Final report: Redox regulation of the cytokine MIF during inflammation

Background:

The current project aimed to collect data in support of potential redox regulation of the immune regulator macrophage migration inhibitory factor (MIF). We have previously identified that the conserved *N*-terminal proline of MIF can be oxidatively modified by hypochlorous acid (HOCl or chlorine bleach), which is produced by neutrophils at sites of inflammation. However, evidence that this modification occurs *in vivo* is missing. Furthermore, it is unknown whether the modification has biological relevance.

Our research questions were:

1. Does hypochlorous acid modify MIF *in vivo*?
2. Does modification by hypochlorous acid alter the biological activity of MIF?

Summary:

Aim 1.*) Establishing sensitive methods to measure hypochlorous acid-modified MIF in biological fluids and tissues*

1.1) Measuring the oxidized N-terminal peptide of MIF using liquid chromatography with mass spectrometry (LC-MS)

We have set up a sensitive liquid-chromatography with mass spectrometry method for the detection of the oxidized peptide and have applied this to a number of biological samples from inflammatory sites. For example, we have used bronchoalveolar lavage fluid samples from children with cystic fibrosis, in which we have previously measured high levels of the HOCl-producing enzyme myeloperoxidase (MPO). We could not detect the oxidized peptide in any of the samples even after the samples were concentrated and enriched for the protein fraction. MIF ELISAs were used to confirm that MIF protein was present in small amounts in these samples.

1.2) Matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI-IMS)

MALDI-IMS work was to be carried out at the Protein Analysis Unit Ludwig at the Maximillian's University Munich (LMU), Germany. Unfortunately, the COVID-19 pandemic prevented travel in 2020.

1.3) Production of antibodies specific for hypochlorous-acid modified MIF for subsequent ELISA

We raised monoclonal antibodies against the oxidized *N*-terminal peptide. Unfortunately while these antibodies reacted with oxidized MIF protein, they also showed reactivity with non-oxidized MIF as indicated by Western blot analysis. This result suggested that when MIF was in a denatured state, the antibody could not distinguish between the unmodified and oxidatively modified forms of the protein. We also used the antibodies in non-denaturing ELISA experiments and showed that when plates were coated with the antibodies directed at oxidized MIF the ELISA readout was much greater for oxidized MIF than for unmodified MIF. This encouraging observation was further scrutinized by using isotype control antibodies as the coating antibody and the same difference in signal was observed for oxidized MIF vs non-oxidized MIF suggesting that the difference in binding to the ELISA plate was not facilitated by the antibodies raised against the oxidized MIF peptide. Further experiments demonstrated that the increased signal for oxidized MIF was due to it binding bovine serum albumin (BSA), which was used in the ELISA to block non-specific binding sites. Increased binding was also observed when we used

human serum albumin, but not observed for milk protein. This is an interesting finding that has implications for the physiological function of oxidized MIF. One might speculate that there may be an important role for the oxidized form of MIF *in vivo*, if greater binding to the major serum protein albumin warrants its efficient distribution throughout the bloodstream.

Aim 2.) Determining the effect of hypochlorous acid-modification on MIF's biological activities

2.1) Determining the effect of hypochlorous acid-modification on MIF's ability to promote biofilm formation in Pseudomonas aeruginosa

MIF was reported to enhance biofilm formation in *Pseudomonas aeruginosa (*PsA) [1]. We have established the assay to measure biofilm formation in our laboratory and tested the effect of MIF and oxidized MIF. In our hands MIF diminished rather than enhanced biofilm formation. Oxidation had no effect.

2.2) Investigating whether MIF and hypochlorous acid-modified MIF can degrade NETs

MIF was reported to have an intrinsic nuclease activity and we hypothesized that this can degrade neutrophil extracellular traps (NETs), structures of DNA speckled with antimicrobial proteins released from neutrophils to trap and kill bacteria. First we investigated whether MIF itself can induce NETs. There was no evidence for MIF being an inducer of NETs. However, we found that both MIF and its oxidized form (MIF-HOCl) enhanced NET formation in neutrophils when this was triggered by phorbol 12-myristate 13 –acetate (PMA) over a 4 h time course as indicated by an increase in Sytox Green fluorescence. When we pre-formed the NETs with PMA for 4h and then added MIF or HOCl-treated MIF and incubated overnight, the Styox Green fluorescence the next day was lower in the MIF and MIF-HOCl-treated samples compared to the controls. DNase was included as a positive control and this also decreased the fluorescence. This result indicated that MIF nuclease activity had degraded NETs and oxidation of MIF had no effect on the nuclease activity.

Additional Aim) Investigating MIF on other neutrophil functions

In lieu of MALDI-IMS experiments and further antibody experiments, we used the remainder of the project's resources to investigate the impact of MIF on other neutrophil functions. Because MIF and neutrophils likely co-localize at inflammatory sites, understanding the interplay between the two is important. MIF may underwrite neutrophil's adoption of a pro-inflammatory phenotype while MIF modification by neutrophil-derived oxidants may be a mechanism by which inflammation is regulated.

We have further investigated the mechanism by which MIF isoforms promote neutrophil survival, which was a previously known, but poorly characterised function of MIF. We found that MIF, MIF2 and their oxidized isoforms all stimulate the release of CXCL8 (and Il-6 in the case of MIF2) from mononuclear cells (PBMCs) via the surface receptor CXCR2 (Figure 1). Cooperative or synergistic action of CXCL8 and MIF delays neutrophil apoptosis via the neutrophil surface receptor CXCR2 and potentially another receptor (Figure 1). We have submitted our findings from this part of the project to the *Journal of Leukocyte Biology* and this has been accepted for publication.

Our investigations also lead to the discovery of a novel MIF function, i.e. its ability to substantially increase HOCl production in phagocytic neutrophils. This was determined using opsonized yeast particles, which are ingested by neutrophils and stimulate the production of HOCl. We included the HOCl-specific fluorescence probe R19S in these experiments, the oxidation of which was monitored using a combination of flow cytometry and live cell microscopy. Alternatively, we monitored HOCl production during phagocytosis of *P. aeruginosa* by measuring oxidation of bacterial glutathione to

the HOCl-specific product glutathione sulfonamide using LC-MS. Collectively, our results suggest that HOCl production is augmented in neutrophils following MIF exposure while the rate of phagocytosis was unaffected. We also measured the production of superoxide $O_2^{\cdot-}$, an upstream oxidant of HOCl, which is produced by the NADPH oxidase in activated neutrophils. We found that MIF primes the NADPH oxidase for superoxide production. In summary, our findings indicate that MIF could play a role in enhancing host defence by increasing the amount of the highly batericidal oxidant HOCl that is produced in response to invading pathogens. However, priming is a double-edge sword and excessive oxidant production can lead to tissue damage. Its ability to boost oxidant production in neutrophils may therefore contribute to the well-recognized pro-inflammatory profile of MIF. A manuscript resulting from this part of the project and from our work on aim 2.2) is currently in preparation and will be submitted to *Redox Biology* in the coming weeks. The key results included in this manuscript are summarized in Figure 2.

Publications resulting from this project:

1) Journal Leukocyte Biology (In print)

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Macrophage migration inhibitory factor inhibits neutrophil apoptosis by inducing cytokine release from mononuclear cells

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2) Redox Biology (In preparation)

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Macrophage migration inhibitory factor (MIF) enhances hypochlorous acid production in phagocytic neutrophils

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