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Tumour necrosis factor alpha causes hypoferraemia and reduced intestinal iron absorption in mice.

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Short title- TNF- α modulates iron transport

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Synopsis

Cytokines are implicated in the anaemia of chronic disease by reducing erythropoiesis and increasing iron sequestration in the reticuloendothelial system. However, the effect of cytokines in particular TNF- α on small bowel iron uptake and iron transporter expression remains unclear. In this study we subjected CD1 male mice to intra-peritoneal injection with TNF- α (10ng/mouse) and then examined the expression and localisation of DMT1, Ireg1, and ferritin in duodenum. Liver and spleen samples were used to determine hepcidin mRNA expression. Changes in serum iron and iron loading of duodenum, spleen and liver were also determined. We report in this study a significant fall in serum iron 3 hours post TNF- α exposure ($p < 0.05$). This was coincident with increased iron deposition in the spleen. After 24 hours exposure there was a significant decrease in duodenal iron transfer ($p < 0.05$) co-incident with increased enterocyte ferritin expression ($p < 0.05$) and re-localisation of Ireg1 from the basolateral enterocyte membrane. Hepatic hepcidin mRNA levels remained unchanged whilst splenic hepcidin mRNA expression was reduced at 24hrs. In conclusion we provide evidence that TNF- α may contribute to anaemia of chronic disease by iron sequestration in the spleen and by reduced duodenal iron transfer which seems to be due to increased enterocyte iron binding by ferritin and a loss of Ireg1 function. These observations were independent of hepcidin mRNA levels.

Keywords- Iron, Tumour necrosis factor alpha, anaemia, murine, Ireg1, ferritin

Abbreviations- ACD- Anaemia of chronic disease, DMT1- divalent metal transporter 1, IL1- interleukin 1, IL6- interleukin 6, INF γ - interferon gamma, Ireg1- iron regulated protein 1, LPS- lipopolysaccharide endotoxin, MT- mucosal transfer, TNF- α - tumour necrosis factor alpha,

Introduction

The anaemia of chronic disease (ACD) or anaemia of chronic inflammation, as it is also commonly referred to, has long perplexed clinicians.[1] It is usually characterised by a normochromic normocytic anaemia with hypoferraemia and hyperferritinaemia in the face of adequate body iron stores.[2] The pathogenesis of ACD is linked to increased circulating levels of pro-inflammatory cytokines found in chronic inflammatory disorders.[3] Cytokines such as tumour necrosis factor alpha (TNF- α), interleukin 1 (IL1), interleukin 6 (IL6) and interferon gamma (INF γ) have a number of diverse actions on the erythropoietic and reticuloendothelial system and have all been implicated in the pathogenesis of ACD.[4]

The role of IL6 in ACD is related to its ability to stimulate hepcidin expression causing hypoferraemia by iron sequestration within the reticuloendothelial system and reducing intestinal iron absorption.[5] The hepatic antimicrobial peptide hepcidin has previously been measured in increased concentrations in the urine of patients with ACD secondary to sepsis.[6,7] Nemeth et al demonstrated that media from endotoxin (LPS) stimulated macrophages induced hepcidin production from cultured human hepatocytes, an effect which was reversed by the addition of IL6 neutralising antibodies.[8] Both in this study and a further study by Kemna et al [9] infusion of IL6 or its induction by LPS infusion led to increased hepcidin levels and resultant hypoferraemia in healthy human subjects. Interestingly, neither study demonstrated an induction in hepatic hepcidin expression by TNF- α .

There is considerable evidence in the literature supporting a role for TNF- α in the anaemia of both chronic inflammation and malignancy.[10,11] In the context of rheumatoid arthritis anti-TNF- α therapy has been shown to improve haemoglobin concentrations in affected patients compared to controls.[12] In another study, bone marrow aspirates from rheumatoid arthritis patients had low levels of haematopoietic progenitor CD34 cells and increased rates of erythroid cell apoptosis.[13] These effects were reversed by *in-vitro* treatment with anti- TNF- α antibodies. In two murine studies TNF- α administration caused hypoferraemia. [14,15] In the latter work this was

attributed to increased iron sequestration within macrophages. A potential mechanism for macrophage iron sequestration was proposed by Ludewicz et al [16] who demonstrated that TNF- α stimulation upregulated the cellular iron import protein divalent metal transporter 1 (DMT1) and reduced the iron exporter iron regulated protein 1 (Ireg1) in a human monocyte cell line.

The majority of work studying the effects of pro-inflammatory cytokines in ACD has focused on the erythropoietic and reticuloendothelial systems. The direct effects of cytokines on small bowel iron absorption have been less well studied. Over the past decade the key proteins involved in inorganic iron trafficking across the small bowel epithelium have been identified [17]. Briefly ferric iron is thought to be reduced to the ferrous form by duodenal cytochrome b (dcytb) at the brush border and is imported into the enterocyte by DMT1. Once in the cell iron may be stored as ferritin or exported across the basolateral membrane by Ireg1 which works in conjunction with the copper-linked oxidase hephaestin generating ferric iron which is transported in the plasma largely bound to transferrin.

In a previous study we demonstrated TNF- α produced rapid changes in the expression and localisation of DMT1, Ireg1 and ferritin in both *in-vitro* and *ex-vivo* enterocyte model systems.[18] This ultimately led to iron sequestration within enterocytes and a reduction of iron export across a Caco-2 cell monolayer. In a similar study, Johnson et al [19] demonstrated reduced DMT1 expression in Caco-2 cells following 72 hours of TNF- α exposure. Interestingly, a previous murine study had shown that small intestinal intra-epithelial lymphocytes produced TNF- α in response to dietary iron, an effect which appeared important in preventing hepatic iron overload.[20] This led us to propose that local intestinal responsiveness to TNF- α could provide enterocyte targets allowing reduced iron absorption in the face of systemic TNF- α excess in chronic inflammation. The *in-vitro* effects observed were hepcidin independent. We therefore wished to extend this study to see if the same effects could be observed in a whole organism.

In this current study, utilising wild type mice, we investigated the effects of TNF- α on small intestinal iron transporter expression and localisation as well as iron absorption. In

addition we have studied the effects of TNF- α on iron levels in serum, spleen and liver with concomitant hepcidin expression.

Materials and Methods

6 week old CD1 male mice (weights 28-32g) (Charles Rivers, Margate, Kent), were subject to intra peritoneal injection with either 0.15M NaCl (control) or TNF- α (10ng/mouse) in 0.15M NaCl. At 3 or 24 hrs post injection mice were anaesthetised and sacrificed by cervical dislocation. Duodenum, liver, spleen and serum were then collected. Serum iron analysis was performed at Birmingham City Hospital, Dept of Biochemistry, whilst small bowel, spleen and liver were divided equally into three portions for: i) RNA extraction, ii) protein extraction iii) immunohistochemistry. All animal experiments were performed under the authority of a UK Home Office licence. Mice were fed CRM diet (Scientific Diet Supplies [SDS], Witham, Essex, United Kingdom).

Real Time PCR

RNA was extracted from small bowel, liver and spleen specimens using Trizol reagent with 1 μ g of RNA subject to reverse transcription utilising a reverse transcription kit (Promega). cDNA was then subject to Real Time PCR as described previously[21]. Briefly, all reactions were performed using 18S ribosomal RNA as an internal standard (PE Biosystems, Roche, USA). Each reaction was performed in triplicate and contained one of the following sets of probes and primers; i) DMT1 (Probe 5' FAM CTG CAT TCT GCC TTA GTC AAG TCT AGA CAG CTA AAC TAMRA 3'), Forward Primer (5'-AGC TGT CAT CAT GCC ACA CAA C -3') and Reverse Primer (5'- GCT TCT CGA ACT TCC TGC TTA TTG 3'), ii) Ireg1 (Probe 5' FAM AGG ATT GAC CAG TTA ACC AAC ATC TTA GCC CC TAMRA 3'), Forward Primer (5'-AGC AAA TAT GAA TGC CAC AAT ACG-3') and Reverse Primer (5'-CAA ATG TCA TAA TCT GGC CAA CAG-3'), iii) ferritin (Probe 5'FAM CCA ACG AGG TGG CCG AAT CTT CCT T TAMRA 3'), Forward Primer (5'-GGA ACA TGC TGA GAA ACT GAT GAA-3') and Reverse Primer (5'-CAT CAC AGT CTG GTT TCT TGA TAT CC-3'), or iv) hepcidin (HEPC1) (Probe 5'-FAM CCT GAG CAG CAC CAC CTA TCT CCA TCA-3'), Forward Primer (5'-CTG TCT CCT GCT TCT CCT CCT T-3') and Reverse

Primer (5'-CTG CAG CTC TGT AGT CTG TCT CAT C-3') in the presence of 1X Mastermix (PE Biosystems), 50 nM 18S 5' and 3' primers and 200 nM 18S probe (5' VIC, 3'-TAMRA labelled) and 0.25 μ l of cDNA (equivalent to 12.5ng of reverse transcribed RNA) in a 25 μ l reaction. Reactions without cDNA were included as negative controls. Gene expression was normalised to the 18S and represented as Δ Ct values. For each sample the mean of the three Δ Ct values was calculated. Comparison of gene expression between control and treated samples was derived from subtraction of control Δ Ct values from treatment Δ Ct values to give a $\Delta\Delta$ Ct value and relative gene expression was calculated as $2^{-\Delta\Delta\text{Ct}}$. Relative gene expression is normalised to 1.0 (100%) of controls. Each experiment was performed in triplicate.

Western Blotting

Whole Duodenum was cut longitudinally and the epithelium removed using a glass slide. The extract was then processed for Western blotting as previously described [21] with monoclonal antibodies against either DMT1 (5 μ g/ml, NRAMP24-A ADI, USA), Ireg1 (5 μ g/ml MTP11-A ADI, USA), ferritin (1:500 ab16875, Abcam, UK), TFR1 (1:500 A11130, Zymed, USA) or Cytokeratin 19 (1:2,000 IF-15, Oncogene Research Products USA), the last for normalisation of epithelial protein loading. Immunoreactive bands were then subject to densitometry using software NIH Image 1.62. Prior to reprobing with the different antibodies the membranes were incubated in stripping buffer containing 2% SDS, 100 mM mercaptoethanol in 62.5 mM Tris-HCL pH 6.8 for 30 min at 70 °C.

Immunocytochemistry

7 μ m sections of small bowel were processed for immunohistochemistry as previously described. Briefly, sections were dewaxed and then incubated in 0.1% Tween 20 containing 1mM EDTA pH 8.0 for 16 hrs at 65⁰C. Sections were then incubated for 1 hr with either DMT1 (10 μ g/ml, NRAMP24-A ADI, USA), Ireg1 (10 μ g/ml MTP11-A ADI, USA) or ferritin (1:500 ab16875, Abcam, UK). Immunoreactivity was detected utilising the avidin-biotinylated secondary antibody method (DAKO ABC kit, DAKO, USA) and visualised with DAB, (diaminobenzidine) reagent. Sections were counterstained with haemotoxylin. Omission of primary antibody was employed as a negative control. Stained sections were scored independently by three observers (SH,

CT and TI) with regards cellular localisation. Images were visualised from paraffin sections using a Nikon Eclipse E600 microscope and digital image taken using a Nikon DXM1200F camera (Surrey, UK). Nikon ACT-1 version 2.62 software was used for image acquisition (Surrey, UK).

Perl's staining

Paraffin sections were dewaxed and incubated in 1% HCl containing 1% ferrous cyanate for 20 minutes. Sections were washed and counterstained with neutral red prior to visualisation. Images were visualised from paraffin sections using a Nikon Eclipse E600 microscope and digital image taken using a Nikon DXM1200F camera (Surrey, UK). Nikon ACT-1 version 2.62 software was used for image acquisition (Surrey, UK).

Radiolabeled iron uptake assay

The length of the duodenum was tied off at both ends and the duodenal segment pre-washed with 0.15M NaCl at 37°C. The segment was then injected with 250µM FeCl₃ (Fe:nitrilotriacetate, 1:2) containing 5µCi of radiolabelled iron ([⁵⁹Fe]Cl₃, specific activity 185 GBq/g, PerkinElmer, UK) in physiological media (125mM NaCl, 3.5mM KCl, 1mM CaCl₂, 10mM MgSO₄, 10mM D-glucose in 16mM HEPES-NaOH buffer, pH 7.4). The chelator nitrilotriacetate was chosen as it has previously been used in iron absorption studies and has been shown to form relatively stable complexes with iron.[22-23] Following a 10 minute incubation, the duodenal segment was removed and the remaining carcass was subject to γ ray counting using a high-resolution bulk sample counter. The count measured was termed mucosal transfer.

Statistics

All experimental errors are shown as 2 standard errors of the mean. Statistical significance was calculated by use of the unpaired Student's t test using SPSS version 10.0 (SPSS Inc, USA). Significance was accepted at P<.05.

Results

Determination of serum iron levels in control and TNF α treated mice

Analysis of serum iron levels in both 3 hr and 24 hr TNF- α treated mice demonstrated a significant decrease in serum iron compared to control mice $P < 0.05$ (Figure 1).

TNF- α mediated repression of iron transfer across small bowel mucosa

No significant change in mucosal iron transfer was observed after 3hr stimulation with TNF- α . (Figure 2). However, a longer exposure to TNF- α (24hrs) did significantly reduce mucosal transfer of iron compared to control mice ($p < 0.05$).

Iron transporter mRNA expression following TNF- α exposure

Consistent with our previous studies Real Time PCR analysis revealed a significant increase in DMT1, Ireg1 and ferritin mRNA in 3 hr TNF- α treated mice, which, with the exception of ferritin, had returned to baseline level by 24 hrs (Figure 3A-C).

As hepcidin release has been shown to be a key mediator of the systemic response of iron metabolism to inflammation we examined whether hepcidin was also modulated by TNF- α . Livers and spleens from these mice were removed, RNA extracted and Real Time PCR performed (Figure 4). In both 3 hr and 24 hr TNF- α treated mice there was no significant modulation in hepatic hepcidin mRNA levels (Figure 4A). In contrast splenic hepcidin levels appeared to be significantly repressed following 24 hour TNF- α exposure ($p < 0.05$) (Figure 4B).

Iron transporter protein expression following TNF- α exposure

To determine whether these transcriptional changes were mirrored at the protein level western blotting was performed. In keeping with the mRNA data, at 3 hours there was a significant increase in DMT1 and Ireg1 protein expression although these had returned to baseline by 24 hours (Figure 5). There was a comparative delay in ferritin expression with protein levels only becoming significant at 24 hours. There was no difference in the protein levels of TFR1 in mouse enterocytes at either time point eliminating this as a potential route of iron ingress into these cells.

Immunolocalisation of iron transporter proteins

Immunohistochemical studies furthermore showed alterations in the localisation of these proteins (Figure 6). Ferritin in control mice was localised predominantly in the apical portion of the villous enterocytes whilst in the treated animals immunoreactivity throughout the enterocyte was apparent. No obvious difference was observed in the crypts where ferritin immunoreactivity was weak and diffuse. The transporter DMT1 was localised as anticipated to the apical border of the villi in both control and TNF- α treated mice and in all mice only very weak patchy cytoplasmic staining in the crypts was observed.

In control mice there was evidence of both apical and basal cytoplasmic staining of Ireg1 in the villous enterocytes with the latter lost following TNF- α treatment. In the crypts of control mice Ireg1 was predominantly preserved on the basolateral borders. This was almost completely lost with only diffuse cytoplasmic immunoreactivity evident 24 hours after TNF- α treatment.

Perl's staining of small bowel, spleen and liver

To examine whether these changes in expression and localisation of ferritin, DMT1 and Ireg1 lead to an increase in enterocyte iron loading Perl's staining was performed on duodenum from both control and TNF- α treated mice (Figure 7). In the villi of control and 3hr TNF- α treated mice there was no discernable staining, however in the 24 hr TNF- α treated mice Prussian blue staining was clearly observed in the apical poles of the villous enterocytes. In none of the mice was there any evidence of crypt Prussian blue staining.

To determine whether iron loading was confined to the small bowel we chose to examine the two major tissue stores of iron, the spleen and liver (Figure 7). In the spleen there was evidence of iron loading in both the 3hr and 24 hr TNF- α treated mice whilst there was no evidence of iron loading in the liver at either time point.

Discussion

In a previous investigation using a Caco-2 cell model we demonstrated TNF- α modulates iron transport ultimately leading to inhibition of iron export from enterocytes.[18] These effects were local and hepcidin-independent. Our findings added to the evidence for a physiological role for TNF- α in small bowel iron absorption and also suggested a potential mechanism for the inhibition of iron absorption in the context of systemic inflammation. [20]

To further clarify the effects of TNF- α on iron regulation a murine model was utilised in the current investigation. In addition to assessing the effect of TNF- α on iron transporter expression and localisation in enterocytes we examined the simultaneous effects on liver and spleen.

In the present study we have been able to demonstrate a rapid fall in serum iron 3 hours after TNF- α injection which was co-incident with an increase in splenic iron loading. It is likely this effect was caused by increased retention of iron by monocytes in response to TNF- α , as previously alluded to by others.[15,16] As expected there was little or no parenchymal hepatic iron deposition in response to TNF- α .

As in our earlier *in-vitro* study [18] an early induction in both duodenal DMT1 and Ireg1 was followed by later fall to baseline levels along with a late up-regulation of ferritin. The induction of ferritin by TNF- α is a recognised phenomenon and is likely to be an adaptive immune response to bacteraemia.[24] However, the relevance of the early induction of DMT1 and Ireg1 is less obvious. Others have shown a similar increase in DMT1 expression in bronchial epithelial cells in response to TNF- α , IFN- γ and LPS.[25] This increase in DMT1 expression was also paralleled by increased ferritin expression. The authors of that study speculated that the increase in DMT1 expression may be a local effect to “de-toxify” bronchial mucosa by reducing the access of potential micro-organisms to iron in the respiratory tract. Whereas the early induction of iron importing proteins at the intestinal brush border seen in our study may seem counter intuitive, it remains possible that this early response is a similar attempt at local detoxification of small bowel luminal contents. Of note, despite the early up-regulation

of both DMT1 and Ireg1 message and protein no significant change in mucosal transfer of iron was seen at 3 hours in TNF- α treated compared to control mice. This implies that in enterocytes at least, the early induction in DMT1 and Ireg1 protein is non-functional and is supported by the observation that there was no discernible enterocyte iron staining at 3 hours.

The mechanisms underlying the observed effects of TNF- α on iron transporter expression are unclear. Interestingly, the 5' untranslated region of both ferritin and Ireg1 genes reveal putative binding sites for the transcription factors NF- κ B and Sp1. Previous work has already implicated Sp1 in the activation of ferritin expression.[26] Recently two studies in fibroblasts and monocytic cell lines respectively, have demonstrated a co-operative effect of Sp1 and NF- κ B in the TNF- α dependent transcription of monocyte chemo-attractant protein 1.[27,28] It is interesting to speculate whether a similar mechanism could be at play in our study. In contrast, the DMT1 promoter region lacks a NF- κ B binding domain but does contain a putative IFN- γ response element. TNF- α has previously been shown to stimulate IFN- γ production in a number of cell types [29] and it is possible that the effects observed on DMT1 expression may be mediated by secondary IFN- γ release. It is also possible that the increase in DMT1 and Ireg1 mRNA may be secondary to IRP mediated post transcriptional stabilisation. However, IRP mediated stabilisation would normally lead to reciprocal changes in DMT1 and Ireg1 expression.

In the current study at 24 hours expression levels of DMT1 and Ireg1 returned to baseline, whilst ferritin remained significantly induced. As in our previous *in-vitro* observations the ultimate effect of TNF- α was to cause enterocyte iron loading and inhibition of iron absorption, which was independent of hepatic hepcidin expression. We propose these effects are secondary to the induction of intracellular ferritin and the post-translational re-localisation of Ireg1 away from its basolateral compartment. Recently work by Nemeth *et al* [30] has demonstrated that hepcidin also exerts its major effect by binding to Ireg1 and causing re-localisation and loss of function of this iron exporter. It is intriguing, therefore, that although TNF- α and hepcidin appear to act independently in our model their ultimate targets and effects are likely to be similar.

Liu *et al* [31] have shown that LPS causes a post-translational down-regulation of Ireg1 in murine splenic macrophages, which is IL6 and hepatic hepcidin dependent. Interestingly their study also revealed that the transcriptional control of Ireg1 in splenic macrophages was IL6 and hepcidin independent and that hepcidin was also synthesised in the spleen.

The relevance of splenic hepcidin production is unknown. In our study we have demonstrated that TNF- α causes a significant repression in splenic hepcidin mRNA expression at 24 hours. It appears, therefore, that TNF- α is able to cause hypoferraemia independent of both hepatic and splenic hepcidin expression.

In summary we provide evidence that TNF- α can potentially cause ACD by the inhibition of small bowel iron absorption in addition to its documented effects on erythropoeisis and the reticuloendothelial system. This appears to be independent of hepcidin but involves effects on enterocyte ferritin expression and Ireg1 localisation.

The anaemia of chronic disease is an important clinical problem with significant prognostic implications. To date, there have been few direct clinical strategies to treat this problem. It has been proposed that hepcidin antagonists may be developed in the future therapy for this condition.[4] In addition to this, TNF- α antagonists which are in widespread clinical use in conditions such as Crohn's disease and rheumatoid arthritis present a possible future therapy for the anaemia of chronic disease.

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Figure Legends

Figure 1 TNF- α causes a decrease in serum iron levels

6 week old male CD1 mice were subject to intra peritoneal injection with either 0.15M saline (control, n=6) or recombinant murine TNF- α (10 ng/mouse, n=6). At 3 or 24 hrs post intra peritoneal injection blood was collected and serum iron levels determined. * (p<0.05). Error bars= 2 standard errors of the mean

Figure 2 TNF- α mediated repression in mucosal transfer.

Mucosal Transfer (the amount of iron exported out of the duodenum) was determined in control, 3hr and 24 hr TNF- α treated mice. Error bars = 2 standard error of the mean. * (p<0.05) Student's t-test. This data is the mean of three independent experiments each set performed in triplicate.

Figure 3 Modulation of DMT1, Ireg1, ferritin and hepcidin mRNA levels by TNF- α .

6 week old CD1 male mice were injected either with 0.15M saline (Control) or 10ng/mouse of TNF- α . Mice were either sacrificed at 3 hrs or 24hrs post injection and

duodenal mRNA levels of A) DMT1, B) Ireg1 and C) Ferritin determined. (n=6 for each time point). *(p<0.05). Error bars= 2 standard errors of the mean.

Figure 4 Modulation of hepcidin mRNA levels by TNF- α .

6 week old CD1 male mice were injected either with 0.15M saline (Control) or 10ng/mouse of TNF- α . Mice were either sacrificed at 3 hrs or 24hrs post injection and hepatic (A) and splenic (B) mRNA levels of hepcidin were determined (n=6 for each time point). *(p<0.05). Error bars= 2 standard errors of the mean.

Figure 5 Protein expression of DMT1, Ireg1, ferritin and TFR1 in response to TNF α stimulation

At 3 hours there is significant increased expression of DMT1 and Ireg1 protein (P<0.05). This is not maintained for 24 hours. At 24 hours there is an increase in ferritin levels (p<0.05). There was no change seen in TFR1 expression. Cytokeratin 19 (CK19) was employed to ensure normalisation of epithelial loading.

Figure 6 Immunolocalisation of ferritin, DMT1 and Ireg1 in control and TNF- α challenged mice.

Control (n=6) and 24hr TNF- α treated mice (n=6) were sacrificed and whole duodenum processed into paraffin blocks, sectioned and subject to immunohistochemistry with antibodies to DMT1 (NRAMP24A), Ireg1 (MTP11-A) and ferritin (ab16875).

Ferritin in control mice was localised predominantly to the apical pole of the villous enterocytes whilst in the TNF- α treated mice immunoreactivity was observed throughout the cytoplasm of all villous enterocytes. Ferritin immunoreactivity was weak and diffuse in all crypts of both control and TNF- α treated mice.

DMT1 was localised to the apical border of the villi in both control and TNF- α treated mice as anticipated. However, in all mice only very weak patchy cytoplasmic staining was observed in the crypts.

Ireg1 was observed in both apical and basal cytoplasmic compartments in control mice whilst in TNF- α treated mice there was mostly apical cytoplasmic staining with little evidence of basal immunoreactivity. In the crypts of control mice Ireg1 was preserved on the basolateral borders. This basolateral immunoreactivity was almost completely

lost in the TNF- α treated mice with only diffuse cytoplasmic immunoreactivity evident. (Original magnification x 60).

Figure 7 Enterocyte Iron loading in TNF- α treated (24 hr) mice

Control mice showed no evidence of Prussian blue staining in duodenum, spleen or liver. In both 3hr and 24hr TNF- α treated mice an abundance of Prussian staining was observed in both duodenal enterocytes and the spleen. However, no hepatic iron loading was observed in TNF- α treated mice. (Original Magnification x40).

Figure 1

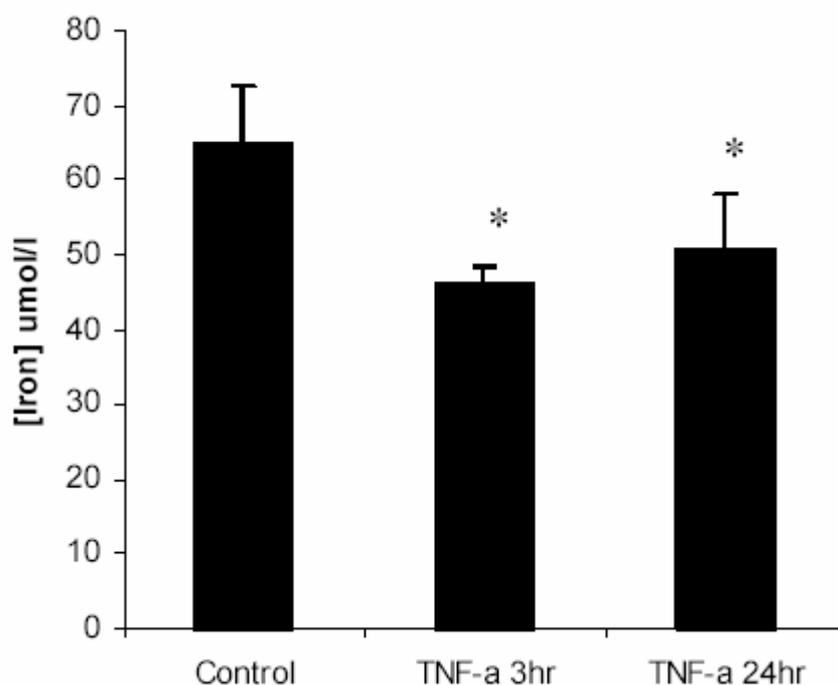


Figure 2

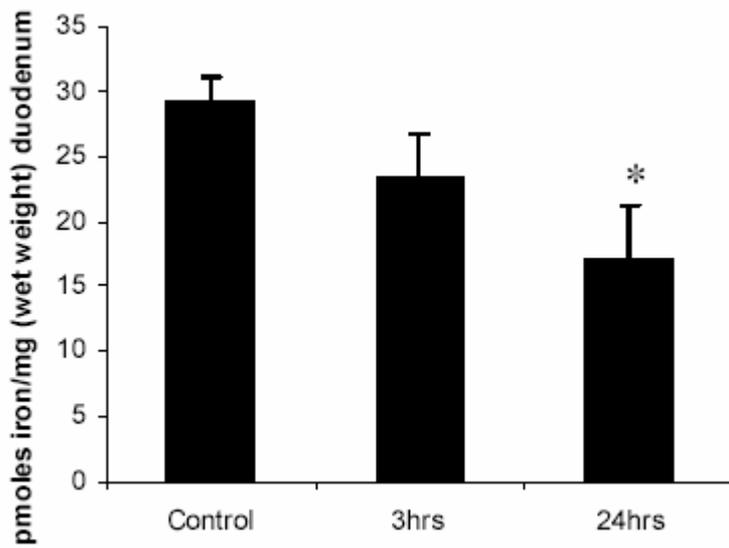


Figure 3

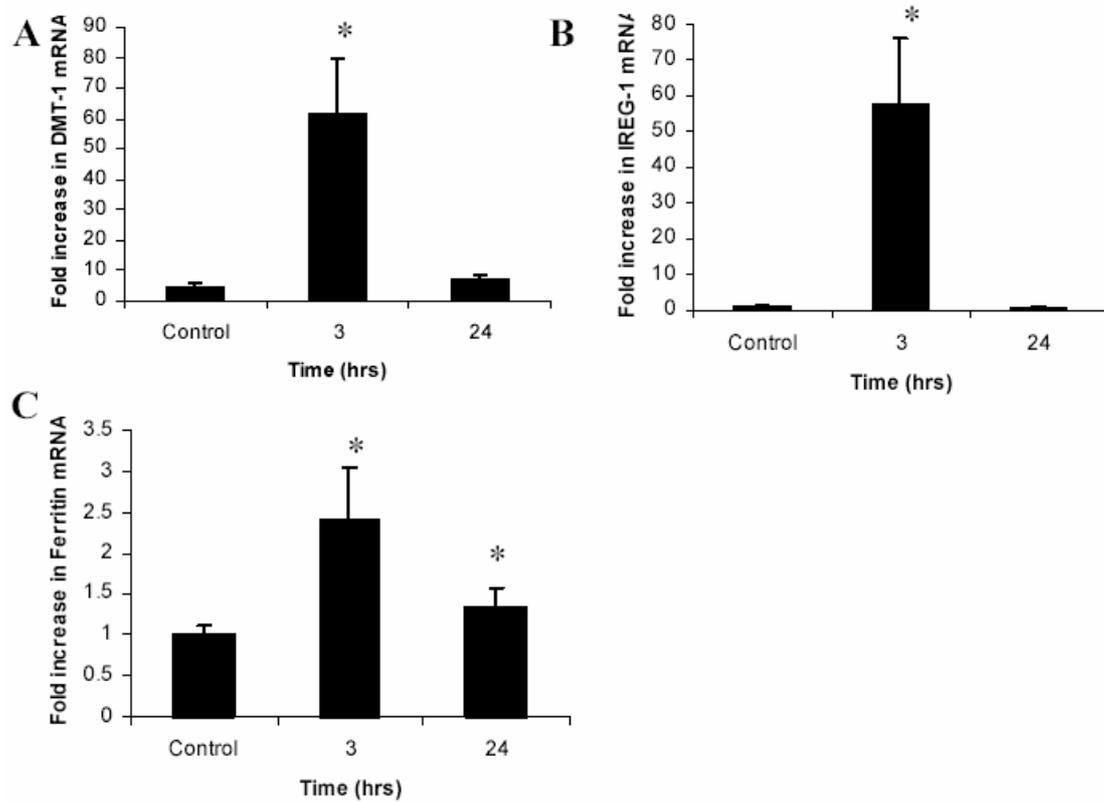


Figure 4

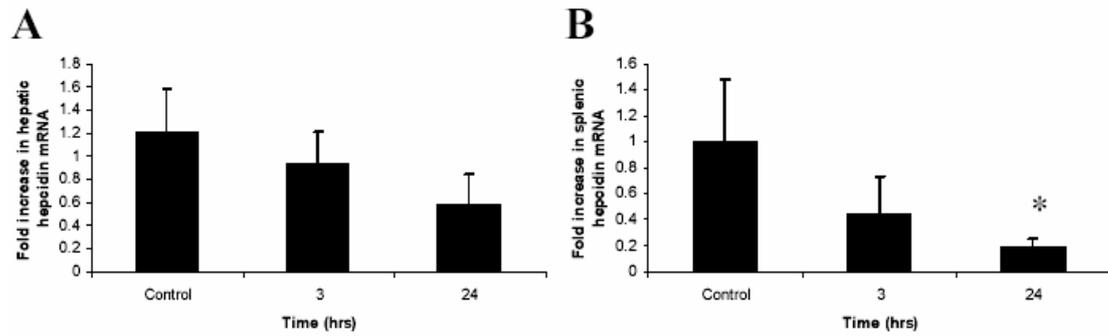


Figure 5

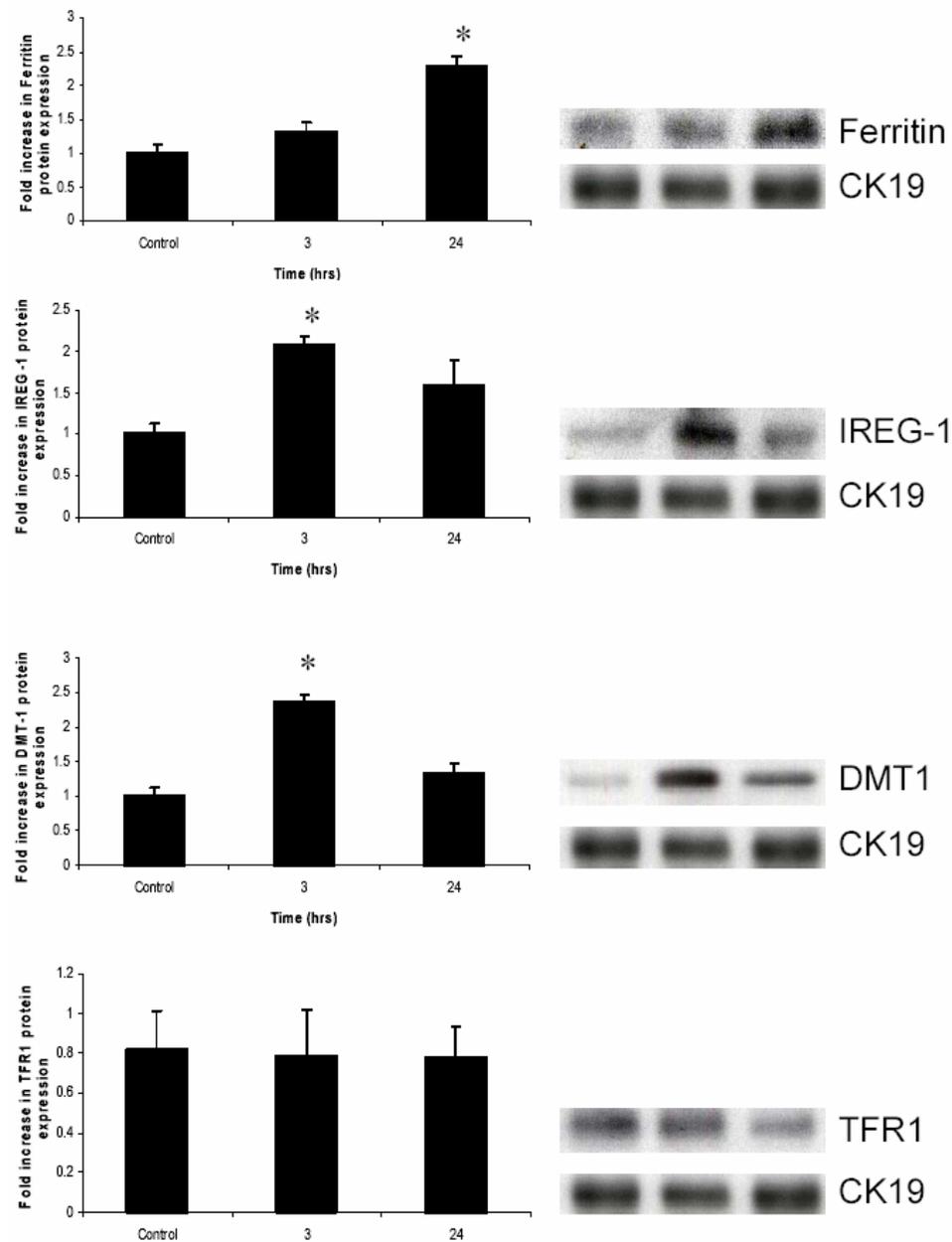


Figure 6

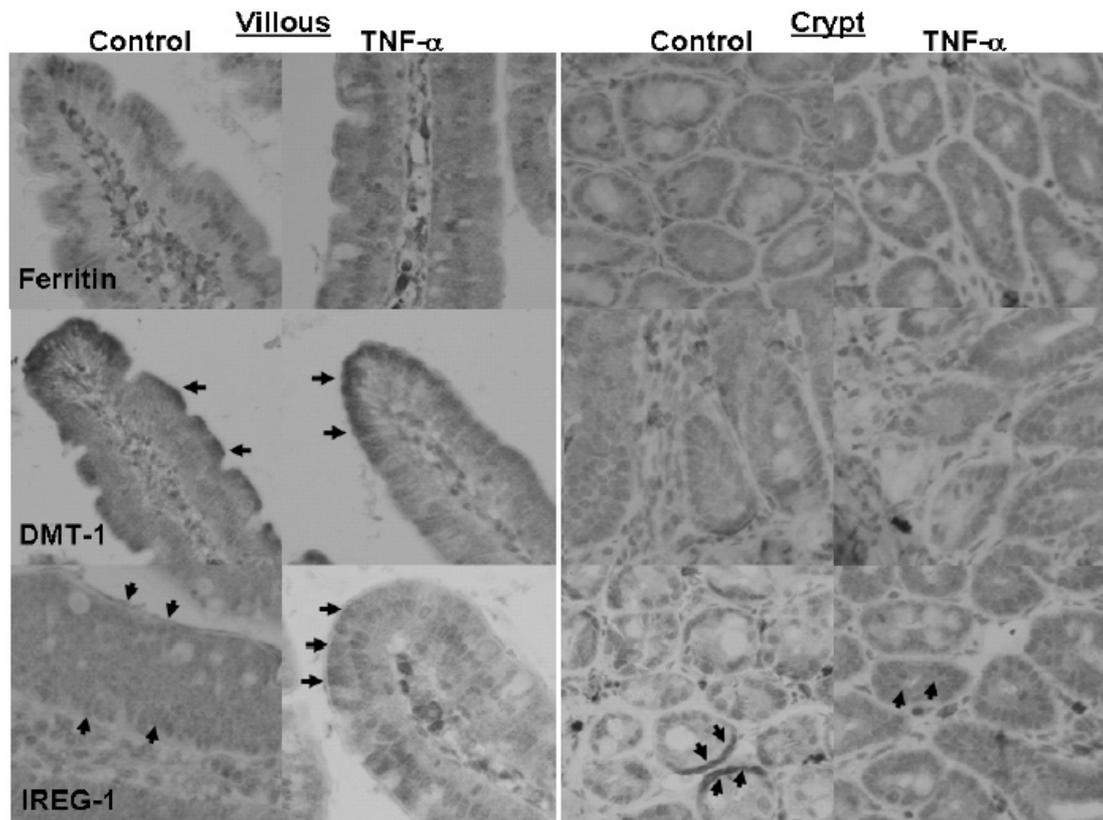


Figure 7

