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Superoxide dismutase from *Helicobacter pylori* suppresses the production of pro-inflammatory cytokines during *in vivo* infection

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Running Head: *Helicobacter pylori* superoxide dismutase suppresses pro-inflammatory cytokines during infection

ABSTRACT

BACKGROUND: *Helicobacter pylori* has undergone considerable adaptation to allow chronic persistence within the gastric environment. While *H. pylori*-associated diseases are driven by an excessive inflammation, severe gastritis is detrimental to colonization by this pathogen. Hence, *H. pylori* has developed strategies to minimize the severity of gastritis it triggers in its host. Superoxide dismutase (SOD) is well known for its role in protecting against oxidative attack; less recognized is its ability to inhibit immunity, shown for SOD from mammalian sources and those of some bacterial species. This study examined whether *H. pylori* SOD (HpSOD) has the ability to inhibit the host immune response to these bacteria.

MATERIALS AND METHODS: The ability of recombinant HpSOD to modify the response to LPS was measured using mouse macrophages. A monoclonal antibody against HpSOD was generated and injected into *H. pylori*-infected mice.

RESULTS: Addition of HpSOD to cultures of mouse macrophages significantly inhibited the proinflammatory cytokine response to LPS stimulation. A monoclonal antibody was generated that was specific for SOD from *H. pylori*. When injected into mice infected with *H. pylori* for 3 months, this antibody was readily detected in both sera and gastric tissues five days later. While treatment with anti-HpSOD had no effect on *H. pylori* colonization at this timepoint, it significantly increased the levels of a range of proinflammatory cytokines in the gastric tissues. This did not occur with antibodies against other anti-oxidant enzymes.

CONCLUSIONS: SOD from *H. pylori* can inhibit the production of proinflammatory cytokine during *in vivo* infection.

INTRODUCTION

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Helicobacter pylori is an important pathogen of the human stomach that has undergone considerable adaptation to facilitate chronic persistence within the gastric environment. This infection triggers a chronic and often severe inflammatory response which is the cause of the main associated pathologies, but excessive inflammation is detrimental to *H. pylori* colonization and gastric bacterial numbers are often decreased in cases of severe gastritis.¹

Presumably for this reason, *H. pylori* has evolved a range of structural modifications in order to minimize the inflammatory response mounted by its host, including alterations to its lipopolysaccharide and flagellin that have resulted in greatly reduced abilities to activate the innate immune receptors Toll-Like Receptor 4 (TLR4) and TLR5 respectively.^{2, 3} The activation of innate immune receptors is an important driver of inflammation, so reducing signaling by these pathways is believed to limit the severity of the gastritic response to *H. pylori* infection, thereby facilitating chronic colonization.

H. pylori also requires attributes that enable it to withstand a range of environmental insults, including low pH from the stomach and attack from reactive oxidative and nitrogen species (ROS and RNS). To survive in this harsh environment, *H. pylori* utilizes a number of different enzymes, including the acid-neutralizing enzyme urease, and the anti-oxidant enzymes superoxide dismutase (SOD), catalase and thiol-peroxidase (Tpx).^{4, 5}

The SOD enzyme protects against superoxide free radicals by converting them into hydrogen peroxide, which is then broken down by catalase into oxygen and water. SOD is therefore commonly considered a virulence factor for many pathogenic bacteria, due to its role in facilitating survival in the host by protecting against oxidative attack.

In the case of *H. pylori*, this would include surviving oxygen free radicals released by neutrophils recruited to the gastritic mucosa. SOD can be differentiated into several families, distinguished by their metal cofactor; SOD cofactored to copper and zinc is designated CuZn-SOD, and this family occurs primarily in eukaryotes. SOD produced by prokaryotic cells generally uses a manganese or iron (Fe) catalytic cofactor.⁶ *H. pylori* is somewhat unusual in only possessing a single Fe-SOD; three different isoforms have been characterized, although they are only differentiated by minor amino acid substitutions and all have the same activity.⁷ *H. pylori* deficient in superoxide dismutase (HpSOD) have an increased susceptibility to oxidative stress and accumulate more free iron *in vitro*, although this was not specific for SOD and also occurs for *H. pylori* lacking other oxidative stress-defensive enzymes including catalase and alkyl hydroperoxide reductase.⁸

In some bacteria, SOD is believed to do more than simply protect against ROS. For example, a number of publications have indicated that *Mycobacterium tuberculosis* may use SOD as a means to suppress the host immune response.^{9, 10} This observation, led us to speculate whether HpSOD may play a similar role in reducing inflammation during *H. pylori* infection, particularly as this enzyme is produced in abundance, has an extracellular presence and is required for colonization of mice as infectivity is reduced when a mutant strain which lacks HpSOD is used.¹¹ In this study, we therefore explored the effect of recombinant HpSOD on the macrophage response to immune stimulation and generated a monoclonal antibody against HpSOD in order to test the hypothesis that HpSOD can modify the host immune response to *H. pylori in vivo*, using a mouse model of infection.

MATERIALS AND METHODS

Helicobacter pylori enzymes

Recombinant HpSOD, catalase and Tpx proteins from *H. pylori* were produced to a high purity as previously described.^{12, 13} The superoxide dismutase activity of HpSOD was measured using a pyrogallol autoxidation inhibition assay, wherein autoxidation and superoxide generation of pyrogallol in a basic EDTA-containing solution is inhibited by the addition of superoxide dismutase. The assay was performed generally as previously described.¹⁴ In brief, 0.3mM pyrogallol (Sigma-Aldrich, Castle Hill, Australia) was added to 50mM Tris-HCl (pH 7.9) containing 1mM EDTA. This initiated autoxidation of the pyrogallol. Some samples also contained a specific concentration of HpSOD which, if active, would inhibit this process. Dithiothreitol (30mM; Sigma-Aldrich) was added after 12 minutes to halt the reaction. Autoxidation was assessed by measuring the change in absorbance at 420nm.

Peritoneal macrophage stimulation assay

Non-induced peritoneal macrophages were obtained from female C57BL/6 mice by flushing the abdomen with ice-cold PBS. Cell suspensions in complete RPMI medium (Invitrogen, Carlsbad, USA), containing 10% (v/v) fetal calf serum, penicillin-streptomycin (Invitrogen) and 1% (v/v) amphotericin B (Sigma-Aldrich) were plated out at 2×10^5 /well in 96-well flat-bottomed plates. Macrophages in some wells were stimulated with lipopolysaccharide (LPS,

Sigma-Aldrich) added to a final concentration of 40 ng/mL, with or without addition of endotoxin-free HpSOD (produced as previously described).¹³ The plates were incubated at 37°C, 5% CO₂ for 18 hours before removal of culture supernatants for measurement of cytokines by ELISA.

Generation of monoclonal antibodies against *H. pylori* enzymes

To generate monoclonal antibodies against HpSOD, *H. pylori* catalase or *H. pylori* Tpx, BALB/c mice were inoculated subcutaneously using 5µg of each recombinant protein in 100µL of Freund's complete adjuvant (Sigma-Aldrich). Two weeks later, splenocytes from these mice were mixed with SP-2 myeloma cells at a ratio of 10:1. Hybridization was induced by slow addition of polyethylene glycol 4000 (61% w/v) in RPMI containing 14.5% (v/v) dimethylsulphoxide. Hybridized cells were then cultured on thymocyte-incubated plates in complete RPMI containing HAT (hypoxanthine-aminopterin-thymidine) supplement. After 10 days incubation, positive hybridomas were selected by performing antigen-specific ELISA using supernatants from the cell cultures. Subcloning of positive wells was performed three times by serial dilution of cell cultures.

Following subcloning, hybridoma clone cultures were amplified for supernatant production in complete RPMI using a miniPERM[®] system (Heraeus Instruments GmbH, Hanau, Germany) according to manufacturer's directions. Antibody was purified from the culture supernatant using protein G chromatography (Thermo Scientific, Waltham, USA) and eluted antibody was transferred into phosphate buffered saline (PBS) using a PD-10 desalting column (GE Healthcare, Buckinghamshire, UK). Immunoglobulin concentration was determined by spectrophotometry using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific).

Bacterial culture

H. pylori (two clinical isolates and mouse-adapted strain SS1),¹⁵ *Helicobacter felis* (CS1),¹⁶ *Helicobacter bilis* (ATCC 51630),¹⁷ and *Citrobacter rodentium* (ICC169) were grown initially on horse blood agar plates at 37°C under microaerophilic (*Helicobacter* spp.) or aerobic (*C. rodentium*) conditions. Cultured organisms were then grown in brain heart infusion broth containing 5% (v/v) horse serum (Sigma-Aldrich) and 1% (v/v) Amphotericin B (Sigma-Aldrich) for 24 hours at 37°C under microaerophilic or aerophilic conditions as above.

Western blots

Bacteria were washed with PBS, centrifuged and their pellets freeze-thawed three times. The bacterial pellets were then lysed by sonication on ice, using a Microson XL 2000 ultrasonic liquid processor (Misonix, Farmingdale, USA). A BCA protein assay (Thermo Scientific) was performed on the lysates, which were diluted to 1 mg/mL and stored at -80°C until required.

For Western blotting, polyacrylamide gel electrophoresis was essentially performed as previously described [32]. Separated lysates were transferred onto Hybond-ECL™ nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) using a Bio-rad Mini Protean II Western blot system. The membrane was blocked with 3% bovine serum albumin (BSA, Sigma–Aldrich) overnight, prior to incubation with 100 ng/mL of purified monoclonal antibodies in 1% BSA for 1 h. After washing, signal was generated by incubating the membrane for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG Fc (Immunopure®, Pierce Biotechnology, USA), diluted 1 in 20,000 in 1% BSA. Signal was visualized with ECL™ Western Blotting Detection Reagents (GE Healthcare).

Infection of mice

C57BL/6 mice were infected with a single dose of 10^7 *H. pylori* SS1 in 100 µL PBS, administered via orogastric gavage. Three months post-infection, mice were injected intraperitoneally with 200 µg of monoclonal antibodies against HpSOD, catalase or Tpx, while controls received an isotype control (anti-sheep CD4 IgG1, Centre for Animal Biotechnology, University of Melbourne). After five days, mice were killed and samples collected for analysis. Sera were collected via cardiac puncture. One half of each stomach was collected for quantification of *H. pylori* colonization by colony-forming assay and cytokine ELISAs. The gastric surface mucus was gently scraped from the other half of the stomach and diluted 1 in 10 in Complete Mini EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), for the analysis of specific antibody levels by ELISA.

ELISAs

For antibody ELISAs, 96-well Maxisorp plates (Nalgene, Franklin Lakes, USA)) were coated overnight at 4°C with 50 µL of 5 µg/mL recombinant *H. pylori* antigen diluted in a buffer of 60mM NaHCO₃ (PDH, Poole, England) and 30mM Na₂CO₃ (Sigma-Aldrich) at pH 9.6. Plates were blocked with 1% (w/v) bovine serum albumin (BSA) in PBS, and mucus scrapings (1:10 then serially diluted 1:4 in 1% BSA/PBS) or sera (1:100 then serially diluted 1:10 in 1% BSA/PBS) added to the wells. After washing, wells were incubated for 1 hour at room temperature with 50 µL of goat anti-mouse IgG-HRP (1:5000; Southern Biotech, Birmingham, USA). Color was developed by addition of 3,3',5,5-tetramethylbenzidine (TMB) (Invitrogen, Camarillo, CA), and the reaction stopped with 2 mol/L H₂SO₄. Absorbance was read at 450 nm and end point titers calculated.

Cytokine concentrations in gastric tissues were determined by sandwich ELISA. ELISA plates were coated with 50 µL of primary anti-mouse cytokine antibody (as indicated in Table 1), blocked with 1% (w/v) BSA in PBS then incubated overnight with 50 µL of stomach homogenate. After washing, wells were incubated with secondary anti-mouse cytokine antibody (Table 1), then incubated with 50 µL of streptavidin-HRP (R&D Systems) at 1:200 dilution. Color was developed as in the previous section. Cytokine concentrations were calculated based on standard curves generated from serial dilutions of recombinant cytokines.

Statistics

Data were log transformed and analyzed by one-way analysis of variance (ANOVA) using IBM SPSS Statistics version 23; p<0.05 was considered significant.

RESULTS

H. pylori* SOD suppresses MIP-2 production by activated macrophages *in vitro

To assess the potential effect of SOD from *H. pylori* on the inflammatory response, we first stimulated mouse peritoneal macrophages with LPS in the presence or absence of recombinant HpSOD, which was shown to be enzymatically active (Figure 1a). The secretion of MIP-2 (the mouse functional homologue of IL-8 and a major cytokine produced during *H. pylori*-induced gastritis) was measured as an indicator of inflammation. The addition of HpSOD to this stimulation assay significantly reduced the LPS-induced secretion of MIP-2 in

a dose-dependent manner (Figure 1b). These data indicated that HpSOD could exert anti-inflammatory activity on immune cells.

H. pylori* SOD suppresses cytokine production in response to *H. pylori* infection *in vivo

To investigate whether the *in vitro* observation made in Figure 1 would translate to effects *in vivo*, we generated a monoclonal antibody against HpSOD (clone 1F11). Characterization of the anti-HpSOD 1F11 antibody showed that it had specificity for SOD from a range of different *H. pylori* strains (including fresh clinical isolates, a laboratory strain and the mouse-adapted strain SS1) and cross-reacted with the closely related *H. felis*, but did not bind the same enzyme from *H. bilis* or the intestinal bacterial pathogen, *C. rodentium* (Figure 2). This narrow specificity range indicated its suitability for subsequent *in vivo* studies. As controls, we also generated and tested monoclonal antibodies against the other *H. pylori* anti-oxidative enzymes catalase (2E11) and Tpx (H1.1); these had similar specificities except the H1.1 antibody which did not detect *H. felis* Tpx (Figure 2).

The 1F11 anti-HpSOD antibody was then used to examine whether HpSOD modified the production of inflammatory cytokines in response to *H. pylori* infection *in vivo*. Mice infected with *H. pylori* for three months were injected intraperitoneally with anti-HpSOD mAb, while control mice either received the antibodies specific for catalase and thiol peroxidase or an isotype control. Five days later, sera and stomachs were collected for analysis. Significant levels of antibodies against all enzymes were present in the sera and gastric secretions of the respective recipient mice (Figure 3a), indicating successful delivery and transfer of mAbs to the site of *H. pylori* infection.

It has been shown previously that SOD-deficient *H. pylori* mutants are defective in their ability to colonize mice.¹⁸ In the current study, neither anti-HpSOD treatment, nor the other antibodies tested, had any significant effect on the colonization levels of an established *H. pylori* infection (Figure 3b), at least within the timeframe of this experiment.

In contrast, the gastric tissues from *H. pylori* infected mice treated with the anti-HpSOD monoclonal antibody, but not with antibodies against catalase or Tpx, contained significantly increased levels of a range of gastric cytokines, relative to the isotype control treated group. These included the macrophage-related pro-inflammatory cytokines MIP-2, KC and TNF α , the pleiotropic cytokine IL-6, the Th1-type cytokine IFN γ and the Th2-type cytokine IL-13 (Figure 4). Levels of the Th17-type cytokines IL-17A and IL-17F remained unchanged

regardless of treatment (Figure 4). Hence, these data show that SOD production by *H. pylori* suppresses the secretion of a large number of pro-inflammatory cytokines during gastric infection.

DISCUSSION

The inflammatory response to *H. pylori* is both a key feature of its infection and the cause of its resulting pathogenicity. In this study we present data indicating that superoxide dismutase produced by this bacterium can reduce the production of inflammatory cytokines. *In vitro* we showed that HpSOD can dampen the MIP-2 response of murine macrophages to LPS stimulation. Moreover, the administration of an anti-HpSOD monoclonal antibody to chronically infected mice resulted in a broad increase in cytokine production within the stomach, comprising both proinflammatory cytokines as well as Th2-associated IL-13. This effect was completely specific for anti-HpSOD treatment, as the injection of antibodies targeting other antioxidant enzymes had no effect. This suggests that regulation of the immune response is a specific function of HpSOD rather than a broader effect on the redox environment. Although no increase in gastritis was observed in this study, this was likely due to the short length of treatment that available antibody allowed. It is possible, given the observed increased levels of proinflammatory cytokines in the stomach, that sustained treatment of *H. pylori* infected mice with anti-HpSOD antibodies would result in increased severity of gastritis.

The anti-SOD monoclonal antibody used in this study displayed a relatively high degree of species specificity, with antibody binding observed with the gastric helicobacters *H. pylori* and *H. felis*, but not the enteric helicobacter *H. bilis*, nor an unrelated enteric Proteobacteria *C. rodentium*. Pairwise sequence alignment indicates only 14% homologous identity between HpSOD and both human and mouse extracellular SODs, compared with 65% homology between HpSOD and *H. bilis* SOD (Needleman-Wunsch Global Align Protein Sequences, NCBI). Thus, the observed effects would indeed be expected to derive from specific activity against HpSOD rather than, for example, the production of a cross-reactive effect against, in this case, mouse SOD.

Although the anti-HpSOD antibody appeared to inhibit the anti-inflammatory effect of SOD *in vivo*, there was no difference in *H. pylori* colonization between treated and untreated mice. While a previous study indicated that SOD-deficient *H. pylori* mutants are incapable of

gastric colonization,¹⁸ these bacteria lacked both intracellular and extracellular SOD, which completely eliminates oxidative defenses. In our current *in vivo* trial, only the extracellular fraction of the enzyme would be affected by antibody treatment suggesting that the inability of SOD mutant *H. pylori* to colonize the mouse stomach results from a deficiency in intracellular SOD, possibly reflecting an inability to neutralize endogenous superoxide production. Moreover, this finding also supports the notion that the release of HpSOD during infection by *H. pylori* may be a mechanism to control the severity of gastritis, rather than directly facilitating the organism's survival within the gastric mucosa.

Immunosuppressive effects of SOD derived from other bacteria, specifically *Mycobacterium tuberculosis*, have also been described. For example, infection of mice with Fe-SOD-impaired mutant strains of *M. tuberculosis* induced a greater inflammatory response than did wild-type bacteria strains,⁹ while Bacillus Calmette-Guérin (BCG) vaccine strains that overexpress Fe-SOD failed to induce protective immunity against *M. tuberculosis*.¹⁰ The Fe-SODs of *H. pylori* and *M. tuberculosis* are both present extracellularly, despite the lack of membrane localization sequences.^{19, 20} Both organisms are instead thought to passively release SOD during bacterial autolysis. Thus, while the primary role of HpSOD is currently believed to be defense against free radical damage, extracellular HpSOD may also have an important secondary immunosuppressive function that regulates the inflammatory response following colonization. Indeed, it is possible that SOD-induced immunosuppression plays a key role in the ability of both *H. pylori* and *M. tuberculosis* to establish chronic infections. SOD appears to be capable of exerting an anti-inflammatory effect even when located at a mucosal surface, as occurs during *H. pylori* infection. Using a variety of experimental colitis models, three independent studies have shown that administration of *Lactobacillus spp.* expressing heterologous SOD can significantly reduce colonic inflammation.²¹⁻²³

The main evidence supporting an anti-inflammatory effect has come from the study of mammalian SOD. The role of the superoxide anion, and by association SOD, in inflammation has been most comprehensively studied in neutrophils. Stimulation of neutrophils by a xanthine oxidase/hypoxanthine superoxide-generating system resulted in a significant increase in their secretion of the proinflammatory cytokines MIP-2 and TNF- α .²⁴ Moreover, these effects were also shown *in vivo*, when the generation of superoxide by injection of mice with xanthine/xanthine oxidase resulted in an increased local neutrophil influx that, significantly, could be abolished by the introduction of SOD.²⁵ The presence of extracellular mammalian Cu/Zn SOD has also been correlated with reduced inflammation following

exposure to LPS,²⁶ and the failure of neutrophil-mediated clearance of *Listeria monocytogenes*.²⁷

There is also some evidence that SODs can affect macrophage activity. MIP-2 production by LPS-stimulated mouse macrophages is attenuated by extracellular Cu/Zn SOD,²⁶ and the data presented here indicate the same effect occurs with HpSOD. Although the specific anti-inflammatory mechanism of SOD is not completely understood, it is thought that the superoxide radical is able to activate the key cytokine transcription master regulator NF- κ B, since superoxide stimulation enhances NF- κ B activity, and this effect is neutralized by mammalian SOD.^{24, 28}

Together, these data, and particularly the selective cytokines affected by HpSOD neutralization, provide pointers towards the mechanism behind the activity of this enzyme *in vivo*. It is notable that treatment of *H. pylori* infected mice with anti-HpSOD antibody affected cytokines such as MIP-2, TNF- α , IL-6 and IL-13 but not the IL-17 cytokines. Interestingly, the cytokines that were affected can all be produced by NF- κ B-dependent pathways, for example following TLR activation. In contrast however IL-17 cytokines, which were not increased, are typically not induced via NF- κ B but rather mediate their effects by inducing activation of an NF- κ B pathway.^{29, 30} Our new results are therefore consistent with HpSOD being secreted by *H. pylori* in order to down-regulate NF- κ B activation pathways with resulting reduction in the production of associated proinflammatory cytokines. Given that severe gastritis is detrimental to *H. pylori* colonization,¹ this is likely to be a strategy used by these bacteria to allow chronic colonization of its host.

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FIGURE LEGENDS

FIGURE 1: HpSOD inhibits the secretion of MIP-2 by LPS-stimulated macrophages

a) Recombinant HpSOD was shown to be enzymatically active by its ability to inhibit the autooxidation of pyrogallol in a dose dependent manner. b) Peritoneal macrophages (5×10^5 /mL) from C57BL/6 mice (n=3) were stimulated with LPS (40 ng/mL) for 18 hours, then MIP-2 concentrations in culture supernatants quantified by ELISA. Results show individual data points and mean concentrations. Addition of HpSOD (60 or 120 μ g/mL) significantly inhibited the secretion of MIP2 in response to LPS stimulation (* ANOVA).

FIGURE 2: Representative reactivity of monoclonal antibodies against lysates from different bacterial species and strains

Purified monoclonal antibodies against *H. pylori* SOD (clone 1F11), catalase (2E11) or thiol peroxidase (H1.1) (all IgG1 at 100 ng/mL) were incubated with various bacterial lysates that had been subjected to SDS-PAGE and then transferred to nitrocellulose by western blot. After washing, membranes were incubated with a HRP-conjugated goat anti-mouse IgG secondary antibody, before developing with an ECLTM Western Blotting Detection Kit. Samples 1 and 2 were clinical isolates of *H. pylori*.

FIGURE 3: Injection of monoclonal antibodies against *H. pylori* enzymes into *H. pylori* infected mice

Groups of C57BL/6 mice (n=6/7), infected three months earlier with 10^7 *H. pylori* SS1, were injected intraperitoneally with an IgG1 monoclonal antibody (mAb) against either *H. pylori* superoxide dismutase (HpSOD; clone 1F11, n=6), catalase (Kat; clone 2E11, n=6) or thiol peroxidase (Tpx; clone H1.1, n=6). Infected control mice were treated with isotype control antibody (anti-sheep CD4 IgG1, n=7). Five days later, samples were collected for analysis.

A) Antibody levels in gastric scrapings and sera were determined by ELISA. All three antibodies were specifically detected in the sera and gastric mucosal secretions of mice receiving injections of those antibodies (*one-way ANOVA on log-transformed end-point titers).

B) *H. pylori* colonization levels were quantified by colony-forming assay (CFU, colony-forming units). The antibody treatments had no significant effect on *H. pylori* colonization (*one-way ANOVA on log-transformed CFU).

FIGURE 4: Gastric cytokine levels in *H. pylori* infected mice treated with anti-HpSOD

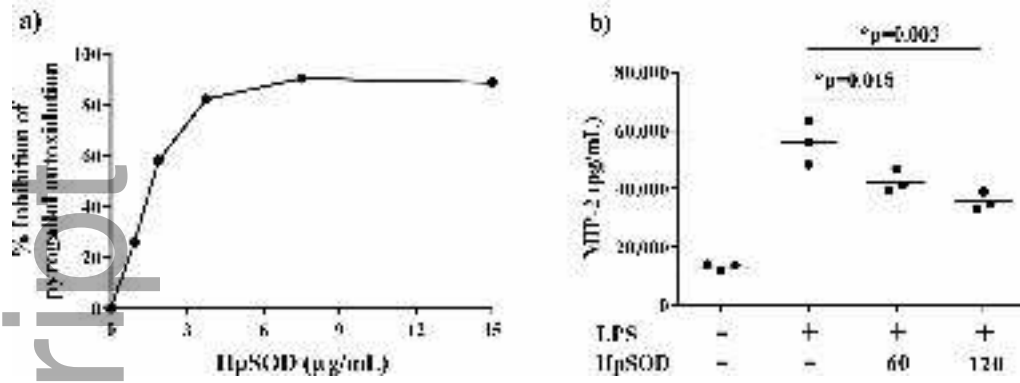
Groups of C57BL/6 mice (n=6/7), infected three months earlier with 10^7 *H. pylori* SS1, were injected intraperitoneally with an IgG1 monoclonal antibody against either *H. pylori* superoxide dismutase (HpSOD; clone 1F11, n=6), catalase (Kat; clone 2E11, n=6) or thiol

peroxidase (Tpx; clone H1.1, n=6). Infected control mice were treated with isotype control antibody (anti-sheep CD4 IgG1, n=7). Five days later, half stomachs were homogenized and cytokine levels quantified by ELISA. Stomachs from mice treated with anti-HpSOD had significantly higher levels of TNF- α , IL-6, KC, MIP-2, IFN- γ and IL-13 compared with those from infected mice receiving isotype controls (*ANOVA). Neither the anti-catalase nor anti-thiol peroxidase monoclonal antibodies had any significant effect on cytokine concentrations.

TABLE 1: Source of cytokine ELISA antibodies

Cytokine	Primary Antibody	Secondary Antibody	Supplier
IFN γ	Rat anti-mouse IFN γ (2 μ g/mL)	Biotin rat anti-mouse IFN γ (1 μ g/mL)	BD Pharmingen (Franklin Lakes, NJ)
IL-6	Rat anti-mouse IL-6 (1 μ g/mL)	Biotin rat anti-mouse IL-6 (0.5 μ g/mL)	eBioscience (San Diego, CA)
IL-13	Rat anti-mouse IL-13 (1 μ g/mL)	Biotin rat anti-mouse IL-13 (0.5 μ g/mL)	eBioscience
IL-17A	Rat anti-mouse IL-17A (1 μ g/mL)	Biotin rat anti-mouse IL-17A (0.5 μ g/mL)	eBioscience
IL-17F	Goat anti-mouse IL-17F (0.8 μ g/mL)	Biotin goat anti-mouse IL-17F (0.2 μ g/mL)	R&D Systems
MIP2	Rat anti-mouse CXCL2 (2 μ g/mL)	Biotin goat anti-mouse CXCL2 (0.075 μ g/mL)	R&D Systems
KC	Rat anti-mouse CXCL1 (2 μ g/mL)	Biotin goat anti-mouse CXCL1 (0.2 μ g/mL)	R&D Systems
TNF α	Rat anti-mouse TNF α (2 μ g/mL)	Biotin rat anti-mouse TNF α (0.25 μ g/mL)	Biolegend (San Diego, CA)

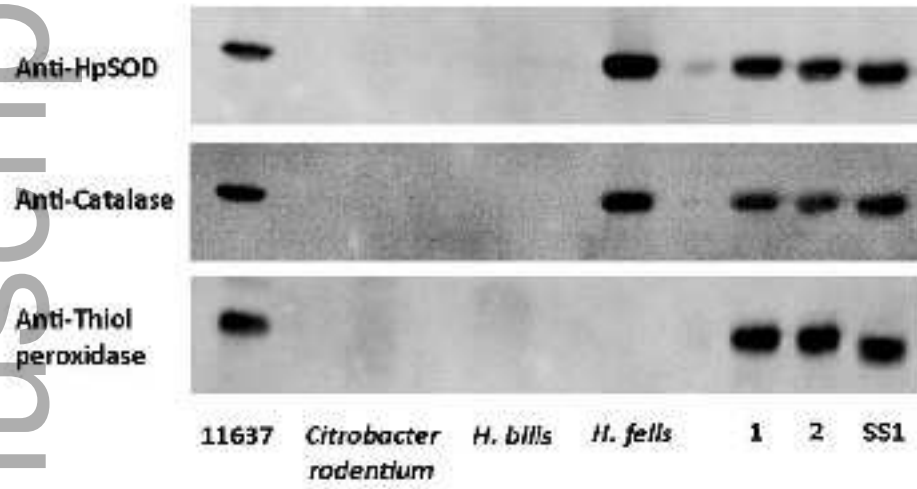
Figure 1



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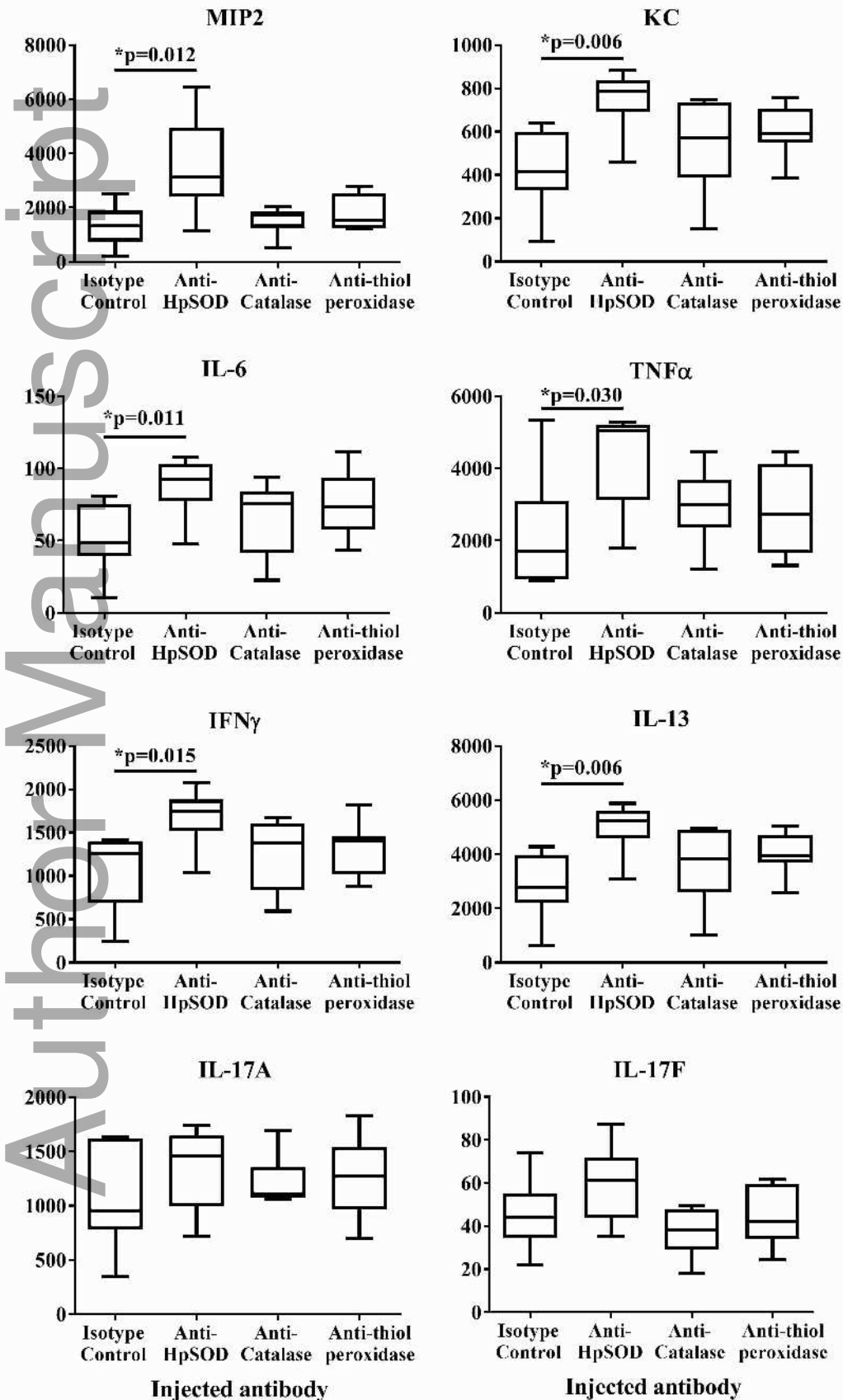
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Figure 2



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Figure 4



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