evolutionary closer to zebra finch and pigeon IL-1 $\beta$ s than to that of  
262 any domestic avian including chicken, turkey, duck, goose and quail (Figure 1A).  
263 Computational analysis revealed that HfIL-1 $\beta$  retains six cysteine residues, and Cys<sup>25</sup> and Cys<sup>27</sup>  
264 as well as Cys<sup>187</sup> and Cys<sup>241</sup> are predicted to form disulfide bonds. The crystal structure of HfIL-  
265 1 $\beta$  revealed 15  $\beta$ -strands and an  $\alpha$ -helix. The house finch and chicken IL-1 $\beta$  (PDB entry, 2wry)  
266 structures share a very similar structural fold with a root mean square deviation (RMSD) of 0.53  
267 Å (Figure 1B). Based on high level of sequential and structural identity between house finch and  
268 chicken IL-1 $\beta$ , we predicted that cross-reactivity would exist based on the anti-ChIL-1 $\beta$  antibody  
269 used in further biological assays.

270

### 271 3.2. Immunoblot analysis of rHfIL-1 $\beta$

272 For biological function characterization, rHfIL-1 $\beta$  with a polyhistidine tag fused at the N-  
273 terminus was purified from *E. coli* BL21 as a soluble form. The endotoxin concentration was  
274 0.07 endotoxin units (EU) per  $\mu$ g protein, which was acceptable for further cellular assay. Prior  
275 to the initiation of the biological assays, purified rHfIL-1 $\beta$  was confirmed by immunoblot  
276 analysis using anti-polyhistidine antibody as well as verified binding reactivity of anti-ChIL-1 $\beta$   
277 antibody made against rHfIL-1 $\beta$ . As shown in Figure 2A, two bands were detected

278 approximately 19 kDa, the predicted size of HfIL-1 $\beta$  containing polyhistidine tag (1.1 kDa)  
279 along with 25 kDa using anti-polyhistidine antibody under reducing conditions. Blotting with  
280 anti-ChIL-1 $\beta$  antibody resulted in a single 25 kDa of rHfIL-1 $\beta$ , which is higher than the  
281 calculated size but identical to that of rChIL-1 $\beta$  (positive control), which is the mature form  
282 containing a polyhistidine tag expressed from *E. coli* under the same conditions (Figure 2B).

283

### 284 3.3. Tissue distribution of HfIL-1 $\beta$

285 The relative abundance of HfIL-1 $\beta$  in tissues was examined at the mRNA and protein  
286 levels using qRT-PCR and immunoblotting, respectively. The mRNA expression of HfIL-1 $\beta$   
287 was normalized to transcript of GAPDH as an endogenous reference gene and calculated as a  
288 fold change relative to the lowest level of brain (arbitrarily set at 1.0). HfIL-1 $\beta$  was expressed at  
289 varying levels in all tested tissues with the highest expression in the lung and proventriculus and  
290 the lowest level in the brain and heart (Figure 3A). Since mRNA expression does not necessarily  
291 predict protein expression, tissue-specific expression pattern of HfIL-1 $\beta$  proteins was determined  
292 by immunoblotting using anti-ChIL-1 $\beta$  antibody (Figure 3B). Prominent expression of HfIL-1 $\beta$   
293 protein was observed approximately 35 kDa in the liver, bursa and gizzard, which is slightly  
294 higher molecular weight than the theoretical size of precursor HfIL-1 $\beta$  of 30 kDa. Also, less  
295 intense bands are shown in the lung and proventriculus; however, no such band was detected in  
296 the brain, which is consistent with its lowest mRNA expression. In addition to the 35 kDa band,  
297 a very weak 60 kDa band was observed in the gizzard (data not shown).

298

### 299 3.4. Effect of HfIL-1 $\beta$ on cell proliferation



300 The proliferative effects of the HfIL-1 $\beta$  on primary cells were investigated, resulting in a  
301 small but statistically significant induction of splenocyte proliferation following treatment with  
302 0.01  $\mu$ g/ml rHfIL-1 $\beta$  for 12 hr (Figure 4), although there was no significant difference after 24 hr  
303 (data not shown). The enhanced splenocyte proliferation was abolished when adding anti-ChIL-  
304 1 $\beta$  antibody thus neutralizing HfIL-1 $\beta$ ; in contrast, control IgG had no effect. However, co-  
305 stimulation of HfIL-1 $\beta$  with Con A had a negligible effect on splenocyte proliferation relative to  
306 Con A alone (data not shown). Contrary to splenocytes, there was no significant proliferation in  
307 HfIL-1 $\beta$ -stimulated hepatocytes.

308

### 309 3.5. Modulation of gene expression and nitric oxide production by HfIL-1 $\beta$ in splenocytes

310 The effect of HfIL-1 $\beta$  on Th1/Th2 cytokine expression was evaluated in splenocytes  
311 stimulated with rHfIL-1 $\beta$  for 6 and 12 hr (Figure 5). The most pronounced induction of cytokine  
312 expression was shown at 12 hr post-stimulation. Treatment with HfIL-1 $\beta$  alone enhanced its  
313 own gene transcription by > 2 fold. Of the Th1 cytokines, IL-2 was remarkably increased  
314 approximately 383-fold by addition of rHfIL-1 $\beta$  compared to Con A alone, while no significant  
315 difference of IFN- $\gamma$  expression was observed. The addition of rHfIL-1 $\beta$  alone induced IL-10  
316 production, a Th2 cytokine. rHfIL-1 $\beta$  also led to elevated iNOS level (3-fold), irrespective of  
317 Con A stimulation. Up-regulation of iNOS mRNA expression results in the production of NO by  
318 splenocytes treated with 0.1  $\mu$ g/ml rHfIL-1 $\beta$  both in the absence and presence of Con A for 6 hr  
319 (Figure 6) and 12 hr (data not shown). Transcription of chemokine CXCL1 was increased 10-  
320 fold and 18-fold in the presence and absence of Con A stimulation, respectively.

321

### 322 3.6. Expression of SAA, LEAP2, and IL-1 $\beta$ in hepatocytes

323 Induction of an acute phase protein (SAA) and an antimicrobial peptide (LEAP2) in  
324 hepatocytes stimulated with HfIL-1 $\beta$  was observed (Figure 7). HfIL-1 $\beta$  treatment induced  
325 transcripts of SAA and LEAP2 by incubation with 0.1 and 0.01  $\mu$ g/ml rHfIL-1 $\beta$ , respectively,  
326 both in the presence and absence of Con A. The transcription of SAA was enhanced 2.2-fold,  
327 which was further enhanced by 8.8-fold in the presence of Con A. In contrast to the induction of  
328 IL-1 $\beta$  in stimulated splenocytes, IL-1 $\beta$  transcription was not changed in hepatocytes. With  
329 induction of acute phase and antimicrobial responses, significant production of NO by  
330 hepatocytes was observed when treated with 0.1 $\mu$ g/ml rHfIL-1 $\beta$  both in the presence and  
331 absence of Con A (Figure 6).

332

#### 333 4. Discussion

334 Although many reports described the potency of IL-1 $\beta$  in immune responses following  
335 viral, bacterial, and protozoal infections, little is known regarding the role of house finch IL-1 $\beta$   
336 in the host immune system. In this study, we identified and cloned the full-length HfIL-1 $\beta$  from  
337 house finch spleen and demonstrated the biological functions of its active form. Phylogenetic  
338 analysis revealed the evolutionary relationships among avian IL-1 $\beta$ s where HfIL-1 $\beta$  clustered  
339 with homologues of flying birds (zebra finch and pigeon), while separated from that of land-  
340 based birds (chicken, turkey, and quail) as well as waterfowl (duck and goose). Despite  
341 considerable phylogenetic distance between house finch and chicken IL-1 $\beta$  in the avian clade the  
342 tertiary structure of HfIL-1 $\beta$  was highly similar to that of ChIL-1 $\beta$ , with the  $\beta$ -strands and  $\alpha$ -helix  
343 located in almost identical regions. Sequence analysis revealed that HfIL-1 $\beta$  lacks the aspartic  
344 acid residue that is critical to form active HfIL-1 $\beta$  as a result of proteolytic cleavage, but retains  
345 conserved alanine at position 108 that represents the initial residue for expression of mature form  
346 similar to other avian IL-1 $\beta$ s (Wu et al., 2007). These high sequential and structural identities

347 suggest that HfIL-1 $\beta$  is likely cross-reactive with anti-ChIL-1 $\beta$  antibody as substantiated by  
348 immunoblot analysis showing that chicken-specific antibody recognized HfIL-1 $\beta$ . Purified  
349 rHfIL-1 $\beta$  was detected at higher molecular weight (25 kDa) than its theoretical value (19 kDa)  
350 which could be caused by unfolding in the presence of a reducing agent. Such unfolding under  
351 reducing conditions would be expected from the potential intra-chain disulfide bond formed  
352 between Cys<sup>187</sup> and Cys<sup>241</sup> that is likely to be predominantly detected by anti-ChIL-1 $\beta$  antibody.  
353 Since precursor HfIL-1 $\beta$  contains two potential disulfide bonds, Cys<sup>25</sup> and Cys<sup>27</sup> as well as  
354 Cys<sup>187</sup> and Cys<sup>241</sup>, its molecular weight would be higher than calculated under reducing  
355 conditions, as 35 kDa shown in Figure 3. This possibility has been experimentally confirmed by  
356 detecting the expected size of mature HfIL-1 $\beta$  (19 kDa) in the absence of a reducing reagent  
357 (data not shown), which is consistent with earlier studies reporting that disulfide bond of murine  
358 IL-1 $\beta$  resulted in varying gel mobility depending on the presence of a reducing reagent (Gunther  
359 et al., 1991).

360 IL-1 $\beta$  is primarily produced by monocytes, macrophages, and dendritic cells as well as B  
361 lymphocytes and natural killer (NK) cells in low amounts. Due to an instability element in the  
362 coding region of IL-1 $\beta$ , mRNA would be poorly translated into protein (Bufler et al., 2004). The  
363 present study showed that HfIL-1 $\beta$  is expressed in a broad range of tissues, mainly in the  
364 digestive tract (proventriculus, gizzard, duodenum, and ileum), immune tissues (liver, bursa, and  
365 spleen) and respiratory (lung) tract. However, the levels of IL-1 $\beta$  mRNA expression were not  
366 congruent with changes of its protein production similar to previous reports of human IL-1 $\beta$   
367 (Schindler et al., 1990a). LPS rapidly increased IL-1 $\beta$  transcript for a short time while the  
368 administration of IL-1 $\beta$  itself sustained its own production long term (Schindler et al., 1990b).  
369 Although we did not measure its continuous production through 24 hr, a small but significant

370 induction of IL-1 $\beta$  transcript was observed in splenocytes stimulated with HfIL-1 $\beta$  alone at 12 hr  
371 post-stimulation, but not with Con A. These results are in accordance with a relatively short  
372 half-life of IL-1 $\beta$  mRNA and a rate-limiting step in the processing of IL-1 $\beta$  to prevent its  
373 continuous and overwhelming activation which would result in deleterious effect on the host.

374 IL-1 $\beta$  is involved in a variety of cellular activities as both a growth factor for B cell  
375 proliferation and stimulator for the generation of Th17 cells which also co-stimulate T cell  
376 proliferation (Dinarello, 2009). Accordingly, we observed the effect of a low concentration of  
377 HfIL-1 $\beta$  (0.01  $\mu$ g/ml) in promoting the proliferation of splenocytes in vitro. Whereas TNF- $\alpha$  and  
378 IL-6 are important factors in the priming phase of liver regeneration, IL-1 $\beta$  is known to be a  
379 potent inhibitor of liver regeneration and hepatocyte proliferation (Sparna et al., 2010). In  
380 contrast to previous reports, we did not observe significant changes with proliferation of  
381 hepatocytes after culture with HfIL-1 $\beta$ . Further work is needed to elucidate the regulatory  
382 function of HfIL-1 $\beta$  in the proliferation and regeneration of hepatocytes which may reveal the  
383 role that IL-1 $\beta$  plays in the pathogenesis of acute inflammatory liver injuries.

384 Through high affinity interaction with cell surface receptor, IL-1 $\beta$  induces Th1 adaptive  
385 cellular responses and triggers the production of acute phase proteins as well as other pro-  
386 inflammatory cytokines (Dinarello, 1996; 1999; Chung et al., 2009). In the current study,  
387 production of the Th1 cytokine IL-2 was elevated by HfIL-1 $\beta$  treatment in Con A-stimulated  
388 splenocytes indicating that it would stimulate T cell proliferation in conjunction with IL-2  
389 release (Schultz, 1987). Previous studies have shown that IL-1 $\beta$  inhibits IL-10 production by  
390 memory T cells in vitro and in vivo while IL-10 counter-regulates the action of IL-1 $\beta$  (Zielinski  
391 et al., 2012). Contrary to previous findings, transcript of IL-10 was enhanced following  
392 stimulation with HfIL-1 $\beta$  which may indirectly occur via PGE2 production by IL-1 $\beta$  (Benbernou

393 et al., 1997). This induction of IL-10 transcript may consequently result in B cell proliferation  
394 and antibody production (Itoh and Hirohata, 1995). Enhancement of iNOS was observed after  
395 HfIL-1 $\beta$  treatment both in the presence and absence of Con A, which is also associated with  
396 PGE2 activation (Benbernou et al., 1997). Further, increased NO production was accompanied  
397 by the expression of iNOS mRNA. In accordance with previous findings (Nogawa et al., 1998),  
398 NO produced by iNOS may not only modulate the formation of PGE2, but also enhance COX-1  
399 activity thereby facilitating the development of fever as well as acting as a mediator of  
400 inflammation. These data are indicative of the molecular mechanisms that regulate the balance  
401 in the expression of Th1 and Th2 cytokines providing the fundamental aspects of the immune  
402 response of wild birds. Consistent with previous findings where ChIL-1 $\beta$  stimulation induced  
403 the expression of CXCL1 in a dose-dependent manner in the chicken fibroblast cell line CEC-32  
404 (Weining et al., 1998), chemokine CXCL1 was markedly upregulated regardless of Con A  
405 stimulation which is able to attract neutrophils and lymphocytes thereby contributing to  
406 inflammatory processes (Batra et al., 2012).

407 The administration of HfIL-1 $\beta$  also augmented the production of acute phase protein in  
408 hepatocytes, similar to previous reports demonstrating that IL-1 $\beta$ , IL-6, and TNF- $\alpha$  circulate to  
409 the liver and induce an acute phase response which is a systemic inflammatory reaction to  
410 disrupt the host's homeostasis (Gabay and Kushner, 1999; Bresnahan and Tanumihardjo, 2014).  
411 Interestingly, the expression of antimicrobial peptides (AMPs) is generally regulated by  
412 inflammatory factors such as IL-1 $\beta$ , TNF- $\alpha$  and LPS (Bando et al., 2007). Of the AMPs, LEAP2  
413 (liver expressed antimicrobial peptide-2) was initially described to be predominantly produced in  
414 the liver and inhibited bacteria and fungi in vitro (Krause et al., 2003). In our study, LEAP2 was  
415 upregulated by HfIL-1 $\beta$ -stimulated hepatocytes suggesting that HfIL-1 $\beta$  modulates the

416 expression of LEAP2 directly or indirectly thus perhaps controlling innate cellular immunity. In  
417 addition, our data corroborate previous findings demonstrating that IL-1 $\beta$  is a major component  
418 of NO production by hepatocytes (Kitade et al., 1996).

419 In addition to the gene expression profiling of HfIL-1 $\beta$ -stimulated immune cells, the  
420 expression pattern of IL-1 $\beta$  in the sera following infection with MG is provided (Park et al., Data  
421 in Brief, submitted). Based on the previously reported data regarding up-regulation of IL-1 $\beta$   
422 mRNA expression after MG infection, IL-1 $\beta$  production would be an expected pro-inflammatory  
423 response to the pathogenesis of MG infection. However, IL-1 $\beta$  mRNA expression levels do not  
424 necessarily reflect the secretion of biologically active protein. The data (Figure 1. in Park et al.,  
425 submitted) revealed two forms (35 and 60 kDa) of putative precursor IL-1 $\beta$ s in sera of control  
426 birds, while more intense bands (25 and 60 kDa), possibly representing mature and dimeric  
427 precursor of IL-1 $\beta$ s, appeared in sera of MG-infected birds. These results raise the question of  
428 how precursor IL-1 $\beta$  is secreted in the blood. Although precursor IL-1 $\beta$  remains primarily  
429 cytosolic and its cleavage is an obligatory step to release precursor IL-1 $\beta$  in the extracellular  
430 milieu, the precursor IL-1 $\beta$  can also be released into extracellular space independent of  
431 processing by enzymes in the presence of some ICE inhibitors (Chin and Kostura, 1993). Given  
432 the elevated production of IL-1 $\beta$  as well as secretion of its bioactive form after MG infection,  
433 these data indicate that IL-1 $\beta$  may be a key cytokine in the pathogenesis of the inflammatory  
434 response and in mediation of host immune responses against MG in house finches. In this  
435 context, further investigation regarding the processing mechanisms leading to the production of  
436 active HfIL-1 $\beta$  and associated enzymatic counterparts that are relevant to the pathogenesis of  
437 MG infection is necessary.

438           In conclusion, we cloned and expressed HfIL-1 $\beta$ , and explored its basic functions  
439 including proliferative effect on splenocytes and hepatocytes, differential mRNA expression  
440 profiles of not only Th1/Th2 cytokines and chemokine but also acute phase protein and  
441 antimicrobial peptide by activated immune cells. Furthermore, the additional data extend  
442 previous findings by demonstrating that up-regulation of IL-1 $\beta$  mRNA expression after MG  
443 infection is accompanied by the bioactive form of IL-1 $\beta$ . Collectively, this study will help us to  
444 better understand the functional role of HfIL-1 $\beta$  in the host immune response along with its  
445 biological importance in the inflammatory response of wild birds against MG infection.

446

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450

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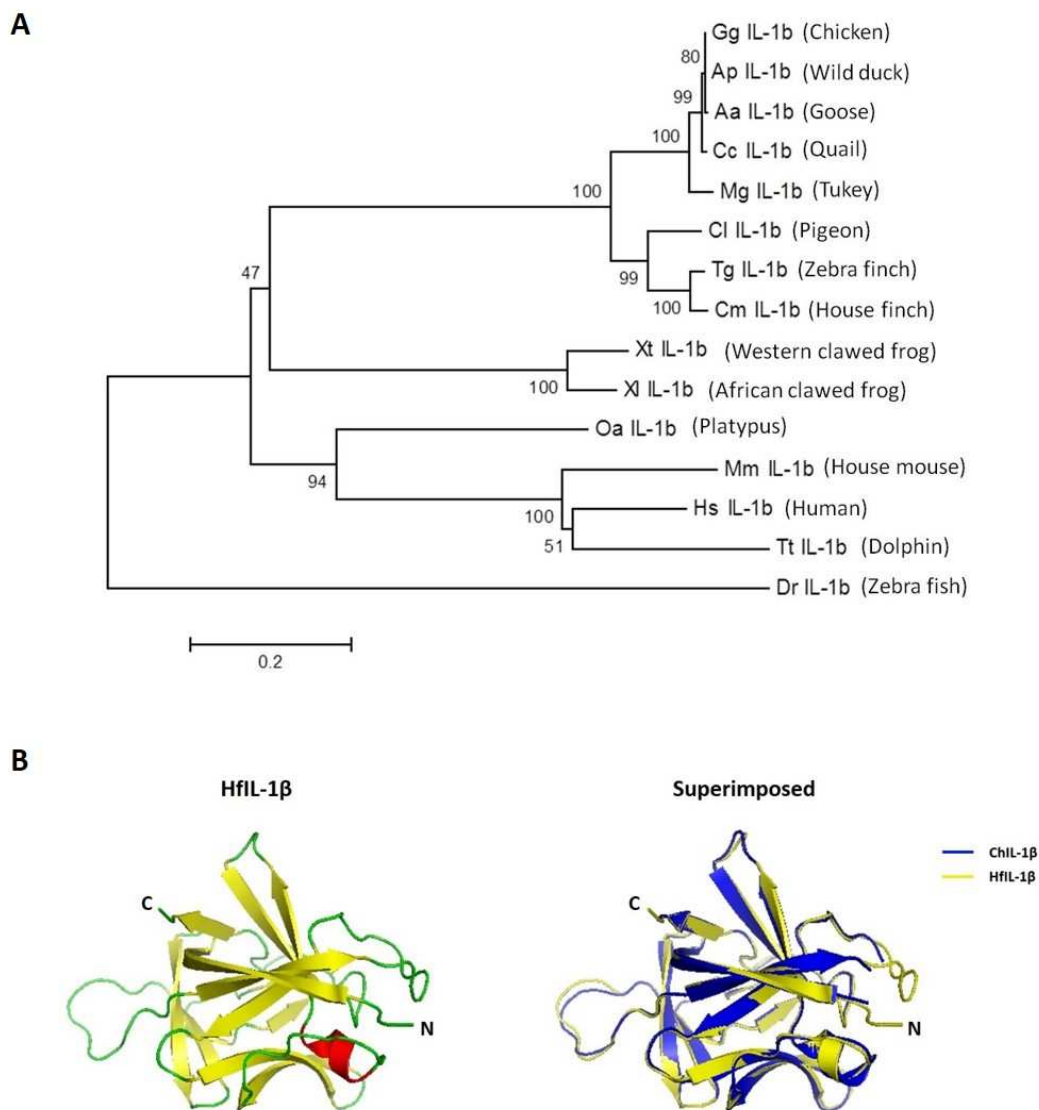
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599 gamma or IL-10 and are regulated by IL-1beta. *Nature* 484, 514-518.

600 **Table 1.** Primers used for gene cloning and qRT-PCR analysis.

<b>Primer Name</b>	<b>Nucleotide Sequence (5'→3')</b>	<b>Application</b>
<b>pHfIL-1<math>\beta</math>_F</b>	AGATCTATGGCATTGTGCCCTGATTTGGAC	Gene cloning
<b>mHfIL-1<math>\beta</math>_F</b>	AGATCTGCACCTGTTTTCCGCTACACT	
<b>HfIL-1<math>\beta</math>_R</b>	CCCGGGTCAGCGCCCACTCAGCTCATA	qRT-PCR
<b>IL-1<math>\beta</math>_F</b>	GGAGGAAGCTGACATCAG	
<b>IL-1<math>\beta</math>_R</b>	TGTCCAGGCGGTAAAAGATG	
<b>IFN-gamma_F</b>	CAAAGGACCATGTCAGGAACA	
<b>IFN-gamma_R</b>	TGAGCCATCAGAAAGGTTTGC	
<b>IL-2_F</b>	TCTTGACTTTTACACACCGAATGAC	
<b>IL-2_R</b>	TCCTCCTCTTCCACATCTTGTTTC	
<b>IL-10_F</b>	AGCACCAGCGCAGCATGA	
<b>IL-10_R</b>	TCATCGTGGCTCTCAGGTTCA	
<b>iNOS_F</b>	TGCCACAAACAATGGTAATATAAGG	
<b>iNOS_R</b>	TGTTCCACACACGGAAATCG	
<b>CXCL1_F</b>	CTGCGAGATGGCAGAGAAGTG	
<b>CXCL1_R</b>	GGCCTTGTCCAGAATTGTCTTG	
<b>SAA_F</b>	TGGGTCTGCATCGCATTG	
<b>SAA_R</b>	TGCATCCCGGACAAACTGT	
<b>LEAP2_F</b>	ATGCACTGGTGGAAAGTGA	
<b>LEAP2_R</b>	GACACTCCTCTCCAGAAG	
<b>GAPDH_F</b>	GGAGCGTGACCCCAGCAACA	
<b>GAPDH_R</b>	CACACGCTTGGCACCACCCT	

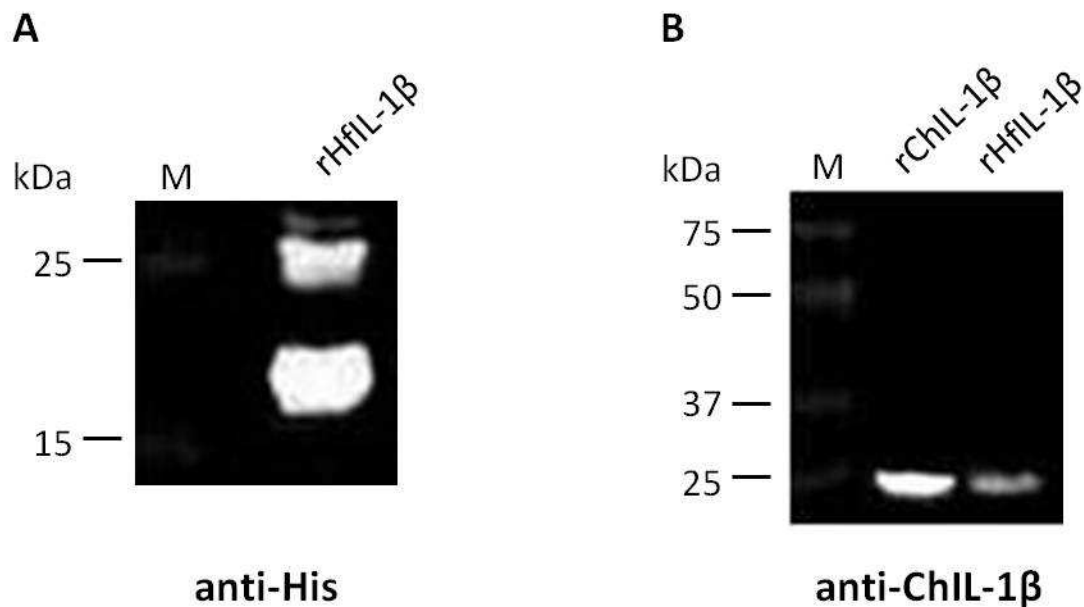
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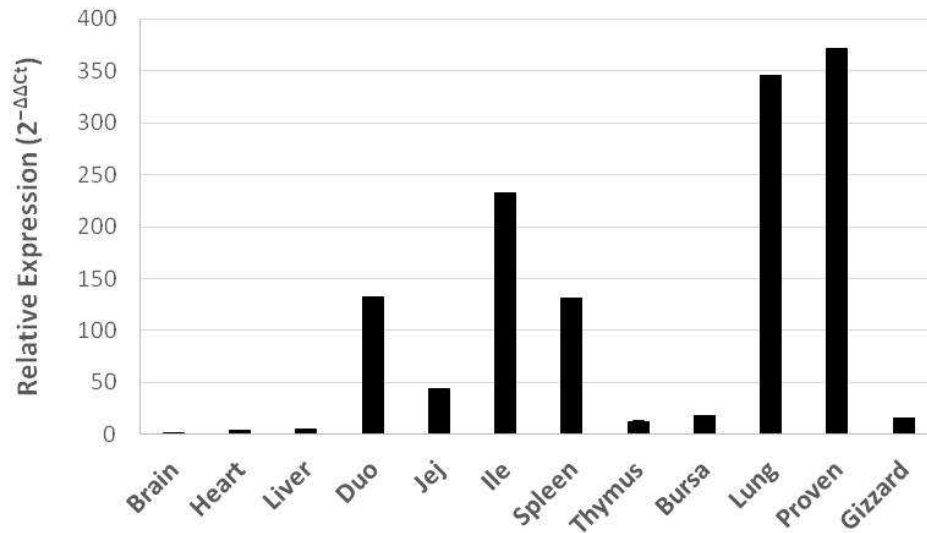
602

603 **Figure 1.** Phylogenetic and structural analysis of HfIL-1 $\beta$ . (A) A phylogenetic tree was  
 604 constructed using multiple alignments with amino acid sequences encoded precursor form of  
 605 HfIL-1 $\beta$  within MEGA 4 program. The clades were validated by 1,000 bootstrap replications,  
 606 which were represented by percentage in branch nodes. The scale bar represents a genetic  
 607 distance of 0.2. (B) Ribbon diagram of HfIL-1 $\beta$  and the superimposed HfIL-1 $\beta$  and ChIL-1 $\beta$ . A  
 608 ribbon diagram of the three-dimensional structure of HfIL-1 $\beta$  has shown (left). The  $\alpha$ -helix and  
 609  $\beta$ -strands indicate as helix and arrows, respectively, and the N- and C- termini are labeled. The  
 610 X-ray structure of HfIL-1 $\beta$  (yellow) is superimposed onto that of ChIL-1 $\beta$  (blue, right).

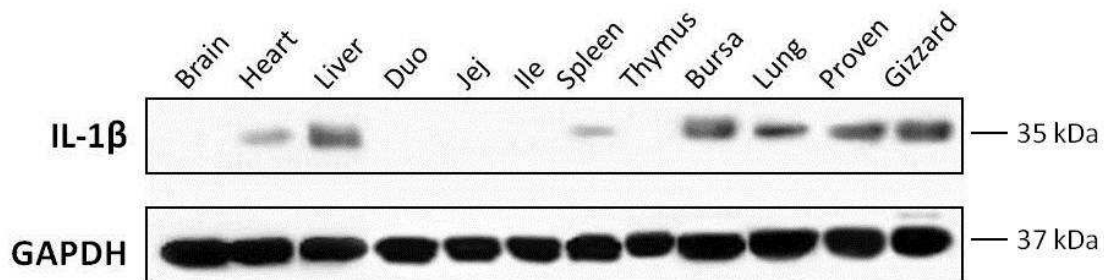


611  
612 **Figure 2.** Immunoblot analysis of purified rHfIL-1 $\beta$ . (A) rHfIL-1 $\beta$  expressed from *E.coli* BL21  
613 was detected with polyhistidine antibody. (B) Immunoblot analysis of purified rHfIL-1 $\beta$  was  
614 performed using anti-ChIL-1 $\beta$  antibody, M, protein molecular weight marker (kDa); lane 1,  
615 ChIL-1 $\beta$  (1 ??g) as a positive control; lane 2, purified rHfIL-1 $\beta$  (1 ??g).

A

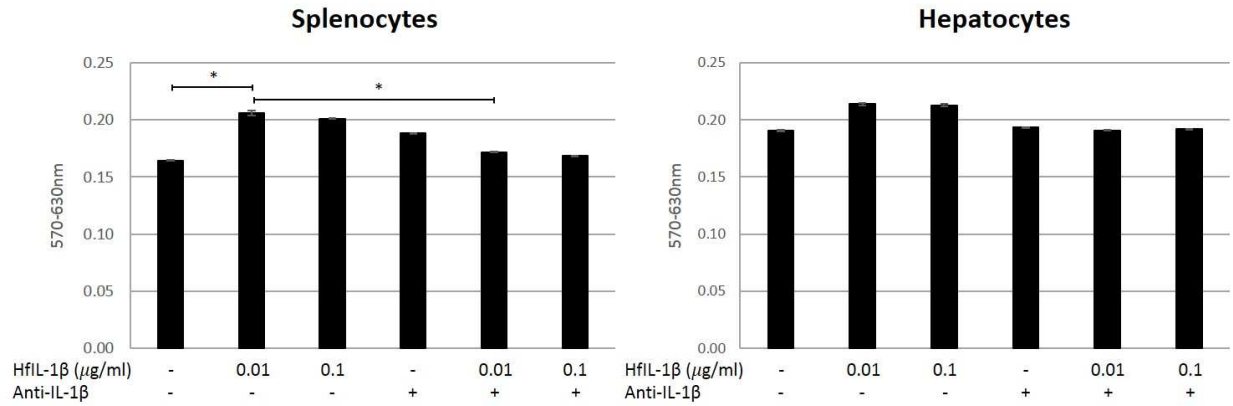


B



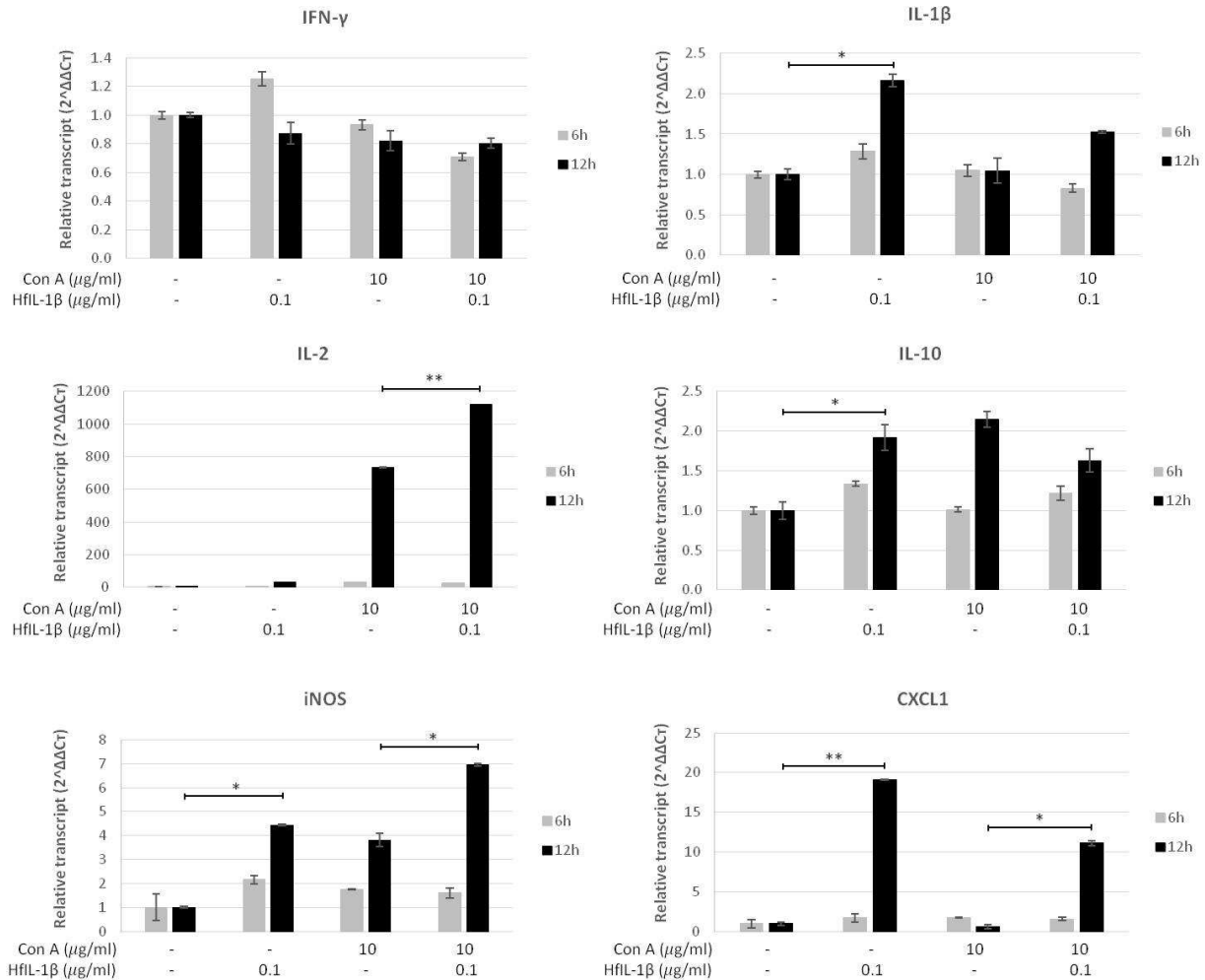
616

617 **Figure 3.** Expression pattern of mRNA and protein of HfIL-1 $\beta$  in various tissues of clinically  
 618 healthy house finches. (A) mRNA expression of HfIL-1 $\beta$  in the different tissues was determined  
 619 by qRT-PCR. Data was normalized to the expression level of GAPDH and represented as fold  
 620 change relative to that of brain. Error bars indicate the SEM. (B) HfIL-1 $\beta$  protein expression  
 621 from various healthy house finch tissues was assessed by immunoblotting using anti-ChIL-1 $\beta$   
 622 antibody, with GAPDH used as a loading control. (Duo, duodenum; Jej, jejunum; Ile, ileum;  
 623 Proven, proventriculus)



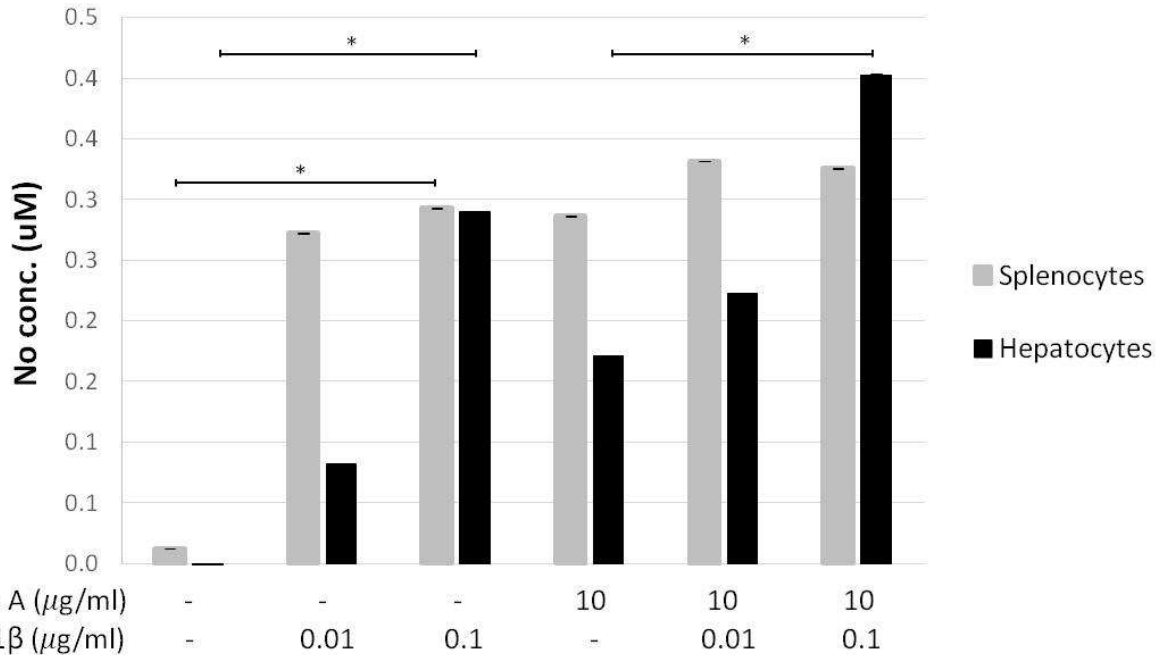
624

625 **Figure 4.** The effect of HfIL-1β on house finch cell proliferation in vitro. Splenocytes ( $2 \times 10^5$   
 626 cells/well) were incubated with medium alone, rHfIL-1β (0.01 and 0.1 μg/ml), rHfIL-1β (0.01  
 627 and 0.1 μg/ml) with anti-ChIL-1β antibody for 12 hr (left). Corresponding proliferation assay  
 628 was conducted on hepatocytes (right). Anti-ChIL-1β antibody alone was used as a negative  
 629 control. Data represent the mean  $\pm$  SEM of two independent experiments performed in triplicate  
 630 and asterisks indicate statistically significant differences ( $p < 0.05$ ).



631

632 **Figure 5.** mRNA expression of Th1/Th2 cytokines and chemokine following stimulation of  
 633 splenocytes with HfIL-1β. Splenocytes (1×10<sup>6</sup> cells/well) were stimulated with medium alone,  
 634 rHfIL-1β (0.1 μg/ml) alone, Con A (10 μg/ml) alone, Con A plus rHfIL-1β (0.1 μg/ml) for 6  
 635 and 12 hr. The expression of Th1/Th2 cytokines and a chemokine was evaluated by qRT-PCR.  
 636 Data are presented as the mean ± SEM of two independent experiments performed in triplicate.  
 637 Asterisks indicate significant differences (\* p < 0.05, \*\* p < 0.01).



638

639

**Figure 6.** Nitric oxide release from HfIL-1 $\beta$ -stimulated splenocytes and hepatocytes.

640

Splenocytes or hepatocytes ( $1 \times 10^6$  cells/well) were stimulated with medium alone, r HfIL-1 $\beta$

641

(0.01 and 0.1  $\mu\text{g/ml}$ ) alone, Con A (10  $\mu\text{g/ml}$ ) alone, Con A plus rHfIL-1 $\beta$  (0.01 and 0.1  $\mu\text{g/ml}$ )

642

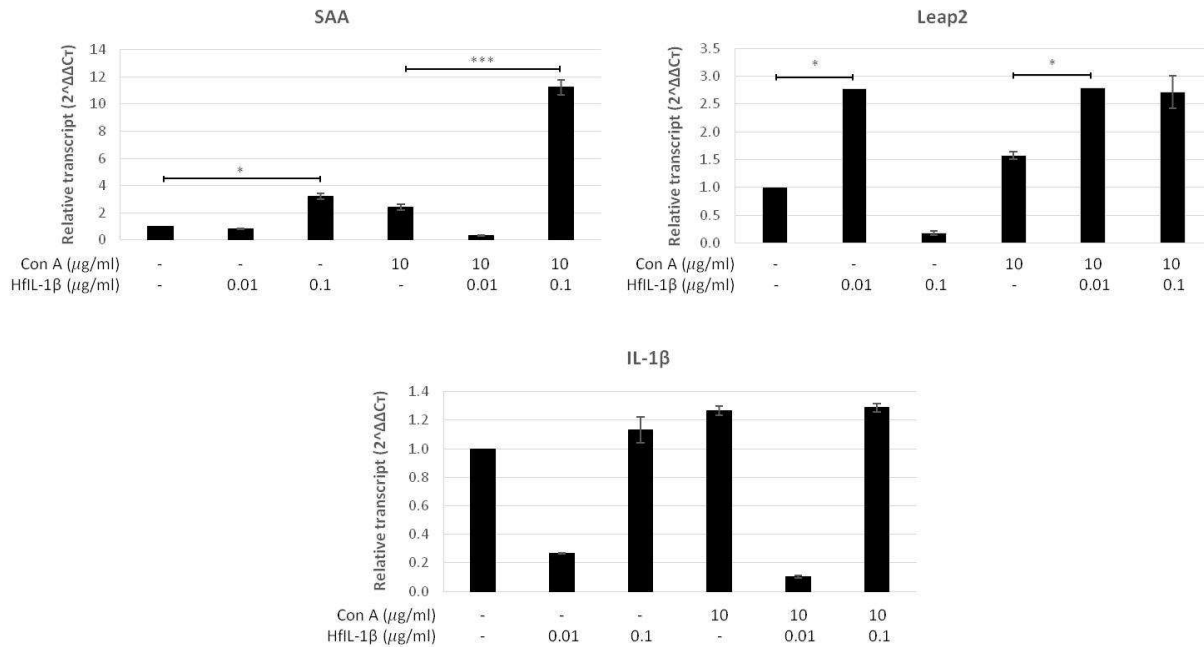
for 6 hr. The levels of NO were determined by Griess assay. Data are presented as the mean  $\pm$

643

SEM of two independent experiments performed in triplicate and statistically significant

644

difference indicated by asterisks ( $p < 0.05$ ).



645  
 646 **Figure 7.** Production of acute phase protein and antimicrobial peptide by rHfIL-1β-stimulated  
 647 hepatocytes. Hepatic cells ( $1 \times 10^5$  cells/well) were treated with medium alone, Con A (10 μg/ml)  
 648 alone, rHfIL-1β (0.01 and 0.1 μg/ml) or rHfIL-1β (0.01 and 0.1 μg/ml) with Con A for 6 hr.  
 649 mRNA expression was measured with qRT-PCR and then values were normalized to GAPDH  
 650 and graphed relative to medium alone. Data are presented as the mean  $\pm$  SEM of two  
 651 independent experiments performed in triplicate and significant differences indicated by asterisks  
 652 (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

## Identification and functional characterization of the house finch interleukin-1 $\beta$

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### Highlights

- Full-length house finch IL-1 $\beta$  was cloned, expressed, and its basic biological roles explored.
- House finch IL-1 $\beta$  modulates the expression of Th1/Th2 cytokines and nitric oxide production by activated immune cells
- House finch IL-1 $\beta$  enhances the expression of acute phase protein and antimicrobial peptide by activated immune cells.