International Immunology, Vol. 27, No. 6, pp. 281–287
doi:10.1093/intimm/dxu144
Advance Access publication 7 January 2015

Analysis of IL-22 contribution to hepcidin induction and hypoferremia during the response to LPS *in vivo*

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Received 20 May 2014, *accepted* 23 December 2014

Abstract

The anaemia of chronic disease (ACD) results from inflammation-mediated up-regulation of the iron regulatory hormone hepcidin, with the consequent sequestration of iron limiting its availability for erythropoiesis. The inflammatory cytokine IL-6, a regulator of hepcidin, has been implicated in this process. Recent *in vivo* and *in vitro* studies indicate that IL-22 is also able to stimulate hepcidin expression. We aimed to determine if IL-22 had a role in causing the hypoferremia associated with the inflammatory response. Wild-type and *Il22*-knockout mice were subjected to an acute inflammatory stimulus via administration of LPS and the response of hepcidin and iron homeostasis was analysed. In the absence of IL-22, there was a response of hepcidin, resulting in a reduction in serum iron levels. However, the hypoferremic response to LPS was slightly blunted in mice lacking IL-22, suggesting that, during LPS-mediated inflammation, IL-22 may play a minor role in mediating the hypoferremic response. These results may have implications for the treatment and management of the ACD.

Keywords: anaemia of chronic disease, hepcidin, IL-22, inflammation, iron

SHORT COMMUNICATION

Introduction

The liver-expressed peptide hormone hepcidin, the key regulator of systemic iron homeostasis, is implicated in many iron-associated disorders, including hereditary haemochromatosis and anaemia. By modulating the surface expression of the iron transporter ferroportin, it controls the bioavailability of iron in the serum, resulting in cellular iron sequestration (1). This limits the erythropoietic iron supply by reducing duodenal iron absorption and macrophage iron recycling. Hepcidin itself is regulated by many factors including iron, hypoxia, inflammatory cytokines, bone morphogenetic proteins and erythroid regulatory factors (2–4). The anaemia of chronic disease (ACD) can be associated with chronic infections, inflammation or cancer and is largely mediated by IL-6-mediated up-regulation of hepcidin (5). Following an acute inflammatory stimulus, wild-type mice show a significant increase in hepcidin mRNA expression and consequent reduction in serum iron (5, 6). In contrast, *Il6*$^{-/-}$ mice show no reduction in serum iron and actually show a reduction in hepcidin mRNA expression (5). This suggests that other components produced during the inflammatory response may be suppressing hepcidin expression in competition with the stimulatory actions of IL-6. Indeed the inflammatory cytokine tumour necrosis factor alpha (TNF-α) can suppress hepcidin expression in hepatocytes via a mechanism unrelated to IL-6 (5). Upon binding its receptor, IL-6 activates the signalling intermediate signal transduction and activator of transcription 3 (STAT3), which then binds to a conserved STAT-binding motif in the proximal hepcidin promoter to drive transcription of the gene (7, 8).

Recently, another inflammatory cytokine, IL-22, has been implicated as a positive regulator of hepcidin, also signalling via STAT3 (9). Administration of an IL-22 receptor agonist in mice resulted in a significant increase in hepatic hepcidin expression and the consequent hypoferremic response (10). While the role of IL-6 in the hepcidin and hypoferremic response to inflammation has been firmly established, the role of IL-22 in this process has not. We investigated the response of hepcidin and iron homeostasis to acute administration of LPS in *Il22*-knockout mice. Our results suggest that IL-22 may play a minor role in mediating the hepcidin and hypoferremic response to LPS but does not play the major role in mediating this response *in vivo*.

Methods

Animals

Animal studies were approved by the QIMR Berghofer Animal Ethics Committee. Animals had free access to water and food

under standard conditions and received humane care. Il22-knockout mice (*Il22*$^{-/-}$) on the BALB/c background, obtained from Genentech through a collaboration with Professor Mark Smyth and Dr Michele Teng (QIMR Berghofer), and wild-type BALB/c mice (Animal Resource Centre, Western Australia) were maintained on standard laboratory chow. Eleven-week-old or 8-week-old male mice (n = 3–7 per group) were either injected intraperitoneally with 1 μg g^{-1} LPS (from *E. coli* 055:B5; Sigma-Aldrich, Castle Hill, Australia) or vehicle (saline) control, sacrificed after 6 h and blood and tissues taken for analysis. During the 6-h treatment mice were fasted but had free access to water.

Measurement of iron indices

Serum iron and transferrin saturation were measured using the iron/TIBC reagent set (Pointe Scientific, Canton, MI, USA) according to the manufacturer's instructions. Hepatic iron concentration (HIC) was measured using the method of Torrance and Bothwell (11).

Real-time quantitative PCR analysis

Quantitative real-time PCR (qPCR) on liver and spleen RNA was performed essentially as previously described (12), except using the Viia 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The expression of all target genes was determined relative to the geometric mean of *Actb*, *Hprt* and *Polr2a*. Primer sequences were as previously described (6, 13–15), except: *Polr2a* (forward: AGCTGGTCCTTCGAATCCGC, reverse: CTGATCTGCTCGATACCCTGC) and *Il22* (forward: CCCAGTCAGACAGGTTCCAG, reverse: TCTGGATGTTCTG GTCGTCA).

Western blotting

Western blotting was performed to determine the levels of total-STAT3, phosphorylated-STAT3 and prohepcidin in liver homogenates as previously described (6, 16).

Cytokine analysis

The serum concentration of the inflammatory cytokines IL-6, IL-12p70, IFN-γ, MCP-1, TNF-α and IL-10 were determined using the BD cytometric bead array: mouse inflammation kit (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry was used to stain formalin-fixed liver sections for Ly6G antibody (clone NIMP-R14; Abcam, Melbourne, Australia), a neutrophil marker, by the QIMR Berghofer HistoTechnology Facility. Slides were scanned using the Aperio ScanScope AT Turbo (Leica Biosystems, Nussloch, Germany) and staining quantified using positive pixel count analysis on the Aperio ImageScope software (Leica Biosystems).

Statistical analysis

Variables were compared between groups using two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test or two-tailed Student's *t*-test in GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Due to the assumption of equal variance between groups not being satisfied for all of the outcome variables, a variance stabilizing transformation was performed for variables comprising the natural log of (1 + x), where x is the variable. When a variance stabilizing transformation was performed, the raw datau are presented on a log-scale. Values equal to 0 are presented in a grey colour on the x-axis of log-scale graphs. A P value <0.05 was considered statistically significant.

Results and discussion

Characterization of the inflammatory response to LPS administration in mice lacking IL-22

The inflammatory response to LPS was assessed in 11-week-old wild-type and *Il22*$^{-/-}$ mice by measuring the mRNA expression of the inflammatory cytokine genes *Il22* and *Il6* in the spleen by qPCR. As expected, the *Il22*$^{-/-}$ mice did not express *Il22* mRNA (Fig. 1A). There was a significant 48-fold increase in the expression of *Il22* mRNA in wild-type mice injected with LPS compared with saline (Fig. 1A), consistent with an appropriate inflammatory response. *Il6* mRNA was significantly up-regulated by LPS injection in both wild-type and *Il22*$^{-/-}$ mice and its expression was similar in the wild-type and *Il22*$^{-/-}$ mice (Fig. 1B).

The serum concentration of six key inflammatory cytokines was measured using a cytometric bead array (Fig. 1C–H). The expression of all six cytokines was elevated in the mice injected with LPS (Fig. 1C–H). There was a statistically significant increase in the serum concentrations of the cytokines IL-6 (Fig. 1C), TNF-α (Fig. 1G) and IL-10 (Fig. 1H) in both wild-type and *Il22*$^{-/-}$ mice injected with LPS. These results suggest that the inflammatory response to LPS was similar in the wild-type and *Il22*$^{-/-}$ mice.

The inflammatory response to LPS in the liver was assessed by staining liver sections with the neutrophil marker Ly6G. Livers from LPS-treated mice showed greatly increased staining for Ly6G, suggesting that inflammatory cells were being recruited to the liver following LPS stimulation (Fig. 2A). Both the basal and LPS-induced numbers of Ly6G-positive cells were marginally higher in the *Il22*$^{-/-}$ mice compared with wild-type (Fig. 2B; *t*-test: $P < 0.01$ and $P = 0.05$ respectively). These results suggest that inflammatory cell infiltration in response to LPS in the livers of *Il22*$^{-/-}$ mice is equal to or even greater than in the wild-type mice. This could be explained by the previously reported hepatoprotective effects of IL-22 (17).

The response to LPS in the liver was also assessed by measuring the mRNA expression of two inflammatory markers, serum amyloid A (*Saa*) and orosomucoid (*Orm*) by qPCR (Fig. 2C and D). There were statistically significant increases in the expression of *Saa* and *Orm* following LPS injection in both the wild-type and *Il22*$^{-/-}$ mice (Fig. 2C and D). However, there were no differences in expression between wild-type and *Il22*$^{-/-}$ mice. The STAT3 signalling pathway in the liver was activated following LPS administration in both wild-type and *Il22*$^{-/-}$ mice, as shown by increased levels of phospho-STAT3 (Fig. 2E–G). However, the levels of phospho-STAT3 relative to both actin and total-STAT3 were higher in the LPS-treated wild-type compared

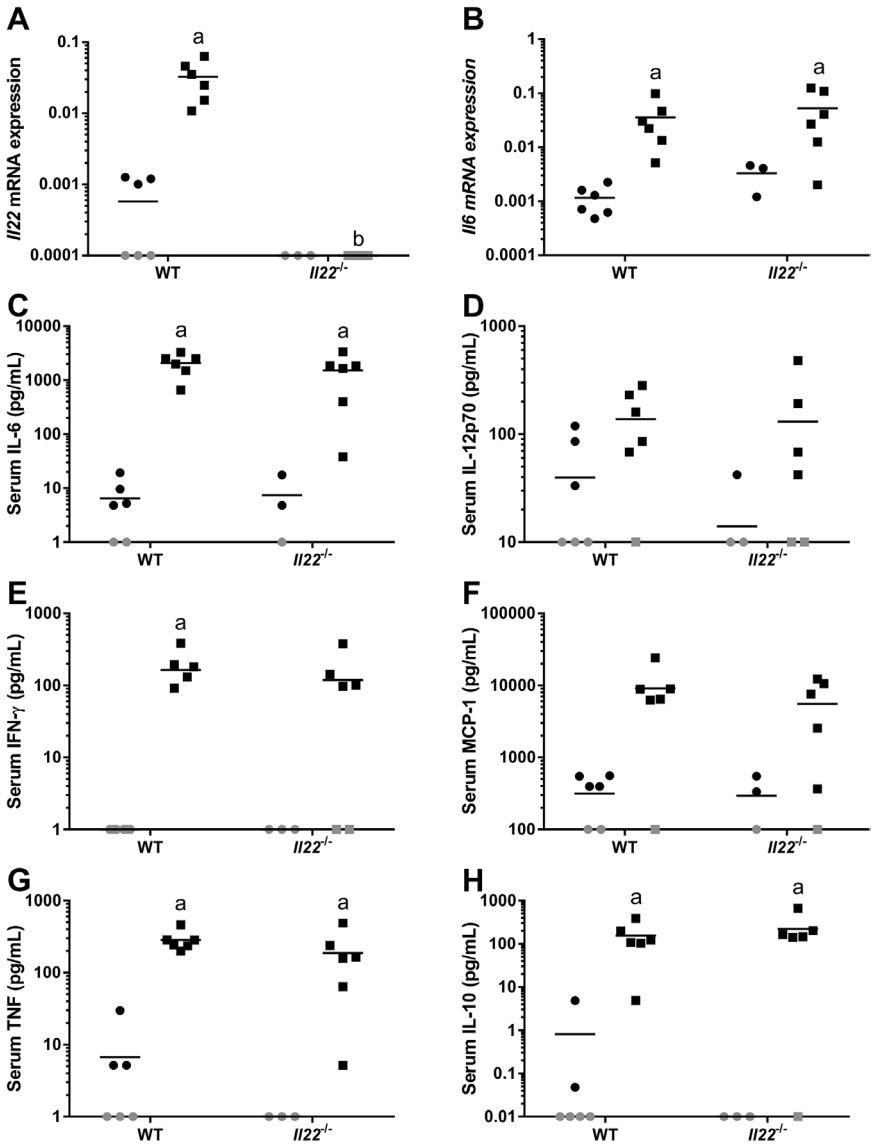

Fig. 1. Inflammatory cytokines are elevated in *Il22*$^{-/-}$ mice following LPS stimulation. Eleven-week-old wild-type (WT) and *Il22*$^{-/-}$ mice (n = 3–6 per group) were injected with either saline control (circles; WT, n = 6; *Il22*$^{-/-}$, n = 3) or LPS (squares; WT, n = 6, *Il22*$^{-/-}$, n = 6) and sacrificed after 6 h. qPCR was used to determine the mRNA expression levels of *Il22* (A) and *Il6* (B) in the spleen relative to the geometric mean of *Actb*, *Hprt* and *Polr2a*. The serum concentration of the inflammatory cytokines IL-6 (C), IL-12p70 (D), IFN-γ (E), MCP-1 (F), TNF-α (G) and IL-10 (H) were measured using a cytometric bead array. Results are expressed as dot plots with a line indicating the mean. Zero values are plotted on the x-axis of the log-scale graphs as grey shapes. Statistically significant differences (two-way ANOVA with Tukey's multiple comparison test; $P < 0.05$) are denoted as (a) compared with relative control and (b) compared with relative wild-type.

with the LPS-treated *Il22*$^{-/-}$ mice, being statistically significant for phospho-STAT3/total-STAT3 (Fig. 2G; t-test: $P < 0.05$). These results suggest that although the *Il22*$^{-/-}$ mice appear to have an appropriate response to LPS in terms of inflammatory cytokine production and inflammatory cell infiltration in the liver, they may have a slight deficiency in signalling through the STAT3 pathway.

Analysis of the hypoferremic response to LPS in mice lacking IL-22

To determine the iron status of mice lacking IL-22, we measured the iron content of the liver, the primary site for the storage of iron. The HIC was significantly elevated in the *Il22*$^{-/-}$ mice compared with wild-type, being 1.4 and 1.7 times higher in the control and LPS-treated mice, respectively (Fig. 3A). These results suggest that the *Il22*$^{-/-}$ mice may have some deficiency in the mechanisms regulating iron homeostasis, resulting in increased iron stores. The hypoferremic response to acute LPS administration was assessed by measuring serum iron concentration and transferrin saturation. LPS administration resulted in a significant decrease in the serum iron and transferrin saturation of both genotypes ($P < 0.05$, two-way ANOVA; Fig. 3B and C). While there were no differences in transferrin saturation between genotypes, the serum iron levels were higher in the *Il22*$^{-/-}$ mice compared with wild-type following LPS injection (t-test: $P < 0.05$). As

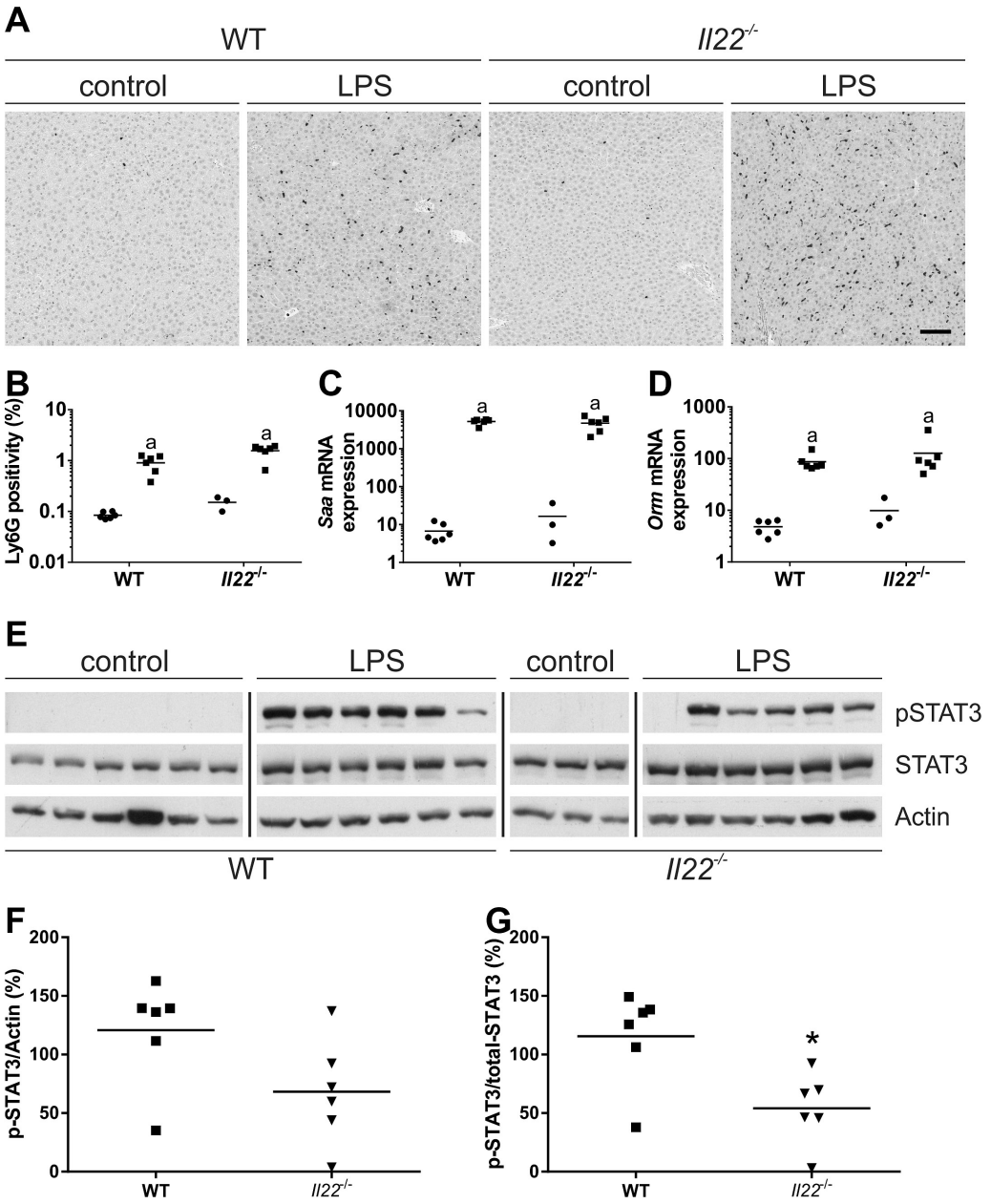

Fig. 2. *Il22*$^{-/-}$ mice have an appropriate inflammatory response to LPS in the liver. Eleven-week-old wild-type (WT) and *Il22*$^{-/-}$ mice (n = 3–6 per group) were injected with either saline control (circles in B–D; WT, n = 6; *Il22*$^{-/-}$, n = 3) or LPS (squares in B–D; WT, n = 6, *Il22*$^{-/-}$, n = 6) and sacrificed after 6 h. (A) Immunohistochemistry analysis was used to estimate the number of inflammatory cells in the liver by staining formalin-fixed liver sections with Ly6G antibodies. (B) The percentage of Ly6G positive staining was quantified using positive pixel count analysis. qPCR was used to determine the mRNA expression levels of (C) *Saa* and (D) *Orm* in the liver relative to the geometric mean of *Actb*, *Hprt* and *Polr2a*. (E) Western blotting was used to determine the levels of phosphorylated-STAT3 (pSTAT3), total STAT3 and actin in the liver. Protein levels were quantitated by densitometry and levels of pSTAT3 displayed relative to actin (F) and total-STAT3 (G) for the LPS-treated mice. Statistically significant differences (two-way ANOVA with Tukey's multiple comparison test; $P < 0.05$) are denoted as (a) compared with relative control. For t-tests, *$P < 0.05$. Scale bar = 100 μm.

the potential effect of IL-22 deficiency on the hypoferremic response to LPS in these mice was marginal, we subjected a younger group of 8-week-old mice (n = 5–7 per group) to the same LPS stimulation. Similar to the 11-week-old mice, the 8-week-old *Il22*$^{-/-}$ mice also had a statistically significant elevation in HIC compared with wild-type, being 1.3 and 1.2 times higher in the control and LPS-treated mice, respectively (Fig. 3D). The 8-week-old *Il22*$^{-/-}$ mice were also able to mount a hypoferremic response, showing a significant reduction in serum iron following LPS stimulation (Fig. 3E). However, again they did not reduce serum iron to the same extent as wild-type mice, having significantly higher serum iron levels than wild-type following LPS treatment (Fig. 3E). These results suggest that after LPS stimulation, mice lacking IL-22 can mount

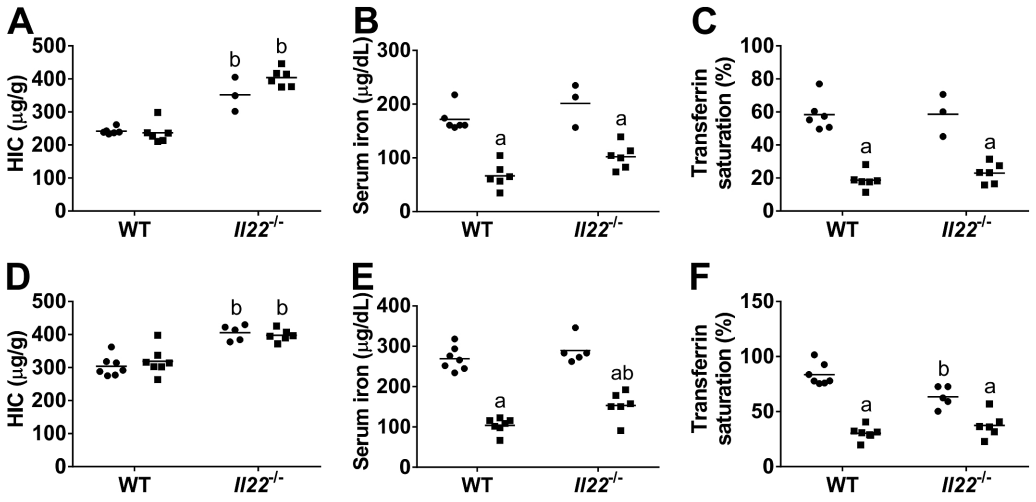

Fig. 3. *Il22*$^{-/-}$ mice have elevated hepatic iron and can reduce serum iron following LPS stimulation. Eleven-week-old (A–C) and 8-week-old (D–F) wild-type (WT) and *Il22*$^{-/-}$ mice (n = 3–7 per group) were injected with either saline control (circles) or LPS (squares) and sacrificed after 6 h. (A, D) HIC, (B, E) serum iron concentration and (C, F) transferrin saturation were measured. Results are expressed as dot plots with a line indicating the mean. Statistically significant differences (two-way ANOVA with Tukey's multiple comparison test; $P < 0.05$) are denoted as (a) compared with relative control and (b) compared with relative wild-type.

an iron-withholding response, but may not be able to reduce their serum iron to quite the same extent as wild-type mice.

Analysis of the hepcidin response to LPS in mice lacking IL-22

To determine whether hepcidin was responding to LPS in the absence of IL-22, we measured the expression of the hepatic hepcidin 1 gene (*Hamp*: hepcidin antimicrobial peptide) by qPCR in 11-week-old wild-type and *Il22*$^{-/-}$ mice. LPS injection resulted in significant up-regulation of *Hamp* mRNA expression (Fig. 4A). However, the magnitude of LPS-mediated hepcidin induction was less in the *Il22*$^{-/-}$ mice (1.6-fold) compared with wild-type (2.8-fold), and following LPS stimulation, *Hamp* mRNA expression was significantly lower in the *Il22*$^{-/-}$ mice compared with wild-type (Fig. 4A). We also measured the levels of prohepcidin protein in the livers of mice by western blotting (Fig. 4B and C). There was a strong positive correlation between the levels of prohepcidin protein and *Hamp* mRNA in the liver (r = 0.881, $P < 0.0001$). In agreement with the qPCR analysis, the levels of prohepcidin protein increased with LPS stimulation in both wild-type and *Il22*$^{-/-}$ mice, however, this did not reach statistical significance in the *Il22*$^{-/-}$ mice, perhaps due to the low number of mice in the control group (Fig. 4B and C). As with the qPCR analysis, the levels of prohepcidin were significantly lower in the LPS-stimulated *Il22*$^{-/-}$ mice compared with wild-type (Fig. 4B and C). As hepcidin responds to iron, we calculated the *Hamp*/HIC and prohepcidin/HIC ratios to determine whether the *Il22*$^{-/-}$ mice had a deficiency in hepcidin relative to iron stores. Because of the higher HIC values in the *Il22*$^{-/-}$ mice, the *Hamp*/HIC and prohepcidin/HIC ratios were lower than in wild-type, being statistically significant in the LPS treated groups (Fig. 4D and E). These results collectively suggest that hepcidin does respond to LPS stimulation in the *Il22*$^{-/-}$ mice, but that the degree of induction is less than that in wild-type mice. The levels of *Hamp* mRNA were also measured in the 8-week-old mice. In these mice, the hepcidin response to LPS was similar to wild-type, with both having a robust, statistically significant increase in *Hamp* expression (Fig. 4F). The *Hamp*/HIC ratios in the 8-week-old *Il22*$^{-/-}$ control mice were slightly lower than in wild-type, although this did not reach statistical significance (Fig. 4G; t-test, P = 0.07).

In conclusion, we have shown that mice lacking IL-22 can mount a hypoferremic response to acute LPS stimulation by up-regulating hepcidin and reducing serum iron. However, the reduction in serum iron was not as great in mice lacking IL-22 than in wild-type mice, suggesting that there may be some deficiency in the inflammatory regulation of hepcidin. Analysis of the older 11-week-old mice suggested that the induction of hepcidin was blunted and this was related to a relative reduction in STAT3 phosphorylation. However, despite a blunted hypoferremic response in the younger 8-week-old *Il22*$^{-/-}$ mice, the hepcidin response following LPS stimulation in these mice appeared normal. These results suggest that there may be a moderate role for IL-22 in mediating the hypoferremic response to LPS *in vivo*. This coupled with the moderately higher hepatic iron levels observed in the *Il22*$^{-/-}$ mice points to a potential role for IL-22 in hepcidin regulation and in maintaining iron homeostasis. The moderate effect of IL-22 on the hypoferremic response contrasts with the much greater effect of IL-6; mice lacking IL-6 did not increase liver hepcidin expression or reduce serum iron levels during an acute turpentine-mediated inflammatory insult (5). Taken together, these studies show that, although, IL-22 can regulate hepcidin transcription (9, 10), and appears to have a moderate effect on the hypoferremic response to LPS, it does not play a major role in mediating the hypoferremic response *in vivo*. IL-6, however, appears to be essential for this process, and, in its absence IL-22 cannot compensate. In this study, we have studied a specific model of LPS-mediated acute inflammation. Whether IL-22-mediated hepcidin induction is

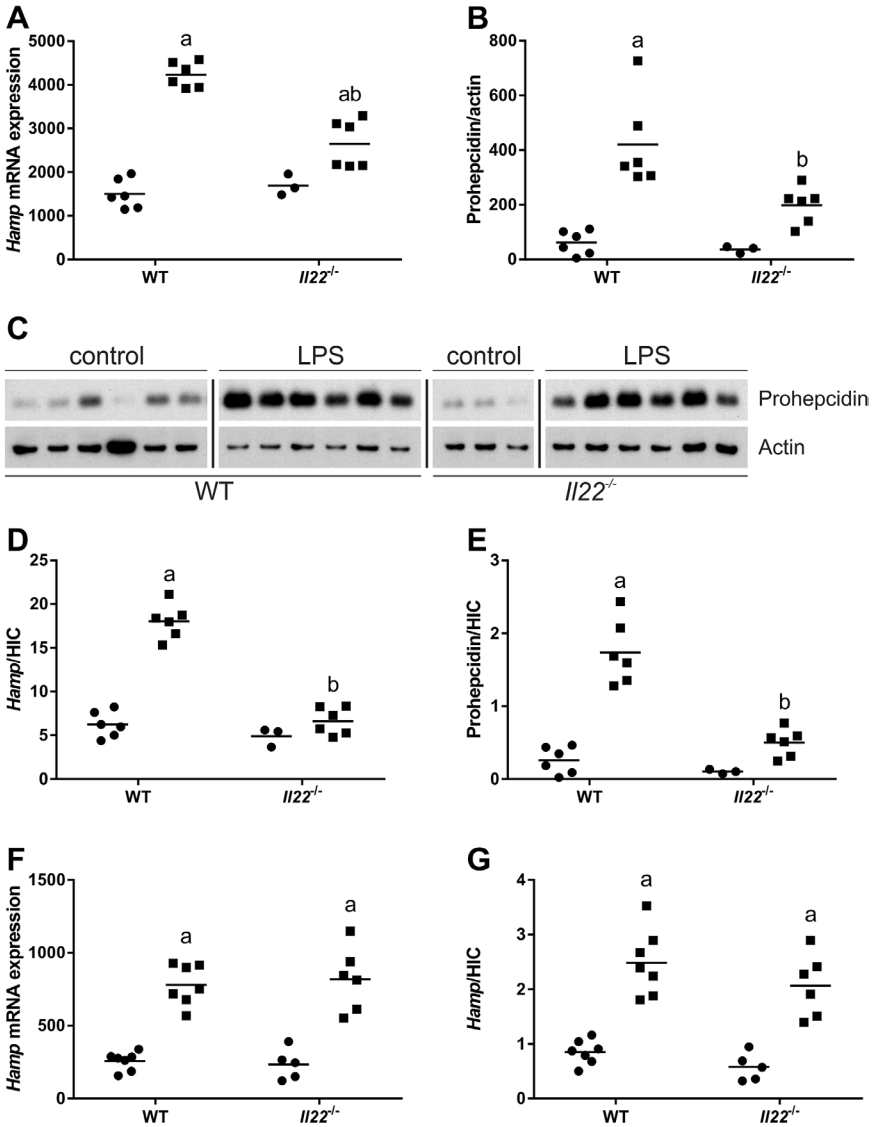

Fig. 4. *Il22*$^{-/-}$ mice have a blunted hepcidin response following LPS stimulation. Eleven-week old wild-type (WT) and *Il22*$^{-/-}$ mice (n = 3–6 per group) were injected with either saline control (circles) or LPS (squares) and sacrificed after 6 h. qPCR was used to determine the mRNA expression levels of *Hamp* (A) in the liver relative to the geometric mean of *Actb*, *Hprt* and *Polr2a*. Western blotting was used to determine the levels of prohepcidin in the liver (B and C). The ratios *Hamp*/HIC (D) and prohepcidin/HIC (E) were calculated. Eight-week-old WT and *Il22*$^{-/-}$ mice (n = 5–7 per group) were injected with either saline control (circles) or LPS (squares) and sacrificed after 6 h. The mRNA expression of *Hamp* was determined by qPCR (F) and ratio of *Hamp*/HIC was calculated (G). Results are expressed as dot plots with a line indicating the mean. Statistically significant differences (two-way ANOVA with Tukey's multiple comparison test; $P < 0.05$) are denoted as (a) compared with relative control and (b) compared with relative wild-type.

involved in the host response to other models of infection, inflammation and the ACD remain to be determined. IL-22 has been shown to be critical for the immunity to intestinal infections such as *Citrobacter rodentium* in mouse models (18). Whether IL-22-mediated hepcidin induction and hypoferremia are important components for mediating this immunity and the immunity to other pathogens would be of interest and may inform the future management and treatment of these conditions and other causes of the ACD.

Funding

Project Grants (1031325 and 1048000 to V.N.S.) from the National Health and Medical Research Council (NHMRC) of Australia. D.F.W. is the Gastroenterological Society of Australia Senior Research Fellow. V.N.S. is the recipient of a NHMRC Senior Research Fellowship (1024672).

Acknowledgements

The authors thank Eriza Secondes, Lesa Ostini, Kylie James and Ashraful Haque for technical assistance and Professor Mark Smyth, Drs Michele Teng and Liam Town for their collaboration and provision of the *Il22* gene-targeted mice. The authors declared no conflict of interests.

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