ROLE OF REDOX-ACTIVE TRACE METAL (RATM) OXIDANTS IN
THE REGULATION OF TOLL-LIKE RECEPTOR 4 (TLR 4)
SIGNALLING–MEDIATED INFLAMMATORY
PHENOTYPE IN SYNOVIAL FIBROBLASTS

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and
Pharmaceutical Sciences

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By
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ROLE OF REDOX-ACTIVE TRACE METAL (RATM) OXIDANTS IN THE REGULATION OF TOLL-LIKE RECEPTOR 4 (TLR 4) SIGNALING–MEDIATED INFLAMMATORY PHENOTYPE IN SYNOVIAL FIBROBLASTS

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University of Missouri-Kansas City, 2018

ABSTRACT

While the involvement of oxidant stress in rheumatoid arthritis (RA) has been suspected, the role of redox-active trace metals (RATM) as exogenous pro-oxidants in the pathogenesis of RA has not yet been investigated. Evidence suggests that oxidant-induced Toll-like receptor 4 (TLR4) activation plays a significant role in initiating “sterile” inflammation. Here, we investigate for the first time the role of RATM-induced oxidant stress in the molecular mechanism of the pathogenesis of RA. Potassium peroxychromate (PPC) (Cr⁵⁺), cuprous chloride (Cu⁺), and ferrous chloride (Fe⁺²) RATM agents were used as exogenous sources of reactive species. LPS-EK as a TLR4 specific agonist was used as a positive control for TLR4 activation. Given the importance of synovial fibroblasts in the development of RA, HIG-82, a rabbit fibroblast like-synoviocytes (FLS) cell line, was used as a model system in the studies proposed in this dissertation research. The expression of TLR4 in HIG-82 was confirmed by quantitative PCR (RT-PCR) and Western blots. Intracellular reactive oxygen species (iROS) production was visualized and quantified by fluorescence imaging microscopy and flow cytometry (FC), respectively. Activation of TLR4 signaling pathway was determined by measuring the expression of TLR4 and the downstream signaling proteins. Either ELISA kits or FC quantified levels of TNF-α, interleukin (IL-1β), and HMGB1
(as pro-inflammatory agents), and IL-10 (as an anti-inflammatory mediator) released into the culture medium. Proliferation index of FLS and examination of the effects of RATM on apoptosis and autophagy-related protein levels were quantified by FC and Western blots. We found that (1) RATM induced iROS production, which was attenuated by pretreatment with antioxidants (2) Similar to TLR4 specific agonist LPS-EK, RATM significantly increased the activity of TLR4, which was blocked by pretreatment with TLR4 signaling inhibitor (CLI-095). (3) To our surprise, RATM increased proliferation of FLS and protected cells against apoptosis through activation of autophagy which is in agreement with the pathophysiological changes that occur in active RA. (4) RATM exogenous RS activate TLR4-mediated different down-stream signaling cascades that lead to an increased production of pro- and anti-inflammatory mediators in FLS, and (5) Further studies reveal that RATM exogenous RS treatment increased the expression of all three major MAPK; (Extracellular signal-regulated protein kinase (ERK), the c-Jun N-terminal Kinase (JNK), and P38 MAPK pathways). Moreover, RATM concurrently increased the expression of AP-1 nuclear protein through TLR4 stimulation. Taken together, our findings indicate that TLR4 has mediated RATM-induced inflammatory phenotype through AP-1 pathway activation in synovial fibroblasts. Therefore, oxidant stress through TLR4 activation may initiate and propagate inflammatory processes that maintain many chronic diseases. The design of dual-functioning antioxidants possessing both metal chelating and oxidative stress scavenging properties will be an essential milestone in pharmacotherapy and could help us live free of many chronic diseases. For the first time, we present evidence that supports a connection between exogenous and endogenous reactive species in enhancing inflammatory phenotype in synovial fibroblasts which is likely responsible for the initiation, propagation, and maintenance of RA.
The faculty listed below, appointed by the Dean of the School of Graduate Studies have examined the dissertation titled “Role of Redox-Active Trace Metal (RATM) Oxidants in the Regulation of Toll-Like Receptor 4 (TLR4) Signaling-Mediated Inflammatory Phenotype in Synovial Fibroblasts” presented by Asmaa Alsousi, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

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2.5.5. Targets and consequences of RS .......................................................... 16
2.2.6. Oxidant stress and inflammation .......................................................... 20
2.2.7. Interdependence between oxidant stress and inflammation .............. 20
2.2.8. Mechanisms of oxidant stress pathology in RA .............................. 22
2.3. Toll-Like Receptors (TLRs) Link Oxidant Stress with Inflammation .... 22
  2.3.1. TLR ligands ......................................................................................... 24
  2.3.2. TLRs endogenous ligands. ................................................................. 24
  2.3.3. TLRs and RA ..................................................................................... 24
    2.3.3.1 TLR2 ............................................................................................. 25
    2.3.3.2 TLR3 ............................................................................................. 25
    2.3.3.3 TLR9 ............................................................................................. 26
    2.3.3.4 TLR4 ............................................................................................. 26
  2.3.4. TLRs and oxidant stress ................................................................. 28
  2.4. TLR4 Signaling ...................................................................................... 28
    2.4.1. TLR4-mediated AP-1 signaling pathway .......................................... 31
      2.4.1.1. AP-1 .......................................................................................... 32
      2.4.1.2. The stress-activated protein kinase/mitogen-activated protein kinase (SAPK/MAPK) pathways. .......................................................... 34
    2.4.2. Key pro-inflammatory mediators of TLR4 activation ...................... 36
      2.4.2.1. High mobility group box 1 (HMGB1) ........................................ 36
2.4.2.2. Inflammatory cytokines and markers ........................................ 40

3. CENTRAL HYPOTHESIS AND SPECIFIC AIMS ........................................ 46

3.1. Rationale .............................................................................................. 46

3.1.1. TLR4 and oxidant stress ................................................................. 47

3.2. Specific Aims ..................................................................................... 49

3.2.1. Specific aim 1 .................................................................................... 52

3.2.2. Specific aim 2 .................................................................................... 52

3.2.3. Specific aim 3 .................................................................................... 53

3.2.4. Specific aim 4 .................................................................................... 53

3.3. Significance ......................................................................................... 54

4. GENERAL MATERIALS AND METHODS ............................................... 55

4.1. Chemicals and Reagents .................................................................... 55

4.2. Equipment ........................................................................................... 61

4.3. Redox-Active Trace Metals (RATM) as Sources of Reactive Species ... 62

4.3.1. Potassium peroxychromate (PPC) (K$_3$CrO$_8$) ................................. 62

4.3.2. Cuprous chloride (CuCl) ................................................................. 63

4.3.3. Ferrous chloride (FeCl$_2$) ............................................................... 63

4.3.4. Ascorbic acid (AA) ......................................................................... 64

4.4. Generation of Oxidants ...................................................................... 66

4.4.1. Synthesis/ Preparation of PPC ......................................................... 66
4.4.2. Preparation of FeCl₅ solution ......................................................... 67
4.4.3. Preparation of CuCl solution .......................................................... 67
4.4.4. Preparation of ascorbic acid (AA) solution ........................................ 67
4.4.5. Preparation of CuAA solution ......................................................... 67
4.5. Treatment Protocols of RATM as Oxidants .............................................. 69
  4.5.1. Treatment of RATM as oxidants ...................................................... 69
  4.5.2. Treatment with blockers and inhibitors ............................................. 69
4.6. Cell Culture and Live Cell Counting ...................................................... 70
4.7. Culture Media Preparation (HAM F-12 Kaighn’s Modification) ................ 71
4.8. Determination of Cell Viability and Optimized Concentrations of Oxidants .. 72
  4.8.1. MTT reduction assay for optimal seeding ........................................... 72
  4.8.2. MTT reduction assay of oxidants ..................................................... 72
  4.8.3. Lactate dehydrogenase (LDH) release assay ..................................... 73
4.9. Preparation of Whole-Cell Protein Extract ............................................ 74
4.10. Preparation of Nuclear and Cytoplasmic Extract .................................... 74
4.11. Immunoblot (IB)/Western Blot (WB) Analysis ...................................... 75
4.12. ELISA Quantifications ........................................................................ 76
4.13. Flow Cytometry ............................................................................... 77
  4.13.1. Quantification of iROS production by flow cytometry ....................... 78
4.13.2. Measurement of cell-cycle phase specific cell death using propidium
iodide: cell cycle flow cytometry analysis .......................................................... 78

4.13.3. CellTrace™ CFSE cell proliferation assay ............................................. 79

4.13.4. FAM FLICA™ poly caspase assay ......................................................... 80

4.13.5. Quantification of total (HMGB1, TLR4, and MRP8/14) protein levels by
flow cytometry ........................................................................................................ 81

4.13.6. Quantification of total and phosphorylated ERK, JNK and P38 by flow
cytometry .............................................................................................................. 82

4.14. Fluorescence Microscopy .............................................................................. 82

4.14.1. Measurement of intracellular ROS (iROS) by Fluorescence Microscopy
Imaging .................................................................................................................. 83

4.14.2. Immunofluorescence (IF) of HMGB1 (Immunohistochemistry) .......... 83

4.15. Quantitative Real-Time Polymerase Chain Reaction (RT- qPCR) .......... 83

4.16. Silencing TLR4 Gene (siRNA Technique) .................................................. 87

4.16.1. Design and synthesis of siRNA ............................................................... 87

4.16.2. siRNA transfection .................................................................................. 87

4.17. Statistical Analysis ....................................................................................... 88

4.18. Solutions ........................................................................................................ 88

4.18.1. Cell culture solutions ............................................................................... 88

4.18.2. Immunoblotting solutions ...................................................................... 88

4.18.3. Flow cytometry solutions ...................................................................... 90
4.18.4. RNA extraction solutions ................................................................. 90

5. REDOX-ACTIVE TRACE METALS (RATM) AS PRO-OXIDANTS .................. 91

5.1. Introduction .......................................................................................... 91

5.2. Results .................................................................................................. 91

5.2.1. HIG-82 rabbit synovial fibroblasts optimal seeding is 8 x 10^3/well in 96-
well plate ................................................................................................. 91

5.2.2. Determination of the working concentrations for each RATM using MTT
and LDH assays ......................................................................................... 93

5.2.3. Confirmation of the expression of TLR4 in FLS .................................. 95

5.2.4. RATM upregulated TLR4 expression in FLS ...................................... 97

5.2.5. RATM induced-intracellular ROS (iROS) decreased after 4h of treatment ..
.................................................................................................................. 98

5.2.6. RATM-induced iROS increase decreased by antioxidants treatment. .... 101

5.2.7. CuCl increased iROS production in FLS cells in the presence of ascorbate.
.................................................................................................................. 103

5.2.8. RATM-induced intracellular ROS (iROS) production in FLS cells is TLR-
4 dependent ............................................................................................ 106

5.3. Discussion ........................................................................................... 108

6. EFFECT OF RATM ON HMGB1 RELEASE AND MARKERS OF INFLAMATORY
PHENOTYPES ............................................................................................... 119

6.1. Introduction .......................................................................................... 119
6.2. Results ........................................................................................................................................... 119

6.2.1. RATM pro-oxidants did not increase HMGB1 mRNA in synovial fibroblasts ................................................................. 119

6.2.2. RATM pro-oxidants increased HMGB1 protein expression in a time-dependent manner .......................................................... 121

6.2.3. RATM pro-oxidants increased extracellular HMGB1 release into the cytoplasm .................................................................... 122

6.2.4. RATM pro-oxidants effect on the mRNA of inflammatory cytokines .......................................................... 127

6.2.5. RATM pro-oxidants-induced inflammatory cytokine release is TLR4-dependent .......................................................... 129

6.2.6. RATM-effect on MRP8 and MRP14 mRNA ................................................................................................. 130

6.2.7. RATM upregulated total MRP8/14 protein expression .......................................................................................... 134

6.3. Discussion ............................................................................................................................................. 135

7. EFFECT OF RATM ON CELL PROLIFERATION AND CELL DEATH ........................................... 146

7.1. Introduction .................................................................................................................................................. 146

7.2. Results ...................................................................................................................................................... 147

7.2.1. Redox-active trace metal (RATM) oxidants treatment increased cell proliferation ............................................................................. 147

7.2.2. Redox-active trace metal (RATM) oxidants decreased polycaspase activity .................................................................................. 154
7.2.3. Reduced apoptosis correlates with enhanced autophagy following RATM oxidants treatment ................................................................. 158

7.2.4. P62/SQSTM1 and other autophagy markers are reduced with autophagy inhibitor U0126 .............................................................................. 161

7.3. Discussion ...................................................................................... 162

8. EFFECT OF RATM PRO-OXIDANTS ON TLR4-COUPLED AP-1 SIGNALING PATHWAY ............................................................. 168

8.1. Introduction ..................................................................................... 169

8.2.1. Redox-active trace metal (RATM) oxidants activated total JNK, ERK 1/2, and P38 .............................................................................. 169

8.2.2. Redox-active trace metal (RATM) oxidants activated phospho-JNK, ERK1/2, and P38 ........................................................................ 170

8.2.3. TLR4 siRNA optimization ............................................................... 179

8.2.4. Phospho-ERK1/2, -JNK and -P 38 expression after knockdown of TLR4 gene using siRNA ................................................................. 182

8.2.5. c-Fos and c-Jun (AP-1) protein levels after knockdown of TLR4 gene using siRNA. .............................................................................. 184

8.3. Discussion ...................................................................................... 187

9. RESEARCH SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS ........... 192

9.1. Research Summary ........................................................................... 192

9.2. Conclusions .................................................................................... 193
9.3. Future Directions ........................................................................................................... 194

9.3.1. Using human cell line and in vivo transgenic animal model ......................... 195

9.3.2. Investigation of the nature of interaction between RS and TLR4 .......... 196

9.9.3. To study the synergistic effects of HMGB1 and/or LPS and RATM on TLR4 activation and inflammatory cytokine production ........................................ 198

APPENDIX .................................................................................................................................. 202

A-1. Milestones in the recognition of RA history ................................................................. 199

A-2. List of drugs used to treat RA .................................................................................. 201

A-3. Different reactive oxygen species (ROS) and their properties ....................... 202

A-4. Different reactive nitrogen species (RNS) and their properties .................. 204

A-5. Different reactive chlorine species (RCS) and their properties ................... 206

REFERENCE LIST .................................................................................................................. 207

VITA ........................................................................................................................................ 247
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Oxidant stress is a common denominator in many diseases</td>
<td>2</td>
</tr>
<tr>
<td>2.1. Direct and indirect effects of oxidant stress</td>
<td>18</td>
</tr>
<tr>
<td>2.2. Structure of TLR4 and AP-1 signaling pathway</td>
<td>33</td>
</tr>
<tr>
<td>2.3. Structure of HMGB1</td>
<td>37</td>
</tr>
<tr>
<td>2.4. Different types of HMGB1 receptor and non-receptor mechanisms</td>
<td>39</td>
</tr>
<tr>
<td>2.5. The imbalance between pro- and anti-inflammatory cytokines in RA</td>
<td>40</td>
</tr>
<tr>
<td>3.1. A simplified graphical representation of a putative mechanism for exogenous RATM-induced AP-1 activation in synovial fibroblasts</td>
<td>50</td>
</tr>
<tr>
<td>4.1. RATM oxidants used in the study</td>
<td>65</td>
</tr>
<tr>
<td>4.2. ROS generated by RATM oxidants</td>
<td>66</td>
</tr>
<tr>
<td>5.1. Determination of the optimal seeding of HIG-82 synovial fibroblasts in 96-well plate</td>
<td>92</td>
</tr>
<tr>
<td>5.2. Effects of RATM oxidants on the viability of FLS</td>
<td>94</td>
</tr>
<tr>
<td>5.3. Expression levels of TLR4 mRNA and protein in FLS</td>
<td>96</td>
</tr>
<tr>
<td>5.4. Time course of RATM oxidants-mediated TLR4 expression in FLS</td>
<td>97</td>
</tr>
<tr>
<td>5.5. Immunofluorescence and flow cytometry time course of RATM oxidants-mediated intracellular ROS (iROS) in FLS</td>
<td>100</td>
</tr>
<tr>
<td>5.6. Immunofluorescence representation of the levels of intracellular ROS (iROS) in HIG-82 in FLS</td>
<td>102</td>
</tr>
<tr>
<td>5.7. Immunofluorescence representation of the levels of intracellular ROS (iROS) following CuCl and Ascorbic acid co-treatment in FLS</td>
<td>104</td>
</tr>
</tbody>
</table>
5.8. Flow cytometry analysis of the levels of intracellular ROS (iROS) following CuCl and Ascorbic acid co-treatment in FLS .......................................................... 105

5.9. Immunofluorescence representation of the levels of intracellular ROS (iROS) following pretreatment with CLI-095 ................................................................. 107

6.1. Effect of RATM oxidants on gene expression of HMGB1 in FLS ................. 121
6.2. Analysis of the effects of RATM oxidants on HMGB1 protein expression .......... 124
6.3. Effect of RATM oxidants on HMGB1 release ............................................. 126
6.4. Effect of RATM oxidants on gene expression of TNF-α, IL-1β, and IL-10 in FLS ..... 129
6.5. Role of TLR4 in RATM oxidants-induced production of TNF-α, IL-1β, and IL-10 .... 132
6.6. Effect of RATM oxidants on MRP gene expression in FLS .......................... 135
6.7. Effect of RATM oxidants on MRP protein expression in FLS .................... 137
7.1. Effect of RATM oxidants on the expression of the proteins of proliferation at 24 and 48 h .......................................................................................................................... 150
7.2. Effect of synchronization and cell cycle arrest on proliferation in FLS ......... 153
7.3. Effect of RATM oxidants on the activity of polycaspases at 2 h in FLS .......... 155
7.4. Effect of RATM oxidants on the activity of polycaspases activity at 24 h in FLS ...... 156
7.5. Time-course effects of RATM oxidants treatment on autophagy in FLS ............ 160
7.6. Effect of autophagy inhibition on the markers of autophagy in FLS ............... 161
8.1. Flow cytometry analysis of the effects of RATM oxidants on total JNK protein expression in FLS ........................................................................................................... 173
8.2. Flow cytometry analysis of the effects of RATM oxidants on total ERK protein expression in FLS ............................................................................................................. 175
8.3. Flow cytometry analysis of the effects of RATM oxidants on total P38 protein expression in FLS ............................................................................................................. 176
8.4. Flow cytometry analysis of the effects of RATM oxidants on phosphorylated MAPKs expression in FLS. ................................................................. 178
8.5. Role of siRNA TLR4 in TLR4 gene expression in FLS .............................................. 180
8.6. Role of siRNA TLR4 in TLR4 protein expression in FLS ........................................ 181
8.7. Role of siRNA TLR4 in the expression of ERK1/2, JNK, and P38 proteins following PPC (10 nM) treatment ........................................................................................................... 183
8.8. Role of siRNA TLR4 in the expression of c-Jun and c-Fos proteins following PPC (10 nM) treatment ........................................................................................................... 185
8.9. AP-1 signaling pathway ............................................................................................ 186
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sources of reactive species</td>
<td>15</td>
</tr>
<tr>
<td>2. Different types of antioxidants</td>
<td>16</td>
</tr>
<tr>
<td>3. Concentrations and effects of reactive species</td>
<td>17</td>
</tr>
<tr>
<td>4. Pattern Recognition Receptors (PRR) families and subfamilies</td>
<td>23</td>
</tr>
<tr>
<td>5. TLRs Ligands</td>
<td>30</td>
</tr>
<tr>
<td>6. Endogenous Ligands</td>
<td>31</td>
</tr>
<tr>
<td>7. HMG protein families</td>
<td>36</td>
</tr>
<tr>
<td>8. Important cytokines involved in RA pathogenesis</td>
<td>41</td>
</tr>
<tr>
<td>9. Treatment protocols of oxidants</td>
<td>69</td>
</tr>
<tr>
<td>10. Sequence similarity percentage of TLR4 between human and other species</td>
<td>71</td>
</tr>
<tr>
<td>11. Antibodies (Abs) used in WB and their dilutions</td>
<td>77</td>
</tr>
<tr>
<td>12. Antibodies used in flow cytometry and Immunofluorescence and their dilutions</td>
<td>85</td>
</tr>
<tr>
<td>13. RT-PCR primers</td>
<td>86</td>
</tr>
<tr>
<td>14. TLR4 siRNA</td>
<td>87</td>
</tr>
<tr>
<td>15. Supportive studies of HMGB1 role in RA</td>
<td>140</td>
</tr>
<tr>
<td>16. RATM- induced changes of PI and cell cycle distribution in HIG-82 cells detected by flow cytometry</td>
<td>153</td>
</tr>
<tr>
<td>A-1. Milestones in recognition of RA history</td>
<td>202</td>
</tr>
<tr>
<td>A-2. List of current treatments of RA</td>
<td>204</td>
</tr>
<tr>
<td>A-3. Different reactive oxygen species (ROS) and their properties</td>
<td>205</td>
</tr>
<tr>
<td>A-4. Different reactive nitrogen species (RNS) and their properties</td>
<td>207</td>
</tr>
</tbody>
</table>
A-5. Different reactive chlorine species (RCS) and their properties ........................................... 208
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ANCA</td>
<td>Anti-neutrophilic cytoplasmic antibodies</td>
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<td>AP-1</td>
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<td>AP-3</td>
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<td>APC</td>
<td>Antigen presenting cells</td>
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</tr>
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<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>DMAPs</td>
<td>Damage-associated molecular patterns</td>
</tr>
<tr>
<td>DMARDS</td>
<td>Disease-modifying anti-rheumatic drugs</td>
</tr>
<tr>
<td>DNA</td>
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</tr>
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<td>Fetal bovine serum</td>
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<tr>
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<td>Fibroblasts-like synoviocytes</td>
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<tr>
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<td>Fenton-Weiss-Haber reaction</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>Glutathione</td>
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<td>H2DC-FDA</td>
<td>5-(and-6)-chloromethyl-2',7’-dichlorodihydrofluorescein diacetates</td>
</tr>
<tr>
<td>HDACs</td>
<td>Histone Deacetylases</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High mobility group box1</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel diseases</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 associated kinase</td>
</tr>
<tr>
<td>iROS</td>
<td>Intracellular ROS</td>
</tr>
<tr>
<td>iTAOC</td>
<td>Intracellular total antioxidant capacity</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitory nuclear κB</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS binding protein</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-reach repeats</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MD-2</td>
<td>Myeloid differential protein-2</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MEK/K</td>
<td>MAPK kinase kinase</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MMP3</td>
<td>Matrix metalloproteinase-3</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mtROS</td>
<td>Mitochondrial ROS</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiating primary response gene 88</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl cysteine</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF</td>
<td>Nuclear fraction</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NLPs</td>
<td>NOD-like receptors</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NLR pyrin domain containing 3</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal NOS</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide binding and oligomerization domain</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>OAS</td>
<td>Oligoadenylate synthase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotides</td>
</tr>
<tr>
<td>OS</td>
<td>Oxidant stress</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PI</td>
<td>Proliferation index</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3 kinase</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PN</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PPC</td>
<td>Potassium peroxychromate</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride or polyvinylidene difluoride</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RAGE</td>
<td>The receptor of advanced glycation products</td>
</tr>
<tr>
<td>RATM</td>
<td>Redox-active trace metals</td>
</tr>
<tr>
<td>RCS</td>
<td>Reactive chlorine species</td>
</tr>
<tr>
<td>RIG</td>
<td>Retinoic acid-inducible gene</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-like receptor</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>RONS</td>
<td>Reactive oxygen/nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RS</td>
<td>Reactive species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SA</td>
<td>Specific antigen</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>STAT3</td>
<td>Single transducer and activator of transcription</td>
</tr>
<tr>
<td>TAB</td>
<td>TAK binding protein</td>
</tr>
<tr>
<td>TACE</td>
<td>TNF-α-converting enzymes</td>
</tr>
<tr>
<td>TAK</td>
<td>Transforming growth factor β activated kinase</td>
</tr>
<tr>
<td>TAOC</td>
<td>Total antioxidant capacity</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substance</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TIM3</td>
<td>T cell immunoglobulin and mucin domain 3</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin-1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR adaptor protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumor necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TRAM</td>
<td>TIRF-related adaptor molecule</td>
</tr>
<tr>
<td>TRAPE</td>
<td>Tetradecanoyl phorbol acetate responsive element</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adaptor-inducing interferon-β</td>
</tr>
<tr>
<td>TrX</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyltransferase-mediated dUTP-nick end</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UBC13</td>
<td>Ubiquitin-conjugating enzyme 13</td>
</tr>
<tr>
<td>UEV1A</td>
<td>Ubiquitin-conjugating enzyme E2 variant 1</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Vit C</td>
<td>Vitamin C</td>
</tr>
<tr>
<td>Vit. E</td>
<td>Vitamin E</td>
</tr>
</tbody>
</table>
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Dedication

I would like to dedicate this dissertation work to my three gorgeous daughters (Heba, Hala, and Hada), who have shared with me almost every moment of the journey, for their unconditional love and support, and to them, I say “Education is exceptionally essential, and with it, you can reach undefined limits.”
CHAPTER 1
INTRODUCTION

Living in an era free of chronic diseases may sound like a bizarre idea, but it is possible. It has been nearly forty-five years since the first evidence was presented for a correlation between the presence of highly reactive species and oxidant stress (OS) as the leading cause of tissue injury and damage. Each day, newly published articles enrich the biomedical literature with proposals that OS is involved in initiating different disease processes. Oxidant stress occurs when there is an imbalance between the production of reactive species and the ability of the body to counteract their harmful effects on cellular structures and functions. Trace metals-induced OS is a common denominator of many diseases (Fig. 1.1) that result partially from decreased antioxidant capacity. We are constantly exposed to trace metals as oxidants through our food and environment. For example, ferrous products are used to fortify food and to treat iron deficiency anemia. One significant feature of trace metals is that their accumulation in tissues is very slow. Therefore, acute effects are very rarely reported, but chronic exposure can lead to a buildup of higher concentrations and thus disease initiation. Accumulation of these metals in our body could initiate a variety of diseases.

This study uses rheumatoid arthritis (RA) as a chronic disease model and trace metal ions as exogenous oxidants in the form of ferrous, cuprous, and peroxycromate ions to potentially mimic the initiation of RA. With no cure to date, RA may be triggered by oxidant stress and thus perhaps prevented. While trace metal ions are essential for health, their accumulation in our bodies may initiate inflammation under certain circumstances. RA, as a common chronic autoimmune systemic inflammatory disease, is a leading cause of disability that results in significant health and social care costs (Lajas et al., 2003). RA primarily affects
the small joints of hands and feet (Gay et al., 1993). Despite its profound impact on the body, RA is still a disease with no unique definitive features. Perhaps chronic arthritis is the oldest disease with explicit evidence (Hormell, 1940). RA is the most common type of arthritis in the USA. More than 21% of US adults have self-reported or doctor-diagnosed arthritis which affects 1.3 million adults (Helmick et al., 2008).

Given the destructive nature of the disease and the percentage of the population that is affected, research in this area is indispensable to reveal the complexities of the disease pathology and to understand cellular and molecular entities which may trigger the onset, propagation, and maintenance of RA. A dearth of information on the etiopathogenesis of RA hinders our ability to develop effective therapeutic strategies, which represents a significant gap in our knowledge. Thereby, the planned research will provide new insights into the etiology and pathogenesis for RA that may be useful to develop a novel treatment.

Figure 1.1. Oxidant stress is a common denominator in many diseases
CHAPTER 2

REVIEW OF LITERATURE AND BACKGROUND

2.1. Rheumatoid Arthritis (RA)

RA is “an autoimmune disease that causes pain, swelling, and stiffness in the joints, and may cause severe joint damage, loss of function, and disability. The disease may last from months to a lifetime and symptoms may improve and worsen over time.” according to Weisman (Weisman, 2014).

2.1.1. History. Several researchers claimed that RA was a modern disease which only emerged in the mid-nineteenth century, following the industrial revolution (Parish, 1963). Others believed that RA was a disease of the New World, which was transmitted to the Old World by European explorers and settlers. They argued that the disease was not unique to the New World and could be tracked back to Sydenham during the 1600s (Pemberton and Osgood, 1934). Disease patterns were collected from fossils dating back to six hundred million years ago (Pemberton and Osgood, 1934). The oldest pathologic specimen was collected from dinosaur fossils aged one hundred million years old (Hormell, 1940). Human lesions in Egyptian mummies were explained in the writings of Sir Marc Armand Ruffer (Ruffer, 1913).

While the disease goes back to the earliest of the 19th century, the term “rheumatoid arthritis” was first proposed by Garrod in the 1850s (Comer and Scott, 1994). (Appendix A-1) provides an overview of milestones in RA history and identification.

2.1.2. Clinical features and epidemiology of RA. A patient is classified to have RA according to the criteria developed and revised by the American Rheumatism Association (Arnett et al., 1988). The characteristic signs of joint inflammation in RA are tenderness and swelling of the joints with frequently reduced mobility. There is always the destruction of
cartilage and bone in the affected joints, but the involvement of non-joint organs occurs as well.

In humans, RA occurs in all ethnic groups and affects approximately 1% of the worldwide population. It is 2-3 times more prevalent in women than men, but this preponderance disappears in the elderly (Symmons, 2002; Firestein, 2003). It is uncommon in men younger than 35 years.

The prevalence of RA in the United States has declined over recent decades and is now estimated to be 0.6% in people aged 18 years and older (Helmick et al., 2008). In European countries, it has been reported to range from less than 0.2% in Yugoslavia (Stojanovic et al., 1998) to as high as 0.8% in the United Kingdom (Symmons et al., 2002).

RA prevalence increases with age and reaches its highest level in women older than 65 years, suggesting the involvement of hormonal factors (Symmons et al., 2002). However, it may occur at any age (Gibofsky, 2012).

Osteoarthritis (OA) is the most common joint disorder in the United States, where 30.8 million adults are estimated to have osteoarthritis. RA, as the second most common type of arthritis in the USA, affects 1.3 million adults, whereas juvenile arthritis affects 294,000 children, spondylarthritides affects 2.4 million adults, systemic lupus erythematosus affects 322,000 adults, systemic sclerosis affects 49,000 adults, and primary Sjögren's syndrome affects 3.1 million adults (Helmick et al., 2008).

Higher rates of RA have been reported in several native American-Indian and Alaskan-Native populations (Del Puente et al., 1989; Jacobsson et al., 1993; Ferucci et al., 2005), and among Mexican Americans (Rasch et al., 2003), while lower rates have been reported in African (Brighton et al., 1988) and Asian groups (Shichikawa et al., 1999) compared to whites.
Despite its profound impact on the body, RA is still a disease without a unique definitive description.

RA is not a fatal disease per se, but RA patients have a gap in mortality compared to the general population. The leading causes of early death are increased cardiovascular complications, infection, and cancer (Sokka et al., 2008; Dadoun et al., 2013). The increased burden of cardiovascular diseases in RA suggests that more attention needs to be focused on effective prevention and management (Myasoedova and Gabriel, 2010).

2.1.3. Pathology of RA. RA as a clinical syndrome bridges several disease subsets with inflammatory cascades, leading to persistent synovial inflammation and to cartilage, joint, and bone damage (Scott et al., 2010), which explains the other synonym (degenerative joint disease). RA is characterized by synovial hyperplasia, pathophysiological immune responses and progressive erosion of joint tissue.

Different types of cells are involved in the pathogenesis of RA. These include T helper (Th) cells, antigen presenting cells, endothelial cells and resident fibroblasts of the synovial membrane (Muller-Ladner and Pap, 2005).

Destruction of cartilage and bone in the affected joints is mainly due to chronic inflammation of the synovium associated with the proliferation of synovial cells and infiltration with activated immune-inflammatory cells (Scott et al., 2010). The thickened intima (innermost membrane) is a result primarily of activated macrophages and fibroblast-like synoviocytes (FLS) (Tak et al., 1997). One hallmark of RA is the massive hyperplasia of the synovial membranes. Synovial fibroblast hyperplasia has been shown to precede inflammation (Qu et al., 1994) and to determine the outcome of disease in most affected
individuals. Such synovial hyperplasia at the synovium-cartilage junction is referred to as “pannus” formation.

FLS play a crucial role in joint damage as well as in the propagation of inflammation (Mor et al., 2005). It appears that in response to pro-inflammatory cytokines such as tissue necrosis factor-α (TNF-α), FLS produce large amounts of matrix metalloproteinases (MMP), which are critical drivers of extracellular matrix (ECM) destruction (Chakraborti et al., 2003). Activation of FLS (e.g., upon stimulation by innate immune mediators) is an early step in the development of RA (Lafyatis et al., 1989; Whitaker et al., 2013). Once activated, FLS attach to cartilage and bone, causing progressive erosion of articular structures by producing a variety of cytokines, chemokines, and extracellular matrix-degrading enzymes that mediate interactions with the microenvironment of neighboring cells. However, endogenous factors in RA transform FLS to a tumor-like phenotype, which is directly and indirectly linked to RA development and progression that eventually leads to bone erosion (Lowin and Straub, 2015).

While the pathogenesis of RA is only partially understood, the involvement of immune cells and their respective pro-inflammatory mediators remains a common hallmark of the disorder (Smith and Haynes, 2002; Tan et al., 2017).

2.1.4. Possible causes and risk factors of RA. Though there are potentially several causes of RA, the generally accepted model is an integrated model which describes the disease as a product of cross-talk between T and B lymphocytes, macrophages, and synovial fibroblasts within the synovium (McInnes and Schett, 2011). While both innate and adaptive immune responses are involved in RA, studies have described the importance of tissue reaction to the immune stimulation rather than the immune system itself, as causing the tissue damage.
Genetic and environmental factors, among other triggers such as air pollution and smoking, are recognized as factors that may contribute to the initiation of the disease. They may promote inappropriate immune reactions, which result in inflammation of synovial structures (McInnes and Schett, 2011). Genetic studies suggest a contribution of as much as 60% to RA susceptibility risk (MacGregor et al., 2000; van der Woude et al., 2009). The four-fold incidence increase in monozygotic twins compared to dizygotic twins along with familial clustering support the presence of genetic components in RA pathogenies (Silman and Pearson, 2002). Data suggests that the presence of shared epitopes (Gregersen et al., 1987) or genes (John et al., 1998; Cantagrel et al., 1999) are associated with disease severity rather than its incidence.

Thus, the two major genetic risk factors associated with RA are (1) the shared epitope of the major histocompatibility complex (MHC) associated with the alleles of the HLA-DRB1 gene (Padyukov et al., 2004), and (2) the non-MHC, non-HLA A allele PTPN22 R620W (Kallberg et al., 2007) with about 30% of the risk for RA associated with the MHC region (Deighton et al., 1989; Hasstedt et al., 1994).

However, genetic factors cannot always explain the development of RA. The effect of the systematic OS in RA is enhanced by exposure to air pollutants and cigarette smoke (Yanbaeva et al., 2007; Faux et al., 2009; Farhat et al., 2011), in which risk correlates with the extent of exposure (Costenbader and Karlson, 2006). Mucosal compartments of active smokers have higher levels of citrullinated proteins, in which antibodies against them are a reliable marker in RA (Verpoort et al., 2007; Kilsgard et al., 2012). Higher prevalence rates of RA are observed in regions with higher air pollution within the United States (Hart et al., 2009). Other
potential environmental risk factors include coffee intake, alcohol consumption, vitamin D status, and oral contraceptives (Liao et al., 2009).

Even infection (Carty et al., 2004) was once considered a triggering factor for RA. Bacterial products of mycobacterial hsp-65 were suggested to be involved (Celis et al., 1997). Likewise, Epstein-Barr virus has been detected in RA patients (Takeda et al., 2000) but there was no existence of substantial evidence to link any infectious agent with RA.

Other possible causes for RA include: autoimmunity directed against components of synovium and cartilage; and dysregulated production of cytokines, causing transformation of synovial cells into independently proliferating cells with infiltrating nature.

Cells can transduce stress into biochemical signals. Several cellular functions are influenced by the presence of stress such as mechanical stress (shear stress) (Nagel et al., 1999; Hochleitner et al., 2000), heat stress (Vance et al., 1989), cytokine stress (Brennan et al., 1992; Farahat et al., 1993), and OS to which cells are exposed in the synovial membrane of RA (Schett et al., 2001). The cellular responses to these stress factors in the joint are usually cellular activation, proliferation, and chronic inflammation.

Despite the devastating impact of RA, the molecular/biological mechanism of its initiation, propagation, and maintenance remains unclear. However, OS has been implicated in RA pathogenesis (Filippin et al., 2008) and has been suggested to play a role in the pathogenesis of inflamed joints (McCord, 1974; Parkes et al., 1991; Miesel and Zuber, 1993). Several epidemiological, biological, environmental, and genetic studies provided evidence to implicate oxidant stress in the pathophysiology of many diseases including RA (Biemand et al., 1984; Halliwell, 1995; Mapp et al., 1995; Ozturk et al., 1999; Jikimoto et al., 2002; Mateen et al., 2016b). Epidemiological data suggest that dietary consumption of antioxidants may
protect against RA (Heliovaara et al., 1994; Bae et al., 2003; Lahiri et al., 2012). Besides, biological studies indicated that sera and synovial fluids of RA patients have full oxidative enzyme activity associated with decreased antioxidant levels and increased damage to hyaluronic acid (Grootveld et al., 1991), oxidative DNA damage, and increased intracellular levels of the promutagenic 8-oxohydrodeoxyguanosine (Bashir et al., 1993). Furthermore, ROS production can be detected in the joints of RA patients by direct measurement using electron spin resonance (Allen et al., 1989). Thioredoxin as a cellular catalyst is overexpressed in synovial cells of RA patients (Maurice et al., 1999). Furthermore, treatment of RA patients with biological agents resulted in a reduction of oxidant stress markers (Biniecka et al., 2011b; Hirao et al., 2012). Moreover, up to date, methotrexate is used as the first line of treatment for RA. The used doses of methotrexate in RA are much lower than its oncological doses, and its efficacy is not related to its anti-proliferative activity only. It has been proposed that methotrexate suppress the generation of active oxygen metabolites induced by IL-6, which itself is generated by TNF-α stimulation (Sung et al., 2000).

2.1.5. Current clinical protocols of treatment for RA. There are different drugs used for the treatment of RA: (i) Nonsteroidal anti-inflammatory drugs (NSAIDs), which are used to help ease the pain and inflammation, (ii) Corticosteroid medications, which are potent and quick-acting medications that are used to control inflammation and slow joint damage, (iii) Disease-modifying antirheumatic drugs (DMARDs), which work to modify the course of the disease through slowing the progression of RA and saving the joints from permanent damage, (iv) Biologics (Biologic response modifiers) which are a new subset of DMARDs which target specific parts of the immune response, (v) JAK inhibitors which are involved in the body of immune response, and (vi) Surgery, as an option for daily function, mobility, and
independence, which may involve synovectomy (removal of the inflamed synovium), tendon repair, joint fusion, and total joint replacement. (Appendix A-2) Lists current drugs used to treat RA.

2.2. Oxidant Stress (OS)

Early reports of free radical existence emerged in the early 20th century. Initially, free radical studies were restricted to industrial research associated with rubber, plastic, oil, paint, and food. Subsequently, the involvement of free radicals in biological systems was uncovered in 1968 when the enzyme Superoxide Dismutase (Erythrocuprein) was discovered (McCord and Fridovich, 1969).

Our routine use of oxygen continuously generates free radicals. They are independent molecular species with one or more unpaired electrons in their outer atomic orbitals. The fact that electrons are more stable when arranged in pairs makes free radicals highly reactive and tend to react with other molecules to generate more stable species. Generally, a free radical can either donate its unpaired electron to molecules or “steal” electrons from other molecules. Cells continuously produce RS at tolerable concentrations under basal conditions. When there is an imbalance between the generation of reactive species (RS) and their removal by natural antioxidant defenses in our systems, oxidant stress is developed (Roberts et al., 2009). However, evidence indicates that the disruption of redox signaling is sometimes more important than the reactive species-antioxidant imbalance or the induced damage by such an imbalance (Jones, 2006).

2.2.1. Generation of reactive species (RS). Cells generate reactive species by several physiological and environmental stimulants simply by regular use of oxygen. When cells use oxygen to generate energy, by-products are created by mitochondria. These by-products are
capable of damaging cells. Both oxygen and nitrogen molecules contain two uncoupled electrons that can undergo reduction and thus form different oxygen/nitrogen metabolites. Other biologically relevant RS include halogen-derived radicals, which are critical in the bactericidal action of phagocytic cells. Thus, the term RS includes the three different classes of RS, which are relevant in biology. (i) reactive oxygen species (ROS), (ii) reactive nitrogen species (RNS), (iii) reactive chlorine species (RCS), including radical as well as non-radical species.

ROS are either oxygen-centered free radicals, such as hydroxyl, or peroxyl radicals, or oxygen-centered non-radical species, such as singlet oxygen, hydrogen peroxide (H$_2$O$_2$) or peroxynitrite (Bergamini et al., 2004). (Appendix A-3) Lists examples of reactive oxygen species (ROS) and their properties. Superoxide anion, hydrogen peroxide, and hydroxyl radicals are among the main ROS produced by cells (Droge, 2002). Reduced oxygen forms are unstable, destructive, and have a very short lifetime in the range of $10^{-5}$ to $10^{-8}$ sec (Pryor, 1986). RS do not cross membranes unless there is a high number of anion transporters (Mao and Poznansky, 1992). Though the cellular respiration through the electron transport chain in mitochondria involves the transformation of cellular, molecular oxygen into water, approximately 5% of the formed RS “HO’” hydroxyl radical, “O$^{-2}$”superoxide escapes the respiratory chain reactions and causes damage to the biological macromolecules such as DNA and proteins (Kelly et al., 1998). Among ROS, superoxide (‘O$^{-2}$’) is a pro-inflammatory species that damage cells such as endothelial cells and the extracellular matrix (ECM). When superoxide interacts with free iron or copper ions, it converts into more reactive ROS like OH· or combines with NO to produce peroxynitrite (ONOO') (Afonso et al., 2007) which react immediately with their surroundings (Aslan et al., 2008). Hydrogen peroxide (H$_2$O$_2$ ) is not a
radical by itself, but due to its highly reactive chemical structure and its ability to cross cell membranes, it is usually included in the list of ROS (Stephens et al., 2009).

Nitrogen also produces free radicals (RNS). (Appendix A-4) Lists examples of reactive nitrogen species (RNS) and their properties. While nitric oxide (NO) is a free radical, it is a beneficial metabolite too. Other potential RNS develop during reactions to form nitric oxide (Alderton et al., 2001).

Examples of reactive chlorine species (RCS) and their properties are listed in (Appendix A-5).

2.2.2. Sources of RS. Cells produce RS by several physiological and environmental stimulants known as oxidants. RS are produced by both endogenous and exogenous sources (Halliwell, 2011).

Endogenous sources in mammals include the seven isoforms of NADPH oxidase family (NOXs) (Jiang et al., 2011); enzymes of the mitochondrial electron transport system; the flavoenzyme ERO1 in the endoplasmic reticulum; xanthine oxidase; lipoxygenase; cyclooxygenases; cytochrome P450s; a flavin-dependent demethylase; oxidases for polyamines and amino acids; and nitric oxide synthases. In addition, free metals such as free copper or iron ions released from iron-sulfur clusters, and haem groups or metal storage proteins may generate free radicals via conversion of superoxide anion and/or H₂O₂ to hydroxyl radicals (Fenton reaction) (Imlay, 2008) (Table 1). Cellular respiration involves the reduction of oxygen into the water with the formation of reactive species. Oxidizing enzymes are a second source of ROS within cells. Enzymes such as tryptophan dioxygenase, diamine oxidase, xanthine oxidase, and cytochrome P450 reductase generate oxygen radicals while others, such as guanylyl cyclase and glucose oxidase generate H₂O₂ (Fridovich, 1974, 1978).
Exogenous sources such as xenobiotics, heat, UV light, X-ray and γ radiation are good sources of RONS. Many xenobiotics such as quinones, some dyes, some herbicides, transition metals, and aromatic nitro compounds can generate RS (Nohl et al., 1981). In addition, free radicals are generated by external sources such as exposure to X-rays, radiation and electromagnetic fields, ozone, cigarette smoking, medications, stress, allergens, air pollutants, industrial chemicals, dietary factors, and excessive exercise.

Sources of RS in RA include activated phagocytes, which generate ROS (OH, HOCl, and ONOO⁻). Autoantibodies, like antineutrophil cytoplasmic antibodies (ANCA) may induce ROS formation (Ketttritz et al., 1997; Weidner et al., 2001). Activated neutrophils liberate O₂⁻, H₂O₂, elastase, HOCl, and eicosanoids (Robinson et al., 1992). Macrophage-like cells in the pannus (Moilanen et al., 1997), cells in the cartilage, synovial fibroblasts, articular chondrocytes, osteoclasts, and TNF-α, can all trigger oxidative stress.

2.2.2.1. Redox-active trace metals (RATM) as sources of reactive species and oxidant stress. Transition metal ions are crucial elements in many biological processes. RATM are essential for health, but they can become problematic under certain circumstances. Not only free metal ions provoke oxidative stress, but also proteins that bind metal ions such as heme proteins can do so. Metal ions are transformed by oxidation/reduction processes leading to the formation of RS (Halliwell and Gutteridge, 1990). Excessive production of RS contributes to inflammation induction in joints. Among RS, superoxide anion (O₂⁻) plays an essential role in damaging cells and ECM in patients with inflammatory joints (Henrotin et al., 2005). RATM such as chromium (+5) from potassium peroxychromate K₃CrO₈ (PPC), cuprous (+1) from cuprous chloride (CuCl), and ferrous (+2) from ferrous chloride (FeCl₂), are exogenous pro-oxidants and RS generating systems. Not only do trace metals generate reactive species
themselves but in the presence of reduced metals such as ferrous or cuprous ions, or hydrogen peroxide, they can be converted into hydroxyl radicals which are highly reactive (Chance et al., 1979). Although iron and copper are essential cofactors of proteins, their ions are potentially dangerous pro-oxidants.

**2.2.3. Mechanisms of oxidant stress elimination.** The underlying and primary antioxidant mechanisms that scavenge OS in humans are not limited to enzymatic pathways such as superoxide dismutase (SOD), glutathione peroxidases (GPx), and catalases (Nathan and Cunningham-Bussel, 2013), but include thioredoxin and thioredoxin reductases (Holmgren and Lu, 2010), peroxiredoxins and methionine sulphoxide reductases (Weissbach et al., 2002), and pyruvate kinase which is involved in the negative feedback regulation of RS (Anastasiou et al., 2011).

Non-enzymatic pathways include small molecules that react with RS, which are then recycled or replenished. These are either non-enzymatic lipid-soluble substances, such as α-tocopherol (vitamin E), ubiquinol Q10, or carotenoids (α and β carotene), or water-soluble ones such as ascorbate (vitamin C) and oxaloacetate (Burton et al., 1983; Burton and Ingold, 1984) (Table 2).

**2.2.4. Physiological functions of RS.** RS homeostasis is mediated through RS elimination and oxidative damage repairing systems (antioxidant buffering capacity): high concentrations of radicals on the one hand, and deficiency of antioxidant enzymes on the other cause damaging effects on cells.

At low to moderate concentrations, reactive species are beneficial for biological systems and participate in various cellular signaling pathways. When cells are treated with low
concentrations of RS, cells are stimulated as if they were subjected to cytokine treatment or specific tumor promoters (Meier et al., 1989).

**Table 1. Sources of reactive species**

<table>
<thead>
<tr>
<th>Endogenous sources</th>
<th>Exogenous sources</th>
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<tbody>
<tr>
<td>Angiotensin II</td>
<td>Air pollutants</td>
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<tr>
<td>Cytochrome P450s; a flavin-dependent demethylase</td>
<td>Glucotoxicity</td>
</tr>
<tr>
<td>Endosome/lysosome degradation</td>
<td>UV light</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>γ irradiation</td>
</tr>
<tr>
<td>Flavoenzyme ERO1 in the endoplasmic reticulum</td>
<td>X-rays</td>
</tr>
<tr>
<td>Free copper or iron ions when released</td>
<td>Ozone</td>
</tr>
<tr>
<td>Heme groups</td>
<td>Infectious microbes</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>Industrial chemicals</td>
</tr>
<tr>
<td>Lipoxygenases and Cyclooxygenases</td>
<td>High-calorie diet</td>
</tr>
<tr>
<td>Metal storage proteins</td>
<td>Hyperoxia</td>
</tr>
<tr>
<td>Mitochondria respiratory chain</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>NADPH oxidases</td>
<td>Cigarette smoke</td>
</tr>
<tr>
<td>Nitric oxide synthases (NOS)</td>
<td>Nanoparticles (NP)</td>
</tr>
<tr>
<td>Oxidases for polyamines and amino acids</td>
<td>Xenobiotic</td>
</tr>
<tr>
<td>Peroxisomes</td>
<td>Heat</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>Trace metals</td>
</tr>
</tbody>
</table>

The responses were further prevented either by adding antioxidants or by antioxidant overexpression (Manna et al., 1998). Studies cite the physiological concentrations of RS as a mean to increase cytosolic calcium concentrations, which, in turn, tune the reductive state of cell thiols and control the activity of several protein kinases (Guyton et al., 1996). Protein kinases such as Src and Syk tyrosine kinases are activated with the ability of the transcriptional activators to target DNA and stimulate gene expression. Nevertheless, hypoxia itself causes down-regulation of the synthesis of cytochrome oxidase (Simon et al., 1977).
In the immune system, RS has a physiological role in signaling, in addition to their primary functional role to kill microorganisms by phagocytes. Reactive species are required for antibacterial autophagy in macrophages and some epithelial cells (Huang et al., 2009).

2.2.5. **Targets and consequences of RS.** Reactive species are essential in maintaining and modulating vital physiological functions. Some growth factors, cytokines, and neurotransmitters utilize reactive species as secondary messengers in executing physiological functions. However, excessive production of RS can be detrimental and can have direct as well as indirect effects (Fig. 2.1).

### Table 2. Different types of antioxidants

<table>
<thead>
<tr>
<th></th>
<th>Enzymatic</th>
<th>Non-enzymatic</th>
<th>Exogenous</th>
<th>Synthetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endogenous</td>
<td>Non-enzymatic</td>
<td>Natural</td>
<td>Synthetic</td>
</tr>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>Glutathione (GSH)</td>
<td></td>
<td>Vitamins</td>
<td>Butylated hydroxy anisole (BHA)</td>
</tr>
<tr>
<td>Glutathione peroxidases (GPx)</td>
<td>Bilirubin</td>
<td></td>
<td>Minerals</td>
<td></td>
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<tr>
<td>Catalases (CAT)</td>
<td>Albumin</td>
<td></td>
<td></td>
<td>Butylated hydroxyl toluene (BHT)</td>
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<tr>
<td>Thioredoxin and thioredoxin reductases</td>
<td>Lipid-soluble</td>
<td>Water-soluble</td>
<td></td>
<td></td>
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<tr>
<td>Peroxiredoxins</td>
<td>A tocopherol (vit E)</td>
<td>Ascorbate (vit C)</td>
<td></td>
<td></td>
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<tr>
<td>Methionine sulphoxide reductases</td>
<td>Ubiquinol Q10</td>
<td></td>
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<tr>
<td>Pyruvate kinase</td>
<td>Carotenoids (α and β carotene)</td>
<td>Oxaloacetate</td>
<td></td>
<td>Tertiary butylated hydroxyl quinine (TBHQ)</td>
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</table>
Table 3. Concentrations and effects of reactive species

<table>
<thead>
<tr>
<th>Concentrations of reactive species and their effects</th>
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</thead>
<tbody>
<tr>
<td><strong>Basal</strong></td>
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<tr>
<td>Constitutive antioxidant levels</td>
</tr>
<tr>
<td>RS/antioxidant balance</td>
</tr>
<tr>
<td>Growth and development</td>
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</table>

The biological effects of RS may be highly specific. RS may display atomic specificity rather than molecular specificity. RS can be cytotoxic if the signal is too long or too strong furthermore if the signal is at the wrong time or place.

RS react covalently and often reversibly with certain atomic elements (Nathan, 2003). ROS react with one of the least abundant atoms in biological macromolecules (Sulphur). In fact, ROS react with a specific subset of Sulphur atoms, mostly in the side chains of cysteine or methionine residues in proteins (Ferrer-Sueta et al., 2011). ROS regulate a number of proteins and DNA physiologically as molecular targets. Such proteins include tyrosine and serine/threonine phosphatases, serine/threonine kinases, zinc-finger proteins, forkhead box O family proteins, histone deacetylases, and signal-regulating proteins.

Mitochondrial ROS oxidize bases in DNA itself and promote transcription rather than oxidizing DNA-binding proteins (Al-Mehdi et al., 2012). As a result, mitochondrial-dependent ROS promote oxidation and are considered to be mediators in signaling between mitochondria and the nucleus (Finkel, 2012). Furthermore, ROS are involved in the formation of DNA breaks in target gene-regulatory regions (Ju et al., 2006), one more reason to understand the potential mutagens in ROS.
RS affect the most sensitive constituents in the biological membranes, which are the polyunsaturated fatty acids in the form of phospholipids (Spiteller, 2006). The unsaturated fatty acid double bonds represent sites of enhanced activities. Lipid peroxidation generates highly reactive aldehydes such as malondialdehyde (MDA). Many lipid peroxidation reactions include epoxidation of cholesterol-polyunsaturated fatty acid esters, plasminogen, and sphingolipids — lipid nitration by RNS such as peroxynitrite and nitrogen dioxide results in the formation of readily detectable biomarkers. Such biomarkers are considered important factors in the pathogenesis of diseases such as Alzheimer’s disease (Williamson et al., 2002).

Protein peroxidation is usually measured through the formation of protein carbonyls. These carbonyls can form adducts known as CO proteins. RS can oxidize amino acid side
chains into ketone or aldehyde derivatives (Stadtman and Oliver, 1991). Several amino acids in protein are oxidized, especially sulfur, methionine, and aromatic containing amino acids. Protein nitration of tyrosine residues causes an alteration in protein functions (Cassina et al., 2000). The level of protein 3-nitrotyrosine in tissue is used as an indicator of the nitro-derived oxidations in human and animal models (Aslan et al., 2003).

Glyco-oxidation (glycation) occurs when free amino-containing lipids or lysine and arginine amino acids and DNA continue through a series of rearrangements to generate reactive products with different crosslinking, pigmentation, and fluorescent properties (Brownlee et al., 1988). When oxidation is associated with glycation, the generated products are called glycol-oxidation products. The advanced glycation end-product leads to protein cross-linking and destruction of cellular structures (Vasan et al., 2003).

RS are highly reactive with nucleic acids especially DNA, leading to several structural modifications at purine bases and sugar components (Ehlers et al., 1999). These effects are mutagenic, especially in proliferating cells. In case of purine bases, modification occurs in guanine, where it is converted into its 8 (OH) derivative, to become highly mutagenic. In pyrimidine bases, the most sensitive positions are positions five and six which convert into glycol derivatives (Bergamini et al., 2004). Investigation of the presence of carboxymethyl-lysine, a glycol-oxidation product, in different tissues is used as a biomarker (Tezel et al., 2007).

RS are major causes of apoptosis (programmed cell death). Apoptosis, as a physiological phenomenon, is induced by two major pathways, intrinsic or extrinsic (Reed, 2004). DNA oxidative damage can lead to DNA cross-linking with proteins and strand breaks as well as base modifications. These changes are identified by measuring the levels of 8-
hydroxy-2-deoxyguanosine (8-OH-dG) as a marker of DNA oxidative damage (Shigenaga et al., 1994).

**2.2.6. Oxidant stress and inflammation.** Inflammation is a highly complicated regulated sequence of events in the vascularized connective tissues which can be initiated in response to exogenous and endogenous stimuli, such as pathogens, mechanical, and chemical agents, and autoimmune responses (Biswas, 2016). At the site of inflammation, inflammatory cells release enzymes, reactive species, and chemical mediators which induce tissue injury and oxidative stress. Two stages of inflammation are recognized: acute (short-term) and chronic (long-term) inflammation. Acute inflammation is a part of the innate protective response of the host to tissue injury and infection (Kain et al., 2014). The essential goal of the inflammatory response is to protect, clear the host from the invading organism, and repair any cellular injury. However, excessive or unregulated prolonged inflammatory process can induce tissue damage resulting in many chronic diseases (Biswas, 2016).

**2.2.7. Interdependence between oxidant stress and inflammation.** In a few human diseases such as overexposure to ionizing radiation or chronic α-tocopherol deficiency that results in neurodegeneration (Muller and Goss-Sampson, 1990), oxidative changes are considered a direct cause of disease. However, overproduction of RS in mammalian systems causes both toxic and adaptive responses. Oxidative changes are considered either a direct cause of disease, or secondary to the primary process. Naviaux proposed an interesting hypothesis in which he suggested that RS and chronic oxidative stress changes are not the cause but rather the symptoms of the disease (Naviaux, 2012).

OS and inflammation are firmly related processes (Biswas, 2016). There is growing evidence of the involvement of RS, as a major pathological factor, in over a hundred chronic
disorders ranging from scleroderma to cardiomyopathy, hearing loss and cancer (Poprac et al., 2017). Toxic responses to oxidative stress are involved in brain injury, lung injury, sepsis, premature labor, fetal death, inflammatory breast cancer, and Parkinson’s disease (Chan, 2005). Furthermore, oxidative/nitrative processes are critical in glaucoma pathogenesis (Aslan et al., 2008). Moreover, oxidative stress is involved in the pathogenesis of chronic obstructive pulmonary disease and chronic degenerative diseases (Kirkham and Rahman, 2006). When oxidative damage involves lipoproteins, oxidized LDL will migrate into the intima and media layers of the artery causing hypertrophy and infarction of the arterial wall with foam cells leading to atherosclerosis (Podrez et al., 2002; Wu et al., 2006). Even though type one and two diabetes are noticeable by hyperglycemia as a hallmark, they are associated with an increase in ROS (Baynes and Thorpe, 1999). Nicotine as a major constituent of tobacco is metabolized by cytochrome P450 into toxic metabolites and induces oxidative stress (Walters et al., 2005), which results in liver damage and lung cancer (Jin et al., 2012).

OS is one of the main causative elements in cardiac hypertrophy development. RS activate hypertrophy signaling kinases and transcription factors (Maulik and Kumar, 2012). The significance of oxidants stress in the pathophysiological mechanisms involved in the development of Heart Failure (HF) have been considered and assessed (Takimoto and Kass, 2007). Antioxidant therapy has shown benefits in cardiovascular diseases and aging (Losonczy et al., 1996). Also, OS is one of the primary mechanisms observed during skeletal muscle changes under chronic conditions (Rudkowski et al., 2004; Marin-Corral et al., 2009) and cancer cachexia (Busquets et al., 2005). It is accepted that OS initiates cancer as well as contributes to its progression (Halliwell, 2007).
2.2.8. Mechanisms of oxidant stress pathology in RA. The synovial environment is a key in RA pathogenesis. Compared to other tissues, the synovial environment is usually hypoxic and devoid of antioxidants. This type of environment is exceptionally detrimental especially when the generation of RS from cellular oxidative phosphorylation and activated phagocytes results in more RS production by several mechanisms (Afonso et al., 2007). When the produced RS exceed the physiological capacity, oxidative stress results, and damages nucleic acids, proteins, and lipids.

2.3. Toll-Like Receptors (TLRs) Link Oxidant Stress with Inflammation

Innate immunity is a rapid and remarkable non-specific first line immune defense. It is comprised of cells and mechanisms for recognizing preserved microbial patterns, lysing, and phagocytizing, and/or signaling danger (Bergman, 2010). The classical innate defense mechanism is phagocytosis. Other innate defenses include protein and peptide secretions such as inflammatory cytokine secretion. They are small, soluble “cell signaling” molecules (sometimes referred to as the polypeptides of the immune system), which are released during an innate response by a cell to act upon the same cell or another cell, as an immunomodulation agent. In fact, synovial activation is driven by proinflammatory cytokines in cytokine-dependent pathways as well as by cytokine-independent pathways that involve TLRs (Ospelt et al., 2004).

The connection between OS and inflammation has only recently been linked to the activation of Toll-like receptors (TLRs) (Gill et al., 2010). TLRs are the best well-characterized pattern recognition receptors (PRR) that play a significant role in the defense system of the host (Table 4) (Janssens and Beyaert, 2003). The PRR family includes nuclear
binding oligomerization domains and retinoic acid-inducible genes (Creagh and O’Neill, 2006).

The mammalian TLR family consists of 13 members. There are ten molecules identified in humans (TLR1 to TLR10) and twelve members in mice (TLR1 to TLR9, TLR11 to TLR13) and are referred to as Toll-Like receptors (TLRs), which are homologous to the Drosophila Toll receptor (Takeda et al., 2003). In order for the immune system to launch an effective response to eliminate a pathogen, the system must first sense the presence of that pathogen and then establish a protective immune response.

Table 4. Pattern Recognition Receptors (PRR) families and subfamilies (Sims et al., 2010; Kigerl et al., 2014)

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<table>
<thead>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Toll-like receptors (TLRs)</td>
</tr>
<tr>
<td>2</td>
<td>Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)</td>
</tr>
<tr>
<td>3</td>
<td>C-type lectin receptors (CLRs)</td>
</tr>
<tr>
<td>4</td>
<td>Retinoic acid-inducible gene-1: (RIG)-1-like receptors (RLRs)</td>
</tr>
<tr>
<td>5</td>
<td>Pyrin and HIN domain-containing (PYHIN) family members: the absent in melanoma 2-like receptors (ALRs) consisting of AIM2 and IFI16</td>
</tr>
<tr>
<td>6</td>
<td>Oligoadenylate synthase (OAS)-like receptors (OLRs)</td>
</tr>
</tbody>
</table>

Different types of stimuli activate TLRs: pathogens (Akira et al., 2006), cytokines, and stress-induced in cells (Koedel et al., 2007). TLR signaling is activated by distinct microbial pathogens known as pathogen-associated molecular patterns (PAMPs). Besides, TLRs sense non-pathogen stimuli and endogenous danger signals that are known as damage-associated molecular patterns (DAMPs) in “sterile” inflammation. These include endogenous substances, which are released as a result of inflammation and injury such as nutritional lipids and heat shock proteins (Shi et al., 2006). The release of damaged or stressed cells further initiates a
form of sterile inflammation independent of infection (Tsan and Gao, 2004). Each TLR detects distinct PAMPs.

TLRs are classified into two main groups according to their ligands: TLRs 1, 2, 4, and 6 bind lipid-based ligands and TLRs 3, 7, 8, and 9 bind nucleic acid-based ligands (Brentano et al., 2005a). The detection of any invading microorganism by TLRs causes the activation of several genes that encode pro-inflammatory cytokines and chemokines.

2.3.1. TLR ligands. In 1998, TLR4 was first identified as the signal transducer for lipopolysaccharide (LPS), a Gram-negative bacterial-cell wall component (Poltorak et al., 1998). TLRs have also been known to recognize other microbial components (Table 5).

2.3.2. TLRs endogenous ligands. TLRs can recognize several endogenous ligands (Table 6).

2.3.3. TLRs and RA. Synovial fibroblasts (FLS), found in the lining of the synovium, can integrate many stimuli and cause disease. Early treatments of RA that targeted the immune system or that combined powerful biological therapies resulted in increased complications and a risk of infection (Weisman, 2002). Dysregulation of innate immune recognition could be associated with autoimmunity (Franchimont et al., 2004). Indeed, administration of endogenous exterior RNA or DNA has been shown to activate TLRs and to induce immune reactions (Hurst and von Landenberg, 2008). A recent study suggests that distinct TLR polymorphisms are associated with the development and clinical characteristics of RA (Lee et al., 2014). The expression of most of the TLRs has been detected in RA synovial membranes. Available data on the involvement of TLRs in RA suggests a potential involvement of the activation of the TLR-signaling pathway in arthritis by oxidants and raises the question of how this could happen.
2.3.3.1. TLR2. Stimulation of cultured synovial fibroblasts with proinflammatory cytokines IL-1β, TNF-α or LPS resulted in a significant increase in TLR2 mRNA. This up-regulation resulted from direct exposure to microbial components or secondarily to the inflammatory cascade. Synovial tissues from RA joints express TLR2 predominantly at sites of attachment and invasion into cartilage and bone. The elevation in TLR2 expression may be a direct result of the presence of inflammatory mediators in the joint (Seibl et al., 2003). However, TLR2<sup>−/−</sup> mice crossed with IL-1Ra<sup>−/−</sup> mice have produced more severe arthritis (Abdollahi-Roodsaz et al., 2008b). In the IL-1-Ra<sup>−/−</sup> model, TLR2 appears to be protective. Nevertheless, in an alternative model of arthritis (the streptococcal cell wall-induced model) TLR2-deficient mice showed reduced disease severity (Abdollahi-Roodsaz et al., 2008a). These findings highlight the variability of results among different models.

2.3.3.2. TLR3. TLR3 is expressed intracellularly within the endoplasmic reticulum, endosomes, multivesicular bodies, and lysosomes (Blasius and Beutler, 2010). An infectious etiology of RA has been a hypothesis for a long time, which encouraged scientists to study the potential role of viral RNA in the pathogenesis of RA. In light of this, it is quite interesting that TLR3 expression increased in the synovium of RA patients (Roelofs et al., 2005). In her study, Ospelt presented TLR3 as one of the most abundant TLRs in the synovium in early stages of RA and demonstrated increased levels of TLR3 expression in RA compared to levels present in osteoarthritis (OA) (Ospelt et al., 2008). Several other studies have shown that TLR3 protein expression is upregulated in RA synovial tissues. The expression of TLR3 and its activation in vitro by poly (I-C) (TLR-3 ligand) and by necrotic RA synovial fluid cells indicates that RNA released from necrotic cells might act as an endogenous TLR3 ligand to stimulate proinflammatory gene expression in RA (Brentano et al., 2005b).
Interestingly, the activation of innate immunity through TLR3 promoted osteoclastogenesis by upregulation of the receptor activator of nuclear factor kappa-B ligand (RANKL) in RA synovial fibroblasts. This activation was the first evidence of TLR3 pathway involvement in bone destruction in RA (Kim et al., 2009). Conflicting data about the role of TLR3 in RA has been reported. TLR3 activation in mice has suppressed arthritis in collagen-induced arthritis (CIA) and K/BxN serum transfer models (Yarilina et al., 2007). In contrast, upregulation of TLR3 has been associated with increased disease severity in rat pristine-induced, and CIA models, while the down-regulation by small interfering RNA improved disease symptoms (Thwaites et al., 2014). The role of TLR3 was further confirmed in the rat model of pristane-induced arthritis. In this model, decreasing TLR3 expression due to a miR-26a mimic decreased disease symptoms (Jiang et al., 2014).

2.3.3. TLR9. Synthetic oligodeoxynucleotides (ODN) that contain CpG motifs are TLR9 ligands (Table 5) which activate the human immune system and contribute to the development of some autoimmune diseases such as Systemic Lupus Erythematosus (SLE) and RA (Bhattacharjee and Akira, 2006). However, TLR2−/− mice crossed with TLR9−/− mice did not affect the incidence and severity of IL-1-Ra−/− autoimmune arthritis (Abdollahi-Roodsaz et al., 2008b). This outcome further suggests that there is no native TLR9 activation present in this model that could modulate the disease expression.

2.3.3.4. TLR4. Within the TLR family, TLR4 is the most critically important. Increased expression of TLR4 in synovial compartments, suggests that TLR4 may play a vital role in the pathogenesis of RA (Seibl et al., 2003; Radstake et al., 2004a). Synovial fibroblasts express TLRs 1-6, but not TLRs 7-10. TLR4 is among the most abundant TLRs in synovial fibroblasts and is highly expressed in early as well as longstanding RA (Ospelt et al., 2008).
Many of the endogenous ligands for TLRs (Table 6) such as hyaluronan, heat shock proteins, and fibronectin are commonly found in the joints of arthritic patients and can bind to TLR4 (Gaston, 1997). Tenascin C, an extracellular matrix glycoprotein, causes persistent synovial inflammation through TLR4 in experimental models of RA (Midwood et al., 2009). Individuals with a particular TLR4 polymorphism have decreased RA susceptibility (Radstake et al., 2004b). A mouse model that was deficient for the adaptor protein, “myeloid differentiation primary response gene 88” (MyD88), did not develop streptococcal cell wall-induced arthritis, which is dependent on the activation of the innate immune system by TLR ligands (Joosten et al., 2003). Furthermore, the TLR4 Knockout in IL-1Ra mouse driven model of arthritis resulted in a less severe phenotype (Abdollahi-Roodsaz et al., 2008b), in which TLR4 was linked to disease development. This data was consistent with using naturally occurring TLR4 antagonists from Bartonella quintana as therapeutic effectors in both the IL-1Ra−/− model and the CIA model (Abdollahi-Roodsaz et al., 2007). Additionally, the injection of peptidoglycans, CpG DNA or dsRNA of Staphylococcus into joints of mice resulted in the form of arthritis (Deng et al., 1999). Human synovial membrane cultures from RA patients were found to express TLR4 and to release endogenous TLR ligands that may contribute to destructive inflammation in the joints (Sacre et al., 2007). Thus, TLR4 is believed to be involved in RA.

TLRs may be involved in mediating oxidant stress response (Gill et al., 2010). The overarching question is how oxidants are sensed, and how they propagate and maintain inflammation in FLS pathogenesis.

2.3.4. TLRs and oxidant stress. To address the question of how oxidants may be sensed and propagated to maintain inflammation in FLS pathogenesis, one should note the
emergence of non-pathogen-associated sensing by TLRs, which has increased its biologic significance especially TLR4 (Karki and Igwe, 2013) and TLR2 (Paul-Clark et al., 2009), so that they now are maybe considered “general” surveillance receptors for danger signals (Brown et al., 2011”). However, among the TLR family members, TLR4 is the most likely implicated in RA (more details are provided in section 3.1.1).

2.4. TLR4 Signaling

TLRs are members of the interleukin-1 receptor (IL-1R) superfamily and share homology in their cytoplasmic (Toll/IL-1R (TIR) domain. TLRs as type I transmembrane proteins that include: an ectodomain of a leucine-rich repeat domain in the extracellular region which mediates the recognition of pathogen-associated molecular patterns. Furthermore, TLRs have a transmembrane region, and a cytosolic Toll-IL-1 receptor (TIR) domain in the intracellular regions that activate the downstream signaling pathway (Kawai and Akira, 2011). The TLR4 pathway is activated by PAMP structures that are shared by pathogens and their products, and by DAMPs, which are ligands derived from redox-stressed cells and released from dead or damaged cells.

Matzinger first suggested that antigen-presenting cells can recognize endogenous molecules as danger or alarm, and initiate innate immune responses. These molecules were named DAMPs (Seong and Matzinger, 2004). Oppenheimer later established the term alarmin (Oppenheimer and Yang, 2005). Recently, various environmental factors including metals, nanoparticles, and ozone have been shown to also activate TLR4 pathways (Lucas and Maes, 2013). In “sterile” inflammation, the cellular response may be driven by ligands derived from redox-stressed or damaged cells (DAMPs), such as the high mobility group box 1 (HMGB1) protein, oxidized lipids, and proteins modified by oxidation or nitration that act as TLR4
ligands (Akira and Takeda, 2004). LPS is a classical PAMP that shows potent stimulatory activity and induces TLR4 expression (Takeuchi and Akira, 2001), which occurs through binding to LPS binding protein (LBP). The resulting complex attaches to cluster of differentiation 14 (CD14) (Fig. 2.2). (Poltorak et al., 1998). CD14 enhances TLR4 signaling by facilitating its transport to lipid rafts. Afterward, MD2 is recruited to promote TLR4 translocation into the cell membrane (Shimazu et al., 1999).

LPS binding studies (Lu et al., 2008) have shown that upon ligand binding, TLR4 undergoes dimerization to initiate intracellular cascades. This process occurs through recruitment of TIR domain-containing adaptor proteins such as myeloid differentiating primary response gene 88(MyD88), TIR adaptor protein (TIRAP), TIR-domain–containing adaptor–inducing interferon-β (TIRF), and TRIF-related adaptor molecule (TRAM).

The recognition of the CD14/TLR4/MD-2 complex by LPS activates one of two distinct signaling pathways, the MyD88-dependent or the MyD88-independent (TRIF-dependent) pathways (Fig. 2.2). Both pathways can activate the AP-1 pathway resulting in the induction of inflammatory cytokines.

Our laboratory has focused on the MyD88-dependent pathway. In the MyD88-dependent pathway (Fig. 2.2), TLR4 dimerization recruits and activates the association of MyD88, which in turn recruits IL-1 receptor-associated kinase (IRAK) 4 that contains both a death domain (DD) and a kinase domain, thus allowing the association of IRAK1. IRAK4 is then responsible for phosphorylation and activation of IRAK1, which stimulates recruitment of tumor-necrosis-factor receptor-associated factor 6 (TRAF6) to the receptor complex.
Table 5. TLRs Ligands (Kawai and Akira, 2011)

<table>
<thead>
<tr>
<th>TLR</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Triacyl lipopeptides</td>
</tr>
<tr>
<td></td>
<td>Lipoproteins</td>
</tr>
<tr>
<td>TLR2</td>
<td>Triacyl lipopeptides</td>
</tr>
<tr>
<td></td>
<td>Lipoproteins, LTA of group B <em>Streptococcus</em>, PGN</td>
</tr>
<tr>
<td></td>
<td>Peptidoglycans</td>
</tr>
<tr>
<td></td>
<td>tGPI-mutin (<em>Trypanosoma</em>)</td>
</tr>
<tr>
<td></td>
<td>Structural protein</td>
</tr>
<tr>
<td></td>
<td>Zymosan, β-glucan</td>
</tr>
<tr>
<td></td>
<td>Mannan</td>
</tr>
<tr>
<td></td>
<td>Porins of <em>Neisseria</em></td>
</tr>
<tr>
<td>TLR3</td>
<td>Double stranded (ds) RNA</td>
</tr>
<tr>
<td></td>
<td>Viral RNA</td>
</tr>
<tr>
<td>TLR4</td>
<td>Lipopolysaccharide (LPS) of Gram-negative bacteria</td>
</tr>
<tr>
<td></td>
<td>Structural protein</td>
</tr>
<tr>
<td></td>
<td>Mannan of <em>Candida albicans</em></td>
</tr>
<tr>
<td></td>
<td>Glycoinositolphospholipids (<em>Trypanosoma</em>)</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen</td>
</tr>
<tr>
<td></td>
<td>Heat-shock protein 60 and 70</td>
</tr>
<tr>
<td></td>
<td>The fusion protein of Respiratory Syncytial Virus (RSV) and envelope</td>
</tr>
<tr>
<td></td>
<td>proteins of Mouse Mammary Tumor Virus (MMTV)</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin of flagellated bacteria</td>
</tr>
<tr>
<td>TLR6</td>
<td>Diacyl lipopeptides</td>
</tr>
<tr>
<td></td>
<td>Zymosan, β-glucan</td>
</tr>
<tr>
<td>TLR7</td>
<td>Single-stranded (ss) RNA</td>
</tr>
<tr>
<td></td>
<td>Viral, fungal RNA</td>
</tr>
<tr>
<td></td>
<td>Synthetic compounds related to nucleic acids</td>
</tr>
<tr>
<td>TLR8</td>
<td>Single-stranded (ss) RNA</td>
</tr>
<tr>
<td></td>
<td>Viral, fungal RNA</td>
</tr>
<tr>
<td></td>
<td>Synthetic compounds related to nucleic acids (imidazoquinolines)</td>
</tr>
<tr>
<td>TLR9</td>
<td>Unmethylated CpG DNA</td>
</tr>
<tr>
<td></td>
<td>Viral, fungal, parasital-DNA</td>
</tr>
<tr>
<td></td>
<td>Hemozoin (<em>Plasmodium</em>)</td>
</tr>
<tr>
<td>TLR10</td>
<td>Triacylated lipopeptide agonists</td>
</tr>
<tr>
<td></td>
<td>Avian influenza virus (H5N1)</td>
</tr>
<tr>
<td></td>
<td>Bind to the TLR1/2 ligand PAM₃CSK₄ incorporation with TLR2</td>
</tr>
<tr>
<td></td>
<td>Whole <em>Borrelia burgdorferia</em></td>
</tr>
<tr>
<td>TLR11</td>
<td>Profilin- like molecule (<em>Toxoplasmodium</em>)</td>
</tr>
<tr>
<td></td>
<td>Non-determined parts of Uropathogenic bacteria</td>
</tr>
</tbody>
</table>
Table 6. TLRs Endogenous Ligands

<table>
<thead>
<tr>
<th>Endogenous molecule</th>
<th>TLR involved</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock proteins (hsp 60, hsp70, and hsp90)</td>
<td>TLR2 and TLR4</td>
<td>(Vabulas et al., 2001; Asea et al., 2002; Bulut et al., 2002; Dybdahl et al., 2002; Vabulas et al., 2002)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>TLR4</td>
<td>(Smiley et al., 2001)</td>
</tr>
<tr>
<td>Fibronectin extra domain A</td>
<td>TLR4</td>
<td>(Okamura et al., 2001)</td>
</tr>
<tr>
<td>Surfactant protein A</td>
<td>TLR4</td>
<td>(Guillot et al., 2002)</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>TLR4</td>
<td>(Johnson et al., 2002)</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>TLR4</td>
<td>(Termeer et al., 2002)</td>
</tr>
<tr>
<td>β-defensin 2</td>
<td>TLR4</td>
<td>(Biragyn et al., 2002)</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>TLR4</td>
<td>(Lucas and Maes, 2013)</td>
</tr>
<tr>
<td>Substance P</td>
<td>TLR4</td>
<td>(Lucas and Maes, 2013)</td>
</tr>
<tr>
<td>Oxidized low-density lipoproteins</td>
<td>TLR4</td>
<td>(Lucas and Maes, 2013)</td>
</tr>
<tr>
<td>S100 proteins (MRP8/14)</td>
<td>TLR4</td>
<td>(Lucas and Maes, 2013)</td>
</tr>
<tr>
<td>HMGB1</td>
<td>TLR2 and TLR4</td>
<td>(Park et al., 2004b)</td>
</tr>
<tr>
<td>mRNA</td>
<td>TLR3</td>
<td>(Kariko et al., 2004)</td>
</tr>
</tbody>
</table>

Phosphorylated IRAK1 and TRAF 6 then dissociate from the receptor and form a complex with transforming growth factor β activated kinase (TAK1), TAK1-binding protein. Following degradation of IRAK1 at the plasma membrane, the remaining complex (TRAF6, TAK1, TAB1, and TAB2) translocate to the cytosol, where it associates with two ubiquitin ligases, ubiquitin-conjugating enzyme 13 (UBC13), and ubiquitin-conjugating enzyme E2 variant 1 (UEV1A), to degrade TRAF6 and activate TAK1.

TAK1, in turn, phosphorylates both the inhibitor of nuclear factor-κB (IκB)-Kinase (IKK) complex and MAPK. In the present study, we have focused on the TLR4/AP-1 signaling pathway (Akira and Takeda, 2004).
2.4.1. TLR4-mediated AP-1 signaling pathway. The signaling pathways of TLRs leading to cytokine production are integrated by adapter molecules such as MyD88 and TRAF6 that eventually activate NF-κB and AP-1.

Activated T cells are considered one of the major inflammatory components that affect the severity of RA; however, other cells such as synovial fibroblasts are the main culprits. A key signaling molecule that was initially identified on activated T cells and as a regulator of T cell function is the receptor activator of NF-κB ligand (RANKL). The most critical transcription factor complexes that are activated by RANKL/TRAF signals are NF-κB and Fos/AP-1. The activation of TLR has led to the initiation of MAPK signaling cascade through MyD88, resulting in the activation of AP-1 in macrophages (Okamoto et al., 2008).

2.4.1.1. AP-1. AP-1 is a redox-regulated transcription factor combined in a dimeric complex of two oncogenic proteins, Fos and Jun. The AP-1 binding site is known as the tetradecanoyl phorbol acetate responsive element (TPARE). Fos proteins (Fos, FosB, Fra-1, and Fra-2) can only heterodimerize with members of the Jun family, while Jun family members (Jun, JunB and JunD) can homodimerize and heterodimerize with Fos members. Thus, AP-1 proteins consist of either Fos-Jun heterodimers or Jun-Jun homodimers (Zenz et al., 2008). Many valuable insights regarding the specific functions of AP-1 proteins in development and disease have been obtained from genetically modified mice and derived cells. AP-1 activity can affect the severity of RA at a level different from osteoclastogenesis.

In addition to osteoclast-mediated bone erosion, several molecules are secreted by synovial fibroblasts and contribute to matrix degradation. Of particular importance are matrix
metalloproteinases (MMPs), which are regulated by AP-1 and degrade components of the ECM. Signals that lead to activation of Jun are also implicated in RA, especially JNK.

2.4.1.2. The stress-activated protein kinase/mitogen-activated protein kinase (SAPK/MAPK) pathways. SAPK/MAPK family proteins are enzymes of highly conserved serine/threonine kinases that are involved in signaling cascades in all eukaryotic cells. Different MAPK pathways coordinate and integrate responses to various stimuli via a cascade of sequential kinase activations by simultaneous Tyr and Thr phosphorylations within conserved Thr-X-Tyr motives in the kinase subdomain VIII activation loop. These stimuli include hormones, growth factors, cytokines, transforming growth factor (TGF)-β-related agents, agents that act through G-protein–coupled receptors, and through PAMPs, DAMPs, and environmental stressors.

Activated MAPKs exert effects on cell physiology where they regulate cell proliferation, differentiation, survival, and apoptosis, motility and senescence (Chang and Karin, 2001; Kyriakis and Avruch, 2012).

2.4.1.2.1. SAPK/MAPKs as therapeutic targets for RA. MAPKs are key regulators of proinflammatory cytokine production in the signaling cascades downstream of TLRs, as well as B-cell antigen receptor (BCR) (Harnett et al., 2005), T-cell antigen receptor (TCR)/CD28 (Liu, 2005), IL-1, IL-17 (Kolls and Linden, 2004) and TNFα receptors.

SAPK/MAPKs are classified into distinctive groups. In mammalian cells, seven different MAPK pathways have been described: extracellular signal-regulated kinases 1 and 2 (ERK1/2) (P42/44MAPK), the c-Jun N-terminal Kinase (JNK) (also known as stress-activated kinase SAPK) pathway, P38 MAPK, ERK5, ERK3/4, ERK7/8, and the Nemo-like kinase (NLK) (Moens et al., 2013). There are at least three main classes of MAPKs (i) Extracellular signal-regulated protein kinase (ERK) (P42/44MAPK) cascades, (ii) the c-Jun N-terminal Kinase (JNK) pathway, (iii) P38 MAPK (Pearson et al., 2001), which are known by their core
signaling modules and play an important role in RA pathogenesis. They appear to control the response of cells to inflammation and stress.

**ERK MAPKs.** ERK1/2 plays an essential role in regulating IL-6, IL-23 and TNF-α production by macrophages that are stimulated by LPS (Goodridge et al., 2003). On the other hand, stimulation of FLS by epidermal growth factor (EGF) in RA patients results in regulation of the cyclooxygenase (COX)-2-dependent prostaglandin E2 (PGE2) (Nah et al., 2010). ERK1/2 signaling is associated with maintenance of RA. Of interest is the examination of ERK signaling cascade especially that ERK signaling appears to be implicated as a critical mediator in arthritic diseases (Barchowsky et al., 2000; Yamamoto et al., 2003).

**JNK MAPKs.** JNK MAPKs in RA appears to regulate MMP-mediated cartilage destruction downstream from IL-1 and TNFα. They promote extracellular matrix degradation by stimulating MMP expression in synovial fibroblasts and chondrocytes (Han et al., 2001; Liacini et al., 2002; Liacini et al., 2003). ROS has been implicated in JNK upregulation, which in turn activates Jun family proteins (Pulverer et al., 1991). JNKs are activated by oxidative stress and are likely to be involved in the trace metal–oxidant activation of cells.

**P38 MAPKs.** P38 MAPK is the mammalian homolog of the yeast high osmolarity glycerol (HOG) response kinase and is expressed throughout the body. Four known P38 MAPK genes encode four different proteins: P38 α (MAPK14), β (MAPK11), γ (MAPK12), and δ (MAPK13). Each homolog has its distinct function, and each can be activated *in situ* by PAMPs and DAMPs. P38 α (MAPK14) was the first isoform to be identified as a phosphorylated protein in response to LPS. cDNA cloning revealed that it was related to *Streptomyces cerevisiae* MAPK HOG1 (Han et al., 1994; Herskowitz, 1995). The
P38 MAPKs pathway is known to contribute to RA pathogenesis through regulation of chemotaxis and angiogenesis (Duch et al., 2012).

Because AP-1 activity is induced in RA by inflammatory cytokines and has a complex impact on osteoclast differentiation and production of soluble mediators of bone erosion. It is anticipated that several AP-1 components or signaling pathways leading to AP-1 activation may provide valuable drug targets for future therapy of RA.

2.4.2. Key pro-inflammatory mediators of TLR4 activation. 2.4.2.1. High mobility group box 1 (HMGB1). High mobility group (HMG) proteins are essential in regulating nuclear functions. HMG proteins can either distort, bend or modify the structure of DNA-transcription factors or DNA-histones complexes. HMG protein families (Table 7) have been re-named with systematic reference to their structural properties (Bustin, 2001).

<table>
<thead>
<tr>
<th>HMG family</th>
<th>Molecular weight</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGA</td>
<td>Approximately 10KDa</td>
<td>contains AT-hooks</td>
</tr>
<tr>
<td>HMGB</td>
<td>Approximately 25 KDa</td>
<td>contains HMG boxes</td>
</tr>
<tr>
<td>HMGN</td>
<td>10-25 KDa</td>
<td>binds directly to nucleosomes between DNA gyres and histone octamer</td>
</tr>
</tbody>
</table>

The HMGB family includes four family members: HMGB1, HMGB2, and HMGB3 (previously known as HMG4 or HMG2b), and HMGB4.

High mobility group box1 (HMGB1) is an abundant, non-histone, chromosomal protein (Johns, 1982), also known as HMG-1, amphotericin, and P30 (Bustin, 2001). It was purified and named after its high electrophoretic mobility in polyacrylamide gels forty years ago (Goodwin and Johns, 1977). HMGB proteins are classified into three groups based on their size, DNA binding properties, and sequence similarities: the HMGB1/2 family, the
HMGB14/17 family, and the HMGB I/Y family (Goodwin et al., 1973; Sparrow and Wells, 1992; Maher and Nathans, 1996). HMGB Y is found only in chickens (Goodwin et al., 1981). HMGB1 and HMGB2 are closely related homologs with 80% sequence homology (Sparrow and Wells, 1992).

2.4.2.1.1. HMGB1 structure. HMGB1 is a single polypeptide chain of 215-amino acid (aa), a protein of 30 kDa, with three domains, two positively charged DNA-binding domains (box A and box B), each about 75 aa in length, and a negatively charged C-terminal domain (Fig. 2.3), which is the longest in HMGB1 and the shortest in HMGB3 (Stott et al., 2010). Box B is the protein transduction domain while the purified (box A) acts as an antagonist, even though it has a short half-life.

![Figure 2.3. Structure of HMGB1](image)

2.4.2.1.2. HMGB1 functions. HMGB1 is an abundant nuclear protein with important functional activities depending upon its location in the cell.

When inside the nucleus, nuclear HMGB1 functions as a structural protein (Bustin et al., 1978). As a DNA chaperone, HMGB1 participates in DNA replication, recombination, transcription, and repair. HMGB1 also interacts with, and enhances, activities of some transcription factors such as p53 (Goodwin and Johns, 1977; Jayaraman et al., 1998; Banerjee and Kundu, 2003) and p73 (Stros et al., 2002; Uramoto et al., 2003). HMGB1 knockout mice
are incompatible with life due to hypoglycemia, while HMGB1\( ^{+} \) \textit{in vitro} cell lines grow normally (Calogero \textit{et al.}, 1999).

Cytosolic HMGB1 mediates autophagy, a programmed survival pathway, induced by environmental and cellular stress (Tang \textit{et al.}, 2012). As a nuclear protein, HMGB1 binds loosely to chromatin and rapidly leaks into the medium when the membrane integrity is compromised (Falciola \textit{et al.}, 1997; Muller \textit{et al.}, 2001).

Extracellular HMGB1 functions as an extracellular signaling molecule (DAMP) in inflammation, immunity, cell migration, and tissue regeneration (Lotze and Tracey, 2005; Dong Xda \textit{et al.}, 2007; Ellerman \textit{et al.}, 2007). It is considered a proinflammatory cytokine itself (Andersson \textit{et al.}, 2000) when secreted by activated immune cells, delivers signals through cell surface receptors, and induce inflammatory responses in immune and endothelial cells (Lotze and Tracey, 2005; Ulloa and Tracey, 2005).

2.4.2.1.3. \textit{HMGB1 receptors}. HMGB1 binds to the cellular receptor of advanced glycation products (RAGE) (Hori \textit{et al.}, 1995). RAGE, as a well-characterized cellular receptor for HMGB, is a transmembrane protein expressed on the surface of different cell types such as endothelium, macrophages, neurons, and monocytes (Kokkola \textit{et al.}, 2005). Both TLR 2 and TLR4 have been implicated in activation of macrophages by HMGB1 (Park \textit{et al.}, 2004a). RAGE, TLR2, and TLR4 receptors share common downstream effector molecules such as NF-κB, c-Jun N-terminal Kinase, and P38 mitogen-activated protein kinases that stimulate similar signaling cascades. This sharing makes it difficult to identify the cytokine activity of HMGB1 (Park \textit{et al.}, 2004b; Kokkola \textit{et al.}, 2005). HMGB1 also acts through TLR9 signaling through presenting ssDNA or ds RNA to its receptor (Tian \textit{et al.}, 2007).
The suppressing activity of HMGB1 is mediated through binding to the bimolecular receptor complex CD24 and Siglec 10 (humans) or Siglec G (mice) (Chen et al., 2009a) (Fig. 2.4).

HMGB1 also acts through non-receptor mechanisms. HMGB1 transports plasmid DNA into cells for transfection (Rubartelli and Sitia, 1995). Furthermore, T cell immunoglobulin and mucin domain 3 (TIM3) antagonizes HMGB1 to suppress immune responses through inhibition of nucleic acids recruitment (Chiba et al., 2012).

**Figure 2.4. Different types of HMGB1 receptor and non-receptor mechanisms**

2.4.2.1.4. **HMGB1 and TLRs.** Once released, HMGB1 functions as one of the most important DAMP danger signals that activate PRRs-including TLR 2 or 4 in “sterile” inflammation- and is a critical mediator of inflammation (Hori et al., 1995; Park et al., 2004b). Hepatocytes actively release HMGB1 in response to oxidative stress in the absence of cell
death. The exact pathway is not clear, but several studies have suggested the involvement of TLR4 activation and calcium signaling (Tsung et al., 2007). In other cells, HMGB1 release has been linked to either oxidation-reduction (Hoppe et al., 2006), post-translational modifications as phosphorylation (Youn and Shin, 2006), or acetylation (Bonaldi et al., 2003). Recent findings have shown that HMGB1 is released in an acetylated form and that the inhibition of histone deacetylases (HDACs) promotes the extracellular release, which suggests a role of HDACs in regulating HMGB1 (Bonaldi et al., 2003).

2.4.2.2. Inflammatory cytokines and markers: Cytokines are crucial regulators for biological processes including cell growth, proliferation, differentiation, and inflammation. Various cytokines have been implicated in the pathogenesis of RA, which are responsible for inflammation and joint destruction (Table 8). The cytokine network is divided into two groups, the pro-inflammatory (PrI) and the anti-inflammatory cytokines. The balance between these two groups is an important therapeutic goal (Fig. 2.5) (Mateen et al., 2016a).

![Figure 2.5. The imbalance between pro- and anti-inflammatory cytokines in RA](attachment://2.5.png)
In previous studies, TNF-α and IL-1 β have been studied as representatives of – proinflammatory cytokines, and IL-10 as an anti-inflammatory cytokine representative.

Table 8. Important cytokines involved in RA pathogenesis

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Receptors</th>
<th>Type</th>
<th>Cellular sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>p5, p75</td>
<td>PrI</td>
<td>Monocytes, macrophages, fibroblasts, mast cells, and NK cells</td>
</tr>
<tr>
<td>IL-1</td>
<td>IL-1RI, IL-1RII</td>
<td>PrI</td>
<td>Monocytes, fibroblasts, B cells, chondrocytes</td>
</tr>
<tr>
<td>IL-6</td>
<td>IL-6Ra, gp130</td>
<td>both</td>
<td>B cells, T cells, fibroblasts, monocytes, macrophages endothelial cells, keratinocytes, chondrocytes</td>
</tr>
<tr>
<td>IL-7</td>
<td>IL-7Rα and γc</td>
<td>PrI</td>
<td>Endothelial cells, fibroblasts, stromal cells, epithelial cells, keratinocytes</td>
</tr>
<tr>
<td>IL-18</td>
<td>IL-18Rα, IL-18Rβ</td>
<td>PrI</td>
<td>Macrophages, fibroblasts, dendritic cells, chondrocytes</td>
</tr>
<tr>
<td>IL-33</td>
<td>T1/ST2</td>
<td>both</td>
<td>Macrophages, fibroblasts, dendritic cells</td>
</tr>
</tbody>
</table>

2.4.2.2.1. TNF-α. TNF-α plays a crucial role in many inflammatory diseases and is a key player in driving inflammation in 50–70% of RA patients in which cells of the macrophage lineage are the primary source (Feldmann, 2009). TNF-α binds to two surface receptors, TNF receptor 1 (TNFR1), and TNF receptor 2 (TNFR2).

TNF-α regulates the expression of other proinflammatory cytokines such as IL-1 and enhances chemotaxis at the site of inflammation. Mice with genetic overexpression of TNF-α spontaneously develop RA-like lesions in the joints with bone destruction (Palladino et al., 2003). In a rodent model of RA, for instance, aberrant TNF-α expression in the synovium contributed to disease progression. Meanwhile, arthritic changes in response to TNF-α signaling inhibition were partially mediated by other pro-inflammatory cytokines, most notably IL-1 (Palladino et al., 2003). TNF-α also plays a dominant role in enhancing COX-2
expression, PGE<sub>2</sub> production, MMP-9 activity, and increased HMGB1 release in this fibroblast cell line (Alsousi AA <em>et al.</em>, 2017).

2.4.2.2. IL-1β. The IL-1 family includes 11 members. IL-1β is the predominant form of IL-1 family and shares approximately 26% amino acid sequence homology with IL-1α. IL-1β is the main therapeutic target for the most abundant inflammatory diseases.

IL-1β, a 17-kDa peptide, is produced as an inactive precursor. The inactive precursor is cleaved by caspase-1 via a protein complex into the active cytokine (Dinarello <em>et al.</em>, 1987). IL-1β is produced predominantly by cells of the innate immune system such as macrophages and monocytes. Other cells, including endothelial cells, keratinocytes, astrocytes, B lymphocytes and activated T lymphocytes also produce IL-1β (Miossec, 1987, 2001).

IL-1β mainly acts through single transmembrane domain associated with IgG-like extracellular domain receptors: type I (IL-1RI), and type II (IL-1RII) receptors. IL-1RI has a conserved region of 212 amino acids in the cytoplasmic tail, which is known as the Toll/IL-1R domain. Type I receptor is the functional receptor that exerts the biological effects of IL-1β. Type II acts as a decoy receptor, thereby preventing its interaction with type I (IL-1RI) receptor (Dinarello, 2013). Upon IL-1β binding, IL-1RI undergoes a conformational change required for the recruitment of downstream signaling molecules.

The systemic effects of IL-1β are exerted in many physiological processes including the CNS, bone marrow, blood vessels, but its local effects on the immune system are essential in RA. A large amount of evidence in experimental models of arthritis and humans has confirmed the role of IL-1β in RA pathogenesis (Johnson <em>et al.</em>, 1998; van den Berg and Bresnihan, 1999; Arend, 2001; Abramson and Amin, 2002). Furthermore, IL-1β stimulates
osteoclast differentiation and activity, via receptor activation of a nuclear factor kappa-β ligand (RANKL) expression in the synovial fibroblasts of RA patients, a mechanism involved in bone erosion (Gravallese et al., 2000; Horai et al., 2000). Also, IL-1β has an intimate relationship with TNF-α in an amplification loop, that includes IL-1β, TNF-α, and IL-6 through modulation of synovial leucocyte infiltration and pannus organization (Strand and Kavanaugh, 2004). TNF-α and IL-1β play a prominent role in the exacerbation of inflammation in RA as evidenced by increased expression of COX-2, PGE2 production, and enhancing the MMP-9 activity following direct treatment with either TNFα and/or IL-1β (Alsousi AA et al., 2017). IL-1β augments the production of T and B-lymphocytes, production of prostaglandin E2 (PGE2) and the proliferation of fibroblasts (Funk, 2001). Experimental evidence suggests that treatment with antagonists that block IL-1β and TNF-α action result in a total arrest of bone resorption leading to improved signs and symptoms of RA.

2.4.2.2.3. IL-10 is a pleiotropic cytokine produced by different cell types including synovial fibroblasts. The IL-10 gene is partially transcribed constitutively and is regulated by posttranscriptional RNA degradation mechanisms (Moore et al., 2001).

IL-10 interacts with the IL-10 receptor (IL-10R) which is composed of two subunits IL-10R1 and IL-10R2. IL-10R1 functions as a ligand-binding subunit with high-affinity while IL-10R2 is an accessory subunit for signaling. Furthermore, IL-10 as an anti-inflammatory cytokine contributes to the suppression of proinflammatory cytokine production and will usually increase during inflammation to surpass proinflammatory cytokine production. IL-10 potently inhibit IL-1α, IL-1β, IL-6, IL-12, IL-18, granulocyte-macrophage-colony-stimulating factor (GM-CSF), and TNF-α by activated macrophages, which is very critical to its activity as an anti-inflammatory cytokine (Moore et al., 2001). The ratio of a proinflammatory cytokine to
anti-inflammatory cytokine could represent the balance between the two types and their biologically relevant functions. Therefore, the extent of inflammation could correlate on a high ratio of TNF-a and IL-1β to IL-10 (You et al., 2011).

2.4.2.2.4. The S100 proteins (MRPs), also known as alarmins, are low molecular weight proteins which are characterized by two calcium-binding sites and belong to the damage–associated molecular patterns (DAMP) family (Foell et al., 2007). There are at least 21 of the S100 proteins that are secreted through active non-classical secretion (tubulin-independent pathway) from necrotic and stressed cells to signal in the early phase of damage (Rammes et al., 1997). The most familiar S100 protein is the S100A8/A9 complex, also known as myeloid-related protein (MRP8/ MRP14), calprotectin, L1 protein or calgranulin A and B. It is expressed in granulocytes and monocytes (Hessian et al., 1993).

Successful management of RA requires both proper measurement of disease activity and early diagnosis. Inflammatory markers are used to detect residual disease activity. Conventional markers of inflammation such as Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are non-specific markers (Pincus, 2005). Complexes of MRP8 (encoded by Mrp8) and MRP 14 (encoded by Mrp14) are the physiologically relevant forms of these proteins (Vogl et al., 2004) and are considered as an alternative laboratory biomarker in inflammatory diseases. MRPs are important contributors to inflammation in several disorders and are associated with inflammatory damage in RA (Youssef et al., 1999; Chen et al., 2009b). The MRP8/14 complex protein is released during the activation of leukocytes and is strongly upregulated in inflammatory diseases such as sepsis, RA, inflammatory bowel diseases, vasculitis, and cancer (Foell and Roth, 2004).
In this study, the involvement of RATM oxidant stress in RA will be investigated. In the next chapter, the past issue will be addressed through the hypothesis and specific aims.
CHAPTER 3
CENTRAL HYPOTHESIS AND SPECIFIC AIMS

The importance of the immunoregulatory function of RS in many human diseases is not evident. The altered biomolecular properties of cellular functions observed in patients suffering from chronic diseases implicate a role for OS in regulating immune responses. Hence, insights into the molecular and cellular mechanisms involved in oxidant stress derangements would be critical in the development of new therapeutic approaches that inhibit the destruction of cartilage and bone in RA.

The objectives of the present set of experiments are: (i) to study the changes induced by exogenous RATM as pro-oxidants in rabbit fibroblast-like synovial (FLS) cells through TLR4 activation, (ii) to investigate the mechanism by which RS generated by RATM as oxidants are sensed by TLR4, a membrane receptor, and (iii) to determine how RS may initiate, propagate, and maintain inflammatory phenotypes in FLS.

3.1. Rationale

The purpose of this research/project is to provide a new understanding of the potential response of RATM as signaling oxidants within FLS, which is an important yet poorly understood model system in RA pathogenesis.

FLS- present in the lining of the synovium-can integrate many stimuli, which may cause diseases. Early treatments of RA that targeted the immune system, or used a combination of powerful biological agents, resulted in increased complications and risk of infection (Weisman, 2002). However, successful management of RA using ethylenediaminetetraacetate (EDTA) metal chelation therapy has been reported (Bamonti et al., 2011). The oxidant stress profile, e.g. (serum ROS, GSH, and GSSH levels and TAC) of the patient improved following
treatment, thereby confirming the ability of chelation therapy to decrease the production of RS. During treatment, the amounts of used RA drugs (methotrexate and prednisolone) were reduced with no sign of blocked joints or walking impairment symptoms, which is a reliable indicator that such a chelation treatment may be useful.

Common pathway(s), by which trace metal-induced-oxidant stress may activate inflammation in synovial fibroblasts to potentially initiate, propagate and maintain RA, have not been explored to any extent. Our knowledge of the relationship between oxidant stress and inflammation has only recently been linked to the activation of Toll-like receptors (TLRs).

Dysregulation of innate immunity could be associated with autoimmunity (Franchimont et al., 2004). Indeed, external administration of endogenous RNA or DNA has activated TLRs and induced immune reactions (Hurst and von Landenberg, 2008). A recent study suggests that distinct TLR polymorphisms are associated with the development and clinical characteristics of RA (Lee et al., 2014). TLRs as PRRs play a significant role in the host defense system and may be involved in RA as the majority of TLRs especially, TLR4 has been detected in RA synovial membranes.

3.1.1. TLR4 and oxidant stress. TLRs are the best PRRs that are expressed both intracellularly and extracellularly (Akira and Takeda, 2004). Previous studies on TLR2 (Seibl et al., 2003; Abdollahi-Roodsaz et al., 2008a), TLR3 (Brentano et al., 2005b; Kim et al., 2009; Jiang et al., 2014; Thwaites et al., 2014), and TLR9 (Bhattacharjee and Akira, 2006; Abdollahi-Roodsaz et al., 2008b) have shown a correlation between these receptors and the pathophysiology of RA. However, TLR4 appears to be the most critically important. TLR4 is the most highly expressed TLR in human synovial fibroblasts, which is maintained in long-standing (chronic) RA (Ospelt et al., 2008). Thus, synovial fibroblasts may serve to integrate
many inflammatory stimuli that contribute to initiating the disease process. In fact, TLR4 is the only TLR that appears to respond to a wide range of non-canonical ligands such as nickel (Schmidt et al., 2010), cobalt (Raghavan et al., 2012), and high mobility group box 1 (HMGB1) (Kim et al., 2013). A previous study has shown that ROS generated from potassium peroxychromate (PPC) decomposition activate NF-kB mainly through a TLR4-dependent pathway in vivo and in vitro. This activation was attenuated by prior treatment with antioxidants (Karki and Igwe, 2013). Thus, inflammation induced by oxidants has many of the features associated with the classical activation of the innate immune system. Oxidants may be considered as another non-canonical ligand for TLR4. The exact molecular mechanism(s) for these effects is unknown.

Previous work in our laboratory has shown that TLR4 is involved in NF-kB activation in HEK-293 cells stably transfected with mouse TLR4 (Karki and Igwe, 2013). Pro-oxidant activation of TLR4 differs from that of TLR2, through TLR2 and TLR4 share the TIRAP adaptor protein, which is essential for the MyD88-dependent signaling pathway of both receptors (Horng et al., 2002). However, the TLR2 gene locus lacks a binding site for the myeloid differentiation factor-2 (MD2) co-receptor. This co-receptor is located upstream of the TLR4 gene where MD2 is directly involved in ligand binding and subsequent receptor activation. The absence of MD2 in the TLR2 locus may be a major difference in responses between the two receptors to pro-oxidant effects. It has been shown that low-level expression of TLR2 in cells can be up-regulated through TLR4 signaling (Xiang et al., 2010).

During the time of the current study, another investigation in our laboratory studied the mediatory role of TLR4 in oxidant-induced NF-kB activation in macrophages (Zhang and Igwe, 2018), another biologically relevant cell model. Interestingly, exogenous pro-oxidants
promoted extracellular disulfide HMGB1-induced NF-kB activation through the TLR4 signaling pathway with the potential to initiate “sterile” inflammatory responses (Zhang et al., 2015a). Thus, TLR4 can be considered a potential therapeutic target for numerous inflammatory diseases, including RA, leading us to focus our study efforts on TLR4, which is expressed in synovial fibroblasts. However, the exact contribution of reactive species to the onset of RA is not fully understood. Potential oxidant-induced signaling has not been explored in synovial fibroblasts. Hence, our primary objective is to determine the role of RS/TLR4-coupled activation of AP-1 in synovial fibroblasts, which may advance the development of effective therapies for RA.

Therefore, we will test the Central Hypothesis that oxidants generated by redox-active trace metals (RATM) will stimulate TLR4 to mediate inflammatory phenotypes through upregulation of activator protein 1 (AP-1)-signaling in synovial fibroblasts (Fig. 3.1).

The central hypothesis will be tested through the following specific aims:

3.2. Specific Aims

3.2.1. Specific aim 1. In this specific aim, we will test the hypothesis that FLS express TLR4, which is activated by RATM-derived pro-oxidants. We will achieve this aim through three sub-aims
Figure 3.1. A simplified graphical representation of a putative mechanism for exogenous RATM-induced AP-1 activation in synovial fibroblasts

(i) First, the expression levels of TLR4 and the characterization of its interaction with RATM-generated pro-oxidants in FLS will be determined.

Rationale: Several research groups have studied the expression of TLR4 in human RA tissues or cells. A higher expression of TLR4 in human synovial tissues has been reported in RA patients compared with patients with osteoarthritis (OA) and healthy individuals (Radstake et al., 2004a). However, precise TLR4 expression in different organs of rabbits was only reported in the lung, bone marrow, heart, kidney, liver, spleen, and thymus (Kajikawa et al.,
Therefore, the presence of TLR4 receptors in synovial fibroblast will be evaluated by PCR, Western blot and FC. These data will confirm the expression of TLR4 receptors in rabbit FLS and add to the validity of the model.

(ii) **Second, the effective concentrations of RATM as pro-oxidants will be established.**

**Rationale:** RATM such as Cu\(^+\), Fe\(^{2+}\), and Cr\(^{5+}\) as exogenous oxidants are essential constituents for vital enzyme functions (Bergman, 2010), but may also sensitize cells to initiate and maintain inflammation. Thus, first, the cytotoxic potential of RATM will be assessed. To measure cell viability, cells will be treated with RATM pro-oxidants and labeled with MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reagent for 24 hours. Since the MTT assay reflects mitochondrial function rather than cell death, Pierce\textsuperscript{TM} Lactate Dehydrogenase (LDH) cytotoxicity assay will be used to confirm the MTT results.

(iii) **Third, the RATM-mediated generation of intracellular reactive oxygen species (iROS) and the resolution whether production of ROS is TLR4-dependent or not will be determined.**

**Rationale:** In this sub-aim, the possible involvement of RS and oxidant stress induction through TLR4 expression in FLS pathogenesis will be highlighted. RATM-mediated production of reactive species as exogenous pro-oxidants will be confirmed using CellROX\textsuperscript{®} oxidative stress deep red dye; a fluorogenic probe designed to measure ROS in live cells. The release of ROS after RATM
treatment - represented by the emitted fluorescence - will be measured using both fluorescence microscopy and fluorescence cell-sorting flow cytometry. The second part of this sub-aim will focus on determining the effects of RATM on TLR4 activation, which will be evaluated by immunofluorescence and Western blots.

3.2.2. Specific aim 2. In this aim, we will test the hypothesis that RATM-mediated ROS/TLR4 coupled activation will induce the release of HMGB1 and markers of inflammatory phenotypes. RATM-induced TLR4 signaling in synovial fibroblasts can result in the expression of genes encoding inflammatory mediators, which can bind to their respective receptors expressed on different cells to promote inflammatory phenotypes.

Rationale: High mobility group box 1 (HMGB1) protein is essential in the regulation of nuclear functions. However, extracellular HMGB1 (ex HMGB1) induce acute and chronic inflammation (Andersson et al., 2018). Generally, TLR4 signaling activates the canonical inflammatory pathways resulting in the induction of a battery of inflammatory mediators. TNF-α, interleukin IL-1β, IL-10, and HMGB1 released into culture supernatants will be quantified using commercial ELISA kits.

Reverse transcription-quantitative PCR and/or Western blots will also be used to quantify mRNA and protein expressions respectively of these inflammatory mediators. Activation of the inflammatory marker MRP 8/14 will also be measured using flow cytometry. This approach will reveal whether FLS respond differently to oxidant stimulation to further clarify our understanding of the role of oxidants in the pathogenesis of RA.

3.2.3. Specific aim 3. In SA 3, we will test the hypothesis that RATM has a direct effect on cell proliferation and cell death in FLS.
**Rationale:** As a stress marker, HMGB1 can function as a signal for tissue damage. HMGB1 plays a vital role as a mediator of autoimmune, acute and chronic inflammatory diseases (Magna and Pisetsky, 2014). In RA, the importance of tissue reaction to immune stimulation, rather than the immune system itself in causing damage, is considered a major cause of tissue damage. To evaluate possible direct regulatory effects of RATM on cell proliferation and cell death, the exogenous RATM agents (Cu+, Fe²⁺, and Cr⁵⁺) will be used in combination with HMGB1 to determine their role in cell death in the FLS model using FC and Western blots. The regulatory effects of RATM on the proliferative capacity of FLS will be analyzed by proliferation assays. The results obtained from these experiments will provide further insight into the role of RATM-induced ROS/TLR4-coupled activation by RATM in synovial fibroblasts.

3.2.4. **Specific aim 4.** In SA 4, we will test the hypothesis that RATM-induced TLR4 activation will stimulate the activator protein (AP-1) signaling pathway.

**Rationale:** Following activation of TLR4 receptors, production of pro and anti-inflammatory mediators can be induced through multiple signaling pathways such as the mitogen-activated protein kinase (MAPK) pathway through AP-1, and nuclear factor-κB.

RATM-induced TLR4 signaling in synovial fibroblasts results in the release of both pro- and anti-inflammatory mediators, but the exact molecular mechanism(s) for these effects are unknown. The transcription factor activator protein 1 (AP-1) family is composed of homo- and hetero-dimeric complexes, which consist of Jun, Fos, activating transcription factor, and musculoaponeurotic fibrosarcoma proteins. These dimers are involved in different cellular processes, such as proliferation, apoptosis, and differentiation. Although members of the Jun and Fos families share a high degree of structural homology, the individual growth factors,
neurotransmitters, polypeptide hormones, bacterial and viral infections, as well as a variety of physical and chemical stresses employ AP-1 to translate external stimuli both into immediate and long-term changes of gene expression. These stimuli activate mitogen-activated protein kinase (MAPK) cascades that enhance AP-1 activity; for example, through phosphorylation of distinct substrates. To evaluate the possible role of TLR4 in FLS, cells will be transfected with TLR4 siRNA to confirm the role of TLR4 in oxidant-mediated AP-1 signaling. Activation of the AP-1 pathway will be determined by measuring the expression of target proteins using Western blots and FC. The results will provide additional information about the effects of RATM on the regulatory mechanisms of FLS, which may have an impact on developing therapeutic agents with trace metal chelating capacity and oxidant scavenging properties in a directed combinatorial therapy.

3.3. Significance

Significant health care costs of RA have created a critical need for finding a better intervention for the disease. Thus, at the completion of the proposed study, it is expected that major cellular components involved in the ROS/TLR4-coupled activation pathway will be identified. These results are expected to have a positive impact on finding new strategies for developing potential therapeutic agents for the management of RA. In addition to advancing public health, the novelty of the proposed work will provide new insights into the potential interaction of common trace metal-generated oxidants in the signaling pathway in FLS; a critical but yet poorly understood system.
CHAPTER 4
GENERAL MATERIALS AND METHODS

4.1. Chemicals and Reagents

**Albumin Bovine Serum** (Cat # A7888. Sigma-Aldrich, St. Louis, MO, USA).

**Alpha-Tubulin Monoclonal Antibody** (Cat. # 66031-1. Proteintech, Rosemont, IL, USA).

**Anti-JNK1+JNK2+JNK3 Antibody** [EPR16797-211] (Cat. # ab179461. Abcam, Cambridge, MA, USA).

**Anti-MRP8+MRP14 antibody [27E10]** (Cat. # ab17050. Abcam, Cambridge, MA, USA).

**Beclin-1 (D40C5) Rabbit mAb** (Cat. # 3495. Cell Signaling Technology, Inc. Danvers, MA, USA).

**Bolt™ 4-12% Bis-Tris Plus**, 12-well and 15 well (Cat. # NW00102BOX and NW04125BOX. Invitrogen, Thermo Fisher Scientific; MA, USA).

**Bullseye EvaGreen q PCR Master Mix** (Cat. # BEQPCR-R. MIDSCI; Valley Park, MO, USA).

**Cell Trace™ Proliferation Kit** (Cat. # C34554. Thermo Scientific; Rockford, IL, USA).

**CellRox® Deep Red** oxidative stress reagent (Cat. # C10422. Life Technologies, CA, USA).

**C-Fos (9F6) Rabbit mAb** (Cat. # 2250S. Cell Signaling Technology, Inc. Danvers, MA, USA).

**Chicken anti-HMGB1 Polyclonal Antibody** (Cat. # 326052233. HMGBiotech IBL International Corp. Toronto, ON, Canada).

**C-Jun (60A8) Rabbit mAb** (Cat. # 9165S. Cell Signaling Technology, Inc. Danvers, MA, USA).

**CLI-095 TLR4 Signaling Inhibitor** (Cat. # tlrI-cli95. InvivoGen; CA, USA).
CuCl (Cat. # 229628, Sigma-Aldrich, St. Louis, MO, USA).

Diethyl Pyrocarbonate (DEPC) (Cat. # D-5758. Sigma-Aldrich, St. Louis, MO, USA).

Dimethyl Sulphoxide (DMSO) HYBRID-MAX ® culture grade (Cat. #D2650. Sigma-Aldrich, St. Louis, MO, USA).

Dulbecco’s Phosphate Buffered Saline (DPBS) (1X) (Cat. #14190-136.Gibco®, Life Technology, Canada).

Ebselen (Cat. # ALX-270-097. Enzo Life Sciences; NY, USA).

Ethyl Alcohol USP, dehydrated 190 proof-95%. (AAPER Alcohol and Chemical Co., Shelbyville, KY, USA)

EUK 134 (Catalog # 10006329. Cayman Chemical, MI, USA).

FeCl2 (Cat. # 380024. Sigma-Aldrich, St. Louis, MO, USA).

Fetal Bovine Serum (FBS) (Hybridoma tested sterile-filtered, Sigma, St. Louis, MO, USA).

Fetal Bovine Serum (FBS), (Cat. # S11150. Atlanta Biologicals, Lawrenceville, GA, USA).

Fluorescein Goat anti-Mouse IgG (H+L) Secondary Antibody (Cat. # F2761. Life Technologies, CA, USA).

Fluorescein Goat anti-Rat IgG (H+L) Secondary Antibody (Cat. # A10528. Life Technologies, CA, USA).

Formaldehyde (Cat. # BP228-100. Fisher Scientific, Pittsburg, PA.USA).

Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, FITC (Cat. # F2765. Life Technologies, CA, USA).

HIG-82, rabbit synoviocytes (Cat. # CRL-1832. ATCC; Manassas, VA, USA).

High-Capacity cDNA Reverse Transcription Kit (Cat. # 4368814. Applied Biosystems; Rockford, IL, USA).
HMGB1 Antibody (Cat. # 3935. Cell Signaling Technology, Inc. Danvers, MA, USA).

HMGB1 ELISA Kit (IBL International Corp. Toronto, ON, Canada).

HMGB1 Polyclonal Antibody (Cat. # 10829-1-AP. Proteintech, Rosemont, IL, USA).

HRP-Conjugated β actin Monoclonal Antibody (Cat. # HRP-60008. Proteintech, Rosemont, IL, USA).


Hydrogen Peroxide (H₂O₂) (Cat. # 16911. Sigma-Aldrich, St. Louis, MO, USA).

IAXO-101 (CD14/TLR4 antagonist) (Cat. # IAX-600-001. AdipoGen® Life Sciences; Innaxon, UK).

IBI Tri-Isolate RNA Pure Kit (200 rxns) (Cat. # IB47632. IBI Scientific, IA, USA).

Isopropyl Alcohol (2-Propanol) (ACS plus) (Cat. # A416-1. Fisher Scientific, NJ, USA).

Jurkat Whole Cell Lysate. (Cat. # sc-2204. Santa Cruz Biotechnology, Inc, Dallas, TX, USA)

Lactate Dehydrogenase (LDH) Cytotoxicity Assay kit (Cat. # 601170. Cayman Chemical, MI, USA).

Lamin B1 Monoclonal Antibody (Cat. # 66095-1. Proteintech, Rosemont, IL, USA).

L-Ascorbic Acid (AA) (Cat. # A4544. Sigma-Aldrich, St. Louis, MO, USA).

LC3B (D11) XP® Rabbit mAb (Cat. # 3868. Cell Signaling Technology, Inc. Danvers, MA, USA).

Live Cell Imaging Solution (1X) (Cat # 14291DJ. Life Technologies, NY, USA).

LPS-EK Ultrapure from Escherichia coli K12 (Cat. # tlrI-eklps. InvivoGen; CA, USA).

Methanol (HPLC grade) (Cat. # A452-4. Fisher Scientific, NJ, USA).

2-Mercaptoethanol (Cat. # M-6250. Sigma-Aldrich, St. Louis, MO, USA).

MOBS SDS Running Buffer (Cat. # B0001. Novex, Life Technologies, CA, USA).
**MTT Reagent** (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide), (Cat. # M6494. Life Technologies, CA, USA).

**Native PAGE™ 20X Cathode Buffer Additive** (Cat. # BN2002. Novex, Life Technologies, CA, USA).

**Non-Fat Dry Milk** (Carnation, Nestle, Solon, OH, USA).

**Nuclear Extract Kit** (Cat. # 40410. Active Motif, CA, USA).

**NuPAGE MES SDS Running Buffer** (20X) (Cat. # B0002. Novex, Life Technologies, CA, USA).

**NuPAGE MOPS SDS Running Buffer** (20X) (Cat. # B0001. Novex, Life Technologies, CA, USA).

**Nutrient Mixture F-12 Ham** (Kaighn’s modification (Cat. # N3520. Sigma-Aldrich, St. Louis, MO, USA).

**OPTI-MEM ® I Reduced Serum Medium** modification of MEM Eagle’s (Cat. # 31985. Gibco®, Invitrogen Life Technologies™; Carlsbad, CA, USA).

**P38 MAPK (D23E1) XP® Rabbit mAb** (Cat. # 8690. Cell Signaling Technology, Inc.; Danvers, MA, USA)

**P62/SQSTM1 Rabbit mAb** (Cat. # 18420-1-AP. Cell Signaling Technology, Inc.; Danvers, MA, USA).

**PageRuler™ Prestained Protein Ladder** (Cat. # 26616. Thermo Scientific; Rockford, IL, USA).

**Penicillin-Streptomycin Solution** (100 X) (Cat. # 30-002 Cl. Corning, Gibco®, Life Technology, Canada).
P44/42 MAPK (Erk1/2) Rabbit mAb (Cat. # 9102. Cell Signaling Technology, Inc.; Danvers, MA, USA).


Phospho-P38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb (Cat. # 4511. Cell Signaling Technology, Inc. Danvers, MA, USA)

Phospho-P44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (Cat. # 4370. Cell Signaling Technology, Inc., Danvers, MA, USA).

Phospho-SAPK/JNK (Thr183/Tyr185) (G9) mouse mAb (Cat. # 9255. Cell Signaling Technology, Inc.; Danvers, MA, USA).

Pierce ® RIPA Buffer (Cat. # 89900. Thermo Scientific; Rockford, IL, USA).

Pierce LDH Cytotoxicity Assay Kit (Cat. # 88953. Thermo Scientific; Rockford, IL, USA).

Pierce™ BCA Protein Assay Kit (Cat. # 23235. Thermo Scientific; Rockford, IL, USA).

Ponceau S Solution (Cat. # P7170. Sigma-Aldrich, St. Louis, MO, USA).

Potassium Chromate (K₂CrO₄) (Cat. # 31250. Sigma-Aldrich, St. Louis, MO, USA).

Potassium Hydroxide (Cat. # 306568. Sigma-Aldrich, St. Louis, MO, USA).

Potassium Peroxychromate (PPC) is not commercially available. It was prepared in our laboratory according to the published protocol (Miesel et al., 1995).

Propidium Iodide Aqueous Solution (1mg/ml) (Cat. # P3566. Life Technologies, CA, USA).

Propidium Iodide Nucleic Acid Stain (Cat. # P3566. Invitrogen by Life Technologies, CA, USA).

Proteinase K (recombinant) PCR Grade (Cat. # E00491. Thermo Scientific, Rockford, IL, USA).
PVDF Transfer Membrane (Cat. # 88518. Thermo Scientific; IL, USA).

Rabbit Anti-murine TNF-α Polyclonal Antibody (Cat. # 500-P64. PeproTech; NJ, USA).

Rabbit Interleukin 1 Beta (IL1-β) ELISA kit (Cat. # MBS763764. MyBiosource.com).

Rabbit Interleukin 10 (IL-10) ELISA kit (Cat. # MBS283713. MyBiosource.com).

Rabbit Tumor Necrosis Factor-alpha (TNF-α) ELISA kit (Cat. # MBS2602362. MyBiosource.com).

Recombinant Murine TNF-α, Animal Free (Cat. # AF-315-01A. PeproTech; NJ, USA)

Restore PLUS Western Blot Stripping Buffer (Cat. # 46430. Thermo Scientific, Waltham, MA, USA).

RNase Staining Buffer (BD Bioscience, San Jose, CA, USA).

RT-PCR primers (IDT Integrated DNA Technologies, Skokie, Illinois, USA).

Silencer Select Negative Control No. 1 siRNA (Cat. # 4390843. Ambion by Life Technologies CA, USA).

Sodium Bicarbonate Solution (7.5%), (Cat. # S8761. Sigma-Aldrich, St. Louis, MO, USA).

Sodium Dodecyl Sulfate (SDS), Sigma-Aldrich, St. Louis, MO, USA).

SuperSignal ® West Pico Chemiluminescent Substrate (Cat. # 34079. Thermo Scientific; Rockford, IL, USA).

TBE Running Buffer (5X) (Cat. # LC6675. Novex, Life Technologies, CA, USA).

TLR 4 siRNA (Custom Designed. Invitrogen by Life Technologies, CA, USA).

TLR4 (Cat. # NB100-56566. Novus Biologicals, Littleton, CO, USA).

TLR4 (Cat. # NB100-56589. Novus Biologicals, Littleton, CO, USA).

TLR4 (M-300) (Cat. # sc-30002. Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Triton X-100 (Cat. # T-8787. Sigma-Aldrich, St. Louis, MO, USA).

Trypan Blue Stain (0.4%) (Cat. # T10282. Life Technologies, CA, USA).

Trypsin-EDTA (1X) (0.25%) (Cat. # 25200-072. Gibco®, Life Technologies, Canada).

Buffered Formaline Phosphate (10%) (Cat. # SF100-4. Fisher Scientific, Fair Lawn, NJ, USA).

Ultra-Leaf™ Purified Anti-mouse TLR4 (CD284)/MD2 complex (Cat. # 117612. BioLegend Inc.; CA, USA).

4.2. Equipment

- Bio-Tek Microplate Reader (Burlington, VT, USA).
- Countess® Automated Cell Counter, (Invitrogen™, Thermo Fisher Scientific, USA).
- FACSCantoTM II Flow Cytometer (BD Biosciences, San Jose, CA).
- Fuji LAS-4000 Imaging System, (Fujifilm, Stamford, CT, USA).
- Gilson Pipetman, models P-2, P-20, P-200, P 1000, P-5000, (Rainin Instrument Company, Woburn, MA).
- Isotemp CO₂ Incubator (Model 3533), (Thermo Fisher Scientific, Marietta, OH, USA).
- NALGENE™ Cryo 1 °C Freezing Container, cat # 5100-0001, (Thermo Scientific, Marietta, OH, USA).
4.3. Redox-Active Trace Metals (RATM) as Sources of Reactive Species

4.3.1. Potassium peroxychromate (PPC) (K₃CrO₈). The red peroxychromates were discovered by Riesenfeld in 1905 (Riesenfeld et al., 1905). Potassium tetraperoxychromate (V) is also known as potassium peroxychromate (PPC). The structure of PPC (K₃[Cr (O₂)₄]) was determined by the work of Stomberg and Brosset (Rolf and Cyrill, 1960). Spectroscopic and magnetic investigations using crystals were first reported on PPC in 1981 (Dalal et al., 1981). Chromium exists in a series of oxidation states with valences ranging from -2 to +6; the most important stable states are 0 (elemental), +3 (trivalent), and +6 (hexavalent). Trivalent Cr is an essential dietary mineral in low doses and is required for normal glucose metabolism. Cr (VI) compounds are amongst the earliest chemicals to be classified as carcinogens (Valko et al., 2005). The geometrical arrangement of oxygen atoms surrounding the central chromium atom as dodecahedral presented the unusual oxidation state +V of the chromium (Fig. 4.1 A-C). The
decomposition of 0.1 mM peroxochromate anion, \( \text{CrO}_8^{3-} \) with chromium +V is complete in less than one min in a HEPES-buffered solution and occurs more slowly in Tris- or PBS solutions. The decomposition type and yield of products in slightly alkaline solutions are very complex and is dependent on the composition of the medium (Worner et al., 1979). PPC decomposition was used to study ROS and their effects on cellular and biochemical functions (Hodgson and Fridovich, 1974; Baird et al., 1977).

4.3.2. **Cuprous chloride (CuCl)** (Fig. 4.1 E). Copper compounds were known as early as 400 BCE and were used to treat mental, pulmonary, and other diseases (Mason 1979). Copper is found in all tissues in small amounts. Studies of dietary copper intake, excretion, balance, and its concentrations in cadavers were reported as early as in the 1930s (Chou and Adolph 1935; Tompsett 1934; Tompsett 1935). The body of a 70 kg healthy adult contains about 110 mg copper. Dietary copper is absorbed through intestinal mucosa with the highest copper concentrations in the liver followed by the brain, kidney, and heart (Turnlund 1998). The concentrations range from 10 to >100 μM with 20 % found in the nucleus. (Linder 1991).

Copper as a transition metal has three oxidation states: Cu\(^0\), oxidized cupric form Cu\(^{+2}\), and reduced cuprous form Cu\(^{+}\) (Uauy et al., 1998). Copper metal ion is not only a ubiquitous metal in the environment, but also functions as an essential cofactor for maintaining normal biological functions required for growth, development, and maintenance including cytochrome c oxidase, tyrosinase, p-hydroxyphenyl pyruvate hydrolase, dopamine beta hydroxylase, lysyl oxidase, and Cu-Zn superoxidase dismutase (Cu Zn SOD) (Nordberg 2007). Furthermore, copper is important in angiogenesis, nerve myelination and endorphin action (Linder 1991). High tissue levels of copper have been found in different types of human cancers, such as breast (Huang et al., 1999), prostate, colon (Nayak et al., 2003), lung and brain (Turecky et
Copper ions are associated with DNA bases in chromatin especially guanine (Bryan et al. 1981) and are considered hallmarks of apoptosis (Burkitt et al. 1996).

**4.3.3. Ferrous chloride (FeCl₂) (Fig. 4.1 D).** Iron as the fourth abundant element in the earth’s crust belongs to the family of transition elements that include Cu, Cr, Mn, Co, Ni, and Zn. Iron as an essential trace element for cellular functions acts directly either as a donor or as an acceptor in many electron transfer reactions. The body of an average adult contains 4.5 gram, mostly in hemoglobin. Premenopausal women usually have lower body iron content.

About 65% of iron in living organisms is bound to the hemoglobin protein; 25% is bound to ferritin and hemosiderin (iron-storage proteins), and about 10% is part of oxygen-carrying proteins myoglobin and cytochromes (Winter et al., 2014).

Disturbances in iron metabolism lead either to deficiency or to overload, both with severe consequences. In case of iron overload, iron accumulates in the soft tissues, mainly in the heart and liver (Andrews 1999). Iron loading in fibroblasts caused a significant increase in iron versus control and the uptake rate of both Fe²⁺ and Fe³⁺, was comparable with myocytes. Furthermore, the efflux of iron to the medium from fibroblasts after 72 h of treatment was about 20 % with 20 μg/ml (Liu et al., 2003). These findings highlight the differential ability of fibroblasts to handle iron.

**4.3.4. Ascorbic acid (AA).** Ascorbic acid (Vitamin C) is an essential micronutrient and is believed to have an antioxidant function in living systems, which is frequently considered responsible for its protective effects against cardiovascular disease and specific types of cancers (Park et al., 2018). However, its use as a therapeutic agent against cancer remains debated. Ascorbic acid stimulates cell death, nuclear fragmentation and internucleosomal DNA cleavage in human myelogenous leukemia cell lines (Gillberg et al., 2017). The apoptosis-
inducing activity of AA has been credited to its pro-oxidant activity and is inhibited by catalase, antioxidants like N-acetylcysteine and GSH, Ca^{2+} and Fe^{3+} depletion but stimulated by hydrogen peroxide, Cu^{2+} and iron chelators (Sakagami and Satoh, 1997). Several studies have explored the pro-oxidant properties of ascorbate (Valko et al., 2005). The different and contradicting results suggest that vitamin C plays a different role in each cell type.

**Figure 4.1. RATM oxidants used in the study.** (A) Structure of tetra peroxychromate anion in the structure of K₃[Cr (O₂)₄]. The [Cr- (O₂)₄]⁻³ ion has a central chromium ion bonded to four peroxide groups, and the interatomic distances in picometer (pm) are given according to (Cage and Dalal, 2001). (B) The crystal structure of K₃[Cr (O₂)₄]. (C) Unit cell in the structure of K₃[Cr (O₂)₄]. (D) Chemical structure of FeCl₂. (E) Crystal structure of CuCl.
4.4. Generation of Oxidants (Fig. 4.2)

All solutions were prepared fresh before each experiment.

4.4.1. Synthesis/Preparation of PPC. PPC is not available commercially; therefore, it was synthesized from 30% aqueous hydrogen peroxide (H₂O₂) and potassium chromate according to the published protocol (Miesel et al., 1995). Briefly, 4.0 g of potassium chromate was added to an Erlenmeyer flask placed in a salted ice bath to ensure continuous cooling, and a solution of 4.0 g KOH in 50 ml H₂O was added in a dropwise fashion. The mixture was cooled down under constant stirring until a thick slurry was formed without freezing. To the thick yellow slurry, 25 ml of 30% H₂O₂ was slowly added under constant stirring. The color of the slurry changed from yellow to a dark brown while evolving bubbles. Cooling was continued with occasional stirring for 2 to 3 hrs. At the end of the reaction, crystals of PPC had formed according to the following equation.

\[
2 \text{K}_2\text{CrO}_4 + 9 \text{H}_2\text{O}_2 + 2 \text{KOH} \rightarrow 2 \text{K}_3\text{CrO}_3 + \text{O}_2 + 10 \text{H}_2\text{O}
\]

PPC crystals were filtered, washed twice with distilled H₂O, and dried overnight at 37°C. Consistently a yield of more than 80% was obtained. Our Laboratory characterized the product by elemental and infrared analyses, which demonstrated a purity of at least ≥ 98%.

A stock solution of 100 μM PPC was prepared fresh in PBS (1X) prior to use in each experiment then diluted to the required concentration to ensure that the released ROS from PPC decomposition have much longer t₁/₂ in solution since the aqueous decomposition rate of PPC is 80 μM/min in carbonate and pyrophosphate buffers at pH 10.2 (Hodgson and Fridovich, 1974).
4.4.2. **Preparation of FeCl\(_2\) solution.** The stock solution of FeCl\(_2\) was prepared fresh just before use in culture grade sterilized water (molecular biology grade) and diluted in PBS to the desired concentrations of 375 nM and 10 \(\mu\)M as needed.

4.4.3. **Preparation of CuCl solution.** The stock solution of CuCl in dimethyl sulphoxide (DMSO) was prepared fresh just before use and diluted in PBS to the desired dilution of 50 \(\mu\)M to containing 0.0001 DMSO in its final dilution.

4.4.4. **Preparation of ascorbic acid (AA) solution.** A working solution was prepared fresh in PBS just before use to the desired concentration of 100 \(\mu\)M.

4.4.5. **Preparation of CuAA solution.** A working solution of CuCl as described in (4.4.3) was mixed with two volumes of the working solution of AA, as described in (4.4.4) to provide a desired ratio of Cu: AA (1:2) in the final dilution.
Figure 4.2. ROS generated by RATM oxidants. (A) PPC can form different species of ROS. (B) Iron can form ROS through iron-catalyzed Haber-Weiss reaction. (C) Copper forms ROS via Haber-Weiss reaction. (D) The cytotoxicity of copper and ascorbate combination is attributed to the continuous formation of hydroxyl radicals through recycling of Cu^{2+} back to Cu^{+}. 
4.5. Treatment Protocols of RATM as Oxidants

4.5.1. Treatment of RATM as oxidants. In the current study, two distinct lengths of treatments were used. Short and long-term treatments were used, both at empirical low concentrations.

Table 9. Treatment protocols of oxidants

<table>
<thead>
<tr>
<th>Treatment time</th>
<th>The concentration of redox-active trace metals (RATM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h short-term treatment</td>
<td>PPC (10 nM)</td>
</tr>
<tr>
<td></td>
<td>FeCl₂ (10 µM)</td>
</tr>
<tr>
<td></td>
<td>CuCl (50 µM)</td>
</tr>
<tr>
<td></td>
<td>CuAA (50 µM CuCl with 100 µM AA)</td>
</tr>
<tr>
<td>24 h and 48 h long-term treatment</td>
<td>PPC (5 nM and 10 nM)</td>
</tr>
<tr>
<td></td>
<td>FeCl₂ (375 nM and 10 µM)</td>
</tr>
<tr>
<td></td>
<td>CuCl (50 µM)</td>
</tr>
<tr>
<td></td>
<td>CuAA (50 µM CuCl with 100 µM AA)</td>
</tr>
<tr>
<td></td>
<td>LPS-EK 100 ng/ml</td>
</tr>
</tbody>
</table>

4.5.2. Treatment with blockers and inhibitors. Cells were pre-incubated for 30 min with EUK-134 (10 µM), or Ebselen (5 µM), for two h with low endotoxin azide-free (LEAF) affinity purified rat IgG2a, κ-isotype anti-mouse TLR4/MD-2 complex polyclonal antibody (pAb), or with CLI-095 (5 µM) before treatment with any RATM.

EUK-134, a superoxide dismutase (SOD) and catalase (CAT) mimic [CAT/SOD] inhibitor and a synthetic serum-stable scavenger for oxidant species (Rong et al., 1999; Lawler et al., 2014), and Ebselen (hydrogen peroxide potent scavenger) (Nakamura et al., 2002). The two free radical quenching-antioxidants were used to clarify the role of RATM as pro-oxidants. Anti-TLR4/MD-2 pAb, a neutralizing Ab for TLR4, was used to block interaction with its putative ligands. CLI-095, a TLR4 signaling inhibitor that binds to TIR domain 54 of TLR4 (Matsunaga et al., 2011), was used to block signal transduction following TLR4 activation.
4.6. Cell Culture and Live Cell Counting

HIG-82 (ATCC® CRL-1832™) is a rabbit synovial fibroblast derived from the intra-articular soft tissue of the knee joint of a young female New Zealand rabbit. HIG-82 is an established cell line that retains the activatable phenotype (Georgescu et al., 1988). Cells were cultured and maintained in HAM F-12 Kaighn’s modification with sodium bicarbonate supplemented with 10% fetal bovine serum (FBS), Penicillin/streptomycin (100 units/ml), at 37 °C in an atmosphere of 5% CO₂/95% air/100% humidity. The cells were passaged every three days following trypsinization at a split ratio of 1: 2. Cells, at 70-80% confluency, were incubated overnight then treated with Opti-MEM® Reduced Serum Medium immediately before treatment with any RATM. Cells were seeded at either 1 X 10⁶ cells/ 60 mm dish, 5 X 10⁵ cells/well in a 6-well plate, 2 X 10⁵ cells/well in a 12-well plate, or 8 X 10³ cells/well in a 96-well plate for all the experiments.

To determine numbers of live cells for seeding, following trypsinization, cells were counted using Countess® Automated Cell Counter. Live and dead cells were counted using 0.4% Trypan Blue dye. Dead cells will have compromised cell membrane integrity and therefore will take up the dye while live cells will exclude the dye.

The rationale for selecting a rabbit cell line is that TLR4 and MD-2 proteins from rabbits are more similar to their human counterparts than mice and rats with 72 and 70% amino acid homology, respectively (Kajikawa et al., 2005; Turin and Riva, 2008). Sequence similarity percentage between amino acid sequences of human TLR4 and other species are shown in (Table 16) (Vaure and Liu, 2014). The hypervariable region displays 57% similarity with the human sequences, which is higher than in mice and rats’ counterparts. Therefore, rabbit TLR4 may recognize human pathogens better than mouse TLR4 (Kajikawa et al., 2005).
Of the species studied, only primates (85-98%) and horses (58%) share greater identity than rabbits when considering the hypervariable region in the extracellular domain of human TLR4 (Hajjar et al., 2002). The greater similarities between the rabbit and human TLR4, suggest that the human immune response is better modeled in rabbits than in either mice or rats. This matter is especially important when human diseases are studied with animal models.

Table 10. Sequence similarity percentage between human TLR4 and TLR4 in other species.

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Chimpanzee</th>
<th>Mouse</th>
<th>Rat</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distal extracellular domain</td>
<td>100</td>
<td>66</td>
<td>66</td>
<td>77</td>
</tr>
<tr>
<td>Hypervariable region</td>
<td>98</td>
<td>48</td>
<td>39</td>
<td>57</td>
</tr>
<tr>
<td>Proximal extracellular domain</td>
<td>100</td>
<td>63</td>
<td>64</td>
<td>63</td>
</tr>
<tr>
<td>Transmembrane domain</td>
<td>100</td>
<td>70</td>
<td>68</td>
<td>75</td>
</tr>
<tr>
<td>Proximal cytoplasmic domain</td>
<td>99</td>
<td>90</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Distal cytoplasmic domain</td>
<td>100</td>
<td>26</td>
<td>38</td>
<td>85</td>
</tr>
</tbody>
</table>

4.7. Culture Media Preparation (HAM F-12 Kaighn’s Modification)

Ninety percent of the final required volume of autoclaved water was measured at room temperature (RT). HAM F-12 Kaighn powder was added with gentle stirring. The original packaging was rinsed with a small amount of water and added to the solution. 33.3 ml of sodium bicarbonate solution (7.5% w/v) was added for each liter of final volume. The medium was stirred until all powder had dissolved, and the pH was adjusted using 1N HCl or 1 N NaOH to pH 7.4. Sterile water was added to bring the solution to its desired volume. Filtration with a membrane of 0.22 mm porosity was used to sterilize the medium. The liquid medium was stored at 4 °C in the dark until used.
4.8. Determination of Cell Viability and Optimized Concentrations of Oxidants

4.8.1. MTT reduction assay for optimal seeding. This assay is based on the observation that living cells will convert the tetrazolium salt into a colored formazan product. The optimal seeding of HIG-82 synovial fibroblasts was determined using MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (Loo and Rillema, 1998). Ten different serial dilutions of cells were plated in a 96-well plate ranging from $2 \times 10^3$ to $2 \times 10^7$ cells per well. The cells were grown in a culture medium consisting of Nutrient Mixture F-12 medium with 10% FBS and antibiotics at 37 °C, and 5% CO$_2$ in 100% humidified incubator for 20 h. The cells were labeled with 20 µl MTT reagent (5 mg/ml) in PBS per well for another four h at 37 °C (total 24 h). After the incubation, the medium containing MTT reagent was removed, and 200 µl DMSO was added to each well to dissolve the violet crystals. Cells were then incubated for another 10 minutes. Cell viability was determined by measuring the absorbance at 540 nm in a Bio-Tek microplate reader. The LDH assay, as described in (4.8.3), was conducted to confirm MTT seeding results. Calculations were performed as was recommended in the protocol. The average of the blank absorbance value was subtracted from each average sample absorbance to yield corrected absorbance values. The corrected sample absorbance (Y-axis) was plotted versus concentration (X-axis), and the IC80 was determined by locating the X-axis value (nM or µM). The percentage of proliferation was calculated by the ratio of absorbance at 540 nm between control and treated cells.

4.8.2. MTT reduction assay of oxidants. To determine the mitochondrial reducing capacity of HIG-82 synovial fibroblasts, cells were seeded at a density of $8 \times 10^3$ cells/well in a 96-well plate. The cells were grown in culture medium consisting of Nutrient Mixture F-12 medium with 10% FBS and antibiotics at 37 °C and 5% CO$_2$ in 100% humidified incubator
overnight. Cells were then treated for 20 h with each of the RATM oxidants: ferrous chloride (FeCl₂), cuprous chloride (CuCl) with L-Ascorbic acid (CuAA), LPS-EK, and PPC at varying concentrations. The cells were then labeled and incubated with MTT as previously described in (4.7.1) for a total of 24 h. The sample absorbance value for the negative control (0 µM) corresponds to 100 % cell viability.

4.8.3. Lactate dehydrogenase (LDH) release assay. Because the MTT assay reflects mitochondrial function rather than cell death, a Lactate dehydrogenase (LDH) cytotoxicity assay was conducted to confirm MTT results. LDH is a cytosolic enzyme present in different cell types. The damage of the plasma membrane releases LDH into the cell culture medium. Extracellular LDH in the medium is quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD⁺ reduction to NADH. Diaphoresis uses NADH to reduce a tetrazolium salt (INT) to a red formazan product that is measured at 490 nm. The level of formazan formation is directly proportional to the amount of LDH released into the medium, which is indicative of cytotoxicity. HIG-82 synovial fibroblasts cells were seeded at a density of 8 x 10³ cells/well in a 96-well plate. The cells were grown overnight in culture medium consisting of Nutrient Mixture F-12 medium with 10% FBS and antibiotics at 37 °C, and 5 % CO₂ incubator. The cells were treated with trace metal oxidants FeCl₂, CuCl (with and without Ascorbic acid), and PPC at concentrations that produce higher than 80% viability for 24 h per manufacturer’s instructions. The level of LDH released into the culture medium was determined from a standard curve.

\[
\text{% Cytotoxicity} = \frac{(\text{LDH level of treatment} - \text{LDH level of control})}{\text{Total LDH level}} X 100\%
\]
4.9. Preparation of Whole-Cell Protein Extract

Approximately 5 X10^5 cells/well were seeded in a 6-well plate, then incubated overnight in a complete medium to reach 70-80% confluence. The complete medium was removed and replaced with Opti-MEM® I Reduced Serum Medium prior to treatment with RATM oxidants or LPS-EK. Following treatments, cells were rinsed with 0.5 ml PBS followed by the addition of 200 μl of radioimmunoprecipitation assay (RIPA) buffer, which was supplemented prior to use with 1% proteinase and phosphatase inhibitor cocktails (1:100). Cells were scraped into the buffer using a cell scraper and transferred into Eppendorf tubes. The total lysate was centrifuged at 12,000 x g for 20 min at 4 °C to remove debris. The supernatant was then transferred into Eppendorf tubes and stored at -80 °C as a total protein extract. The total protein was quantified spectrophotometrically using the Micro BCA protein assay reagent kit, and absorbance was measured at 562 nm. Aliquots of total cell extracts containing 15-20 μg total protein were mixed with a loading buffer in a total volume of 20 μl and denatured at 95 °C for 5 min. Samples, ready for electrophoresis were run immediately or were stored at -80 °C until used.

4.10. Preparation of Nuclear and Cytoplasmic Extract

Cells were grown in (60 x 15) mm Petri-dishes at 2 x 10^6/dish overnight to 70% confluence. The cells were treated with culture medium (control), or pro-oxidants for 24 h in Opti-MEM® I Reduced Serum Medium, after which they were washed. A nuclear fraction (NF) and a cytoplasmic fraction (CF) were prepared using a nuclear extract kit as per manufacturer’s instructions. Briefly, cells were collected in ice-cold PBS supplemented with phosphatase inhibitors. Cells were resuspended in hypotonic buffer to swell the cell membrane and make them fragile. Detergent was added to cause leakage of the cytoplasmic proteins into
the supernatant. After collection of the CF by centrifugation at 14,000 × g for 10 min at 4°C, the nuclei were lysed, and nuclear proteins were solubilized in detergent-free lysis buffer; supplemented with protease inhibitor cocktail. NF and CF were subjected to an immunoblot assay to determine the protein expression of HMGB1.

4.11. Immunoblot (IB)/Western Blot (WB) Analysis

Protein aliquot samples containing 15-20 μg total protein were mixed with 5 x SDS sample loading buffer in a total volume of 20 μl and denatured at 95 °C for 5 min. Equal amounts of denaturated total protein were loaded per lane along a protein molecular weight ladder. Loaded proteins were fractionated in precast Tris-glycine electrophoresis gels (4-12%) or (12 %), using a Novex X-Cell II electrophoresis cell system run at 165 volts for 50 min. Following fractionation, samples were transferred onto a polyvinylidene difluoride (PVDF) membrane using an electrophoretic transfer cell. Immunoblotting was commenced by blocking non-specific binding sites on the membrane with a blocking buffer consisting of 5% non-fat milk in PBS or 1-5% BSA in PBS, for 90 min at room temperature; followed by washing with (TBST) for 30 min. The membrane was incubated overnight at 4 °C with an affinity-purified primary antibody diluted (1: 250 to 1:1000), (Table 17). The blot was washed in TBST for 30 min and incubated for two h at room temperature with HRP-coupled anti–β-actin pAb (1:500) for normalization. Following incubation, the blot was washed, and total immunoreactivity was detected using the SuperSignal West Pico Chemiluminescent substrate (ECL) substrate kit. Signals were visualized using Fuji LAS-4000. The optical density (OD) ratio of the detected relevant protein signal to β-actin protein was obtained by densitometric analysis. the β-actin signal was used to normalize for loading errors.
To determine the expression of more than one protein on the same membrane, Restore PLUS Western Blot Stripping Buffer™ was used for reblotting. Ten (10) ml of the stripping buffer were added to the membrane, which was incubated for 5-10 min with agitation at RT. After removing the stripping buffer, the membrane was washed 3x for 5 min each in TBS, followed by blocking, and then incubation with the primary and secondary antibody as described above.

4.12. ELISA Quantifications

Cells were grown overnight in a complete medium in 6-well plates at 5 x 10^5 cells/well. The cells were treated with Opti-MEM® I Medium (control) or RATM oxidants for either 6, 12, or 24 h, in which the culture media supernatant was collected, centrifuged to remove cell debris, aliquoted, and stored at -80 °C until used for ELISA. The contents of HMGB1, TNF-α, IL-1β, and IL-10 levels released to the media were quantified using HMGB1, TNF-α, IL-1β, and IL-10 ELISA kits as per manufacturer’s instructions. The fluorescence readings were determined by measuring the absorbance in a Bio-Tek microplate reader. The fluorescence intensities data were compared among different treatments and respective controls.
Table 11. Antibodies (Abs) used in WB and their dilutions

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha- tubulin mAb</td>
<td>1:5000 diluted in 1% BSA in TBST</td>
</tr>
<tr>
<td>Anti-JNK1+JNK2+JNK3 Ab</td>
<td>1:1000 diluted in 1% BSA in TBST</td>
</tr>
<tr>
<td>Beclin-1 mAb</td>
<td>1:1000 diluted in 1% BSA in TBST</td>
</tr>
<tr>
<td>c-Fos mAb</td>
<td>1:1000 diluted in 1% BSA in TBST</td>
</tr>
<tr>
<td>c-Jun mAb</td>
<td>1:1000 diluted in 1% BSA in TBST</td>
</tr>
<tr>
<td>Goat Anti-Rabbit IgG, HRP conjugated</td>
<td>1:20,000 diluted in TBST</td>
</tr>
<tr>
<td>Goat Anti-Rabbit IgG, HRP conjugated</td>
<td>1:20,000 diluted in TBST</td>
</tr>
<tr>
<td>HMGB1 Ab</td>
<td>1:1000 diluted in 5% BSA in TBST</td>
</tr>
<tr>
<td>HRP-conjugated ACTB (β actin)</td>
<td>1:5000 diluted in 5% non-fat milk in TBST</td>
</tr>
<tr>
<td>Lamin B1 mAb</td>
<td>1:5000 diluted in 5% non-fat milk in TBST</td>
</tr>
<tr>
<td>LC3B (D11) XP® Rabbit mAb</td>
<td>1:1000 diluted in 5% BSA in TBST</td>
</tr>
<tr>
<td>P38 MAPK (D23E1) XP® Rabbit mAb</td>
<td>1:1000 diluted in 5% BSA in TBST</td>
</tr>
<tr>
<td>P44/42 MAPK (Erk1/2) Rabbit mAb</td>
<td>1:1000 diluted in 5% BSA in TBST</td>
</tr>
<tr>
<td>P62/SQSTM1 Rabbit Ab</td>
<td>1:1000 diluted in 5% BSA in TBST</td>
</tr>
<tr>
<td>Phospho-P38 MAPK (D3F9) XP® Rabbit mAb</td>
<td>1:1000 diluted in 5% BSA in TBST</td>
</tr>
<tr>
<td>Phospho-P44/42 MAPK (Erk1/2) (D13.14.4E) XP® Rabbit mAb</td>
<td>1:2000 diluted in 5% BSA in TBST</td>
</tr>
<tr>
<td>Phospho-SAPK/JNK (G9) mouse mAb</td>
<td>1:2000 diluted in 5% non-fat milk in TBST</td>
</tr>
<tr>
<td>TLR4 (M-300)</td>
<td>1:500 diluted in 5% non-fat milk in TBST</td>
</tr>
<tr>
<td>TLR4 (NB100-56580)</td>
<td>1:1000 diluted in 5% non-fat milk in TBST</td>
</tr>
<tr>
<td>TLR2</td>
<td>1:5000 diluted in 1% BSA in TBST</td>
</tr>
</tbody>
</table>

4.13. Flow Cytometry

4.13.1. Quantification of iROS production by flow cytometry. To confirm the initial fluorescence microscopy data, flow cytometry (FC) was used to determine the time-dependent fluorescence intensity of iROS with the same dye CellROX®. We used Opti-MEM® I Reduced Serum Medium and DPBS with extremely gentle scraping instead of trypsin to quantify iROS by FC for the following reasons. When culture medium is changed, cells switch from relative hypoxia to hyperoxia and suffer culture shock. Cells usually respond to this by altering ROS
production. As well as the use of trypsin in sub-culturing depletes GSH levels (Clement et al., 2001).

HIG-82 synovial fibroblasts cells were grown overnight in 6-well plates at 5 x 10^5 cells/ well. Cells were treated with vehicle (control), or FeCl₂, CuCl and PPC for a specified time point in Opti-MEM® I Reduced Serum Medium, after which cells were incubated with CellROX® Deep Red reagent (1 mM) for 30 min at 37 °C followed by subsequent two washes in PBS. DPBS was added, and cells were incubated for 15-30 min, after which cells were very gently scraped and resuspended for flow cytometry.

Flow cytometry acquisition and analysis were conducted using FACSCanto™ II flow cytometer. The fluorescence intensity corresponding to iROS-labeled CellROX® Deep Red reagent was determined using the Allophycocyanin (APC) filter at excitation/emission wavelengths of 645/660 nm. Unstained cells were used as negative controls. For each parameter investigated, at least 10^4 events (cells) were analyzed per sample. The fluorescence intensities data was compared for different treatments.

4.13.2. Measurement of cell-cycle phase specific cell death using propidium iodide: cell cycle flow cytometry analysis. For cell cycle withdrawal studies, HIG-82 synovial fibroblasts were seeded at 5 x 10^4 cells/well in 6-well plates and grown in Nutrient Mixture F-12 Ham with sodium bicarbonate and 10% fetal bovine serum (FBS) overnight at 37 °C in a 100% humidified incubator. The medium was replaced with fresh, warmed Nutrient Mixture F-12 Ham with 1% FBS for synchronization to arrest cells in the G0/G1 phase by serum deprivation. After 24 h of synchronization, cells were treated with RATM pro-oxidants or HMGB1 for 24 h in Opti-MEM® I Reduced Serum Medium. The medium was collected and saved, and the cells were washed with PBS, followed by addition of 150-μl trypsin-EDTA
(0.25% trypsin/EDTA) to each well. The medium from corresponding wells was added back to inactivate trypsin. Cells were pooled and centrifuged in the same tube at 496 g for 6 min. Ethanol fixation stabilizes the stained samples and conserves the separation of living and dead cells in the bivariate FCM histograms. To fix the cells in 70% ethanol, cell pellets were washed with ice cold 1 x PBS, then suspended in 300 µl PBS, and fixed by dropwise addition of 2 volumes of absolute cold ethanol (700 µl) while vortexing the cell suspension.

Cells were kept for two h at 4 °C if samples were to be processed on the same day, or stored at −20 °C for future analysis/assay. Fixed cells were transferred to room temperature (RT) and centrifuged to discard ethanol. Cells were then washed in PBS, resuspended in 100 µl PBS containing five µl Propidium iodide (1 mg/ml) and ten µl RNase-DNase free (10 mg/ml) and incubated in the dark (covered with aluminum foil) for 30 min at RT. Fluorescence-activated cell sorting (FACS) analysis was performed using FACSCantoTM II flow cytometer. The proliferation index (PI) which represents the matter of cell proliferation was calculated using the following equation (Guo et al., 2011):

$$PI = \frac{(S+G_2M)}{(S+G_2M + G0/G1)} \times 100\%$$

The sub-G0 fraction of events represented the apoptotic population of cells.

4.13.3 CellTrace™ CFSE cell proliferation assay. Carboxyfluorescein diacetate succinimidyld ester (CFSE) reagent diffuses readily into cells and binds covalently to intracellular amines resulting in stable, well retained fluorescent staining. It is colorless and non-fluorescent until the acetate groups are removed by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyld ester. The succinimidyl ester group reacts with intracellular amines forming conjugates that are well retained. The dye-protein adducts formed in cells are retained throughout development and meiosis.
Cells were cultured overnight in a 12-well plate at $2 \times 10^5$ cell/well. The culture medium was removed and replaced with the CFSE-diluted in 1 μM PBS loading solution for 20 min at 37°C. CFSE loading solution was removed, and cells were washed twice with fresh culture medium containing 1% FBS. Complete fresh medium was added, and cells were incubated for 30 min to allow the reagent to undergo acetate hydrolysis. Cells were treated with vehicle control or pro-oxidants for 24 and 48 h. At the end of treatments, cells were washed with DPBS, detached with 0.25% trypsin. Cells were resuspended in PBS then fixed for further analysis with FC.

FC analysis was conducted on FACSCantoTM II flow cytometer. Fluorescence intensity corresponding to proliferation was determined using the FITC filter with excitation/emission of 492/519 nm. Unstained cells were used as negative controls. For each parameter investigated, at least $10^4$ events (cells) were analyzed per sample. The fluorescence intensity data were compared among different treatments.

4.13.4. FAM FLICA™ poly caspase assay. The FAM FLICA Poly Caspase kit was used to assess caspase activation to quantify apoptosis in vitro. The FLICA reagent FAM-VAD-FMK enters each cell and irreversibly binds covalently to active caspase enzymes, leading to its retention within the cell. Unbound FAM-VAD-FMK FLICA reagent diffuses out of the cell and is washed off. Apoptotic and pyroptotic cells will retain higher concentrations of FLICA. The green fluorescent signal is a direct measure of the active caspase enzyme activity present in the cell at the time the reagent is added. Cells that contained the bound FLICA were analyzed by flow cytometry.

Cells were seeded at $5 \times 10^5$ cells/well in a six-well plate and grown overnight in Nutrient Mixture F-12 Ham with sodium bicarbonate and 10% fetal bovine serum (FBS) at 37
°C in a 100% humidified incubator. The medium was replaced with fresh, warmed Opti-MEM® I Reduced Serum Medium immediately before treatment. The FAM-FLICA working solution was added directly to the overlay media at a v/v ratio of 1:30. Cells were incubated for 30-60 min at 37 °C, mixed gently every 10-20 min to disperse the reagent. Cells were washed, and RATM pro-oxidant treatments were applied to cells for 2 and 24 h. Ninety (90) % ethanol was used as a positive apoptosis control.

Upon completion of treatment and to avoid losing non-adherent cells during washing, all overlay media and wash buffer were combined and centrifuged. Washed cell pellets were recombined with the adherent samples before the analysis by flow cytometry. FAM-FLICA excites at 492 nm and emits at 520 nm.

4.13.5. Quantification of total (HMGB1, TLR4, and MRP8/14) protein levels by flow cytometry. HIG-82 synovial fibroblasts cells were grown overnight in 6-well plates at 5 X 10^5 cells/well. Cells were treated in Opti-MEM® I Reduced Serum Medium (control), or FeCl2, CuCl and PPC for 24 h. Washed cells were incubated with DPBS for 15-30 min at 37 °C, followed by transfer into 1.5 ml vials for FC. After fixation in 4 % formaldehyde in PBS for 10 min at room temperature (RT), cells were permeabilized on ice with 0.2 % Triton X-100 in PBS for 30 min. After rinsing in PBS, cells were blocked in 5% BSA at room temperature (RT) for 30 min, followed by incubation for three h at 4 °C with the primary antibody (Table 18). Cells were then incubated with FITC-conjugated goat anti-mouse, anti-rabbit, or anti-rat IgG for one h. Acquisition and analysis of flow cytometry data were conducted on a FACSCantoTM II flow cytometer.

The fluorescence intensity corresponding to each antibody was determined using the FITC filter with excitation/emission of 495/519 nm. Unstained cells were used as negative
controls. For each parameter investigated, at least $10^4$ events (cells) were analyzed per sample. The fluorescence intensity data were compared among different treatments.

4.13.6. Quantification of total and phosphorylated ERK, JNK, and P38 by flow cytometry. Cells were treated as indicated above in (4.13.5), except for ERK quantification where cells were permeabilized with Methanol. After rinsing in PBS, cells were blocked in 5% BSA at room temperature (RT) for 30 min, or followed by incubation for 3 h at 4 °C with any of the following primary antibodies: (P38 MAPK Rabbit mAb, Phospho-P38 MAPK (Thr180/Tyr182) Rabbit mAb, P44/42 MAPK (Erk1/2) Rabbit mAb, Phospho-P44/42 MAPK (Erk1/2) (Thr202/Tyr204) Rabbit mAb, Phospho-SAPK/JNK (Thr183/Tyr185) (G9) mouse mAb, or anti-JNK1+JNK2+JNK3 antibody, with shaking. After rinsing in PBS, cells were incubated with FITC-conjugated goat anti-mouse, anti-rabbit, or anti-rat IgG (Table 18) for one h. Acquisition and analysis of flow cytometry data were conducted on a FACSCantoTM II flow cytometer.

The fluorescence intensity corresponding to each antibody was determined using the FITC filter with excitation/emission of 495/519 nm. Unstained cells were used as negative controls. For each parameter investigated, at least $10^4$ events (cells) were analyzed per sample. The fluorescence intensity data were compared among different treatments.

4.14. Fluorescence Microscopy

4.14.1. Measurement of intracellular ROS (iROS) by Fluorescence Microscopy Imaging. HIG-82 synovial fibroblasts cells were treated with different trace metal pro-oxidants at subthreshold concentrations in Opti-MEM® I Reduced Serum Medium, followed by CellROX® Deep Red reagent per manufacturer’s instructions. Cells were incubated with
CellROX® (5µM) and NucBlue live cell stain for 60 min at 37 °C followed by washes in PBS and fixation for 10 min with 4 % paraformaldehyde.

After subsequent washes with PBS, images were acquired using fluorescence microscopy at excitation and emission wavelengths of 644/665 nm for CellRox® and 405/410-550 nm for NucBlue®. Images were analyzed using the Image J software (Version 1.44, http://image.nih.gov/ij/).

4.14.2. Immunofluorescence (IF) of HMGB1 (Immunohistochemistry). HIG-82 synovial fibroblasts cells were seeded in 8-well Lab-Tek™ II chamber slide (Nalge Nunc International, NY) and grown overnight, followed by serum starvation with Opti-MEM®I Reduced Serum Medium. The cells were treated with FeCl₂ (375 nM and 10 µM), CuCl (50 µM), and PPC (5 and 10 nM) for 24 h. After fixation in 4 % formaldehyde in PBS for 10 min at RT, cells were permeabilized with 0.2 % Triton X-100 in PBS for one h. After rinsing in PBS, cells were blocked in 5% BSA at room temperature (RT) for one h followed by overnight incubation with shaking at 4 °C with primary antibody (1:100, rabbit polyclonal, HMGB1).

After rinsing in PBS, cells were incubated in the dark with FITC-conjugated goat anti-rabbit IgG for one h and NucBlue R live cell stain for 10-30 min while shaking. After subsequent washing with PBS, images were acquired using fluorescence microscopy at excitation and emission wavelengths: ~ 495/519 nm for FITC and 405/410-550 nm for NucBlue R.

4.15. Quantitative Real-Time Polymerase Chain Reaction (RT- qPCR)

Cells were grown overnight in 6-well plates at 5 X 10⁵ cells/well in the culture medium. After removal of culture medium, cells were treated in Opti-MEM® I Reduced Serum Medium (control), or FeCl₂, CuCl, and PPC. Total RNA was extracted using (IBI Tri-Isolate total RNA
purification kit) according to the manufacturer’s instructions and was quantified in a Nanodrop spectrophotometer. An aliquot of RNA sample with the A260/280 nm absorbance ratio of 1.8 or above was reverse transcribed in a 20 μl reaction volume using High-Capacity cDNA Reverse Transcription Kit according to the manufacturer’s instructions. The cDNA was amplified using real-time PCR Step-One plus™ Real-Time PCR System via standard fluorescence methodology and thermal cycling conditions following the manufacturer’s recommendations, including validation of each gene amplification testing by the identification of single peaks in melting curves.

The real-time PCR reaction mixture contained 2.5 μl cDNA, 10 μl of the RT Real-Time™ SYBR Green PCR master mix, 0.5 μl of each primer pair (10 μM), and 6.5 μl of RNase-free water to a complete reaction volume of 20 μl. Ct values were normalized to β-actin as a reference gene.

The primers for β-actin, HMGB1, TLR4, TNF-α, IL-1β IL-10, and MRP8 and MRP14 were purchased from IDDT (see Table 19). Gene expression levels were calculated relative to controls and expressed as $2^{-ΔΔCt}$. The RT-PCR amplicons were loaded onto 1% agarose gels at 5 μl/well and fractionated at 125 V. Images of the gels were acquired using the Fuji LAS-4000.
Table 12. Antibodies used in flow cytometry (FC) and immunofluorescence (IF) and their dilutions

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Dilution</th>
<th>FC (diluted in blocking buffer)</th>
<th>IF (diluted in blocking buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-JNK1+JNK2+JNK3 Ab</td>
<td></td>
<td>1:250</td>
<td></td>
</tr>
<tr>
<td>Anti-MRP8 + MRP14 antibody (ab17050)</td>
<td></td>
<td>1:50</td>
<td></td>
</tr>
<tr>
<td>Fluorescein Goat anti-Mouse IgG (H+L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary Antibody</td>
<td></td>
<td>2 µg/ml</td>
<td></td>
</tr>
<tr>
<td>Fluorescein Goat anti-rat IgG (H+L) secondary Ab</td>
<td></td>
<td>2 µg/ml</td>
<td></td>
</tr>
<tr>
<td>Goat anti-rabbit IgG (H+L) secondary Ab</td>
<td></td>
<td>1:500</td>
<td>1:200</td>
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<tr>
<td>HMGB1 Ab</td>
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<td></td>
<td>1:100</td>
</tr>
<tr>
<td>HMGB1 polyclonal Ab</td>
<td></td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>P38 MAPK (D23E1) XP® Rabbit mAb</td>
<td></td>
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<tr>
<td>P44/42 MAPK (Erk1/2) Rabbit mAb</td>
<td></td>
<td>1:50</td>
<td></td>
</tr>
<tr>
<td>Phospho-P38 MAPK (D3F9) XP® Rabbit mAb</td>
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<tr>
<td>Phospho-P44/42 MAPK (Erk1/2) (D13.14.4E) XP® Rabbit mAb</td>
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<td>1:800</td>
<td></td>
</tr>
<tr>
<td>Phospho-SAPK/JNK (G9) mouse mAb</td>
<td></td>
<td>1:400</td>
<td></td>
</tr>
<tr>
<td>TLR4 (NB100-56566)</td>
<td></td>
<td>5 µg</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Primer Sequence</td>
<td>Reverse Transcription</td>
<td>Denaturation</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------</td>
<td>-----------------------</td>
<td>------------------</td>
</tr>
</tbody>
</table>
| IL-1β | Fwd: 5'-TAC AAC AGG AGC TTC CGG CA-3'  
        | Rev: 5'-GGC CAC AGG TAT CTT GTC GT-3'  | 50 ºC for 10 min 95ºC for 1min | 95ºC for 15 Sec | 60 ºC | 95 ºC |
| IL-10 | Fwd: 5'-GAG AAC CAC AGT CCA GCC AT-3'  
        | Rev: 5'-CAT GGC TTT GTA GAC GCC TT -3' | 50 ºC for 10 min 95ºC for 1min | 95ºC for 15 Sec | 55 ºC | 95 ºC |
| TNF α | Fwd: 5'-AGA TGG TCA CCC TCA GAT CAG-3'  
        | Rev: 5'-GAA GAG AAC CTG GGA GTA GAT GAC-3' | 50 ºC for 10 min 95 ºC for 1min | 95ºC for 15 Sec | 61 ºC | 95 ºC |
| HMGB1 | Fwd: 5'-CCA TTG GTG ATG TTG CAA AG-3'  
        | Rev: 5'-CTT TTT CGC TGC ATC AGG TT-3' | 50 ºC for 10 min 95 ºC for 1min | 95ºC for 15 Sec | 53 ºC | 95 ºC |
| TLR4  | Fwd: 5'-TCC ATA AAA GCC GAA AGG TG-3'  
        | Rev: 5'-CTG AGC AGG GTC TTT AC-3' | 50 ºC for 10 min 95 ºC for 1min | 95ºC for 15 Sec | 52 ºC | 95 ºC |
| β actin | Fwd: 5'-TGC TTC TAG GCG GAC TGT TA-3'  
        | Rev: 5'-CGT CAC ATG GCA TCT CAC GA-3' | 50 ºC for 10 min 95 ºC for 1min | 95ºC for 15 Sec | 55 ºC | 95 ºC |
| MRP8  | Fwd: 5'-CAT GGC GAC TGA TCT GGA AA-3'  
        | Rev: 5'-CGT AGA GGG CGT GGT AAT TT-3' | 50 ºC for 10 min 95 ºC for 1min | 95ºC for 15 Sec | 55 ºC | 95 ºC |
| MRP14 | Fwd: 5'-CTT TGG CAG GGT TTC ACA GGA AT-3'  
        | Rev: 5'-ACT GGT GGA AGA CGT TGA TG-3' | 50 ºC for 10 min 95 ºC for 1min | 95ºC for 15 Sec | 55 ºC | 95 ºC |
4.16. Silencing TLR4 Gene (siRNA Technique)

4.16.1. Design and synthesis of siRNA. Two siRNAs (Table 14) targeting TLR4 (GenBank accession no. AY101394) were synthesized using Custom Silencer® Select siRNA (Ambion by Life Technologies). The siRNAs are 21 nucleotides (nt) in length with a 3′ overhang of two thymidines. To avoid cross-silencing of non-target genes, all of the designed siRNA sequences were blasted against the rabbit genome database. A predesigned Silencer Select Negative Control No. 1 siRNA (Cat # 4390843) that does not have any significant similarity with this target gene was used as a negative control (NC).

Table 14. TLR4 siRNA

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′-&gt;3′ siRNA 513</td>
<td>Sense: GAAGUUCACCCGUUGGUUUt</td>
</tr>
<tr>
<td></td>
<td>Antisense: AAACCAACCUTFUAGCACUUc</td>
</tr>
<tr>
<td>siRNA 514</td>
<td>Sense: CACUCAACCAACCUCCUUUAtt</td>
</tr>
<tr>
<td></td>
<td>Antisense: UAAAGGAGGUUGUGAGUGGAt</td>
</tr>
<tr>
<td>Negative control (NC)</td>
<td>NC No.1 siRNA(Cat # 4390843). The sequence was not provided by (Ambion by Life Technologies)</td>
</tr>
</tbody>
</table>

4.16.2. siRNA transfection. Cells were transfected with siRNA using Lipofectamine® RNAiMax. Briefly, cells were seeded in 6-well plates at a density of 3×10^5 cells/well in antibiotic-free medium for 12 h before transfection.

The transfection mixture was prepared by mixing a 1.25-μl aliquot of 20 μM synthetic siRNA and a 1-μl aliquot of Lipofectamine® RNAiMax with two separate aliquots of 100 μl of serum-free OPTI MEM® I and then combining the two solutions at room temperature for 30 min to form a complex. The cells were washed with PBS, and 200 μl of the transfection mixture was added to each well along with 800 μl of serum-free OPTI MEM® I to produce a final concentration of 50 nM siRNA per well.
The transfected cells were incubated for 24 h before being harvested for RNA and protein. After the initial 24 h incubation period, the medium was replaced with fresh OPTI MEM® I medium, and cells were treated with RATM pro-oxidants and incubated for an additional two h.

4.17. Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) from at least 3–7 independent experiments. One group of data was analyzed using two-tailed unpaired Student’s t-test. Remaining data were analyzed by one or two-way analysis of variance (ANOVA), followed by Tukey’s post hoc tests. GraphPad software (7) was used. P values of ≤ 0.05 were considered significant for all tests.

4.18. Solutions

4.18.1. Cell culture solutions. Complete growth HAM F-12 Kaighn’s modified Medium. 445 ml of freshly prepared HAM F-12 Kaighn’s solution was mixed with 50 ml fetal bovine serum and 5 ml penicillin/streptomycin mixture (10,000 U/ml penicillin and 10 mg/ml streptomycin), to make 500 ml of HAMF-12 medium which were stored at 4 °C.

1% HAM F-12 Kaighn's modified Medium.: 490 ml of freshly prepared HAM F-12 Kaighn’s solution was mixed with 5 ml fetal bovine serum and 5 ml penicillin/streptomycin mixture (10,000 U/ml penicillin and 10 mg/ml streptomycin), to make 500 ml of HAMF-12 medium, which was stored at 4 °C

1X Phosphate-buffered Saline (PBS). One phosphate buffered saline PBS tablet was dissolved in 200 ml ultrapure water to make 1 x PBS.

4.18.2. Immunoblotting solutions. 1M Tris -HCl pH 6.8. 121.14 g of Tris base was dissolved in 800 ml dH2O; the pH was adjusted to 6.8 by slow addition of 1 N HCl with stirring. The final volume was brought to 1 L with dH2O, which was autoclaved and stored at RT.
20% SDS. 20 g of SDS were dissolved in 80 ml dH₂O in a ventilated fume hood, and the volume was adjusted to 100 ml with dH₂O.

3 M NaCl Solution. 175.3 g NaCl were dissolved in 800 ml of ddH₂O and then was adjusted to 1 L of dd H₂O.

1 X Tris-Buffered Saline with 0.1% Tween 20 (TBST). 50 ml 3 M NaCl, 20 ml 1 M Tris-HCl, and 930 ml dH₂O were mixed, and 1 ml of Tween 20 was added with gentle stirring to make 1 L TBST.

Protein loading buffer-5x sample loading buffer (5X LB). To prepare 50 ml, 25 mg bromophenol (0.05%) was added to 10 ml of 1 M Tris-HCl. 10 ml glycerol (20%) were added and mixed while stirring. The mixture was transferred to the fume hood to add 25 ml of SDS (20%) and mixed for several minutes. Finally, 5 ml β-mercaptoethanol (10%) were added and stirred efficiently. The solution was aliquoted and stored at –20 °C until use.

1 X Phosphate-buffered saline with 0.1% Tween 20 (PBST). 1 ml Tween 20 was added to 999 ml of 1 X PBS.

1 % BSA in PBST. 1 g bovine serum albumin (BSA) was dissolved in 80 ml 1 X PBST then was adjusted to 100 ml.

5% Bovine Serum Albumin (BSA). 5 g bovine serum albumin (BSA) was dissolved in 80 ml 1 x TBST then was adjusted to 100 ml.

5% Non-fat Milk. 5 g non-fat dry milk powder were dissolved in 80 ml 1X TBST then was adjusted up to 100 ml.

Running Buffer. 20 ml of 20 X NuPAGE MOPS SDS running buffer was mixed with 480 ml ultrapure water to make 1 X working solution.
Transfer Buffer. 10X transfer buffer was made by dissolving 30.25g Tris base and 144 g glycine into 1 L double distilled (dd) H₂O. 100 ml of 10X transfer buffer was diluted with 700 ml dd H₂O, and 200 ml methanol was added to make 1 L working solution containing 20% of methanol.

4.18.3. Flow cytometry solutions. 10% FBS with 1% sodium azide in PBS. 10 ml FBS was diluted with 80 ml PBS to make 100 ml of 10% FBS/PBS. 1 g sodium azide was dissolved then the solution was adjusted to 100 ml 10% FBS/PBS.

3 % BSA in PBS. 3 g BSA were dissolved in 80 ml 1 X PBS then made up to 100 ml.

3% BSA with 1% sodium azide. 1 g sodium azide was dissolved in 100 ml 3 % BSA/PBS.

1 M Tris pH 8.0. 12.11 g Tris base was dissolved in 80 ml dH₂O. The pH value was adjusted to pH 8.0 with HCl, and the volume was adjusted to 100 ml with dH₂O.

1.0 M Tris pH 8.5. 12.11 g Tris base was dissolved with 80 ml H₂O. The pH value was adjusted to pH 8.5 with HCl, and the volume was adjusted to 100 ml with dH₂O.

6 M NaCl. 175.38 g NaCl were dissolved in 400 ml dH₂O then was adjusted to 500 ml.

0.5 M EDTA pH 8.0. 186.1 g of disodium EDTA (Na₂EDTA) was suspended in 800 ml dH₂O. The pH was adjusted to 8.0 with NaOH, and a clear solution was obtained. The volume was adjusted to 1 L with dH₂O.

Tris-EDTA buffer Preparation. To make a 50 ml Tris-EDTA (TE) buffer, 4.9 ml dH₂O were mixed with 500 μl of 1 M Tris pH 8.5 and 100 μl of 0.5 M EDTA pH 8.0.

4.18.4. RNA extraction solution: 70% Ethanol in diethylpyrocarbonate (DEPC)-treated H₂O. Dilute 700 ml 100 % ethanol (200 proof) by addition of 300 ml DEPC-treated H₂O to make 1 L of 70 % ethanol in DEPC-treated H₂O.
CHAPTER 5
REDOX-ACTIVE TRACE METALS (RATM) AS PRO-OXIDANTS

5.1. Introduction

The current understanding of the mechanism(s) responsible for RATM-mediated inflammation is minimal and requires further study. Therefore, we sought to determine if RATM-induced oxidant stress would provoke the expression of various proinflammatory cytokines and if so, which signaling pathway(s) may be involved, and whether this signaling pathway may potentially contribute to the pathogenies of RA. The results from this study will provide essential information regarding the possible molecular mechanism(s) underlying the redox-active trace metal oxidant stress-mediated inflammation in synovial fibroblasts.

5.2. Results

5.2.1. HIG-82 rabbit synovial fibroblasts optimal seeding is 8 x 10^3 / well in 96-well plate. Previously HIG-82 cells were seeded at 7 x 10^5 cells / 25 cm^2 (Georgescu et al., 1988). An initial experiment was performed to determine the proper seeding. The optimum seeding of HIG-82 synovial fibroblasts was determined using the MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Eight different serial dilutions of cells were seeded in a 96-well plate ranging from 2 x 10^3 to 20 x 10^3 cells per well. Using the MTT cytotoxicity assays (Fig. 5.1-panel A), optimal seeding for the HIG-82 cells was determined as 8 x 10^3/ well in a 96-well plate, which is the highest available seeding before evading linearity. LDH assay was used as well to confirm the results (Fig. 5.1-panel B).
Figure 5.1. Determination of the optimal seeding of HIG-82 synovial like-fibroblasts (FLS) in 96-well plate. Synovial fibroblasts were seeded at serial dilutions of cells in a 96-well plate ranging from $2 \times 10^3$ to $20 \times 10^3$ cells/well. Cells were grown in culture medium consisting of Nutrient Mixture F-12 medium with 10% FBS and antibiotics at 37 °C, 5% CO$_2$ and 100% humidified incubator for 20 h. Cells were labeled and incubated with (A) MTT reagent for four h at 37 °C, after which the supernatant was removed, and the formazan crystals were dissolved in DMSO. Cell viability was determined by measuring absorbance at 540 nm in a Bio-Tek microplate reader. (B) LDH assay was used to confirm MTT results.
5.2.2. Determination of the working concentrations for each RATM using MTT and LDH assays. To avoid using toxic concentrations of RATM as exogenous pro-oxidants and to mimic the exposure to the physiological concentrations, the viability of FLS was determined for each RATM over a range of five different concentrations within 24 h of treatment. The used concentrations allowed at least 80 % cell viability at subthreshold oxidant concentrations (Fig. 5.2-panel A and B). The MTT assay was used to determine cell viability. At 5 nM PPC, ≥ 80% of cells remained viable with similar viability at 375 nM FeCl₂. 10 nM PPC and 10 µM FeCl₂ were used to examine the effects of higher concentrations. At 50 µM CuCl, ≥ 80 % of cells remained viable. LPS-EK at 100 ng/ml provided ≥ 80 % viability. For each RATM pro-oxidant, at least five independent experiments were performed.

Subsequently, the LDH assay was used to confirm the MTT assay results (Fig. 5.2-panel C). It was observed that PPC (5 nM), FeCl₂ (375nM and 10 µM), and LPS-EK (100 ng/ml) did not increase LDH release into the culture medium, compared to vehicle control. In contrast, PPC (10 nM), CuCl (50 µM), and CuAA resulted in the release of LDH. However, data showed that the cytotoxicity induced by all RATM ranged from only 0-10 % of the vehicle control (PBS).
Figure 5.2. Effects of RATM oxidants on the viability of FLS. Cells were grown in culture medium consisting of Nutrient Mixture F-12 medium with 10% FBS and antibiotics at 37 °C, 5% CO₂ and 100% humidified incubator overnight. Cells were treated with each of the RATM oxidants ferrous chloride, cuprous chloride, CuAA, and PPC at different concentrations for 20 h. Cells were labeled and incubated with (A) MTT reagent for four h at 37 °C, after which the supernatant was removed, and the formazan crystals were dissolved in DMSO. (B) LPS-EK MTT cell viability assay was assessed by measuring absorbance at 540 nm in a Bio-Tek microplate reader. (C) LDH assay. The p-value ≤ 0.05 (*) and ≤ 0.0001 (#) were considered significant using t-test and one ANOVA followed by Tukey’s multiple comparison tests.
5.2.3. Confirmation of the expression of TLR4 in FLS. TLR4 is expressed in synovial fibroblasts derived from trauma, RA, or OA patients, which constitutively express mRNA for TLRs 1–6. Consistent with the basal expression pattern in cultured synovial fibroblasts, TLR4 was most abundant among the TLRs measured in the synovium (Ospelt et al., 2008). However, HIG-82 cells are derived from a rabbit synovial fibroblasts cell line, which expresses TLR4 complex and responds to TLR4 agonist stimulation. To determine the role of TLR4 in RATM oxidants-induced AP-1 activation, the expression of TLR4 in HIG-82 cells was confirmed at both the mRNA and protein levels using RT-PCR and flow cytometry, respectively (Fig. 5.3). Expression of TLRs mRNA including TLR4 mRNA was determined in cells as described in Figure (5.3-panel A). Lane 4; HEK Blue mTLR4 cells are stably transfected cells with TLR4 which used as positive control cells (Fig. 5.3-panel B). HIG-82 cells were genotyped by assessing the expression of TLR4 using RT-PCR. A single PCR product as expected at 195 bp was amplified in the presence of the primers (Table 19).

This result revealed that TLR4 is the most abundant TLR expressed in HIG-82 cell line. These observations confirmed that HIG-82 synovial fibroblasts constitutively express TLR4 and are considered a valid model.
Figure 5.3. Expression levels of TLR4 mRNA and protein in FLS. (A) Comparative expression levels of TLR4 protein by Western blot analysis. (B) RT-quantitative PCR confirmation of the expression of TLR4 mRNA in HIG-82 cells, DNA gel electrophoresis of TLR4 RT-quantitative PCR product. (C) Quantitative FC analysis of TLR4 protein expression in HIG-82 cells
5.2.4. RATM upregulated TLR4 expression in FLS. Treatment with PPC (5 nM), FeCl$_2$ (375nM), and CuCl (50 µM) for 6, 12, 24, and 48 h increased TLR4 protein expression over untreated control cells incubated under the same conditions in a time-dependent manner (Fig. 5.4- panel A). PPC significantly upregulated TLR4 protein expression at 12, 24, and 48 h by 1.6-, 1.8-, and 2.5- fold respectively. FeCl$_2$, and not CuCl, significantly increased TLR4 expression at 24 h. (Fig. 5.4- panel B). Therefore, 24 h was selected as a treatment duration time for subsequent experiments unless otherwise indicated.

**Figure 5.4. Time course of RATM oxidants-mediated TLR4 expression in FLS** (A) Cells were treated with vehicle control (C), or PPC, FeCl$_2$, CuCl and LPS-EK (T) at different time points (0, 6, 12, 24 and 48 h). Western blots determined the TLR4 protein expression levels. The histogram (B) represents the optical density (OD) ratios of TLR4 immunoblot signals normalized to those of β-actin from the same tests. The data represent five independent experiments [n=5, * P < 0.05, two-way ANOVA with Tukey’s multiple comparison tests].
5.2.5. **RATM-induced intracellular ROS (iROS) decreased after four h of treatment.** Oxidants damage, as bimolecular damage, is caused by a direct attack of reactive species during oxidants stress (Halliwell and Whiteman, 2004). A combination of immunofluorescence microscopy and flow cytometry was used to determine intracellular ROS (iROS) production.

Treatment with PPC, FeCl₂, CuCl, and LPS-EK at different time points increased iROS generation. Immunofluorescence imaging microscopy data ([Fig. 5.5 panel A](#)) confirmed the generation of iROS, which is represented by the CellROX® red dye while blue color reveals the stained nuclei. Thus, ROS generated exogenously by RATM treatment resulted in increased cellular iROS. Immunofluorescence data showed ([Fig. 5.5 panel B](#)) that RATM treatment generated the highest level of iROS after two h, which subsequently decreased significantly after 4h of treatment.

The time course of iROS production by flow cytometry ([Fig. 5.5 panel D](#)) revealed that PPC produced the highest amounts of iROS after 24 h, increased on average by 3.8- fold compared to controls. FeCl₂ (375nM and 10 µM) ([Fig. 5.5 panel E](#)), and CuCl (50 µM) ([Fig. 5.5 panel F](#)) generated maximum iROS after one h of interaction with an average increase of 2.8- and 2.3- fold respectively. LPS-EK induced the highest level of ROS production after 1-2 h of treatment.

The time course of CuCl (50 µM) ([Fig. 5.5 panel F](#)) showed that iROS generation starts as early as 30 min and reaches its maximum after 1h, continues to generate ROS up to 4 h, followed by a decrease to control levels. The data is in agreement with those of Xiao et al. (Xiao *et al.*, 2010), where copper complexes were able to induce iROS after one h of treatment and continued to be detected after four h of treatment.
Figure 5.5. Immunofluorescence and flow cytometry time course of RATM oxidants - mediated intracellular ROS (iROS) levels in HIG-82 FLS. Cells were treated with PPC, FeCl₂, CuCl, and LPS-EK at different time points in OPTI-MEM media. Following the removal of the incubation media, (A) Cells were stained with CellRox® Deep Red Reagent and NucBlue® Live Ready Probes Reagent (to counterstain cellular nuclei) for 30 min at 37 °C followed by PBS washes and fixation with 4% paraformaldehyde for 15 min. After subsequent rinses with PBS, images were acquired using fluorescence microscopy or cells were incubated with CellRox® Deep Red Reagent for 30 min. Cells were rinsed with PBS, scraped and resuspended into warm DPBS per the manufacturer's instructions for flow cytometric analysis. (B) Shows quantitative analyses of immunofluorescence following treatments with vehicle (control), or oxidants resulting in changes in fluorescence intensity after different periods (2, 4, and 6 h) Panel (C) shows representative flow cytometry tracings following treatments with media (control), and pro-oxidants for 1.5 h Panel (D) shows quantitative analyses of flow cytometry tracings following treatments with vehicle (control) and PPC (5nM) which show changes in fluorescence intensity after different times of treatment. (E) Shows quantitative analyses following treatments with vehicle (control), and FeCl₂ (375nM and 10 µM), (n = 3–4; *p ≤ 0.05). (F) Shows quantitative analyses following treatments with vehicle (control), and CuCl (50 µM).
5.2.6. RATM-induced iROS increase decreased by antioxidants treatment.

Treatment with exogenous oxidants including, PPC (5 nM), FeCl₂ (375 nM), CuCl (50 µM), and LPS-EK (100 ng/ml) for 4 h increased intracellular ROS generation. The presence of exogenous oxidants resulted in a transfer of oxidants tone to the intracellular environment.

However, prior incubation of cells with EUK-134 (10µM) (superoxide dismutase/catalase mimetic agent), or Ebselen (5µM) (hydrogen peroxide potent scavenger) for 30 min before treatment with pro-oxidants decreased iROS level (Fig. 5.6 panel A). Representative semi-quantitative histograms (Fig. 5.6 panel B) clearly shows a decrease in iROS by more than 50% following each treatment when compared to treatment with the oxidant alone. We had observed that EUK (10µM) pretreatment, in general, is more significant in abolishing iROS compared to Ebselen (5µM) pretreatment. PPC iROS generation went down from almost 2.5 to 0.5-fold and from more than a 2.5-fold to a 1.25-fold for FeCl₂ and from a 2.75-fold to a 1.25-fold for CuCl.
Figure 5.6. Immunofluorescence representation of the levels of intracellular ROS (iROS) in HIG-82 FLS. (A) Immunofluorescence representation of the accumulated levels of iROS following cell stimulation with RATM oxidants. Cells were treated with PPC, FeCl₂, and CuCl and/or in combination with EUK-134 or Ebselen (EB). Cells were stained with CellRox® Deep Red Reagent, and NucBlue® Live Ready Probes® Reagent (to counterstain cellular nuclei) for 30 min at 37 °C followed by subsequent PBS washes and fixation with 4% paraformaldehyde for 15 min. After subsequent rinses with PBS, images were acquired using a fluorescence microscope and analyzed using the ImageJ1 software. (B) Representative semi-quantitative histogram of A. The data represent three independent experiments carried out in duplicates. # p ≤ 0.0001, and *p ≤ 0.05.
5.2.7. CuCl increased iROS production in FLS cells in the presence of ascorbate.

To further answer the question of whether a combination of ascorbate (AA) and Cu$^{2+}$ (CuCl) increase iROS production compared to Cu$^{+2}$ alone, fluorescence microscopy testing of CuAA co-incubation for 4 h at the ratios of 1:1, 1:2, 1:3, and 1:4 clearly showed that AA addition to CuCl in the ratio of 1:2 yielded the highest (3.6-fold) enhancement of iROS generation over CuCl alone under the same incubation conditions (Fig. 5.7- panel A and Fig. 5.7- panel B). These results were further confirmed by flow cytometry (Fig. 5.8).
Figure 5.7. Immunofluorescence representation of the levels of intracellular ROS (iROS) following CuCl and Ascorbic acid co-treatment. (A) Immunofluorescence representation of cells treated with CuCl and/or in combination with AA in different ratios. Cells were stained with CellROX® Deep Red Reagent and NucBlue Live Ready Probes® Reagent (to counterstain cellular nuclei) for 30 min at 37 °C followed by PBS washes and fixation with 4% paraformaldehyde for 15 min. After rinsing with PBS, images were acquired using fluorescence microscopy and analyzed using the ImageJ1 software. (B) Representative semi-quantitative histograms. The data represent three independent experiments carried out in duplicate. # p ≤ 0.0001, **p ≤ 0.01.
Figure 5.8. Flow cytometry analysis of the levels of intracellular ROS (iROS) following CuCl and Ascorbic acid co-treatment. (A). Flow cytometry representation of cells, which were treated with CuCl alone or in combination with AA (CuAA) at different ratios. Cells were stained with CellRox® Deep Red Reagent for 30 min at 37 °C followed with PBS rinsing, then scraped and resuspended into warm DPBS per the manufacturer's instructions for flow cytometric analysis. (B) Quantitative analyses of tracings following treatments with the vehicle, CuCl, and CuAA. The data represent three independent experiments. *p ≤ 0.05, **p ≤ 0.001.
5.2.8. RATM-induced intracellular ROS (iROS) production in FLS cells is TLR-4 dependent. To address the question of whether TLR4 signaling plays a role in pro-oxidant-induced iROS production, cells were treated with PPC (5 nM), FeCl₂ (375nM and 10µM), CuCl (50 µM), and CuAA at a 1:2 ratio for 4 and 24 h following a 2 h pre-incubation with a specific TLR4-signaling inhibitor (CLI-09-095) (Matsunaga et al., 2011).

Data shows that the use of a TLR4 signaling blocker decreased iROS generation by more than 50% suggesting that iROS production is TLR4 signaling dependent. (Fig. 5.9- panel A and panel B).
Figure 5.9. Immunofluorescence representation of the levels of intracellular ROS (iROS) following pretreatment with CLI-095. Cells were preincubated with CLI-095 for two h before treatment with PPC, FeCl₂, and CuCl alone or in combination with ascorbic acid (CuAA). Cells were stained with CellRox® Deep Red Reagent and NucBlue® Live Ready Probes Reagent (to counterstain cellular nuclei) for 30 min at 37 °C followed by subsequent PBS washes and fixation with 4% paraformaldehyde for 15 min. After rinsing with PBS, images were acquired using fluorescence microscopy and analyzed using the ImageJ software. (A) Merged representative pictures (B) Semi-quantitative histograms. The data represent three independent experiments carried out in duplicate. *p ≤ 0.01.
5.3. Discussion

Excessive RS production is mainly associated with RA-related pathogenic cellular and molecular outcomes. RS are well known to trigger protein and DNA damage (Ueda et al., 2002; Lee et al., 2003), telomere attrition (Schonland et al., 2003), epigenetic modifications (Lee and Lee, 2006), cell senescence (Goronzy et al., 2005), osteoclast activation and T-cell hyporesponsiveness (Cemerski et al., 2003).

In this study, the hypothesis was that RATM as exogenous pro-oxidants might contribute to the pathogenesis of RA through the generation of ROS and interaction with TLR4 leading to inflammation. To test this hypothesis, HIG-82 synovial fibroblasts were used as a culture model, because they had been shown to express TLR4 at both the mRNA and protein levels. This data is consistent with Kajikawa work who reported the expression of TLR4, MD2, and CD14 on the cell surface as well as in the cytoplasmic pool of multiple rabbit cells (Kajikawa et al., 2005). However, Kajikawa did not report any data regarding rabbit synovial fibroblasts.

The novelty of this study lies in the confirmation of new insights on the interaction of a common potential oxidant, RATM, an important yet poorly understood synovial fibroblast signaling pathway. The generation of RS from RATM and the expression of TLR4 in synovial fibroblasts was confirmed. The TLR4 signal transduction inhibitor CLI-095 was used to characterize the role of TLR4 in ROS/TLR4 signaling to confirm that TLR4 is involved in sensing oxidants in vitro in synovial fibroblasts.

Three RATM were tested in the form of, potassium peroxychromate (PPC, Cr⁵⁺), cuprous chloride (Cu⁺), and ferrous chloride (Fe⁺²). These oxidants were selected to provide insights on the potential interactions of environmental trace-metals within synovial fibroblasts.
In addition, they were selected as exogenous pro-oxidants and ROS generating systems excluding H$_2$O$_2$ which has been used extensively as an oxidant for the following reasons: (i) H$_2$O$_2$ is uncharged, thus, lipid soluble with rapid diffusion through membranes by unknown mechanisms, and (ii) H$_2$O$_2$ has been used at high concentrations to study its effects on signaling pathways; concentrations that are considered physiologically impractical.

Initially, MTT and LDH were used to determine optimal seeding and then working concentrations of treatments. The chemical nature of each RATM further rationalized the resultant optimal MTT and LDH concentration. The peroxychromate anion (CrO$_8^{3-}$), consists of a central chromium atom in the oxidation state (+V) surrounded by four peroxy anions (O$_2^-$) in a dodecahedral arrangement. PPC resulted in the lowest viability concentration (5 nM) which was not surprising for the following reasons; (i) Peroxychromate anions with chromium in the oxidation state of (+V) decompose spontaneously in neutral or alkaline aqueous solutions to generate a robust number of pro-oxidants in biological systems. An aqueous solution of PPC is stable for a minimum of 2 h, and it decays to yield robust ROS and chromate (VI) at a physiological pH (Miesel and Weser, 1989). PPC decay in aqueous solutions releases several RS including superoxide radicals (Hodgson and Fridovich 1974), hydroxyl radicals (Edwards and Quinn 1982; Paschen and Weser 1975; Shi and Dalal 1994), singlet oxygen (Peters et al., 1975; Baird et al., 1977), possibly superoxide (Miesel and Weser, 1989), and hydrogen peroxide (Paschen and Weser 1975) which provide sufficient oxidative activity. H$_2$O$_2$ was established as a decomposition product of K$_3$CrO$_8$ in all media utilized, which is considered the long-lived species. When Cr (VI) enters the cell, it can be reduced to Cr (III), resulting in the formation of more ROS (Valko et al., 2005). This result may explain the observation of the high sensitivity of FLS to PPC in this study. (ii) Further explanation is expected due to the
combined effects of both PPC decay and chromate generation of ROS, and (iii) In addition, normal synovial tissues consist of two cell types: macrophage-like synoviocytes (MLS) (Demaziere, 1993, Mulherin et al., 1996), and synovial fibroblasts (type B synoviocytes). Two thirds of synoviocytes are synovial fibroblasts. Thus, a synovial environment is critical. Compared to other tissues, the synovial environment is usually hypoxic and devoid of antioxidants (Biniecka et al., 2011a). This type of environment is exceptionally detrimental (Afonso et al., 2007). Interestingly, the use of low concentrations of PPC in synovial fibroblasts resulted in detectable levels of RS release and TLR4 expression in contrast to the use of low concentrations of PPC in RAW-Blue cells where it resulted in undetectable levels of SEAP release.

PPC has previously been used as a source of ROS to examine its effects on cellular functions (Hodgson and Fridovich, 1974; Baird et al., 1977). PPC decays readily into the same products that are produced physiologically by activated phagocytes (Miesel and Weser 1989). Therefore, PPC decay represents a simple, fast, and convenient model of inflammation (Miesel and Haas 1993). Indeed, PPC data shows a very robust effect at a lower concentration than other used pro-oxidants. This is because PPC in vitro and in vivo decomposes to release reactive oxygen species that cause lipid peroxidation as an oxidative stress hallmark (Edwards and Quinn, 1982).

Furthermore, PPC was selected not only because of its pro-oxidant characteristics but also because of its characteristic to induce arthritis in mice and rats in a single dose of ROS via inducing a long-lasting self-perpetuating inflammatory process (Miesel et al., 1994; Miesel et al., 1995). Unlike other models of arthritis-induction such as collagen type II or adjuvants-induced arthritis, PPC-induced arthritis lacks immunogenic interferences found in other
models and relies only on the activation of phagocytes and the inhibition of glutathione reductase. The selective inhibition of glutathione reductase by released chromate during the aqueous decay of PPC blocks the enzymatic reduction of oxidized sulfhydryl, which causes chronic inflammation (Miesel et al., 1995). None of the other clinically relevant transition trace metals such as iron (Fe) or copper (Cu) in any of their respective valency states could replace chromium (+V) because of its fidelity in generating reactive molecules \textit{in vivo} and \textit{in vitro}.

The rationale for selecting FeCl$_2$ salt is based on its effects on lipid peroxidation which are due to iron-generated-oxidative stress and not caused by chloride anions as observed in rats injected with sodium chloride (Dougherty et al., 1981). Even with the care of handling iron \textit{in vivo}, experimental evidence suggests that iron can induce cell injury. Observations of higher than normal deposits of iron in rheumatoid synovial membrane resulted in the study of the generation of toxic free radicals that may contribute to RA (Blake et al., 1981). A case study of exacerbation of synovitis after iron-dextran infusion showed that iron could promote oxidative stress as reflected by testing of hepatic functions (Winyard \textit{et al.}, 1987).

FeCl$_2$ at concentrations of (375 nM) and (10 µM) (\textbf{Fig. 5.5 panel E}) generated maximum iROS after 1 h of interaction (\textbf{Fig. 5.5 panel A}). This outcome is in agreement with the documented rapid burst in oxidants produced 60 min exposure to (Fe$^{+2}$) oxidation (Keenan \textit{et al.}, 2009). Iron is unique in its ability to alter its oxidation and redox states in response to ligands (Kruszewski 2003). The ability of the iron atom to carry on one-electron reactions is encrypted by its electronic structure, which is a key element in the formation of ROS.

Iron in biological systems exists in two oxidation states, ferric (Fe$^{+3}$) which is available in an aerobic atmosphere, and ferrous (Fe$^{+2}$) in reducing intracellular environment (Halliwell and Gutteridge 1992), and to a lesser extent in the highly oxidizing ferryl (Fe$^{+4}$) state possibly
formed in PBS (Reinke et al., 1994). Ferrous ions are soluble in biological fluids, and their toxicity had been credited to its reaction with oxygen, thus generating damaging hydroxyl free radicals (Aisen and Listowsky, 1980; Chamnongpol et al., 2002). Iron-mediated oxidant stress is caused by Fe$^{2+}$, which reacts in aqueous media with oxygen to generate ferric ions and superoxide anion radicals (O$_2^\cdot$). This is the principal mechanism for oxidant production; the iron catalyzed Haber-Weiss reaction (Liochev, 1999; Kehrer, 2000; Jones-Lee and Lee, 2005; Keenan et al., 2009) (Fig. 5.5 panel A). O$_2^\cdot$, which forms as the result of the redox enzyme autoxidation, reduces ferric iron through Fenton reactions to generate hydroxyl radicals that oxidize proteins and DNA (McCord and Day; 1978). While O$_2^\cdot$ is known as a reductant of Fe$^{3+}$, it oxidizes Fe$^{2+}$ in some cases, such as the catalytic cycle of iron-containing superoxide dismutase (Miller 2012). Furthermore, O$_2^\cdot$ in vivo is poorly reactive, and H$_2$O$_2$ is almost unreactive at physiological levels but can still cross membranes to react with Fe$^{2+}$ to form a highly reactive OH$, which reacts with almost all macromolecules at all sites (Halliwell and Gutteridge 1992).

The observation that higher concentration of CuCl (50 µM) (Fig. 5.5 panels A and F). was required in the current study is not surprising. Copper transport systems differ between different cell types, and copper uptake varies among cell types. Among these cells, fibroblasts are known to take up copper very well (Harris et al., 1998). Copper is known to be 62-fold more efficient than iron in producing hydroxyl radicals under the same conditions. Given the ability of Cu to produce ROS, a number of copper transporters and chaperones have evolved to protect the cell by regulating the uptake, distribution, and efflux of this active metal (Uriu-Adams and Keen, 2005). This regulation may explain why FLS cells were not as sensitive to Cu as to Fe.
When the concentration of copper inside cells increases due to environmental or pathological causes, toxicity occurs. Copper toxicity is in part a result of its redox reactivity as free Cu or its low molecular weight complexes, which are capable of catalyzing the reaction between superoxide anions and $\text{H}_2\text{O}_2$ to produce hydroxyl radicals (Gutteridge and Halliwell 1992). Besides, copper binds directly to the free thiols of cysteine and results in oxidation causing crosslinking between proteins leading to impaired activity (Cecconi et al., 2002). The effectiveness of copper ions in inducing protein and nucleic acid oxidation is well known; however, because of its ability to bind both proteins and nucleic acids, copper can promote in situ oxidative modifications (Aruoma et al. 1991; Gutteridge and Halliwell 1982; Li et al. 1994; Ueda et al. 1996). Although most of the acute toxicity of Cu were observed in suicide attempts, the mechanisms of these acute toxicities in humans are not well understood. It is reasonable to speculate that these reactions represent oxidative stress at multiple points in the body (Gaetke and Chow 2003).

The initial attempt was to challenge synovial fibroblasts with CuCl$_2$ as pro-oxidant instead of CuCl; but, the observed higher production of ROS with CuCl compared to CuCl$_2$ lead to the selection of CuCl. Copper induction of cellular toxicity through the generation of ROS is higher in its reduced (Cu$^+$) form, which is consistent with previous reports (Gaetke and Chow, 2003; Xiao et al., 2010). The most common basis for cellular copper toxicity is thought to be Cu ions participation in ROS formation via the Haber–Weiss reaction to generate hydroxyl radicals (Bremner 1998) (Fig. 5.2 panel C). In the metal site mechanism, an OH radical is formed near the target biological molecule and reacts at its site of production. Any reductants present in cells such as ascorbate or glutathione may promote the toxicity by replacing the superoxide in the Haber Weiss cycle (Kadiiska et al., 1992).
It has been established that Cu^{2+} is reduced to Cu^{+} by antioxidants. The combination of ascorbate and Cu^{2+} causes damage to molecular structures through the generation of reactive species (Chiou, 1983; Shinar et al., 1983; Kobayashi et al., 1988). In vivo administration of copper and ascorbic acid-generated hydroxyl radicals was confirmed by spin trapping (Kadiiska et al., 1992).

The cytotoxicity of the combination of copper and ascorbate is attributed to the continuous formation of hydroxyl radicals through recycling Cu^{2+} back to Cu^{+} (Samuni et al. 1983; Stoewe and Prutz 1987). Similar cytotoxicity of a mixture of iron salts and ascorbate have been used for decades to stimulate free-radical oxidation of lipids (lipid peroxidation), DNA, and proteins in vitro (Halliwell and Gutteridge, 2015), under certain circumstances by reducing Fe^{3+} to Fe^{2+}.

While ascorbate, in the presence of Cu^{2+}, induces two classes of DNA damage, this damage can be inhibited by catalase and metal chelators, which suggests the involvement of generated radicals (Kobayashi et al., 1988). This generation of RS was further tested in the presence of the antioxidant EUK-134 at ratios of 1:1 and 1:2 of CuAA, where iROS generation was reduced to reach levels of CuCl treatment alone (Fig. 5.7 and 5.8). The rationale for testing different ratios of AA was encouraged by the work of Xiao and coworkers (Xiao et al., 2010), who selected a ratio of 1:4, and by Bhat and coworkers (Bhat et al., 2006) who showed that increased AA concentrations contributed to more ROS production. The importance of such work could add to the conflicting data about the role of ascorbic acid (friend or foe). Ascorbate is often regarded as an important antioxidant, protecting against cancer by scavenging DNA-damaging ROS, while several studies suggested that ascorbate increases DNA damage in humans (Halliwell, 1999). This conflicting data is an indication that we need to change our
thinking about the mechanisms of the antioxidant action of ascorbate *in vivo* depending on what the underlying mechanism is.

Quantification of RS can be challenging. Levels of RS are usually measured by direct methods in whole animals such as L-band electron spin resonance (ESR) and aromatic hydroxylation or by indirect methods such as ESR spin-trapping. Alternatively, oxidant damage can be measured by examining the products of damage to biomolecules such as DNA, lipids, and proteins. To identify RS in cells, many methods have been developed, although limited information is available on what has been measured. Cell culture itself causes oxidant stress either by increased generation of ROS or by hindering adaptive upregulation of cellular antioxidants. For example, free radical reactions taking place in culture may be artifacts caused by oxidation of ascorbate, flavonoids, and dopamine in culture media (Nathan, 2003).

Another example is when catalase is added outside cells; it lowers the intracellular H₂O₂ levels by removing the extracellular H₂O₂. Different fluorescent probes can measure the cellular production of reactive species. Available methods for direct measurements are of limited applicability to humans; therefore, most studies focus on measuring end-products of damage. In this study, reactive species and cellular oxidant stress resulting from RATM activation were measured by a combination of FM and FC to determine the levels of iROS using CellROX® Deep Red reagent. The fluorescence intensity readouts by FM were more sensitive than the FC results for the same treatments when using the same fluorescence-labeling reagent (Fig. 5.7 panels A, B, and F). These differences can be explained by differences in sample preparation methods and data acquisition duration. The FM preparation method estimates both extra- and intra-cellular ROS while the FC method only reads the fluorescence of intact cells. In addition, cells in the FC method were scraped during sample
preparation, which caused a loss for positive fluorescence cells. The FM method relies on human eye observation to detect fluorescence. Also, the duration of observation in FM is longer than in FC, thus possibly contributing to neglect a fraction of positive cells due to fluorescence bleaching (Muratori, 2008).

Two anti-oxidants agents, EUK-134 and Ebselen, were used to confirm that exogenous RATM oxidants cause the generation of cellular iROS. EUK-134 inhibited RS (Fig. 5.6). Ebselen had less of an antioxidant effect during PPC treatment than EUK-134, which generates mainly radical and non-radical ROS. This could be due to: (i) EUK-134 is a synthetic cell-permeable mimetic of superoxide dismutase and catalase (Rong et al., 1999; Lawler et al., 2014). Thus, it diffuses through the plasma membrane to reside within cells and results in inhibiting cellular ROS, (ii) EUK-134 main effect as RS scavenger is to prevent oxidant stress by eliminating oxygen-free radicals and/or hydrogen peroxide (non-radical ROS). Also, EUK-134 is expected to prevent the accumulation of peroxynitrite (RNS) through the removal of its precursor superoxide and, thus, the prevention of both ROS and RNS (Rong et al., 1999). In contrast, Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), a seleno-organic compound with a glutathione peroxidase-like activity, is an antioxidant with poor radical scavenging properties, if at all, but it is an effective scavenger of organic hydroperoxides, particularly, of lipid hydroperoxides (Nakamura et al., 2002).

After having confirmed that trace metals (RATM) generate RS, the pathway (s) by which RATM induce oxidant stress was/were explored. Depending on the origin and level of ROS, the activation of the transcription pathway (s) may occur in discrete subcellular locations or across a large population of cells (Nathan and Cunningham-Bussel, 2013). Thus, it is no surprise that increased iROS by RATM transcends cell membrane injury and includes lipid
and protein peroxidation and nitration, glyco-oxidation, DNA damage, and enzyme activation. This study demonstrates that stimulation of synovial fibroblasts with RATM pro-oxidants resulted in activation of the TLR4 signaling pathway (Fig. 5.4). It did not activate TLR2 (data not shown), a process that was reported to be essential for sensing of oxidants during inflammation (Paul-Clark et al., 2009), and that seems to be induced by oxidant stress in many different diseases (Lucas and Maes, 2013). TLR4 is the only TLR receptor pathway that responds to a wide range of non-canonical ligands such as nickel (Schmidt et al., 2010), cobalt (Raghavan et al., 2012), fibrinogen (Smiley et al., 2001), and high mobility group box 1 (HMGB1) (Park et al., 2004a; Kim et al., 2013). The mechanism behind these activations is not yet known. Our laboratory had shown previously that pro-oxidants and, in specific PPC, can activate NF-kB mainly through a TLR4-dependent pathway in vivo and in vitro, which was attenuated by prior treatment with antioxidants (Karki and Igwe, 2013).

Inflammation induced by oxidant stress has many of the features associated with classical activation of the innate immune system. Therefore, the role of oxidant stress in TLR4 stimulation was assessed (Fig. 5.4). Activation of intracellular signaling requires the interaction between TLR4 and the adaptor molecules containing the TIR domain such as TIRAP and TRAM. A single point mutation is known to eliminate the response to TLR4 activation (Poltorak et al., 1998), while dimerization of TIR domains was sufficient to induce signaling (Daringer et al., 2015). Therefore, the TIR domain is crucial for TLR4 signaling transduction. Pre-incubation with the TLR4 signaling inhibitor CLI-095 markedly decreased cellular oxidant stress (Fig. 5.9), confirming that TLR4 is required for sensing exogenous RATM and confirming its role in generating cellular oxidant stress. CLI-095 is known to strongly covalently bind to the Cys747 residue within the intracellular TIR domain of TLR4.
(Matsunaga et al., 2011; Zhang et al., 2015b), interfering with TLR4 signaling and showed beneficial effects in a mouse sepsis model (Sha et al., 2007). One of the advantages of using CLI-095 is its strong binding to TLR4 with no or marginal binding to the intracellular domains of TLR 2, 3, 5, and 9. This rules out any involvement of other extracellular TLRs’ interaction with exogenous RATM (Takashima et al., 2009). Our results clearly showed that pre-treatment with CLI-095 markedly inhibited oxidant-induced ROS generation (Fig. 5.9), further confirming that the TIR domain of TLR4 is required in oxidant-induced oxidative stress.

In conclusion, this part of the study clearly demonstrates that RATM as pro-oxidants stimulates the TLR4 signaling pathway, resulting in cellular oxidant stress production. Further study of TLR4-induced release of inflammatory cytokines like, TNF-α, and IL-1β, as a pro-inflammatory cytokine and IL-10 as an anti-inflammatory cytokine, is required to characterize the inflammatory phenotype, which will be presented in the following chapter.
CHAPTER 6

EFFECT OF RATM ON HMGB1 RELEASE AND MARKERS OF INFLAMMATORY PHENOTYPES

6.1. Introduction

So far, the ubiquitous nuclear HMGB1 molecule is the best-studied alarmin that activates innate immunity (Andersson et al., 2018). Since the original study demonstrating that extracellular HMGB1 (ex HMGB1) promotes inflammation 19 years ago (Wang et al., 1999), several clinical studies have supported a role for ex HMGB1 in the pathogenesis of RA. Once, released outside the cells, HMGB1 acts as a prototypic DAMP and a promiscuous danger sensor for the immune system and other organs (Yang et al., 2015). Oxidants have been shown to stimulate NF-κB through TLR4 activation (Karki and Igwe, 2013), and a significant outcome of NF-κB activation is the production of cytokines and chemokines. The production of pro-inflammatory and anti-inflammatory cytokines during inflammation dictates the inflammatory response. Therefore, the effect of RATM oxidants on the production of HMGB1, TNF-α, IL-1β and IL-10 in FLS was investigated.

6.2. Results

6.2.1. RATM pro-oxidants did not increase HMGB1 mRNA in synovial fibroblasts. Treatment with PPC (10 nM), FeCl₂ (10 µM), CuCl (50 µM), and CuAA for 0, 2, 6, 12 and 24 h did not affect HMGB1 mRNA levels indicating that HMGB1 release in synovial fibroblasts is unlikely to be linked to increased transcription of the gene (Fig. 6.1). This data is consistent with unchanged levels of HMGB1 mRNA in murine macrophage-like RAW264.7 cells after (1 µg/ml) LPS treatment (Wang et al., 1999), as well as in agreement with Sundberg study (Sundberg et al., 2008), where HMGB1 mRNA remained unchanged in RA patients.
Figure 6.1. Effect of RATM oxidants on gene expression of HMGB1 in FLS.
Cells were treated with PPC, FeCl$_2$, and CuCl alone or in combination with Ascorbic acid (CuAA) at different time points. Relative HMGB1 mRNA expression was quantified at different time points after treatment using RT-qPCR and normalized against β-actin. The data represent 3 independent experiments *P < 0.05.
6.2.2. RATM pro-oxidants increased HMGB1 protein expression in a time dependent manner. Interestingly, was not only the release of HMGB1 modulated by RATM pro-oxidants treatment but also its cellular expression (Fig. 6.2 panel A). Treatment with PPC (10 nM), FeCl₂ (10µM), CuCl (50 µM), and CuAA for 24 h increased the total HMGB1 cellular expression in a time dependent manner. This outcome was consistent with the immunoblot analysis of HMGB1 released from murine macrophage-like RAW 264.7 cells treated with LPS with the highest expression at 24 h (Wang et al., 1999).

Total HMGB1 upregulation was detected as early as four h after RATM treatment and continued to increase over 24 h treatment (Fig. 6.2 panels A and B). Data were further confirmed by FC quantification at 24 h. HMGB1 protein upregulation is concentration (Fig. 6.2 panel C) and time dependent (Fig. 6.2 panels A and B). This data was consistent with HMGB1 release by LPS-activated monocytes at 18 and 30 h (Gardella et al., 2002).
6.2.3. RATM pro-oxidants increased extracellular HMGB1 release into the cytoplasm. Treatment with PPC (5 nM), FeCl₂ (375nM), (10µM), CuCl (50 µM), and CuAA for 24 h increased HMGB1 release, which was visualized by immunofluorescence staining (Fig. 6.3 panel A). The release of total HMGB1 into culture supernatant was significantly higher than control when it was quantified by ELISA. CuAA, in the ratio of 1:2, showed the highest release for HMGB1 by a mean of 3.2- fold (Fig. 6.3 panel D). To further confirm the release of HMGB1 into the cytoplasm, cytoplasmic/nuclear proteins were fractionated and characterized by WB (Fig. 6.3 panel B). The OD quantification ratios (Fig. 6.3 panel C) of the cytoplasmic HMGB1 normalized to β-actin for PPC (5nM, 10nM), CuCl (50µM), FeCl₂ (375nM, 10 µM), and CuAA showed significant increase in HMGB1 release by means of 1.5-, 1.6-, 1.7-, 1.6-, 3.6- and 1.9- fold, respectively.
Figure 6.2. Analysis of the effects of RATM oxidants on the levels of HMGB1 protein expression. HIG-82 cells were treated with PPC, FeCl₂, and CuCl alone and in combination with Ascorbic acid (AA) at different time points. Cells were then further used to (A) Western blots of the total HMGB1 time course at 2, 4, 6, 12 and 24h of treatment. (B) Represents semi-quantitative histograms of the optical density (OD) ratios of HMGB1 normalized to b-actin from the same tests. The data represent 3 independent experiments *P < 0.05, +P < 0.005, # P < 0.0001. (C) Flow cytometric quantitative analysis of total HMGB1 in HIG-82 synovial fibroblasts following 24h of pro-oxidants treatment. (D) Quantitative analyses of C tracings following treatments with vehicle control, and RATM pro-oxidants.
Figure 6.3. Effect of RATM oxidants on HMGB1 release. HIG-82 cells were treated with PPC, FeCl₂, and CuCl alone and/or in combination with Ascorbic Acid (AA). Cells were used to determine (A) immunofluorescence representation of the levels of the released HMGB1 (green) following stimulation with oxidants. (B) Represents Western blots of the cytoplasmic and nuclear fractions of HMGB1. (C) Representative semi-quantitative histogram of the OD ratios of HMGB1 in the cytoplasm immunoblot signals normalized to those of β-actin from the same tests. (D) Total HMGB1 released into the culture supernatant measured by ELISA. The data represent three independent experiments *P < 0.05, +P < 0.005, #P < 0.0001.
6.2.4. RATM pro-oxidants effect on the mRNA of inflammatory cytokines. To confirm the role of ROS/TLR4 coupling on mRNA expression of inflammatory cytokine genes during RATM oxidative stress, mRNA changes were measured at 0, 2, 6, 12 and 24 h after PPC (10nM), FeCl₂ (10µM), CuCl (50µM) and CuAA. Interestingly, no change was noticed in TNF-α mRNA during all RATM pro-oxidants treatment (Fig. 6.4 panel A). Only PPC and CuAA had a detected effect on the transcriptional levels of IL-1β after two h of treatment (Fig. 6.4 panel B). Further measurement for IL-10 mRNA showed changes at 6 h following PPC (10 nM), FeCl₂ (10µM), and CuCl treatment (Fig. 6.4 panel C).
Figure 6.4. Effect of RATM oxidants on gene expression of TNF-α, IL-1β, and IL-10 mRNA in FLS. Cells were treated with PPC, FeCl₂, and CuCl alone or in combination with Ascorbic acid (CuAA) at different time points. Relative mRNA expressions were quantified at different time points following RATM treatment using RT-qPCR and normalized against β-actin. The data represent 3 independent experiments *P < 0.05, +P < 0.01, #P < 0.0001.
6.2.5. RATM pro-oxidants-induced inflammatory cytokine release is TLR4-dependent. To confirm the role of ROS/TLR4 coupling, the time course release of cytokines at 0, 2, 6, 12, and 24 h, was measured. The initial data indicated that TNF-α and IL-1β release was maximum at six h following PPC (10 nM), and FeCl₂ (10µM) treatment, whereas CuCl₂-induced release was at a later point (12 h) (data not shown here). Therefore, these time points were selected to explore RATM effects on cytokine release further. Treatment with RATM pro-oxidants and pre-incubation with CLI-095 for two h significantly decreased TNF-α and IL-1β release into the medium.

Treatment with PPC increased TNF-α, IL-1β, and IL-10 by a mean of 1.2-, 1.5-, and 13.7–fold, respectively (Fig. 6.5 panels A-C), whereas, FeCl₂ treatment increased TNF-α, IL-1β, and IL-10 by means of 2-, 1.4-, and 14.85–fold, respectively. In addition, CuCl treatment increased TNF-α, IL-1β, and IL-10 by a mean of 2.4-, 1.5-, and 5.5–fold, while CuAA treatment increased TNF-α, IL-1β, and IL-10 by a mean of 2.6-, 2.2-, and 3.9–fold. As expected, LPS-EK stimulated TNF-α, IL-1β, and IL-10 production. We further noticed that cytokine expression was higher at six h than 12 h of RATM incubation.

Further incubation with CLI-095 (Matsunaga et al., 2011) reduced the release significantly to the control level. Thus, our data indicated that RATM could induce proinflammatory cytokine release in a TLR4 dependent manner with further implication to the role of HMGB1 release by pro-oxidants. Therefore RATM-mediated TNF-α, IL-1β, and IL-10 production are at least partially due to TLR4. The ratios of TNF-α and IL-1β to IL-10, following stimulation with RATM pro-oxidants, were further calculated as a parameter to represent the balance between these cytokines. The treatment of both PPC and FeCl₂ increased the ratio of TNF-α to IL-10 in a similar or higher than LPS-EK at six h, whereas CuCl and
CuAA were less potent than LPS-EK at 12 h. A similar pattern was noticed when we calculated the ratio of IL-1β to IL-10 at 6 and 12 h.

6.2.6. RATM effect on MRP8 and MRP14 mRNA. To clarify changes in mRNA expression of MRP8 and MRP14 genes during RATM oxidant stress, mRNA changes were measured at 0, 2, 6, 12, and 24 h after PPC (10 nM), FeCl$_2$ (10µM), CuCl (50µM), and CuAA treatments. Interestingly, only FeCl$_2$ activated MRP 14 at the transcriptional level while PPC, CuCl, and CuAA did not have any detected effects on the transcriptional levels of MRP 8 and 14 at the specified time points. (Fig. 6.6 A and Fig. 6.6B).
Figure 6.5. Role of TLR4 in RATM oxidants-induced production of TNF-α, IL-1β, and IL-10. Cells were treated with RATM or LPS-EK. Cytokine released into the culture medium was determined using ELISA kits, according to the manufacturer's instructions. The effect of LPS-EK on each cytokine release was used as a positive control. (A) TNF-α levels at six h for PPC and FeCl₂, while at 12 h for CuCl and CuAA. (B) IL-1β levels at six h for PPC and FeCl₂ while at 12 h for CuCl and CuAA. (C) IL-10 levels six h for PPC and FeCl₂ while at 12 h for CuCl and CuAA. Ratios of TNF-α to IL-10 (D) and IL-1β to IL-10 (E) were calculated. *p ≤ 0.05, +P < 0.001, #P < 0.0001, one way ANOVA with Tukey’s multiple comparison tests.
Figure 6.6. Effect of RATM oxidants on gene expression in FLS. Cells were treated with PPC, FeCl$_2$, and CuCl alone or with combination with Ascorbic acid (CuAA) at different time points. Relative MRP8 (A) and MRP14(B) mRNA expression were quantified at different time points post-treatment using RT-qPCR and normalized against β-actin. The data represent 3 independent experiments (*P < 0.05, +P < 0.001, #P < 0.0001), one-way ANOVA with Tukey’s multiple comparison tests.
6.2.7. **RATM upregulated total MRP8/14 protein expression.** As a result of the observed changes in mRNA expression with FeCl$_2$ treatment at 12h, a 24 h time point was chosen to measure protein expression further. Treatment with PPC (10 nM), FeCl$_2$ (10 µM), CuCl (50µM), and CuAA for 24h upregulated the MRP8/14 protein expression by a mean of 1.4-, 1.8-, 1.8- and 1.3 –fold, respectively (Fig. 6.7A and B).
6.3. Discussion

In this Chapter, the hypothesis that RATM pro-oxidants would promote the release of HMGB1 and inflammatory cytokines, and would contribute to RA initiation was tested. In this study, we have reported for the first time that exogenous RATM pro-oxidants induced HMGB1 release in synovial fibroblasts. The data confirmed the role of oxidant stress in modulating HMGB1 regulation, which may contribute to propagating and maintaining the inflammatory process.

As a stress marker, HMGB1 release can function as a tissue-damaging signal in autoimmune, acute and chronic inflammatory diseases (Magna and Pisetsky, 2014). The unique feature of HMGB1 of acting as an alarm signal for the environment is enabled by extracellular HMGB1 (exHMGB1) attaching to a number of DAMPs and PAMPs. HMGB1 proinflammatory properties were initially recognized in (sepsis, shock) experiments with macrophages and were identified as a late endotoxin mediator (Wang et al., 1999). Subsequently, it was studied in chronic inflammatory and autoimmune conditions (Ulfgren et al., 2004; Popovic et al., 2005) especially RA.

According to the many existing studies (Chirico et al., 2014), ample evidence proposes HMGB1-mediated biology as a central pathogenic mechanism in many “sterile” inflammatory diseases primarily in RA and joint inflammation. More than twenty studies and observations supported an essential role for HMGB1 in RA pathology (Table 15) and had provided a compelling rationale for examining the effect of RATM pro-oxidants on HMGB1 release.
Figure 6.7. Effect of RATM oxidants on MRP8/14 protein expression in FLS. (A) Cells were rinsed with PBS following the removal of the incubation media, scraped and resuspended into warm PBS for flow cytometric analysis. (B) FC representative tracings of (A) following treatments with media (control), and pro-oxidants or LPS-EK. The data represent 4 independent experiments *P < 0.05, +P < 0.001, #P < 0.0001, one way ANOVA with Tukey’s multiple comparison tests.
### Table 15. Supportive studies of HMGB1 role in RA

<table>
<thead>
<tr>
<th><strong>Animal Studies</strong></th>
<th><strong>Human Studies</strong></th>
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<td>Human Studies (continued)</td>
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<td>2</td>
<td>Prominent HMGB1 pannus expression is involved in the destructive cartilage and bone</td>
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<td>penetrating process (Andersson and Erlandsson-Harris, 2004).</td>
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<td>3</td>
<td>Undegraded mammalian DNA activates synovial cells in developing chronic polyarthritis</td>
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<td></td>
<td>(Kawane et al., 2006).</td>
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<td>4</td>
<td>Ex HMGB1 and constitutive nuclear HMGB1 expression, with histological signs of inflammation, were evident in the biopsies of nine patients with RA before and after initiation of anti-TNF mAb therapy (Sundberg et al., 2008). Cytoplasmic and extracellular expression of HMGB1 decreased in five out of nine patients while HMGB1 mRNA remained unchanged.</td>
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<td>5</td>
<td>HMGB1 levels in synovial fibroblasts from patients with RA and pseudogout were 2 SD above</td>
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<td>the mean level in samples from patients with OA (Hamada et al., 2008).</td>
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<td>6</td>
<td>HMGB1 in complex with suboptimal concentrations of IL-1α, IL-1β or LPS induces inflammatory</td>
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<td>cytokine production from RA synovial fibroblast (Wahamaa et al., 2011). HMGB1 is a mediator</td>
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<td>of JIA pathogenesis as well as a biomarker for inflammatory activity (Schierbeck et al.,</td>
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<td>2013).</td>
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<td>HMGB1 in complex with IL-1β amplify the biosynthesis of prostaglandin E (PGE2) through the</td>
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<td>induction of microsomal prostaglandin E synthase-1(mPEGS-1) which is strongly upregulated</td>
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<td>in RA synovium (Leclerc et al., 2013).</td>
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<td>8</td>
<td>HMGB1 is strongly expressed in the synovial fluid of RA patients when compared to OA</td>
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<td>patients and induces the release of proinflammatory cytokines (Taniguchi et al., 2003).</td>
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<td>9</td>
<td>Increased levels of serum HMGB1 were detected in RA patients (Ulloa et al., 2003).</td>
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<td>10</td>
<td>Analysis of the synovial fluids of juvenile idiopathic arthritis patients revealed that</td>
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<td>most of released HMGB1 is in a hyperacetylated form, which signifies active secretion</td>
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<td>with monomethylated indicating a neutrophil origin (Lundback et al., 2016a).</td>
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<td>HMGB1 levels measured during the use of anti-rheumatic treatments</td>
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<td>1</td>
<td>Intra-articular Glucocorticoid treatment reduced HMGB1 levels in RA synovium (af Klint et al., 2005).</td>
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<td>2</td>
<td>The anti-rheumatic effects of gold salts are explained as interference with intracellular HMGB1 transport mechanisms (Zetterstrom et al., 2008).</td>
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<td>3</td>
<td>The anti-rheumatic drug, Methotrexate (MTX) inhibits the interaction between HMGB1 and RAGE, thus inhibiting its inflammatory reaction (Kuroiwa et al., 2013). Decreasing the expression of HMGB1 in RA synovial membrane, promoted MTX–proliferation inhibition in RA (Li et al., 2016).</td>
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In addition, our data suggested that RATM-induced HMGB1 release from FLS cells is not due to cell death, as ≥ 80% of cells remained viable after RATM treatment. This suggestion was confirmed by MTT and LDH assays and is likely due to aberrant regulation of iROS levels instead. This is consistent with the observation that LPS induces HMGB1 release from murine macrophages (Wang et al., 1999), and with induced HMGB1 release from hepatocytes by exogenous hydrogen peroxide (Tsung et al., 2007). Furthermore, it is consistent with HMGB1 alone and/or in conjunction with pro-oxidants increased TNFα and IL-6 released from the TLR4-WT macrophage, but not from the TLR4-KO macrophage. Presumably, the released HMGB1 can also stimulate FLS cells directly by binding to toll-like receptor 4 (TLR4) (Zhang et al., 2015a). In addition to potentially stimulating cells directly, HMGB1 can form immunostimulatory complexes with IL-1β, TLR4 LPS, and other endogenous and exogenous factors to promote inflammatory activity (Hreggvidsdottir et al., 2009; Wahamaa et al., 2011). Furthermore, once released, HMGB1 might generate a positive feedback loop and induce production of several proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 by macrophages and dendritic cells, thereby sustaining an extended inflammatory phenotype.
(Andersson et al., 2000). Therefore, another compelling question regarding a possible functional relationship between TNF receptor stimulation and HMGB1 release in synovial fibroblasts, while therapeutically pertinent in RA, was not clarified, and more experiments were necessary to clarify this. Moreover, the complexity of FLS cell activation (Pap et al., 2000) raises the question as to what extent the inhibition of TNFα alone can influence the long-term outcome of RA. The work of our Laboratory answered these questions. Treatment of FLS with TNF-α increased the levels of extranuclear/cytoplasmic HMGB1 released in a TNF-α concentration-dependent manner while a TNF-α-induced release was inhibited by prior or co-incubation with TNF-α neutralizing antibody suggesting that the release must be due to activation/stimulation of TNF-R (Alsousi AA et al., 2017). Curiously, HMGB1 released in rheumatoid synovitis was not consistently inhibited in vivo by TNF-α receptor fusion proteins as well as novel TNF-α mAb (Infliximab) blocking therapy (Sundberg et al., 2008).

The passive release of HMGB1 occurs instantaneously within the context of necrotic cell death, while active secretion begins with translocating the nuclear HMGB1 in hyperacetylated form to prevent the bi-directional shuttle of HMGB1 between the cytoplasm and nucleus. This secretion is usually followed with pyroptosis or exocytosis of lysosomes which allows the cytoplasmic HMGB1 to reach extracellular spaces (Yang et al., 2015). However, pyroptosis requires the activation function of intracellular caspase-1 in inflammasomes. The RATM-induced release of HMGB1 has shown only a few, if any, signs of apoptosis, which could lead to the conclusion that the released HMGB1 in FLS is not in a fully oxidized form (sulfonyl HMGB1) that is usually seen after apoptosis, or in a fully reduced form that is seen after necrosis. Also, RATM have shown no activation of caspases in FLS, which indicates that pyroptosis is not involved, but instead it could be alternatively via
exocytosis of secretory lysosomes that deliver HMGB1 outside the cells (Gardella et al., 2002). The accumulation of cytoplasmic HMGB1 is vital for additional cellular functions such as promoting autophagy/mitophagy, a self-protective process that allows damaged mitochondria and different microbial intruders to be removed. Indeed our system has shown activation in autophagy. Cytoplasmic HMGB1 can initiate autophagy events by binding to Beclin-1 which is required for the formation of autophagosomes (Yang et al., 2015).

HMGB1 is a multifunctional protein, and the list of disorders in which it is involved is continuously growing. The worldwide information about HMGB1 proposes the time to translate results into therapeutics for clinical trials. Currently, there is enough available data on the successful use of polyclonal or monoclonal anti-HMGB1 antibodies, fragments of HMGB1 itself, and soluble receptors in different experimental inflammatory models (Venereau et al., 2016). So far, two differently designed humanized anti HMGB1 monoclonal antibodies, out of several different conceivable anti HMGB1 antibodies administered to patients, have worked out. (Lundback et al., 2016b) (M. Nishibori et al. Abstracts at the 8th International DAMPs and Alarmins Symposium, Cold Spring Harbor, NY, USA, 2017). Other endogenous antagonists that neutralize extracellular HMGB1(ex HMGB1) or convert ex HMGB1 mediated functions have been reported (Andersson et al., 2018).

In this chapter, the hypothesis that TLR4 mediated exogenous RATM-induced inflammatory cytokines release was tested. Two central proinflammatory cytokines have definitively been shown to contribute to RA, TNF-α, and IL-1β (Barbara et al., 1996; Feldmann et al., 2001). While TNF-α can regulate the expression of other proinflammatory cytokines such as IL-1, it also enhances chemotaxis at the site of inflammation. FLS cells were treated with RATM followed by measurement of TNF-α and IL-10 production as inflammatory
outcomes. FeCl$_2$ and CuCl clearly induced more robust production of TNF-α than PPC. While PPC induced a robust release of IL-1β than TNF-α (Fig. 6.4 panels A and B), both cytokines are pleiotropic with multiple biological effects on different cell types; many of which are not yet fully understood. The master cytokine that triggers inflammation and destruction in joints is TNF-α, as systemic overexpression of the TNF-α gene in the transgenic mouse model hTNFtg (Keffer et al., 1991) is sufficient to initiate chronic synovitis, cartilage destruction, and bone erosion (Zwerina et al., 2004). Thus, TNF-α is a crucial player in driving inflammation in 50–70% of RA patients in which cells of macrophage lineage are the primary source (Feldmann, 2009). This may explain our results of robust production of IL-1β which is not as much production of TNF-α in FLS. The results are in agreement with the fact that there is heterogeneity of the inflammatory process assessed in RA patients where high inter-individual diversity expression patterns of proinflammatory cytokines including TNF-α were observed (Santos Savio et al., 2015).

IL-1β is proposed as the leading player in the degradation of articular cartilage by stimulating both synovial fibroblasts and chondrocytes to secrete matrix metalloproteinases (MMPs), cathepsins, and mast cell proteinases and impairs the synthesis of new matrix components (Ruscitti et al., 2015). It has been shown that IL-1β upregulates the MMPs expression (Alsousi AA et al., 2017). The upregulation of IL-1β increases MMPs, which in turn aggravates synovial inflammation, increases joint destruction, and bone resorption. Furthermore, this upregulation supports the bone resorption, increasing the RANKL expression in cultured synovial fibroblasts and T cells (Ruscitti et al., 2015). Thus, our results suggest that RATM-induced IL-1β proinflammatory cytokine has a possible effect on bone loss and cartilage damage in joints. In fact, TNF-α and IL-1β induced expression of
cyclooxygenase-2 (COX-2), production of prostaglandin PGE2, increased the release of HMGB1, and the activity of metalloproteinase-9 (MMP-9) in FLS cells (Alsousi AA et al., 2017). A synergism between TNF-α and IL-1β showed higher production of PGE2 compared to the control. However, other investigators have shown synergism between IL-1β and TNF-α in primary cultures of articular chondrocytes and airway smooth muscle (Moore et al., 2001). This synergistic effect is of particular relevance, as increased levels of both of these cytokines have been reported at the site of inflammation in RA (Jenkins et al., 2002). While our work (Alsousi AA et al., 2017) was not in complete agreement with previous studies, it may reflect a decreased expression of IL-1 R1 on HIG-82 cells. Nonetheless, the data appear to support a relationship between some malignancies and arthritis (Love and Solomon, 2008; Itatsu et al., 2009).

TLR4 activation/stimulation mediates TNF-α and IL-10 production in systemic inflammation (Adib-Conquy et al., 2003). A specific TLR4 signal transduction inhibitor CLI-095 was used to further characterize the role of TLR4 activation in the ROS/TLR4 signaling. Consistent with that, our results here supported a role for TLR4 in releasing TNF-α and IL-1β from FLS after pro-oxidants treatment since the TLR4 signaling inhibitor significantly decreased TNF-α, and IL-1β release; markers of the TLR4 signaling pathway.

Furthermore, IL-10 as an anti-inflammatory cytokine may contribute to the suppression of proinflammatory cytokine production, and could usually increase during inflammation. The ratios of proinflammatory to anti-inflammatory cytokine production could represent a balance between the two cytokines and their relevant biologic functions (Fig 6.5 panels D and E). Thus, the extent of inflammation could depend on a high ratio of TNF-α and IL-1β to IL-10 (You et al., 2011). Therefore, the data suggest that PPC and FeCl₂ treatments may trigger the
counteracting anti-inflammatory IL-10 more than the CuCl treatment, while both still trigger this release by (5-18) pg/ml whereas triggering the pro-inflammatory phenotype response in FLS by (100-700) pg/ml. Thus, our data present -for the first time- an important mechanism that may explain the intersection between exogenous and endogenous RS in enhancing inflammatory phenotypes that may initiate, propagate, and maintain several chronic diseases such as RA.

The S100 proteins play a role in inflammation as activators of TLRs and RAGE receptors. The MRP8/14 complex (calprotectin) serves as an endogenous activator of the TLR signaling pathway and in particular for TLR4 (Vogl et al., 2007; Tamaki et al., 2011). Thus, in a fashion similar to LPS, MRP8 caused an elevation in levels of TNF-α, IL-1β, and IL-12. Also, MRP8 is an endogenous ligand of TLR4 (Tamaki et al., 2011). MRP antigens reside only in macrophage subtypes in synovial membranes and are only localized into cells that are adjacent to the cartilage-pannus junction and in only inactive disease patients of RA (Youssef et al., 1999).

Plasma calprotectin is a useful clinical biomarker in RA (Abildtrup et al., 2015). In his recent systematic review, Abildtrup presented an evidence that calprotectin blood levels were measurable in sixteen studies during 1988-2013 in patients with RA. Calprotectin levels were higher in patients with RA than controls. Five of these studies measured calprotectin levels in synovial fluid of RA patients and presented high calprotectin concentrations (Berntzen et al., 1991; Burmeister and Gallacchi, 1995; Drynda et al., 2004; De Rycke et al., 2005; Sunahori et al., 2006) suggesting that it is produced locally in the synovium (Abildtrup et al., 2015). Previously, no direct evidence of synovial origin existed. Here the data clearly showed that RATM-induced TLR4 signaling pathway significantly induced the production of MRP8/14.
complex in FLS (Fig. 6.6 panels A and B). This upregulation of the MRP8/14 complex in FLS may provide another possible mechanism for TLR4 activation in synovial fibroblasts other than the possible direct interaction of reactive species with TLR4. MRP8/14 appears to provide a feedback mechanism to amplify the inflammatory response by further activating TLR4.

Overall, the findings indicate that neutralizing the HMGB1 protein may be advantageous in alleviating pain and inflammation and confirming the role of TLR4/ROS-coupled activation in potentially initiating, propagating, and maintaining inflammatory phenotype.
CHAPTER 7
EFFECT OF RATM ON CELL PROLIFERATION AND CELL DEATH

7.1. Introduction

The joint space is lined with macrophages and synovial fibroblasts. The lining is highly vascularized, one or two cells thick (Walsh et al., 1998). RA synovial fibroblasts (RASF), also known as FLS or type B synoviocytes, along with macrophages are the two main cell types in the hyperplastic synovial tissue that degrade cartilage and bone (Muller-Ladner et al., 2007). Synovial hyperplasia is mainly caused by the increased infiltration of inflammatory cells and by the proliferation of fibroblast-like cells (Qu et al., 1994). Thus, hyperplasia of synovial fibroblasts (also called pannus formation) precedes inflammation (Qu et al., 1994) and determines the outcome of RA in most of the affected individuals. This is in contrast to osteoarthritis (OA) where no in vivo hyperplasia is observed (Hashimoto et al., 1998). Hyperplasia and inflammation are the typical pathological changes of RA. During RA, RASF increases in the number and produce inflammatory cytokines and matrix metalloproteinases, which degrade cartilage.

In multicellular organisms, homeostasis is maintained through a balance between cell death and cell proliferation. Three different mechanisms have been proposed to explain the increased synovial fibroblasts population (hyperplasia). Hyper-proliferation and the substantial increase in the number of resident synovial cells are due to (i) massive influx and retention of inflammatory cells such as macrophages and lymphocytes on the one hand. On the other hand, there is (ii) overexpression of in vitro mitotic growth factors, overexpression of proteins that regulate proliferation, and mutations in tumor suppressor gene P53, and (iii) decreased apoptosis (Korb et al., 2009). Eukaryotic cells tend to survive various cellular stressor
conditions such as ER stress, hypoxia, starvation, heat shock, and microbial infection through autophagy (Levine, 2007), a highly regulated and conserved survival strategy. Autophagy is a process of lysosome-mediated intracellular degradation of unfolded/misfolded proteins and organelles that is also important for cellular homeostasis, development, and tumorigenesis (Lamb et al., 2013).

In this current set of experiments, the hypothesis that oxidant stress induced by RATM in FLS through TLR4 stimulation and HMGB1 release will produce profound oxidative adaptations that contribute to RA pathogenesis through changes in the cell life cycle and cell death is tested.

7.2. Results

7.2.1. Redox-active trace metal (RATM) oxidants treatment increased cell proliferation. In order to identify the effect of RATM pro-oxidants on FLS proliferation, flow cytometry was used to detect any changes in the proteins of proliferation at 24 h and 48 h. Oxidants increased proliferation in a concentration and time-dependent manner (Fig. 7.1. panels A-D). Our data indicate that at 24 h, proliferation is higher for PPC and CuCl.

Cell cycle analysis was used to calculate the proliferation index (PI) as shown in (Table 21). The data indicates a significant increase in oxidant-induced PI compared with the control (Figure 2F). However, at 24 h, the number of cells in the S phase were up-regulated in the HMGB1 group compared to the control group. Thus, PI increased in a time-dependent manner with HMGB1 treatment. To examine the correlation between HMGB1 and synovial cells, we used a recombinant HMGB1 to stimulate synoviocytes. We found that HMGB1 could induce PI and proliferation. Our data also show that when HMGB1 (1µg) was added to each pro-oxidant (data not shown here), cell populations % were increased in both S and G2/M phase.
Given these results, we confirmed that HMGB1, as an important damage-associated molecular pattern (DAMP) in the pathogenesis of RA and innate immunity, is closely correlated with the proliferation of synoviocytes (Guo et al., 2011) and that RATM pro-oxidants are more active in stimulating synoviocytes than the recombinant HMGB1 to induce PI and proliferation.
Figure 7.1. Effect of RATM oxidants on the expression of the proteins of proliferation at 24 and 48 h. CellTrace™ Carboxyfluorescein diacetate succinimidyl ester (CFSE) probe was used to quantify proliferation proteins in FLS cells by Fluorescence Cell sorting (FC). (A) Representative FC tracings are showing changes in fluorescence intensity of proliferation proteins after 24 h treatment. (B) Quantitative analyses of FC tracings after 24 h treatment. Representative tracings after 48 h treatment are shown in (C). Quantitative analyses of FC tracings show changes in fluorescence intensity of proliferation proteins after 48 h treatment are shown in (D). (n = 3-5 independent experiments; *p ≤ 0.05, one - way ANOVA with Tukey’s multiple comparison tests).

Table 16. RATM-induced changes of PI and cell cycle distribution in HIG-82 cells detected by flow cytometry (X ± SEM, n = 3)

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<tr>
<th>Group</th>
<th>Proliferation</th>
<th>Cell cycle distribution %</th>
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<tr>
<td></td>
<td>Index (%)</td>
<td>SubG₀</td>
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<tr>
<td>Control</td>
<td>32.7 ± 0.5</td>
<td>0.3 ± 0.08</td>
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<td>HMGB1 (1µg)</td>
<td>35.4 ± 1.7</td>
<td>0.52 ± 0.3</td>
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<td>PPC (10 nM)</td>
<td>35.6 ± 2.3</td>
<td>0.37 ± 0.08</td>
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<td>FeCl₂(10 µM)</td>
<td>37.6 ± 3.3</td>
<td>1.8 ± 0.414</td>
</tr>
<tr>
<td>CuCl (50 µM)</td>
<td>44.9 ± 1.0</td>
<td>1.2 ± 0.367</td>
</tr>
<tr>
<td>CuAA</td>
<td>35.5 ± 0.2</td>
<td>0.63 ± 0.2</td>
</tr>
</tbody>
</table>
Figure 7.2. Effect of synchronization and cell cycle arrest on proliferation in FLS. (A) Treatment with RATM increased cells arrested in G2/M phase. Cells were stained with 100 μl PBS containing 5 μl propidium iodide (1 mg/ml) and 10 μl RNase-DNase free (10 mg/ml), incubated in the dark for 30 min at RT. The proliferation index (PI) represents the measure of cell proliferation calculated using the following equation: PI = (S + G2M)/(S + G2M + G0/G1) X 100 %. The sub-G0 fraction of events represented the apoptotic population of cells. Representative FC tracings show changes in fluorescence intensity after 24 h treatment. (B) Representative quantitative histogram of all populations. (C) Representative quantitative histogram of SubG0 population. (D) Representative quantitative histogram of the G0/G1 population. (E) Representative quantitative histogram of S population. (F) Representative quantitative histogram of G2/M population. (G) Representative quantitative histogram of PI. The data represent three independent experiments. *** p ≤ 0.001, **p ≤ 0.01*p ≤ 0.05, one-way ANOVA with Dunnet’s multiple comparison tests.
7.2.2. Redox-active trace metal (RATM) oxidants decreased poly-caspase activity. An enzymatic family of cysteine proteases (caspases) regulates the degradative phase of apoptosis. To measure the apoptotic activity of the ROS generated from the RATM treatments, we used flow cytometry to determine the activity of caspases after 2 and 24 h treatment. Ninety percent ethanol (90%) was used as positive apoptosis control. To investigate the effect of autophagy inhibition on caspase activity, FLS cells were preincubated with the autophagy inhibitor U0126) at 20 μM for three h before treatment with RATM oxidants in the continued presence of the inhibitor for 24 h.

Our results show a reduced poly-caspase activity after two h treatment compared to the control and the positive control. A marginal non-significant increase in poly-caspase activity was noticed after 24 h treatment (Fig. 7.3). We found that HMGB1 (1µg) alone or in combination with pro-oxidants did not induce poly-caspase activity either, and hence apoptosis. This data again confirms another mechanism for HMGB1 involvement in proliferation by not inducing apoptosis but rather by promoting proliferation. Our data also showed that pre-incubation of FLS with U0126 before treatment with RATM oxidants significantly enhanced poly-caspase activity at 24 h (Fig. 7.4).
Figure 7.3. Effect of RATM oxidants on the activity of polycaspases at 2h in FLS. Cells were treated with PPC, FeCl₂, and CuCl alone and/or in combination with Ascorbic acid (AA). (A) Representative FC tracings are showing changes in fluorescence intensity of polycaspase activity after two h treatment. (B) Represents quantitative analyses of FC tracings of (A). All data represent three independent experiments. There were no statistical differences observed *p < 0.05. One - way ANOVA with Tukey’s multiple comparison tests.
Figure 7.4. Effect of RATM oxidants on the activity of polycaspases at 24 h in FLS. Cells were treated with PPC, FeCl₂, and CuCl alone and/or in combination with Ascorbic acid (AA) (100 μM) in the absence or presence of the autophagy inhibitor U0126. (A) Representative FC tracings are showing changes in fluorescence intensity of poly-caspase activity after 24 h treatment. (B) Quantitative analyses of FC tracings of (A) after 24 h treatment. All data represent 3 independent experiments *p < 0.05, # p < 0.0001, one-way ANOVA with Tukey’s multiple comparison tests.
7.2.3. Reduced apoptosis correlates with enhanced autophagy following (RATM) oxidants treatment. To determine the extent by which RATM treatment could affect cell death and autophagy in FLS, we measured Beclin I, LC3 II/I, and p62/SQSTM1 protein levels after RATM treatment in the presence and absence of U0126 (20 μM). The autophagic flux assay was performed by Western blots for LC3II and Beclin 1 after treatment. RATM up-regulated Beclin I and stimulated the conversion of LC3 I to LC3 II in FLS compared to the untreated control cells over a time course (Fig. 7.5), which suggests enhanced autophagy.
7.2.4. P62/SQSTM1 and other autophagy markers are reduced with autophagy inhibitor U0126. To further determine whether RATM affects cell death and autophagy in synovial fibroblasts, we tested FLS for P62/SQSTM1, Beclin I, and LC3 II and I protein levels after RATM treatment in the presence and absence of the autophagy inhibitor U0126. Autophagy flux assay was performed by Western blots after 24 h treatment with RATM. Our data also showed that the autophagy markers were significantly down-regulated in the presence of the autophagy inhibitor U0126 (Fig. 7.6). Besides, RATM caused a significant increase in P62 expression compared to the control alone, whereas pre-treatment with U0126 reduced the expression of P62 compared to RATM treatment alone.
Figure 7.5. Time-course effects of RATM oxidants treatment on autophagy in FLS. Cells were treated with RATM at different times (0, 2, 4, 6, 12, and 24 h). Proteins were extracted at the end of each treatment, and Western blots were used to determine (A) LC3B I and II protein expression. (B) Representative semi-quantitative histogram of (A) which represents the optical density (OD) ratios of LC3BII immunoblot signals normalized to those of β-actin from the same tests. (C) Beclin-1 protein expression (D) Representative semi-quantitative histogram of (C) that represents the optical density (OD) ratios of Beclin-1 immunoblot signals normalized to those of β-actin from the same tests. The data represent three independent experiments. # p < 0.0001, two-way ANOVA with Tukey’s multiple comparison tests.
Figure 7.6. Effect of autophagy inhibition on the markers of autophagy in FLS. Cells were pre-incubated with the autophagy inhibitor U0126 for three h then treated with RATM at 24 h. Proteins were extracted at the end of each treatment, and Western blots were used to determine (A) Levels of LC3B I and II, Beclin 1, and p62/SQSTM1 protein expression. (B) A semi-quantitative histogram of (A) representing the optical density (OD) ratios of LC3BII, Beclin 1, and p62/SQSTM1 immunoblot signals normalized to those of β-actin from the same blots. The data represent three independent experiments. *p ≤ 0.05, # p < 0.0001, 2-way ANOVA with Tukey’s multiple comparison tests.
7.3. Discussion

The RATM exogenous pro-oxidants; potassium peroxychromate (PPC, Cr$^{+5}$), ferrous chloride (FeCl$_2$, Fe$^{+2}$), and cuprous chloride (CuCl, Cu$^+$) generated ROS in FLS cells through TLR4 signaling pathway and promoted extracellular HMGB1 and inflammatory cytokines release with the potential to initiate “sterile” inflammatory responses (Alsousi and Igwe, 2018). Not only can trace metals generate reactive species themselves, but in the presence of reduced metals such as ferrous, cuprous ions, or hydrogen peroxide, they can also be converted into highly reactive radicals (Chance et al., 1979). Although iron and copper are essential cofactors of proteins, and chromium is essential for lipid metabolism, their ions are potentially dangerous pro-oxidants (Gutteridge and Halliwell, 1992).

Reactive species are not always proapoptotic. Certain cancerous cell lines use ROS to prevent apoptosis by mechanisms that suppress caspase activity and change intracellular pH (Teoh et al., 2005; Urbano et al., 2005). Here, RATM-induced oxidative stress has shown to enhance cell proliferation and decrease apoptosis caspase activity while enhancing autophagy in synovial fibroblasts. Previously, we have shown that RATM agents as exogenous pro-oxidants in the form of potassium peroxychromate (PPC, Cr$^{+5}$), ferrous chloride (FeCl$_2$, Fe$^{+2}$), and cuprous chloride (CuCl, Cu$^+$) induce oxidative stress and may contribute to the pathogenesis of RA through ROS / TLR4-coupled activation (Alsousi and Igwe, 2018).

To understand the role of RATM in the pathogenesis of RA, it is crucial to understand their potential role in proliferation and cell death of the synovial membrane cells under oxidants stress.

Different mechanisms have been proposed to explain the increase in synovial fibroblasts population, which includes hyperproliferation with a substantial increase in the number of resident synovial cells due to overexpression of growth factors and proteins that regulate proliferation, and
mutations in tumor suppressor gene P53 in RA (Korb et al., 2009). The mammalian cell cycle is divided into four stages: a growth phase (G0/G1), a synthesis phase (S), a second growth phase (G2), and mitosis (M) phase. In RA-synovial fibroblasts, the measured DNA synthesis or (S) phase entry demonstrated enhanced activity (Mohr et al., 1975; Qu et al., 1994). Analysis of the (S) phase marker (Proliferating Cell Nuclear Antigen (PCNA)) revealed positive fibroblasts in RA within areas of the hypertrophic synovial lining (Sugiyama et al., 1996; Walsh et al., 1998). Also, data from animal models of arthritis support the finding that the proliferation of synovial fibroblasts contributes to synovial lining thickening (Henderson et al., 1981; Henderson et al., 1982a). The data shows that RATM-induced proliferation of proteins at 24 and 48 h is concentration and time dependent. This is in agreement with the observed increased proliferation in cells during the development of arthritis after an intra-articular challenge in rabbits (Henderson et al., 1982b), in which the labeling index of cellular proliferation in the synoviocytes of the inflamed joints was increased at 24 h after the challenge and started to decrease rapidly after 3 days of the challenge.

Synovial macrophages and fibroblasts are critical in the pathogenesis of RA. Thus, a better understanding of the mechanisms that regulate cellular survival is crucial, not only to enhance our knowledge of the cellular pathogenesis of the disease but also to develop more effective therapeutic approaches. Previous studies on the apoptotic response of synovial fibroblasts in RA patients have shown the absence of clear signs of apoptosis in these cells (Matsumoto et al., 1996) (Firestein et al., 1995). This absence of apoptosis has also been confirmed in animal studies (Perlman et al., 2001). As a conserved cell death pathway, a number of methods have been used to study the apoptotic response of synovial fibroblasts in RA patients compared either to non-arthritic controls or to patients with other degenerative joint diseases such as OA. The majority of these studies
revealed the absence of clear signs of apoptosis in RASF and that apoptosis is rarely found in the synovial tissues of either patients or animal models of RA (Drynda et al., 2005; Mizushima, 2007; Dharmapatni et al., 2009). Our data show that RATM induced few to no signs of apoptosis when we measured polycaspase activity, or when we used cell cycle analysis to determine the apoptotic population represented by subG0. FLICA polycaspase reagent, which measures the intracellular process of apoptosis instead of a side effect and eliminates the false positives that often, may be seen with Annexin V and TUNEL staining. Interestingly, we confirmed that RATM were not effective either alone or in combination with recombinant disulfide high mobility group box-1 in inducing apoptosis, which mimics potential in vivo pathogenesis of RA (Franz et al., 2000; Perlman et al., 2001; Drynda et al., 2005; Xu et al., 2013). While the previously reported data showed a positive macrophage (Firestein et al., 1995), and fibroblast (Nakajima et al., 1995) apoptosis, other reports revealed that only a few cells displayed apoptosis (Matsumoto et al., 1996).

Our results are consistent with the reports of insufficient apoptosis in the synovial lining that contribute to RA synovial tissue hyperplasia. The resistance of RASF to apoptosis is identified as one of the critical characteristics of RA (Matsumoto et al., 1996), even with indisputable evidence of DNA strand fragmentation in the synovium of RA patients (Firestein et al., 1995; Perlman et al., 2001). Apoptotic rates that are lower than usual are considered the immediate cause of synovial hyperplasia (Muller-Ladner et al., 1996; Muller-Ladner et al., 2007). Although RASF demonstrated the expression of several death surface receptors of the TNF-receptor family (Drynda et al., 2005; Morel et al., 2005; Wang et al., 2006), evidence indicated that they are relatively resistant to receptor-induced apoptosis. TNF-α does not induce apoptosis in RASF at all but instead promotes proliferation (Korb et al., 2009).
Alterations in apoptosis not only affect the RASF population but also infiltrating inflammatory cells. T cells in RA have elevated levels of antiapoptotic proteins of the Bcl-family (Schirmer et al., 1998). In addition to the effects of RASF on T cell survival, RASF also contributes to the B cells attraction and accumulation (Lindhout et al., 1999; Hayashida et al., 2000). However, some data have confirmed that reduced apoptosis in RA is correlated with enhanced autophagy (Xu et al., 2013) and that a complex interplay exists between the processes that regulate autophagy and apoptosis (Eisenberg-Lerner et al., 2009; Schwarten et al., 2009). However, this issue remains incompletely resolved.

In the present study, it was demonstrated that RATM treatment induced autophagy in FLS which express TLR4. When autophagy is upregulated, a cleaved cytosolic form of LC3 (LC3-I) is conjugated with phosphatidylethanolamine to form LC3-phosphatidylethanolamine (LC3II), which is recruited to autophagosomal membranes. Recent studies have shown that p62/SQSTM1 plays an essential role in the pathogenesis of several human diseases, including cancer, Alzheimer's disease, and infectious diseases (Wu et al., 2018). During autophagy, LC3 is processed in the autophagosome membrane. The p62/SQSTM1 forms a complex with LC3-II and transfers substrates for autophagy to autolysosomes for degradation. Activation of autophagy reduces the expression of p62, thus pharmacological and genetic inhibition of autophagy can increase the level of p62 in various cell lines, in tissues such as the liver and CNS, and in different species (Liu et al., 2016a). On the other hand, one of the key forms of transcriptional regulation of p62 is dependent on the NRF2 transcription factor, which belongs to the basic leucine zipper (bZIP) family of transcription factors. In response to oxidative stress, NRF2 binds explicitly to the antioxidant-responsive element (ARE motif), which is located in the p62 promoter to enhance the expression of p62 mRNA (Puissant et al., 2012).
This is consistent with our data, where RATM oxidants caused an increase in P62 protein expression compared to the control, and the pre-treatment with U0126 reduced the expression of P62 compared to RATM treatment alone. RATM treatment induced the expression of Beclin 1, an essential mediator of autophagy, and stimulated the conversion of LC3I to LC3II, another marker of autophagy. This is consistent with the higher expression of Beclin-1 and LC3, as upregulated indicators of autophagy in RA (Xu et al., 2013). Autophagy is activated in RA in a TNF-α-dependent manner to stimulate osteoclasts differentiation and to regulate bone resorption as a critical pathway in joint destruction (Lin et al., 2013b; Lin et al., 2013a).

Previous findings indicated that TNFα and other inflammatory cytokines including IL-1 as well as TLR ligands stimulate autophagy (Shi and Kehrl, 2010; Lin et al., 2013b). Therefore; our findings could be explained by ROS-induced TLR4 activation and the release of inflammatory cytokines. HMGB1 induces autophagy as well (Tang et al., 2010; Liu et al., 2011), whereas MRP8/14 enhances ROS generation and promotes autophagy in a ROS-dependent manner (Wang et al., 2015). Our RATM system has been shown to promote the release of HMGB1 and inflammatory cytokines and to upregulate MRB8/14 protein expression, which could provide additional explanation for our results (Alsousi and Igwe, 2018).

As autophagy has both protective and inductive effects on cell death in RASF, we used flow cytometry cell cycle analysis to explore its role after RATM treatment. The number of dead cells after RATM treatment showed that FLS were resistant to RATM–induced cell death at 24 and 48 h. Caspase activity was not induced at 2 and 24 h of treatment, which indicates that apoptosis is minimal. Thus, the activation of autophagy here exhibits a protective role and contributes to the apoptosis-resistant phenotype similar to what was seen in RASF (Kato et al., 2014). However, further experiments are needed to address the underlying mechanism.
In summary, our studies suggest that RATM as pro-oxidants could play a role in RA pathogenesis by increasing proliferation and promoting autophagy in synovial fibroblasts, which is in agreement with the pathophysiological changes that occur in active RA. These findings may serve as a basis for individualized RA therapy, considering the underlying cause of the pathogenesis.
CHAPTER 8
EFFECT OF RATM PRO-OXIDANTS ON TLR4 - COUPLED AP-1 SIGNALING PATHWAY

8.1. Introduction

Yu et al. (Yu et al., 2010) reported that TLR4 signaling in Kupffer cells (myeloid cells) plays a central role in liver tumorigenesis, while Dapito et al. (Dapito et al., 2012) showed that TLR4 signaling in hepatocytes is responsible for TLR4-dependent tumorigenesis. This is in contrast to reports of LPS-induced TLR4 signaling leading to tumor regression in colorectal cancer (Otto et al., 1996). These findings highlight the importance of understanding the specific involvement of TLR4 in different cell types and suggest that TLR4 signaling may play different roles in different cells.

Meanwhile, our laboratory had shown that TLR4 mediates exogenous oxidant-induced NF-κB activation in the human embryonic kidney (HEK)-Blue mTLR4 artificial cell system (Karki and Igwe, 2013). Further, it confirmed the role of TLR4 in RAW-Blue macrophage cells as an in vitro system, where oxidant-induced NF-κB activation resulted from released ROS stimulated TLR4 (Zhang and Igwe, 2018).

The transcription factor AP-1 is a redox-regulated transcription factor and is composed of homo and heterodimeric complexes of two oncogenic proteins, Fos and Jun activating transcription factor (Zenz et al., 2008). Many valuable insights regarding the specific functions of AP-1 proteins in development and disease have been obtained from genetically modified mice and derived cells.

MAPKs are classified into distinctive groups. There are at least three main MAPK modules; (i) Extracellular signal-regulated protein kinase (ERK) (P42/44MAPK) cascades, (ii) the c-Jun N-terminal Kinase (JNK) (also known as stress-activated kinase SAPK) pathway, and (iii)
P38 MAPK, and other MAP kinases (Pearson et al., 2001). All three major classes of MAPKs play an essential role in the pathogenesis of RA.

8.2. Results

8.2.1. Redox-active trace metal (RATM) oxidants activated total JNK, ERK 1/2, and P38. To identify the effect of RATM pro-oxidants on total and phospho-NK, Western blots, and flow cytometry were used to detect changes. PPC (10 nM) upregulated total JNK protein expression as measured by Western blot and FC at 5, 15, and 60 min, while FeCl$_2$ showed a significant increase at 15 min. Interestingly, CuCl, but not CuAA, increased JNK expression at 10 and 25 min (Fig. 8.1).

For ERK1/2 expression, PPC (10 nM) significantly upregulated total ERK1/2 protein expression as measured by Western blot and FC at 10 and 60 min (Fig. 8.2), whereas FeCl$_2$ showed a significant increase at 5 and 60 min. Interestingly, CuCl and CuAA increased ERK1/2 expression at 25 and 20 min, respectively.

For P38 expression, PPC significantly upregulated total P38 protein expression as measured by Western blot and FC at 10, 15, and 20 min, while FeCl$_2$ showed the highest significant increase at 25 min. Interestingly again, CuAA but not CuCl increased P38 expression at 10 min (Fig. 8.3).
8.2.2. Redox-active trace metal (RATM)-oxidants activated phosho-JNK, -ERK1/2, and -P38. To further confirm the effect of RATM oxidants on MAPK and to determine that the persistence of these activated signals is due to the induction of the activity of kinase enzymes rather than only upregulation of protein expressions. FLS cells were treated with PPC, FeCl₂, CuCl, and CuAA, then MAPK pathways were assayed by phosho – specific antibodies for JNK, ERK1/2, and P38 by Western blot and FC at 0, 5,10, 15, 20, 25, 30, and 60 minutes (Fig. 8.4).

Data clearly showed that PPC (10 nM) and FeCl₂ (10 μM) treatment increased phosho ERK1/2 and P38 and P-JNK. Furthermore, treatment with CuCl (50 μM) upregulated P-JNK, ERK1/2 while the increase in phosphorylated P38 was not significant. In addition, treatment with CuAA significantly upregulated P-JNK at 20 and 60 min along with upregulation of P-ERK1/2 at 5 and 20 min, while the upregulation for P38 was only at 5 min.

Overall the data suggest that RATM oxidants readily activate phosphorylation of the three main MAPK modules with potential implication in the molecular role of these RATM oxidants in clinical RATM.
Figure 8.1. Flow cytometry analysis of the effects of RATM oxidants on total JNK protein expression in FLS. HIG-82 cells were treated with PPC, FeCl₂, and CuCl alone and in combination with Ascorbic acid (CuAA) at different time points. Cells were then further used to run the flow cytometric quantitative analysis of total JNK in HIG-82 synovial fibroblasts treatment. (A, B, C, and D) Representative FC tracings are showing changes in fluorescence intensity of total JNK following treatments with vehicle and RATM oxidants. (E) Quantitative analyses of FC tracings after each treatment. (n = 3 independent experiments; *p ≤ 0.05, two-way ANOVA with Tukey’s multiple comparison tests).
Figure 8.2. Flow cytometry analysis of the effects of RATM oxidants on total ERK1/2 protein expression in FLS. HIG-82 cells were treated with PPC, FeCl$_2$, and CuCl alone and in combination with Ascorbic acid (CuAA) at different time points. Cells were then further used to run flow cytometric quantitative analysis of total ERK1/2 in HIG-82 synovial fibroblasts treatment. (A, B, C, and D) Representative FC tracings are showing changes in fluorescence intensity of total JNK following treatments with vehicle control, RATM oxidants. (E) Quantitative analyses of FC tracings after each treatment. (n = 3 independent experiments; *p ≤ 0.05, two-way ANOVA with Tukey’s multiple comparison tests).
Figure 8.3. Flow cytometry analysis of the effects of RATM oxidants on total P38 protein expression in FLS. HIG-82 cells were treated with PPC, FeCl₂, and CuCl alone and in combination with Ascorbic acid (CuAA) at different time points. Cells were then further used to run flow cytometric quantitative analysis of total P38 in HIG-82 synovial fibroblasts treatment. (A, B, C, and D) Representative FC tracings are showing changes in fluorescence intensity of total P38 following treatments with vehicle control, RATM oxidants. (E) Quantitative analyses of FC tracings after each treatment. (n = 3 independent experiments; *p ≤ 0.05, two-way ANOVA with Tukey’s multiple comparison tests.)
Figure 8.4. Flow cytometry analysis of the effects of RATM oxidants on phosphorylated MAPKs expression in FLS. HIG-82 cells were treated with PPC, FeCl$_2$, and CuCl alone and in combination with Ascorbic acid (CuAA) at different time points. Cells were then further used to run flow cytometric quantitative analysis of phosphorylated MAPKs in HIG-82 synovial fibroblasts. (A) Representative FC tracings are showing changes in fluorescence intensity of p-JNK following treatments with the vehicle, RATM oxidants. (B) Representative FC tracings are showing changes in fluorescence intensity of p-ERK following treatments with the vehicle, RATM oxidants. (C) Quantitative analyses of FC tracings of p JNK after each treatment. (D) Quantitative analyses of FC tracings of pERK after each treatment. (E) Quantitative analyses of FC tracings of p P38 after each treatment. (n = 3 independent experiments; *p ≤ 0.05, # p < 0.0001, two-way ANOVA with Tukey’s multiple comparison tests.)
8.2.3. **TLR4 siRNA optimization.** Negative control siRNA was transfected into cells with Lipofectamine RNAiMax. Subsequent to 24 h of transfection, the transfection efficiency was determined using RT-PCR. The ratio of TLR4 siRNA to Lipofectamine RNAiMax resulted in the highest transfection efficiency was (1:1.25).

In total, two custom designed siRNAs were transfected into FLS cells, with medium alone (blank), negative siRNA or Lipofectamine RNAiMax alone as controls. After 24 h of transfection, TLR4-siRNA-514 effectively reduced TLR4 messenger RNA expression in transfected cells (Fig. 8.5). The TLR4 mRNA expression in the TLR4-siRNA-513, and the negative sequence transfection group was significantly different. However, there was a significant difference between the expression of TLR4 in the TLR4-siRNA-514 group and the negative control group (Fig. 8.6).

At 6 and 24 h post-silencing, the mRNA expression levels of TLR4 in FLS were determined by RT-PCR. All samples were analyzed in duplicates in three separate experiments. The mRNA expression level of TLR4 in FLS from the siRNA-514 showed the highest level of efficiency at 24 h compared with six h. Therefore, 24 h was chosen as an experimental time of treatment. The results were further confirmed by Western blots. As shown in (Fig. 8.6), expression levels of TLR4 in FLS decreased significantly at 24 h post silencing, while no noticeable change in TLR4 levels occurred following transfection with the negative TLR4 siRNA group. The result showed that interference of TLR4 could persist for a long time. Only TLR4-siRNA-514 was used in all subsequent experiments.
Figure 8.5. Role of siRNA TLR4 in TLR4 gene expression in FLS. Cells were transfected with Lipofectamine, siRNA513, and siRNA514 or NC at two different time points (6 and 24 h). Relative TLR4 mRNA expression was quantified post treatment using RT-qPCR and normalized against β-actin. The data represent 3 independent experiments ***P< 0.001, # P < 0.0001, one-way ANOVA with Tukey’s multiple comparison tests.
Figure 8.6. Role of siRNA TLR4 in TLR4 protein expression in FLS following siRNA. (A) At two different time points (6 and 24 h), Cells were treated with blank control, siRNA513, siRNA514, Lipfectamine or NC at two different time points (6 and 24 h). The TLR4 protein expression levels were determined by Western blots. The histogram (B) represents the optical density (OD) ratios of TLR4 immunoblot signals normalized to those of β-actin from the same tests. The data represent 3 independent experiments (n=3, ** P < 0.01, # P < 0.001, two-way ANOVA with Tukey’s multiple comparison tests).
8.2.4. Phospho-ERK1/2, -JNK and -P 38 expression after knockdown of TLR4 gene using siRNA. To investigate the potential function of TLR4 in ERK1/2, JNK, and P38, siRNAs were used to knockdown endogenous TLR4 gene expression. WB data for PPC (10 nM) treatment post siRNA transfection showed dysregulation in phosphorylated ERK1/2, JNK and P38 expression (Fig. 8.7).
Figure 8.7. Role of siRNA TLR4 in the expression ERK1/2, JNK, and P38 proteins in FLS following PPC (10 nM) treatment (A). At different time points, cells were treated with vehicle control or PPC. The protein expression levels were determined by Western blots. The histogram (B) represents the optical density (OD) ratios of ERK immunoblot signals normalized to β-actin from same tests. (C) Represents the optical density (OD) ratios of JNK. (D) Represents the optical density (OD) ratios of P38. The data represent three independent experiments (n=3, # P < 0.001, two-way ANOVA with Tukey’s multiple comparison tests).
8.2.5. c-Fos and c-Jun (AP-1) protein levels after knockdown of TLR4 gene using siRNA. To investigate the potential function of TLR4 c-Fos and c-Jun proteins, siRNA was used to knockdown endogenous TLR4 gene expression. Treatment with PPC (10 nM) significantly upregulated c-Jun at 5 min and c-Fos at 10, and 20 min. However, treatment with PPC (10 nM) post the siRNA transfection showed dysregulation in c-Jun and c-Fos expression (Fig. 8.8).
Figure 8.8. Role of siRNA TLR4 in the expression of c-Jun and c-Fos proteins in FLS following PPC (10 nM) treatment (A). At different time points, cells were treated with vehicle control or PPC. The protein expression levels were determined by Western blots. The histogram (B) represents the optical density (OD) ratios of proteins immunoblot signals normalized to β-actin from same tests. The data represent 3 independent experiments, n=3, * P ≤ 0.05, # P < 0.001, two-way ANOVA with Tukey’s multiple comparison tests.
8.3. Discussion

Whereas TLR mediates the inflammatory immune response, different studies propose an association between TLR activation, uncontrolled inflammation, and tumor development. TLR4 and other TLRs have been detected in the throat, breast, colorectal, gastric, prostate, and lung cancer cell lines (Gu et al., 2018). The silencing of TLR4 signaling in cancer cells had shown to reduce the risk of tumor formation (Ahmed et al., 2013; Liu et al., 2016b). Nevertheless, CD14 ligand LPS-induced TLR4 signaling in cancer cells promoted cell survival and proliferation in hepatocellular carcinoma (HCC) (Wang et al., 2013) supporting distinctive biology of TLR4 signaling in different cell types. There is evidence that TLR4 activation and signaling plays a vital role in the pathogenesis of HCC. It remains highly unclear about the cellular and molecular interplay between TLR4 and the progression of many diseases.

In the present study, the biological effects of TLR4 activation on the AP-1 pathway were investigated. The optimal transfection conditions were determined to ensure that siRNA could efficiently transfect into HIG-82 cells using Lipofectamine RNAiMax. TLR4 mRNA and protein were respectively detected by RT-PCR and Western blot analysis subsequent to transfection of cells. Our data confirmed that TLR4-siRNA-514 was a more efficient siRNA for silencing TLR4 than TLR4-siRNA-513, suggesting that cells transfected with TLR4-siRNA-514 using Lipofectamine RNAiMax were feasible and practical. Thus, providing a reliable tool to study the biological effects of RATM oxidant / TLR4 coupled signaling in FLS.

Previously, we have shown that RATM as exogenous oxidants induces oxidative stress, which may contribute to the pathogenesis of RA through the ROS/TLR4-coupled activation (Alsousi and Igwe, 2018; Alsousi and Igwe, 2019). To understand the role of RATM in the
pathogenesis of RA, it is crucial to clarify their potential role in TLR4 signaling in FLS under oxidant stress.

Mitogen-activated protein kinases (MAPKs) are critical regulators of proinflammatory cytokine production in the signaling cascades downstream of Toll-like receptors (TLRs), as well as B-cell antigen receptor (BCR) (Harnett et al., 2005), T-cell antigen receptor (TCR)/CD28 (Liu, 2005), IL-1, IL-17 (Kolls and Linden, 2004), and TNF-α receptors. The MAPKs (ERK, JNK, and p38) contribute by phosphorylating key transcription factors, such as activator protein-1 (AP-1), that are required for gene transcription (Han et al., 1999; Liacini et al., 2002).

The signal transduction pathways that are activated in RA, in response to elevated levels of proinflammatory cytokines, include JNK, ERK1/2, P38 kinase, and JAK/STAT (Malemud, 2011, 2013), which are activated by MRP complex (Youssef et al., 1999). P38, JNK, and ERKs are constitutively expressed in RA and osteoarthritis (OA) FLS. P38 and ERK1/2 are readily phosphorylated in both RA and OA FLS after interleukin-1 (IL-1) stimulation, while JNK is phosphorylated in RA FLS but not OA FLS after IL-1 stimulation (Han et al., 1999).

This is consistent with our data where RATM oxidants have shown to elevate levels of pro-inflammatory cytokines (Alsousi and Igwe, 2018) and to induce the activity of JNK, ERK1/2, and P38 kinase. More importantly in vitro MRP8/14-mediated gene expression upregulation by macrophages was associated with the activation of several protein kinase-mediated pathways including c-JNK, ERK1/2, and JAK/STAT as well as NF-κB activation (Youssef et al., 1999). This again provides another rationale for our results, where our data has shown that RATM oxidants mediated upregulation of MRP8/14 expression.
MAPKs are highly activated in rheumatoid synovium and contribute to the inflammatory and destructive mechanisms. MAPKs are known to be involved in regulating COX-2 expression (Dean et al., 2003), and we have also shown that TNF-α up-regulated COX-2 expression with increased PGE₂ production in FLS cells leading to activation of MMP-9 expression and enhanced HMGB1 release to potentially propagate and maintain RA pathogenesis (Alsousi AA, 2017). Therefore, it is anticipated that RATM oxidants, by upregulating MAPK are inducing COX-2 expression and hence contributing to the inflammatory process.

JNKs have been shown to play an essential function in cytokine production and ECM degradation by regulating MMP in FLS and animal models of RA (Han et al., 1999; Han et al., 2001). In particular, JNK plays an essential role in cytokine-mediated AP-1 induction and MMP gene expression in FLS (Han et al., 1999; Liacini et al., 2002). Three isoforms of JNK have been characterized, namely JNK1, 2 and 3. JNK1 and 2 are ubiquitous while JNK3 is primarily restricted to nervous tissue (Davis, 2000). JNK2 deficiency has only modest progenitors that do not develop into mature bone-resorbing osteoclasts (David et al., 2002). These data suggest that TLR4 downstream JNK pathway may be implicated in synovial inflammation and joint destruction of RA and could potentially be targeted in diseases like RA. While JNKs are among novel therapeutic targets, a significant challenge is these molecules are essential in many normal cell functions, especially in matrix remodeling and host defence (Nishina et al., 1997; Sabapathy et al., 1999). Thus, blocking all JNK activity, or even a specific JNK1 activity, may potentially affect host defenses or matrix homeostasis. An alternative strategy would be targeting a distinct upstream kinase-like MKK4 or MKK7. This targeting could allow some normal JNK functions while interfering with a subset that is
pathogenic in synovitis. MKK4 and MKK7, two JNK upstream kinases, exhibit some different properties although they can synergistically activate JNKs. TNF and IL-1 mainly activate MKK7 in murine embryonic fibroblasts, while ultraviolet radiation, anisomycin, heat and osmotic shock activate both MKK4 and MKK7 (Tournier et al., 2001). These data suggest that MKK4 and MKK7 contribute separately to the activation of JNKs in response to environmental stress or inflammatory cytokines.

Another downstream signaling pathway in TLR4 activation is the ERKs. The protein kinases are again involved in the regulation of cell survival and cell death. ERK-1, -2, signaling are essential regulators of the biological activity of hemopoietic progenitor cells in the RA synovium (Varfolomeev et al., 2012). The suppression of ERK activation by IL-1 has been used to reduce inflammation and joint destruction in a rat model of adjuvant-induced arthritis (Yamamoto et al., 2003). The impact of inhibiting ERK signaling in arthritis appears to center on the fact that ERK signaling cascade plays a critical role in joint destruction. For example, the production of the protease stromelysin (MMP3) by cultured rabbit synovial fibroblasts has been shown to be dependent on IL-1α-induced activation of ERK (Thiel et al., 2007). Comparable results were obtained as well in human synovial fibroblasts (Tagoe et al., 2008).

Interestingly, the inhibition of P38 MAPK α using the orally–administered P38 inhibitor, SCIO-469, to patients with active RA failed to show efficacy in adult RA clinical trials (Malemud, 2012). Given these disappointing results, further attention should be focused on ERK1/2 and JNK signaling in synoviocytes.

Our data has shown that PPC as RATM induces c-Jun, which regulates a program of pro- and anti-inflammatory gene expression during macrophage activation and thereby influences the severity of arthritis. Thus c-Jun acts as a checkpoint during macrophage
activation and promotes arthritis via differentially regulating cyclooxygenase-2 and arginase-
1 levels and may represent an attractive therapeutic target in rheumatoid arthritis (Hannemann
et al., 2017).

In RA synovial tissues, constitutive expression of the c-Fos gene has been reported
(Dooley et al., 1996). c-Fos protein has been found in fibroblast-like cells in RA synovium.
The pathological importance of c-Fos in RA was supported by the finding of destructive
arthritis in c-Fos transgenic mice. Therefore, c-Fos is considered an appropriate target gene in
the treatment of RA.

In summary, our studies suggest that RATM oxidants play a role in RA pathogenesis
by enhancing the activity of MAPK in synovial fibroblasts and upregulating AP-1 transcription
in synovial fibroblasts. This outcome is again in agreement with the pathophysiological
changes that occur in active RA, which validates this simple model. Our findings may serve as
a basis for individualized RA therapy considering the underlying mechanism of the
pathogenesis.
CHAPTER 9

RESEARCH SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Given the destructive nature of RA and the percentage of the population that is affected by the disease, molecular research in this field is critical to understand the pathological complexities of the disease and to elucidate the cellular and molecular mechanism underlying the initiation, propagation, and maintenance of RA.

9.1. Research Summary

First, ROS activate toll-like receptor 4 (TLR4) expressed in FLS. The detection potentially plays a significant role in the etiopathogenesis of RA. The included redox-active trace metals (RATM) in our study, ferrous (Fe^{2+}), chromium (Cr^{5+}), and cuprous (Cu^+) at the indicated valency states, served as in vitro exogenous sources of reactive species and in particular for reactive oxygen species (ROS), which induced cellular oxidant stress in FLS. Ascorbic acid (Vit C) could be used to increase the generation of RS, therefore enhancing oxidative stress.

Second, TLR4 is necessary for a robust production of oxidant-mediated proinflammatory phenotypes. Activation of TLR4 is involved in oxidant-induced stress and subsequently in the production of robust IL-1β/IL-10 ratios, and HMGB1 release in the FLS cells, ultimately leading to the propagation and maintenance of RA pathogenesis.

Third, RATM’s treatment increases proliferation index in FLS that protects the cells from apoptosis via induction of autophagy through TLR4/ROS-coupled stimulation. Our findings suggest a possible mimicry of RATM molecular interactions to the in vivo pathogenesis of RA.
Fourth, RATM mediated activation of protein kinases including ERK1/2, P38, and JNK. Our mechanistic studies have documented a TLR4-dependent activation in FLS.

9.2. Conclusions

The current study suggests a combination and individualized therapy of robust antioxidants and inhibitors as a viable therapeutic approach for RA, which remains a formidable clinical problem despite the remarkable advances of recent years. Whereas inflammatory cytokines are now firmly established as therapeutic targets, with blockade of TNF-α providing a benchmark for the development of new therapies, it is harder to define what constitutes a “good” clinical target from a preclinical modeling/perspective. TNF-α and IL-1β are known to play a prominent role in the exacerbation of inflammation in RA, as evidenced by increased expression of COX-2 following direct treatment with either TNF-α and/or IL-1β (Alsousi AA et al., 2017). This is in contrast to IL-1β-mediated COX-2, which may be attributed to the low expression of IL-1 type 1 receptor density in our FLS cell line. However, combined treatment with TNF-α and IL-1β at various low concentrations displayed a TNF-α-dependent dominance in inducing COX-2 expression, PGE₂ production and enhancing the MMP-9 activity.

Besides, our results affirm that TLR4 stimulation mediates RATM-induced inflammatory phenotype. Thus, RATM with the potential to initiate oxidant cellular stress can propagate and maintain inflammatory processes in many chronic diseases. To target RATM therapeutically might have the potential to provide health benefits that ameliorate ROS-mediated inflammation.

The understanding that when HMGB1 is released extracellularly after tissue damage or injury, will promote innate and adaptive immune responses and will not maintain their
previous intracellular activities is very critical. Utilization of exHMGB1 antagonists is still somewhat an unmapped pathway. In the absence of a clinical trial using HMGB1 blocking agents, no one can predict what the actual risks and benefits of HMGB1 are in RA therapy.

Interestingly, when RATM are used as sources of oxidant stress, both the cationic part (metal) and the generated reactive species can activate TLR4. These cation-induced TLR responses may be inhibited by soluble expressed TLRs, thereby opening another new therapeutic perspective (Raghavan et al., 2012).

Design of combination therapy of anti-HMGB1-releasing agents, as well as dual–functioning antioxidants that possess both RATM chelating and oxidant-scavenging properties, could be an essential milestone in the pharmacotherapy of rheumatic diseases.

9.3. Future Directions

9.3.1. Using human cell line and in vivo transgenic animal model. Inflammation in chronic diseases such as RA is a vast field to explore, and much work is needed in order to understand the mechanisms underlying the pathology associated with RA comprehensively. The role of RATM in oxidant stress strongly suggest its role in the cellular toxicity in RA. However, our study might show as a tip of the iceberg in the field of RA. As will be explained later, the origin of any cell line used in investigating TLR4 role would be a key player in the successful translation of the study. Furthermore, a future study using human synovial fibroblasts is recommended to ensure acute translational of results and applications as well as a rabbit animal model for any future in vivo studies. Transgenic animal models are considered as an important tool to understand the pathophysiology of the disease. Such a transgenic model for TLR4 provides vital information in the current understanding of TLR4-mediated oxidant
stress in RA. Therefore, in future studies inclusion of TLR4-transgenic animals would be a fundamental approach.

9.3.2. Investigation of the nature of the interaction between RS and TLR4. In future studies, an investigation into the nature of the interaction between reactive species (radical and non-radical) generated from RATM and TLR4 is necessary. The first and most significant source that increases the expression of TLRs is PAMPs and/or DAMPs. It is imperative to reference a well-known extracellular PAMP which induces the activation of TLR4, LPS, a cell wall component of gram-negative bacteria (Takeuchi and Akira, 2001). The critical pathway activated by LPS is the myeloid differentiation primary response protein 88 (MyD88) dependent as well as the independent pathway. The standard step to both pathways is the recognition of the lipid A-region of LPS by TLR4.

LPS binds to the LPS binding protein (LBP), and the resulting complex attaches to another protein known as cluster of differentiation 14 (CD14) (Poltorak et al., 1998). CD14 serves either as a membrane-bound protein in innate immune cells or circulates in plasma in a soluble form. The primary role of CD14 is to enhance TLR4 signaling by facilitating its transport to lipid rafts in the cellular membrane. Subsequently, the co-receptor myeloid differentiation factor-2 (MD-2) is recruited to promote the translocation of TLR4 to the cell membrane (Shimazu et al., 1999). This translocation is followed by endocytosis of the TLR4/MD2 complex. The recognition of the heterotrimer CD14/TLR4/MD-2 to LPS induces the activation of the MyD88-dependent and independent pathways.

Therefore, one of the most exciting aspects would be identifying the nature of the interaction of reactive species (radical and non-radical species) with TLR4.
In this regards, activation of TLR4 can be mediated by two possible mechanisms: direct RATM-cation interaction or direct RS interaction mechanisms. Observations and data from previous reports support both.

9.3.2.1. Direct interaction between RATM oxidants cation and a peptide model of the human TLR4 ectodomain. Observations from the recently published work (Peana et al., 2017). Human toll-like receptor 4 (hTLR4) plays a crucial role in the development of nickel contact allergy (NCA). The fact that mouse and other species lacking the histidine-rich region in TLR4 plasma membrane do not develop nickel hypersensitivity reaction specifies that histidine residues are required for this inflammatory phenotype. The protein, which is located on the plasma membrane of macrophages, fibroblasts, and dendritic cells, can be directly activated by nickel ions binding to the histidine residues in the ectodomain of hTLR4. In particular, the protein region comprising the residue 429–460 is supposed to be responsible for Ni (II) coordination and the induction of hTLR4 dimerization, leading to the interaction with the co-receptor MD2 and subsequent signal transduction that trigger an allergic response, through the nuclear factor (NF)-κB, p38, and interferon regulatory factor 3 (Peana et al., 2017). This structural model of the accepted nickel binding sites in the ectodomain of hTLR4, presented two histidine His-456 and His-458 residues as a potential binding site for nickel ions because of their location at an optimal distance for metal interaction, whereas the other conserved His-431 resulted apart.

In contrast, and even though both mouse TLR4 and rabbit TLR4 lack the histidine-rich region in the TLR4 plasma membrane, they still developed an inflammatory phenotype (Karki and Igwe, 2013; Karki et al., 2014; Alsousi and Igwe, 2018; Zhang and Igwe, 2018; Alsousi and Igwe, 2019). This indicates that reactive species do not require the specific histidine region
to develop inflammatory phenotype. Moreover, SIN-1 (3-morpholinosydnonimine N-ethylacarbamide) which was used in some of these studies as an exogenous peroxynitrite (PN) donor (Zhang and Igwe, 2018), releases superoxide and nitric oxide under physiological condition and activate TLR4 without including any cation. This further unlocks and augment another debate concerning the highly possible direct interaction of reactive species with TLR4.

**9.3.2.2. Direct interaction between reactive species and peptides of the TLR4 ectodomain.** Through examining the docking binding site of TLR4 and LPS (best well-studied agonist for TLR4) (Patra et al., 2018), one could consider that a possible approach of interaction includes a direct interaction of reactive species with TLR4 receptor by oxidizing specific amino acids in TLR4 without actually sitting in the receptor leading to conformation changes. This hypothesis may be favored by the fact that reactive species have relatively small sizes compared to LPS and have a very short half-life.

Personally, I tend to concur more with the second suggestion of the direct interaction between reactive species and TLR4 as presented in the idea of ‘atomic specificity’ (Nathan and Cunningham-Bussel, 2013), where ROS reacts covalently and reversibly in cell signaling with only specific atomic element in biological macromolecules, sulfur, which is one of the least abundant atoms. In fact, ROS do not react with all sulfur atoms, but typically with sulfur atoms in the side chains of cysteine or methionine residues in proteins. The reactivity of these specific cysteinytl thiols is partly affected by neighboring amino acid side chains. The expected conformational change is presumed to be responsible for the induction of TLR4 dimerization, leading to the interaction with the co-receptor MD2 and subsequent signal transduction.

Further mutation experiments and possible crystallography could provide insights into this hypothesis.
9.3.3. To study the synergistic effects of HMGB1 and/or LPS and RATM on TLR4 activation and inflammatory cytokine production. HMGB1 loves company. Testing the effects of the combination of RATM oxidants and extracellular HMGB1 and/or LPS on synovial fibroblasts would add more for the possible synergism effects of pathogen infection and oxidative stress.
# APPENDIX

## A-1. Milestones in recognition of RA history

<table>
<thead>
<tr>
<th>Year/Milestone</th>
<th>Event / Finding</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>600 x 10^6 BCE</td>
<td>The existence of the disease was deduced from fossil studies</td>
<td>(Pemberton and Osgood, 1934)</td>
</tr>
<tr>
<td>100 x 10^6 BCE</td>
<td>A pathologic specimen of dinosaurs was identified</td>
<td>(Hormell, 1940)</td>
</tr>
<tr>
<td>15 x 10^6 BCE</td>
<td>Arthritic bones of an Egyptian crocodile and a camel of the Pliocene Era were described</td>
<td>(Hormell, 1940)</td>
</tr>
<tr>
<td>5000 BCE</td>
<td>Cases of osteoarthritis in humans were documented</td>
<td>(Department et al., 1910)</td>
</tr>
<tr>
<td>500</td>
<td>Lesions in Egyptian mummies compatible with <em>rheumatoid arthritis</em> were reported</td>
<td>(Ruffer, 1913)</td>
</tr>
<tr>
<td>1063</td>
<td>Illness of Constantine IX, described by Psellus had features of RA illness</td>
<td>(Caughey, 1974)</td>
</tr>
<tr>
<td>1466-1536</td>
<td>Appelboom and others reported an illness of Desiderius Erasmus, which had features similar to RA</td>
<td>(Appelboom et al., 1986)</td>
</tr>
<tr>
<td>1400-1700</td>
<td>Several examples of paintings by artists of the Flemish school suggest the possible presence of rheumatoid-like lesions in the European models</td>
<td>(Dequeker, 1977)</td>
</tr>
<tr>
<td>1577-1640</td>
<td>Oil paintings of Rubens suggest that <em>rheumatoid arthritis</em> may have existed a long time ago</td>
<td>(Appelboom et al., 1981)</td>
</tr>
<tr>
<td>1683</td>
<td>Thomas Sydenham gave the first recognizable description forms in the modern era of acute rheumatism (meaning rheumatic fever) and chronic arthritis</td>
<td>(Sydenham, 1734)</td>
</tr>
<tr>
<td>1703</td>
<td>The (buffy clot) which represents the increased sedimentation rate of the red blood cells was observed in rheumatoid arthritis by Musgrave</td>
<td>(Short et al., 1937)</td>
</tr>
<tr>
<td>1708</td>
<td>The Physical Dictionary described Rheumatism as a wandering pain accompanied with small fever, swelling, and inflammation.</td>
<td>(Blankaart, 1708)</td>
</tr>
<tr>
<td>1772-1840</td>
<td>The first credit for a description of RA is given to Augustin-Jacob (Landre-Beauvais) who described in his thesis the disease to be variant of gout</td>
<td>(Short, 1959; Parish, 1963)</td>
</tr>
<tr>
<td>1783-1862</td>
<td>Benjamin Brodie recognized the disease as a cause of articular cartilage deterioration</td>
<td>(Brodie, 1818)</td>
</tr>
<tr>
<td>1800</td>
<td>The first generally accepted description of RA by Lander-Beauvais as a modern disease in his doctoral dissertation where he distinguished RA from gout as a distinctive disease</td>
<td>(Landré-Beauvais, 2001)</td>
</tr>
<tr>
<td>1835-1837</td>
<td>University College hospital records documented 35 cases of rheumatism (10 males and 25 females)</td>
<td>(Comer and Scott, 1994)</td>
</tr>
<tr>
<td>Year/Milestone</td>
<td>Event/Finding</td>
<td>References</td>
</tr>
<tr>
<td>--------------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Mid-nineteenth century</td>
<td>RA was first seen in the mid-nineteenth century</td>
<td>(Comer and Scott, 1994)</td>
</tr>
<tr>
<td>1859</td>
<td>Sir Alfred Baring Garrod, a physician at West London Hospital, did not approve of the term of Heberden's &quot;chronic rheumatism&quot; or Fuller's &quot;rheumatic gout.&quot; He proposed the term rheumatoid arthritis and introduced it into the medical literature, dividing the disease into acute, chronic and irregular forms of generalized and localized types.</td>
<td>(Garrod, 1876)</td>
</tr>
<tr>
<td>1867</td>
<td>Jean-Martin Charcot pointed to RA as not a rare disease, and he made an excellent distinction between gout, rheumatic fever, rheumatoid arthritis, and osteoarthritis.</td>
<td>(Charcot and Loomis, 1881)</td>
</tr>
<tr>
<td>1873</td>
<td>Robert Adams added the description of subcutaneous and intrabursal nodules associated with RA, which again raised the debate whether RA is a variant of rheumatic fever or a distinctive disease.</td>
<td>(Adams, 1873)</td>
</tr>
<tr>
<td>1892</td>
<td>Garrod declares that gout RA had an independent pathology from gout.</td>
<td>(Garrod, 1892)</td>
</tr>
<tr>
<td>1896</td>
<td>George Still identified unrecognized features of rheumatoid arthritis in children (Juvenile rheumatoid arthritis) as Still’s disease.</td>
<td>(Still, 1897)</td>
</tr>
<tr>
<td>1907</td>
<td>The distinction between osteoarthritis and RA was made by Sir Alfred Barring’s son, Sir Archibald.</td>
<td>(Symmons, 1995)</td>
</tr>
<tr>
<td>1922</td>
<td>The term rheumatoid arthritis was adopted officially by the British Ministry of Health as the official designation.</td>
<td>(Parish, 1963)</td>
</tr>
<tr>
<td>1924</td>
<td>RA was reported to be associated with splenomegaly and leucopenia (Felty’s syndrome).</td>
<td>(Hatch, 1945; Gibberd et al., 1965)</td>
</tr>
<tr>
<td>1929</td>
<td>Russell L Cecil concluded that RA is a streptococcal infection. Which is caused in a large proportion of cases by a biologically specific strain of the organism.</td>
<td>(Cecil et al., 1929)</td>
</tr>
<tr>
<td>1931</td>
<td>Cecil and others claimed that they could agglutinate suspensions of bacteria from the serum of 94% RA patients.</td>
<td>(Cecil and Ssell, 1931)</td>
</tr>
<tr>
<td>1932</td>
<td>Martin Dawson reported that RA serum agglutinated other microorganisms than streptococci.</td>
<td>(Dawson et al., 1932)</td>
</tr>
<tr>
<td>1937 and 1940</td>
<td>Douglas Collins and Granville Bennett studies demonstrated the histological distinction between nodules of RA and nodules of rheumatic fever.</td>
<td>(Collins, 1939; Bywaters et al., 1958)</td>
</tr>
<tr>
<td>Year/Milestone</td>
<td>Event/Finding (continued)</td>
<td>References</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1940</td>
<td>Erik Waaler observations lead to the development of what is known as a rheumatoid factor when he noticed that rheumatoid sera enhance the agglutination of sensitized sheep red cells.</td>
<td>(Natvig and Tonder, 1998)</td>
</tr>
<tr>
<td>1941</td>
<td>The term <em>rheumatoid arthritis</em> was adopted officially by the American Rheumatism Association (now the American College of Rheumatology) to replace atrophic and chronic infectious arthritis.</td>
<td>(Hunder and Matteson, 2010)</td>
</tr>
<tr>
<td>1963</td>
<td>Parish proposed a historical approach to RA nomenclature.</td>
<td>(Parish, 1963)</td>
</tr>
<tr>
<td>The early 1970s</td>
<td>RA and rheumatoid spondylitis were distinguished as separate diseases.</td>
<td>(Weisman, 2014)</td>
</tr>
<tr>
<td>The late 1970s and 1980s</td>
<td>The possible role of genetic factors was introduced. HLA-DRB1 was presented to be associated with the disease onset and severity</td>
<td>(Deighton <em>et al.</em>, 1989; Wordsworth <em>et al.</em>, 1989)</td>
</tr>
<tr>
<td>2000</td>
<td>Identification of RA factors, antibodies, and allelic polymorphism provided a base for gene-environment research.</td>
<td>(Weisman, 2014)</td>
</tr>
</tbody>
</table>

**A-2. List of examples of drugs used to treat RA**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mechanism</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NSAIDs</strong></td>
<td>Anti-inflammatory</td>
<td>Heart problems, liver and kidney damage</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naproxen</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Steroids</strong></td>
<td>Anti-inflammatory and pain relief</td>
<td>Diabetes, weight gain, and increased risk of viral or bacterial infections</td>
</tr>
<tr>
<td>Prednisone</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DMARDs</strong></td>
<td>Slow the progression of RA</td>
<td>Teratogenesis, liver damage</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Inhibit dihydrofolate reductase</td>
<td></td>
</tr>
<tr>
<td>Leflunomide</td>
<td>Inhibit pyrimidine synthesis</td>
<td>Teratogenesis, liver damage</td>
</tr>
<tr>
<td>Hydroxychloroquine</td>
<td>Block TLRs</td>
<td>Rare ocular toxicity</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>Folate depletion</td>
<td>Anemia in G6PD deficiency</td>
</tr>
<tr>
<td><strong>Biologic agents</strong></td>
<td>CTLA4 blocker</td>
<td>Opportunistic infections</td>
</tr>
<tr>
<td>Abatacept</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adalimumab, Certolizumab</td>
<td>TNF blocker</td>
<td></td>
</tr>
<tr>
<td>Anakinra</td>
<td>IL-1α and IL-1β</td>
<td></td>
</tr>
<tr>
<td>Etanercept</td>
<td>TNF inhibitor</td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>Anti-human TNF antibody</td>
<td></td>
</tr>
<tr>
<td>Rituximab</td>
<td>Anti-CD20</td>
<td></td>
</tr>
<tr>
<td>Tocilizumab</td>
<td>Anti-IL-6 antibody</td>
<td></td>
</tr>
<tr>
<td>Tofacitinib</td>
<td>JAK1/3-STAT inhibitor</td>
<td></td>
</tr>
</tbody>
</table>
### A- 3. Different reactive oxygen species (ROS) and their properties

<table>
<thead>
<tr>
<th>ROS</th>
<th>Nature and properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide anion (O$_2$$^•$)</td>
<td>A primary reduced form of molecular oxygen. Mild oxidant and reductant with limited biological activity and limited membrane permeability. Less reactive than OH$^•$ undergoes dismutation to form H$_2$O$_2$ spontaneously or by enzymatic catalysis. Half-life is $10^{-6}$ sec</td>
</tr>
<tr>
<td>Hydroxyl radical (OH$^•$)</td>
<td>It is considered the most reactive ROS. Extremely reactive with most cellular components; causes DNA and protein modifications, and lipid peroxidation. Half-life is $10^{-9}$ to $10^{-10}$ sec</td>
</tr>
<tr>
<td>Hydroperoxyl (HO$_2$$^•$)</td>
<td>The simplest reactive peroxyl radical. It is the protonated form of superoxide. About 0.3% of any superoxide present in the cytosol of a typical cell is in the protonated form.</td>
</tr>
<tr>
<td>Peroxyl, RO$_2$$^•$ (ROO$^•$)</td>
<td>It can react with each other or abstract H$^•$ from molecules and initiates fatty acid peroxidation and can promote tumor development. Most peroxides are stable at room temperature, but they can decompose by heating or exposure to UV or by addition of metal ions such as iron. Half-life is 17 sec</td>
</tr>
<tr>
<td>Alkoxyl, RO$^•$</td>
<td>Oxygen centered radicals formed during the breakdown of organic peroxides. It can induce damage to DNA. Half-life is $10^{-6}$ sec</td>
</tr>
<tr>
<td>Carbonate CO$_3$$^•$</td>
<td>Carbonate radicals are powerful one-electron oxidizing agents and oxidize a range of biomolecules such as hyaluronic acid.</td>
</tr>
<tr>
<td>Carbon dioxide CO$_2$$^•$</td>
<td>A strong one-electron reductant, commonly used in aqueous radiation chemistry as a reducing agent for metalloproteins or as an intermediate in the formation of the superoxide anion.</td>
</tr>
<tr>
<td>Singlet oxygen (O$_2$ $^{1\Sigma}$ g $^+$)</td>
<td>This state rapidly decays to the $^1$O$_2$ $\Delta$g state, therefore only $\Delta$g is considered in biological systems. Compared to other reactive species, singlet oxygen is mild and nontoxic for mammalian tissue</td>
</tr>
<tr>
<td>Hydrogen peroxide (H$_2$O$_2$)</td>
<td>A classic example of ROS and the least reactive molecule. A weak oxidizing, reducing agent, and lipid soluble (membrane permeable); reacts slowly with reducing agents such as thiols to initiate radical reactions and lipid peroxidation. Half-life: stable</td>
</tr>
<tr>
<td>Organic peroxides (R-OOH)</td>
<td>Decomposition of alkyl peroxides results in peroxyl and alkoxyl radicals, which are good oxidizing agents. Half-life: stable</td>
</tr>
<tr>
<td><strong>ROS</strong></td>
<td><strong>Nature and properties (continued)</strong></td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>Singlet oxygen ($^1$O$_2$ ∆g)</td>
<td>An electronically high-excited non-radical state of oxygen. Highly reactive and a potent oxidizing agent that reacts with biological molecules to cause DNA and tissue damage. It is involved in cholesterol oxidation. Half-life is 10$^{-6}$ sec</td>
</tr>
<tr>
<td>Hypobromous acid (HOBr)</td>
<td>A weak acid, but with high reactivity and ability to damage biomolecules, both directly and by decomposing to form bromine. Half-life: stable (min)</td>
</tr>
<tr>
<td>Hypochlorous acid (HOCl)</td>
<td>Highly reactive and powerful oxidizing agent. It can damage biomolecules both directly and by decomposing to form chlorine. Half-life: stable (min)</td>
</tr>
<tr>
<td>Peroxynitrite (ONOO$^-$)</td>
<td>The potent oxidant is likely produced <em>in vivo</em> by rapid reaction between two relatively unreactive but biologically ubiquitous free radicals, nitric oxide and superoxide anion</td>
</tr>
<tr>
<td>Ozone (O$_3$)</td>
<td>An irritating, non-free radical triatomic blue gas and poorly soluble in water. It is not generated <em>in vivo</em>, but it is a powerful oxidizing agent. It can form free radicals. It adds directly across double bonds in lipids to generate ozonides and oxidizes proteins. Half-life: seconds</td>
</tr>
</tbody>
</table>
## A- 4. Different reactive nitrogen species (RNS) and their properties

<table>
<thead>
<tr>
<th>Free Radicals</th>
<th>RNS</th>
<th>Nature and properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitric oxide or Nitrogen Monoxide (NO(^{+}))</td>
<td>Formed in a rapid reaction between (\cdot O^2^-) and NO; lipid soluble; potent oxidizing agent causing direct protein nitration, DNA fragmentation, and lipid oxidation. Much of the (NO(^+)) generated \textit{in vivo} is removed by interaction with hemoglobin. Half-life: sec or min (depending on the environmental medium).</td>
<td></td>
</tr>
<tr>
<td>Nitrogen dioxide (NO(_2^+)), Nitric dioxide</td>
<td>A gaseous oxidizing radical formed by peroxyl and NO reaction. It is unreactive towards saturated aliphatic compounds but adds to double bonds and oxidizes ascorbic acid. Half-life: seconds</td>
<td></td>
</tr>
<tr>
<td>Nitrite and Nitrate (NO(_3^-))</td>
<td>Oxidation of NO(^+) leads to the formation of nitrite. Nitrite is then converted into nitrate by reacting with oxyhemoglobin in whole blood.</td>
<td></td>
</tr>
<tr>
<td>Nitrous acid (HNO(_2))</td>
<td>Atmospheric Nitrous acid is generated from heterogeneous reactions of nitrogen dioxide and water on ground surfaces such as soil, walls of buildings, and airborne particle surfaces. Photolysis of HNO(_2) gas is a source of hydroxyl radical. High concentrations of HNO(_2) may be associated with decreased lung functions and some respiratory symptoms. HNO(_2) is often presumed to cause base substitutions in organisms with double-stranded DNA as a direct consequence of oxidative deamination of adenine and cytosine residues. Half-life: seconds</td>
<td></td>
</tr>
<tr>
<td>Nitrosyl cation (NO(^+))</td>
<td>Strong oxidizing agent. Half-life: seconds</td>
<td></td>
</tr>
<tr>
<td>Nitroxyl anion (NO(^-))</td>
<td>Nitroxyl anion (NO(^-)) may be a natural co-product of NO synthase. NO(^-) exerts general signaling functions alongside NO(^+) in the cardiovascular and autonomic nervous systems. Half-life: seconds</td>
<td></td>
</tr>
<tr>
<td>Dinitrogen tetroxide, N(_2)O(_4)</td>
<td>A strong oxidizing agent, water-reactive and air-reactive. Half-life: seconds</td>
<td></td>
</tr>
<tr>
<td>Dinitrogen trioxide, N(_2)O(_3)</td>
<td>Strong irritant to skin, eyes and mucous membranes. It is the anhydride of the unstable nitrous acid and produces it when mixed water. Dinitrogen trioxide promotes nitrosative deamination of purines and pyrimidines as well as thiol-containing peptides and amines. Half-life: seconds</td>
<td></td>
</tr>
<tr>
<td>Peroxynitrite (ONOO(^-)), officially called oxoperoxonitrate</td>
<td>Cytotoxic and potent oxidizing, hydroxylating and nitrating molecule. Lipid soluble. It oxidizes and nitrates DNA resulting in DNA strand breaks. At physiological pH, any remaining ONOO(^-) protonates and rearranges to form nitrate plus NO(_2^+). Gaseous, unreactive, with greater stability, readily diffuses through the plasma membranes and has an effect</td>
<td></td>
</tr>
<tr>
<td>RNS</td>
<td>Nature and properties (continued)</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>On neuronal transmission as well as synaptic plasticity. Half-life: $10^3$ second</td>
<td></td>
</tr>
<tr>
<td>Peroxynitrous acid (ONOOH)</td>
<td>Very unstable. At physiological pH decomposes to yield nitrate (NO$_3^-$) which react with a broad range of biological substrates including nitration of tyrosine residues in proteins, oxidation of DNA, lipids, sulfhydryls, and methionine. Half-life: Fairly stable</td>
<td></td>
</tr>
<tr>
<td>Nitronium (nitryl) cation (NO$_2^+$)</td>
<td>It is stable enough to exist in normal conditions, but it is generally reactive and used as an electrophile in the nitration of other substances.</td>
<td></td>
</tr>
<tr>
<td>Alkyl peroxynitrites (ROONO)</td>
<td>They form by the reaction of NO$_2^-$, which is present in cigarette smoke, with organic peroxyl radicals.</td>
<td></td>
</tr>
<tr>
<td>Nitryl (nitronium) chloride (NO$_2$Cl)</td>
<td>Nitryl chloride is a member of the Halogen oxides. Half-life: seconds</td>
<td></td>
</tr>
<tr>
<td>Peroxyacetyl nitrate (PAN), CH$_3$C(O)OON$_2$</td>
<td>It is an indicator of photochemical smog. PAN can play a pivotal role as a reservoir of nitrogen dioxide (NO$_2$) which affects the production of tropospheric ozone (O$_3$) in relatively remote areas.</td>
<td></td>
</tr>
</tbody>
</table>
A-5. Different reactive chlorine species (RCS) and their properties

<table>
<thead>
<tr>
<th>RCS</th>
<th>Nature and properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Free Radicals</strong></td>
<td></td>
</tr>
<tr>
<td>Atomic chlorine (Cl(^{•}))</td>
<td>It is formed in the upper atmosphere by several mechanisms. Their significance <em>in vivo</em> is uncertain.</td>
</tr>
<tr>
<td>Hypochlorous acid (HOCl)</td>
<td>It is not a free radical, but it is a powerful oxidizing and chlorinating agent. Its formation at sites of inflammation by neutrophils leads to an attack of primary amines and sulphydryl groups in proteins and to chlorinate purine bases in DNA.</td>
</tr>
<tr>
<td>Nitryl (nitronium) chloride (NO(_{2})Cl)</td>
<td>Nitryl chloride is considered to be soluble (in water) and basics.</td>
</tr>
<tr>
<td>Chloramines</td>
<td>They are long-lived oxidants but are generally less toxic than (HOCl).</td>
</tr>
<tr>
<td>Chlorine gas (Cl(_{2}))</td>
<td>Chlorine gas is reactive; it is not expected to remain in the environment very long after it is released. Cl(<em>{2}) undergoes direct photolysis in the air. Cl(</em>{2}) gas rapidly hydrolyzes to hypochlorous acid. Chlorine (hypochlorite) is a strong base. Half-life: several min</td>
</tr>
</tbody>
</table>
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VITA

Asmaa A. Alsousi graduated from Al-Azhar University, School of Pharmacy-Gaza, Palestine with Excellence and the first degree of Honor in 1999. During her Bachelor degree in Pharmacy, she was awarded the Excellence Scholarship in 1995, 1996, 1998 and 1999. After graduating from Al-Azhar University, she worked as a Teaching Assistant and Laboratory Instructor from 1999 till 2005 at Al-Azhar University-Palestine. During her work at the School of Pharmacy, she was awarded Excellence in Teaching Award Certificate, Al-Azhar University-Palestine, 2005. She earned a Master of Science degree in Pharmaceutical Sciences in 2008, then worked as Lecturer till 2010 in the Pharmacognosy and Phytochemistry Department. In 2012, Alsousi joined the interdisciplinary Ph.D. (iPh.D) program at the University of Missouri-Kansas City (UMKC) with Pharmacology/Toxicology as a primary discipline and Pharmaceutical Sciences as a co-discipline. She finished both her iPh.D. related courses and joined the laboratory of Dr. Igwe in fall 2013.

During her interdisciplinary Ph.D. study, Asma was awarded a 0.25 teaching assistant (TA) from 2015 to 2018. From January 2015 to August 31, 2018, she worked as a student assistant in the Health Sciences Library (HSL) at the University of Missouri-Kansas City. Alsousi was awarded UMKC Women’s Council Graduate Assistant Fund (GAF) in 2013, 2015, 2016, 2017 and 2018. Her GAF award in 2017 was with Outstanding Merit. She was awarded the School of Graduate Studies travel grant in 2015 and 2016. She was awarded the School of Graduate Studies (SGS) Community of Scholars Symposium best Poster Award in Biological and Chemical Sciences Discipline (2016). Alsousi is a member of Golden Key International Honor Society (2013), University of Missouri-Kansas City Honor Society (2014), University of Missouri -Kansas City Phi Kappa Phi Honor Society (2014), and Rho Chi member of Honor Society, University of Missouri-Kansas City (2015). Asmaa also served as
an elected representative for Hazardous Materials Oversight Committee (2014) and the elected representative for Graduate Program Committee (GPC), University of Missouri-Kansas City (2017). Asmaa has presented her iPh.D. research work at the annual meetings of Society for Neurosciences (2015, 2016) and the 48th Annual meeting of Pharmaceutical Graduate Research Meeting (PGSRM) (2016). Asmaa has served as the Graduate Student Council (GSC) President-Elect, University of Missouri-Kansas City (2017)- where she chaired the preparation for the Community of Scholars (CoS) symposium, the Graduate Student Council (GSC) President, University of Missouri-Kansas City (2018), and as the graduate student representative for the Associated Students of University of Missouri (ASUM) (2018).