Targeting the “Rising DAMP”

during a *Francisella tularensis* infection.

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Abstract

Antibiotic efficacy is greatly enhanced the earlier it is administered following infection with a bacterial pathogen. However, in a clinical setting antibiotic treatment usually commences following the onset of symptoms, which in some cases (e.g. Biothreat Agents) may be too late.
In a BALB/c murine intra-nasal model of infection for Francisella tularensis SCHU S4 (F. tularensis) infection, we demonstrate during a time course that pro-inflammatory cytokines and the damage associated molecular pattern, HMGB1, were not significantly elevated above naive levels in tissue or sera until 72h post-infection. HMGB1 was identified as a potential therapeutic target that could extend the window of opportunity for the treatment of tularemia with antibiotics.

Antibodies to HMGB1 were administered in conjunction with a delayed/sub-optimal levofloxacin treatment of F. tularensis. We found in the intranasal model of infection that treatment with anti-HMGB1 antibody, when compared to an isotype IgY control antibody, conferred a significant survival benefit and decreased bacterial loads in the spleen and liver but not the lung (primary loci of infection) four days into infection. We also observed an increase in the production of interferon-γ in all tested organs.

This data demonstrates that treatment with anti-HMGB1 antibody is beneficial in enhancing the effectiveness of current antibiotics in treating tularemia. Strategies, of this type, involving antibiotics in combination with immuno-modulatory drugs are likely to be essential for the development of a post-exposure therapeutic for intracellular pathogens.
Introduction

*Francisella tularensis* is a Gram-negative intracellular bacterium that has the ability to infect several different types of cell and is the causative agent of the disease tularemia [1,2]. Four subspecies of *F. tularensis* have been identified, each varying in the degree of pathogenicity and virulence. *F. tularensis* subspecies *tularensis* (type A strains) are considered to be the most virulent in man and have a low infectious dose of between 1-10 colony forming units (CFU) in humans [3]. *F. tularensis* SCHU S4 is a type A strain with a high mortality rate of between 30-40% if left untreated and has been listed as a Category A biothreat agent as a consequence [4–7].

Current treatments for tularemia are limited. A Live Vaccine Strain (LVS), which is an attenuated form of *F. tularensis* subspecies *holarctica* (or type B strain), has proved to be an effective vaccine. However, as the nature of the attenuation has yet to be defined, LVS remains unlicensed, meaning there is no licensed vaccine available to protect against *F. tularensis* [8,9]. Therefore at present, antibiotics represent the only clinically available treatment for tularemia. The levels of protection afforded by antibiotics are highly dependent upon timely administration following infection [10–12]. This is exemplified by the intranasal mouse model of infection for *F. tularensis* where it was found that the administration of the fluoroquinolone levoflaxacin at 72 h post-infection (p.i.) confers 100% survival but this protection is lost when antibiotic administration is delayed to 120 h p.i (0%) [13]. Therefore a strategy that could increase the antibiotic therapeutic window may be of use for the treatment of *F. tularensis* infections and those caused by other intracellular pathogens.

The ability of *F. tularensis* to subvert the host immune response within murine models of infection has also been previously reported. The organism appears to block the production of pro-inflammatory cytokines up to 72 h p.i., immediately following this immune modulatory event there is a rapid increase in cytokines such as TNF-α, IFN-γ, IL-6 and the chemokine CCL2 that is reminiscent of a “cytokine storm” [14,15]. This has led to the theory that a cytokine release of this type and scale is
damaging to the infected host and is in fact “too much, too late” [16]. In addition, it has been proposed that the resulting hypercytokinemia and bacteremia are indicative of sepsis [16]. Recent studies have focused on markers of sepsis during the course of an infection with *F. tularensis*. High-mobility group protein B1 (HMGB1), a late marker of sepsis, has been found to be released during infection with *F. tularensis* [15,17]. The protein acts as a “Damage Associated Molecular Pattern” (DAMP) secreted by immune cells in response to tissue damage caused by injury, necrosis or infection [18]. Its role as a mediator of inflammation means the protein plays a pivotal role in the generation of a “cytokine storm” and therefore represents a potential target for immunotherapy. In this study we explored the hypothesis that targeting HMGB1 represents a useful therapeutic strategy for the treatment of tularemia. By modulating the levels of HMGB1 present in the host we aimed to dampen the host immune response following infection with *F. tularensis* and potentially increase the efficacy of antibiotics to treat the disease. Here, we report the effects of administering a polyclonal antibody (Ab) raised against HMGB1, in combination with a delayed (sub-optimal) administration of the antibiotic levofloxacin, within a BALB/c mouse model of infection for *F. tularensis* SCHU S4.
Materials and Methods

**Bacterial culture:** *F. tularensis* strain SCHU S4 was cultured from frozen stock for 2 days on Blood Cysteine Glucose Agar (BCGA) with cysteine at 37°C. Subsequently, bacteria were harvested to inoculate 50 ml of Modified Cysteine Partial Hydrolysate (MCPH) broth with cysteine and glucose and incubated at 37°C overnight on a rotary shaker (150 rpm). The suspension was then adjusted using phosphate-buffered saline (PBS) until the optical density at 590 nm was 0.10, where the estimated bacterial density would be $5 \times 10^8$ CFU per ml. Bacterial numbers for challenge were enumerated on agar following serial dilution (1:10) of samples.

**Animal husbandry, challenge and monitoring:** Six to eight week old male BALB/c mice (Charles River, UK) were transferred to a high containment Class III rigid isolator, where they were given unlimited access to food and water. Mice were challenged with *F. tularensis* strain SCHU S4 by the intranasal route via pipette and under light anaesthesia, with 2-bromo-2-chloro-1,1,1-trifluoroethane (Halothane). Mice were checked twice daily and scored for clinical symptoms. Mice were culled at pre-determined humane end points. Survival times were recorded for some mice and others were culled for analysis of tissues at different time points. All procedures and housing were in accordance with the UK Animal (Scientific Procedures) Act (1986).

**Time course of infection:** BALB/c mice were challenged in two separate experiments ($n = 7$ and 5) with *F. tularensis* SCHU S4 (99 and 89 CFU respectively) and animals culled at 1, 2, 3, 4 or 5 days post-infection. The bacterial burden, HMGB1 protein levels in the serum and also the pro-inflammatory cytokine response within the lung, liver and spleen were measured at each time point. In addition, tissue samples from naive mice were used to establish the background levels of each parameter.
Measurement of HMGB1 in serum: Whole blood was microfuged at 7,000 rpm for 20 min and the serum removed and stored at -80°C. Serum HMGB1 was measured by ELISA kit (Shinotest™, supplied through Oxford Biosystems Cadama, UK) in accordance with manufacturer’s instructions. In brief, serum samples and standards were diluted and placed on an antibody-coated plate and incubated at 37°C for 20 – 24 h. The plate was washed 5 times and then coated with a peroxidase-linked anti-HMGB1 monoclonal antibody (supplied with the kit and the same antibody used in treatment) for 2 h at 25°C. Plates were washed prior to the addition of the substrate solution (3, 3’, 5, 5’-tetramethylbenzidine and buffer containing 0.005 mol/L hydrogen peroxide) for 30 min. The reaction was stopped with 0.35 mol/L sulfuric acid and plates were read at 450nm (microplate reader).

Administration of anti-HMGB1 Ab in sub-optimal antibiotic model: Three separate experiments were conducted where BALB/c mice were challenged via the intranasal route. Each animal received either anti-HMGB1 polyclonal Ab or the isotype control IgY via the intraperitoneal route (600 μg; Shinotest™, supplied through Oxford Biosystems Cadama, UK) at 48 and 72 h post-infection (p.i). Groups of mice receiving either the anti-HMGB1 or the isotype control Ab were culled at 96 h to examine the effects of the treatment alone. Additionally, a second group of mice received a combination therapy of the antibody and the antibiotic levofloxacin (Table 1). In these groups the antibiotic was administered once daily via the intraperitoneal route at 40 mg / kg commencing 96 h p.i and continuing for a period of seven days. Where mice were culled, they were exsanguinated using cardiac puncture following terminal anaesthesia. Blood was extracted as above and placed into 1.5 ml eppendorf tubes. Lungs, spleen and liver were removed and placed into universal tubes filled with 2 ml of PBS.

Measurement of bacterial burdens and pro-inflammatory cytokines: The lung, liver, spleen and blood were all processed at less than 1 h post-mortem. All organs were weighed and collected in 2mls PBS then disrupted through a 40 μm cell sieve and the resulting homogenate collected. Subsequently, 100 μl aliquots of the cell suspension were used for enumeration of bacteria on agar following serial
dilution in PBS. For cytokine analysis, 200 μl aliquots of cell suspension were centrifuged for 5 min at 2,000 rpm. Supernatants were removed for cytokine analysis and stored at -80°C. The cytokines IL-6, MCP1, TNF-α, IL-10, IFN-γ and IL-12p70 were measured via Cytometric Bead Array (Becton Dickinson™) in the lung, liver and spleen according to manufacturer’s instructions, with the additional step of fixing the samples in 4% paraformaldehyde in PBS for at least 24 h at 4°C. Briefly, prior to fixing, samples were incubated with the combined capture bead cocktail, and PE detection antibodies for 2 h. Samples were then washed in FACS buffer and resuspended in the 4% paraformaldehyde in PBS. Cytokine concentrations were measured via quantification of PE fluorescence of samples in reference to a standard curve using a BD FACS CANTO flow cytometer. The cytokine standards were treated in the same manner and simultaneously with the test samples negating any effects of fixation.

Statistical analysis: All graphs were generated using Graphpad PRISM V4.0. All statistical comparisons were made using PASW (SPSS release 18.0); some data manipulation and basic calculations were performed using Microsoft Excel 2007. Data from cytometric bead arrays were analysed by fitting a quadratic regression to the standard curves and reading the samples as unknowns. Cytokine and bacterial burden data was converted to ‘per gram of tissue’ for comparison. Analyses included stratified logrank tests and univariate, repeated measures or multivariate linear models. Where necessary, data was transformed to the logarithm of 10 for data to better fit the requirement for equal variance between groups in these tests (tested by Levene’s tests of equal variance).
Results

Rapid increases in serum levels of HMGB1 precede death.

To determine the inflammatory kinetics of intranasal *F. tularensis* strain SCHU S4 infection, BALB/c mice were infected with 99 CFU (experiment 1) and 89 CFU (experiment 2). Mice were culled at 1, 2, 3, 4 or 5 days p.i with bacterial load enumerated and the pro-inflammatory cytokine response assessed in the lung, liver and spleen (Figure 1 and 2 respectively). The lung, being the primary site of infection, showed the highest bacterial burden d1 and d2 p.i. (Figure 1A) with a steady increase over time. The infection spread to the liver and spleen, with detectable bacteria at d2 p.i. (Figures 1B and 1C) followed by more rapid increases in bacterial load between d3 - d5 as the infection progressed (P<0.001; for all organs). At d5 p.i only 4/7 (experiment 1) and 1/5 (experiment 2) mice were still alive, however all surviving mice had high loads (10^9-10^10 CFU/g of tissue) of bacteria in all organs.

The ability of *F. tularensis* to suppress the host inflammatory response was evident within the BALB/c model of infection. Pro-inflammatory cytokines IL-6, MCP1, TNF-α, IL-10, IFN-γ and IL-12p70 were measured in all three organ types across all five time points. IL-6, MCP1, TNF-α and IFN-γ were found to be below the limits of detection at d0, d1 and d2 p.i despite the presence of bacteria (Figure 2). However, it was found that the amounts of IL-6, MCP1, TNF-α and IFN-γ present in the lung, liver and spleen then increased rapidly and significantly across d3 – d5 p.i (P<0.001 for all four cytokines). In contrast, the amount of IL-10 and IL-12p70 present remained low and relatively constant throughout the time course of infection with no obvious correlation with bacterial load (P=0.052, for IL-10 and P=0.298, for IL-12p70).

The quantities of the “sepsis marker” HMGB1 present in the blood was also examined. The amount of HMGB1 in the serum of infected animals remained constant from d1 to d3 p.i. and comparable to that for naive blood samples (P>0.05). However, significant increases in HMGB1 levels were detected at
Treatment of intranasal *F. tularensis* infection with HMGB1 neutralising antibody increases host survival and correlates with increased IFN-γ.

Three experiments were performed where mice were infected with estimated challenge doses of 89 CFU (experiment 1), 77 CFU (experiment 2) and 103 CFU (experiment 3) by the intranasal route. Mice were treated with two doses of either 600 µg of anti-HMGB1 Ab or 600 µg matched isotype (IgY) control Ab at d2 and d3 p.i by the intraperitoneal route. Preliminary data indicated that effective suppression of HMGB1 levels required once daily dosing (data not shown). At d4 p.i. a subset of animals were culled for bacterial and cytokine analysis. The remaining mice received 7 daily intraperitoneal doses of levofloxacin; a sub-optimal regimen as opposed to the 14 days of treatment required for complete clearance of the organism [13]. We observed significantly increased median time to death of mice treated with the anti-HMGB1 Ab when compared to their isotype-treated counterparts (P=0.004) and found a suitable level of repeatability between experimental runs (P=0.263) (Figure 4).

In the subset of mice culled at d4 p.i, ELISA was used to identify whether levels of HMGB1 had been reduced. We found that the amount of circulating HMGB1 was significantly lower in the treatment group when compared to the control (P=0.003). Overall the serum HMGB1 levels were approximately 4-fold (1.54, 6.42 95% CI) lower in the anti-HMGB1 Ab treated mice when compared the IgY treated mice.

The bacterial burden and the quantity of cytokines present were also assessed d4 p.i in the lung, liver and spleen of these animals (Figures 5 and 6 respectively). We subjected this data to repeated measures (by organ) univariate analysis and found that the treatment had a significant effect on bacterial numbers overall (P=0.010). Further independent analysis of each organ showed significantly reduced bacterial loads in the livers (P=0.005) and spleens (P=0.006) in the mice receiving anti-HMGB1 Ab treatment; however this was not observed in the lung (P=0.312). This decrease in
bacterial burden between groups correlated with a significant change in levels of IFN-γ. Overall it was consistently found that the treatment groups receiving the anti-HMGB1 polyclonal Ab had significantly increased amounts of IFN-γ (P<0.001) present within the infected organs compared to those receiving the isotype control Ab. Despite monitoring a range of pro-inflammatory cytokines, no other significant differences were found. To further determine where differences in cytokine production occurred during infection, the data was further split by organ and re-analysed using univariate linear models. We found that IFN-γ was increased in all three organs of anti-HMGB1 Ab treated mice (Lung P<0.001; Liver P<0.001; Spleen P=0.022) (Figure 6). The quantities of chemokine MCP-1 present were not affected by anti-HMGB1 Ab treatment in the lung and spleen (P=0.674 and P=0.069 respectively) but a reduction was noted in the liver when compared with equivalent data from the isotype control group (P=0.035). No significant differences were seen in the levels of IL-6, IL-10, TNF-α and IL-12p70 cytokines at the organ level.
Discussion

The control of acute bacterial infections by the host requires the activation of the innate immune system and the subsequent initiation of an inflammatory response. However, during lethal infectious disease, the induction of inflammation can be the principle cause of mortality due to immunopathology. There is a fine line between the host mounting an appropriate immune response to clear infection and mounting an overactive immune response which can lead to host tissue damage and mortality [19]. Evidence indicates that *F. tularensis* has evolved to disrupt this balance. The initial delay in the inflammatory response allows the pathogen to colonise and grow within the host. This ultimately results in a hypercytokinemia and a bacteremia that is characteristic of sepsis [14,15]. Therefore, the targeting of therapeutics towards key immunomodulators involved in the inflammatory response may represent an effective strategy for the treatment an established, symptomatic infection.

In these studies we demonstrate that during an intra-nasal BALB/c mouse model of *F. tularensis* SCHU S4 that there is an absence of pro-inflammatory cytokines during the first 2 days of infection. Thereafter, there is a rapid increase of IL-6, TNF-α, IFN-γ and MCP-1 within the lungs, liver and spleen and an increase in the DAMP, HMGB1 within the blood. This is the first time this has been demonstrated within the BALB/c murine model. Importantly, we also provide evidence that targeting HMGB1 with an antibody, in combination with an antibiotic, levofloxacin, can increase host survival time and this survival correlates with an increase in secretion of IFN-γ.

During two detailed time course studies we show that, despite there being detectable bacteria at the site of infection (lung) at d1 p.i and in the spleen and liver on d2 p.i, the levels of pro-inflammatory cytokines are not measurable until d3p.i in all organs. Following this initial release, levels rapidly increase indicative of a cytokine storm [18,20]. These findings are supported by previously published research with different strains of mice and/or *F. tularensis* [21–23].
In addition to monitoring the amount of cytokine released in the tissues, the quantities of the DAMP, HMGB1, were also assessed in the blood during the whole time course. It was found that HMGB1 was only detected in the sera of infected mice between 72 – 96 h p.i. Previous studies using different models of *F. tularensis* infection, again validate our data with release of this protein reported by other methods, such as western blot [14,24].

Once HMGB1 is secreted into the extracellular milieu it acts in a similar manner to other pro-inflammatory cytokines. The protein binds several receptors, most notably the Receptor for Advanced Glycation Endproducts (RAGE), which is expressed on several different cell types including macrophages and endothelial cells [25]. Following an intracellular signalling cascade, NF-κB is activated and release of other pro-inflammatory cytokines occurs leading to a positive feedback loop, which contributes to a “cytokine storm” [26]. What separates HMGB1 from other proteins, is that it is a late mediator of sepsis [27,28]. Whilst strategies targeting TNF-α (previously believed to be the key cytokine) have failed to treat sepsis [29], targeting HMGB1 has shown some encouraging results. Preliminary studies have demonstrated that anti-HMGB1 antibodies could protect the host following lethal LPS exposure and that the efficacy of the therapy was dose dependent [30].

In these studies we sought to determine whether controlling/dampening down the “overactive” inflammatory response, by targeting HMGB1, was a suitable strategy to increase the effectiveness of antibiotic treatments. Our study utilized an adapted version of a previously published *F. tularensis* levofloxacin antibiotic model [13], to test our hypothesis of whether anti-HMGB1 Ab therapy could extend the window for successful antibiotic treatment and thus increase overall host survival. We used a sub-optimal antibiotic dosing regimen (commencing antibiotic treatment at 96 h for a total of 7 days) to establish whether firstly anti-HMGB1 Ab therapy could increase the antibiotic window during acute disease and secondly whether the therapy could aid in bacterial clearance.

Since HMGB1 levels are raised between 72 h and 96 h p.i., we concluded that administration of anti-HMGB1 Ab should commence at 48 h p.i, as HMGB1 release was most likely commencing in the tissue at this time point and would allow time for the antibody to take effect. An additional
administration of anti-HMGB1 Ab was given at 72 h in order to reduce the quantities of HMGB1 present prior to antibiotic administration at 96 h. Previous studies in our lab have shown that the effect of a single antibody dose of anti-HMGB1 Ab lasted 24 h (unpublished data). We found that anti-HMGB1 Ab treatment was not detrimental to the host, and provided a statistically significant survival benefit, reducing the bacterial burden in the liver and spleen. This reduction in bacterial burden following treatment with anti-HMGB1 Ab may be due to the increased phagocytic ability of macrophages that has been previously reported during Pseudomonas aeruginosa infection [31].

The most surprising and interesting data generated in these studies was the increase in IFN-γ in anti-HMGB1 Ab treated groups compared to isotype control treated mice. In all three independent experiments, anti-HMGB1 Ab caused an increase in IFN-γ in all of the organs analysed. This increase is unlikely to be solely due to changes in bacterial load, as bacterial burden in the lung, was not found to be significantly different between treatment groups. In addition, the levels of pro-inflammatory cytokines tend to correlate with bacterial load. Therefore, an increased bacterial load would also be expected in the anti-HMGB1 Ab treated mice, and this is certainly not the case, since decreases in bacterial load were observed in the spleen and liver post therapeutic treatment.

Indeed the increase in cytokines seems to be specific to IFN-γ, as quantities of MCP-1 (another pro-inflammatory marker) decrease in the liver following treatment with anti-HMGB1 Ab and other cytokines measured remain unchanged between groups.

The effect on IFN-γ in these studies may indicate a further mechanism of action by the therapeutic antibody, in addition to the previously mentioned, increased phagocytic ability of macrophages. Indeed it is well reported that IFN-γ is critical for protection against tularemia (38-40). Our data suggests that treatment with anti-HMGB1 Ab leads to a more appropriate immune response by increasing levels of IFN-γ and decreasing MCP-1. The exact role of the increase of IFN-γ in this model is yet to be determined, but is most likely due to increased nitric oxide production in macrophages resulting in enhanced bacterial killing.
In our experiments administration of anti-HMGB1 Ab in combination with sub-optimal antibiotic dosing did not lead to bacterial clearance, with mice succumbing to infection several days following the seven day antibiotic treatment regime. Clearly, in order to make these findings clinically relevant it would be advantageous to conduct further work examining the efficacy of this therapeutic strategy by administering anti-HMGB1 Ab at the same time as antibiotics (i.e. from 96 h post-infection). Nonetheless, this study demonstrates proof of concept that targeting an immuno-stimulatory DAMP (such as the HMGB1 protein) with an appropriate antibody therapy can increase the window of opportunity of antibiotics within an acute model of infection. In addition, the apparent increase in IFN-γ, known to be important for the clearance of a range of infectious diseases, following the modulation of the amounts of HMGB1 present in vivo, highlights the potential for this antibody-antibiotic combination therapeutic regimen for the treatment of diseases caused by other intracellular pathogens.
Table 1:

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* Number of animals culled for analysis 96 h post-infection prior to the administration of levofloxacin.

Figure Legends

Figure 1: Bacterial colonisation in organs post intranasal infection with *F. tularensis* strain SCHU S4.

Bacterial numbers were enumerated on supplemented BCGA plates for three organs: lung (A), liver (B) and spleen (C). Each data point represents a single mouse and lines indicate the median. Data shown is for two independent experiments. The calculated dose for experiments 1 and 2 were 99 and 89 CFU respectively. 7 mice (experiment 1) or 5 mice (experiment 2) were culled at d1-4 p.i. 4 mice (experiment 1) or 1 mouse (experiment 2) were culled at d5 p.i (remaining survivors). An additional 5 mice were used to establish a baseline/naive concentration (d0).

Figure 2: Organ cytokine profile post intranasal infection with *F. tularensis* strain SCHU S4

Levels of IL-6, MCP-1, IFN-γ, TNF-α, IL-10 and IL-12p70 were measured via CBA. Cytokine concentrations are displayed as pg/g per organ: lung, liver and spleen. Each data point represents a single mouse and the lines indicate the median. Data shown is for two independent experiments. The calculated dose for experiments 1 and 2 were 99 and 89 CFU respectively. 7 mice (experiment 1) or 5
mice (experiment 2) were culled at d1-4 p.i. 4 mice (experiment 1) or 1 mouse (experiment 2) were culled at d5 p.i (remaining survivors). An additional 5 mice were used to establish a baseline/naive concentration (d0).

Figure 3: Serum HMGB1 release in mice post intranasal infection with *F. tularensis* strain SCHU S4

Levels of serum HMGB1 (ng/ml) were assed via ELISA. Each data point represents a single mouse and the lines indicate the median. Data shown is for two independent experiments. The calculated dose for experiments 1 and 2 were 99 and 89 CFU respectively. 7 mice (experiment 1) or 5 mice (experiment 2) were culled at d1-4 p.i. 4 mice (experiment 1) or 1 mouse (experiment 2) were culled at d5 p.i (remaining survivors). An additional 13 mice were used to establish a baseline/naive concentration (d0).

Figure 4: Survival of mice following anti-HMGB1 Ab treatment in combination with Levofloxacin.

Male BALB/c mice were challenged with 87 CFU (experiment 1), 77 (experiment 2) and 103 CFU (experiment 3) *F. tularensis* SCHU S4 via the intranasal route. Groups were treated with either 600 µg of anti-HMGB1 Ab or 600 µg of an isotype control (IgY) at 48 and 72 h p.i. Levofloxacin (40mg/kg) was administered via the i.p route at 96 h p.i and continued once daily for 7 days for both groups. Each data point represents the percentage number of survivors in each group at the corresponding time point. In the first experiment 7 animals received anti-HMGB1 Ab and 17 animals received isotype control IgY; in the second and third experiments 10 animals received anti-HMGB1 Ab and 10 animals received isotype control IgY. Data was analysed by stratified logrank test.

Figure 5: Bacterial colonisation in anti-HMGB1 and IgY Ab treated mice

Male BALB/c mice were challenged with 87 CFU (experiment 1), 77 (experiment 2) and 103 CFU (experiment 3) *F. tularensis* SCHU S4 via the intranasal route. Mice were culled at 96 h p.i and bacteria enumerated on supplemented BCGA for the lung, liver and spleen. Groups were treated with either 600 µg of anti-HMGB1 Ab or 600 µg of an isotype control Ab (IgY) at 48 and 72 h p.i. Each
data point represents a mouse and the line represents the median. In the first experiment, 4 animals received anti-HMGB1 Ab and 7 animals received isotype control IgY Ab; in the second and third experiments 5 animals received anti-HMGB1 Ab and 5 animals received isotype control IgY Ab. Data was analysed by univariate linear models of the log10 transformed data.

Figure 6: Cytokine production in anti-HMGB1 and IgY Ab treated mice

Male BALB/c mice were challenged with 87 CFU (experiment 1), 77 (experiment 2) and 103 CFU (experiment 3) F. tularensis SCHU S4 via the intranasal route. Mice were culled at 96 h p.i and IFN-γ and MCP-1 cytokines assayed for the lung, liver and spleen. Groups were treated with either 600 µg of anti-HMGB1 Ab or 600 µg of an isotype control (IgY) Ab at 48 and 72 h p.i. Each data point represents a mouse and the line represents the median. In the first experiment, 4 animals received anti-HMGB1 Ab and 7 animals received isotype control IgY Ab; in the second and third experiments 5 animals received anti-HMGB1 Ab and 5 animals received isotype control IgY Ab. Data was analysed by univariate linear model of the log10 transformed data. Significance markers have been included to show differences associated with the different treatments. Levels of IL-6, IL-10, TNF-α and IL-12p70 were also determined but not shown.

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