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Effect of bioactive particulates and lipopolysaccharide on immune regulation via Class A scavenger receptor and Toll – like receptor 4

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2005

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Antigen presenting cells (APC) express a wide range of surface pattern recognition receptors (PRR) that involve in pathogen recognition, phagocytosis, antigen presentation, and induction of adaptive immunity. It is known that vaccine adjuvants may act as conserved microbial ligands, and function via initiation and manipulation of innate immune responses through certain PRRs. Meanwhile, signaling pathways mediated via various PRRs may contribute differently to the pathogenicity of exposure to microbial ligands and bioactive particulates.

Class A scavenger receptor (SR-A) and Toll – like receptor 4 (TLR-4) are two types of PRRs expressed on the cell surface of APCs. The present study utilized human and murine macrophage cell lines to investigate signaling mechanism and potential ligands, including lipopolysaccharide (LPS), silica, and airborne particulate matter PM1648, for the two PRRs. Biological potency of synthetic lipid A compounds was evaluated with an in vitro human cell assay system, and the length of compound secondary fatty acyl chains was found to play a critical role in compound immunological potency. With biological inhibition assays using monoclonal antibodies, we demonstrated that lipid A compounds were signaling through TLR-4. With direct binding assay and binding inhibition studies, we indicated a two – step binding mechanism of LPS to TLR-4 receptor. A possible cross – reactivity between TLR-4 and Fc receptor CD64 was also investigated.

It is clear that PRRs may function individually or cooperatively to direct adaptive immune responses following innate ligand recognition. Therefore, the possible cooperative mechanism between TLR-4 and SR-A for up-regulating murine APC activity in response to LPS, silica, and PM1648 was investigated. Using blocking antibodies and receptor deficient murine macrophages, we demonstrated that silica uses both TLR-4 and SR-A for signaling in order to activate macrophages efficiently, while LPS and PM1648 use TLR-4 for macrophage activation. APCs stimulated with silica sent signals other than soluble cytokines to T cells for activating host immune responses. SR-A appeared to be involved in negative regulation of cell activation by LPS and PM1648, as well as in mediating endotoxin and particulate direct binding and clearance.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><strong>Overview of Immune Response, Vaccination, and Immune Cell Surface Receptors</strong>&lt;br&gt;1. Innate Immunity and Adaptive Immunity&lt;br&gt;2. Overview of Importance and Application of Vaccination and Adjuvant&lt;br&gt;3. Lipopolysaccharide (LPS), Silica, and Airborne Particulate Matter (PM)&lt;br&gt;3.1. Lipopolysaccharide (LPS)&lt;br&gt;3.2. Silica&lt;br&gt;3.3. Airborne Particulate Matter (PM)&lt;br&gt;4. Surface Receptors on Antigen Presenting Cells (APC)&lt;br&gt;4.1. Toll-like Receptors (TLR)&lt;br&gt;4.2. Scavenger Receptors&lt;br&gt;Research Objectives</td>
<td>1&lt;br&gt;1&lt;br&gt;2&lt;br&gt;4&lt;br&gt;5&lt;br&gt;6&lt;br&gt;9&lt;br&gt;13&lt;br&gt;13&lt;br&gt;18&lt;br&gt;25</td>
</tr>
<tr>
<td>II</td>
<td><strong>Biological Potency Evaluation of Synthetic Lipid A Compounds with <em>in vitro</em> Human Cell Assay System</strong></td>
<td>28&lt;br&gt;28&lt;br&gt;35&lt;br&gt;40&lt;br&gt;61</td>
</tr>
<tr>
<td>III</td>
<td><strong>Characterization of Specific Involvement of Toll-like Receptor 4 in Signaling Pathways for LPS and Synthetic Lipid A Compounds</strong></td>
<td>70&lt;br&gt;70&lt;br&gt;75&lt;br&gt;82&lt;br&gt;99</td>
</tr>
<tr>
<td>IV</td>
<td><strong>The Role of Scavenger Receptor and Toll-like Receptor 4 in Murine APC Activation by Bioactive Particulates and Lipopolysaccharide</strong></td>
<td>106&lt;br&gt;106&lt;br&gt;110&lt;br&gt;119&lt;br&gt;143</td>
</tr>
<tr>
<td>V</td>
<td><strong>Conclusions and Future Directions</strong></td>
<td>153</td>
</tr>
<tr>
<td></td>
<td><strong>Literature Cited</strong></td>
<td>164</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>3-1</td>
<td>Surface expression (MFI values) of CD16, CD32, CD64, and TLR-4 upon MonoMac-6 cells</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>3-2</td>
<td>Effect of HTA125 on surface expression levels of CD64 and CD32 upon MonoMac-6 cells</td>
<td>94</td>
</tr>
</tbody>
</table>

iv

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## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1-1</td>
<td>Lipopolysaccharide (LPS) from <em>Salmonella Minnesota</em> R595</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>Crystalline and amorphous forms of silica</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>1-3</td>
<td>Mechanisms of silica pathogenicity</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>1-4</td>
<td>Functions of Toll-like receptors (TLRs) in initiation and regulation of adaptive immunity</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>1-5</td>
<td>Structural comparison between Toll-like receptor (TLR) family and Interleukin – 1 receptor (IL-1R) family</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>1-6</td>
<td>Specificities of pathogen-associated ligands for members of TLR family</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>1-7</td>
<td>Different groups of scavenger receptors that have been identified to be involved in recognition of specific microbial ligands</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>1-8</td>
<td>Biological functions identified for macrophage scavenger receptors</td>
<td>22</td>
</tr>
<tr>
<td>II</td>
<td>2-1</td>
<td>Structure of the major hexa-acyl component of monophosphoryl lipid A</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2-2</td>
<td>General structure of aminoalkyl glucosaminide 4-phosphates (AGPs)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td>Chemical structures of AGP compounds: RC-522, RC-524, RC-527, RC-529, RC-545, and RC-590</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>2-4</td>
<td>Concentrations of PMA for cell pretreatment</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>2-5</td>
<td>Time periods for PMA pretreatment (MonoMac-6 cells)</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>2-6</td>
<td>Time periods for PMA pretreatment (MonoMac-6 cells, continued)</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>2-7</td>
<td>Time periods for PMA pretreatment (U937 cells)</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>2-8</td>
<td>Time periods for cell stimulation (MonoMac-6 cells)</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>2-9</td>
<td>Time periods for cell stimulation (U937 cells)</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>2-10</td>
<td>Cytokine secretion from activated MonoMac-6 cells</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>2-11</td>
<td>Cytokine secretion from activated U937 cells</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>2-12</td>
<td>MonoMac-6 cell activation with LPS, <em>E. coli</em> DNA, PHA, and MPL stimulation</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>2-13</td>
<td>Dose response curves obtained from MonoMac-6 and U937 cells stimulated with AF or 0.2% TEOA-formulated AGPs</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>2-14</td>
<td>Potency evaluation of AF formulated AGPs</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>2-15</td>
<td>Potency evaluation of AGPs in 0.2% TEOA formulation</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>2-16</td>
<td>Effect of PMA co-stimulation with LPS and AGPs on cytokine secretion</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>2-17</td>
<td>Comparison of biological activities between FITC conjugated LPS and unlabeled LPS from <em>E. coli</em> serotype 0111:B4</td>
<td>60</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Chapter</th>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>3-1</td>
<td>Surface expression of TLR-4 receptor by MonoMac-6 cells</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>3-2</td>
<td>A typical direct binding curve of PE-HTA125 with human MonoMac-6 cell line</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>3-3</td>
<td>Histograms illustrating percentage of inhibition generated by HTA125 and mouse IgG2a isotype from MonoMac-6 cells stimulated with LPS, MPL adjuvant, and AGP compounds RC-527 and RC-529</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>3-4</td>
<td>Direct binding of Alexa Fluor 488 conjugated LPS with MonoMac-6 cells</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>3-5</td>
<td>Line graph illustrating typical direct binding curve of Alexa Fluor 488 conjugated LPS with human MonoMac-6 cell line</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>3-6</td>
<td>Histograms illustrating percentage of inhibition generated by unlabeled LPS or HTA125 from MonoMac-6 cells stained with Alexa Fluor 488 conjugated LPS</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>3-7</td>
<td>Line graph illustrating inhibition of HTA125 direct binding to MonoMac-6 cells by mouse isotypes and monoclonal mouse anti-human IgG FcR</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>3-8</td>
<td>FACS analysis of CD16, CD32, and CD64 surface expression on MonoMac-6 cells</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>3-9</td>
<td>Line graph illustrating inhibition of HTA125 direct binding to MonoMac-6 cells by human immunoglobulin</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>3-10</td>
<td>FACS analysis of CD14 surface expression on MonoMac-6 cells</td>
<td>98</td>
</tr>
<tr>
<td>IV</td>
<td>4-1</td>
<td>Flow cytometry analysis of TLR-4 and SR-A expression on mouse bone marrow derived macrophages</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>4-2</td>
<td>IL-6 dose response curves obtained from mouse bone marrow derived macrophages (Balb/c W/T) stimulated with LPS or PM1648</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>4-3</td>
<td>IL-12 p40 dose response curves obtained from mouse bone marrow derived macrophages (Balb/c W/T) stimulated with LPS or PM1648</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>4-4</td>
<td>Histograms illustrating blocking effect of MTS510 on cell activation by LPS and PM1648 stimulation</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>4-5</td>
<td>Histograms illustrating no effect of 2F8 on cell activation by LPS and PM1648 stimulation</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>4-6</td>
<td>Line graph illustrating IL-6 release from TLR-4 $^{-/-}$ mouse BMDM cells stimulated with LPS or PM1648</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>4-7</td>
<td>Histograms illustrating IL-6 production from wild type and SR-A$^{-/-}$ mouse BMDM cells stimulated with LPS or PM1648</td>
<td>130</td>
</tr>
<tr>
<td>Chapter</td>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>IV</td>
<td>4-8</td>
<td>Histograms illustrating percentage of Th1 cytokine increase / decrease from BMDM cells exposed to silica, PM1648, or LPS</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>4-9</td>
<td>Histograms illustrating percentage of Th2 cytokine increase / decrease from BMDM cells exposed to silica, PM1648, or LPS</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>4-10</td>
<td>Histograms illustrating percent inhibition of IL-13 secretion from BMDM cells exposed to LPS</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>4-11</td>
<td>Histograms illustrating percent inhibition of IL-13 secretion from BMDM cells exposed to PM1648</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>4-12</td>
<td>Histograms illustrating percentage of inhibition obtained from cell pretreatment with MTS510, 2F8, or with antibody combination</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>4-13</td>
<td>Histograms illustrating IL-13 production from wild-type and TLR-4⁻/- mouse BMDM cells stimulated with LPS, silica, or PM1648</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>4-14</td>
<td>Histograms illustrating T cell cytokine production from wild type and SR-A⁻/- mouse BMDM cells stimulated with LPS, silica, or PM1648</td>
<td>142</td>
</tr>
</tbody>
</table>
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CHAPTER 1

Overview of Immune Response, Vaccination, and Immune Cell Surface Receptors

Innate Immunity and Adaptive Immunity

Immunologists have been trying for decades to solve the mystery of how host organisms detect and dispose the infectious agents without harming self tissues. Up to date, several distinct immune-recognition systems in vertebrate animals have been identified to help partially unfold the mystery. These systems fall into two broad categories: the “innate immunity” and “adaptive immunity”.

The innate immune system serves as the first line of host defense that detects the presence and the nature of an infection, initiates and defines the cell activating signals, hence directs the adaptive immune responses. The adaptive immune system, on the other hand, functions through the generation of random and diverse antigen-specific receptors, including both T cell – and B cell – receptors (TCR and BCR), via the mechanism of “clonal selection and expansion”. Furthermore, this mechanism also leads to the generation of immunological memory, which is essential for the long-term immune protection and for a successful vaccination.

The specialized phagocytes of immune system, including both macrophages and dendritic cells (DC), are responsible for detection and clearance of infectious agents and infected host cells, as well as for initiation and connection between innate and adaptive immune responses. They are involved in initial capture and processing of potential antigens (innate immunity), as well as in antigen presentation and activation of antigen-specific T and B lymphocytes (adaptive immunity). In addition to the efficient endocytic
and phagocytic activities, these phagocytes also function as antigen-presenting cells (APC) that are able to induce and regulate local and systemic immune responses by acting as potent secretory cells which release inflammatory cytokines and chemokines.

**Overview of Importance and Application of Vaccination and Adjuvant**

Attacks using biological or chemical agents to poison or infect humans and animals have been reported since the 14th century. Today, it still remains as a serious threat to the world. One recent event (fall of 2001) involved letters filled with *B. anthracis* spores that were mailed to government leaders in the U.S indicating that individuals or groups are still actively engaged in obtaining and developing biological agents for terrorism use in this country. Therefore, development of vaccines, antisera, and therapeutic agents against a wide range of organisms, toxins, and viruses has received a great deal of attention in the research field due to the heightened concerns about bioterrorism and the use of biowarfare agents.

In the meantime, effective vaccination provides significant benefits to human health. Vaccines have been well recognized as one of the most effective and widely used pharmaceuticals. The contribution of vaccination to smallpox eradication is a good example. Today in the United States, vaccines that can provide effective protection against eleven diseases, including polio, measles, whooping cough, diphtheria, and hepatitis types A and B, etc, are recommended for use. Data of statistical analysis indicated that the annual morbidity resulting from the above diseases has decreased by more than 95% for each indication, with the widespread use of these vaccines \(^{(31)}\).
Moreover, such great results may still be significantly improved by the combinational use of effective adjuvants.

Adjuvants are being developed and utilized to enhance vaccine-induced protective immunity by increasing the immunogenicity of certain vaccines, enhancing responses in low responder populations, such as the elderly and the immunosuppressed, and modulating inappropriate immune responses. The combinational use of vaccine and adjuvant can also reduce the amount of antigen required for protective levels of immunity, therefore provides an economic benefit as well. Research data indicated that adjuvant activity could be mediated through a number of mechanisms, such as providing a general inflammatory stimulus, activating antigen presenting cells (macrophages and dendritic cells) and lymphocytes, increasing phagocytosis activity, controlling the speed of antigen release, optimizing cytotoxic T cell responses by facilitating MHC-I presentation, and enhancing long-term immunological memory, etc.

Up to date, only two types of adjuvants are officially approved and licensed for clinical use in human: aluminium salts and MF-59 as part of an inactive flu vaccine. Some other products are in the late stage of clinical testing, such as monophosphoryl lipid A (MPL) and the saponin derivative QS-21. Many more potential products are in clinical development or pre-clinical testing stage, including *Mycobacterium vaccae*, cytokine/growth factors, CpG motifs, syntex adjuvant formulation/2 (SAF/2), muramyl tripeptide (MTP) and liposomes.

It has been well understood that the immune cells and cytokines of the innate immune system play an essential role in the long-term, protective immunity induced by vaccination. Various immune cells of innate immune system express pattern recognition
receptors (PRR) on cell surface, such as Toll-like receptors (TLRs) and scavenger receptors (SRs), that usually function by direct binding to specific and highly conserved microbial components. With cell surface PRRs, the host innate immune system can detect microbial infections or vaccines containing highly conserved microbial ligands, and activate antigen-presenting cells (APCs), resulting in phagocytosis, antigen presentation, and the secretion of inflammatory cytokine and chemokines. Therefore, naïve T- and B-lymphocytes of the adaptive immune system may be stimulated, and antigen-specific T cell receptors (TCR) and B cell receptors (BCR) may be generated by clonal selection and expansion. Meanwhile, cytokines and chemokines may continue directing the immune response against the microbes by guiding the antigen-specific lymphocyte maturation, leading to a long-term, antigen-specific adaptive immunity, which is essential for a successful vaccination. This mechanism indicates that certain adjuvant candidates may be able to enhance vaccine-induced protective immunity by mimicking or acting as conserved microbial ligands, and triggering innate immune responses through certain cell surface receptors (PRRs).

**Lipopolysaccharide (LPS), Silica, and Airborne Particulate Matter (PM)**

Available research results indicated that lipopolysaccharide and its active lipid A component, as well as certain bioactive particulates, including silica and PM, can effectively activate the innate immune system and lead to inflammation. Therefore, they all possess a potential adjuvant effect.
I. Lipopolysaccharide (LPS)

LPS is the endotoxin that is localized on the surface of bacterial cells, and together with phospholipids and proteins, forms the outer membrane of Gram-negative bacteria. LPS can produce adverse physiological reactions, including circulatory and metabolic failures, and often lethal shock in animals. These effects have been attributed largely to the release of several chemical mediators such as interleukins, tumor necrosis factor (TNF), and eicosanoids from activated macrophages. It is also well known that LPS is able to activate the complement cascade and prime polymorphonuclear leukocytes or monocytic macrophages to release these various mediators. The in vitro culture of polymorphonuclear leukocytes or peritoneal macrophages with LPS promotes an enhanced respiratory burst and release of eicosanoids: events considered important in the defense against bacterial infections as well as in the progress of tissue damage.

LPS is composed of three distinct structural parts: 1) O-antigenic repeating polysaccharide, 2) core-oligosaccharide, and 3) a lipophilic component designated lipid A (Figure 1-1). There are considerable variations in the amounts of polysaccharide in LPS so that some LPS consists predominantly of lipid A and is so called “R-form” LPS, whereas others have abundant polysaccharide (the O-side chain) therefore is called “S-form” LPS. Among the three structural parts of LPS, lipid A has been well known as a potent stimulator of host immune system, therefore it has the potential to act as an adjuvant for vaccine antigens. Meanwhile, due to its immunostimulating capability, lipid A may also provide general protections against infections by functioning as a strong inducer of non-specific resistance (NSR).
However, even though the lipid A component of LPS is so well known to be responsible for the endotoxic activities, the polysaccharide moiety influences the elimination kinetics, mediates specific LPS binding to macrophages and lymphocytes, and is essential for activation of the alternate pathways of the complement system.

Figure 1-1. Lipopolysaccharide (LPS) from *Salmonella Minnesota* R595. Adapted from Reference (31).

II. Silica

"Silica" is the general name given to a variety of substances that are primarily made up of two elements: silicon and oxygen. The chemical formula used for silica is SiO$_2$. Under natural conditions, these oxygen and silicon atoms bond together to form a large macromolecular structure, and the fundamental unit of this structure is the silicon centered tetrahedron SiO$_4^{4-}$. These fundamental
units are connected through the oxygen atoms that are shared between the two neighboring tetrahedral. Therefore, silica does not exist as a discrete molecule.

There are two natural forms of Silica: the crystalline form and the amorphous form (Figure 1 - 2). When found in the crystalline forms, the tetrahedra fundamental units are arranged in an orderly sequences which results in a repeatable pattern. However, in the amorphous forms, these units are randomly oriented so that no structural periodicity can be observed \(^{(35)}\).

![Diagram of Silica units](image)

**Figure 1 - 2.** Crystalline and amorphous forms of silica. Adapted from Reference \(^{(35)}\).

The pathogenic effect of exposure to crystalline silica on lung disease has been known since the 18\(^{th}\) century. Among the many diseases associated with silica inhalation, chronic and acute silicosis are probably the best known. However, after decades of research work, scientists have learned that there are many other different physical disorders associated with silica exposure. Some
typical examples include progressive systemic sclerosis, systemic lupus erythematosus (SLE), rheumatoid arthritis, dermatomyositis, glomerulonephritis (GN) and vasculitis.

Available studies indicated that common mechanisms mediated the many different pathogenic responses to silica exposure. Among the involved mechanisms, the interaction of silica particles with the immune system has been known to play an important role in silica pathogenicity, especially the activation of alveolar macrophages by silica stimulation (Figure 1 - 3). Once inhaled and contacted with cell membrane, silica particles can be engulfed by macrophages. After that, two different pathways may follow: the “successful” pathway or the “unsuccessful” pathway. When the engulfed particle is successfully cleared and disposed from the lung without causing cell death, the clearance process follows the “successful” path. On the other hand, if the particles activate the macrophages and eventually cause cell death, the whole process will then follow the "unsuccessful" path. In this case the particles will be released from the dead cells and deposited in the lung. In the meantime, cytokines, chemokines and oxidants (ROS, RNS) will be released into the medium as well, sending signals to target cells and initiating an ingestion-reingestion cycle, resulting in the recruitment of more macrophages and polymorphonuclear (PMN) cells to the site. The establishment of such a prolonged cell recruitment will cause inflammation, which becomes a critical step for silica - associated diseases (Figure 1-3).

In addition, proteins or phospholipids adsorbed onto the particle surface may be denatured and rendered antigenic properties due to the structural changes.
Thus, free particle inhalation may produce novel antigens, resulting in possible autoimmune responses (Figure. 1 - 3). Furthermore, silica has also been found to possess adjuvant effects. Silica inhalation may induce immune dysfunctions in several ways, such as depressing phagocytic capacities of the reticuloendothelial system and increasing polyclonal antibody synthesis, especially IgA and IgG (18, 21, 35).

![Mechanisms of silica pathogenicity: the activation of alveolar macrophages accounts for the many pathogenic responses induced by silica exposure. Adapted from Reference 35.]

**Figure 1 - 3.** Mechanisms of silica pathogenicity: the activation of alveolar macrophages accounts for the many pathogenic responses induced by silica exposure. Adapted from Reference 35.

### III. Airborne Particulate Matter (PM)

The general term “particulate matter” (PM) refers to a mixture of different types of particles in ambient air, with different sizes and chemical composition, and originating from many different sources. The concept of “PM” has been commonly applied in health-related research fields. In urban air, significant variations of particle size can be frequently observed, including the tiniest particles sized in nanometers, others equivalent to molecular clusters, and some...
very large particles, such as pollen grains and windblown sand. Therefore, the
general concept of “PM” covers a variety of particle-size categories. The different
sizes, together with other characteristics of the particles, have been considered to
have biological significance, and have been well related to particle toxicity. For
example, particle size and shape may determine the amount of inhaled particles
that will be deposited in the lung, and these parameters may also define the
regions of the respiratory system where the inhaled particles will affect.

Evaluating health effects of PM in ambient air is difficult and complicated
due to the highly diverse size, shape, and concentrations of the particles.
However, scientific studies have developed new techniques and knowledge
regarding PM health effects in susceptible subpopulations during the past years.
In addition to previously observed adverse health effects, recent studies have
identified new pathogenic responses associated with PM exposure, particularly on
cardiovascular health effects and for children and older adults with asthma.
Several common themes have been recognized and reported lately (20, 23, 25),
including the connection between PM exposure and exacerbation of existing
asthma conditions among children and older adults, and the adverse health effects
of PM exposure on population in general, and more specifically, on those with
cardiovascular and respiratory illnesses.

It has been well known that inflammation may lead to a series of systemic
effects, such as acute-phase responses with increase blood viscosity and
coagulability. Therefore, the initiation and continuous presence of an
inflammation response has been an important issue for mechanistic studies of PM
pathogenicity. Available results also suggest that inflammation is a key pathogenic and physiological feature for chronic respiratory diseases, such as asthma and chronic obstructive pulmonary disease (COPD), because the chronic and repeated inflammatory reactions may result in remodeling of the airway passages, leading to irreversible lung disease. Thus, inflammation may be involved in both acute and chronic effects.

Recent studies indicated that exposure to several types of particles could induce inflammation responses in humans \(^{25,32}\). Study results have shown connections between exposure to concentrated ambient particles (CAPs) and altered pulmonary or systemic inflammatory markers, such as cytokines, chemokines, and adhesion molecules in healthy humans. Such observations suggested that exposures to the CAP particles may have an influence on endothelial and leukocyte activation, which is a key initial step in leukocyte recruitment, due to the critical functions of these soluble molecules in cell recruitment to atherosclerotic lesions and inflamed airways. In addition, more recent evidence (H. Geng et al, 2005) indicated that phagocytic activity of alveolar macrophages (AMs) was impaired by prolonged exposure to airborne fine particulate matter PM\(_{2.5}\) (particle size \(\leq 2.5 \, \mu m\) in aerodynamic diameter). Moreover, production of inflammatory factors, such as nitrogen oxide (NO), interleukin-8 (IL-8), and tumor necrosis factor - \(\alpha\) (TNF-\(\alpha\)), was induced following PM\(_{2.5}\) exposure as well. Thus, these data well linked respiratory disorders, such as asthma morbidity, to PM\(_{2.5}\) exposure, due to the fact that AMs are specialized cells responsible for removing dusts / particles from the airways,
and for eliminating microbial infections from the lung by granulocyte recruitment
and immune activation with cytokine secretion from AMs.

Up to date, research efforts focusing on the pathogenicity of PM exposure
have been made mainly in the fields of physiological and cellular mechanisms.
The molecular mechanistic basis for the observed health effects induced by the
bioactive particles is not yet completely understood, but starts receiving a great
deal of attentions in the research field. This subject is likely to become more
important as the research community focus more on the field of molecular
epidemiology for the bioactive particulate induced health consequences. During
the past few years, significant research progresses have been made on the
mechanisms of PM - induced health effects, based on the novel advancement of
cellular – molecular research tools and the better understanding of underlying
biological mechanisms. However, how to accurately evaluate and interpret the
vast amount of resulting research data still remains as a problem. Thus, there have
been a variety of important but yet unanswered questions, such as interpreting
changes in protein products of gene activation, cellular receptor signaling, and the
metabolic products of PM-associated compounds and the biological cascades they
stimulate.
Surface Receptors on Antigen Presenting Cells (APC)

Antigen presenting cells express a large variety of pattern recognition receptors (PRRs) on cell surface. These receptors enable the APC cells to detect and bind highly conserved infectious or endogenous ligands prior to phagocytosis activity, intracellular signal transduction, and altered biosynthesis and secretion of cytokines / chemokines, which can further direct the specific immune responses. There is a wide range of ligands, from either endogenous or exogenous origins, for APC surface PRRs, which may include proteins, polysaccharide, lipid, and nucleic acid. Due to the critical functions of the PRRs, pattern recognition of pathogen-associated molecules has become an important research topic in innate immunity. To date, the best known among the APC surface PRRs are the Toll-like receptors (TLR) and scavenger receptors (SR-A).

I. Toll-like receptors (TLR)

The identification and characterization of the Toll-like receptor (TLR) family is one of the most important discoveries in the field of innate immunity. Mammalian TLR proteins share sequence similarity with the *Drosophila* Toll protein, which was originally identified in the *Drosophila* embryo for its crucial role in defining the dorsal-ventral pattern formation. The cytoplasmic domains of Toll protein and the mammalian interleukin-1 receptor (IL-1R) were found to be homologous, leading to the hypothesis that similar receptors might be encoded in the mammalian genome. In 1997, the first human Toll protein was discovered, cloned, and termed “hToll” by Medzhitov and Janeway (58). Since then, additional mammalian Toll-like proteins have been identified and been given the general
term of “TLR”, and the very first “hToll” was renamed as “TLR-4”. Available studies have indicated that these receptors possess critical functions in detecting microbial infection, activating anti-microbial genes, as well as initiating and directing the adaptive immune responses. More recent studies have shown that TLRs play crucial roles that affect many aspects of the immune system, such as recognizing highly conserved microbial components or molecular features, engaging differential signaling pathways, guiding dendritic cells (DCs) maturation, and directing differentiation of T helper (T_H) cells (2). Figure 1 - 4 summarizes some of the vital roles for TLRs in the regulation of adaptive immunity.

Figure 1 - 4. Functions of Toll-like receptors (TLRs) in initiation and regulation of adaptive immunity. The innate immune system detects microbial infections by cell surface TLRs binding to highly conserved microbial ligands, leading to phagocytosis, APC activation, up-regulation of cell surface co-stimulatory molecules (CD80/86) and MHC-II complex. Thus, naïve T- and B-lymphocytes are activated and antigen - specific TCR /BCR are generated. The TLR - induced secretion of inflammatory cytokine and chemokines, such as IL-12, direct the immune response by guiding the maturation and differentiation of activated T cells towards T helper (T_H) effector cells. Adapted from reference (2).
Structurally, TLRs belong to the family of type I transmembrane receptors. All TLR family members possess an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain. A diagram of TLR family members and their structure comparison with IL-1 receptors is shown in Figure 1 - 5. To date, at least ten TLR receptors have been identified in mammalian species, and each one of them appears to have distinct functions in innate immune recognition. During the past few years, a variety of TLR ligands have been identified, and results indicate that TLR ligands are highly diverse in structure and origin. Based on the available information, a proposed mechanism for specific ligand recognition by TLRs is shown in Figure 1 - 6. According to the identification and characterization studies, several common themes have been recognized regarding TLR functional mechanisms and their specific ligands. First, most TLR ligands are highly conserved microbial components or surface structures that can signal the presence and the nature of the infection. Second, most of the individual TLR receptors can recognize several ligands, and these ligands can be structurally related or unrelated. Third, for some TLRs, including TLR-4, ligand recognition and signal initiation require other accessory proteins to form a structural complex with the TLR. Finally, available data from binding studies indicate that direct binding with ligands is usually the first and critical step for mammalian TLR-mediated ligand recognize and signaling, suggesting that these receptors function as pattern-recognition receptors (PRR) (2, 3). However, the actual and detailed mechanism of ligand recognition by TLRs remains unknown.
Figure 1 - 5. Structural comparison between Toll-like receptor (TLR) family and Interleukin - 1 receptor (IL-1R) family. An intracellular TIR (Toll/IL-1R) domain is conserved for all members from both families. An unique extracellular leucine-rich repeat (LRR) domain is conserved for members in the TLR subfamily. Adapted from Reference (3).
As described earlier, human Toll-like receptor 4 (TLR-4) was the first discovered and characterized mammalian Toll protein, and it has been implicated in innate recognition and signaling of lipopolysaccharide (LPS). Surface expression of TLR-4 has been found on a variety of cell types. Macrophages and dendritic cells, the two major types of antigen presenting cells in the innate immune system, express TLR-4 on cell surface as well. Available studies indicated that recognition and signaling of LPS is mediated through TLR-4. However, the process of LPS recognition via TLR-4 is complicated, requiring several accessory proteins. The current understanding of this mechanism indicates that LPS-binding protein (LBP) is the first serum protein that binds to LPS, and then transfer LPS monomers to CD14 (4, 5). CD14 is a glycosyl-phosphatidylinositol - linked membrane protein widely expressed on mononuclear cells, or, it can be secreted and expressed as a soluble serum protein as well. CD14 has been well known as a high-affinity receptor for LPS (3). However, due
to the fact that membrane CD14 lacks a transmembrane domain, it is unlikely that membrane CD14 can transmit signals directly into the cells. MD-2, a small glycosylated protein that also lacks a transmembrane region, is another critical component of the LPS receptor complex. The expression of MD-2 is found on the surface of immune cells as well. Available data indicated that MD-2 was associated with the ectodomain of TLR-4, suggesting that it was included in the LPS signal complex formation⁹. Even though the precise function of MD-2 is still unknown, previous studies have shown that MD-2 was required for efficient LPS recognition via TLR-4²⁴. Meanwhile, other studies have shown direct interactions between TLR-4 and LPS, however, such interactions still required involvements of CD14 and MD-2 for optimal LPS signaling⁸. Additionally, it has been found that CD14, TLR-4, and MD-2 could act in a simultaneous manner, when co-expressed in HEK293 cells, to bind radioactive labeled LPS⁶. However, the precise molecular mechanism of TLR-4-mediated recognition of LPS remains unclear, and has become one of the most challenging issues in Toll biology today.

II. Scavenger receptors

The scavenger receptors were first discovered in 1979, during the process of trying to understand how cholesterol from low-density lipoproteins (LDL) accumulates in macrophages in atherosclerotic plaques¹¹. Since then, a few different groups of scavenger receptors have been identified, including Class A collagenous scavenger receptors, Class B CD36 family, Drosophila Class C
macrophage specific scavenger receptor named dSR-CI, mucin-like scavenger receptor family CD68/macroialin, and an endothelial cell-specific scavenger receptor LOX-1 (Figure 1 - 7).

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Receptor structure</th>
<th>Cell membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>SR-A</td>
<td>SR-A II</td>
</tr>
<tr>
<td>Gram-positive bacteria</td>
<td>MARCO</td>
<td>SR-C II</td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td>SR-C I</td>
<td>LOX-1</td>
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<tr>
<td>Bacterial DNA</td>
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**Figure 1 - 7:** Different groups of scavenger receptors that have been identified to be involved in recognition of specific microbial ligands. Adapted from Reference (12).

The Class A scavenger receptors (SR-A) are mainly expressed in macrophages and related cells as membrane receptors. SR-As are able to recognize a variety of ligands, including chemically modified or altered molecules, and the modified lipoproteins that are pertinent to the development of vascular disease (13). The Class A SRs can be broadly classified as three different...
SR-A types, including type I, II, and III. Type III is a nonfunctional splice variant type. Another distant receptor member, MARCO, is also included in this class.

Type I and II SR-A receptors are multi-domain trimeric molecules composed of three identical protein chains, which preferentially bind acetyl-LDL. An N-terminal cytoplasmic domain and glycosylated fibrous coiled coil extracellular domains are conserved for members of both SR-A type I and II. In addition, both types have 23 (human) or 24 (bovine, rabbit, murine) uninterrupted Gly-X-Y tripeptide repeats that form a collagenous triple helix. There are five highly conserved basic amino acids among the C-terminal 22 amino acids of the collagenous-like domain. These basic amino acids have been found to have essential functions in binding with both oxidized LDL and acetyl-LDL.\(^{11}\)

The concept of a "macrophage scavenger receptor" refers to a broad range of cell surface molecules involved in receptor-mediated endocytosis of selected polyanionic ligands. Other roles for some of these receptors have been identified, including phagocytosis of apoptotic cells and invading microbes, as well as functions in cell adhesion and host defense initiation (Figure 1 - 8). Two of the many themes that have been drawn from previous studies of macrophage SRs are particularly relevant to this dissertation. First, the ligand-binding properties of most macrophage SRs are quite broad and overlapping, therefore the biological specificity of these receptors has to be determined not only by ligand structure and its corresponding signal transduction pathways, but also by other considerations. These may include distribution and availability of the various SRs, their ability and specificity to interact with other receptors, and their relative
affinities for the various ligands. Second, among the distinct classes of macrophage SRs, ligand binding usually occurs without conserved protein sequences. In order to better understand the mechanisms involved in individual SR's broad and specific ligand recognition, and in the features shared by the unrelated molecules, it might require the examination of tertiary structures of the multiple SRs to solve the mystery.
Figure 1 - 8. Biological functions identified for macrophage scavenger receptors. Adapted from Reference (13).
In 1991, Hampton and colleagues were the first to identify the involvement of SR-A in the binding of lipid A, the active component of LPS\textsuperscript{(14)}. Their data showed that SR-A could mediate clearance and detoxification of plasma endotoxin. Expression of both type I and type II SR-A was also found to be up-regulated by intracerebral administration of LPS or cytokines\textsuperscript{(11)}. Subsequently, the binding of lipoteichoic acid (LTA) of Gram-positive bacteria to SR-A was reported as well\textsuperscript{(15)}. Haworth tested the \textit{in vivo} relevance of the data of Hampton and his coworkers, and the data generated from these \textit{in vivo} studies were consistent with Hampton's \textit{in vitro} work\textsuperscript{(16)}. SR-A deficient mice (SR-A\textsuperscript{−/−}) were found to be more susceptible to LPS-induced shock after they had been primed by the mycobacterial vaccine Bacille Calmette Guerin (BCG) to activate their macrophages. The studies also showed that these results were accompanied by overproduction of cytokines, such as TNF-\textgreek{a} and IL-6, as well as other pro-inflammatory mediators, perhaps due to an imbalance between SR-A-dependent clearance of LPS and TLR-4-dependent pathways of secretory stimulation. However, the opposite findings have been reported as well, namely that SR-A\textsuperscript{−/−} mice were more resistant to endotoxic shock\textsuperscript{(17)}. There are several possible explanations for the above contradictory results, and on-going studies are trying to solve the mystery.

Overall, the differential responses initiated by clearance of endogenous host ligands and exogenous microbial invaders are poorly understood, but remains as a key issue for immunological studies that try to understand the mechanisms of immunosuppression and autoimmunity, as well as immune activation. It has been well accepted that SR, as well as other pattern recognition receptors (PRR), such as TLRs,
may function individually or cooperatively to play a critical role in defining and directing various adaptive immune responses following innate recognition via these PRRs.

Lipopolysaccharide has been well identified as a ligand for both SR-A and TLR-4 receptors. However, knowledge regarding silica and PM receptors and their signaling pathways is still very limited, but has drawn significant attention in the research field. Therefore, it would be of interest to examine whether these two different PRRs could influence one another through a cross-talk mechanism as well as ligand-induced responses while cells are stimulated with LPS or bioactive particles. A detailed comparison of innate and adaptive immune activation also remains to be made.
RESEARCH OBJECTIVES

The research objectives and corresponding specific aims of this dissertation are listed as follows.

Major Objective #1:

Evaluate the biological potency of synthetic lipid A compounds for potential vaccine and adjuvant development.

Specific Aims:

1. Develop a reproducible in vitro cell assay system using human monocytic cell lines U937 and MonoMac-6.
2. Evaluate biological potency of synthetic lipid A compounds with the above cell assay system by measuring quantitative cytokine secretion from activated human cell lines.

Major Objective #2:

Characterize specific involvement of Toll-like receptor-4 (TLR-4) in lipopolysaccharide (LPS) signaling pathways.

Specific Aims:

1. Perform inhibition study with the above cell assay system, using monoclonal anti-human-TLR-4 as receptor blocker.
2. Verify and characterize human TLR-4 surface expression on U937 and MonoMac-6 cell lines using fluorescence conjugated TLR-4 specific monoclonal antibodies.

3. Generate fluorescence-conjugated lipopolysaccharide (LPS) direct-binding curves with U937 and MonoMac-6 cell lines.

4. Verify LPS-specific binding to the two human cell lines by detecting binding inhibition with non-labeled LPS and monoclonal anti-TLR-4 antibody, therefore further characterizing TLR-4 functions in LPS signaling and cell activating pathways.

**Major Objective #3:**

Test the hypothesis that both silica and urban particulates, as well as LPS, can up-regulate immune response by increasing the activity of antigen-presenting cells (APC) via TLR-4 and/or scavenger receptors (SR-A).

**Specific Aims:**

1. Verify surface expression of TLR-4 and scavenger receptors (SR-A) on murine bone marrow derived macrophage cells.

2. With a murine in vitro antigen presenting cell (APC) assay, perform inhibition studies using specific monoclonal antibodies as receptor blockers to determine the functions and cooperation mechanisms between TLR-4 and SR-A receptors for up-regulating APC response to bioactive particulates and to LPS.
The experimental studies addressing the above objectives and specific aims are presented in Chapters 2, 3 and 4 of this dissertation. A summary of conclusions and future directions is presented in Chapter 5.
CHAPTER 2
Biological potency evaluation of synthetic lipid A compounds with \textit{in vitro} human cell assay system

Introduction

As described in Chapter 1, LPS and its active lipid A component are potent stimulators of host immune systems. The lipid A portion of LPS is highly conserved in all Gram-negative bacteria, whereas the O-antigen portion is variable in LPS from different species of bacteria. Previous studies indicated that the pro-inflammatory effects of LPS were due to the biological activity of lipid A component, therefore, lipid A has the potential to act as an adjuvant for vaccine antigens. Moreover, due to its immunostimulating capability, lipid A may provide general protections against infections by functioning as a strong inducer of non-specific resistance (NSR). However, due to the highly endotoxic activities, the clinical and medicinal use of LPS and lipid A is very limited.

Because of the potentially beneficial and clinical use of lipid A, a considerable amount of research work has been performed focusing on LPS and its active lipid A component during the past two decades. One of the most significant achievements was conducted by Dr. Edgar Ribi and his colleagues at Ribi ImmunoChem Inc. (Hamilton, MT) in the early 80’s. They found that the toxicity of lipid A could be separated from its beneficial functions of inducing cytokines and other effector molecules, which were required for adjuvant activities, by modifying lipid A structure. Based on their discovery, the natural product of monophosphoryl lipid A (MPL™ Adjuvant) was derived from LPS, and it has been included in several vaccine products for on-going clinical or pre-clinical
testing. For example, it is currently under consideration for regulatory approval as an adjuvant in a prophylactic hepatitis B virus vaccine in the United States.

Monophosphoryl lipid A (Figure 2 - 1) is an attenuated derivative of LPS from *Salmonella minnesota* R595. Isolated from its parent molecule LPS, MPL is an immunoadjuvant fraction with only one signal phosphate group. Available research data indicate that MPL has potent adjuvant and immunostimulating activities, but does not possess the many endotoxic properties from its parent molecule LPS. Adjuvant evaluation studies performed in several clinical or pre-clinical trials have shown that MPL™ Adjuvant is immunologically potent with clinical safety when administrated to humans at its active doses, measured by human cytokine induction and cytotoxic T cell activation, etc.

Based on the knowledge and experience with MPL Adjuvant, scientists at Corixa Corporation have developed a library of novel synthetic glycolipids, the aminoalkyl glucosaminide 4-phosphates (AGPs). These molecules are lipid A mimetics structurally related to the major hexaacyl component (Figure 2 - 1) present in the low toxicity 3-O-deacylated monophosphoryl lipid A (MPL™ Adjuvant) derived from *Salmonella minnesota* R595 lipid A. With the general structure as shown in Figure 2 - 2, the AGPs are synthetic lipid A compounds in which the reducing sugar in Figure 2 - 1 has been replaced with an aminoalkyl aglycon unit. The structures of a few representative AGP compounds that will be tested in this project are shown in Figure 2 - 3.
Figure 2 - 1. Structure of the major hexa-acyl component of monophosphoryl lipid A. Adapted from Reference (7).

Figure 2 - 2. General structure of aminoalkyl glucosaminide 4-phosphates (AGPs). Adapted from Reference (7).
Figure 2-3. Chemical structures of AGP compounds: RC-522, RC-524, RC-527, RC-529, RC-545, and RC-590.
In order to develop and select compounds as potential novel clinical products, research work has been performed at Corixa Corporation focusing on the functional mechanism of MPL and AGP compounds. Biological potency of AGP compounds was also evaluated with different in vitro or in vivo models. After synthesis, AGP compounds may be formulated in either of two ways. For the so-called “AF” formulation, AGP triethyl amine (TEA) salt is dissolved in dipalmitoyl phosphatidylcholine (DPPC) with water and sonicated for suspension. For the “TEOA” formulation, AGP TEA salt is dissolved in 0.2% tri-ethanolamine (TEOA) with water. Cytokine-inducing potency of AGP compounds with both formulations have been evaluated in a human setting with human whole blood as well as human PBMC cultures. The induction of the innate immunity by various AGPs was also examined in two distinct murine models. Data obtained from the above studies strongly suggested that Toll-like receptor 4 (TLR-4) was required for signal transduction and cell activation induced by MPL or AGP compounds, and the AGP fatty acyl chain length had significant effects on the stimulatory capacity of these compounds. However, no in vitro cell assay system is available at this stage for reproducible evaluation of AGP biological potency. Therefore, one major objective of this study was to develop the basic parameters of a reproducible in vitro cell culture system using human monocytic cell lines MonoMac-6 and U937 for synthetic lipid A compound potency evaluation.

In vitro cell assays using human cell lines or cloned cell lines provide a predictable and accurate means for assessing research compounds. Although human whole blood and human PBMC cultures have been used as test systems for adjuvant and synthetic lipid A compound evaluation, whole blood contains polymorphonuclear as well
as mononuclear white blood cells. Meanwhile, donor-to-donor variation adds a measure of unpredictability to the results. A predictable in vitro human cell assay system can be applied to better characterize and compare the activation of mononuclear cells present in human whole blood by stimulants. Such a system also could be adapted for development of potency assays for synthetic lipid A mimetic, such as AGPs.

Derived from a human histocytic lymphoma, the U937 cell line may serve as a study model for monocytic differentiation. Because of its well defined adhesive properties, this cell line, with the basic phenotype corresponding to normal monoblasts (3), has been frequently applied in assessing monocytic cell adhesion to human umbilical vein endothelial cell (HUVEC) monolayers (2). U937 cells may be rendered the phenotypic properties similar to normal monocytes and macrophages by proper induction of differentiation. Previous studies reported that the inducers for such differentiation might include phorbol 12-myristate 13-acetate (PMA), cholecalciferol (Vitamin D<sub>3</sub>), retinoic acid, or a cocktail of interferon gamma (IFN-γ), IL-6, and GM-CSF (3, 4).

MonoMac-6, on the other hand, is another human monocytic cell line that has been frequently used to analyze the behavior of monocytes in vitro (7, 8). Such utilization of MonoMac-6 cells is mainly due to its derivation from a human monoblastoma (5, 6), and its mature monocytic properties, including CD14 antigen expression, phagocytosis activity, and the ability of cytokine secretion when stimulated. Available studies indicated that MonoMac-6 cells appeared to be arrested at a more mature stage of monococyte differentiation compared to U937 cells, whose differentiation was found to be associated with growth cessation in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle (3, 4).
Based on the available knowledge, the above two human cell lines were selected to develop a reproducible in vitro cell culture system for lipid A compound and adjuvant evaluation in this study.
Materials and Methods

Cell Lines and Culture Medium

U937 cells

U937 cells were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). Cells were grown in RPMI 1640 (Sigma) with 2 g/L sodium bicarbonate and 2 mM glutamine (Sigma) supplemented with 10% heat inactivated fetal bovine serum (FBS, HyClone), 100 µg/ml gentamicin (Sigma) and 10 mM HEPES buffer (Sigma).

MonoMac-6 cells

Mono Mac 6 cells were obtained from Deutsche Cell Bank (Germany), and were grown in RPMI 1640 (Sigma) plus 10% heat inactivated fetal bovine serum (HyClone), 2 mM L-glutamine, 10.0 units/ml Penicillin, 10.0 µg/ml Streptomycin (Penicillin/Streptomycin, Sigma), 0.2x non-essential amino acid solution (L-Alanine, L-Asparagine, L-Aspartic acid, L-Glutamic acid, Glycine, L-Proline, and L-Serine. Sigma), 150 µg/ml oxaloacetate, 50 µg/ml pyruvate, and 8.2 µg/ml bovine insulin (OPI medium supplement, Sigma).

Culture conditions and Cell bank

Cells were grown in respective culture media in T75 culture flasks in a 37 °C CO₂ incubator. Neither cell line was adherent; therefore, after harvesting by centrifugation, cells were resuspended in fetal bovine serum with 6% dimethyl sulfoxide (DMSO) at 2.0 x 10⁶ cells/ml, and frozen at -70°C in 1.0 ml/vial aliquots.
AGP compounds and cell stimulants

MPL™ Adjuvant and AGP compounds used in this study were kindly provided by Corixa Corporation (Montana Facility). The AGP library for potency evaluation included RC524, RC526, RC527, RC529, RC534, RC545, and RC590.

Phorbol 12-myristate 13-acetate (PMA), phytohaemagglutinin (PHA), cholecalciferol sulfate (Vitamin D3), and E. coli DNA (from E. coli serotype 055:B5) were obtained from Sigma.

LPS was applied in this study as a positive control for cell activation measurement.

Unlabeled LPS preparation was obtained from Sigma (E. coli serotype 0111:B4), and fluorescence conjugated (Alexa Fluor 488) LPS was purchased from Molecular Probes. For the purpose of further appropriate inhibition tests, both labeled and unlabeled LPS were from the same E. coli serotypes (0111:B4), and had gone through the same purification procedures. The two LPS preparations were both chromatographically purified by gel filtration after an initial phenol extraction.

Cell culture preparation for in vitro assay

Fresh cell cultures were prepared weekly for each in vitro cell assay. For each preparation, one vial containing 2.0 x 10^6 cells in 1.0 ml of each cell stock, was quickly thawed in 37 °C water bath, added to 9.0 ml of respective culture medium and centrifuged for 5 minutes at 1500 rpm in order to remove DMSO. After centrifugation, supernatant was removed, and the cell pellet was resuspended in 35 ml of pre-warmed culture medium and transferred to a T75 culture flask. Cells were incubated in a 37 °C CO₂ incubator for three days, then fed once with 15 ml fresh medium, and incubated for another 24 hours. After overnight incubation, cell culture was ready for assay.
Cell culture pre-stimulation

Cells were harvested from a T75 culture flask, transferred to a 50 ml centrifuge tube, and centrifuged at 1500 rpm for 5 minutes. After removing supernatant, cells were resuspended in 5.0 ml of fresh culture medium, counted, and cell concentration was adjusted to $2.5 \times 10^6$ cells/ml. Cells then were distributed to 96-well cell culture plate at 100 µl/well with $2.5 \times 10^5$ cells/well.

Phorbol 12-myristate 13-acetate (PMA, SIGMA catalog #P-8139) powder was dissolved in dimethyl sulphoxide (DMSO) to reach a concentration at 1.0 mg/ml, aliquot and stored at -70 °C as stocks. For cell pretreatment, the PMA stock was diluted in respective culture medium to various concentrations, and added to the cell cultures at 100 µl/well. Control wells received fresh culture medium only. The plate was then incubated in a 37 °C CO$_2$ incubator. At the end of each different “pre-stimulation” period, cells were stimulated with LPS at 10 ng/mL for 3 hours, and cell activation was measured by quantitative cytokine secretion with Luminex 100™ Analyzer (Luminex Corporation, Austin, TX). The most appropriate pretreatment (the combination of PMA concentration and incubation time period) was identified based on the highest cytokine data.

Human in vitro cell assay

With the appropriate cell pre-stimulation identified from the previous step, stock solutions of various stimulants were diluted in respective fresh culture medium and added to the pretreated cells at 100 µL/well for cell stimulation. In order to reach the desired final stimulant concentration in each well, a 3-fold concentration of each stimulant had to be prepared for addition to the plate. After stimulant addition, the 96-well culture plate was incubated in the 37 °C CO$_2$ incubator for various time periods in order to determine
an appropriate stimulation period for each cell line. At the end of incubation, the plate
was centrifuged at 1100 rpm for 3 minutes, and 200 μL supernatant was harvested from
each well. Cell activation was measured by quantitative cytokine secretion with a
Luminex 100 Analyzer, and the most appropriate stimulation time period for each cell
line was identified based on the highest cytokine data.

**Evaluation of AGP biological potency with the developed human cell assay system**

Based on the cytokine data obtained from the previous steps, a Standard Operational
Procedure (SOP) was developed for an *in vitro* cell assay system using human U937 and
MonoMac-6 cells (see the section of “Discussion & Conclusions”). Biological potency of
AGP compounds, including RC522, RC524, RC527, RC529, RC545, and RC590, was
evaluated following the obtained SOP by measuring quantitative cytokine secretion from
the cells stimulated with various AGP compounds.

**Measurement of human cytokine release**

Cell culture samples collected from the previous step were thawed at room temperature,
and Luminex 100 Analyzer was applied for the measurement of human cytokines. A
Fluorokine MAP Human Base Kit (R&D systems Catalog #LU000) was applied for
quantitation of multiple cytokines released from every single cell sample. A combination
of five human cytokines, including TNF-α, IL-1β, IL-6, IL-8, and IL-10, was measured
due to previous data from our laboratory that suggested such a cytokine combination
could be simultaneously quantified using the above system without further sample
dilutions.
For experimental operation, standards provided by test kit were included in each measurement. The median value of relative fluorescence units (RFU) was collected from all samples with the Luminex machine and its installed “IS” software. A set of standard curves was generated, one curve for each cytokine, based on the RFU values of the standards and their concentrations with a four parameter logistic (4-PL) curve fit (RFU values vs. Standard pg/ml values). Multiple secreted cytokine values (pg/ml) of each test sample were calculated based on sample RFU value and the available standard curve for each cytokine.

Statistics

For the stimulant concentration – dependent cytokine data, a one – way analysis of variance (ANOVA) was applied for data analysis with a post hoc comparison using Dunnett test. Other analysis included one sample, one – tailed unpaired t test, Student – Newman – Keuls test, and Bonferroni test. Sample size for each experiment is available in the figure legends.
Results

PMA concentration for pre-stimulation

A wide concentration range of PMA, from 5 ng/ml to 0.005 ng/ml, was tested in order to determine the appropriate cell pretreatment. PMA at various concentrations was added to the cells and co-incubated with LPS at 10 ng/ml for 3 hours in a 37 °C CO₂ incubator. Samples were harvested after incubation and cytokine secretion was measured. Both MonoMac-6 and U937 cells pretreated with PMA at 5.0 or 2.5 ng/ml had the highest levels of cytokine release. Cytokine data obtained from cells pre-stimulated with lower PMA concentrations were close to background levels obtained from cells without pretreatment (Figure 2 - 4). Therefore, PMA concentration at 2.5 ng/ml was selected for cell pre-stimulation.
Figure 2-4. Concentrations of PMA for cell pretreatment.

PMA was diluted in respective culture medium to 5, 2.5, 0.5, 0.25, 0.05, 0.025, 0.005, and 0 ng/ml. PMA was then added to MonoMac-6 and U937 cells and co-incubated with LPS (E. coli serotype 0111:B4) at 10.0 ng/ml for 3 hours in a 37 °C CO₂ incubator. Samples were harvested and cytokine secretion was measured. Upper panel: TNF-α (filled bars) and IL-6 (empty bars) secretion (pg/ml) from MonoMac-6 cells; Lower panel: IL-8 secretion (pg/ml) from U937 cells. Data are shown as means ± STDEV, n = 5. Statistical analysis by 1 – factor ANOVA followed by Newman-Keuls multiple comparison to multiple controls. (*) = statistical significance at $p < 0.05$. 

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Time period for PMA pretreatment

Various time periods of pretreatment were tested with PMA on both U937 and MonoMac-6 cell lines. The two cell lines had different requirements for a pre-stimulation period. For MonoMac-6, cells pretreated for 3 hours had the most cytokine secretion, whereas 24 hour pretreatment caused the lowest cell response (Figure 2 - 5 and Figure 2 - 6). On the other hand, overnight pre-stimulation was required by U937 cells for activation (Figure 2 - 7). Flow cytometry data from our laboratory indicated that overdose (over 5 ng/ml) or over-exposure (overnight incubation) with PMA could cause cell death due to PMA toxicity (data not shown).
Figure 2 - 5. Time periods for PMA pretreatment (MonoMac-6 cells).

MonoMac-6 cells were pretreated with PMA (2.5 ng/ml) for 0, 3, and 24 hours. Cytokine secretion (IL-1 beta and IL-6) was measured after LPS (*E. coli* serotype 0111:B4, 1.0 ng/ml) stimulation for 22 hours. Data are shown as means ± STDEV, n = 4. Statistical analysis by two-way ANOVA followed by post test of Bonferroni. (*) = statistical significance at p < 0.05.
Figure 2 - 6. Time periods for PMA pretreatment (MonoMac-6 cells).

MonoMac-6 cells were pretreated with PMA (2.5 ng/ml) for 0, 3, and 24 hours. Cytokine secretion (IL-8 and IL-10) was measured after LPS (E. coli serotype 0111:B4, 1.0 ng/ml) stimulation for 22 hours. Data are shown as means ± STDEV, n = 4. Statistical analysis by two-way ANOVA followed by post test of Bonferroni. (*) = statistical significance at p < 0.05.
Figure 2 - 7. Time periods for PMA pretreatment (U937 cells).

U937 cells were pretreated with PMA (2.5 ng/ml) for 0, 3, and 24 hours. Cytokine secretion (IL-1β and IL-8) was measured after LPS (E. coli serotype 0111:B4, 1.0 ng/ml) stimulation for 22 hours. Data are shown as means ± STDEV, n = 4. Statistical analysis by two-way ANOVA followed by post test of Bonferroni. (*) = statistical significance at p < 0.05.
Time period for cell stimulation

With proper respective PMA pretreatment, MonoMac-6 and U937 cells were stimulated with LPS, MPL™ adjuvant, and AGPs for various time periods in order to determine an appropriate stimulation period for each cell line. MonoMac-6 released more TNF-alpha with 5 hour stimulation, but more IL-6, IL-8, IL-10, and IL-1 beta were released with 22 hour stimulation (Figure 2 - 8). Similar observations were obtained with U937 cells (Figure 2 - 9), therefore, 22 hours incubation was selected for both cell lines as an optimum stimulation period.
Figure 2 - 8. Time periods for cell stimulation (MonoMac-6 cells).

MonoMac-6 cells were pretreated with PMA (2.5 ng/ml) for 3 hours, and stimulated with AF formulated RC-522 for 5 or 22 hours. TNF-α and IL-6 secretion was measured with Luminex 100 Analyzer. Top panel: AF-RC522 at concentrations of 1000, 100, 10, and 1 ng/ml; Data are shown as means ± STDEV, n = 3. Statistical analysis by two - way ANOVA followed by post test of Bonferroni. (*) = statistical significance at p < 0.05. Middle and bottom panels: AF-RC522 at 10000 ng/ml. Data are shown as means ± STDEV, n = 3. Statistical analysis was performed with one - tailed unpaired t - test. (*) = statistical significance at p < 0.05.
Figure 2 - 9. Time periods for cell stimulation (U937 cells).

U937 cells were pretreated with PMA (2.5 ng/ml) overnight, and stimulated with 0.2% TEOA formulated RC-527 for 6 or 22 hours. TNF-α and IL-6 secretion was measured with Luminex 100 Analyzer. Top panel: U937 cells were stimulated with TEOA-RC527 at 100, 10, 1, and 0.1 ng/ml; Data are shown as means + STDEV, n = 3. Statistical analysis by two-way ANOVA followed by post test of Bonferroni shows no significant difference (p < 0.05) between groups of 6 and 22 hours for TNF-α production, but shows significant difference for IL-6 production. (*) = statistical significance at p < 0.05. Bottom panel: U937 cells were stimulated with TEOA-RC527 at 100 ng/ml. Data are shown as means ± STDEV, n = 3. Statistical analysis was performed with one-tailed unpaired t-test. (*) = statistical significance at p < 0.05.
Cytokine secretion in response to lipopolysaccharide (LPS) stimulation

LPS was applied as a positive control stimulant in this study. A combination of five human cytokines was measured from each sample with a Luminex 100 Analyzer, including TNF-alpha, IL-1 beta, IL-6, IL-8, and IL-10. Both cell lines had a dose response to LPS stimulation, but within a different LPS concentration range. MonoMac-6 cells exhibited dose responses within a LPS concentration range from 10 ng/ml to pg/ml levels (Figure 2 - 10), whereas U937 required a higher concentration range starting from 50 ng/ml for activation (Figure 2 - 11). PHA (Sigma), E. coli DNA (Sigma), and MPL (Corixa) were also tested with MonoMac-6 cells, and dose response was obtained with each stimulant (Figure 2 - 12).
Figure 2 - 10. Cytokine secretion from activated MonoMac-6 cells.

Cytokine secretion (IL-1β and IL-10) from MonoMac-6 cells stimulated with LPS at 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 ng/ml. Cells were pretreated with PMA (2.5 ng/ml) for 3 hours, and stimulated with LPS (E. coli serotype 0111:B4) for 22 hours. Data are shown as means ± STDEV, n = 5. Statistical analysis was performed with one-way ANOVA followed by Dunnett’s multiple comparison to a single control group. All cytokine data obtained from LPS stimulation at concentrations above 0.156 ng/mL showed significant difference (p < 0.05) over control value obtained from group treated with medium only.
Figure 2 - 11. Cytokine secretion from activated U937 cells.

Cytokine secretion from human U937 cells stimulated with LPS at: 50, 40, 30, 20, 15, 10, 7.5, 5.0, 2.5, 1.25, 0.625, 0.312, 0.16, 0 ng/ml. Cells were pretreated with PMA (2.5 ng/ml) overnight, then stimulated with LPS (E. coli serotype 0111:B4) for 22 hours. Data are shown as means ± STDEV, n = 5. Statistical analysis was performed with one - way ANOVA followed by Dunnett's multiple comparison to a single control group. All cytokine data obtained from LPS stimulation at concentrations above 2.5 ng/mL showed significant difference (p < 0.05) over control value obtained from group treated with medium only.
Figure 2-12. MonoMac-6 cell activation with LPS, *E. coli* DNA, PHA, and MPL stimulation.

TNF-α and IL-6 secretion from human MonoMac-6 cells stimulated with LPS, *E. coli* DNA, PHA, and MPL™ Adjuvant. Concentrations of all stimulants: 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, and 0.0 ng/ml. Data are shown as means ± STDEV, n = 5. Statistical analysis was performed with one-way ANOVA followed by Dunnett’s multiple comparison to a single control group. All cytokine data obtained from cell activation with stimulant’s concentrations above 0.312 ng/mL showed significant difference (p < 0.05) over control values obtained from groups treated with medium only.

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Cytokine secretion in response to AGP stimulation

AGP compounds, RC522, RC524, RC527, RC529, RC545, RC590 in AF formulation, and RC524, RC527, RC545, RC590 in 0.2% TEOA formulation, were tested for potency evaluation using the above *in vitro* cell assay systems. Levels of secreted cytokines were measured with a Luminex 100 Analyzer. Both cell lines exhibited dose responses to AGP stimulations, and typical dose response curves obtained from individual experiments are shown in Figure 2 - 13. For both AF and 0.2% TEOA formulated AGPs, RC-522 and RC-527 were the most potent compounds, whereas RC-524 and RC-545 had less but similar potency. RC-529 was the least potent AGP compound among those tested (Figure 2 - 14 and Figure 2 - 15).
Figure 2 - 13. Dose response curves obtained from MonoMac-6 and U937 cells stimulated with AF or 0.2% TEOA-formulated AGPs.

Top panel: MonoMac-6 cells stimulated with AF-RC522 at 1000, 100, 10, and 1 ng/ml; Middle panel: MonoMac-6 cells stimulated with AF-RC529 at 10000, 1000, 100, 10, and 1 ng/ml; Bottom panel: U937 cells stimulated with TEOA-RC527 at 100, 10, 1, and 0.1 ng/ml.

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Figure 2-14. Potency evaluation of AF formulated AGPs.

MonoMac-6 cells were pretreated with PMA at 2.5 ng/ml for 3 hours, and stimulated with AF-AGPs at 100 ng/ml for 22 hours. TNF-alpha, IL-6, and IL-1β were measured. Data are shown as means ± STDEV, n = 5. Statistical analysis was performed by 1-factor ANOVA followed by Newman-Keuls multiple comparison to multiple controls. (*) = statistical significance at p < 0.05.

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Figure 2 - Potency evaluation of AGPs in 0.2% TEOA formulation.

MonoMac-6 cells were pretreated with PMA (2.5 ng/ml) for 3 hours, and stimulated with TEOA-AGPs at 5.0 μg/ml for 22 hours. Cytokine secretion of TNF-alpha, IL-6 (top panel), and IL-1β (bottom panel) was measured with Luminex Analyzer. Data are shown as means ± STDEV, n = 5. Statistical analysis was performed by 1-factor ANOVA followed by Newman–Keuls multiple comparison to multiple controls. (*) = statistical significance at p < 0.05.
Effect of co-incubation with PMA

PMA co-incubation during the stimulation period was tested. MonoMac-6 cells were pretreated with PMA at 2.5 ng/ml for 3 hours. At the end of the pretreatment period, the assay was either processed with addition of stimulants directly to the cells, or 200 μl culture supernatants were removed from each well, and stimulants were diluted in fresh culture medium and added to the wells for stimulation without PMA. All samples were harvested after 22 hours incubation, and cytokine release was measured using the Luminex system. PMA presence during the cell stimulation period significantly increased the secretion of TNF-α, IL-1β, and IL-6 (Figure 2 - 16). However, compared to control samples that were treated with culture medium only, PMA alone did not increase cytokine background.
Figure 2 - 16. Effect of PMA co-stimulation with LPS and AGPs on cytokine secretion.

MonoMac-6 cells were pretreated with PMA (2.5 ng/ml) for 3 hours, then stimulated with LPS or AGPs (TEOA) with or without continued PMA presence. AGP concentration: 5.0 μg/ml; LPS concentration: 10 ng/ml. Data are shown as means ± STDEV, n = 3. Statistical analysis was performed with one-tailed unpaired t-test. For samples stimulated with AGPs or LPS, all cytokine data obtained with continued PMA presence (solid bars) showed significant difference (p < 0.05) over data obtained without continued PMA presence (empty bars). However, there is no significant increase of cytokine production from samples treated with PMA only, as compared to samples treated with medium only.

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Comparison of biological functions between fluorescence-conjugated and non-conjugated LPS

FITC-LPS was tested for binding to the surface of MonoMac-6 cells. Biological activity of FITC labeled LPS was tested and compared with unlabeled LPS. Due to possible loss or change of LPS biological functions during labeling and purification procedures, or due to serotypes, the labeled and unlabeled LPS preparations for comparison study were from the same \textit{E. coli} serotype (\textit{E. coli} 0111:B4, SIGMA catalog number L3012 and F3665), and were both purified by a two-step process of gel filtration after an initial phenol extraction.

Cytokine data obtained using the above cell culture system showed a typical dose response from MonoMac-6 cells stimulated with FITC-LPS. However, FITC-labeled LPS had about 20 - 25\% reduction of biological functions compared with unlabeled LPS (Figure 2 - 17). A comparison study was also performed between Alexa - conjugated LPS (\textit{Molecular Probes}) and unlabeled LPS from the same \textit{E. coli} serotype 055:B5, and similar data were obtained (data not shown). The FITC-LPS obtained from \textit{SIGMA} contained 3 \( \mu \)g of FITC in every milligram of LPS, so the percentage of FITC in this LPS preparation was 0.3\%, which could not account for the 20\% loss of biological activity. Therefore, this comparison study suggested that a minor loss of biological activity could result from the FITC conjugation procedure.
Figure 2-17. Comparison of biological activities between FITC-conjugated LPS and unlabeled LPS from *E. coli* serotype 0111:B4. MonoMac-6 cells were pretreated with PMA (2.5 ng/ml) for 3 hours, then stimulated with FITC conjugated LPS (empty triangles) or unlabeled LPS (filled squares) for 22 hours. Cytokine secretion (TNF-α, IL-6, and IL-1β) was measured for biological activity comparison. Data are shown as means ± STDEV, n = 3. Statistical analysis by two-way ANOVA followed by post test of Bonferroni. (*) = statistical significance at $p < 0.05$. 

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Monocytes are one of the essential components of the immune response system due to their activities of phagocytosis, antigen presentation, and cytokine secretion. On the other hand, the involvement of monocytes in various disease pathologies has been known as well. For example, the severe clinical syndromes, septicemia and shock, are due to the augmented monocyte activation in response to LPS. The LPS – induced cell activation leads to extensive cytokine productions, such as IL-1, IL-6, and tumor necrosis factor - alpha (TNF-α), which are responsible for initiating the disease states (1). Hence, representative monocytic cell lines could be applied as appropriate research tools for detailed analysis of monocyte functions in either physiological or pathological mechanisms.

In the current study, an in vitro cell assay system was developed with human monocytic cell lines MonoMac-6 and U937. Both cell lines could be activated by phorbol 12-myristate 13-acetate (PMA) to respond to LPS, MPL™ Adjuvant, and AGP compounds. Responses were measured by quantitative cytokine secretion. Potency of two formulations of AGP compounds was evaluated with this cell assay system. Biological functions of fluorescence conjugated and unlabeled LPS were tested and compared as well. Based on our data, MonoMac-6 cell line is recommended for further cell assay development and application.

Cell pretreatment

Effect of pretreatment on cell response was previously tested with human cell lines THP-1, K562, and U937 in our laboratory. Because adherence has been considered
a marker of differentiation for monocytic cells (4,16,17), 4-beta-phorbol-12-myristate 13-acetate (PMA), phytohaemagglutinin (PHA), and cholecalciferol sulfate (Vitamin D3) were applied to pretreat the above cell lines for various time periods in order to determine if these non-adherent cells could be rendered adherent. THP-1 cells were rendered adherent after 24 hour pretreatment only with PMA, but neither U937 nor K562 could be induced to adhere to the culture plate by any of the above pretreatments. Previous data also had indicated that without proper pretreatment, neither THP-1 nor K562 cells could be activated by MPL or LPS as expected, suggesting that pre-stimulation is required for cell activation. Adherence did not correlate with activation by MPL in THP-1 cells (data not shown).

The pretreatment effect of PMA, PHA, and vitamin D3 on THP-1 cell activation was investigated and compared early in our laboratory. THP-1 cells were pretreated overnight with PMA at 5 ng/ml, PHA at 10 pg/ml, or Vitamin D3 at 2 pg/ml. After pretreatment, cells were stimulated with MPL at 20 pg/ml for 22 hours. Cytokine (IL-8) secretion was then measured with R&D Human IL-8 ELISA kit. After PMA pretreatment and MPL stimulation, IL-8 secretion from THP-1 cells was 6,600 pg/ml, whereas with PHA or Vitamin D3 pretreatment, IL-8 secretion from THP-1 cells was close to the un-pretreated sample levels (both around 800 pg/ml). Based on these available data, PMA was selected for further investigations in our current study.

Previous data from Merck Research Laboratories indicated that addition of LPS at 10 ng/ml to MonoMac-6 cells induced very little production of TNF-α (57). However, co-incubation of LPS with PMA significantly increased TNF-α secretion, whereas PMA alone is inactive (5), suggesting PMA is required for the completion of TNF-α precursor
processing and secretion in LPS-treated MonoMac-6 cells. Our development data are in agreement with these findings, and the current study extended these findings by testing both U937 and MonoMac-6 cell lines. PMA pretreatment was required for both cell lines to respond to LPS, MPL, and AGPs. Co-stimulation of PMA with stimulants was significant for cell responses, whereas PMA alone did not trigger cell activation.

**Structural requirements for biological potency of AGP compounds**

Based on the discovery and development work of Ribi ImmunoChem with MPL Adjuvant, a library of novel synthetic glycolipids known chemically as \( \omega \)-aminoalkyl 2-amino-2-deoxy-4-phosphono-\( \beta \)-D-glucopyranosides (aminoalkyl glucosaminide 4-phosphates, or AGPs) was developed by scientists at Corixa Corporation. Since then, a considerable number of studies have been conducted at Corixa Corporation to investigate receptor mediated signaling pathways employed by AGP compounds. Using receptor negative cell lines transfected with TLR-4, the cells were stimulated with AGP compounds, and cell activation was measured by cytokine secretion. Results obtained from the above study strongly suggested that a combination of TLR-4 and MD-2 was required by AGPs for signal transduction \(^9\). Furthermore, potency evaluation studies indicated that the secondary fatty acyl chain length of AGPs played an important role in determining compound biological potency \(^7,31\). AGP compounds that possess 10 carbon secondary acyl chains appeared to be the most potent TLR-4 agonists. For example, compound RC-524 with C10 acyl groups was approximately 5 times more potent than RC-529 (C14 acyl groups), measured by TNF-\( \alpha \) and IL-1\( \beta \) secretion from AGP – treated human whole blood in vitro \(^7\).
Similar studies were performed using *in vitro* human PBMC culture systems, and data again indicated that AGP compounds carrying 9, 10, 11, or 12 carbons in their secondary fatty acids were generally 20 to 30-fold more potent compared to compounds with 6, 7, or 8 carbons in their secondary positions. RC-527, another AGP compound that carries ten-carbon fatty acids in all three secondary positions, showed the highest biological potency in this study (7). Furthermore, two *in vivo* infectious disease models, using influenza virus or *Listeria monocytogenes*, were applied to investigate the relationship between AGP protective capability (non-specific resistance) and its chemical structure. Results obtained from this *in vivo* study were very similar to the above *in vitro* findings, indicating that AGPs carrying 9 – 12 carbons at their secondary positions provided the most effective protection against infection in mice, compared to compounds with either short (6 – 8 carbons) or long (14 carbons) secondary fatty acid chains.

Data generated from our current *in vitro* cell assay study are in agreement with the previous findings. Both AF formulated and 0.2% TEOA formulated AGPs showed similar biological potency patterns when evaluated with the developed *in vitro* cell assay. Dose responses were obtained from both cell lines by stimulation of AGPs with either formulation (Figure 2 - 13). Compound RC-527, both AF and 0.2% TEOA formulated, appeared to be the most potent AGP, followed by compound RC-522 (Figure 2 - 14 and 2 - 15). Compound RC-524 and RC-545 exhibited less but similar potency. Again, compound RC-529, which has 14 carbon acyl chains at all three secondary positions, was the least potent compound. Our data indicated that *in vitro* samples stimulated with RC-529 secreted about the same amount of cytokines compared to samples treated with PMA only. Based upon our cytokine data, RC-527 was 15, 17, and 62-fold more potent.
compared to RC-529 when measured by TNF-α, IL-1β, and IL-6 secretion, respectively. Another potent AGP RC-522 appeared to be 18, 16, and 45-fold more potent compared to RC-529, when measured with the above three cytokines as well.

Data obtained from the current and previous studies indicated that certain structural parameters had significant effects on AGP biological activity. Such information regarding chemical structure and biological potency would greatly help identify key structural characteristics that are responsible for the high immune potency. Chemical structure is the key component that could directly impact compound solubility in aqueous solutions, as well as on its stability by increasing resistance to enzymatic or chemical degradation. Different secondary fatty acid chain length of AGPs would affect the acyl chain spacing, distance, and their packing tightness in the solution, in a way that is either energetically favored or disfavored, therefore resulting in different immune stimulating capacities. On the other hand, AGP structural flexibility in solution would vary due to compound conformational variations caused by different acyl chain length/spacing. The different conformation and flexibility would not only affect compound biological potency, but also potentially lead to certain specificity of AGP-induced immune responses. Because different conformations and steric flexibility would allow or limit binding access for certain cell surface receptors and molecules, resulting in increased or decreased specificity for certain signaling pathways, therefore triggering only particular and beneficial responses. Because AGPs are synthetic chemical compounds, their structures can be designed and modified for clinical and medicinal needs. This is a great advantage for AGP compound series because the research work focusing on structure, potency, and specificity of induced responses could lead to the discovery of novel
adjuvant candidates as potential clinical products with high efficiency and desired specificity, but low toxicity and fewer side effects.

**Cytokine combination measured with Luminex 100 Analyzer**

The Luminex Analyzer provided an efficient system that allows simultaneous measurement of several cytokines in a single sample with a relatively small volume. However, one disadvantage of such a system is that only one dilution factor is allowed for the single test sample, therefore, it is difficult to keep all cytokine data within each proper standard curve range due to significant variations within cytokines released from the same sample. The combination of cytokines that we chose for initial studies included IL-1β, IL-6, IL-8, IL-10, and TNF-α. IL-8 levels were frequently higher than the highest standard curve value, and could not be calculated as accurately as other cytokines with the same dilution factor. Therefore, IL-8 is not recommended to be included in this one-sample, multiple-cytokine assay of *in vitro* cell supernatants using the Luminex Analyzer.

**Effect of DMSO on cell pretreatment**

As described in Materials and Methods, PMA applied in this study for cell pretreatment was dissolved in DMSO for storage at –20 °C. Previous data indicated that DMSO could act as a stimulant for certain cell lines. PMA stock was diluted about 400,000-fold in culture medium for cell pre-stimulation in this study, therefore, only trace amounts of DMSO was actually added to the cells. However, the effect of DMSO on cell pretreatment and stimulation was not tested in this study.
Preference of MonoMac-6 for *in vitro* cell assay system

Compared to U937 cells, MonoMac-6 cells showed increased adhesion to stimulated and unstimulated HUVEC in previous studies, indicating that MonoMac-6 is a more mature monocytic cell line \(^{(4)}\). Therefore, compared to the relatively immature U937 cells, MonoMac-6 cells may serve as a better endotoxin indicator because this cell line represents a more advanced and mature stage of myelomonocytic development \(^{(3)}\).

Untreated MonoMac-6 cells have been found to be closely related to human monocytes from many aspects, such as expression of NaF-sensitive non-specific esterase, production of reactive oxygen, phagocytosis activity, surface expression of CD14 and many other monocyte-specific cell surface molecules, as well as cytokine productions upon stimulation \(^{(4)}\). MonoMac-6 cells could produce a variety of human cytokines, such as IL-1 \(\alpha/\beta\), IL-6, and TNF-\(\alpha\), after being induced with lectin phytohaemagglutinin A (PHA, a known T cell activator). Maximum cytokine levels were found 24 hours after stimulation with kinetic studies \(^{(1)}\).

Data from our current study is consistent with the previous findings. MonoMac-6 cells require less time for PMA pre-stimulation compared with U937 cells (3 hours vs. overnight) in our study. MonoMac-6 cells also had higher levels of cytokine secretion than U937. Therefore, MonoMac-6 cell line is recommended as a better human cell line for use in an *in vitro* cell assay system. If necessary, an *in vitro* biological assay could be completed within a one-day time period using MonoMac-6 cells with a 5-hour cell stimulation period. Overnight incubation is preferred for higher cytokine levels. Based on the above data, a brief outline of a proposed human *in vitro* cell assay procedure using MonoMac-6 cells is described as follows:
Materials

1. MonoMac-6 cells (Deutsche Cell Bank, Germany)

2. Cell culture medium and supplements:
   - RPMI 1640 (SIGMA Catalog #5886) with 10% heat inactivated fetal bovine serum (HyClone)
   - L-glutamine 2 mM (SIGMA, catalog #G7513)
   - Penicillin (10.0 units/ml) & Streptomycin (10.0 µg/ml) (Penicillin/Streptomycin, SIGMA catalog #P7539)
   - Non-essential amino acid solution (L-Alanine, L-Asparagine, L-Aspartic acid, L-Glutamic acid, Glycine, L-Proline, and L-Serine. 0.2x. SIGMA catalog #M7145)
   - Oxaloacetate (150 µg/ml), pyruvate (50 µg/ml), and bovine insulin (8.2 µg/ml), (OPI medium supplement, SIGMA catalog #O5003).

3. 4-beta-phorbol-12-myristate 13-acetate (PMA, SIGMA catalog #P-8139), stock 10 µg/ml in DMSO and store at –20 °C.

4. Lipopolysaccharide (LPS, SIGMA catalog #L-3012), stock 100 µg/ml in PBS and store at –20 °C.

5. Fluorokine MAP Human Base Kit (R&D systems, catalog #LU000)

6. Luminex 100 Analyzer (Luminex Corporation)
Experimental procedures

1. Start fresh cell culture weekly in T75 culture flask with 35 ml culture medium.
2. Incubate cell culture in a 37 °C CO₂ incubator for three days, then feed once
   with 15 ml fresh culture medium, and incubated again overnight.
3. Harvest cells from culture flask, reuspend in fresh medium, count, and
   distribute to 96-well culture plate at 100 μl/well with 2.5 x 10⁵ cells/well.
4. Dilute PMA to 5 ng/ml in culture medium and added to the cells on the
   culture plate at 100 μl/well (PMA final concentration at 2.5 ng/ml in each
   well).
5. Incubate the plate in a 37 °C CO₂ incubator for three hours.
6. Dilute stimulants in fresh culture medium at 3-fold of desired concentrations
   and add 100 μl to cells.
7. Incubate the plate in a 37 °C CO₂ incubator for 20 - 22 hours. (An incubation
   of 3 hours is optional if a one-day time period assay is preferred.)
8. Spin the plate at 1100 rpm (BECKMAN, Allegra ™ 6R Centrifuge) for 3
   minutes, harvest 200 μl supernatant from each well, and store all supernatant
   samples at −20 °C.
9. Thaw samples at room temperature. Quantitate human cytokines, including
   TNF-α, IL-1 β, IL-6, and IL-10, with Fluorokine MAP Human Base Kit and
   Luminex 100 Analyzer.
CHAPTER 3

Characterization of Specific Involvement of Toll-like Receptor-4 in Signaling Pathways for LPS and Synthetic Lipid A Compounds

Introduction

Lipopolysaccharide (LPS, or endotoxin) is the major component of the outer membrane of Gram-negative bacteria. LPS can be highly toxic when introduced into the bloodstream of an animal, and clinical symptoms may include fever, multi-organ failure, septic shock, and even death in some cases \(^{(5)}\). A variety of biochemical pathways can be activated with LPS stimulation, resulting in the above severe clinical syndromes. Among the many complicated mechanisms, one of the most important phenomena is the LPS-induced activation of monocytes and other phagocytes, because this is one of the major events that under certain circumstances can lead to the serious, complicated, or sometimes even life-threatening consequences, such as septic shock \(^{(4)}\). Therefore, signal transduction pathways and cell surface receptors for LPS recognition by monocytes and phagocytes have received a great deal of attention in the research field. The search for the specific membrane receptors for LPS on these cells has led to the discovery of the involvement of Toll-like receptor 4 (TLR-4) and CD14.

It has been well understood that human Toll-like receptors can recognize, and usually function by direct binding with highly conserved microbial molecular features, and initiate intracellular signal transduction. Therefore, these receptors are critical components of the host innate immune systems. Various immune cells of the innate immune system, such as macrophages and dendritic cells (DCs), express Toll-like
receptors on cell surface. With these surface receptors, the host innate immune system can detect microbial infections or vaccine/adjuvant that contains highly conserved microbial ligands, and activate antigen-presenting cells (APCs), resulting in phagocytosis, up-regulation of major histocompatibility complex (MHC) and cell surface co-stimulatory molecules, antigen presentation, as well as the secretion of inflammatory cytokines and chemokines. Thus, naïve T- and B-lymphocytes of the adaptive immune system may be stimulated, and antigen-specific T cell receptors (TCR) and B cell receptors (BCR) may be generated by clonal selection and expansion. Meanwhile, cytokines and chemokines may further direct the immune responses against the microbes by guiding the antigen-specific lymphocyte maturation, leading to an effective, antigen-specific adaptive immunity as well as a long-term immunological memory. Such an effect is also essential for a successful vaccination. In 1997, the first human homologue of Drosophila Toll was discovered and cloned, later designated as “TLR-4”. Since then, a great deal of research work has been performed focusing on TLR-4 functions and its signaling mechanism. Results of these studies indicated that TLR-4 played a key role in signaling of host cells in response to LPS (see Chapter I) and to other bacterial cell surface components, such as lipoteichoic acid (LTA) from Gram-positive bacteria.

CD14, a glycosyl phosphatidylinositol-anchored protein of 55 kDa is another well characterized LPS receptor (8). CD14 is normally expressed as a membrane-bound cell surface receptor (mCD14) on monocytes and macrophages. On polymorphonuclear granulocytes, CD14 was found to be expressed in a 10-fold-lower amount compared to monocytes and macrophages (4). CD14 can also be secreted and expressed in the serum as a soluble receptor (sCD14). In patients with acute infections, the concentration of sCD14
can be three- to four-times – higher compared to the healthy individuals\(^4,7,8\). Membrane-bound CD14 lacks an intracellular domain, therefore, it is unlikely that CD14 could act alone to transmit signals directly into the cell. Rather, CD14 appears to be part of a multicomponent LPS receptor complex, which is responsible for the initiation and transduction of intracellular signaling for LPS, leading to cell activation\(^8\). In the meantime, available research data also indicated that CD14-negative cells, such as endothelial or epithelial cells, were non-responsive to LPS stimulation. However, these cells can be activated by LPS with the addition of sCD14. The mechanism behind such a phenomenon is not yet completely understood, but with data showing that sCD14 did not simply serve as a shuttle for LPS transfer in this case\(^8,12\).

The current understanding of LPS signaling mechanism indicates that the interaction of LPS with CD14 requires LPS binding protein (LBP). LBP is an acute-phase protein that is synthesized by hepatocytes, and is normally expressed as a soluble serum protein. LBP can form complexes with LPS through high-affinity attachment to the lipid A moiety, which in turn transfer LPS to mCD14, and catalyzes LPS recognition by mCD14. The LPS – CD14 complexes then initiate the intracellular signaling by binding and aggregating cell surface TLR-4 receptors. Another small glycosylated protein, MD-2, has also been shown to be part of the TLR-4 signaling complex for its optimal function\(^2,33\). It has been known that TLR-4 mediates LPS intracellular signaling via different pathways, which lead to the induction of various genes that function in host defense. Two major signaling pathways have been identified, including the common MyD88 – dependent pathway and the specific Trif – dependent pathway\(^28,29\).
LPS signaling via these pathways results in the activation of NF-κB transcription factor as well as extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase. Therefore, intracellular signaling of LPS activates monocytes and macrophages, and mediates the production of inflammatory cytokines such as TNF-α, IL-1β, IL-6, and other cytokines \(^{4,5,28}\).

In Chapter II of this study, we demonstrated that \textit{in vitro} stimulation with LPS or synthetic lipid A compounds could activate human monocytic cell lines (MonoMac-6 and U937) and result in production of cytokines. In order to better characterize the specific involvement of TLR-4 in signaling pathways for LPS, and possibly for synthetic lipid A compounds as well, human MonoMac-6 cell line was again selected as an experimental model in this chapter. Inhibition studies were performed with the previously developed \textit{in vitro} cell assay system, using monoclonal anti-human-TLR-4, clone HTA125, as receptor blocker to identify TLR-4 function in macrophage activation when stimulated with LPS and other lipid A compounds. HTA125 is a monoclonal antibody that has been used to detect or block human TLR-4 in previous studies \(^{8,10,11}\), therefore, fluorescence-conjugated HTA125 was applied in this project to verify and characterize TLR-4 receptor surface expression on human MonoMac-6 cells. Meanwhile, binding studies have been of great value in unraveling the interactions of LPS with the cell surface \(^{4,7,8}\), so in the present study, fluorescence-conjugated LPS was used for cell staining in order to generate LPS direct-binding curves with MonoMac-6 cells, followed by verifying LPS-specific binding to the cell lines by detecting binding inhibition with non-labeled LPS and HTA125. The significance of CD14 and LBP involvement in LPS direct binding to cell surface was also investigated during the development of a cell staining procedure. By
comparing inhibition data generated from biological assay and LPS direct binding assay, we further characterized TLR-4 functions in signaling and cell activation pathways for LPS and lipid A compounds. In particular, the consistent appearance of high levels of fluorescent background during our studies of identifying surface expression of TLR-4, as well as evidence of inhibition during co-staining using flow cytometry immunoreagents led us to characterize the interaction of HTA125 with the human cell line.
Materials and Methods

Materials

Antibodies and Immunoreagents

Antibodies were purchased from commercial sources: Phycoerythrin (PE) conjugated anti-human TLR4, clone HTA125, IgG2a (eBioscience); Purified functional-grade anti-human TLR4, clone HTA125, IgG2a (eBioscience); Fluorescein isothiocyanate (FITC) conjugated HTA125 (BIOCARTA); Biotinylated mouse anti-human TLR4 clone HTA125 (BD Biosciences); FITC mouse anti-human CD14, clone 1.BB.211, IgG2a (U.S. Biological); FITC mouse anti-human CD14, clone Tuk 4, IgG2a (Cell Sciences); PE mouse anti-human CD14, clone CRIS-6, IgG1 (BioSource International); PE mouse anti-human CD14, clone MφP9, IgG2b (BD Biosciences); APC mouse anti-human CD14, clone M5E2, IgG2a (BD PharMingen); FITC and unlabeled mouse anti-human IgG Fc receptor CD16, clone 3G8, IgG1 (PharMingen); FITC and unlabeled mouse anti-human IgG Fc receptor CD32, clone FL18.26, IgG2b (PharMingen); FITC and unlabeled mouse anti-human IgG Fc receptor CD64, clone 10.1, IgG1 (PharMingen); PE, FITC and unlabeled mouse isotype IgG1, clone MOPC 21 (PharMingen); mouse IgG2a, clone G155-178 (PharMingen); mouse IgG2b, clone 27-35 (PharMingen). Purified human immunoglobulin IgG1, IgG2, IgG3, IgG4, and IgG Fc were purchased from Fitzgerald Industries. Other immunoreagents were obtained commercially: PE conjugated NeutrAvidin (Molecular Probes) and FITC streptavidin (Pierce); Mouse Ig was obtained from pooled normal mouse serum, and fractionated in 50% ammonium sulfate followed by serial ultrafiltration in PBS. Rabbit polyclonal anti-
human TLR4 was purchased from Torrey Pines Biolabs, Inc, and FITC-goat anti-rabbit Ig was purchased from PharMingen. Recombinant soluble human CD14 and LPS binding protein (LBP) were purchased from R&D Systems.

**Human MonoMac-6 cell line and culture conditions**

MonoMac 6 cells were obtained from Deutsche Cell Bank (Germany), and were grown in RPMI 1640 (SIGMA) plus 10% heat inactivated fetal bovine serum (HyClone), 2 mM L-glutamine (SIGMA), 10.0 units/ml Penicillin, 10.0 μg/ml Streptomycin (Penicillin/ Streptomycin, SIGMA), 0.2x non-essential amino acid solution (SIGMA), 150 μg/ml oxaloacetate, 50 μg/ml pyruvate, and 8.2 μg/ml bovine insulin (OPI medium supplement, SIGMA).

Cells were grown in the above culture media in T75 culture flasks in a 37 °C CO2 incubator. The MonoMac-6 cell line is not adherent, therefore after harvesting by centrifugation, cells were resuspended in fetal bovine serum with 6% dimethyl sulphoxide (DMSO) at 2.0 x 10⁶ cells/ml, and frozen at -70 °C in 1.0 ml/vial aliquots.

**Stimulants**

Phorbol 12-myristate 13-acetate (PMA, Sigma) powder was dissolved in dimethyl sulphoxide (DMSO, Sigma) to reach a concentration of 1.0 mg/mL, and stored in aliquots at -70°C. Cell pretreatment procedure was described in Chapter 2.

For the purpose of appropriate inhibition test and accurate data comparison, both labeled and unlabeled LPS used in this study were from the same *E. coli* serotype (055:B5), and both preparations went through the same purification procedures. Unlabeled LPS from *E. coli* serotype 055:B5 was obtained from Sigma, and dissolved in
PBS and stored frozen in aliquots at a concentration of 10.0 µg/mL. Alexa Fluor 488 conjugated LPS (E. coli serotype 055:B5) was obtained from Molecular Probes.

MPL™ Adjuvant and AGP compounds used in this study were kindly provided by Corixa Corporation (Montana Facility).

**Flow Cytometry (FACSCalibur, Becton Dickinson)**

Flow cytometry was applied for analysis of fluorescence - conjugated antibody staining and labeled LPS direct binding to cells, as well as calculation of mean fluorescence intensity (MFI) of each sample with the installed computer software “Cell Quest”. LPS direct binding curves were generated for each cell line based on sample MFI values (MFI vs. LPS concentrations). For direct binding inhibition, percentage of inhibition was calculated based on sample MFI values as well, and inhibition curves were generated according to the values of percent inhibition and inhibitor concentrations.

**Luminex 100 Analyzer (Luminex Corporation)**

A Luminex 100 Analyzer was applied in this study for measurement of human cytokines as described in Chapter 2.

**Methods**

**Inhibition study with *in vitro* human cell assays**

Biological inhibition assays were performed based on the previously developed *in vitro* human cell assay using MonoMac-6 cells, as described in Chapter 2. After the appropriate cell pre-stimulation with PMA, monoclonal anti-human-TLR-4 (HTA125) and other potential inhibitors were diluted in cell culture medium and added to the cells at
50 µL/well. The antibody isotype control, mouse IgG2a, was also included in the assay as a potential inhibitor for comparison with HTA125. Cells in uninhibited-control wells received fresh medium only. The pre-stimulated cells were incubated with the inhibitors in the 37 °C CO₂ incubator for two hours. At the end of the incubation period, stimulants, including LPS, MPL™ adjuvant, and AGP compounds (TEOA-RC-527 and TEOA-RC-529) were diluted in fresh culture medium and added to the cells at 50 µL/well for cell stimulation following the developed cell assay procedure. After proper incubation, samples were harvested and cytokine secretion was measured with the Luminex system as described previously.

Verification of TLR-4, CD14, and FcγR surface expression on MonoMac-6 cells

For direct staining, cells were cultured, harvested, and pre-stimulated following the developed in vitro cell assay procedure. At the end of cell pre-stimulation period, the 96-well cell culture plate was centrifuged at 1100 rpm for 3 minutes, and the supernatant was removed from each well. Cells were washed twice by filling each well with 300 µL staining buffer (PBS plus 2.5% fetal bovine serum and 0.025% sodium azide), and removing the liquid after centrifugation. After washing, fresh staining buffer was added to the plate at 100 µl/well to resuspend the cells. Fluorescence (FITC, PE, or APC) conjugated monoclonal antibodies and appropriately labeled mouse isotype controls were added to the cells for staining. The plate was incubated at 4 °C in the dark for 30 minutes. At the end of the incubation period, fresh staining buffer was added to the plate at 175 µL/well. The plate was centrifuged at 1100 rpm for 3 minutes, and supernatant was removed from each well. Cells were washed again by filling each well with 300 µL staining buffer and removing the liquid after centrifugation. After washing, cells were
resuspended with 200 μL/well of cold (4 °C) 1% paraformaldehyde (PFA), and
transferred to individual sample acquisition tubes. Another 200 μL/tube of cold (4 °C)
1% PFA was added to all samples for fixation. Samples were analyzed with a flow
cytometer (FACSCalibur, Becton Dickinson), and mean fluorescence intensity (MFI) of
10,000 or 20,000 cells per sample was measured with the installed software “CellQuest”.
Fluorescent HTA125 direct binding curves were generated based upon MFI values
obtained from samples stained with HTA125 at different concentrations (MFI values vs.
fluorescent HTA125 concentrations). Appropriate HTA125 concentrations within the
linear section of the direct binding curve were identified as inhibitable concentrations,
which would be applied in further inhibition studies.

For indirect staining, cells were treated as above, incubated with biotinylated
monoclonal antibody or unlabeled primary antibody, washed, and fluorescence -
conjugated NeutrAvidin or secondary antibody was added and incubated for 30 minutes.
Cells were then washed, and resuspended in fixative and treated as above.

Inhibition of HTA125 direct binding to cell surface

Various mouse immunoglobulin isotypes and monoclonal mouse anti-human
FcγR antibodies were applied to competitively inhibit PE – HTA125 direct binding to
MonoMac-6 cells, at a concentration of PE – HTA125 that had been found previously to
lie within the linear section of the PE - HTA125 direct binding curve. Each unlabeled
inhibiting antibody was diluted in staining buffer and added to the cells simultaneously
with PE – HTA125. The uninhibited control samples received PE – HTA125 only. After
incubation at 4 °C following the cell staining procedure as previously described, all
samples were analyzed with a flow cytometer (FACSCalibur, Becton Dickinson) and MFI
value of each sample was measured with "CellQuest". Percentage of inhibition was calculated using the difference of MFI of uninhibited control samples minus MFI of inhibited samples, divided by MFI of uninhibited control samples.

**Generation of fluorescence - conjugated LPS direct binding curves**

Alexa Fluor 488 conjugated LPS (*E. coli* serotype 055:B5, *Molecular Probes*) was applied to stain MonoMac-6 cells in order to generate a linear LPS direct-binding curve with the cell line. Cells were harvested and pretreated with PMA as previously described. At the end of the pretreatment period, the plate was centrifuged at 1100 rpm for 3 minutes, and culture supernatant was removed from all wells. Alexa - conjugated LPS was diluted in RPMI 1640 media (*SIGMA*) containing 1.0 µg/ml of LPS binding protein (R&D Systems) and 1.0 µg/ml of human soluble CD14 (R&D Systems). Diluted LPS was then added to the plate at 100 µl/well for cell staining. The highest concentration of fluorescent LPS for this binding test was 50 µg/ml. The plate was incubated at 37 °C in the dark for 45 minutes, and washed twice with staining buffer (PBS 1.0x + 2.5% FBS + 0.025% Azide). Cells in each well were resuspended in 400 µl of cold (4 °C) staining buffer, and LPS direct binding was detected with a flow cytometry (FACSCalibur, *Becton Dickinson*). A LPS direct binding curve with MonoMac-6 cells was generated based on flow cytometry data (MFI values vs. LPS concentrations). Appropriate LPS concentrations within the linear section of the direct binding curve were identified as inhibitable concentrations, which would be applied in further inhibition studies using unlabeled LPS and anti-human TLR-4 monoclonal antibody as potential inhibitors.
Inhibition of LPS direct binding with unlabeled LPS and anti-human TLR-4

Unlabeled LPS (\textit{E. coli} serotype 055:B5) and monoclonal anti-human Toll-like receptor 4 (clone HTA125) were applied to inhibit Alexa - conjugated LPS direct binding to MonoMac-6 cells. After PMA pretreatment and removal of supernatant, unlabeled LPS or anti-TLR4 (HTA125) was diluted in culture media and added to the plate at 100 µl/well for pretreatment. Control samples received fresh culture media only. The plate was incubated in the 37 °C CO\textsubscript{2} incubator for 2 hours, and supernatant was removed after centrifugation. Cells were then stained with Alexa - conjugated LPS at inhibitable concentrations identified from previous step, following cell staining procedure as previously described. All samples were analyzed with a flow cytometer (FACSCalibur, \textit{Becton Dickinson}) and MFI value of each sample was measured with “CellQuest”.

Percentage of inhibition was calculated using the difference of MFI of uninhibited control samples minus MFI of inhibited samples, divided by MFI of uninhibited control samples.

Statistics

For inhibition data obtained from cell assays, statistical analysis was performed with a one – tailed unpaired $t$ - test. Sample sizes for each experiment are available in the figure legends. Computer software “Cell Quest” for flow cytometry was applied for analysis of fluorescence conjugated antibody staining and calculation of mean fluorescence intensity (MFI) of each sample. MFI values were also applied to calculate and compare the expression levels of each cell surface marker.
Results

Verification of TLR-4 surface expression

Surface expression of TLR-4 by MonoMac-6 cell line was verified by flow cytometry. MonoMac-6 cells stained with PE-HTA125 had weak positive binding signals (30–40 mean channel fluorescence, or MCF) when compared with mouse IgG2a isotype background. A representative histogram is shown in Figure 3-1.

Figure 3-1. Surface expression of TLR-4 receptor by MonoMac-6 cells. MonoMac-6 cells were stained with PE-HTA125 (2.0 μg/mL). Histogram peaks from left to right: unstained cells (3.1 MFI); PE mouse IgG2a isotype background (clone G155-178, 19.71 MFI, black filled); PE-HTA125 (37.4 MFI, gray). MFI was measured using a FACSCalibur with computer software “Cell Quest”, counting 20,000 cells per sample.
Cell staining with rabbit polyclonal anti-human TLR-4 followed by FITC-goat-anti-rabbit Ig had fluorescence results similar to PE-HTA125 staining (data not shown). Fluorescence background obtained from mouse IgG2a isotype was high, as shown in Figure 3-1, up to 19.71 MFI, which was significantly higher than unstained cells (3.1 MFI) or background due to labeled IgG1 (3 MFI) or IgG2b (7 MFI) mouse isotype antibodies. FITC-HTA125 (BIOCARTA) and PE-HTA125 (BD) from different commercial sources were also tested, and similar results were obtained (data not shown). Direct binding of PE-HTA125 was nearly linear (R=0.98) up to 5.0 µg/mL, leading to the selection of 5.0 µg/mL as the HTA125 concentration to use for further inhibition studies. A typical direct binding curve of PE-HTA125 with MonoMac-6 cell line is shown in Figure 3-2.

![Figure 3-2](image)

**Figure 3-2. A typical direct binding curve of PE-HTA125 with human MonoMac-6 cell line.** MonoMac-6 cells were pretreated with PMA at 2.5 ng/mL for 3 hours. After removal of PMA supernatant and washing, PE-HTA125 was diluted in staining buffer and added to the cells at a concentration range of 20, 15, 10, 5, 2.5, 1.0, and 0.0 µg/mL. MFI values were measured using a FACSCalibur with computer software “Cell Quest”, counting 20,000 cells per sample.
Utility of HTA125 and mouse IgG2a isotype in biological inhibition assays

Functionality of TLR-4 receptor on MonoMac-6 cells was assessed by an in vitro cell assay coupled with receptor blocking antibodies, and cytokines were induced by the TLR-4 agonist LPS, as well as other lipid A compounds. Using concentrations of the stimulants in the middle section of the dose-response curves previously generated in Chapter 2, stimulants were co-cultured with HTA125 to determine if this antibody could inhibit TLR-4-based cytokine induction. Data indicated that HTA125 was a successful inhibitor for LPS as well as for other synthetic lipid A stimulants (Figure 3-3). Using MonoMac-6 cells, cytokine induction by 0.5 ng/mL LPS was inhibited from 24% to 86% by HTA125, depending upon the types of cytokine and HTA125 concentrations. In the same experiments using the same concentrations as HTA125, mouse IgG2a, an isotype control matched to HTA125, inhibited LPS (0.5 ng/mL)-induced IL-10 production up to 18%, but not LPS (0.5 ng/mL) – induced IL-1β and IL-6. Curiously, IgG2a isotype enhanced the production of TNF-α up to 21%. Similar inhibition results were obtained when other stimulants were tested, including MPL adjuvant and two synthetic lipid A compounds (AGPs) with different biological potency: TEOA-RC-527 (the most potent compound) and TEOA-RC-529 (the least potent compound). As shown in Figure 3-3, the significant reduction of cytokine secretion by co-incubation with HTA125 strongly suggested that TLR-4 receptor played a critical role in LPS, MPL and AGP signaling pathways.
Figure 3-3. Histograms illustrating percentage of inhibition generated by HTA125 and mouse IgG2a isotype from MonoMac-6 cells stimulated with LPS, MPL adjuvant, and AGP compounds RC-527 and RC-529. MonoMac-6 cells were pretreated with PMA at 2.5 ng/mL for 3 hours, then stimulated with LPS (0.5 ng/mL), MPL (5.0 µg/mL), AGP compound TEOA-RC-527 (0.5 µg/mL) or TEOA-RC-529 (5.0 µg/mL) for 5 hours. In addition, each stimulant was co-incubated in the presence of monoclonal antibody HTA125 which has specificity for human TLR-4 receptor, or mouse IgG2a which is the antibody isotype control for HTA125. Top panel: percentage of inhibition measured with TNF-α secretion; Bottom panel: percentage of inhibition measured with IL-6 secretion. Data are shown as means ± STDEV, n = 5. Statistical analysis was performed with one-tailed unpaired t-test. Percentage of inhibition generated by HTA125 (solid bars) showed significant difference (p < 0.05) over control values generated by mouse IgG2a isotype control (empty bars).
Direct binding of fluorescence - conjugated LPS with MonoMac-6 cells

Direct binding of fluorescent LPS to MonoMac-6 cell line was tested. A typical histogram analysis of Alexa Fluor 488 LPS binding peaks is shown in Figure 3-4. Linear binding curve was obtained from this test with Alexa-LPS staining (Figure 3-5), calculated based upon flow cytometry data (MFI values vs. LPS concentrations).

According to the linear binding curve, a concentration of Alexa-LPS at 5.0 μg/mL was selected as inhibitable concentration for LPS direct binding, and was applied for further inhibition studies.

![Figure 3-4. Direct binding of Alexa Fluor 488 conjugated LPS with MonoMac-6 cells.](image)

MonoMac-6 cells were pretreated with PMA for 3 hours. After removal of supernatant, Alexa - LPS was diluted in RPMI 1640 with 1.0 μg/mL LBP and 1.0 μg/mL soluble CD14 for cell staining at 37 °C in the dark for 45 minutes. Alexa-LPS concentration (from left to right): 0 (Black filled), 1, 5, 10, and 20 μg/ml (heavy gray).
Figure 3-5. Line graph illustrating typical direct binding curve of Alexa Fluor 488 conjugated LPS with human MonoMac-6 cell line. Cells were pretreated with PMA at 2.5 ng/ml for 3 hours. After removal of supernatant and washing, Alexa - LPS was diluted in RPMI1640 media (SIGMA) containing 1 µg/ml LBP and 1 µg/ml soluble human CD14, and added to the cells. Alexa-LPS concentrations for cell staining: 50, 40, 30, 20, 10, 5, 1.0, 0.5, 0.0 pg/ml (Based upon whole molecule weight). MFI values were measured using a FACSCalibur with computer software “Cell Quest”, counting 20,000 cells per sample.

Inhibition of LPS direct binding with unlabeled LPS and anti-human TLR-4

In order to verify LPS-specific binding to human MonoMac-6 cells, unlabeled LPS from the same *E. coli* serotype (055:B5) as Alexa-LPS was co-incubated with Alexa-LPS (5.0 µg/ml). Percentage of inhibition was calculated using mean fluorescence intensity units, compared to uninhibited control samples. Using MonoMac-6 cells, an average of 56% inhibition was achieved by a 10-fold concentration of unlabeled LPS (50 µg/ml) pre-incubation, and an average of 38.5% inhibition was obtained by pre-incubation of a 5-fold concentration (25 µg/ml) of unlabeled LPS. The time period for unlabeled LPS pretreatment was 1 hour, and Alexa-LPS was directly added to the cells without removing the unlabeled LPS pretreatment.
A competitive inhibition of LPS direct binding to the cell surface by anti-human TLR-4 antibody (clone HTA125) pretreatment was also investigated. However, the percentage of inhibition obtained from this procedure was lower compared to the previous experiments using unlabeled LPS as the inhibitor (Figure 3-6). Using MonoMac-6 cells, an average of 18% and 22% inhibition was achieved by monoclonal anti-TLR4 pretreatment at 50.0 and 70.0 μg/ml, respectively, followed by Alexa-LPS staining at 5.0 μg/mL.

![Figure 3-6](image)

**Figure 3-6.** Histograms illustrating percentage of inhibition generated by unlabeled LPS or HTA125 from MonoMac-6 cells stained with Alexa Fluor 488 conjugated LPS. Cells were pretreated with PMA at 2.5 ng/ml for 3 hours. After removal of supernatant and washing, Alexa - LPS was diluted in RPMI1640 media (SIGMA) containing 1 μg/ml LBP and 1 μg/ml soluble human CD14, and added to the cells for cell staining with the presence of unlabeled LPS (50 μg/mL) or HTA125 (50 μg/mL). Uninhibited control samples received Alexa – LPS only. MFI values were measured using a FACSCalibur with computer software “Cell Quest”, counting 20,000 cells per sample. Percentage of inhibition was calculated using the difference of MFI of uninhibited control samples minus MFI of inhibited samples, divided by MFI of uninhibited control samples. Data are shown as means ± range, n = 4. Statistical analysis was performed with one – tailed unpaired t – test. (*)= statistical difference at p< 0.05. Percentage of inhibition generated by unlabeled LPS (filled bar) showed significant difference (p < 0.05) over percentage of inhibition generated by unlabeled HTA125 (empty bar).

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General inhibition of mouse IgG2a isotype antibodies by mouse immunoglobulin

Because labeled mouse IgG2a isotype antibodies appeared to possess high background binding to MonoMac-6 cells, we first sought to verify that epitopes of mouse Ig immunoglobulin molecules were involved in this background phenomenon, rather than nonspecific interactions of cells with fluorescent molecules of labeled antibodies. Therefore, MonoMac-6 cells were incubated simultaneously with FITC mouse IgG2a and unlabeled mouse Ig (0.5 mg/mL or 1 mg/mL) which resulted in ablation of the FITC signal. In a similar experiment, labeled anti-CD14 that possessed the mouse IgG2a isotype was blocked by mouse Ig. Low background fluorescence due to FITC mouse IgG1 (2.4 MFI) and FITC mouse IgG2b (2.8 MFI) was not affected by addition of mouse Ig, nor was the fluorescent signal of FITC anti-CD14 of IgG1 isotype (91.5 MFI) affected by simultaneous addition of mouse Ig (93.4 MFI). However, MFI of APC-IgG2a (33.2 MFI) was lessened by addition of mouse Ig to 10.4 MFI. Further investigations also found that labeled HTA125 (mouse IgG2a) could be blocked by mouse Ig. In this respect, co-incubation with mouse Ig significantly decreased MFI of PE-HTA125 as well, from 30.3 MFI to 3.9 MFI.

To verify that IgG2a epitopes were shared by HTA125 and mouse IgG2a, unlabeled HTA125 was added to MonoMac-6 cells together with PE-IgG2a (clone G155-178). PE IgG2a fluorescence (MFI 15.3) was inhibited 63%, 62%, and 57% by 50, 20 and 10 μg/mL HTA125, respectively.
Characterization of inhibition of HTA125

Direct binding of PE-HTA125 was lessened competitively by a number of different inhibiting antibodies, including various mouse immunoglobulin isotypes and mouse anti-human IgG Fc receptor CD16, CD32, and CD64. Each potential inhibitor was diluted in staining buffer to 50, 20, 10, 5, and 1 μg/mL, and added to MonoMac-6 cells simultaneously with PE-HTA125 in order to achieve an inhibition curve. Unlabeled mouse immunoglobulin of IgG1, IgG2a, or IgG2b isotypes was incubated simultaneously with PE-HTA125, and relative inhibition was calculated. IgG2a isotype (clone G155-178) had the highest inhibition (up to 93%) of HTA125 binding. Mouse IgG1 (clone MOPC-21) and IgG2b (clone 27-35) isotypes had no significant effects on HTA125 binding. Mouse IgG2a was about 17-fold more inhibitory than IgG1 or IgG2b, based upon relative inhibition at 5 μg/mL. Figure 3-7 shows typical data obtained from this inhibition study.

To determine if a specific FcγR might be involved in this phenomenon, mouse monoclonal antibodies with specificity for anti-human IgG Fc receptors CD16 (FcγRIII), CD32 (FcγRII), or CD64 (FcγRI) were incubated simultaneously with FITC-HTA125. Maximal inhibition by anti-CD64 (clone 10.1, isotype IgG1) was 50%, and maximal inhibition by anti-CD32 (clone FL18.26, isotype IgG2b) was 20% - 25%. Anti-CD16 (clone 3G8, isotype mouse IgG1) had no significant inhibition effect on HTA125 direct binding (Figure 3-7). Based upon relative inhibition (5 μg/mL concentrations), anti-CD64 was almost 3-fold more competitive for HTA125 than anti-CD32.
Figure 3-7. Line graph illustrating inhibition of HTA125 direct binding to MonoMac-6 cells by mouse isotypes and monoclonal mouse anti-human IgG FcR. Unlabeled mouse immunoglobulin isotypes and mouse anti-human IgG Fc receptor CD16, CD32, and CD64 were each diluted in staining buffer to 50, 20, 10, 5, 1, and 0.0 µg/mL, and added to the cells simultaneously with PE conjugated HTA125. Uninhibited control samples received PE – HTA125 only. Mean fluorescence intensity (MFI) was measured on a FACSCalibur using computer software “Cell Quest”, counting 20,000 cells per sample. Percentage of inhibition was calculated using the difference of MFI of uninhibited control samples minus MFI of inhibited samples, divided by MFI of uninhibited control samples. Mouse IgG1 clone MOPC-21 (empty squares); Mouse IgG2a clone G155-178 (empty triangles); Mouse IgG2b clone 27-35 (empty circles); Mouse IgG1 anti-CD16, clone 3G8 (filled circles); Mouse IgG2b anti-CD32, clone FLI8.26 (filled squares); Mouse IgG1 anti-CD64, clone 10.1 (filled triangles). Data are shown as percentage of inhibition vs. inhibitor concentrations (µg/mL) from one representative experiment of 3 reproducible experiments.
Presence of human IgG Fc receptors upon MonoMac-6 cells

Using FITC – conjugated monoclonal mouse anti-human IgG Fc receptors (FcγR), including CD16, CD32, and CD64, MonoMac-6 cells were stained and assessed for FcγR density after culture with or without PMA-induced differentiation. All samples were analyzed with a flow cytometer (FACSCalibur), and MFI values for each marker were calculated with “Cell Quest” software. Flow cytometry analysis found no positive binding signals with FITC anti-CD16 staining, when compared with its antibody isotype background. When stained with FITC anti-CD32 or FITC anti-CD64, MonoMac-6 cells showed positive binding signals with both antibodies, and the density of CD32 was higher than CD64 (Figure 3-8). MFI values for each marker are reported in Table 3-1, which indicates that PMA pretreatment increased surface expression of both CD32 and CD64, but the pretreatment effect on such increase was not significant. MFI values of PE - TLR-4 staining are also included in Table 3-1, which shows that PMA pretreatment increased surface TLR-4 expression as well, even though the percentage of increase was not significant.

Table 3-1. Surface expression (MFI values) of CD16, CD32, CD64, and TLR-4 upon MonoMac-6 cells. Cells were stained with FITC anti-CD64 (clone 10.1), FITC anti-CD32 (clone FL18.26), FITC anti-CD16 (clone 3G8), or PE anti-TLR-4 (clone HTA125). MFI values were measured on a FACSCalibur using computer software “Cell Quest”, counting 20,000 cells per sample. Percentage of increase was calculated using the MFI difference between the PMA pretreated samples and the untreated samples, divided by MFI of the untreated samples. Data are from one representative experiment of 5 reproducible experiments.

<table>
<thead>
<tr>
<th>Fluorescence conjugated antibodies</th>
<th>MFI without PMA-induced differentiation</th>
<th>MFI with PMA-induced differentiation</th>
<th>Percentage of increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC anti-CD16</td>
<td>3.6</td>
<td>3.6</td>
<td>0.0%</td>
</tr>
<tr>
<td>FITC anti-CD32</td>
<td>30.4</td>
<td>31.4</td>
<td>3.0%</td>
</tr>
<tr>
<td>FITC anti-CD64</td>
<td>9.9</td>
<td>10.5</td>
<td>6.0%</td>
</tr>
<tr>
<td>PE anti-TLR-4</td>
<td>40.5</td>
<td>42.2</td>
<td>4.0%</td>
</tr>
</tbody>
</table>

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Figure 3-8. FACS analysis of CD16, CD32, and CD64 surface expression on MonoMac-6 cells. Frequency histograms illustrating FITC intensity for MonoMac-6 cells stained with FITC conjugated monoclonal mouse anti-human IgG Fc receptors. Top panel: cells stained with FITC anti-CD16 (clone 3G8, gray line) or FITC-conjugated mouse IgG1 isotype control (clone MOPC-21, black filled). Middle panel: cells stained with FITC anti-CD32 (clone FL18.26, gray line) or FITC-conjugated mouse IgG2b isotype control (clone 27-35, black filled). Bottom panel: cells stained with FITC anti-CD64 (clone 10.1, gray line) or FITC-conjugated mouse IgG1 isotype control (clone MOPC-21, black filled).
Because anti-CD64 was capable of inhibiting HTA125, and CD64 was expressed upon MonoMac-6 cells (Figure 3-8, bottom panel), we determined if a reciprocal relationship would exist such that HTA125 could inhibit anti-CD64 direct binding to the cell surface. The effect of HTA125 on anti-CD32 direct binding was also tested for reference. Unlabeled HTA125 was simultaneously incubated with FITC-conjugated anti-CD64 or FITC-conjugated anti-CD32, resulting in a slight increase in CD64 MFI values, but a slight decrease in CD32 MFI values (Table 3-2).

Table 3-2. Effect of HTA125 on surface expression levels of CD64 and CD32 upon MonoMac-6 cells. Cells were stained with FITC anti-CD64 (clone 10.1) or FITC anti-CD32 (clone FL18.26) with or without the presence of unlabeled HTA125 at 50.0 or 10.0 μg/mL. MFI values were measured on a FACSCalibur using “Cell Quest” software, counting 20,000 cells per sample. Percentage of gain or loss was calculated using the MFI difference between the HTA125 treated samples and the untreated samples, divided by MFI of the untreated samples. Data are from one representative experiment of 3 reproducible experiments.

<table>
<thead>
<tr>
<th>FITC-conjugated antibodies</th>
<th>No HTA125</th>
<th>HTA125 50 μg/mL</th>
<th>HTA125 10 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MFI value</td>
<td>MFI value</td>
<td>MFI value</td>
</tr>
<tr>
<td>Anti-CD64</td>
<td>8.4</td>
<td>10.2</td>
<td>+ 21.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD32</td>
<td>26.1</td>
<td>23.8</td>
<td>- 9%</td>
</tr>
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<td></td>
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Inhibition of HTA125 by human immunoglobulin isotypes

The effects of human immunoglobulins on HTA125 binding to MonoMac-6 cells were investigated and shown in Figure 3-8. Human IgG1, IgG2, IgG3, IgG4, and IgG Fc were diluted in fresh staining buffer to 50, 10, 2.0, 0.4, and 0.0 µg/mL, and added to the cells together with biotinylated HTA125 at 1.2 µg/mL. Cells were subsequently stained with PE-NeutrAvidin at 5.0 µg/mL. Maximal inhibition for each human immunoglobulin was 91% for human IgG Fc and human IgG1, 83% for IgG4, 28% for IgG3 and only 3% for IgG2. Human IgG Fc provided about 90% inhibition at all concentrations tested, whereas low concentrations of IgG3 did not inhibit HTA125. No concentration of human IgG2 had any inhibitory effect upon HTA125 binding. Relative inhibition was calculated using 2 µg/mL for each human immunoglobulin isotype. Human IgG Fc was 90-fold more competitive than human IgG2 and IgG3, whereas human IgG1 and IgG4 were about 75-fold and 35-fold more competitive, respectively.
Figure 3-9. Line graph illustrating inhibition of HTA125 direct binding to MonoMac-6 cells by human immunoglobulin. Human IgG1 (filled circles), IgG2 (filled squares), IgG3 (filled triangles), IgG4 (empty squares), and IgG Fc (empty triangles) were diluted in fresh staining buffer to 50, 10, 2.0, 0.4, and 0.0 μg/mL, and added to MonoMac-6 cells together with biotinylated HTA125 at 1.2 μg/mL. Cells were stained with PE conjugated NeutrAvidin at 5.0 μg/mL. MFI of each sample was measured with a FACSCalibur using computer software “Cell Quest”, counting 20,000 cells per sample. Percentage of inhibition was calculated using the difference of MFI of uninhibited control samples minus MFI of inhibited samples, divided by MFI of uninhibited control samples. Data are shown as percentage of inhibition vs. inhibitor concentrations (μg/mL) from one representative experiment of 3 reproducible experiments.
Regulation of TLR-4 expression by LPS pretreatment

In order to determine the effect of LPS pretreatment on TLR-4 surface expression, MonoMac-6 cells were pretreated with LPS for 24 hours. Data showed that PE-HTA125 fluorescence signal (MFI) was lessened only 18% by prior 24-hour stimulation with LPS from *E. coli* 055:B5 (10 ng/mL). When the experiment was repeated, TLR-4 surface expression was lessened 28% by 50 ng/mL LPS, and 17% by 10 ng/mL LPS. In separate experiments, PE-HTA125 signal was decreased 23% and 29% after a 24 hour exposure to 10 ng/mL LPS. In addition, TLR-4 expression upon MonoMac-6 cells was lessened a maximum of 7% if PMA pretreatment was not used. Therefore, TLR-4 expression was lessened slightly but consistently by 24 hour exposure to the TLR-4 ligand LPS.
CD14 surface expression upon MonoMac-6 cells

CD14 expression was utilized as a positive control for these investigations and results are reported here in order to verify, by comparison, the relatively weak fluorescence obtained using HTA125 to detect TLR-4. Monoclonal anti-CD14 antibodies with various clone numbers and mouse isotypes were utilized. FITC-conjugated anti-human CD14 clone Tuk 4 (2.5 µg/mL, murine IgG2a, 18 MFI) had greater fluorescence than IgG2a isotype control (clone G155-178, 12.6 MFI) and unstained cells (3.0 MFI). PE-anti-human CD14 (clone CRIS-6, 1.5 µg/mL) with a mouse IgG1 isotype was much brighter (142 MFI) compared to IgG1 isotype stained (2.8 MFI) or unstained cells (2.6 MFI). PE-anti-human CD14 (clone MφP9, 2.25 µg/mL) with a mouse IgG2b isotype was very bright also (192 MFI) compared to IgG2b-stained (11 MFI) or unstained (3.1 MFI) cells. A typical histogram illustrating CD14 surface expression on MonoMac-6 cells was shown in Figure 3-10.

Figure 3-10. FACS analysis of CD14 surface expression on MonoMac-6 cells. Frequency histogram illustrating PE intensity for MonoMac-6 cells stained with PE-conjugated mouse anti-human CD14 (clone CRIS-6, black line) or MonoMac-6 cells stained with PE-conjugated mouse IgG1 isotype control (clone MOPC-21, black filled).
Discussion

In order to study human TLR-4 receptor, we used predominantly the human monocytic cell line MonoMac-6. MonoMac-6 cell line was derived from a human monoblastoma and is capable of producing IL-1α, IL-1β, IL-6, TNF-α and IL-8 following LPS stimulation. MonoMac-6 has been consistently more sensitive to LPS than U937, for which we found similar results (See Chapter II). MonoMac-6 cell line has also been shown to express CD14. Other studies also indicated that pretreatment with calcitriol for two days could significantly enhance MonoMac-6 cell surface expression of CD14, and TNF induced a moderate increase of CD14 surface expression as well. Previous binding studies with flow cytometry analysis detected direct binding of labeled rough or smooth LPS with MonoMac-6 cells. For LPS concentrations lower than 100 ng/ml, this direct binding process required the presence of both CD14 and LPS-binding protein (LBP). For LPS concentrations higher than 100 ng/mL, binding appeared to be partially CD14-dependent, but LBP was no longer required for this process. Further inhibition studies indicated that the CD14-dependent direct binding of labeled LPS to MonoMac-6 cells could be inhibited by a 1000-fold excess of unlabeled LPS.

Similar results were obtained from our current study, and we further investigated the TLR-4 signaling mechanism by comparing inhibition data obtained from in vitro biological assays and LPS direct binding assays. By using monoclonal anti-human-TLR4, our data clearly indicated that LPS, as well as synthetic lipid A compounds (MPL and AGPs), were signaling through TLR-4 receptor. With antibody (HTA125) blocking,
TLR4-based cytokine induction was significantly reduced from MonoMac-6 cells, up to an 86% decrease in TNF-α with LPS stimulation (0.5 ng/mL), and up to a 92% decrease in IL-6 with AGP compound RC-527 stimulation (50 ng/mL) (Figure 3-3). Compared to HTA125, the antibody isotype control, mouse IgG2a had no significant inhibition effects toward LPS and lipid A compound stimulation.

However, inhibition data from LPS direct binding assays were more curious. Previous studies (4, 8) indicated that there were a few critical differences between LPS direct binding assay and normal antibody staining assay. In order to obtain ideal binding signals, LPS binding assays had to be performed at 37 °C instead of 4 °C, and cell culture basal media, such as RPMI 1640, had to be applied instead of using a PBS-based staining buffer in order to mimic a physiological condition for LPS binding. Moreover, LPS direct binding to cells appeared to be partially or completely dependent on the presence of LPS-binding protein (LBP) and soluble CD14 in the staining media (4, 8).

Based on the available information, with LBP and CD14 added to the basal media, we successfully generated LPS direct binding curves with human MonoMac-6 cell line using Alexa-conjugated LPS, and LPS binding was nearly linear up to 40 µg/mL as shown in Figure 3-2.

In order to verify specific-LPS binding to cells versus non-specific fluorescence staining, unlabeled LPS from the same E. coli serotype as the labeled-LPS was applied to inhibit Alexa-LPS direct binding, and the ideal inhibition data indicated LPS specific binding to the cells. With only a 10-fold excess of unlabeled LPS (not a 1000-fold concentration as reported previously), Alexa-LPS direct binding to MonoMac-6 was inhibited up to 56% (Figure 3-6). Furthermore, unlabeled anti-human TLR-4 monoclonal
antibody (HTA125) was used to inhibit Alexa-LPS direct binding in order to verify LPS specific binding to TLR-4 receptor versus non-specific cell surface binding. However, data from this inhibition study was less successful. HTA125, up to 70 μg/mL, inhibited Alexa-LPS (5.0 μg/mL) direct binding to MonoMac-6 cells by only 22% (Figure 3-6).

The different inhibition data obtained from using unlabeled LPS and unlabeled HTA125 indicate a two-step binding mechanism of LPS to TLR-4 receptor for triggering signal transduction pathways. This is very similar to that reviewed by Medzhitov in 2001 (2), proposing that signaling by LPS through TLR-4 occurs after transfer of LPS by lipopolysaccharide binding protein (LBP) to CD14 with subsequent engagement of a receptor complex composed of TLR-4, CD14 and MD-2, and aggregation of human TLR-4 on the cell surface by the receptor complex can initiate signaling (61). Our inhibition data confirmed that unlabeled LPS could competitively inhibit the primary binding step: Alexa-LPS direct binding with LBP and CD14, maybe MD-2 as well, on cell surface to form a multi-unit complex. This effective inhibition reduced the signal of Alexa-LPS detected on cell surface. Since the transfer and binding of LPS-multi-unit complex to TLR-4 receptor, the secondary LPS binding step, is required for signaling, therefore HTA125 could effectively inhibit LPS signaling by blocking TLR-4 receptor, as indicated by our in vitro inhibition assay data. However, this antibody can only block the TLR-4 receptor that is required for the LPS secondary binding step, but it’s not effective to inhibit LPS binding to LBP, CD14 and MD-2 to form the multi-unit complex on cell surface. Therefore, compared to unlabeled LPS, unlabeled HTA125 is less effective for inhibiting LPS direct binding to the cell surface because it only inhibits the LPS.
secondary binding step but not the primary step, while this antibody is still an efficient TLR-4 blocker that can inhibit LPS signaling through the receptor.

Because TLR-4 has been well identified as the major signaling receptor for LPS, it has been suggested that TLR-4 is involved in LPS uptake and clearance as well. However, a recent study conducted at Immunology Department of Scripps Research Institute (Tobias, et al. 2004) found no involvement of TLR-4 in cellular LPS uptake mechanisms. According to their experiments using monocytes or endothelial cells, no difference in LPS uptake and intracellular LPS distribution was observed between TLR-4 /− and wild-type cells. After investigating the role of CD14 and SR-A in LPS clearance and detoxification mechanisms, this study concluded that instead of TLR-4, LPS uptake in CD14-positive monocytes was predominantly mediated through a CD14 – dependent pathway. In CD14-negative endothelial cells, on the other hand, SR-A was found to play a critical role in LPS clearance and detoxification (33).

Indeed, LPS has been well identified as a ligand for binding to scavenger receptors (11,13), and our inhibition data obtained from this study was in agreement with this finding, suggesting that besides TLR-4, other cell surface receptors might also be involved in LPS direct binding as a ligand, but not necessarily in LPS signaling. HTA125, with specificity for human TLR-4, was unable to block LPS direct binding to other surface receptors, which included scavenger receptors. However, unlabeled LPS could competitively inhibit labeled LPS direct binding to all types of cell surface receptors. Therefore compared to HTA125, unlabeled LPS appeared to be a more effective inhibitor for blocking Alexa-LPS binding to cell surface. It is also possible that our inhibition data could be extended into support for the above findings obtained at
Scripps Research Institute. The primary binding step of LPS with CD14, MD-2, and LBP forms the multi-unit complex, and there might be double functions and outcomes for this complex formation. The transfer and binding of this complex to TLR-4 would initiate LPS signaling, or, this complex itself could trigger CD14-mediated pathways for LPS uptake, internalization and intracellular transport, leading to LPS clearance and detoxification. Certainly, further investigations are required before this can be proposed.

MonoMac-6 cells express other surface molecules characteristic of monocytes. MonoMac-6 cells have been found to express FcγRI (CD64) and FcγRII (CD32) \(^{68,69}\), which is in agreement with our data. In addition, U937 cells expressed FcγRI (CD64) and FcγRII (CD32) \(^{70}\). We found a low density of CD64, but a higher density of CD32 upon MonoMac-6 cells (Figure 3-8). Human monocytic cells THP-1 \(^{71}\) have been found to phagocytose using CD64 and CD32, with CD32 phagocytosis occurring predominantly with human IgG3 or IgG1 isotypes depending upon CD32 polymorphism \(^{72}\). In general, human FcγRI (CD64) and FcγRII (CD32) prefer human IgG1 and IgG3 isotypes as reviewed by de H ass \(^{73}\). FcγRI (reviewed by Ravetch and Kinet, 1991) \(^{74}\) was proposed to be the human equivalent of mouse FcR that bound mouse IgG2a \(^{75}\). For example, CD64 can bind ricin-labeled monomeric antibody \(^{76}\). FcγRI (CD64) in mice, prefers mouse IgG2a and is inhibited by IgG \(^{77}\). Expression of FcγRI (CD64) by MonoMac-6 and U937 cells was increased by IFN-γ but not TNF-α or IL-6, although expression of FcγRII (CD32) was unchanged or decreased by these cytokines \(^{69}\).

In our experiments, PMA was required to increase the receptivity of MonoMac-6 cells and U937 cells to LPS (See Chapter 2). PMA can induce MonoMac-6 differentiation \(^{78,79}\) toward a macrophage phenotype including increased cytokine

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secretion (53). The effect of PMA upon FcR/TLR-4 co-engagement in MonoMac6 cells is not significant in our hands, because PMA pretreatment altered CD16, CD32, and CD64 expression by only 3 – 6% based upon MFI values.

Development of monoclonal antibody HTA125 was reported by Shimazu et al (38). Other monoclonal antibodies with specificity for human TLR-4 have been reported, including clone HTA1216 (80) and clone H-80 (81), which has been identified also as a polyclonal antibody (82). Polyclonal anti-human TLR-4 extracellular peptide has been used (83). HTA125 was utilized as an inhibitor of cytokine induction by TLR-4 agonists in THP-1 cells (39,41,51), human PBMC or monocytes (40,42,48,49,50), transfected CHO cells (43), oral epithelial cells (47), HUVEC (84), human liver stellate cells (52) and gingival fibroblasts (44). HTA125 has been used also for flow cytometry to detect cell surface TLR-4 expression (45,46). We found that HTA125, a mouse monoclonal antibody with IgG2a isotype, engages TLR-4 as well as FcR on MonoMac6 cells. Binding of mouse IgG2a to U937 cells has been described (75), which was inhibited by human IgG. We also found that human IgG1 would inhibit HTA125 binding to MonoMac6 cells, which is similar to reported human IgG1 inhibition of mouse IgG2a binding to U937 cells (85). In our hands, human IgG3 did not inhibit HTA125 as well as human IgG4, although others using U937 cells found that IgG3 was a strong inhibitor of mouse IgG2a (85). The pattern of inhibition of HTA125 by mouse and human antibodies suggests that HTA125 may exhibit Fc docking upon CD64 (86).

It is possible that our isotype inhibition data and anti-FcγR inhibition data can be extended into support for a cross-reactivity between TLR-4 and CD64, as defined by an epitope recognized by HTA125. Further studies are warranted before this can be
proposed. It is more likely that the HTA125 Fc region, which possesses an IgG2a isotype, engages CD64 when CD64 is in close proximity to TLR-4 in concurrence with active site engagement of TLR-4. This phenomenon is very similar to that described by Kurlander (91) who used U937 cells to elucidate a “3-component complex of antibody bound by its Fab portion to antigen and by its Fc fragment to an Fc receptor”.

In conclusion, with biological inhibition assays using monoclonal anti-human TLR-4 (HTA125), we demonstrated that both MPL and synthetic Lipid A compounds (AGPs) were signaling through TLR-4 receptor, similar to their parent molecule LPS. Meanwhile, with direct binding assays and binding inhibition studies, we have shown a two-step binding mechanism of LPS to TLR-4 receptor, which might first involve a complex formation of LPS/LBP/CD14/MD-2, then initiate signal transduction by complex binding to and aggregating cell surface TLR-4. Furthermore, our inhibition data suggested that besides TLR-4, other receptors might be involved in LPS direct binding to the cell surface. A possible cross-reactivity between TLR-4 and CD64 as defined by an epitope recognized by monoclonal antibody HTA125 was also investigated. The findings from the present study would be helpful for a more comprehensive understanding regarding the mechanism of TLR-4 – mediated recognition of bacterial LPS and synthetic Lipid A compounds, which in turn activates innate immune responses. Such information could lead to development of novel TLR-4 agonists and antagonists, as candidates for potential new clinical products, which function through pharmacological manipulation of innate immune responses via pattern recognition receptors.
CHAPTER 4
The role of scavenger receptor and Toll-like receptor 4 in murine APC activation by bioactive particulates and lipopolysaccharide

Introduction

It is well known that silica possesses adjuvant effects\(^{(35)}\), and silica exposure has been related to the increase of polyclonal antibody synthesis, especially IgA and IgG\(^{(50)}\). Such activity has also been suggested to be involved in the prevalence of autoimmune diseases in patients with silicosis. Research focusing on the pathogenicity of silica exposure has provided some explanations regarding the pathogenic mechanism for fibrosis and auto-immune disease based on certain recent findings, including the understanding of silica – induced T cell activation that bias a Th1 response\(^{(18,35)}\). A recent study\(^{(22)}\) tested the hypothesis that silica and urban particulate matter PM1648 (Standard Reference Material 1648) could activate antigen presenting cells (APCs), resulting in an up-regulation of human immune response as measured by T cell cytokine production. Their results showed that silica could up-regulated both Th1 and Th2 lymphocyte-derived cytokine production. On the other hand, PM1648 exposure mainly triggered a Th2 response.

Lipopolysaccharide (LPS), the highly conserved component of the outer membrane of Gram-negative bacteria, is another exogenous immunomodulator that possesses adjuvant effect. LPS exposure triggers host innate immune responses, leading to the activation and differentiation of T cells, more towards Th1 effector cells\(^{(2,31)}\). It has been known that LPS can bind to several receptors on macrophages, including toll –
like receptor 4 (TLR-4), CD14, and Class A scavenger receptor (SR-A), which in turn stimulate macrophages to release various inflammatory mediators.

It has been known that inhalation of particulate matter (PM) may influence the respiratory health of both children and adults. However, the potential involvement of LPS contamination on particulate surface in the PM related health consequence has not been extensively investigated. Previous research work by Soukup, et al.\textsuperscript{(104)} showed that the coarse respirable particle fraction (PM$_{2.5-10}$) could trigger cytokine production from human airway macrophages (AM). More importantly, they found that the endotoxin associated with these particles was mainly responsible for this phenomenon, due to the fact that the PM – induced cytokine secretion required the presence of LPS binding protein (LBP), and cytokine production could be inhibited by polymyxin B and anti-CD14. Furthermore, critical involvement of TLR-4 receptor in PM$_{2.5-10}$ – induced cell activation was also observed, leading to the conclusion that AM activation was most likely mediated by endotoxin attached to the PM – particles. It has been shown that ambient urban air contains a variety of bacteria, including both Gram-positive and Gram-negative, at a relatively high level, even though most bacteria in the air do not grow on conventional media\textsuperscript{(108)}. However, certain bacterial components, such as endotoxin, could preferentially attach to the surface of the particles. The bacterial contamination on the surface of various particulates has not been well qualitatively and quantitatively measured. The biological activities between the particle itself and endotoxin contamination have not been well evaluated. Furthermore, the macrophage receptors and signaling pathways employed by the particulate or the attached endotoxin have not been investigated and compared.
Because TLR-4 has been identified as the major signaling receptor for LPS, it has been suggested that TLR-4 is involved in LPS uptake and clearance as well. However, a recent study\(^{(33)}\) found no involvement of TLR-4 in cellular LPS uptake mechanisms. According to their experiments using monocytes or endothelial cells, no difference of LPS uptake and intracellular LPS distribution was observed between TLR-4\(^{-/-}\) and wild type cells. After investigating the role of CD14 and SR-A in LPS clearance and detoxification mechanisms, this study concluded that LPS uptake in CD14-positive monocytes was predominantly mediated through a CD14-dependent pathway instead of via TLR-4. However, in CD14-negative endothelial cells, SR-A was found to play a critical role in LPS clearance and detoxification\(^{(33)}\). Indeed, scavenger receptors expressed by macrophages have been considered to play an important role in the immune responses against bacterial infection by mediating ligand binding and phagocytosis. Gene deletion studies indicated that macrophage SR-A receptors protected mice from LPS-induced endotoxemia, showing that RAW264.7 cells increased SR-A transcript levels in response to LPS challenge\(^{(96)}\).

These results have led us to hypothesize that both pattern-recognition receptors, SR-A and TLR-4, could recognize silica and urban particles, as well as LPS as their ligands, and function together through a cross-talk mechanism to up-regulate immune responses by increasing the activity of antigen-presenting cells. Based on this hypothesis, we proposed that specific monoclonal antibodies could be applied to block either receptor, or both, on murine antigen presenting cells, followed by cell stimulation with the receptor ligands, including the bioactive particles and LPS. Observation of macrophage activation could be achieved by a number of approaches, including
measuring macrophage cytokine secretion, or, by adding antigens (ovalbumin) and spleen T cells to the macrophage culture, and measuring APC activity by T cell cytokine production. Alternatively, studies could also be performed using macrophages from receptor null animals, and data could be compared to the results obtained from the studies using blocking antibodies to inhibit receptors on wild-type cells. Macrophage cytokine measurement could provide information regarding signals that activated macrophages send to, and eventually activate T cells. On the other hand, the T cell cytokine profile could also provide information regarding different T cell responses (Th1 and Th2) caused by either receptor, or both, when certain ligands were bound. Therefore, a possible co-functional mechanism with the two receptors and their specific ligands could be further determined. Previous studies (18, 22) identified positive surface expression of SR-A on mouse bone marrow derived macrophages, therefore this cell line was selected as antigen presenting cells for this in vitro study.
Materials and Methods

Materials

Mice

Wild-type Balb/c and C57BL/6 mice, as well as TLR-4−/− mice in Balb/c background were all purchased from the Jackson Laboratory (Bar Harbor, ME). SR-A−/− mice in C57BL/6 background were kindly donated by Dr. Kobzik (School of Public Health, Harvard University). For T-cell enrichment preparation, DO11.10 TCR transgenic mice in Balb/c background and OT-II TCR transgenic mice in C57BL/6 background were also obtained from the Jackson Laboratory. T cells from both transgenic mice express an ovalbumin – specific αβ TCR, which recognizes ovalbumin C-terminal peptide 329 – 337 as a specific epitope presented on mouse MHC II complex. Therefore, all cells applied in the APC assay in this study were syngeneic. All animals were housed, maintained, and bred under pathogen – free conditions at the University of Montana animal facility. All mice were between 8 and 10 weeks of age at the time of use.

Murine cell line and culture condition

Mouse bone marrow derived macrophages were harvested from tibia and femur of sacrificed mice at 8 - 10 weeks of age. Cells were cultured in 75-cm² tissue culture flasks (Corning) at about 3.0 x 10⁷ cells/flask, and incubated in a water-jacketed incubator (Forma Scientific) at 37°C in the presence of 5% CO₂ overnight for stromal cell elimination. Culture medium for mouse bone marrow derived macrophages contained RPMI 1640 (Cellgro) supplemented with 25 mM HEPES buffer, L-glutamine, 10% fetal calf serum, 1.0 mM sodium pyruvate, 0.055 mM 2-Mercaptoethanol, and antibiotics (100
U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 0.25 µg/ml amphotericin B (Gibco BRL)). The culture medium was replaced every 3 days, and macrophage colony stimulating factor (M-CSF) was added to the cells with fresh medium at 15 ng/mL each time. Cells were ready for assay after culture medium replacement and incubation for 7 days.

**Bioactive particulates and LPS**

Silica (min-U-sil-5, average size 5 µm) was obtained from Pennsylvania Glass Sand Corporation (Pittsburgh, PA), and acid-washed to prepare a bioactive powder form. Urban particulate matter PM1648 (Standard Reference Material 1648) was obtained from the U.S Department of Commerce, National Institute of Standards & Technology (Gaithersburg, MD). It consists of natural atmospheric particulate matter collected in the St. Louis, Missouri area. The material was collected over a period in excess of 12 months and, therefore, is a time-integrated sample. The material was removed from the filter bags, combined in a single lot, screened through a fine-mesh sieve to remove extraneous materials and thoroughly blended in a V-blender, then packaged into bottles sequentially numbered. Lyophilized LPS powder (*E. coli* serotype 0111:B4) was purchased from Sigma (St. Louis, MO).

Endotoxin contamination on both particulates was measured with a Limulus Assay Gel Clot Test Kit (BioWhittaker, Walkersville, MD). Particulates were serially diluted in sterile water and exposed to Limulus amebocyte lysate following the manufacturer’s directions.

For experimental use, silica and PM1648 particles were suspended in sterile PBS (1.0x solution), and sonicated for 1 minute to make a stock suspension at a concentration of 5
mg/mL. Particles were further diluted in culture medium and used for cell stimulation at various final concentrations at μg/mL level. Lyophilized LPS powder was dissolved in sterile PBS (1.0x solution), and filter sterilized to prepare a stock solution at a concentration of 1.0 mg/mL. The stock solution was further diluted in culture medium and used for cell stimulation with various final concentrations at ng/mL level.

**Monoclonal antibodies and isotype controls**

Phycoerythrin (PE) conjugated rat anti-mouse TLR-4/MD2 (clone MTS510) was purchased from eBioscience (San Diego, CA). PE conjugated rat IgG2a κ isotype control (clone R35-95) was purchased from PharMingen (San Diego, CA). Fluorescein isothiocyanate (FITC) conjugated rat anti- mouse CD204 (murine scavenger receptor class A, clone 2F8) was purchased from Serotec (Raleigh, NC), and its isotype control, FITC labeled rat IgG2b κ (clone A95-1) was purchased from PharMingen. Purified rat anti-mouse CD16/CD32 monoclonal antibody (clone 2.4G2) that was used to block Fc gamma receptors during cell staining procedure was obtained from BD Biosciences (San Jose, CA).

Unlabeled, affinity purified rat anti-mouse TLR-4/MD2 (clone MTS510) was purchased from eBioscience, and affinity purified rat anti-mouse CD204 (clone 2F8) was purchased from Serotec for using as blocking antibodies in murine macrophage assays and APC – T cell assays. The unlabeled antibody isotype controls, purified rat IgG2a κ (clone R35-95) and rat IgG2b κ (clone A95-1), were obtained from BD PharMingen (San Diego, CA).

**Flow cytometry (FACS Aria, Becton Dickinson)**

Flow cytometry was utilized for analysis of fluorescence conjugated antibody staining and calculation of mean fluorescence intensity (MFI) of each sample was accomplished.
with the installed computer software (FACS Diva, BD Biosciences). MFI values were also applied to calculate and compare the expression levels of each cell surface marker. Control samples included unstained cells, as well as cells that were stained with properly labeled isotype control antibodies only (negative control). The instrument was calibrated with BD™ CompBeads Anti-Rat Ig κ (BD Biosciences) that was used to optimize fluorescence compensation settings for multicolor flow cytometric analyses.

**Murine cytokine assays**

Mouse IFN-γ, IL-2, IL-10, IL-6, and IL-12 p40 levels in culture supernatants were quantified using OptEIA ELISA sets (PharMingen), and mouse IL-13 level was quantified using DuoSet ELISA Development System (R&D Systems, Minneapolis, MN), according to the manufacture's protocols. Samples were tested with different dilution factors for different cytokine assays, based upon the amount of cytokine secretion and each kit's standard curve range. ELISA microtiter plates were analyzed with a Spectra Max™ plate reader (Molecular Devices, Sunnyvale, CA), and the resulting data were processed by Soft Max™ software (Molecular Devices) using a 4-parameter curve fit. Data were expressed as picograms per milliliter of medium (pg/mL).
Methods

Verification of SR-A and TLR-4 surface expression on murine bone marrow derived macrophages (BMDM)

For verification of SR-A and TLR-4 surface expression on murine bone marrow derived macrophages, cells from wild type Balb/c mice were cultured and harvested as previously described. Cells were distributed into 1.2 ml microcentrifuge tubes at one million cells per tube, centrifuged at 4000 rpm for 3 minutes, and supernatants were removed. Cells were washed once with staining buffer (PBS with sodium azide and BSA), and then resuspended with 100 μL/tube of staining buffer containing antibodies to block Fc gamma receptors (CD16 and CD32) at 60.0 μg/mL. After 10 to 15 minutes incubation at 4°C, fluorescence conjugated antibodies (clone MTS510 and 2F8) and appropriately labeled antibody isotype controls were added to the cells for staining at 1.0 or 2.0 μg per million cells. Samples were incubated at 4°C in the dark for 15 minutes. At the end of the incubation period, fresh staining buffer was added to the samples at 750 μL/tube, and all samples were centrifuged at 4000 rpm for 3 minutes. Supernatant was removed from each tube, and cells were washed again by filling each tube with 500 μL fresh staining buffer and removing the liquid after centrifugation. After washing, cells were resuspended with 300 μL/tube of cold (4°C) 1% paraformaldehyde (PFA), and transferred to individual sample acquisition tubes for flow cytometry analysis.

In order to investigate the effect of LPS, silica, and PM1648 pretreatment on cell surface receptor expression, BMDM cells from wild type Balb/c mice were harvested, diluted in culture medium and distributed into 6 - well cell culture plates at one million cells per well. Stock solutions of LPS, silica, or PM1648 were prepared as described above,
diluted in culture medium, and added to the cells. Control samples received fresh medium only. Cells were then incubated at 37°C in a CO₂ incubator for 2, 24, or 48 hours. At the end of each incubation period, cells were harvested from the plate, washed, and stained with fluorescent antibodies as described previously.

**Murine in vitro macrophage assay using mouse bone marrow derived macrophages (BMDM)**

Mouse bone marrow derived macrophages (BMDM) from wild-type or receptor knock-out mice were harvested, counted, and cell concentration was adjusted to 2 x 10⁶ cells/mL. Cell suspension was distributed into 1.2 mL centrifuge tubes, and fresh culture medium containing silica, PM1648, or LPS was added to the tubes at equal amounts as the cell suspension volume in each tube. In order to reach the desired final stimulant concentration in each tube, a 2-fold concentration of each stimulant was prepared for addition to the samples. Control samples received fresh medium only. After addition of stimulants, all samples were incubated on a rotator in a 37°C CO₂ incubator for two hours. At the end of the incubation period, samples were transferred to a 96-well cell culture plate at 100 µL/well (1 x 10⁵ cells per well), and another 150 µL/well of fresh culture medium was added to all samples. The plate was incubated for another 24 hours at 37°C in a CO₂ incubator. At the end of the incubation period, the plate was centrifuged at 1500 rpm for 10 minutes, and culture supernatants (200 µL/well) were harvested and stored at -20°C for further ELISA analysis. Mouse macrophage cytokines, including IL-6 and IL-12 p40, were measured with commercial mouse cytokine ELISA kits (PharMingen, R&D Systems) as described previously.

In order to investigate the functions of TLR-4 and SR-A receptors on macrophage activation, affinity purified rat anti-mouse TLR-4/MD2 (clone MTS510) and rat anti-
mouse CD204 (clone 2F8) were applied as blocking antibodies in this study. Certain samples using wild-type cells received a one-hour pre-incubation on the rotator, for keeping cells suspended, with or without the monoclonal antibodies specific for the receptors prior to stimulant addition. After incubation with or without the blocking antibodies and their proper isotype controls, the \textit{in vitro} macrophage assay was performed following the same procedure as described above.

\textbf{Determination of function and cooperation mechanism between TLR-4 and SR-A receptors with murine antigen presenting cell (APC) assay}

1. \textbf{Preparation of Mouse Spleen T-cell Enrichment from DO11.10 or OT-II TCR Transgenic Mice}

Fresh mouse spleen T cells were prepared for each APC assay. Spleens were harvested from healthy DO11.10 or OT-II mice, then minced and washed to prepare a fresh cell suspension. SpinSep\textsuperscript{TM} mouse T Cell Enrichment Cocktail Kit from StemCell Technologies (Catalog #17061) was applied for T cell separation, following the commercial kit’s instructions and procedures. After the separation and washing procedure, the obtained T cells were counted, resuspended in mouse cell culture medium, and cell concentration adjusted to $4 \times 10^6$ cells/mL. The purity of mouse T cells obtained from this separation procedure was previously tested by cell staining and flow cytometry analysis, and data showed that up to 97% of the obtained cells were positive when stained with fluorescence conjugated anti-mouse CD3.

2. \textbf{Mouse \textit{in vitro} APC assay}

Mouse BMDM from wild-type or receptor knock-out mice were harvested, counted, and cell concentration was adjusted to $2 \times 10^6$ cells/mL. Cells were distributed into 1.2 mL
centrifuge tubes, and incubated for one hour with or without monoclonal antibodies specific for the receptors in a 37°C CO₂ incubator on a rotator to prevent adherence. After incubation, fresh culture medium containing silica, PM1648, or LPS was added to the tubes at equal amount as the cell suspension volume in each tube. In order to reach the desired final stimulant concentration in each tube, a 2-fold concentration of each stimulant was prepared for addition to the samples. Control samples received fresh medium only. After addition of stimulants, all samples were incubated for two hours on a rotator in a 37°C CO₂ incubator. At the end of the incubation period, samples were transferred to a 96-well cell culture plate at 100 μL/well (1 x 10⁵ cells per well).

Ovalbumin from chicken egg white (Grade V, Sigma Catalog #A5503-10G) was diluted in fresh cell culture medium and added to the plate at 50 μL/well to reach a final concentration of ovalbumin at 10 mg/mL in each well. The plate was incubated for another two hours at 37°C in a CO₂ incubator to allow cell adherence and ovalbumin (antigen) uptake. After incubation, splenic T cells were added to the plate at 100 μL/well to give 4 x 10⁵ T cells in each well. The plate was incubated again at 37°C in a CO₂ incubator for 48 (+ 4) hours. At the end of the incubation period, the plate was centrifuged at 1500 rpm for 10 minutes, and culture supernatant (200 μL/well) was harvested and transferred to a new 96-well plate. All samples were stored at -20°C.

Murine cytokine secretion, including IFN-γ, IL-2, IL-10, and IL-13, was measured with commercial mouse cytokine ELISA kits (PharMingen, R&D Systems) as described previously.
3. Determination of receptor function and cooperative mechanism using blocking antibodies in mouse APC assay

In order to further determine the function of each receptor and their possible cooperative mechanism on immune system regulation, the above two monoclonal antibodies were again applied as inhibitors in the APC assay. Certain samples, using wild-type BMDM cells, received a one hour pre-incubation on the rotator with or without the monoclonal antibodies specific for the receptors, prior to stimulant addition. After incubation with or without the blocking antibodies and their proper isotype controls, mouse APC assays were performed following the same procedure as described above.

Various combinations of the two blocking antibodies, as well as proper isotype controls, were tested with the murine APC assay. T cell cytokine secretion under different experimental conditions were calculated and compared. These included APC assays without blocking antibody, with antibody isotype controls only, with MTS510 alone, with 2F8 alone, and with both antibodies combined at appropriate concentrations as identified from previous steps. Meanwhile, control samples were performed in each assay for comparison. This included samples of T cells only, macrophages only, and macrophages plus T cells with or without the presence of ovalbumin.

Statistics

For the stimulant concentration – dependent cytokine data, a one – way analysis of variance (ANOVA) was used with a post hoc comparison using a Dunnett test. Other analysis included one sample, one – tailed unpaired t - test, Student – Newman – Keuls test, and Bonferroni test. Sample size for each experiment is available in the figure legends.
Results

Surface expression of SR-A and TLR-4 receptors on murine bone marrow derived macrophages (BMDM)

In order to verify the presence of SR-A and TLR-4 receptors upon mouse BMDM (from wild – type Balb/c mice), cells were stained with fluorescence conjugated monoclonal antibodies specific for SR-A or TLR-4, and the resulting fluorescence signals were analyzed with flow cytometry.

Mouse BMDM cells specifically bound the two fluorescence conjugated monoclonal antibodies specific for SR-A and TLR-4, respectively. When stained with 1.5 μg per million cells of PE-MTS510 (anti-mouse TLR-4/MD-2), mouse BMDM cells showed positive signals (2623 MFI) when compared with rat PE-IgG2a isotype background (336.1 MFI). A positive signal was also obtained when cells were stained with 1 μg per million cells of FITC-2F8 (anti-mouse-CD204) (7776 MFI compared with 407.8 MFI from antibody isotype control rat FITC-IgG2b) (Figure 4-1).
Figure 4-1. Flow cytometry analysis of TLR-4 and SR-A expression on mouse bone marrow derived macrophages. Top panel: frequency histogram overlay illustrating PE intensity for BMDM cells stained with PE-conjugated antibody isotype only (black filled) or cells stained with PE-conjugated MTS510 (anti – TLR-4, gray line). Bottom panel: frequency histogram overlay illustrating FITC intensity for BMDM cells stained with FITC-conjugated antibody isotype only (black filled) or cells stained with FITC-conjugated 2F8 (anti – SR-A, gray line).
Mouse BMDM activation with silica, PM1648, and LPS

Mouse BMDM cells from wild-type Balb/c mice were stimulated with silica, PM1648, and LPS in order to investigate the biological effects of the three stimulants on mouse macrophage activation. Two macrophage cytokines, IL-6 and IL-12 p40, were measured with ELISA assays as macrophage activation markers. Dose-response curves were obtained with both cytokines when cells were stimulated and activated by LPS or PM1648 (Figure 4-2 and Figure 4-3). However, no measurable cytokines were detected when cells were stimulated with silica (data not shown).
Figure 4-2. IL-6 dose response curves obtained from mouse bone marrow derived macrophages (Balb/c W/T) stimulated with LPS or PM1648. Top panel: cells were stimulated with LPS (E. coli serotype 0111:B4) at 50, 40, 30, 20, 10, 5, 1, and 0.0 ng/mL. Bottom panel: cells were stimulated with PM1648 at 1000, 500, 250, 125, and 0.0 μg/mL. Data are shown as means ± SD, n = 5. Statistical analysis was performed with one-way ANOVA followed by Dunnett’s multiple comparison to a single control group. All cytokine data obtained from samples with LPS or PM1648 stimulation showed significant increase (p < 0.05) over control values obtained from samples treated with medium only.
Figure 4-3. IL-12 p40 dose response curves obtained from mouse bone marrow derived macrophages (Balb/c W/T) stimulated with LPS or PM1648. Top panel: cells were stimulated with LPS (*E. coli* serotype 0111:B4) at 50, 40, 30, 20, 10, 5, 1, and 0.0 ng/mL. Bottom panel: cells were stimulated with PM1648 at 1000, 500, 250, 125, and 0.0 µg/mL. Data are shown as means ± SD, n = 5. Statistical analysis was performed with one-way ANOVA followed by Dunnett's multiple comparison to a single control group. All cytokine data obtained from samples with LPS or PM1648 stimulation showed significant increase (*p* < 0.05) over control values obtained from samples treated with medium only.
Effects of blocking antibodies of TLR-4 or SR-A on macrophage activation

In order to investigate the functional roles of TLR-4 and SR-A on macrophage activation by LPS or PM1648, mouse BMDM cells were incubated with or without blocking antibodies specific for each receptor prior to LPS or PM1648 stimulation, and macrophage cytokine secretion was measured as previously described. Because the previous IL-6 and IL-12 p40 results were very similar, the remaining macrophage cytokine studies focused on IL-6 production. Since silica exposure did not trigger IL-6 and IL-12 p40 secretion from mouse BMDM cells, macrophage cytokine studies using blocking antibodies and receptor deficient cells only focused on PM1648 and LPS stimulation. Investigation of receptors and signaling pathways employed by silica will be described in the APC assay section.

Based upon the dose response curves shown in Figure 4-2 and 4-3, LPS at 10 ng/mL and PM1648 at 200 μg/mL were selected, and inhibition curves were obtained using monoclonal anti-TLR4 (MTS510) when cells were stimulated with either LPS or PM1648 (Figure 4-4). However, cell activation by LPS or PM1648 were not blocked with monoclonal anti-SR-A (clone 2F8) (Figure 4-5). No inhibition was obtained from antibody isotype controls, nor was cell activation observed from samples treated with antibodies only (data not shown).
Figure 4-4. Histograms illustrating blocking effect of MTS510 on cell activation by LPS and PM1648 stimulation. BMDM cells from wild-type Balb/c mice were pretreated with monoclonal anti-TLR-4/MD-2 (MTS510) for 1 hour, then stimulated with LPS at 5.0 ng/mL (top panel) or PM1648 at 200 μg/mL (bottom panel), and IL-6 secretion was measured with ELISA assay. Samples with stimulant alone or antibody alone were included as controls. Data are shown as means ± SD, n = 3. Statistical analysis was performed with one-way ANOVA followed by Dunnett’s multiple comparison to a single control group. (*) = statistical significance at p < 0.05 compared to appropriate control group (uninhibited control).
Figure 4-5. Histograms illustrating no effect of 2F8 on cell activation by LPS and PM1648 stimulation. BMDM cells from wild-type Balb/c mice were pre-treated with monoclonal anti-SR-A (clone 2F8) for 1 hour, then stimulated with LPS at 5.0 ng/mL (top panel) or PM1648 at 200 µg/mL (bottom panel), and IL-6 secretion was measured with ELISA assay. Samples with stimulant alone were included as uninhibited controls. Data are shown as means ± SD, n = 3. Statistical analysis was performed with one-way ANOVA followed by Dunnett’s multiple comparison to a single control group (uninhibited control). Statistical analysis revealed no significant difference in IL-6 levels between sample groups with and without 2F8 pretreatment.
IL-6 production using BMDM cells from TLR-4 " Balb/c mice

In order to investigate the role of TLR-4 receptor in signaling pathways for BMDM activation by LPS and PM1648, IL-6 production was measured using BMDM cells from TLR-4 " Balb/c mice. Cells from wild-type Balb/c mice were included in the assay as a positive control. Data obtained from this study showed that IL-6 secretion using BMDM cells from TLR-4 " mice was significantly reduced with LPS stimulation, compared to the results obtained from wild-type BMDM cells (Figure 4 – 6). Furthermore, when stimulated with PM1648, IL-6 production at each concentration of PM1648 was decreased by approximately 50% using BMDM cells from TLR-4 " mice, compared to the wild-type cells.
Figure 4-6. Line graph illustrating IL-6 release from TLR-4 deficient mouse BMDM cells stimulated with LPS or PM1648. Mouse BMDM from TLR-4 ^{−/−} (filled squares) or wild type Balb/c (empty triangles) mice were stimulated with LPS at 20, 10, 5, 2.5, 0 ng/mL (top panel) or PM1648 at 1000, 500, 250, 125, 0 µg/mL (bottom panel). IL-6 secretion was measured with ELISA assay. Data are shown as means ± SD, n = 3. Statistical analysis by two-way ANOVA followed by post test of Bonferroni. All cytokine data obtained from TLR-4 ^{−/−} cells, with either LPS or PM1648 stimulation, showed significant decrease (p < 0.05) compared to control values obtained from wild type BMDM cells.
IL-6 production using BMDM cells from SR-A \(^{+/−}\) C57BL/6 mice

In order to further test the role of SR-A in response to LPS and PM1648 stimulation, BMDM cells from SR-A \(^{+/−}\) (C57BL/6) mice were utilized to measure IL-6 production. Cells from wild-type C57BL/6 mice were also included as a positive control. IL-6 secretion from SR-A \(^{+/−}\) BMDM cells with LPS stimulation was apparently increased as compared to results obtained from wild-type BMDM cells, even though the amount of increase did not achieve statistical significance (Figure 4-7). However, at each concentration of PM1648 used for cell stimulation, IL-6 production from SR-A \(^{+/−}\) cells was significantly increased compared to the secretion from wild-type cells (Figure 4-7).
Figure 4-7. Histograms illustrating IL-6 production from wild type and SR-A⁻ mouse BMDM cells stimulated with LPS or PM1648. Mouse BMDM from SR-A⁻ (empty bars) or wild type C57BL/6 (filled bars) mice were stimulated with LPS at 20, 10, 5.0 ng/mL (top panel) or PM1648 at 500, 250, 125 μg/mL (bottom panel). IL-6 secretion was measured with ELISA assay. Data are shown as means ± SD, n = 3. Cytokine data obtained from SR-A⁻ cells with PM1648 stimulation (bottom panel) showed significant difference (p < 0.05) over control values obtained from wild type BMDM cells, by one tailed, unpaired t-test. (*) = statistical significance at p < 0.05.
Murine in vitro APC assay

Murine in vitro APC assays were performed using mouse BMDM cells from wild-type Balb/c mice, as antigen presenting cells, to investigate the biological effects of silica, PM1648 particulates, and LPS on APC activation. Four mouse T cell cytokines, including IFN-\(\gamma\), IL-2, IL-10, and IL-13, were quantified, as APC activation markers, with ELISA assays.

The data in Figure 4-8 show the results of IFN-\(\gamma\) and IL-2 production, the two Th1 cytokines, following exposure to the three stimulants. Silica at 200 \(\mu\)g/mL increased IFN-\(\gamma\) secretion by 105\% \(\pm\) 16\%, indicating an up-regulation of the APC activity. However, PM1648 exposure at the same concentration showed no significant effect on IFN-\(\gamma\) production. On the other hand, although LPS at 10 ng/mL did tend to decrease the amount of IFN-\(\gamma\) production by calculating the mean percentage of decrease, statistical analysis revealed no significant changes in IFN-\(\gamma\) levels compared to the control samples. Similar results were obtained with measurement of IL-2 secretion.

A different activation pattern was obtained when mouse Th2 cytokines, IL-10 and IL-13, were quantified (Figure 4-9). All three stimulants increased the amount of both IL-10 and IL-13 secretion, showing an up-regulation of APC activity.
Figure 4-8. Histograms illustrating percentage of Th1 cytokine increase / decrease from BMDM cells exposed to silica, PM1648, or LPS. BMDM from wild-type Balb/c mice were treated with silica (200 μg/mL), PM1648 (200 μg/mL), or LPS (10 ng/mL) for 2 hours, followed by addition of albumin and T cells from DO11.10 mice. After incubation for 48 hours, cytokine production was measured and the percentage of increase / decrease compared to control samples was calculated. Top panel: IFN-γ production; Bottom panel: IL-2 production. Data are shown as mean ± SD, n=3. (*) = statistical significance at p < 0.032 compared to appropriate control, by one - sample t test.
Figure 4-9. Histograms illustrating percentage of Th2 cytokine increase / decrease from BMDM cells exposed to silica, PM1648, or LPS. BMDM from wild-type Balb/c mice were treated with silica (200 μg/mL), PM1648 (200 μg/mL), or LPS (10 ng/mL) for 2 hours, followed by addition of albumin and T cells from DO11.10 mice. After incubation for 48 hours, cytokine production was measured and the percentage of increase compared to control samples was calculated. Top panel: IL-10 production; Bottom panel: IL-13 production. Data are shown as mean ± SD, n=3. (*) = statistical significance at p < 0.032 compared to appropriate control, by one – sample t test.
Inhibition of APC activation by LPS with monoclonal anti-SR-A (clone 2F8) and anti-TLR-4 (clone MTS510)

Blocking antibodies were used to determine the functions and possible cooperative mechanisms between TLR-4 and SR-A receptors for up-regulating APC response to LPS exposures. Because LPS exposure mainly increased Th2 cytokines from Balb/c BMDM, this study therefore focused on IL-13 and IL-10 production. When BMDM cells from wild type Balb/c mice were pretreated with MTS510 at 10 μg/mL, 5.0 μg/mL, or 2.5 μg/mL, followed by LPS stimulation at 10 ng/mL, a reduction of IL-13 secretion was observed and an inhibition curve was obtained based on the antibody concentrations applied. However, when cells were pretreated with 2F8 (2.0, 1.0, or 0.5 μg/mL) to block the SR-A receptor, no significant inhibition effect was observed (Figure 4-10). The antibody isotype controls were included in the assay and showed no effects. Similar inhibition results were also obtained with IL-10 measurement (data not shown).
**Figure 4-10.** Histograms illustrating percent inhibition of IL-13 secretion from BMDM cells exposed to LPS. BMDM cells from wild type Balb/c mice were pretreated with MTS510 (10, 5.0, or 2.5 µg/mL) or 2F8 (2, 1, or 0.5 µg/mL) for 1 hour, followed by LPS stimulation at 10 ng/mL for 2 hours. After incubation with ovalbumin and spleen T cells from DO11.10 mice for 48 hours, IL-13 production was measured and percentage of inhibition, compared to uninhibited controls, was calculated. Data are shown as mean ± SD, n=3. (*) = statistical significance at $p < 0.032$ compared to appropriate control, by one-sample $t$ test.
Inhibition of APC activation by PM1648 with monoclonal anti-SR-A (clone 2F8) and anti-TLR-4 (clone MTS510)

When cells were pretreated with the same antibodies but exposed to PM1648, an inhibition pattern similar to LPS exposure was obtained. When BMDM cells from wild-type Balb/c mice were pretreated with MTS510 at 10 µg/mL, 5.0 µg/mL, or 2.5 µg/mL, followed by PM1648 stimulation at 200 µg/mL, a reduction of both IL-13 and IL-10 secretion was observed. Again, no significant inhibition effect was observed with PM1648 stimulation after cell pretreatment with monoclonal anti-SR-A 2F8 (2.0, 1.0, or 0.5 µg/mL) (Figure 4-11).
Figure 4-11. Histograms illustrating percent inhibition of IL-13 secretion from BMDM cells exposed to PM1648. BMDM cells from wild-type Balb/c mice were pretreated with MTS510 (10, 5.0, or 2.5 \( \mu \)g/mL) or 2F8 (2, 1, or 0.5 \( \mu \)g/mL) for 1 hour, followed by PM1648 stimulation at 200 \( \mu \)g/mL for 2 hours. After incubation with ovalbumin and spleen T cells from DO11.10 mice for 48 hours, IL-13 production was measured and percentage of inhibition compared to uninhibited controls was calculated, compared to uninhibited control samples. Data are shown as mean ± SD, n=3. (*) = statistical significance at \( p < 0.032 \) compared to appropriate control, by one-sample t test.
Inhibition of APC activation by silica with monoclonal anti-SR-A (clone 2F8) and anti-TLR-4 (clone MTS510)

A different inhibition pattern was obtained when BMDM cells from wild type Balb/c mice were pretreated with the same antibodies but exposed to silica. Because silica stimulation increased both Th1 and Th2 cytokines with in vitro APC assay using BMDM cells from Balb/c mice, this inhibition study was measured with both Th1 and Th2 cytokine productions. When determined with Th1 cytokines (IFN-γ or IL-2), MTS510 or 2F8 pretreatment each decreased cytokine secretion from BMDM cells. More importantly, when BMDM cells were pretreated with the combination of the two antibodies, an increased inhibition was observed. When inhibition was measured with Th2 cytokines (IL-10 and IL-13), similar inhibition patterns were obtained, showing that each antibody inhibited APC activation by silica exposure, and the percentage of inhibition was increased when both antibodies were applied (Figure 4-12).
Figure 4-12. Histograms illustrating percentage of inhibition obtained from cell pretreatment with MTS510, 2F8, or with antibody combination. BMDM cells from wild type Balb/c mice were pretreated with MTS510 at 10 μg/mL, 2F8 at 2 μg/mL, or with combination of both antibodies for 1 hour, followed by silica exposure at 200 μg/mL for 2 hours. After incubation with ovalbumin and spleen T cells from DO11.10 mice for 48 hours, IFN-γ (top panel) and IL-13 (bottom panel) production was measured and percentage of inhibition was calculated, compared to uninhibited control samples. Data are shown as mean ± SD, n=3. (*) = statistical significance at $p < 0.05$ by Dunnett test. All inhibition percentage showed significant difference ($p < 0.05$) compared to uninhibited controls. Percentage of inhibition obtained from cell pretreatment with antibody combination showed significant difference ($p < 0.05$) over inhibition percentage obtained from cell pretreatment with each individual antibody.
Murine in vitro APC assays using BMDM cells from wild-type and TLR-4 \(^{-/-}\) Balb/c mice

In order to further investigate the role of the TLR-4 receptor in APC activation by silica, PM1648 particulates, and LPS stimulation, murine in vitro APC assays were performed using mouse BMDM cells from wild type compared to TLR-4 \(^{-/-}\) Balb/c mice. IL-13, a Th2 cytokine was selected as the activation marker for this assay since only Th2 - dominant responses to LPS and PM1648 were observed (Figure 4-9). Data showed that IL-13 production was significantly decreased from TLR-4\(^{-/-}\) cells stimulated with LPS, silica, or PM1648, compared to IL-13 secretion from wild type cells (Figure 4-13).

**Figure 4-13.** Histograms illustrating IL-13 production from wild-type and TLR-4 \(^{-/-}\) mouse BMDM cells stimulated with LPS, silica, or PM1648. Mouse BMDM cells from TLR-4 \(^{-/-}\) (empty bars) or W/T Balb/c (filled bars) mice were stimulated with LPS (10 ng/mL), silica (200 µg/mL), or PM1648 (200 µg/mL). After incubation with OVA (antigen) and spleen T cells from DO11.10 transgenic mice in Balb/c background for 48 hours, IL-13 secretion was measured with ELISA assay. Data are shown as means ± SD, n = 3. (*) = statistical significance at \(p < 0.05\), by one tailed, unpaired \(t\) - test.
Murine in vitro APC assay using BMDM cells from wild-type and SR-A \textsuperscript{−/−} C57BL/6 mice

In order to further investigate the role of SR-A receptor in mouse APC activation with silica, PM1648 particulates, and LPS stimulation, murine in vitro APC assays were performed using mouse BMDM cells from wild type or SR-A \textsuperscript{−/−} in the background of C57BL/6 mice. Two types of mouse T cell cytokines, IFN-γ (Th1) and IL-13 (Th2), were selected as activation markers for this assay, and were quantified with ELISA assays. No statistical significance was achieved between wild type and SR-A \textsuperscript{−/−} cells with IFN-γ production when cells were stimulated with LPS at 10 ng/mL, even though the mean of IFN-γ production did tend to lessen from SR-A \textsuperscript{−/−} cells. When cells were stimulated with silica, IFN-γ production from SR-A \textsuperscript{−/−} cells was significantly decreased compared to the wild-type cells. The two types of cells produced almost the same amount of IL-13 when stimulated with LPS at 10 ng/mL. Compared to the wild-type cells, IL-13 production was significantly decreased from SR-A \textsuperscript{−/−} cells with silica stimulation at 200 μg/mL, but not with PM1648. PM1648 stimulation did tend to increase the average IL-13 secretion from SR-A \textsuperscript{−/−} cells, but again the difference did not achieve statistical significance when compared to the wild type cells (Figure 4-14).
Figure 4-14. Histograms illustrating T cell cytokine production from wild type and SR-A<sup>−/−</sup> mouse BMDM cells stimulated with LPS, silica, or PM1648. Mouse BMDM cells from SR-A<sup>−/−</sup> (empty bars) or W/T C57BL/6 (filled bars) mice were stimulated with LPS (10 ng/mL), silica (200 μg/mL), or PM1648 (200 μg/mL). After incubation with OVA (antigen) and spleen T cells from OT-II transgenic mice in C57BL/6 background for 48 hours, IFN-γ (top panel) and IL-13 (bottom panel) secretion was measured with ELISA assays. Data are shown as means ± SD, n = 3. (*) = statistical significance at p < 0.05, by one tailed, unpaired t-test.
Discussion

TLR-4 and SR-A are two types of pattern recognition receptors expressed on antigen presenting cells. LPS has been identified as a ligand for both receptors, and it has been known that LPS signals through TLR-4 receptor. LPS exposure could result in macrophage activation and cytokine secretion, followed by activation of the adaptive immune system. On the other hand, different types of bioactive particles can lead to different health consequences. A previous study has shown involvement of SR-A in silica-induced apoptosis using Chinese hamster ovary cells transfected with SR-A (24). Several recent controlled-exposure studies in humans indicated that exposure to certain types of PM particles could induce inflammatory responses (20, 22, 23, 25). Alveolar macrophage (AM) apoptosis induced by airborne particulate matter PM1648 was reported to be mediated through SR-A (112). Moreover, exposure to airborne fine particulate matter PM$_{2.5}$ was clinically related to respiratory disorders, such as asthma morbidity (111). However, the molecular mechanistic basis for the observed health effects induced by the bioactive particles is not yet completely understood. This subject is likely to become more important as the research community focus more on the field of molecular epidemiology for the bioactive particulate induced health consequences.

Our results demonstrated that BMDM exposure to bioactive particulates and LPS could increase their APC activity in vitro, as measured by Th cytokine production. Meanwhile, since selective blocking of TLR-4 or SR-A receptors with monoclonal antibodies was able to inhibit certain T cell cytokine secretion, together with cytokine data obtained from TLR-4 or SR-A deficient animals, we have demonstrated that signaling pathways for LPS and bioactive particulates, including silica and PM1648,
were mediated through different receptor combinations. Silica uses both SR-A and TLR-4 to activate APC efficiently, while both LPS and PM1648 use TLR-4 for macrophage activation. However, our further investigations suggested that LPS and PM1648 might bind to SR-A as receptor ligands, even though macrophage activation appeared to be mediated through TLR-4 and maybe some other receptors. Furthermore, by using SR-A deficient cells, our data also suggested that SR-A might function as a negative regulator in BMDM activation by LPS and PM1648.

LPS has been used as a stimulant control in this project, and data obtained from the current study was in agreement with previous findings from human studies (see Chapter 2 and 3). LPS-stimulated macrophage cytokine production (IL-6 and IL-12 p40) was dependent on TLR-4 since mouse BMDM cells from TLR-4/- Balb/c mice did not respond to LPS stimulation (Figure 4-6). However, both wild type and SR-A-/- BMDM cells from C57BL/6 mice were activated to a similar extent in response to LPS stimulation, and indeed, SR-A-/- cells tended to have a higher cytokine production than the wild-type cells (Figure 4-7). This finding indicated that SR-A might play a role of negative regulation on cell activation by LPS. Therefore, even though LPS has been well identified as a ligand for direct binding to both cell surface receptors, our results indicated that BMDM activation by LPS was mediated through TLR-4 receptor, and SR-A might function as a negative regulator in this process, possibly by its function of LPS clearance and detoxification. Data from murine in vitro APC assays with selective monoclonal antibody inhibition confirmed the above finding. BMDM cells from wild-type Balb/c mice mainly produced Th2-associated cytokines (IL-10 and IL-13) in response to LPS challenge. Pre-incubation of TLR4-specific monoclonal antibody
MTS510 with the cells resulted in reduction of cytokine production, and an antibody dose-dependent inhibition curve was obtained (Figure 4-10, top panel). Meanwhile, when SR-A specific monoclonal antibody 2F8 was applied in the same procedure, no inhibition effect was observed (Figure 4-10, bottom panel). Therefore, with the APC inhibition assay data we again demonstrated that between the two receptors, BMDM activation by LPS was mediated through TLR-4.

Available studies have indicated that LPS and its lipid A component, as well as synthetic MPL adjuvant whose structure was based on active lipid A component, had the capacity to mainly induce a Th1 immune response, characterized by cell-mediated immunity and production of Th1 cytokine (IFN-γ) \(^{(31)}\). However, with our mouse in vitro APC assay using wild type Balb/c BMDM cells, our data showed that LPS stimulation mainly triggered a Th2 response (Figure 4-8 and 4-9). A previous study conducted at Yamanouchi Pharmaceutical Corporation (Ibaraki, Japan) provided a possible explanation for the conflicting results, and suggested that the addition of ovalbumin (OVA) to the assay system could play a role in determining the different Th responses \(^{(26)}\). Their study investigated the effect of antigen dose on immune response, with Balb/c and C57BL/6 mice being sensitized by aluminum hydroxide gel (alum)–precipitated OVA then challenged with aerosolized OVA. Their data showed that sensitization with different doses of OVA could elicit different Th responses. Sensitization with high dose OVA mainly induced Th1–type cytokine production in the lungs of C57BL/6 mice, whereas Balb/c mice mainly generated Th2–type cytokines in response to the same sensitization. More interestingly, they found that a wide range of OVA sensitization dose (100 ng – 1 mg) did not affect the types of Th responses from splenocyte cultures of
either mouse type. A Th1 type response was always triggered in C57BL/6 mice, whereas a Th2 type response was always observed from Balb/c mice when their splenocyte cultures were tested in vitro. This study therefore concluded that C57BL/6 and Balb/c mice had different susceptibilities to OVA – sensitization because the antigen dose range could determine the types of Th responses in the lungs of the two different groups of mice, but independent of the splenic Th cytokine responses \(^{(26)}\). With one of our murine APC assay systems using BMDM cells from Balb/c mice, OVA, at a high concentration of 10 mg/mL, was added to the assay culture system with spleen T cells from transgenic DO11.10 mice in Balb/c background. Therefore, such a culture system could well exhibit a Th2 dominant response to LPS challenge. On the other hand, with another APC assay system using C57BL/6 BMDM cells and spleen T cells from transgenic OT-II mice in C57BL/6 background, we obtained both Th1 and Th2 cytokines in response to LPS stimulation (Figure 4-14).

Our data also indicated that PM1648 could up-regulate Th2 cytokine production (IL-10 and IL-13), but it had no significant effect on Th1 cytokine secretion from mouse in vitro APC assays, with either Balb/c – DO11.10 or C57BL/6 – OT-II systems. These results were very similar to a previous report showing that PM1648 could increase IL-4 production from human alveolar macrophage (HAM) and peripheral blood monocytes, but did not affect Th1 cytokines \(^{(22)}\). More importantly, due to the absence of SR-A on blood monocytes, their data also suggested that PM1648 might bind and signal through some other cell surface receptors for inducing Th2 cytokine production. This proposal may provide explanations for the above phenomenon that PM1648 exposure primarily triggers a Th2 cytokine response and it can affect both HAM and peripheral blood.
monocytes (22). Similar as LPS, we found that SR-A might play a role in negatively regulating cell activation by PM1648. This result is apparently in agreement with the previous findings. With both APC and macrophage cytokine assays, we also demonstrated that one of the signaling pathways for BMDM activation by PM1648 was TLR-4 - dependent.

The SR-A blocking antibody 2F8 had no inhibitory effect towards Th2 cytokine production induced by PM1648 (Figure 4-11, bottom panel). In contrast, MTS510, the TLR4 - specific monoclonal antibody, inhibited PM1648 - induced Th2 cytokine production up to approximately 45% (Figure 4-11, top panel). Similar results were obtained from both macrophage cytokine assays and in vitro mouse APC assays using receptor deficient cells. Both wild type and SR-A" cells produced macrophage cytokines (Figure 4-7, bottom panel) and Th2 - associated cytokines (Figure 4-14, bottom panel) in response to PM1648 stimulation. However, PM1648 challenge induced significantly less macrophage cytokines (Figure 4-6, bottom panel) as well as Th2 cytokines (Figure 4-13) from TLR-4" cells compared to the wild type cells. Taken together, these data clearly indicated that PM1648 signaling was in part mediated through TLR-4 receptor. However, as previous studies suggested (112), some other scavenger receptors, such as MARCO, might be involved in PM1648 signaling since BMDM activation by PM1648 exposure was not completely blocked by using either blocking antibody (MTS510) or TLR-4 deficient cells.

The possibility of cell activation by LPS contamination on PM1648 particles was taken into consideration. Residual endotoxin on PM1648 particulates was detected with a Limulus assay at 1.04 EU/mg. However, with the PM1648 concentrations used for cell
stimulation in this study, the concentration of possible LPS contamination was at low pg/mL levels, from 1.3 to 5.2 pg/mL. Previous results (Wang, et al., 1997) have shown that LPS stimulation at such low concentrations would not be able to activate macrophage cells and induce cytokine secretion. Therefore, the low level of LPS concentration on PM1648 suggested that BMDM cells were not activated by LPS contamination, but rather the stimulation was from some other component of PM1648.

Interestingly, PM1648 stimulation resulted in a significant increase of macrophage cytokine production, with a dose-dependent manner, from SR-A−/− BMDM cells compared to wild type C57BL/6 BMDM cells (Figure 4-7, bottom panel). As shown in the same figure, a similar cytokine increase was obtained when the SR-A−/− cells were stimulated with LPS, even though the increase in this case did not reach statistical significance. A similar observation was obtained from a previous study (13), showing that instead of activating a cytokine response, SR-A – mediated LPS uptake actually suppressed cytokine production in response to LPS exposure, suggesting that LPS binding to SR-A might result in various consequences, determined by the extent of activation of the macrophages. As described earlier, our data also suggested that SR-A might play a role in negatively regulating mouse BMDM activation in response to LPS and PM1648, probably due to the SR-A mediated endotoxin and particle clearance.

However, another potential role of SR-A receptor, which was just described in a recent study (Jozefowski and Kobzik, 2004), might also be involved in the above phenomenon. This study found that SR-A on murine macrophages might be able to affect the different directions of Th responses by generating H₂O₂ to suppress IL-12 production from macrophages while the cell surface SR-A receptor was engaged with ligands. A
similar observation was obtained from a recent study (Migliaccio, Holian, et al. unpublished data), showing that with the presence of ovalbumin in the culture system, IL-12 production was significantly decreased from BMDM cells (from w/t Balb/c mice) treated with silica, a known ligand for SR-A receptor. This may help to explain the tendency of PM1648 exposure to bias a Th2 cytokine response in our APC assays, with either Balb/c or C57BL/6 cells. Meanwhile, it may also help to explain the increase of macrophage cytokine that we obtained with SR-A-/- BMDM cells. Even though our current study indicated that both LPS and PM1648 did not signal through SR-A receptor for APC activation, SR-A has been well known to play a role in immune response against bacterial infection by mediating pathogen binding and phagocytosis, and LPS has been identified as a ligand for direct binding with SR-A. Similarly, our present data also suggested that PM1648 particulate might be involved in direct binding with SR-A as well. The engagement of LPS and PM1648 to cell surface SR-A receptor could result in generation of H$_2$O$_2$ followed by suppression of certain cytokine productions, which might include both IL-12 and IL-6. With the SR-A-/- cells, we propose that due to the absence of surface SR-A receptor and the receptor – ligand engagement, LPS and PM1648 treatment did not result in H$_2$O$_2$ production, therefore the SR-A-/- macrophages could operate free of the H$_2$O$_2$ suppressing influence, enhancing the production of IL-12 and IL-6 as compared to the wild type cells.

Investigation of surface receptors for silica signaling pathways rendered different results. Both Th1 and Th2 responses were obtained when BMDM cells from wild type Balb/c mice were treated with silica (Figure 4-8 and 4-9). A different inhibition pattern was obtained with APC antibody - inhibition assays. MTS510 and 2F8 each
demonstrated an inhibitory effect on cell activation by silica, when activation was measured with either Th1 or Th2 cytokines (Figure 4-12). When cells were pretreated with the combination of both antibodies, an additive effect of inhibition was observed (Figure 4-12). Similar results were obtained from APC – T cell assays using receptor deficient APC cells, showing significant reductions of both IFN-γ and IL-13 from SR-A⁻/⁻ BMDM cells (Figure 4-14) and a reduction of IL-13 from TLR-4⁻/⁻ BMDM cells as antigen presenting cells (Figure 4-13). Taken together, the current study indicated that both TLR-4 and SR-A receptors play a role in silica signaling pathways for APC activation.

Limulus assays were again performed for detection of residual endotoxin on silica particulates in order to confirm the above conclusion that silica signals through both receptors. No detectable amount of LPS was found on silica particulates with the Limulus assay (data not shown). In addition, the macrophage cytokine assays performed in the present study found no measurable amount of cytokines (IL-6 and IL12 p40) from cells stimulated with silica, whereas dose response curves were obtained from cells activated by LPS stimulation (Figure 4-2). Therefore, macrophage cytokine secretion would be well expected if a significant amount of LPS contamination existed on silica. Taken together, these data confirmed the Limulus assay results, indicating that no LPS contamination on silica particles was activating macrophages.

However, the low amount of macrophage cytokine secretion, below detectable levels of ELISA assays, from silica – treated BMDM cells raises another question. Such a low amount of cytokine production was obtained from cells of wild-type mice as well as receptor deficient mice, from either Balb/c or C57BL/6 background. Since silica
stimulation did trigger both Th1 and Th2 responses in APC - T cell assays, the low macrophage cytokine data suggested that macrophages activated by silica treatment sent signals other than cytokines, IL-6 and IL-12 p40 in this case, to T cells and eventually activated T cells. Recent studies (Hamilton, et al., unpublished data) supported this hypothesis. In their experiments following the APC - T cell assay protocol, BMDM cells from wild-type Balb/c mice were incubated with OVA and spleen T cells in routine cell culture plates. However, insertion of “filter trans-wells” that contained BMDM cells being treated with silica to the culture plate wells did not increase, in some cases even decreased, T cell cytokine production (IFN-γ and IL-13) compared to the wells without “trans-well” insertions. Since “trans-wells” only allow soluble materials to pass through the filter, their data therefore indicated that signal factors, perhaps cell surface bound, other than soluble cytokines from silica - treated APC cells contributed to T cell activation. Further studies are required for investigation of signal factors sent by BMDM cells stimulated with silica.

In conclusion, the present study demonstrated that silica uses both TLR-4 and SR-A receptors for signaling through a possible “cross-talk” cooperative mechanism in order to activate macrophages efficiently, while endotoxin and PM1648 use TLR-4 for macrophage activation, even though SR-A might be involved in negative regulation of the cell activation process, as well as in mediating endotoxin and particulate direct binding and clearance. Such findings might be well related to the different health implications caused by silica and PM1648 exposure, as silica has been related mainly to fibrosis in the lung, whereas PM exposure has been known to exacerbate asthma. The different receptor combinations, signaling mechanisms and pathways employed by the
two particulates for activating macrophages, which in turn initiate innate and adaptive immune responses, might contribute differently to lung pathogenicity upon exposure to the two different bioactive particulates, and might lead to different health consequences.
CHAPTER 5
Conclusions & Future Studies

The innate immunity is the first line of host defense system that enables multicellular organisms to rapidly and efficiently respond to infectious agents. One of the most important components of this quick – reaction force is a sensitive and accurate detection system, that consists of a set of immune cells and receptors expressed on the surface of these cells that can recognize particular molecular features associated with the pathogens and initiate signal transduction. Therefore, these receptors are named as pattern recognition receptors (PRR). Antigen presenting cells (APC), including macrophages and dendritic cells, express a wide range of PRRs involved in pathogen recognition, phagocytosis, antigen destruction or presentation to T cells, as well as induction of adaptive immunity. The current study focused on the signaling mechanisms and potential ligands for two types of PRRs, Toll-like receptor 4 (TLR-4) and scavenger receptor (SR-A).

TLRs recognize a variety of structural components that are unique and highly conserved in pathogens. The interactions between TLR and its specific ligands activate the host defense system with both innate and adaptive immune responses. After TLR – mediated recognition of pathogen components, a variety of immune cell types may be activated to initiate a series of defensive actions against the invading pathogen, including phagocytosis, production of reactive oxygen intermediates, up-regulation of MHC complex and surface co-stimulatory molecules, and secretion of inflammatory cytokines. These cytokines may activate natural killer cells, and furthermore, they may initiate and direct the adaptive immune system. Thus, TLRs possess critical functions in innate
immunity with short-term anti-microbial effects, as well as in adaptive immunity with long-term effects and immunological memory (31). In addition, recent studies have revealed relationship between vaccine adjuvant activity and TLR functions, indicating that most vaccine adjuvants function via TLR-mediated recognition and signaling pathways. Therefore, development of TLR agonists and antagonists as potential adjuvant candidates has become a novel and effective approach in the adjuvant research field, since it has been clear that the initiation and manipulation of innate immune responses through certain TLRs could play a critical role in a successful vaccination with long-term protective immunity (33, 34).

So far, the known ligands that signal through TLRs are mainly from exogenous origins, but there are reports of endogenous ligands as well as intracellular ligand-receptor interactions rather than surface engagement through TLRs (37, 38). Among the many identified TLR ligands, LPS from Gram-negative bacteria is one of the best understood ligands for TLR-4 receptor, and the mechanism and pathways for LPS signaling through TLR-4 to initiate innate immune responses have been illustrated (28, 29, 30). In addition, MPL adjuvant, developed by Ribi ImmuneChem Research Inc., is an immunoactive lipid A fraction derived from the LPS of Salmonella Minnesota R595 through sequential steps of acid and base hydrolysis. Pre-clinical and clinical studies focusing on the functional mechanism of MPL adjuvant have shown that the immunological activity of MPL adjuvant was mediated through TLR-4 receptor, which is very similar to its parent molecule LPS. Based on the experience and knowledge from MPL studies, scientists at Corixa Corporation developed a library of novel synthetic glycolipids, the so-called “AGP” compounds. More importantly, some AGP compounds
have shown very similar adjuvant activities as those induced by MPL, suggesting that the synthetic compounds and MPL adjuvant might share the same or similar intracellular signaling pathways.

The adjuvant activity, immune potency and efficacy, as well as clinical safety of MPL adjuvant have been well evaluated during the past 15 years in human clinical trials with various disease models, including cancer, autoimmune diseases and infectious diseases. Study results indicated that MPL adjuvant was safe and efficacious for use in human vaccines in these clinical trials. Meanwhile, pre-clinical studies also demonstrated that some leading AGP compounds were as effective as MPL adjuvant for certain disease models, and in some cases even improved clinical safety due to their synthetically designed chemical structures and high purity (7, 31).

In order to further characterize immunological mechanisms for AGP activity, it was first necessary to evaluate AGP biological potencies with a consistent and reproducible assay system, and identify certain connections between AGP structural characteristics and its biological potency. Chapter Two of this study focused on the development of a reproducible in vitro cell assay system using human monocytic cell lines MonoMac-6 and U937, and this system was applied for evaluation of AGP biological potencies. Data reported in Chapter Two are in excellent agreement with previous work accomplished at Corixa Corporation using in vitro or in vivo models, indicating that secondary fatty acid chain length played an important role in AGP potency. AGP compounds that carry 9, 10, 11, or 12 carbons in their secondary fatty acids were immunologically much more potent compared to compounds with either short (6 – 8 carbons) or long (14 carbons) secondary fatty acid chains. Compounds with ten-
carbon fatty acids in all three secondary positions showed the highest biological potency in numerous evaluation studies. Such results suggested that certain structural parameters had significant effects on AGP biological activity. Critical structural characteristics, such as acyl chain length, spacing, distance, and the resulting packing tightness in the solution, could directly affect compound solubility, stability, and its energetically favored or disfavored conformation in aqueous solution, therefore leading to different immune stimulating capacities. The conformational changes resulting from acyl chain length / spacing would be involved in the specificity of AGP - induced immune responses as well, since structural flexibility would affect the binding access of cell surface receptors and molecules to the compounds. Hence, manipulation of certain structural parameters could have a critical impact on a compound's adjuvant activities. Because AGPs are synthetic chemical compounds, their structures can be designed and modified for clinical and medicinal needs. Certain structural parameters can be identified as key components that are responsible for compound biological potency, the ability to bias and direct immune responses, as well as safety profiles. Such characterization and development work would help further discovery of immune stimulants and adjuvants that could become leading candidates for new clinical products with high efficiency and specificity, but with low toxicity and fewer side effects.

Chapter Three explored the TLR-4 signaling functions on AGP compound activity through biological inhibition assays, direct binding assays, as well as binding inhibition studies. By using monoclonal anti-human-TLR4 (clone HTA125), we demonstrated that both MPL adjuvant and AGP compounds were signaling through TLR-4 receptor, similar to their parent molecule, LPS.
Even though TLR-4 receptor has been well recognized as the primary recognition molecule for LPS, the detailed and actual mechanism of ligand binding and signaling through the receptor remains unclear. In addition, although TLR-4 is a pattern recognition receptor, like scavenger and mannose receptors, its functions on LPS clearance are unknown. The current understanding of the signaling mechanism involves LPS binding protein (LBP), CD14, and a small glycosylated protein, MD-2. LBP first binds and transfers LPS to CD14 for signal complex formation, which in turn initiates intracellular signaling of LPS via membrane TLR-4. Additional evidence also suggested that MD-2 was required in the TLR-4 signaling complex for optimal function\(^\text{(33)}\). Our LPS direct binding assay indicated that addition of soluble CD14 and LBP was required for Alexa-LPS direct binding to cell surface, which was consistent with the above description. The inhibition data reported in Chapter Three, using unlabeled LPS and HTA125 to inhibit Alexa-LPS direct binding to human MonoMac-6 cells, indicated a two-step binding mechanism of LPS to TLR-4, which was also in agreement with the above description. The mechanism might first involve a complex formation of LPS/LBP/CD14/MD-2, which in turn initiate signaling by complex binding to and aggregating cell surface TLR-4. These inhibition data also suggested that besides TLR-4, other receptors, such as SR-A, might be involved in direct binding of LPS to the cell surface.

The term “scavenger receptor (SR)” covers a wide range of structurally related or unrelated membrane molecules expressed mainly by macrophages and endothelial cells. To date, numerous polyanionic ligands for SR receptors have been identified. Among these, the endocytosis of modified low-density lipoprotein (LDL) by Class A SR (SR-A) has been the best characterized. The understanding of the role of SR-A receptor in the
The innate immune system has been developed mainly based on the following evidence. First, binding studies have shown that certain conserved molecular features associated with pathogens, such as LPS from Gram-negative and LTA from Gram-positive bacteria, could compete with some other known ligands for direct binding to SR-A. Such observations suggested that SR-A could function as a PRR, activating innate responses by direct binding to conserved bacterial structures. Second, SR-A is primarily expressed on the cell surface of macrophages, and macrophages are the main players of innate immune system that functions as the first line of anti-microbial defense. Additional evidence indicates that SR-A may function as an adhesion molecule in vitro, and similar to other PRR molecules, SR-A may also directly bind to modified – host components and to exogenous ligands, such as LPS and LTA, and in turn mediate endocytosis and phagocytosis.

However, the intracellular signaling pathways induced by SR-A have been poorly defined, as the ligands utilized in many in vitro and in vivo studies could bind to a range of SR-A molecules. This makes characterization studies of signal pathways mediated via certain receptors very difficult to perform. The structural basis for receptor – ligand interactions, as well as cooperative mechanisms of SR-A with other receptors, therefore, also remains unclear. Chapter Four of this study investigated the functions and cooperative mechanisms between SR-A and TLR-4 receptors for up-regulating mouse APC responses to LPS, as well as to silica and PM1648, the two bioactive particulates that have shown implications for adverse health effects, such as silicosis and asthma. The pathogenic responses to the bioactive particulates have been suggested to be mediated through certain immune system dysfunctions.
In vitro inhibition assays using specific monoclonal antibodies to block either receptor, or both, on mouse macrophages, followed by cell stimulation with the bioactive particles and LPS were performed in Chapter Four. Similar studies using cells from receptor deficient animals were performed as well. By comparing macrophage cytokine and T cell cytokine secretion from either wild type or receptor deficient cells, we concluded that signaling pathways for LPS and the two bioactive particulates were mediated through different receptor combinations. Silica needs both SR-A and TLR-4 to "turn on" APC activity efficiently, whereas both LPS and PM1648 use TLR-4 for macrophage activation. Furthermore, SR-A appeared to be involved in direct binding to LPS and PM1648, and might even function as a negative regulator in macrophage activation when challenged by LPS and PM1648. The different receptor combinations and signaling mechanisms employed by the two particles for activating macrophages might contribute differently to lung pathogenicity upon exposure to the particles, as silica exposure is mainly related to fibrosis, whereas PM has been known to exacerbate asthma. Further in vivo studies with receptor deficient animals being exposed to the particles would help confirm the present in vitro findings by observation and measurement of the resulting in vivo pathogenic responses to the two types of particles.

Macrophage SR-A receptors include a wide range of surface molecules that are involved in receptor-mediated endocytosis of selected polyanionic ligands. Some of these receptors also function in phagocytosis of apoptotic cells and bacteria. A previous study (96) showed that LPS exposure induced SR-A surface expression on mouse macrophages. This phenomenon has been related to the observed resistance towards endotoxemia in mice. Additional murine in vivo studies have shown that in LPS – induced lung injury,
SR-A could play a protective role by uptake and clearance of LPS and apoptotic cells\(^{(97)}\). Another previous study conducted at Scripps Research Institute (Tobias, et al, 2004) found no involvement of TLR-4 in cellular LPS uptake mechanisms\(^{(33)}\). Instead, their data indicated that LPS uptake in CD14-positive monocytes was predominantly mediated through a CD14 – dependent pathway. In CD14-negative endothelial cells, on the other hand, SR-A was found to play a critical role in LPS clearance and detoxification. Our data presented in Chapters Three and Four are consistent with the previous studies, showing that SR-A might be involved in LPS direct binding to the cell surface, but LPS binding to SR-A did not transmit signals, suggesting a host protective role of SR-A that mediates a disposal system for toxic and pathogenic substances.

Significant numbers of studies have been performed focusing on the effects of endotoxin exposure on TLR-4 and SR-A expression\(^{(96, 97, 99 – 103)}\). However, there is very limited information regarding the effects of particulate exposure on the biosynthesis and function of these two receptors. The present study investigated the pretreatment effects of the two bioactive particulates, silica and PM1648, on surface expression levels of TLR-4 and SR-A, and our data indicated that the particulate exposure could increase expression of both receptors (data not shown).

It has been well accepted that the physical contact between particulates and the cell membrane is the first and critical step for macrophage activation in response to particle exposure. Particulates could be uptaken by immunocompetent cells for clearance, and such engulfment might trigger different biochemical pathways, leading to cell activation. In addition, particulates could be rendered antigenic properties by adsorbing proteins, phospholipids, or metal ions on particle surface. SR-A has been suggested to
possess double functions in host defense system, including a host – protective role by mediating a disposal system for toxic and pathogenic substances, as well as the other critical function in both innate and adaptive branches of the immune response. Indeed, characterization studies have shown that SR-A played a role in exogenous antigen presentation\(^{(98)}\). Therefore, it would be expected that particle exposure could trigger SR-A biosynthesis pathways for its enhanced particle clearance and potential antigen presentation functions.

Available data also suggested that there might be receptors other than SR-A involved in particle signaling\(^{(18, 22)}\). The present study indicated that TLR-4 was involved in silica and PM1648 signaling for mouse APC activation, but its detailed functional mechanism is not yet clear. Based on the available knowledge, TLR-4 mediated LPS signaling pathways fall into two categories: the common MyD88 – dependent pathway and the specific Trif – dependent pathway. The question of whether the same pathways are required for TLR-4 mediated particle signaling has not been answered, and this question is worth the research effort in the future. In addition, it will be necessary to further investigate if other novel pathways and adaptor proteins are involved in TLR-4 mediated intracellular signaling mechanisms for the bioactive particles. The present study also indicated that both silica and PM1648 pre-exposure could increase TLR-4 surface expression (data not shown). However, the mechanism of TLR-4 up-regulation by bioactive particulate exposure again remains unknown. Therefore, the biological significance of differential TLR-4 expression needs to be examined as well.

Previous studies indicated that silica exposure could result in SR-A – mediated caspase activation in macrophages cells, leading to subsequent apoptosis and necrosis\(^{(18)}\).
The present study also indicated that both TLR-4 and SR-A were required for signaling to activate mouse APC cells by silica exposure. However, our macrophage cytokine tests found no detectable amount of IL-6 and IL-12 p40 from BMDM cells stimulated with silica, suggesting that macrophages stimulated with silica sent signals other than soluble cytokines to T cells. Previous “trans-well” study results were in agreement with our current data, indicating that signal factors other than macrophage cytokines from silica treated cells, perhaps membrane bound, had contributions to T cell activation. However, information is not available at this point regarding the mechanism implicated in the immune responses initiated by silica exposure. It would be of interest to examine and identify the signal factors sent by the silica - treated macrophages to the T cells that eventually turn on the host defense reaction.

The identification and functional characterization of cell surface pattern recognition receptors (PRR) in murine and human models have brought our understanding of the innate and adaptive immune system to a new level, but the picture is far from complete. The function of these receptors in host defense is so important and fundamental, but our knowledge and understanding regarding these receptors is still quite limited due to the complication that the PRR function is actually related to most aspects of the mammalian immune system. Hence, knock – out mutations of these critical receptors are most likely to cause immunodeficiency. On the other hand, over expression of the receptors might result in inflammatory or autoimmune disorders. In the mean time, PRRs have also become crucial research targets for immune interventions due to the importance of their effects on initiating and directing the adaptive immune responses. For example, information obtained from these studies might be potentially useful.
therapeutically, as it might lead to the development of novel agonists or antagonists of
PRRs as candidates for potential new adjuvant products that function through
pharmacological manipulation of innate immune responses. The work presented in this
dissertation focused on two pattern recognition receptors, TLR-4 and SR-A. Although
progress has been made in characterizing and understanding these two pattern recognition
receptors, there are still many more unanswered fundamental questions, such as the
detailed mechanisms of ligand recognition, cooperative mechanisms among different
receptors, and the different cellular and immune responses induced by various receptor –
ligand engagements. In the future, the answers to these questions will greatly help us to
better understand the complicated action force that the host immune system utilizes to
detect and dispose invading pathogens without destroying self tissues.


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