An Entirely Cell-based System to Generate Single-chain Antibodies against Cell Surface Toll-like Receptors

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Immunology in the Graduate School of Duke University

2008
ABSTRACT

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Abstract

The generation of recombinant antibodies (Abs) using phage display is a proven method to obtain a large variety of Abs that bind with high affinity to a given antigen (Ag). Traditionally, the generation of single chain Abs depends on the use of recombinant proteins in several stages of the procedure. This can be a problem, especially in the case of cell surface receptors, because Abs generated and selected against recombinant proteins may not bind the same protein expressed on a cell surface in its native form and because the expression of some receptors as recombinant proteins is problematic. To overcome these difficulties, we developed a strategy to generate single chain Abs that does not require the use of purified protein at any stage of the procedure. In this strategy, stably transfected cells are used for the immunization of mice, measuring Ab responses to immunization, panning the phage library, high throughput screening of arrayed phage clones, and characterization of recombinant single chain variable regions (scFvs). This strategy was used to generate a panel of single chain Abs specific for the innate immunity receptor Toll-like receptor 2 (TLR2). Once generated, individual scFvs were subcloned into an expression vector allowing the production of recombinant antibodies in insect cells, thus avoiding the contamination of recombinant Abs with microbial products. This cell-based system efficiently generates Abs that bind native molecules displayed on cell surfaces, bypasses the requirement of
recombinant protein production, and avoids risks of microbial component contamination. However, an inconvenience of this strategy is that it requires construction of a new library for each target TLR. This problem might be solved by using non-immune antibody libraries to obtain antibodies against multiple TLRs. Non-immune libraries contain a wide variety of antibodies but these are often low affinity, while immune libraries, derived from immunized animals, contain a high frequency of high affinity antibodies, but are typically limited to a single antigen. In addition, it can be difficult to produce non-immune libraries with sufficient complexity to select Abs against multiple Ags. Because the re-assortment of $V_H$ and $V_L$ regions that occurs during antibody library construction greatly increases library complexity, we hypothesized that an immune antibody library produced against one member of a protein family would contain antibodies specific for other members of the same protein family. Here, we tested this hypothesis by mining an existing anti-hTLR2 antibody library for antibodies specific for other members of the TLR family. This procedure, which we refer to as homolog mining, proved to be effective. Using a cell-based system to pan and screen our anti-hTLR2 library, we identified single chain antibodies specific for three of the four hTLR2 homologs we targeted. The antibodies identified, anti-murine TLR2, anti-hTLR5, and anti-hTLR6, bind specifically to their target, with no cross-reactivity to hTLR2 or other TLRs tested. These results demonstrate that combinatorial re-assortment of $V_H$ and $V_L$ fragments during Ab library construction increases Ab repertoire complexity,
allowing antibody libraries produced by immunization with one antigen to be used to obtain antibodies specific to related antigens. The principle of homolog mining may be extended to other protein families and will facilitate and accelerate antibody production processes.

In conclusion, we developed an entirely cell-based method to generate antibodies that bind to native molecules on the cell surface, while eliminating the requirement of recombinant proteins and the risk of microbial component contamination. With homolog mining, this system is capable of generating antibodies not only against the original immunized Ag, but also against homologous Ags. In combination, this system proved to be an effective and efficient means for generating multiple antibodies that bind to multiple related Ags as they are displayed on cell surfaces.
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<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
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<tr>
<td>ALI</td>
<td>acute lung injury</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchial alveolar lavage</td>
</tr>
<tr>
<td>BRASIL</td>
<td>biopanning and rapid analysis of selected interactive ligand</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCL-</td>
<td>chemokine (C-C motif) receptor ligand-</td>
</tr>
<tr>
<td>CCR-</td>
<td>chemokine (C-C motif) receptor-</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>chemokine (C-X3-C motif) receptor 1</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell;</td>
</tr>
<tr>
<td>ExMAC</td>
<td>exudate macrophage;</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunoabsorbent assay</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GFP</td>
<td>green-fluorescent protein</td>
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<tr>
<td>hTLR</td>
<td>human Toll-like receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>IL-</td>
<td>interleukin</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
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<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LTA</td>
<td>lipoteichoic acid</td>
</tr>
<tr>
<td>MCP-</td>
<td>monocyte chemotactic protein-</td>
</tr>
<tr>
<td>MFI</td>
<td>mean florescent intensity</td>
</tr>
<tr>
<td>moDC</td>
<td>monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>mTLR</td>
<td>murine Toll-like receptor</td>
</tr>
<tr>
<td>NK</td>
<td>nature killer</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear</td>
</tr>
<tr>
<td>scFv</td>
<td>single-chain variable fragment</td>
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<tr>
<td>SEAP</td>
<td>secretory alkaline-phosphatase</td>
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<tr>
<td>s.c.</td>
<td>sub-cutaneous</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow-fluorescent protein</td>
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Acknowledgment

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Part I

1. Introduction

1.1 Phage-displayed single-chain antibody (scFv) system

The generation of recombinant antibodies by the creation of single chain Ab libraries and their screening by phage display [1, 2] has been in used for nearly two decades and many modifications have been reported to fit different needs of Ab generation [1, 3, 4]. The basic principle, however, is unchanged: an antibody library is generated from the antibody producing cells of pools of human donors or from animals immunized with a target antigen. Typically the target antigen is expressed as a recombinant protein and used for selection and screening of this library. As summarized in Figure 1, the processes involved are often simple and fast, and multiple Ab clones can be obtained in a very short period of time. For these reasons, screening phage display libraries has been a powerful tool for generating both research and therapeutic reagents [5]. Several recombinant Abs are currently approved as therapeutics, while many additional scFv-derived molecules are in the latter phases of clinical trials [5, 6].
Figure 1: Overview of phage-display scFv system.

1.2 Monoclonal Ab production methods: hybridoma vs. scFv

Traditionally hybridomas [7] have been the predominant method used for obtaining monoclonal antibodies, even after the introduction of phage-displayed single chain Abs. Although both systems have proven to be powerful tools to generate Abs, there are several essential differences between hybridomas and single chain Abs. First, generation of antibodies is always at the cellular level in the hybridoma system, whereas phage-displayed scFv systems utilize several molecular cloning techniques to obtain genes expressing V\text{H} and V\text{L} fragments to produce recombinant antibodies from selected genes. Because these recombinant Abs are produced and maintained using standard molecular biology techniques, they provide greater genetic stability than hybridomas and can be easily re-engineered into alternative forms, including conversion into humanized Abs, isotype switching, and affinity maturation. Second, hybridomas may be screened for specificities without a selection step to enrich Ag-binding fractions in the Ab pool, whereas, due to the vast complexity of scFv libraries, a selection step is necessary for single chain Ab libraries to enrich the desired Ab clones to a sufficient level for a feasible screening. Third, a process of re-assortment occurs during the formation of scFvs wherein the heavy chain from one antibody can join to the light chain from a different antibody, thereby creating a unique antibody that was not present in the immune repertoire of the donor animal. This combinatorial re-assortment can create antibodies recognizing epitopes that might not have elicited an antibody response due
to tolerance, as well as antibodies that bind to antigens that are too toxic to use as immunogens. Because of the re-assortment and high complexity of phage-displayed scFv libraries, it is also possible to use a naïve library to generate Abs, while immunization is almost always required for hybridomas. Another important difference is the relative speed of these two systems. Because single chain Abs are handled at the molecular level in prokaryotic systems and immunization can be skipped in some cases, the number of phage antibodies that can be generated and screened is vast compared to the number of hybridoma cells that can be produced and examined in the same period of time, or from the same number of immunized animals. It is possible to generate Abs in a very short period of time [8] (within 2 weeks from a mouse spleen to Abs [9]); whereas mAbs generated from hybridomas are always handled at the cellular level and require several weeks or even months from immunization, colony isolation, and screening before one can obtain the desired Abs.

In addition to these differences, displaying scFvs on the surface of bacteriophage M13 [10] has several advantages relative to traditional hybridoma technology. These are summarized in Table.1.
<table>
<thead>
<tr>
<th></th>
<th>Hybridoma</th>
<th>Phage-displayed scFv system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex mammalian cell culture</td>
<td>Required. Ab is produced in cellular level</td>
<td>Not required. Recombinant Ab cloned and engineered in <em>E.coli</em></td>
</tr>
<tr>
<td>Stability of Ab gene</td>
<td>Less stable and may need enrichment after several passages of cell culture</td>
<td>More stable even after long-term culture, molecularly defined</td>
</tr>
<tr>
<td>Availability of Ab gene</td>
<td>Require additional cloning steps</td>
<td>Readily available for analysis or further manipulation</td>
</tr>
<tr>
<td>Library complexity</td>
<td>Lower</td>
<td>Higher, due to re-assortment of VH and VL fragment</td>
</tr>
<tr>
<td>Total time required</td>
<td>Long (at least months)</td>
<td>Short (as short as 2 weeks)</td>
</tr>
<tr>
<td>Need for immunization</td>
<td>Yes</td>
<td>Not necessary in some cases</td>
</tr>
<tr>
<td>Obtaining tolerated or toxic Ags</td>
<td>Highly unlikely</td>
<td>Possible, because re-assorting VH and VL generates new Abs</td>
</tr>
<tr>
<td>Labor required</td>
<td>Higher, with highly sterile cell culture technique required</td>
<td>Lower, only requires basic bacterial culture technique</td>
</tr>
<tr>
<td>Cost of Ab production</td>
<td>Higher</td>
<td>Lower</td>
</tr>
</tbody>
</table>
1.3 Advantages of cell-based methods

Both currently available methods for generating monoclonal antibodies, hybridomas and recombinant Abs, rely heavily on the use of recombinant proteins at several stages of their respective procedures, including immunizations, library enrichment, clone screening, and characterization of antibody specificity and affinity. For many cell surface molecules, the use of recombinant proteins for Ab generation may not be feasible. Some surface molecules, such as G protein coupled receptors, cannot be expressed in recombinant forms that retain their native conformation. Moreover, molecules with large extracellular domains may contain complicated structures that are not properly formed during recombinant expression, or post-translational modifications that may be critical but absent or altered in purified recombinant proteins. Even if the purification procedures completely preserve the original structure, many standard screening practices, such as immobilization of recombinant proteins on plastic, may significantly alter protein conformation. These problems are more severe for surface receptor proteins, whose structures often have extensive post-translational modifications. Purified recombinant receptors may also expose their transmembrane domains, which may further alter their structure. For this reason, it is often necessary to molecularly engineer constructs consisting of only the extracellular/hydrophilic domains of the Ags in order to achieve expression, purification and immobilization. However, this truncation may also affect the antigen structure itself. In summary, the conformation of
some structurally complex surface proteins can only be preserved when they are on a bi-lipid membrane. Therefore, Abs selected on the basis of binding to a purified recombinant protein may not bind the same protein when it is in its native context, particularly Ags expressed on the cell surface. Many cell surface Ags have been shown to have important biological effects when tested with genetic approaches, or with chemically synthesized agonists/antagonists, but characterizing their expression levels or patterns in living cells remains difficult because of a lack of reliable reagents for flow cytometry or other cell-based assays. Many of the currently available Abs for these cell-surface receptors are polyclonal. Polyclonal Ab quality and specificity varies from batch to batch, often making these Abs unreliable. In the immune system, the studies of many chemokine receptors and toll-like receptors have been hampered by this problem for years. The recent boom in antibody-based immune therapy [6, 11] has greatly boosted the exploration of new antibodies against these critical antigens. For example, an antibody cross-reactive to both human and mouse TLR2 (clone T2.5 [12]), and an antibody against human TLR4/MD2/CD14 complex (clone HTA-125 [13, 14]) were developed. A monoclonal anti-murine CCR2 antibody was developed by Mack et al [15, 16], although it is not commercially available at this time. A few newly developed antibodies against human or murine CCR5 or CCR7 are now commercially available (eBiosciences and BD Pharmingen), but their performance in flow cytometry has not yet been confirmed. Most of these Abs were generated using hybridomas and traditional
protein-based selection and screening, indicating that traditional methods may be in
some instances capable of generating Abs binding to these important immune targets in
their native form. Nevertheless, reliable Abs are still unavailable for many important
surface Ags. For example, despite the key role that the chemokine receptors CCR2 and
CX3CR1 play in the immune system (as demonstrated by gene targeting studies [17-19]),
there are still no commercially available Abs capable of identifying cells expressing these
receptors by flow cytometry. There is no clear explanation for why some surface Ags
are more difficult targets for Ab generation than others, but the fact that structurally
complex proteins may adopt different structures once they leave the cell surface may
explain most of the difficulties. Another technical concern about using recombinant
proteins is that some proteins are highly insoluble or toxic to the expression host cells
and therefore cannot be purified at a desired level. In conclusion, despite the fact that
using purified proteins for immunization, Ab selection and screening are all well-
established methods, for reasons described above, purified proteins often fail to mimic
native structures on the cell surfaces. Therefore, developing an entirely cell-based
system for Ab generation, which allows us to bypass the requirement of and problems
raised from purified proteins, is highly desired. Even though traditional protein-based
methods have been proven effective for many Ags, cell-based methods may still be
better alternatives because they avoid any potential, yet to be identified, problems due
to the usage of purified recombinant proteins.
1.4 Antibodies against TLRs

Toll-like receptors (TLRs) encompass a family of cell surface receptors that recognize conserved pathogen-associated molecular patterns (summarized in Table 3) [20, 21]. TLRs appear to be structurally complex proteins [22, 23] that are not readily amenable to standard Ab identification methods. To date, 13 mammalian TLRs have been identified and shown to play important roles in modulating both innate and adaptive immune functions during infections, inflammatory events, and autoimmune diseases [24-27]. TLRs appear to be vital for immune response, yet studies of TLR biochemistry and function have been severely limited due to a lack of reagents. Production of recombinant TLRs has proven to be difficult and most existing antibodies are either unreliable or not useful for biochemical analysis due to their failure to bind native TLR proteins (NIAID Biodefense Workshop Summary: http://www3.niaid.nih.gov/about/organization/dait/conferences.htm). It is critical to identify antibodies that recognize native TLRs expressed on the surface of cells in order to perform functional studies and explore the activity of TLRs in physiologic settings.

Here, we developed a strategy to generate recombinant anti-TLR antibodies that specifically recognize these receptor proteins in their native conformation (Figure 2). This strategy does not require recombinant proteins, instead utilizing antigens expressed on the surface of stable transfectants for immunizations, measuring Ab responses in immunized mice, library panning, clone screening, and scFv clone characterization. This
cell-based system was able to generate multiple scFvs that bind to cell-surface TLR2 while no such scFvs were generated using standard protein-based methods. These results demonstrate that the use of an entirely cell-based strategy to generate recombinant Abs against cell surface TLRs is feasible and suggests that such a strategy is applicable to a wide variety of cell surface proteins.
Table 2: Summary of Toll-like receptors

<table>
<thead>
<tr>
<th>Name</th>
<th>Ligands</th>
<th>Expression (mRNA/protein)</th>
<th>Function/Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Triacyl lipopeptide</td>
<td>protein: monocytes (low level)</td>
<td>Associates with TLR2 to recognize lipopeptide</td>
</tr>
<tr>
<td>TLR2</td>
<td>Lipopeptide LTA*, Peptidylglycan Zymosan, LPS (some species), HSP* 22, 60, 70, HMGB-1*</td>
<td>protein: monocytes, granulocytes, and macrophages</td>
<td>Interacts with microbial lipoproteins and peptidoglycans. Response to LPS from some bacteria species.</td>
</tr>
<tr>
<td>TLR3</td>
<td>dsRNA</td>
<td>mRNA: DC, upregulate on endo/epithelium Protein: fibroblasts (low level)</td>
<td>Interacts with dsRNA, induces production of type I interferons; MyD88-independent response to poly (I:C)</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS*, HSP* 22, 60, 70 HMGB-1*, Mannan, Fibrinogen, Mrp8/Mrp14*, Hyaluronan fragments</td>
<td>Protein: monocytes, DC, macrophages, neutrophils. Upregulated on endothelium</td>
<td>Complex with CD14 and MD2 to interacts with LPS/LPS-binding protein complex</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>mRNA: leukocytes, prostate, liver, lung</td>
<td></td>
</tr>
<tr>
<td>TLR6</td>
<td>diacyl lipopeptide LTA</td>
<td>mRNA: leukocytes, ovary, lung</td>
<td>Protein sequence most similar to hTLR1. Associates with TLR2 to recognize bacterial lipopeptide.</td>
</tr>
<tr>
<td>TLR7</td>
<td>ssRNA imidazoquinoline</td>
<td>mRNA: spleen, lung, placenta; macrophages</td>
<td>Less related to other TLRs</td>
</tr>
<tr>
<td>TLR8</td>
<td>ssRNA</td>
<td>mRNA: leukocytes, lung</td>
<td>Function of TLR8 in human is similar to TLR7 in mouse</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG-DNA</td>
<td>mRNA: leukocytes protein: DC, B cells (intracellular, low)</td>
<td>Recognize bacterial DNA. Similar to TLR3.</td>
</tr>
<tr>
<td>TLR10</td>
<td>Unknown</td>
<td>mRNA: lymphoid tissues</td>
<td>Most closely related to TLR1 and TLR6, appears to be functional but its ligand is still unknown.</td>
</tr>
</tbody>
</table>

* LPS: lipopolysaccharide; LTA: Lipoteichoic acid; HSP: heat-shock protein; HMGB: High-mobility group box; Mrp: myeloid-related protein

Adapted from Akira et al [28, 29], and “Toll-Like Receptor (TLR) Antibodies Overview” (http://www.ebioscience.com/ebioscience/whatsnew/trl.htm)
Figure 2: Step-by-step procedures for comparing protein-based vs. cell-based assays
2. Materials and methods

2.1 Mice

All animal experiments were conducted in accordance with National Institutes of Health guidelines using protocols approved by the Animal Care and Use Committee at Duke University.

2.1.1 Immunization

WT BALB/c mice (Charles River Laboratories, Wilmington, MA) and TLR2⁻/⁻ C57BL/6 mice [30] (The Jackson Laboratory, Bar Harbor, ME) were immunized subcutaneously at two sites on the back with 10⁸ hTLR2-transfected 300.19 cells. The mice were boosted with 5x 10⁷ cells IP at 14 day intervals. 7 days after the 5th boost, blood was collected and serum serially diluted in FACS buffer for analysis by ELISA or flow cytometry. ELISA measurements of serum IgG titers were performed as described previously [31, 32].

2.1.2 Sera harvest

Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (173mg/7mg per kg bodyweight). Blood was collected from the orbital sinus using heparinized Natelson capillary tubes. The tube was directed into the orbital sinus, inserted into the medial canthus of the eye and directed caudally and slightly dorsally.
After sufficient blood is collected, blood around the eye was wiped away with gauze. Pressure was applied after blood collection to prevent hematomas.

2.1.3 Spleen mRNA harvest

Spleens were harvested from immunized BALB/c and TLR2−/− C57BL/6 mice that displayed significant anti-hTLR2 IgG titers. Fresh spleens were harvested from mice and homogenized in Trizol (Invitrogen, Carlsbad, CA), then enriched for mRNA using Dynal magnetic oligo(dT) beads (Invitrogen). The resulting mRNA was used for preparation of an anti-hTLR2 scFv library using the reagents supplied in the Recombinant Phage Antibody System (GE Healthcare, Buckinghamshire, UK).

2.1.4 Mouse lung cells for Ab evaluation

8-12 week-old C57BL/6 mice received 12.5 ug LPS intranasally. 24 hours following treatment, lung parenchymal cells were harvested as described previously [33]. Briefly, lungs were perfused with 3 ml HBSS, minced and digested with 1 mg/ml of collagenase for 40 min at 37°C. Cells were dissociated by passing through a 70-um mesh strainer. DC and macrophages were enriched by centrifugation over a 17% Metrizamide cushion at room temperature. Red blood cells were lysed using AKC buffer (0.15 M NH₄Cl, 10mM KHCO₃, 0.1 mM EDTA, pH7.2-7.4). The resulting single cell suspensions were subjected to staining and flow cytometry analysis to identify neutrophils, DC, macrophages, and monocytes as described [33].
2.2 Cell lines

Several cultured cell types were used throughout the procedures. 300.19 cells were used for cell-based immunizations. HEK 293 cells, wild type and transfected, and its transfectants were used for cell-based procedures of sera evaluation, library selection (BRASIL), clone screening, verification of phage and scFv binding. THP-1 cells, CaCo-2 cells, MonoMac 6 cells, and J774.1A cells were used for endogenous human or mouse TLR2 staining.

2.2.1 300.19 cells for immunization

Murine pre-B- cell line 300.19 [34] (kindly provided by Dr. T. Tedder, Duke University, Durham, NC, USA) was used for immunization as described by Kearney, J et al [34].

2.2.1.1 hTLR2 transfection by electroporation

To establish stable transfectants, 12 x 10^6 cells were resuspended with 0.2 ml RPMI 1640 medium supplemented with 10% FBS and electoporated in a 0.4 cm gap cuvette with 10 ug hTLR2-HA-pUNO plasmid. The power was set at 0.2 kV and 960F (Gene Pulser apparatus; Bio-Rad, Richmond, CA, USA). The pulsed cells were plated at 10^5, 8.3x10^4, 1.6x10^4, and 0.4x 10^4 cells/well in flat-bottom 96-well microplates, and 48 h later cells were selected for hTLR2 with 10 ug/ml blasticidin. hTLR2 expression in
selected clones was tested by detecting HA-tag expression in Western blots (HRP-conjugated anti-HA-tag Ab).

2.2.1.2 Culture maintenance

The transfected 300.19 cells were maintained in complete medium (RPMI 1640 supplemented with 10% FBS, 10mM L-glutamine, 50 mM 2-mercaptoethanol (Gibco-BRL, Gaithersburg, MD, USA) and 10 ug/ml blasticidin for selection) in 75 cm² flasks and subjected to a 1:10 dilution every 2-3 days.

2.2.2 HEK cells for sera evaluation, library selection, clone screening and verifications

The Human Embryonic Kidney (HEK) 293 cell line was one of the most extensively used cell lines currently used. This cell line was obtained from ATCC (Manassas, VA) and chemically transfected with several different plasmid for TLR expression.

2.2.2.1 Chemical transfection of TLR-expressing plasmid

Stable hTLR2-HEK transfectants were established using SuperFect by the manufacturer’s instructions. Briefly, 60ul of SuperFect transfection reagent was made by vigorously mixing 5ug hTLR2-HA-pUNO plasmid in 300 ul serum-free MEM and incubated at room temperature for 10 min, added to 3ml of MEM supplemented with 10% FBS. A 70%-confluent 10cm dish of HEK293 cells was resuspended and washed
once with MEM (with no serum or antibiotics added), resuspended in the above transfection reagent and incubated at 37°C, 5% CO₂ for 2 hours. Cells were then gently washed and resuspended with complete medium (MEM supplemented with 10% FBS, 2 mM L-glutamine, 10 units/ml penicillin and 1 mg/ml streptomycin) and seeded in a 15 cm cell culture dish. 48 h later cells were selected for hTLR2 with 10 ug/ml blasticidin. Selected single colonies were picked by pipeting and transferred to one well of 96-well culture plate for further growth. hTLR2 expression in selected clones was tested by detecting HA-tag expression in Western blots (Sections 2.2.4 and 2.9). hTLR1-HA-pUNO, hTLR5-HA-pUNO, hTLR6-HA-pUNO and mTLR2-HA-pUNO plasmids were chemically transfected into HEK293 cells with the same procedures.

2.2.1.2 Culture maintenance

The transfected HEK293 cells were maintained in complete medium composed of Minimum Essential Medium (MEM; Gibco) supplemented with 10% FBS, 2 mM L-glutamine, 10 units/ml penicillin, 1mg/ml streptomycin (1/100 of 100X cell-culture L-glu/Pen/Strep mix; Sigma), and 10 ug/ml blasticidin for selection.

2.2.3 Cell lines with endogenous TLR expression

The following 4 cell lines were used to test the ability of our scFv for detection of endogenous TLR2 expression.
2.2.3.1 THP-1 cells

Human monocyte/macrophage cell line, THP-1 [35], was obtained from ATCC. It is maintained with RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 10 units/ml penicillin, 1 mg/ml streptomycin and, 50 mM 2-mercaptoethanol.

2.2.3.2 Caco-2 cells

Human intestinal epithelial cell line, CaCo-2 [36], was obtained from ATCC. It is maintained with RPMI-1640 supplemented with 20% FBS in MEM, 1mM% Sodium Pyruvate (Gibco), 1% non-essential amino acids(Gibco, MEM), 10 mM HEPES (Gibco, pH=7.3), 2 mM L-glutamine, 10 units/ml penicillin and 1 mg/ml streptomycin. Cells were resuspended by trypsinization (0.25%) and subjected to a 1:10 subculture every 3-4 days.

2.2.3.3 MonoMac6 cells

Human monocyte/macrophage cell line, MonoMac6 [37], was obtained from ATCC. It is maintained in RPMI 1640 supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 2 mM L-glutamine, 10 units/ml penicillin and 1 mg/ml streptomycin, 1% HEPES, 0.5% of additional 45% glucose, 9ug/ml bovine insulin.

2.2.3.4 J774A.1 cells

Murine monocyte/macrophage cell line, J774A.1 [38], was obtained from ATCC. It is maintained in Dulbecco’s Minimum Essential Medium (Gibco DMEM, w/o glucose).
supplemented 10% FBS, 2 mM L-glutamine, 10 units/ml penicillin and 1 mg/ml streptomycin. Cells were resuspended by trypsinization (0.25%) and subjected to a 1:10 subculture every 3-4 days.

**2.2.4 Verification of TLR expression on transfected HEK cells**

All of the TLR genes on the expression vector were C-terminally tagged with an HA-tag (hemagglutinin epitope YPYDVPDYA [39]). The expression of TLRs in the transfectants thus can be verified by detecting the expression level of HA-tag with Western blots. TLR transfected 300.19 or HEK cells were lysed with 1 ml RIPA cell lysis buffer (0.1% SDS, 1% Nonidet P-40, 0.5% Na-deoxycholate, in PBS) with protease inhibitor at 4°C for 30 minutes. Genomic DNA in the lysates was sheared by passing through a 22G needle for more than 10 times. Genomic DNA was then pelleted by 10000g centrifugation for 10 min at 4°C, and the resulting supernatants containing both cytosolic and membrane proteins were analyzed Western blotting as described in Section 2.9. TLR transfected clones with the highest expression level were selected and used as the reagents for other procedures in this cell-based Ab generation system.

**2.3 Library construction**

As summarized in Figure 3, RNA from each spleen of TLR-responding mice was reverse transcribed with random hexamers and used as template for separate PCR reactions for heavy chain and light chain variable fragments (V<sub>H</sub> and V<sub>L</sub>). The V<sub>H</sub> and V<sub>L</sub>
PCR products were pooled in equimolar amounts for assembly and cloned into pCANTAB 5E. The library of resulting phagemids was transformed into TG-1 cells (Stratagene, La Jolla, CA) in thirty separate electroporations, performed with a BioRad Gene Pulser II at 1700 V, with a resistance of 200 ohms and capacitance of 25 uF. The electroporated bacteria were pooled and plated on 2xYT plates supplemented with 2% glucose and 0.1 mg/ml ampicillin (2xYT/AG). Colonies of phagemid clones were scraped into 2xYT and pooled to make library stocks.

Figure 3: Library construction strategy

This figure is kindly provided by Dr. Barbara Lipes (Duke University, Durham, NC, USA)
2.4 Phage rescue and purification

Rescues with M13K07 (New England Biolabs, Ipswich, MA) were performed to obtain M13 phage particles for selections and screening. Two types of phage preparation were made. An unpurified raw phage preparation in bacterial culture medium was used for screening of individual TLR-binding Ab clones. After identified TLR-binding Ab clones, a highly purified phage preparation in PBS was made and tested for its binding ability and specificity.

2.4.1 Raw phage preparation for screening

Individual phage clones were rescued for screening in sterile 2 ml/ well 96-well plates (Continental Lab Products, San Diego, CA). 400 ul of 2xYT/AG in each well was inoculated with a phage clone and shaken at 200 rpm for 16 hours at 30°C. Aliquots from the wells of this master plate were used to inoculate a rescue plate containing 400 ul of 2xYT/AG supplemented with 2x10^10 phage/ml of M13K07 helper phage. The rescue plate was shaken at 37°C for 4 hours. Bacteria were pelleted at 3000g for 10 minutes, resuspended in 400 ul of 2xYT/AK and shaken for 16 hours at 30°C. The bacteria were pelleted as above and the supernatant containing phage particles was transferred to a fresh plate for screening assays.
2.4.2 Phage purification

We generated phage-displayed scFv as a preliminary reagent to confirm Ab bindings before producing soluble scFv. To obtain concentrated scFv-phage preparations, a 12 ml 2xYT/AG culture was inoculated from frozen stocks and grown at 37 °C to an OD$_{600}$ of 0.7. M13K07 helper phage were added and the culture was shaken at 200 rpm for 30 minutes at 37°C. Bacteria were pelleted at 3500g for 10 minutes and the medium replaced with 50 ml of 2xYT supplemented with ampicillin and kanamycin. The culture was shaken at 37°C for 30 minutes, then at 30°C for 16 hours. The culture was centrifuged as above, and the supernatant subjected to multiple precipitations in 0.2 volumes of ice-cold 20% PEG-8000/2.5 M NaCl to purify and concentrate the phage particles. Phage particles were pelleted at 10,000g for 10 minutes. Following the last precipitation, the phage were resuspended in 1 ml PBS and stored at 4°C. Phage titers were determined by infections of TG-1 cells with serially diluted phage.

2.5 Purified recombinant TLR protein production

Purified recombinant proteins are not required in this cell-based Ab generation system. However, they are required for direct comparison of traditional protein-based procedures and the newly-developed cell-based methods. The putative extracellular domain of human TLR2 (hTLR2-ECD) was expressed in Sf9 insect cells using a baculovirus vector construct, sTLR2-ECD-PVL1393 (a kind gift from Dr. Yoshio Kuroki, Sapporo Medical University School of Medicine, Japan) as described previously [40, 41].
The HIS-tagged soluble hTLR2-ECD was purified from culture medium by NTA agarose (Qiagen) following the manufacturer’s instructions.

2.6 ELISA

ELISA (Enzyme-linked Immunosorbent Assay) is an essential assay for traditional protein-based Ab generation. It is used in (1) evaluation of the sera after immunization, (2) evaluation of the total library affinity against immunized Ag (hTLR2), and (3) screening for positive phage-displayed scFv clones. We converted these procedures into cell-based methods, but we also performed a traditional ELISA-based assays for comparison.

2.6.1 Sera evaluation

Immunized mouse sera were evaluated by both ELISA and flow cytometry. ELISA measurements of serum IgG titers with recombinant protein were performed as described previously [31, 32], and flow-cytometry-based sera evaluation is described in Section 2.7.1. However, ELISA was only used for the purpose of comparison to cell-based assays. In most instances mouse sera were evaluated with the cell-based methods (Section 2.7.1) instead of traditional ELISA methods.

2.6.2 Pooled phage ELISA

We performed ELISA on enriched pooled phage to evaluate the library affinity against TLRs. Pools of phage corresponding to the initial library of M13 scFv phage or
each round of iterative enrichment were subjected to ELISA to evaluate their total binding affinities against purified recombinant hTLR2 proteins. \(5 \times 10^{10}\) phage/ml of pooled phage preparations were purified as described above (Section 2.4.2) and used as primary Ab reagent against polystyrene immobilized recombinant hTLR2 proteins. 0.25 ug/ml HRP-conjugated anti-M13 phage coat protein Ab (Amersham) was then applied as a secondary reagent, followed by signal development with OPD (Pierce) substrate.

### 2.6.3 ELISA-based Ab clone screening

The screening of individual hTLR2-binding Ab clones was essentially performed as described in 2.6.2 except that individual raw phage clone preparations, instead of pooled phage, was used as primary Ab.

### 2.7 Flow cytometry

Flow cytometry is the most extensively utilized technique in this cell-based Ab generation system. It is used for (1) sera evaluation, (2) initial scFv-phage clone screening, (3) phage clone binding verification, (4) scFv binding verification, and (5) biotinylated-scFv binding verification. One previous study suggested that using highly purified phage-displayed scFv could potentially be used as a staining reagent in flow cytometry [42]. However, to achieve high-throughput screening, we need to employ raw phage preparations, which contain considerable amounts of bacterial culture components and are not normalized for phage titers. We therefore sought to develop
and optimize flow cytometry procedures for phage-displayed scFv in both forms. The FACS buffer we used throughout the flow cytometry procedures is serum-free FACS buffer (PBS with 0.5% BSA, 10 mM EDTA, 10 mM HEPES) unless otherwise noted. All Ab incubations were in 100 ul volume for 30 min on ice and followed with 2 washes of 200 ul FACS buffer.

### 2.7.1 Serum evaluation

hTLR2-HEK and YFP-HEK cells were grown in MEM to 70% confluence, harvested into FACS buffer by gentle pipetting, washing, and resuspending. Equal numbers of cells were mixed and plated in 96-well U-bottom plates at 5 x10⁵ cells/well. Cells were pelleted by 500g centrifugation for 3 min and resuspended in serial diluted mouse sera as primary staining reagents. After incubation, the cells were washed x2 in 200ul FACS buffer, resuspended in 0.5 ug/ml allophycocyanin(APC)-conjugated goat-anti-mouse IgG F(ab)’₂ (Jackson Immuno Research Labs, West Grove, PA), incubated, washed x2 in 200ul FACS buffer, resuspended in 200ul FACS buffer and subjected to flow cytometric analysis using a BD LSRII flow cytometer. Data were analyzed using Flowjo software (TrecStar Inc, Ashland, OR). To determine hTLR2-specific binding, hTLR2-HEK and YFP-HEK cell populations were gated separately based on YFP fluorescence in the FITC channel, and the mean fluorescence intensity (MFI) of allophycocyanin was determined for each population. hTLR2-specific staining was calculated as the hTLR2-HEK MFI minus the YFP-HEK MFI.
2.7.2 High throughput cell-based Ab clone screening

The procedures of flow cytometry for high throughput cell-based Ab clone screening were essentially as above (Section 2.7.1) except with different staining reagents. For primary staining, 100 ul of TLR-HEK/YFP-HEK cell suspension in FACS buffer was mixed with 100 ul unpurified phage supernatants, obtained from the corresponding wells of the rescued 96-well phage plate, as the primary staining reagent. After 2 washes, 100 ul of 1 ug/ml mouse-anti-M13 mAb (Amersham) was used for secondary staining. After 2 washes, 100 ul of 0.5 ug/ml allophycocyanin-conjugated goat-anti-mouse IgG F(ab)’2 was used for tertiary staining. After 2 washes, cells were resuspended in 200 ul FACS buffer and subject to flow cytometry analysis. An automatic plate loader for sample loading was used to accelerate and ease the sample loading process.

2.7.3 Purified phage staining

The procedure of flow cytometry to verified TLR-binding Ab clones in the form of phage-displayed scFv were performed essentially as above (2.7.2) except that 100 ul of $10^{12}$ phage/ ml purified scFv-phage preparation (see 2.4.2) was used as primary staining reagent.

2.7.4 Purified scFv staining

The procedures of flow cytometry to verify TLR-binding Ab clones in the form of purified scFv were essentially as above (2.7.1) except with different staining reagents.
0.5 μg/ml purified scFv (see Section 2.10) preparation was used as primary staining reagent, mouse-anti-V5-tag Ab (Invitrogen) was used as secondary staining reagent, and 0.5 μg/ml allophycocyanin-conjugated goat-anti-mouse IgG F(ab)'2 (Jackson ImmunoResearch Labs) was used as tertiary staining reagent.

2.7.5 Biotinylated-scFv staining

The detection of endogenous TLR expression on cell lines or primary cells was performed with Ab clones in the form of biotinylated scFvs. The procedures were essentially as above (Section 2.7.1) except with different staining reagents. 0.5 μg/ml purified biotinylated scFv (see Section 2.10) preparation was used as primary staining reagent, and 0.2 μg/ml allophycocyanin-conjugated streptavidin (BD pharmingen) was used as secondary staining reagent.

2.8 Library selection (panning/ enrichment)

We employed BRASIL[43] for cell-based library enrichment, in comparison to traditional protein-based immunotube selection.

2.8.1 Immunotube selection

Recombinant hTLR2 (50 μg in 1 ml PBS) was adsorbed to NUNC immunotubes. After rinsing with PBS, the experimental tube and a control tube were blocked with 4 ml of 2% milk in PBS (MPBS). An aliquot of 10^{12} freshly prepared M13 phage particles from the hTLR2 scFv library was pre-cleared for one hour at room temperature in the MPBS
control tube then transferred to the target tube with hTLR2 for a one hour incubation. Unbound phage were removed with 20 washes of 4 ml PBS 0.1% Tween-20 followed by 20 washes with 4 ml PBS. A 1 ml aliquot of log-phase TG-1 bacteria was added to the target tube and incubated at 37 °C for 30 minutes to allow infection with the bound phage. The TG-1 cells were plated on 2xYT/AG plates and grown overnight at 30°C. Individual phagemid colonies were rescued in 96-well plates to produce phage particles for screens as in Sections 2.6.3 and 2.7.2.

2.8.2 BRASIL selection

Cell-based selection was performed using the biopanning and rapid analysis of selected interactive ligand (BRASIL) approach of Giodano et al [43] with slight modification. All solutions and incubations were at 4°C unless otherwise noted. HEK293 and hTLR2-HEK cells were harvested with Versene (Invitrogen), washed in Dulbecco’s PBS and resuspended at 10⁶ cells/ml in DMEM (Invitrogen)/ 1% BSA Fraction V (Sigma, St. Louis, MO). An aliquot of anti-hTLR2 scFv phage library containing 5x10⁹ M13 phage particles was added to 150 ul of HEK293 cells suspension and rotated for two hours. This solution was layered on top of 200 ul of a mixture of dibutyl phthalate:cyclohexane (15:1) (Sigma) and spun at 10000g in a microfuge for 10 minutes. The aqueous phase containing pre-cleared phage was used to resuspend a cell pellet containing 1.5x10⁶ hTLR2-HEK cells. This suspension was rotated for 1.5 hours. The cells were washed three times in 200 ul DMEM/1% BSA, then resuspended in 200 ul
DMEM/ 1% BSA and layered over 200 ul of dibutyl phthalate:cyclohexane (15:1). After centrifugation (10000g x 10 min), the tube was flash frozen in liquid nitrogen. The cell pellet with bound phage in the tube tip was removed with a tube cutter (GeneMate). The cell pellet was resuspended in 10 ml of log-phase TG-1 cells. Following a 30 minute incubation at 37°C, the cells were concentrated by centrifugation and plated on two 15 cm 2xYT/AG plates. The plates were incubated for 16 hours at 30°C. The resulting colonies were scraped into 2xYT and used to make a glycerol frozen stock.

2.9 Western blotting

Proteins in cell lysates of TLR transfected HEK cells (see Section 2.2.4) were separated by 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham) by Bio-Rad semi-dry transfer apparatus. The membrane was blocked for 1 hr at room temperature with 5% non-fat milk (Carnation, Solon, OH) and 0.05% Tween-20 (EMD Biosciences) in PBS (MPBS-T: with milk, and PBS-T: without milk), followed by overnight incubation at 4°C with primary Ab diluted in MPBS-T (1:1000 anti-HA-tag antibody (Invivogen). The membrane was washed 3x 15 min vigorously with PBS-T at room temperature. The detection was achieved with ECL substrate (Amersham).
2.10 scFv production

We sub-cloned scFv genes of TLR-binding Ab clones into a Drosophila expression vector. Expression in the Drosophila expression system provides us ostensibly endotoxin-free scFv preparations at high yields.

2.10.1 Cloning of scFv into a Drosophila expression system

ScFv inserts were amplified from pCANTAB 5E by PCR. The PCR was performed using 2ul of TG-1 culture, 200 uM dNTPs, 200 uM pCANTAB 5E primers (scFvGWfwd: GCGGCCCAGCCGGCC; scFvGWrev: CTGGAACCGCGTG), 3 units EasyA DNA polymerase (Stratagene), and manufacturer’s buffer in a volume of 50 ul. After amplification (95°C x 2 min, 30 cycles of 95°C x 30 sec, 58°C for 30 sec, 72°C for 1 min; and 72°C for 10 minutes), the PCR product was ligated with topoisomerase into the Gateway entry vector pCR8Topo (Invitrogen). The Drosophila expression vector pMTBiP/V5His (Invitrogen) was converted to a Gateway destination vector by replacing the multiple cloning sequence with recombination sites for the bacteriophage enzyme LRII (Invitrogen) and a BirA Avitag site (Avidity, Denver, CO).

2.10.2 ScFv expression in S2 cells

After recombining scFv inserts into pMTBiP, plasmids were transfected into Schneider 2 (S2) cells using Cellfectin (Invitrogen). Stable transfectants were obtained by co-transfection with plasmid pCoBlast (Invitrogen) and selection in 25 ug/ml blasticidin.
Expression of scFvs was induced with 0.75 mM CuSO₄ in Sf900-II serum-free medium (Invitrogen) for 36 to 48 hours at 28°C. ScFvs were purified from culture supernatant by ion metal affinity chromatography (Talon resin, Clontech, Mountain View, CA). Purity and quantity were assessed by SDS-PAGE and BCA assay (Pierce, Rockford, IL).

2.10.3 Biotinylation of scFvs

To make biotinylated-scFvs, purified scFvs were subject to *in vitro* BirA biotinylation (Avidity) as described in manufacture’s instructions. Briefly, scFv preparations were dialyzed into 10 mM Tris-HCl, pH 8, mixed with 1/8 volume of manufacture’s biomix buffer A and biomix buffer B. For every 37ug (10nmole) scFv, 2.5 ug birA was added into this mixture, well mixed by vortexing and incubated at room temperature for 30 min.

2.11 DNA fingerprinting

ScFv inserts obtained by PCR from infected bacteria [44] were subjected to restriction fragment length polymorphism or “DNA fingerprint” analysis. PCR was performed under the same conditions as described in Section 2.10.1. Aliquots of the PCR product were digested with Sau3A I or BstN I (New England Biolabs) then fractionated on 2% agarose TAE gels. Ab clones with the same restriction digestion pattern were identified as one unique clone group. Some selected clones were being sequenced to confirm their uniqueness.
2.12 Plasmids and antibodies

2.12.1 Plasmids

hTLR1-HA-pUNO, hTLR2-HA-pUNO, hTLR5-HA-pUNO, hTLR6-HA-pUNO, and mTLR2-HA-pUNO plasmids for 300.19 and HEK transfections were purchased from Invivogen. pCANTAB5 phagemid plasmid was purchased from Amersham/GE Healthcare (Buckinghamshire, UK). Gateway cloning and Drosophila expression system vectors pCR8-Topo and pMTBiP were purchased from Invitrogen.

2.12.2 Antibodies

For cell-based Ab generation system, an Ab cross-reactive to both human and murine TLR2 (clone T2.5) was purchased from e-biosciences. Mouse anti-M13 phage coat protein Ab was purchased from Amersham. Mouse anti-V5 tag Ab was purchased from Invitrogen. Allophycocyanin-conjugated goat-anti-mouse IgG F(ab’)2 was purchased from Jackson Immuno Research labs. For characterizing mouse lung cell types, we used Abs against Ly6G (for neutrophil; BD pharmingen), CD11c (for macrophage and DC; e-biosciences), IA/IE (for macrophage, DC, and B cell; BD pharmingen), CD11b (for inflammatory macrophage and DC; BD pharmingen)
3. Cell-based immunization and serum evaluation

Cells expressing human TLR2 were the only immunogen used to treat mice for generation of our scFv immune library. This library was used to generate anti-hTLR2 Ab here, and also anti- hTLR5, anti-hTLR6-, and anti-mTLR2-specific Abs from this hTLR2 immune library in Chapter 7.

3.1 Introduction

3.1.1 Immunization methods

Traditionally, peptide or purified recombinant protein is used as the immunization antigen for Ab generation. However, the structure or post-translational modification in these forms may not recapitulate the same structure exhibited by the antigen on the cell surfaces. To maximize Ab responses to native cell surface receptors, cell-based immunization is preferred. A method established by Kearney et al [34] using a 300.19 murine tumor cell line is well-suited for this purpose since it provides a native protein structure while minimizing Ab response across species.

3.1.2 Mice for immunization

Traditionally, spleenic \( V_\text{H} \) and \( V_\text{L} \) cDNA from a single immunized mouse is used to construct an Ab phage display library since the immune repertoire of one immunized mouse is often sufficient to generate multiple Ab clones against the target Ag. However,
mice with different genetic backgrounds, or even individuals with the same genetic background, often have different immune responses after immunization. Thus reassorting V\textsubscript{H} and V\textsubscript{L} genes from multiple mice of various genetic backgrounds, theoretically, may further increase the library diversity. Similar approaches have been taken in pooling multiple naïve human blood samples to increase total Ab library complexity [45]. Therefore, in order to further boost the diversity of our library, we pooled Ab genes from multiple mice with two different genetic backgrounds. In addition, TLR2-deficient mice [30], which were expected to develop increased anti-hTLR2 immune responses due to a lack of tolerance to epitopes shared with murine TLR2, were included for immunization in an effort to further boost library diversity.

### 3.1.3 Evaluation of the immunization

Recombinant protein-based ELISA is the standard method to evaluate Ag-specific IgG titer in mouse sera after immunization. Serial dilutions of mouse sera were applied as primary Ab to an Ag-coated plate and Ag-specific IgG titers were detected by anti-mouse IgG Ab. Serum samples retaining positive binding signals at higher dilution indicate a higher Ag-specific serum IgG response. However, ELISA has the same problem described above as other protein-based methods, since it measures the IgG response against immobilized proteins, which may not represent the native structure of an Ag. To avoid this problem, we sought to completely eliminate the requirement for recombinant protein throughout the system. Another advantage of converting ELISA to
a cell-based method is that it streamlines and eases the procedures in our entirely cell-based Ab generation system by totally eliminating the requirement for recombinant proteins.

**3.2 Results**

**3.2.1 Cell-based immunization induces serum IgG response against hTLR2**

We immunized mice with cells from the 300.19 mouse tumor cell line stably transfected with hTLR2. This immunization strategy has been shown to result in robust Ab responses against transfected proteins and tumor Ags [34]. The animals immunized included wild type BALB/c mice (*mus musculus*) and TLR2-deficient C57BL/6 mice [30], which were expected to develop increased anti-hTLR2 immune responses due to the lack of tolerance to epitopes shared with murine TLR2. After 6 rounds of immunization, sera from hTLR2 immunized mice, or a control naïve mouse, were harvested and subjected to standard protein-based ELISA for hTLR2-specific IgG titer in the sera. As shown in Figure 4, ELISA detected robust serum Ab responses against hTLR2 in all immunized mice but with no signal detected in serum from non-immunized mice. A comparison of ELISA with cell-based evaluation will be discussed below (Section 3.2.2 and 3.3). This data suggests that cell-based immunization with transfected 300.19 cells results in robust anti-hTLR2 Ab responses. The use of TLR2-deficient mice appears to offer no advantage in terms of overall Ab response.
Figure 4: Protein-based or cell-based assays to evaluate mouse hTLR2-specific IgG responses after immunization.

Sera from 3 TLR2KO BL6 mice and 4 WT BL6 mice were harvested 7 days after the 5th boost. Serial 2-fold dilutions were assayed for binding against recombinant hTLR2 protein by ELISA (a) or against hTLR2-HEK cells by flow-cytometry (b). Both assays utilized labeled anti-mouse IgG as a secondary Ab to detected hTLR2-specific IgG binding. In the cell-based assay, hTLR2-specific binding was measured as the difference in MFI between hTLR2-HEK and control WT-HEK staining. Serum from non-immunized mice was used as a negative control (naïve)
3.2.2 Flow cytometry-based evaluation of serum IgG response is comparable with ELISA-based methods

Currently there are several available cell-based methods for sera IgG evaluation. Whole-cell ELISA [46, 47] is a possible method for our system and one that we tested. Briefly, live hTLR2 transfected HEK cells (hTLR2-HEK), or cell lysates, were absorbed on the PVDF membranes in wells of an ELISA plate as Ag and the same IgG detection procedures for protein-based ELISAs were applied. However, after several trials of this method under various conditions, we were not able to detect any hTLR2-specific IgG response (data not shown). We then sought to develop a flow cytometry-based procedure for evaluating Ag-specific serum Ab responses. In this method (principles illustrated in Figure 5), live hTLR2-HEK cells captured hTLR2-specific IgG present in the serum samples and the bound IgG was then detected by allophycocyanin (APC)-conjugated anti-mouse IgG antibody. WT-HEK cells were used as negative control cells for staining background. The relative binding intensity was determined by the mean florescence intensity (MFI) difference between hTLR2-transfected and non-transfected HEK cells. As shown in Figure 4, both protein- and cell-based assays indicated robust serum antibody responses to hTLR2 in immunized mice with no signal detected in serum from non-immunized mice. The decrease in specific signal with serial dilution was similar between the two assay formats. ELISA assays displayed a greater variability in signal among individual mice but it is not clear if this is biologically significant. Both assays identified WT mouse #4 as having the greatest response to immunization. These
findings demonstrate that determination of serum Ab responses by a flow cytometric assay provides results comparable to protein-based ELISA assays and that immunization with transfected cells results in robust anti-hTLR2 Ab responses. The use of TLR2-deficient mice appears to offer no advantage in terms of Ab response. While eliminating the requirement of purified recombinant Ag protein is an advantage to accelerate and ease the procedures in this system, it is not clear whether the binding signals detected in the flow cytometry-based method represents Ab binding to Ag in its native forms better than would be obtained with protein-based methods.
Polyclonal IgG in mouse sera binds to hTLR2 on transfected cells. A dye-conjugated secondary anti-mouse IgG Ab then develops the signal for mouse IgG-TLR2 specific binding (left), whereas all Abs will be washed out in the WT-HEK control staining (right). MFI differences between these two situations are calculated and defined as relative TLR2 binding signal for each mouse serum sample.

Figure 5: Principle of cell-based sera evaluation
3.3 Discussion

In our hands, immunization with stably transfected cells that express hTLR2 generated impressive responses. Such cell-based immunizations have previously been shown to work well in the generation of mAbs using hybridomas [34]. We were able to measure responses to immunization with flow cytometric assays using sera from the immunized animals, obtaining titers comparable to those determined by ELISA. Evaluating the immune response with both approaches allows determination of the extent of the polyclonal response for a full constellation of native and denatured epitopes. However, the ability to perform both immunizations and evaluations of Ab titers without purified protein is advantageous for many antigens.

It is not clear why the whole cell ELISA assay failed to detect sera IgG response. We performed this assay as reported [46, 47], but some potential problems may have led to the failure, such as: (1) the hTLR2 expression level on the cells may have been too low to be detected, (2) the Ag (TLR) structure may have been altered to an undetectable form under the assay conditions, (3) the membrane (PVDF) may have not been optimized for the purpose of retaining a HEK cells or membrane-bound proteins in the cell lysate. Even though we do not know why the whole-cell ELISA failed to work in our hands, this method was not necessary since the flow cytometry method we developed here is simple and effective for sera evaluation.
The results shown here only demonstrate that our flow cytometry-based method is as effective as, but not more sensitive than, an ELISA-based method. A cell-based method is often preferred over a protein-based method because it reveals the binding of Ab to surface Ag in its native form rather than in the form of immobilized proteins. After immunizing with transfected cells, we expected that a cell-based method for sera evaluation would reveal stronger signals than a protein-based method, since the Ag structure is not altered. However, comparing the results of these two types of assays, we found no significant difference in sensitivity (similar rate of intensity decrease with increasing dilutions) and both assays identified WT mouse number 4 as having the greatest response to immunization. One possible explanation for this similarity is that cell-based immunization generated Abs against a broad spectrum of epitopes on the Ag. Some of these epitopes may have different structures when they are in the forms of recombinant or native proteins, while some of them may have intact structure in both forms. Therefore, comparing ELISA-based with flow cytometry-based methods for sera evaluation, each which only measure total serum affinity against Ag, cannot reveal the difference between these assays’ ability to distinguish Ab specificity against native or immobilized Ags. As we will discuss in Section 4, when we isolate individual Ab clones, we found both Abs that bind to recombinant protein (on ELISA) or to native Ag (on flow cytometry), suggesting the possibility that a cell-based immunization elicited serum Abs against a large spectrum of epitopes in both recombinant and native forms.
4. Cell-based selection of antibody libraries

4.1 Introduction

Library selection, also known as panning or enrichment, is a critical step to enrich the fraction of desired Ab clones to a level that allows positive clones to be found in a reasonable numbers during a screen of individual clones, typically a few hundred. This process is especially important for a non-immune library, but is also required for an immune library. Often, enrichment is achieved with immunotube selection [48, 49] (Figure 6a), a protein-based method similar to the concept of ELISA. However, for structurally complex proteins, such as TLRs, the protein may be structurally distinct from a purified, immobilized protein to a native cell surface protein. Since Ag plays a key role in selection, the difference in structures or post-translational modification may significantly affect the outcome of the enrichment, even though it is the same Ag in terms of amino acid sequences. Therefore, we hypothesized that the traditional protein-based methods for scFv library selection preferentially enrich Ab clones against purified protein Ags whereas cell-based selection enrich Ab clones against native cell surface Ags. To test this hypothesis, we performed a protein-based method (Immunotube) and a cell-based method (BRASIL [43] and Figure 6) for library enrichment against hTLR2. After selection, individual Ab clones were seeded in a 96-well plate and subjected to ELISA screening to search for Ab-binding clones. Positive clones identified by ELISA were
picked and subjected to a flow cytometry test for confirmation of their binding to native cell surface hTLR2.
Figure 6: Principle of immunotube selection (a) and BRASIL [43] selection (b)

This figure is kindly provided by Dr. Barbara Lipes (Duke University, Durham, NC, USA)
4.2 Results

4.2.1 Cell-based methods for library selection enrich the total library binding to hTLR2

The use of recombinant protein to select phage displaying scFvs specific for that protein is simple and rapid using well-characterized methods [49], often requiring only a single round to obtain 1000-fold enrichment for antigen-specific phage [48]. To test if a cell-based method can also efficiently enrich Ab library against hTLR2, we performed parallel selections from our anti-hTLR2 scFv library to compare the efficacy of recombinant protein selection with cell-based selection. A single round of selection for phage that bound to recombinant hTLR2 adsorbed to polystyrene was performed. Absorbance values from ELISA measurements using pools of phage from the selected phage versus the starting library demonstrated a high degree of enrichment for hTLR2 binding (Figure 7a). This enrichment is specific to hTLR2, since no enrichment was seen for binding to a control His-tagged recombinant protein.

Biopanning and rapid analysis of selected interactive ligand (BRASIL [43]), the cell-based selection procedure we employed, relies on differential centrifugation to obtain phage clones that specifically recognize cell-surface antigen. The anti-hTLR2 scFv phage library was pre-cleared by incubating it with parental HEK293 cells in an aqueous solution, which was then layered over a non-polar organic phase and centrifuged. The HEK293 cells and any phage bound to the cells formed a pellet in the lower organic
phase. The pre-cleared phage remaining in the aqueous phase were then incubated with hTLR2-HEK cells. Following washes to remove the bulk of unbound phage, the cells were again centrifuged through the non-polar organic phase. Pelleting the cells through this non-polar solution dissociated phage loosely bound due to non-specific interactions, reducing the background of irrelevant phage. Phage bound to the hTLR2-HEK cells were used to infect bacteria and generate enriched phage stocks for iterative rounds of selections. Three iterative rounds of enrichment were performed. To determine the degree of enrichment for hTLR2-specific phage, ELISA measurements were done to compare binding of pooled phage from each round of selection with the starting library. Three rounds of selection were required to obtain an appreciable degree of enrichment for hTLR2-binding phage clones (Figure 7b). The absorbance value for this third round of cell-based selection was not as high as that of the single round of enrichment with recombinant hTLR2. In conclusion, in terms of total phage pool binding to recombinant hTLR2 protein in ELISA, a cell-based selection method can achieve an appreciable degree of enrichment after 3 rounds of selection. The degree of enrichment is lower than that of traditional protein-based method. However, since ELISA is a protein-based method that only measures the binding against immobilized recombinant proteins, the results here do not reveal the enrichment against cell surface proteins.
Figure 7: Comparison of protein-based and cell-based selections

Equal numbers of phage ($10^{10}$ phage) from the starting M13 library, after immunotube selection (a), or after three rounds of BRASIL selection (b) were assayed for binding to recombinant hTLR2 by ELISA. A labeled anti-M13 antibody was used for detection of bound phage. ExTek: anti-Extracellular-Tie2 antibody control.
4.2.2 Both protein-based and cell-based methods for library selection generated Ab clones against hTLR2 in ELISA assays

Individual phage clones from round 1 of recombinant hTLR2 selection and round 3 of the cell-based selection were assayed for binding to recombinant hTLR2 by ELISA. Numerous phage clones from both selections showed a high degree of specific binding to hTLR2 (Figure 8). Clones from the cell-based selection showed absorbance values in ELISA assays that were comparable to those obtained by recombinant hTLR2 selection. This suggests that although the cell-based phage pool displays a lower degree of enrichment than the pool obtained by recombinant hTLR2 panning (Figure 6), the cell-based selection is nonetheless capable of identifying clones that bind with similar affinities in ELISA assays.

To determine the number of distinct phage clones obtained from each selection, the phage clones identified as binding to hTLR2 in ELISA screens were examined by DNA fingerprinting of the scFv inserts. Screening of 96 protein-selected clones by ELISA identified 21 clones that display significant binding to recombinant hTLR2 (Figure 5). Among these, 12 clones were unique (Table 4). Screening of 154 cell-selected clones by ELISA identified 19 that bound recombinant hTLR2. These 19 positive clones were subjected to DNA fingerprinting to eliminate duplicate clones, and 8 clones proved to be unique (Table 3). One clone from each set of duplicates was used for further analysis (see below). In conclusion, despite a lower degree of enrichment in the cell-based selection, both protein- and cell-based assays are capable of identifying clones that
bind to hTLR2 in ELISA assays.
**Table 3: Relative efficiencies of protein-based versus cell-based selection and screening methods**

<table>
<thead>
<tr>
<th>Selected on</th>
<th>Screened by</th>
<th>Initial screening-Raw phage</th>
<th>Fingerprint</th>
<th>Purified phage binding to cells</th>
<th>scFv binding to cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>ELISA</td>
<td>96</td>
<td>21 (21%)</td>
<td>12/21 (57%)</td>
<td>0/12 (0%)</td>
</tr>
<tr>
<td>Cell</td>
<td>ELISA</td>
<td>154</td>
<td>19 (12%)</td>
<td>8/19 (42%)</td>
<td>7/8 (88%)</td>
</tr>
<tr>
<td>Cell</td>
<td>Flow cytometry</td>
<td>168</td>
<td>78 (46%)</td>
<td>9/23 (39%)</td>
<td>8/9 (89%)</td>
</tr>
</tbody>
</table>

Numbers in fractions indicate “number of positive clones/number of clones tested” and percentages in parentheses indicate positive rates for each test.
Figure 8: ELISA screening for hTLR2-binding Ab clones.

Binding of individual phage clones to recombinant hTLR2 after round 1 of immunotube selection (left) or round 3 of BRASIL selection (right). Phage clones were prepared in 96-well plates and tested in ELISA assays against recombinant hTLR2-extracellular domain (TLR2) or a control protein (ExTek).
4.2.3 Cell-based, but not protein-based, library selection generates Ab clones that bind to cell-surface native hTLR2

We next compared the ability of the hTLR2-binding clones obtained from these two selections to bind cell surface native hTLR2 protein. A concentrated, highly purified stock of M13 phage particles for each clone was prepared and used as the primary staining reagent in flow cytometry [42, 50]. As a negative control, we employed the anti-ExTek phage clone 1D6 (Lipes and Kenan, manuscript in preparation), which binds the extracellular domain of Tie2. Since Tie2 is an endothelial cell-specific receptor tyrosine kinase [51], this phage clone is well suited for use as a control with HEK cells. When tested by flow cytometry, none of the clones obtained by selection with recombinant protein bound to cell-surface hTLR2 (Figure 9a), despite their ability to bind to recombinant hTLR2 in ELISA assays. Performing a second round of selection with recombinant hTLR2 did not produce any additional clones, instead yielding multiple clones representing a small subset of the round one positive clones (data not shown). In contrast, 7 of the 8 unique clones from the cell-based selection were positive for cell surface hTLR2 binding (Figures 9b and Table 34). These results demonstrate that cell-based selection is substantially more efficient than panning on protein-absorbed plates for enriching phage clones that bind native hTLR2 expressed on the cell surface.
Figure 9: Binding of individual phage clones to cell-surface hTLR2 as assessed by flow cytometry.

Clones that tested positive in ELISA (Figure 5) were chosen to be tested by flow cytometry here. Unique ELISA-positive phage clones were incubated at $10^{12}$ phage/ml with hTLR2-HEK cells (black lines) or WT-HEK cells (gray shadow) and examined by flow cytometry (Y-axis: % of maximum cell number. X-axis: log scale of fluorescent intensity for phage staining in APC channel). 8 representative clones from those in Figure 5 are shown here.
4.3 Discussion

A comparison of protein-based versus cell-based selections with the anti-hTLR2 scFv M13 phage library suggests that cell-based selections may provide a distinct advantage for obtaining Abs that bind cell-surface antigens rather than immobilized recombinant protein (Table 3). Selection of phage antibodies with recombinant hTLR2 was rapid and yielded a population of Abs that bound with high specificity to the recombinant antigen. However, none of the antibody phage clones obtained from our selection with recombinant hTLR2 were able to bind the receptor in its native context on the cell surface (Table 3 and Figure 9a). It may have been possible to identify such clones through an exhaustive screen of the clones from our recombinant protein selection. Alternatively, it may have been possible to obtain these clones by modifying the selection method in an effort to allow the protein to adopt a native structure. For example, immobilizing the recombinant protein via an N- or C-terminal tag to magnetic beads instead of adsorbing it to polystyrene may have been beneficial [52]. Nonetheless, the relative ease by which phage clones binding to cell-surface hTLR2 were found with cell-based selection illustrates this approach’s significant advantage of ensuring the selected antibodies will bind the target protein on cell surfaces, and therefore should be considered even when recombinant antigen is available.

Given the rapid development of improved or application extended phage-display scFv systems, many different library selection methods are available. Combining
other selection methods such as fluorescence activated cell sorting (FACS) [42, 50] or magnetically activated cell sorting (MACS) [53] with our cell-based immunizations and screens may offer even greater selection efficiencies than the BRASIL selection method employed here.
5. Cell-based screening of hTLR2-binding Ab clones

5.1 Introduction

ELISA assays is the standard method for screening in both hybridomas and phage-displayed scFvs. As demonstrated in Chapter 4, many Ab clones that bound to Ag in ELISA failed to bind to cell surface native Ag (Figure 9a). Thus, to be certain that Abs bind to cell surface Ags, an additional confirmation step by flow cytometry is needed when using traditional ELISAs as a screening method. In addition, ELISA assays require expression and purification of recombinant protein for use as immobilized Ag. Therefore, to further streamline this cell-based system and also completely eliminate the requirement of recombinant hTLR2 protein, a cell-based method for screening is needed to replace the traditional ELISA method. To achieve this, we developed a high throughput flow cytometric screening method to directly identify scFv clones that bind to cell surface hTLR2.

5.2 Results

5.2.1 Components in fetal bovine serum cause non-specific binding during staining of raw phage preparations

Although scFvs displayed on purified phage are not a standard reagent for flow cytometry staining, they can be used when highly purified and utilized under
appropriate staining conditions [42, 54]. Using phage as a staining reagent tends to produce higher staining background and less cell-viability, presumably due to the nature of phage structure and toxicity. Therefore, it is necessary to carefully optimize phage titers and staining conditions before employing phage as a flow cytometric staining reagent. Moreover, raw phage preparations, which are essentially bacterial culture supernatants, tend to have even higher staining backgrounds and cellular toxicity, presumably due to additional molecules present in the broth from bacterial cultures. For these reasons, no previous studies have been reported that used raw phage preparations as a staining reagent in flow cytometry. For staining on TLRs, the nature of TLR recognizing microbial components may lead to even more problems. Since TLRs recognize microbial components, phage or other components in bacterial cultures may interfere with TLR-Ab recognition, or activate TLRs on the cell surface, which in turn could affect a cell’s viability or phenotype. Thus, to employ flow cytometry in a high throughput cell-based screening method, we must first ensure that: (1) raw phage preparations are capable of generating enough specific binding signal to be detected over a high staining background, and (2) the effects of phage itself or microbial components in the preparation are minimized to allow acceptable cell viability and phenotype changes. We first sought to optimize the staining conditions by testing several concentrations of scFv-phage and staining buffer components. Fetal bovine serum (FBS) is a commonly used stabilizing/blocking reagent in buffers for
many cell-based assays. When used with purified IgG or scFv, FBS does not interfere with the Ab-Ag interaction but instead often decreases staining background because it stabilizes cell surface, Ag and Ab structures, reduces cell death, and neutralizes molecules that potentially cause non-specific binding [55]. However, in initial studies, we found that addition of serum to the raw phage preparations causes non-specific phage binding to cells (Figure 10, right). Therefore, it was critical to use a serum-free staining buffer throughout the process. We did not further investigate which component in FBS caused the non-specific binding, however, we found that replacing 3% FBS with 0.5% BSA (Bovine Serum Albumin) as stabilizing/blocking reagent reduced the non-specific binding to a minimum (Figure 10, left) while maintaining good cell viability. No other buffer components were found to have effects on staining background. ScFv-phage titers were optimized to the range of $10^9$-$10^{11}$ phage/100ul of cell solution in one well (containing $10^5$ cells), with $10^{11}$ phage/100 ul having the highest staining signal and acceptable staining background. All of the following studies were conducted under these optimized staining conditions.
Four potential anti-hTLR2 clones (G11, G12, A5 and B6) and one negative control clone (ExTek) are shown here as representative clones. In the form of purified scFv (Chapter 6), clones G11 and G12 were tested negative whereas clone A5 and B6 were tested positive. Flow cytometry was performed with buffers contains 0.5% BSA (left) or 3% FBS (right). (Y-axis: % of maximum cell number. X-axis: log scale of fluorescent intensity for phage staining in APC channel)
5.2.2 hTLR2-negative YFP-positive HEK cells as an internal negative control

We sought to minimize the required reagents, labor and sample numbers to streamline the system. For this high-throughput screening assay, equal numbers of hTLR2-HEK cells and HEK cells stably transfected with yellow fluorescent protein (YFP-HEK cells) were mixed, stained with individual phage clones in 96-well plates, and subjected to flow cytometric analysis using an automatic plate-loader. The YFP-HEK cells served as an internal negative control for each sample (Figure 11a). By combining target and negative control cells in every well, we ensured that both cell types were exposed to identical staining conditions and also lowered the sample number and the amount of staining reagents that were required. In pilot studies, we demonstrated a clear separation of YFP+/hTLR2- and YFP-/hTLR2+ HEK cells in the FITC channel (Figure 11). Detection of phage binding to cells was accomplished using an anti-M13 Ab and APC-conjugated-anti mouse IgG as secondary and tertiary reagents, respectively. The anti-Tie2 1D6 phage clone was used as a negative control. Previously identified phage clones demonstrated to bind cell surface hTLR2 were used as positive controls.
Figure 11: Flow cytometric screening of phage clones for binding to cell-surface hTLR2.

Representative scatter plots and histograms demonstrating typical results for hTLR2-specific clones (left), non-binding clones (center), and clones that bind to all HEK cells (right). Equal numbers of hTLR2-HEK and YFP-HEK cells were mixed and stained with raw phage preparations of individual clones. hTLR2-HEK cells (black gate) were distinguished from control YFP-HEK cells (gray gate) in the FITC channel as shown and the binding of phage to each population displayed as overlays of histograms (black line - hTLR2-HEK cells; gray shadow - YFP-HEK cells)
5.2.3 High-throughput flow cytometric screening for positive antibody clones

Once a high-throughput, optimized flow cytometry staining condition had been established for raw phage preparations, we employed this staining method to screen a total of 168 cell-selected phage clones. We identified 78 clones that bound specifically to hTLR2-HEK cells (Figure 12 and Table 3). The results shown in Figure 12 are representative of all 96-well plates screened, revealing hTLR2-specific clones (e.g. clones A3 and A4 in this figure), non-binding clones (B4 and B5), and clones that bind to YFP-HEK cells (D5) in various ratios. By eliminating ELISA assays and directly screening with flow cytometry, we were able to rapidly identify large numbers of phage clones that specifically recognize cell surface hTLR2. 23 of these clones were subjected to fingerprinting analysis, resulting in the identification of 9 unique clones (Table 3). When prepared as purified phage, 8 of these 9 unique clones bind to native hTLR2 in a flow cytometric assay (Figure 15 and Table 3). These clones also bind to native hTLR2 when they are prepared as purified scFvs (Figure 15 and Chapter 6). These results demonstrate that flow-cytometric screening of raw phage preparations is a rapid and efficient method to identify Abs that bind to cell surface Ags.
Figure 12: Typical results of flow cytometry-based screening.

Results for 24 out of 96 wells in one screen of the cell-selected library are shown, including hTLR2-binding clones (e.g. A3 and A4), negative clones (e.g. B4 and B5), and WT-HEK binding negative clones (e.g. D5). X axis: Ab-binding signal in APC channel. Y-axis: percentage of cells. Histogram layout as described in Figure 11.
5.3 Discussion

Performing direct screens of selected antibody phage clones by flow cytometry in a 96-well high-throughput format with raw phage preparations further streamlined the identification of clones specific for cell-surface hTLR2. High throughput screening is important when the objective is to obtain Abs for a specific purpose such as receptor modulation or therapeutic applications since Abs with these capabilities typically would be expected to comprise a very small fraction of the total pool of antibodies. Here, we provide an optimized technique for such screens using 96-well preparations of individual phage clones. The use of an internal control cell line expressing YFP provides excellent discrimination of positive phage clones from background. Another critical finding from the optimization of this approach is that use of serum as a stabilizing/blocking agent causes non-specific binding of M13 phage to HEK cells. This background is avoided by use of bovine serum albumin as the blocking agent.

Previous studies have utilized flow cytometry for phage selections, iteratively employing FACS to enrich for phage binding a subpopulation of cells [42, 50] and then individually screening resulting clones by flow cytometry. Screening of phage clones individually appears to be more labor intensive and expensive than the 96-well flow cytometric screening approach used here.

Optimizing raw phage preparations in bacterial culture supernatant as a flow cytometry staining reagent is critical for our cell-based screening method and may be
helpful to facilitate other assay systems that involve phage-displayed scFvs. Our finding that serum (FBS), a commonly used stabilizing/blocking reagent, causes non-specific binding in flow cytometry may be helpful for some other assays using staining reagents other than standard IgG. It is not clear which components in serum cause this non-specific binding, but several TLR ligands have been reported in serum [56] and may be responsible for the increased background we see. Hps60, a TLR2 ligand [57, 58], Mrp8 (myeloid-related protein 8), a TLR4 ligand [59], and HMGB-II (High-mobility Box II) [60], another TLR4 ligand, were found in infected or chronically injured animals or patients. To our knowledge, no studies have investigated the direct interactions between these endogenous TLR ligands and phage or bacterial components. An alternative explanation is that these molecules may stimulate TLR-positive cells and affect cell function, phenotype, or even viability, which in turn interferes with the experimental readout.
6. Endotoxin-free Single-chain antibody production

6.1 Introduction

The preceding flow cytometry measurements were performed using normalized titers of purified phage, allowing us to make preliminary assessments as to which phage-conjugated scFv antibodies bind to cell-surface hTLR2. The most promising candidates were chosen for expression as soluble scFvs. The simplest and standard method for obtaining soluble scFvs is expression from the phagemid vector in a non-suppressor strain of E. coli (Escherichia coli). However, among the Ab clones we have generated to date, many scFvs either failed to express at acceptable levels in E. coli or formed inclusion bodies of incorrectly folded non-functional protein. Furthermore, bacterial component contamination in scFv preparations is problematic when applying this Ab reagent into functional studies. Thus, an efficient and endotoxin-free system to produce scFv is needed.

6.2 Results

6.2.1 TLR ligands are contaminants in scFv produced in bacteria and the contaminants activate TLR signaling

LPS (a bacterial cell wall component in all gram-negative bacteria), lipopeptides and lipoteichoic acid (LTA) are commonly present in recombinant protein preparations purified from E. coli. This contamination of Ab reagents has been tolerated in many
studies since these components usually do not interact with the Ag of interest. When the Abs were used in detecting cell lysates, fixed tissue sections, or recombinant proteins, for which cell function or viability is not an issue, the microbial contaminant was also ignored. However, when Abs are used in staining TLRs on a cell surface, it is critical to determine the affects of these bacterial components on the target cells, since these contaminants may block the access of Ab to TLR, alter TLR structure, or affect cell phenotype/viability and dramatically change the experimental outcome. To test if bacterial components in phage-displayed scFvs or purified scFv preparations activate TLR signaling, we used NF-kB promoter driven secreted alkaline phosphatase (SEAP) reporter assays [61, 62]. This reporter gene was co-transfected into hTLR4-HEK, hTLR2-HEK, or mTLR2 cells. Soluble scFvs (not conjugated with phage) purified from an E. coli expression system were applied to the TLR-HEK transfectant to assess their ability to activate TLR signaling. We found that scFvs purified from E. coli are prone to contamination with endotoxin and lipopeptides, leading to TLR4 and TLR2 activation (Figure 13). When LPS in the Ab preparations is pre-cleared by polymyxin-B [63], their ability to activate hTLR4-SEAP-HEK cells decreased. When a TLR2-blocking Ab (T2.5) is present, the Ab preparation is less effective in activating hTLR2-HEK (Figure 13). This data suggested that TLR4 ligands (LPS) and TLR2 ligands are contaminants in the Ab preparations, causing TLR activations. hTLR2- or mTLR2- SEAP assays of purified phage-conjugated scFv, which is also produced in E. coli, showed similar TLR activation
results (Figure 14 and data not shown). In addition, the fact that anti-Tie2 scFv activated both TLR2 and TLR4 signaling, suggested that the TLR activation we saw here was not due to the specific scFv binding of anti-TLR scFvs to their targets TLRs (Figures 13 and 14). Although we did not test other TLR or TLR ligands, it is likely that other bacteria-derived TLR ligands are present in the Ab preparations. This contamination may not only affect Ab-Ag interactions for TLRs but also cause significant problems in functional assays involving TLRs or other pattern-recognition molecules since even slight amounts of contamination with bacterial components may activate leukocytes and profoundly alter experimental results. In fact, several previously identified “TLR ligands” were proven to be false positives due to LPS or flagellin contamination [64-66]. Therefore, for TLR studies, generating antibodies with an endotoxin-free system is required.
Figure 13: scFv preparations from *E. coli* activate TLR4 and TLR2.

(a) hTLR4/CD14 HEK cells with (black) or without (gray) MD2 co-transfection were cultured with scFv preparation for 24 hours. LPS in some scFv preparations were removed by polymyxin B before being used. (b) hTLR2-HEK cells were cultured with scFv with or without Pam3CSK for 24 hours. ND: not done. T2.5 mAb: anti-TLR2 blocking Ab.
6.2.2 Drosophila expression system efficiently produces TLR ligand-free scFvs

To overcome the problem of bacterial component contaminations, we employed an insect cell expression system. ScFv insert sequences were recovered by PCR amplification from positive phagemid clones, then ligated with topoisomerase into a donor plasmid between recombination sequences for a bacteriophage recombination enzyme. Corresponding recombination sequences were added to a Drosophila expression vector to allow rapid transfer of the scFv inserts into this vector for expression in S2 insect cells. To date, we have successfully expressed dozens of scFv antibodies against various antigens as secreted products in stably transfected S2 cells. There have been no instances in which an antibody failed to express in the Drosophila system, in contrast to the recognized scFv expression problems in the E. coli system. Typical yields for scFvs in the Drosophila expression system are 1-2 mg/L of culture, and range from 0.5 to 5 mg/L. This is ten-fold higher than the typical yield of 0.1-0.2 mg/L from E. coli (data not shown).
Figure 14: ScFv produced in Drosophila system does not activate hTLR2.

Two representative anti-hTLR2 scFv clones were purified from *E. coli* (top) or Drosophila (bottom) and cultured with hTLR2-HEK with (left) or without (right) Pam3CSK stimulation for 24 hr. The resulting culture supernatants were subjected to SEAP assays.
6.2.3 ScFv produced in insect cell system retains binding and specificity to hTLR2

In the case of hTLR2, 5 out of 5 clones from both the protein-based selection and the cell-based selection specifically bind to hTLR2-HEK cells when expressed as scFvs by this Drosophila system (Figures 15, 17 and Table 3) and provoke no endotoxin-mediated stimulation of TLR signaling (Figure 14 and data not shown), demonstrating an efficient, effective, and endotoxin-free method for scFv production.
Figure 15: Representative histograms of flow-cytometric staining profiles obtained from individual clones at each stage of screening and validation.

A5 and B11, two clones that bind cell-surface hTLR2, demonstrate hTLR2-specific staining during flow cytometric screening of raw phage preparations (top), when prepared as purified phage (middle), and when expressed as purified scFvs (bottom).
6.2.4 Staining of endogenous hTLR2 with scFvs

To determine whether scFvs selected against ectopically expressed hTLR2 would bind to the endogenous receptor on human cells, we examined TLR2 expression on two human cell lines: THP-1 cells (a macrophage cell line) and CaCo-2 cells (an intestinal epithelial cell line). Both soluble scFvs tested, A5 and B11, demonstrate specific binding to both THP-1 and CaCo-2 cells, generating a robust signal at scFv concentrations of 0.1 ug/ml (Figure 16). As we will discuss in detail later, one scFv, clone B11, was also capable of detecting endogenous hTLR2 tested on freshly isolated bronchioalveolar (BAL) cells obtained from a patient with known pulmonary inflammation (Chapter 8.2 and Figure 25). These results demonstrate that a cell-based system of phage selection and screening efficiently generates scFvs that bind specifically to endogenous cell surface proteins.
Figure 16: Anti-hTLR2 scFvs specifically bind to endogenous hTLR2.

Flow cytometric results of the binding of two scFv clones to human THP-1 and CaCo-2 cells. The binding of the clones to hTLR2-HEK cells (left) is shown for comparison. In each panel, signals obtained with anti-hTLR2 scFvs (black lines) were compared with signals obtained with an anti-ExTek negative control (gray). Histograms are formatted as in Figure 11.
6.3 Discussion

The methods developed here for rapidly shuttling scFv inserts into a vector for expression in S2 insect cells provides an advantage over expression in E. coli, given the sensitivity of immune system receptors such as TLRs to bacterial components such as lipopeptides and LPS. The yields of functional scFv proteins we obtained from Drosophila were greater than those we typically obtain from E. coli, a finding others have noted [67]. Sub-cloning VH and VL genes into the insect cell expression system also facilitates re-engineering anti-hTLR2 scFv antibodies into other antibody formats such as full-length IgG [68] or humanized IgG. We performed pilot experiments to convert anti-Tie2 and anti-hTLR5 scFv into full-length IgG (Chapter 7 and Figure 27) and these Abs retain their ability to specifically bind to their targets.

For protein stability, scFv is expected to be less stable than full-length IgG. Therefore we performed quality control tests for several scFv preparations after longer storage. We found that the staining intensity of cell surface hTLR2 in flow cytometry with our anti-hTLR2 scFv remained unchanged after a 48 hr incubation at 37°C in cell culture medium (RPMI or MEM), 1 month of storage at 4°C, or 1 year of storage at -80°C (data not shown). Although we did not perform parallel comparison of Ab stability between scFvs and IgGs, these data suggest that the scFv produced in the Drosophila system is stable enough for most research needs.
In conclusion, using a *Drosophila* expression system, we efficiently and successfully generated multiple clones of scFv that are free of bacterial component contaminations. These Ab clones retained their specificity in all formats we tested. Our achievements to this point are summarized in Table 3 and Figure 17.
Figure 17: Representative results of flow-cytometric validation assays of clones obtained by different selection and screening methods.

Validation includes assays of purified phage (top row) or purified scFv (bottom row) for binding to hTLR2-HEK cells (black lines) versus WT-HEK cells (gray shadow). Histograms are formatted as in Figure 11
7. Homolog mining for Abs specific to hTLR2-related proteins

7.1 Introduction

7.1.1 Naïve and immune Ab libraries

ScFvs are usually generated using one of two general strategies. The first involves screening non-immune phage libraries. Non-immune libraries are typically highly complex (10⁹-10¹¹ clones), consisting of scFvs formed from the VHV and VL fragments of a large number of naïve donor animals or humans [8, 45, 69, 70]. The advantage of this strategy is that a single highly complex library can be used to screen for many different targets. The disadvantages of this strategy are that the frequency of scFvs specific for a given Ag are low, thereby necessitating a large amount of screening, and that the affinity of the scFvs identified are often only moderate, making it necessary to employ affinity maturation through mutagenesis to achieve the required affinity [49, 69]. The alternative strategy is to screen immune libraries, which are generated from the B cells of immunized animals or humans after an infection. Immune libraries are typically smaller (10⁵-10⁶ clones) than non-immune libraries but usually contain a much higher frequency of scFvs that bind with high affinity to the Ag used for immunization. For this reason, immune libraries typically yield a greater number of candidate scFvs than non-immune libraries. The disadvantage of using immune libraries is that a new
library must be used for each target Ag. This involves both a dedicated series of immunized animals or patient samples and the effort required for library construction itself.

The utility of immune phage display antibody libraries would be improved if they could be used to obtain antibodies against multiple distinct target antigens. We hypothesized that, due to the reassortment of \( V_H \) and \( V_L \) regions, an immune library generated against one antigen would contain scFvs that recognize members of the same protein family at a reasonable frequency and thereby eliminate the need to generate immune libraries for each family member. Here, we tested this hypothesis by mining an existing anti-human Toll-like receptor-2 (hTLR2) library for scFvs that are specific for other members of the Toll-like receptor (TLR) family.

### 7.1.2 Staining reagents for cell surface native TLRs

TLRs are important mediators of innate immune response, but investigation of these receptors has been hampered by a lack of antibodies capable of binding to cell surface TLRs. Anti-TLR antibody generation has proven problematic because TLRs can be difficult to express as recombinant proteins and because antibodies raised against TLR proteins often fail to recognize these proteins in their native form as expressed on the cell surface [71].

To date, most available Abs against TLR only recognize denatured or purified Ags, thus reliable Abs for flow cytometry or other functional studies are urgently
needed. To study TLR expression, most published studies have measured TLR mRNA levels, or TLR proteins in cell lysates by Western blotting. Recently GFP-labeled flagellin was used as staining reagent for TLR5 to detected endogenous TLR5 expression [72, 73]. A few flagellin-directed Ag targeting strategies for vaccine adjuvant development have been successful so far and are currently in phase I clinical trials. [73]. However, using this strategy for staining has two major problems. First, applying flagellin will activate TLR5 which in turn could change cell phenotype. Second, TLR ligands, except TLR5, are mainly lipid-based or nucleotide-based molecules thus more difficult to be conjugated with GFP to form a stable staining reagent. Nevertheless, the success of the TLR5-targeting strategy for vaccine adjuvant effects suggests the great potential of TLR-specific agonistic/antagonistic antibodies in adjuvant development and Ab-based therapeutic purposes.

In previous work we addressed these problems by establishing an entirely cell-based procedure to produce scFvs that bind to native proteins on the surface of cells [71]. This allowed the generation of numerous scFvs that bind cell-surface hTLR2. In the current study, we sought to extend the use of our existing anti-TLR2 scFv library by using our cell-based system to select scFvs specific for other members of the TLR family from this library. This strategy, which we refer to as homolog mining, proved to be feasible, as we were able to identify scFvs specific for mTLR2, hTLR5, and hTLR6 that did not cross-react with the original immunogen, hTLR2. These results demonstrate that
a single immune library can be mined for recombinant Abs against multiple homologous cell surface proteins using a procedure that efficiently selects for scFvs that bind those proteins in their native form. This strategy should extend the utility of existing immune phage display libraries and provide a marked increase in the efficiency with which Abs against families of complex cell surface proteins can be generated.

<table>
<thead>
<tr>
<th>Table 4: Comparison of Non-immune library with immune library for Ab production</th>
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<tr>
<td><strong>Non-immune library</strong></td>
</tr>
<tr>
<td>Number of Ab donor animals/ humans</td>
</tr>
<tr>
<td>Library complexity</td>
</tr>
<tr>
<td>Generate Abs against multiple Ags?</td>
</tr>
<tr>
<td>Ab affinities</td>
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</table>
7.2 Results

To investigate the potential of obtaining scFvs against molecules related to hTLR2 from our existing anti-hTLR2 library, we applied the procedures for selection and screening described in the above sections and our previous publication [71]. In brief, the library was created from multiple BALB/c and C57BL/6 mice which were immunized with hTLR2-transfected 300.19 cells. Spleens from six high titer animals were used to construct an anti-hTLR2 M13 phage-scFv library, with a theoretical complexity of 3x10^6. We employed the same cell-based selection procedure, BRASIL, which relies on differential centrifugation to obtain Ag-specific phage clones [43]. In this procedure, the scFv phage library is incubated with parental HEK293 cells followed by differential centrifugation to obtain a pool of phage pre-cleared to remove clones binding to parental cell markers. The remaining phage are incubated with target TLR-HEK cells. Loosely associated non-specific phage are dissociated by pelleting the cells through an organic phase. The phage remaining bound to TLR-HEK cells are used to generate enriched phage stocks for iterative rounds of selections. Once enriched, the phage library is arrayed into 96 well plates. The raw phage preparations, in plates, are screened for their capacity to bind target (TLR-HEK) versus control (YFP-HEK) cells in a high throughput flow cytometric assay. The resulting positive clones are subjected to restriction fragment length polymorphism or “DNA fingerprint” analysis to eliminate duplicates, prepared as purified phage, and tested again by flow cytometry to confirm their specific binding
to TLR-HEK cells. The scFv inserts of confirmed clones are subcloned into a Drosophila expression vector, expressed as soluble scFvs, purified, biotinylated, and validated for their capacity to specifically bind TLR on cell surface cells in a flow cytometric assay.

7.2.1 Generation of anti-mTLR2 Abs from a hTLR2 library

7.2.1.1 Library selection and screening

To test homolog mining, we first attempted to identify clones specific for mTLR2, the receptor most closely related to hTLR2. The hTLR2 scFv library was subjected to three iterative rounds of BRASIL selection using mTLR2-HEK cells for positive selection as above. 43 (51%) of the 84 clones screened in a flow cytometric assay were positive for mTLR2 binding (Table 5, Figure 18). DNA fingerprinting of 32 clones revealed 18 (56%) to be unique (Table 5). Nine of these unique clones were used to generate purified phage stocks and tested for their ability to bind mTLR2. Five (56%) of these nine anti-mTLR2 phage clones proved to be positive in this confirmatory test (Table 5, Figure 19), and were subcloned and expressed as purified soluble scFvs in S2 cells. All five clones retained their capacity to specifically bind to mTLR2-HEK cells when expressed as soluble scFvs (Table 5, Figure 19). Importantly, none of these anti-mTLR2 clones demonstrated binding to human TLR2 (Figure 221 and data not shown).
Figure 18: Screening anti-mTLR2 antibody clones.

Raw phage preparations of individual clone were used to stain mTLR2-HEK (black line) or YFP-HEK (gray) cells. 20 representative clones are shown here. Positive clones are indicated in bold.
7.2.1.2 Evaluation of anti-mTLR2 scFvs

To determine whether the anti-mTLR2 scFvs identified are able to detect endogenous mTLR2, these clones were tested for their ability to stain a murine monocyte/macrophage cell line, J774A.1 [38]. Despite the low level of mTLR2 expression in this cell line, all scFvs were able to detect mTLR2 on the surface of these cells (Figure 19). One anti-mTLR2 scFv (D2-3) was also capable of detecting mTLR2 expression in the LPS-induced lung injury as we will discuss in detail in Chapter 8.2 (Figure 26). These findings demonstrate that it is possible to obtain anti-mTLR2 antibodies that have sufficient affinity to detect endogenous mTLR2 from a library raised against hTLR2.
Figure 19: Validation of anti-mTLR2 binding to transfected or endogenous mTLR2.

Purified phage (left) or corresponding scFvs (center) were used as primary Ab to stain on mTLR2-HEK cells (black line) or YFP-HEK (gray). Right panel shows J774A.1 cells stained with anti-mTLR2 scFvs (black line) or with anti-Tie2 scFv (gray).
7.2.2 Generation of anti-hTLR5 and anti-hTLR6 Abs from a hTLR2 library

7.2.2.1 Library selection and screening

Having demonstrated that homolog mining works in principle, we sought to test this procedure with other, less homologous, members of the TLR family. Separate cell-based selections of the hTLR2 library were performed as above for hTLR1, hTLR5 and hTLR6. After 3 rounds of selection, arrays of the enriched clones were screened in a flow cytometric assay. These initial screens yielded 39 (21%) of 186 positive clones for hTLR5 and 11 (6%) of 186 positive clones for hTLR6 (Table 5, Figure 20). No positive clones were obtained for hTLR1 from a screen of 94 clones (Table 5). After elimination of duplicate clones by DNA fingerprinting (Table 5), the activity of clones prepared as purified phage was validated for binding to the selected TLR. When tested by flow cytometry, 2 (12%) of 17 anti-hTLR5 phage clones and 1 (17%) of 6 anti-TLR6 clones bound their respective target (Figure 21 and Table 5). Without antibodies available to be used as a positive controls in screening hTLR5 and hTLR6, it was more difficult to distinguish positive clones from background signals (Figure 20 and data not shown) than it was for previous TLR2 measurements that included a positive control. This resulted in a higher rate of false positives for hTLR5 and hTLR6 (discussed in Section 7.3).
Figure 20: screening of anti-hTLR5 or anti-hTLR6 antibody clones.

Screening anti-hTLR5 (a) or anti-hTLR6 (b) antibody clones. Individual clones of raw phage preparation were used as primary Ab to stain hTLR5- (a, black line), hTLR6- (b, black line), or YFP- (a & b, gray) HEK cells. 20 representative clones were shown here. Positive clones are indicated in bold.
7.2.2.2 Evaluation of anti-hTLR5 and nati-hTLR6 scFv

When prepared as purified scFvs in Drosophila cells, 1 out of 2 (50%) anti-TLR5 scFvs bound to hTLR5-HEK cells (clone 3B5 in Figure 21; Table 5), while 1 out of 1 (100%) anti-hTLR6 scFvs bound to hTLR6-HEK cells (clone 3F7 in Figure 21 and Table 5). In an attempt to obtain more anti-hTLR5 clones, we performed a fourth round of BRASIL selection for hTLR5. This resulted in similar frequency of positive clones in the initial screening step (22 out of 92, or 24%), but a much lower frequency of unique clones than seen after three rounds of selection (2 out of 19, or 11%). One clone, 4A10 in Figure 21 was confirmed in the form of scFv) (Table 5, row hTLR5-HEK R4).
Figure 21: Identification of anti-hTLR5 or anti-hTLR6 antibody clones.

Flow cytometry staining results for two representative hTLR5 (a) or hTLR6 (b) antibody clones. Raw phage preparations (left), purified phage (center), or purified scFvs (right) were used as primary antibody to stain hTLR5- (a, black line), hTLR6- (b, black line) or YFP- (gray) HEK cells.
Table 5: Summary of numbers of clones and positive rates for screening/verification at each step of anti-TLR scFv generation

<table>
<thead>
<tr>
<th>Screened by</th>
<th>Initial Screen - Raw Phage</th>
<th>Fingerprint</th>
<th>Purified phage Binding to cells</th>
<th>scFv binding to cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td># of clones screened</td>
<td># of clones positive</td>
<td># of unique clones</td>
<td># of clones positive</td>
</tr>
<tr>
<td>hTLR2 HEK</td>
<td>168</td>
<td>78 (46%)</td>
<td>9/23 (39%)</td>
<td>8/9 (89%)</td>
</tr>
<tr>
<td>mTLR2-HEK</td>
<td>84</td>
<td>43 (51%)</td>
<td>18/32 (56%)</td>
<td>5/9 (56%)</td>
</tr>
<tr>
<td>hTLR5-HEK R3</td>
<td>186</td>
<td>39 (21%)</td>
<td>18/39 (46%)</td>
<td>2/17 (12%)</td>
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<td></td>
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<tr>
<td></td>
<td>92</td>
<td>22 (24%)</td>
<td>2/19 (11%)</td>
<td>1/2 (50%)</td>
</tr>
<tr>
<td>hTLR6-HEK</td>
<td>186</td>
<td>11 (6%)</td>
<td>6/8 (75%)</td>
<td>1/6 (17%)</td>
</tr>
<tr>
<td>hTLR1-HEK</td>
<td>94</td>
<td>0 (0%)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Numbers in fractions indicate “number of positive clones/number of clones tested” and percentages in parentheses indicate positive rates for each test.

* Results after performing a 4th round of selection (R4) for hTLR5 versus the standard 3 rounds (R3) of selection.
7.2.3 Antibody specificity

A potential pitfall of homolog mining is that it may select for scFvs that bind to protein domains conserved among multiple receptors and thereby only identify clones that cross-react with multiple TLRs. To ensure that the scFvs we identified are specific to individual TLRs, we examined the binding of purified anti-TLR scFvs mined from the hTLR2 library to all of the TLRs we have expressed. The anti-Tie2 scFv, ExTek clone 1D6, was again used as negative control. As shown in Figure 3, all of the scFv clones we identified bound only to their target and displayed no cross-reactivity to other members of the TLR family. In the case of TLR2, scFvs demonstrated no cross-species reactivity. The anti-TLR scFvs isolated from the hTLR2 library thus exhibit exquisite specificity for their target TLR. This result suggests that homolog mining selects for scFvs that recognize epitopes that are not fully conserved among TLRs.
Figure 22: Anti-TLR antibody clones specifically bind their target Ag but not other TLRs.

ScFvs against hTLR2 (clone B11), hTLR5 (clone 3B5), hTLR6 (clone 3F7), mTLR2 (clone B2-3), or Tie2 extra-cellular domain (ExTek, clone 1D6) were used as primary antibodies to stain hTLR1-, hTLR2-, hTLR4-, hTLR5-, hTLR6-, mTLR2- or mTLR5-HEK (black line), with YFP-HEK as negative control cells (gray). All scFv clones obtained to date show the same specificity to their target TLR Ag and the result of one representative clone is shown here for each group. Positive stainings are outlined in bold.
7.2.4 Origin of anti-homolog antibodies

As shown in this chapter, our scFv phage display library, which was raised against hTLR2, contains clones specific for TLRs other than hTLR2. It is not clear if such clones represent antibody molecules formed in vivo after immunization or if they were created during library construction in the course of V\textsubscript{H}-V\textsubscript{L} re-assortment. To address this question, we examined the reactivity of the sera of the individual hTLR2-immunized mice used to construct the phage library. Using a flow cytometric assay we have previously described (Chapter 3 and [71]), serial dilutions of each serum sample were tested for binding to hTLR1, hTLR2, mTLR2, hTLR5, and hTLR6.

As we have shown previously (Chapter 3 and [71]), sera from responsive hTLR2-immunized mice contain detectable anti-TLR2 antibodies at dilutions up to 2^{12} (Figure 4). In contrast, the reactivity of these sera against hTLR1, mTLR2, hTLR5 and hTLR6 is no greater than that of unimmunized mice (Figure 23). Antibodies against these TLRs are either not present in the original antibody repertoire or represent an extremely small fraction of it. This finding strongly suggests that the scFvs we identified are the products of combinatorial re-assortment of heavy and light chain variable fragments.
Figure 23: Sera from hTLR2-immunized mice do not contain detectable levels of IgG against hTLR1, hTLR5, hTLR6 or mTLR2.

Mice were immunized with hTLR2 by a cell-based immunization method and sera were harvested 7 days after the 5th boost. Mice sera were used as primary staining reagents in flow cytometry to test their binding on hTLR2-, mTLR2-, hTLR5-, hTLR6- or hTLR1-HEK, respectively. Ag-specific IgG titer was detected by secondary staining of APC-conjugated goat-anti-mouse IgG antibody. Serum from naïve mice were used as negative control.
7.3 Discussion

The screening of immune scFv phage display libraries is an effective means to generate large numbers of high affinity single-chain antibodies against a single antigen. The major impediment to the routine use of this strategy has been the need to construct a new library for each individual antigenic target. Here, we demonstrate that a novel strategy, homolog mining, can be used to significantly extend the utility of immune scFv libraries. By homolog mining an existing anti-hTLR2 library, we were able to obtain scFvs specific for three of the four hTLR2 homologs we targeted. These results demonstrate that, although not foolproof, homolog mining has the potential to markedly reduce the number of libraries required to generate antibodies against a family of proteins. It appears that homolog mining is widely applicable. Preliminary results in our lab indicate that an anti-Tie2 scFv library can be mined for antibodies against other receptor tyrosine kinases (Lipes and Kenan, manuscript in preparation).

Our findings demonstrate that a significant number of anti-homolog scFvs are present in our immune library. These were most likely generated via the combinatorial re-assortment of anti-hTLR2, and perhaps background, VH and VL fragments during library construction. Consistent with this view, we were unable to detect anti-homolog binding activity in the sera of the mice used for library construction. We have no way to assess the extent to which reassortment increased the diversity and complexity of our library over that of the cells from which it was derived. However, previous studies
suggest that this increase can be significant. In one elegant study, transgenic mice were engineered to express a single immunoglobulin mu heavy chain derived from a vesicular stomatitis virus Indiana serotype-neutralizing (VSV-Ind) antibody [74]. This heavy chain could not undergo rearrangements and was expressed in combination with endogenous kappa or lambda chains. The mice expressed a B cell repertoire biased heavily toward VSV-Ind, with 30 to 60 % of the peripheral B cells showing VSV-Ind specificity. Despite this bias, the mice were able to mount successful B cell responses when immunized with the VSV-New Jersey serotype virus as well as other unrelated antigens such as bacterial porins. These studies showed combinatorial L chain variability alone is capable of generating a complex antibody repertoire. In another study, induction of further V_H and V_L re-assortment with Cre recombinase in a naive scFv library increased its calculated theoretical complexity from $10^7$ to $10^{11}$ [75, 76]. When this library was used in selections for 18 recombinant targets, an average of 6 specific antibodies were obtained per target.

By comparison, even though the number of antibodies contained in our anti-hTLR2 scFv library (3x10^6) is five orders of magnitude lower than the Cre-reassorted library and three to four orders of magnitude lower than the number contained in a highly complex non-immune library [8, 45], we were able to obtain multiple specific phage clones for most targets by screening a single 96-well plate. This finding suggests that immunization with hTLR2 provides numerous affinity matured V_H and V_L regions
directed at TLR epitopes that, when re-assorted, can bind TLRs other than the original immunogen. It would be reasonable to expect that scFvs generated by such re-assortment and selected against a homolog would bind to both the homolog and the original immunogen. However, all of the Abs generated from these selections bound only to their target TLR without cross-reactivity to the library immunogen hTLR2 or any other TLRs tested (Figure 22). One possible explanation for this is that the entirely cell-based techniques for selection and screening employed here allowed the receptors to be fully post-translationally modified and to adopt their native, membrane bound structures, emphasizing subtle structural differences between homologs. Purified recombinant proteins often lack such post-translational modifications, do not fold into their native structures, and may have normally internal epitopes exposed. Homologous domains may therefore adopt more similar structures in these conditions, thereby increasing the potential for antibody cross-reactivity. In selections we have previously performed using recombinant hTLR2 protein, we saw appreciable cross-reactivity with recombinant hTLR4 protein (data not shown), suggesting that cell-based methods offer improved antibody specificity. The impressive level of specificity exhibited by our anti-TLR antibodies establishes the feasibility of using existing immune libraries for selections with related antigens, and suggests that cell-based selections may, in general, yield fewer cross-reactive antibodies than selections with recombinant proteins.
There are several practical aspects of homolog mining and scFv generation that should be emphasized. First, we have found that the ability to generate scFvs that bind to either the immunogen or its homologs is highly dependent on the initial response to immunization. In our hands, libraries generated from mice that display only low-titer responses to the immunogen have failed to generate functional scFvs. Second, the hTLR2 scFv library used in these studies was constructed by pooling material from six mice of two different strains. We believe that the strategy of incorporating multiple immune responses from multiple mouse strains into the library captures a more diverse antibody repertoire than would be obtained from a single animal or mouse strain. A similar approach has been used to harvest immune responses from patients with autoimmune diseases [77] and from convalescent serum [3, 69] to make human immune libraries.

Another practical concern is determining the threshold at which anti-homolog clones are considered positive in initial screens and, thereby, the sensitivity and specificity of this assay. In instances where a control antibody is not available, setting this threshold correctly can be problematic. In addition, the concentration of phage in the raw phage preparations used for screening can vary markedly between wells, leading to large differences in signal that are independent of the actual binding affinity. The relatively high level of background staining seen in our anti-TLR5 and anti-TLR6 screens was likely due to a low level of expression of these TLRs on HEK cells, and
perhaps a lower affinity of the scFvs specific for these TLRs. Thus, the high rate of false positives seen in these screens is not a general feature for this technique, but will depend on scFv affinities, target expression levels, and the criteria determined by the user to select positive clones.

In order to be certain our TLR positive clones were emerging as the result of successful enrichment through our cell-based selections, we screened the original library without enrichment as well as pools of phage resulting from only two rounds of enrichment. No phage clones binding to any TLRs were seen in either instance (data not shown). A fourth round of selection performed for some of the receptors did not yield a more enriched population, but rather a much less diverse pool of positive clones (Table 1, hTLR5-HEK R4 and data not shown) as well as a distinct population of WT-HEK binding clones (data not shown). Thus, three rounds of enrichment were required and optimal to obtain positive clones. Taken together, these results indicate the anti-mTLR2, -hTLR5, and -hTLR6 antibody clones generated were not random results of large scale screening of the anti-hTLR2 library.

The exception to our successful screening results was the hTLR1 selection, in which we did not discover any anti-hTLR1 antibodies among the 94 clones screened. A more exhaustive screen may have revealed some positive clones but the more likely explanation is that a low level of hTLR1 expression in the cells used for panning may have excluded sufficient enrichment during iterative selection. Western blot
measurements showed that expression of hTLR1 in HEK cells was lower than that of the other TLR HEK cell lines utilized (data not shown).

While these studies illustrate a valuable new application for immune antibody libraries, highly complex non-immune antibody libraries remain the best option to obtain antibodies to the widest variety of targets, particularly antigens that are non-immunogenic due to tolerance or agents that are too toxic to use for immunizations [78]. However, construction of non-immune libraries is difficult, requiring extensive reagent optimization to obtain vast amounts of variable region fragments and necessitating hundreds of electroporations. Thus, investigators without access to a non-immune library may choose instead to attempt selections using an immune library against an antigen related to their target of interest
8. TLR expression in the lungs

8.1 Introduction

8.1.1 TLR expressing tissues

In addition to the problem of lacking reliable Ab for cell surface native TLRs, the very low protein expression levels in normal tissues have made detection of TLR proteins difficult. Currently, most studies determine TLR expression by monitoring RNA transcripts. At the RNA level, and protein level in some cases, the expression of TLRs has been extensively studied, although not in an ideal way to determine native, endogenous TLRs on the cell surface. The expression for some TLRs was reviewed by Akira et al ([28, 29]; Table 2). At this time, a few antibodies allow detection of native TLRs on living cells. At least two commercially available antibodies allow the detection of TLR2 and hTLR4, respectively. Human and mouse TLR2 were detected in their native structure in tissues including PBMC, spleen, and lung by histology staining or by flow cytometry [12, 79, 80]. Native human TLR4/MD2/CD14 (clone HTA-125) complex was detected in gingival fibroblasts [14] and peripheral blood mononuclear cells [81]. However, because this Ab is specific to the hTLR4/MD2/CD4 complex, it is not possible to detect the hTLR4 molecule unless it is in a complex with other molecules. There are commercially available antibodies against other TLRs, such as hTLR1, hTLR5 and hTLR6,
but in our hands they only bind to denatured TLRs in Western blots; none of them bind to native TLR protein in flow cytometry (data not shown).

In spite of the lack of antibodies binding to native TLRs on the cell surface, expression of several TLRs has been detected by virtue of mRNA transcript or denatured protein [82-84]. These studies, together with studies using TLR ligands for stimulation, suggest the presence of TLR in several cells or tissues [28, 85, 86]. However, to characterize and track cell populations that respond to TLR stimulation, antibodies that bind to native TLRs on cell surfaces are still required. These studies are critical not only for understanding the mechanism of immune responses but also for identifying potential therapeutic targets for infectious or immune diseases. A recent NIH meeting testified to an urgent need to develop antibodies that bind native TLR proteins (NIAID Biodefense Workshop Summary, http://www3.niaid.nih.gov/about/organization/dait/conferences.htm). Even though a few flow-cytometry compatible antibodies against hTLR2 and hTLR4 are currently available, antibodies against other TLRs are still urgently needed for detecting TLR-expressing cells and for functional studies of TLRs.

8.1.2 TLR expression in the lungs

Lungs are constantly exposed to an environment full of various harmful and benign components. The pulmonary immune system has evolved mechanisms to simultaneously induce tolerance against harmless airborne materials and recognize
invasive pathogens and induce proper immune response to clear them [87, 88]. The toll-like receptor family serves as a first line of defense to recognize infectious microbes and is believed to play a critical role in initiating and regulating immune responses in the lungs [89-91]. Due to the lack of proper antibodies (see Section 8.1.1), the exact cell populations that express TLRs in the lungs are not well understood. Most anti-TLR Abs are not capable of detecting TLRs on primary cells or in situ. TLR2 and TLR4 have been reported to be expressed on leukocytes and epithelial cells in the lungs [91-94]. Many studies also suggest that these two TLRs are expressed on dendritic cells and macrophages [90], the two critical antigen presenting cells for regulating tolerance or protective immune responses. Since currently available antibodies only bind to the human TLR4/MD2/CD14 complex, the expression of isolated TLR4 is uncertain. Here, with a monoclonal antibody we obtained in a side-project (clone name 11B8, isotype: mouse IgG1) for generating anti-hTLR4 antibodies, we sought to determine the expression of hTLR4 in bronchial alveolar lavage cells. In addition to the problem of Ab availability, lung macrophage/DC/monocyte surface marker profiles seem to be distinct from their well-studied counterpart in secondary lymphoid organs [33], and therefore, we could not determine which cell types in the lung express TLRs until a reliable system to characterizing these complicated lung cell types had been developed. Our lab previously established a combination of surface markers to characterize lung myeloid cells [33]. With the understanding of lung myeloid cells phenotypes and the antibodies
obtained in our completely cell-based scFv system (Section 7.2.1), we sought to identify the expression of TLRs on lung myeloid cells during LPS-induced inflammation.

8.2 Results

8.2.1 hTLR4 expression in the lungs

We generated one monoclonal Ab, clone 11B8, against human TLR4 (Figure 24a). With this mAb, we sought to detect TLR4-expressing cell types in the lung. We applied this Ab to stain normal adult human BAL (bronchial alveolar lavage) cells and found that small SSC (side-scatter)-low population expresses higher level of hTLR4 than the SSC-hi population (Figure 24b). We then tested their expression of other myeloid surface markers, including CD11b, CD11c, CD14, CD16 and MHC class II. TLR4+ cells express CD11c, MHC class II, and CD11b, but not CD14 or CD16, suggesting a macrophage-like phenotype (Figure 24c). However, we did not find significant difference in these markers between TLR4+ and TLR4− populations (Figure 24d). Additional surface markers may be required to characterize these TLR4+ cells in human BAL.
Figure 24: Identifying hTLR4+ cells in human BAL

(a) 11B8 mAb (black line) were used as primary Ab to stain on transfected hTLR4 on HEK, or endogenous hTLR4 on THP-1 cells. (Gray: IgG isotype control) (b) anti-hTLR4 Ab (right) or IgG control (left) stained on human BAL cells. (c) BAL cells were stained with Abs as labeled (black line) or IgG isotype control (gray). Events were gated on SSC-low/hTLR4+ cells as in figure b. (d) BAL cells were stained with Abs as labeled. The expressions of these markers were analyzed for SSC-low/ hTLR4-positive cell (black line) or SSC-hi/ hTLR4-negative cells (gray) as gated as in figure b.
8.2.2 hTLR2 expression in the lungs

With the hTLR2 scFv we generated, we sought to detect if hTLR2 is expressed in human BAL cell populations. One scFv, B11, was tested on freshly isolated bronchialveolar cells obtained by lavage from a patient with known pulmonary inflammation. B11 demonstrated specific staining of an SSC (side-scatter)-low cell population (Figure 25a). In addition, we also obtained human BAL cells from recent lung transplant patients and analyzed their expression of TLR2, CD14, CD16, and MHC class II. Similarly, we identified that a SSC-low population expresses hTLR2, and MHC class II, but not CD14 or CD16 (Figure 25b). The SSC-hi/ hTLR2-negative population, however, has the same phenotype in these three markers (Figure 23b). Again, identifying the expression of other surface markers is required to further characterize this hTLR2+ cell type.
Figure 25: Identification of endogenous TLR2 on human BAL cells

(a) Binding of an anti-hTLR2 scFv to human bronchial alveolar lavage (BAL) cells obtained during pulmonary inflammation. Only small SSC-low cells demonstrate specific staining for hTLR2. In each panel, signals obtained with anti-hTLR2 scFvs (black lines) were compared with signals obtained with an anti-ExTek negative control (gray shadows). Histograms are formatted as in Figure 11. (b) Expressions of CD14, CD11c, and MHC class II (black lines) were analyzed on TLR2-/SSC-hi (top) or TLR2+/SSC-low (bottom) cells, with Isotype IgG controls (gray shadows).
8.2.3 mTLR2 expression in the lungs

Using mice, we were able to obtain both the BAL cells and lung parenchymal cells for TLR2 detection. With a previously established surface marker profile (Part II, Section 3; [33]), we were able to characterize multiple myeloid cell types in the lung and detect their expression of TLR2 with the Abs we developed. We tested lung cells in an LPS-induce acute lung injury model, which has increased total cell numbers in the lung [16]. Among several anti-mTLR2 scFv we have generated, one representative clone (D2-3) was used to determine the expression of mTLR2 on these cell types. As shown in Figure 26, this Ab detected expression of mTLR2 on inflammatory monocytes, neutrophils, macrophages, and dendritic cells obtained from the lungs of LPS-treated mice. No such staining was seen on cells obtained from TLR2-deficient mice or when staining was performed with an irrelevant scFv (anti-Tie2 clone ExTek 1D6). These results were confirmed with the commercially available T2.5 mAb (data not shown).
Figure 26: mTLR2 expression on lung cells

Purified anti-mTLR2 scFv was used as primary Ab to stain mouse lung cells (black line). In the left panel, anti-Tie2 antibody was used as a negative control antibody (gray). In the right panel, cells harvested from a mTLR2 knock-out mouse were used as negative control cells (gray). Lungs were harvested from intranasally LPS-treated mouse and myeloid cells populations were identified as described by in Section 2.2 of Part II, and Lin et al [33].
8.3 Discussion

We successfully determined hTLR2 and mTLR2 expression with the Ab we generated, suggesting that the affinity of our Ab is high enough to detect endogenous TLRs despite their reported low expression levels. The affinity of a divalent Ab is generally higher than a single-chain Ab, thus it is possible that when we convert these scFvs into a full-length IgGs, their affinity will be even higher so that we will obtain an even better staining signal. Our preliminary data of converting anti-hTLR5 scFv and anti-Tie2 scFv to a full-length human IgG confirms that these Abs retain their specificity after converting into full-length IgG, but we have not yet determined their relative affinity compared to scFv under the same conditions (Figure 27 and data not shown).

We have detected a distinct TLR2+ and TLR4+ population in human BAL cells, as well as several TLR2+ myeloid cell types in mouse lungs. The only difference we found for this TLR2+/TLR4+ population relative to other cells is their side-scatter signal in flow cytometry. The surface markers we tested did not further separate this TLR2+/TLR4+ cell population from the other cells. Additional markers may be required to further characterize this cell population. We have not yet applied our anti-hTLR5 and anti-hTLR6 Abs for human BAL staining in flow cytometry, an experiment that may be helpful in characterizing the TLR2+/TLR4+ cell populations. Since the majority of cells in normal human BAL are alveolar macrophages, the TLR2+/TLR4+ cell population is very likely a subtype of alveolar macrophages, therefore the myeloid cell markers we used
did not separate them from the TLR2/TLR4-negative cells. In addition to normal human 
BAL, we also stained human BAL cells after lung transplantation. Comparing the 
samples from patients with or without acute transplant rejection, we did not find a 
significant difference in the expression of TLR2 or the percentage of hTLR2+ cells among 
these samples. However, the sample size we have tested so far is too small to reach a 
reliable statistical conclusion.
Figure 27: Abs retain their binding specificity after converted into full-length IgG.

ExTek (anti-Tie2) or 3B5 (anti-hTLR5) scFvs were subcloned and converted into full-length IgGs. Top: Anti-Tie2 IgG stains Tie2-HEK (left, black line) or WT-HEK (gray). Anti-gp120 Ab serves as a negative control Ab (right). Bottom: Anti-hTLR5 IgG (black line) or control IgG (grade shade) stains on hTLR5-HEK transfectants. WT-HEK (black dash) serves as negative control cells.
Part II: Role of CCR2+ monocyte-derived cells in LPS-induced acute lung injury

Abstract

Acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury (ALI), remains one of the deadliest syndromes around the world, despite extensive studies seeking to understand the mechanisms of its pathogenesis. Leukocytes are believed to cause lung damage associated with acute lung injury, however, the specific types of cells involved have not been clearly demonstrated. Neutrophils and macrophages are found in ARDS bronchial alveolar lavage fluid (BALF) and believed to play important roles in ARDS. However, the roles of other myeloid cell types in the lungs during ARDS are not well-characterized. Using flow cytometry, we examined the inflammatory cell types that arise in the lung after intranasal administration of LPS, a commonly used animal model for ARDS/ ALI. We found that LPS instillation results in the accumulation of neutrophils, inflammatory monocytes, exudative macrophages (ExMACs), monocyte-derived DCs (moDCs), and lymphocytes during the acute phase (days 1-3) of ALI. In comparison to WT mice, CCR2-deficient mice given LPS display significantly reduced numbers of lung monocytes, macrophages, and moDCs. CCR2-deficient mice also display reduced level of pro-inflammatory cytokines (IL-6, TNF-α) and reduced accumulation of neutrophils. Since neutrophil recruitment is not directly
dependent on CCR2, we investigated the possibility of CCR2-dependent induction of IL-17 and IL-23, two upstream regulators for neutrophil recruitment and granulopoiesis. We found LPS-induced expression of IL17a and IL23 in WT lung cells but not in CCR2-deficient mice. These findings suggest that inflammatory monocyte-derived cells promote the production of pro-inflammatory cytokines and also the accumulation of neutrophils. Relative to WT mice, CCR2-deficient mice displayed reduced lung immune pathology, improved survival rates, decreased weight loss and faster recoveries after LPS challenge. These findings suggest that CCR2+ monocytes and monocyte-derived inflammatory DCs play a central role in mediating the lung injury, morbidity and mortality associated with LPS-induced acute lung injury.
1. Introduction

1.1 Acute lung injury and acute respiratory distress syndrome

41 years after Ashbaugh et al. first described adult respiratory distress syndrome (ARDS) in 1967 [95], its pathogenic mechanisms are still largely unknown and minimal progress has been made in developing proper treatments. The official definition of acute lung injury (ALI) and ARDS published by American-European Consensus Conference (AECC) is following: A syndrome of inflammation and increased lung capillary permeability associated with a number of clinical, radiological and physiological abnormalities that cannot be explained by, but may coexist with heart failure. Detailed definitions of ARDS with hypoxemia and a decreased rate of oxygen delivery have been discussed and defined in the past few decades [96-98]. The incidence of ARDS/ALI reported ranges from 1.5 to 75 per 100,000 persons per year, depending on the applied definition of ALI and also the specific population [99-103]. A large scale study in Japan estimated that 20,000 to 40,000 cases of ALI, half of which also have ARDS, occur annually in Japan [99]. Recent studies, however, suggested that the incident rate may be underestimated [97, 104] for a variety of reasons. In addition to its relatively high incidence rate, ARDS/ALI also has one of the highest mortalities in the ICU, ranging from 30-70% [104], and currently there are no proven effective treatments to reduce the exceptionally high mortality rate.
Potential causes of ARDS/ALI include: lung infection, sepsis, smoking, and physical injury [97]. All of these events may lead to damage of lung epithelia, which in turn induce further inflammation. Lung edema would result from tissue fluid, and even blood, entering into the lung alveolar space through the damaged epithelial barrier. Uncontrolled inflammation and lung edema block oxygen exchange, which in turn will eventually lead to death, if the inflammation is not resolved in time [105]. Neutrophils are found in the lungs of ARDS/ALI patients [106] and may be responsible for lung damage caused by our own immune system, although some studies showed that depletion of neutrophils does not correlate with improved ARDS symptoms [107-109]. Macrophages are also found during ARDS, although the heterogeneity of macrophage populations leads to controversy in assigning roles of macrophages. As to resident alveolar macrophages, it is generally believed that they contribute to inflammation in an early stage, but assist in repairing tissue and resolving inflammation at later stages [110, 111]. In conclusion, both neutrophils and macrophages are thought to be involved in ARDS/ALI. Although their pathogenic or beneficial role is still unclear, they could be potential targets for ARDS treatment. Current strategies of modulating these two cell types have not been very effective [112], indicating that other cell types are likely to be involved. Inflammatory monocytes are one of the alternative potential targets that we focus on and these will be discussed in more details in the following sections.
1.2 LPS-induced acute lung injury

Many experimental animal models for human ARDS/ALI have been developed in order to understand the complicated mechanisms underlying this deadly disorder. Local LPS (Lipopolysaccharide) treatment (by aerosol or intranasal instillation) and systemic LPS treatment by i.v. or i.p. are currently the most extensively used animal models for human ARDS/ALI. Aerosol or intranasal LPS treatment were reported to cause lung edema, neutrophil accumulation and production of proinflammatory cytokines [99, 113, 114], the same events observed in human ARDS/ALI. Different LPS doses may affect the severity of the symptoms; but while various doses were used in different studies, they all resemble human ARDS/ALI [115, 116] in some presentation of severity. Similar to human ARDS, these LPS-treated animals also have macrophage activation and neutrophil accumulation in the lung. In addition to the accumulation of neutrophils and macrophages, LPS-treated animals also have elevated levels of various cytokines and chemokines, including TNF-α, IL-1β and MCP-1 (Monocyte chemotactic protein-1, or CCL2) [117-119] as well as lung injury as measured by pathologic indices (LDH assays and protein levels in BALF) or observed histologically [117, 119, 120]. In conclusion, the high similarity to human ARDS/ALI symptoms suggests that LPS-treatment is a suitable experimental animal model for human ARDS/ALI.
1.3 Leukocyte populations in the lung during ALI

As discussed above, neutrophils and macrophages are two obvious cell types that accumulate during ARDS/ALI, although their roles in pathogenesis are controversial. Other cell types, however, are not well-characterized but believed to play important roles during ARDS/ALI [121]. For example, Dendritic cells (DC), the most potent type of antigen-presenting cell type, are key cells for both the induction and control of immune responses [122, 123], although their presence and involvement during ARDS/ALI has not been extensively studied. One major obstacle to elucidating cell populations during lung inflammation was the lack of well-defined surface markers for lung myeloid cell types. Traditionally, granulocytes such as neutrophils were identified by the expression of Gr-1 [124, 125], but recent studies identified Gr-1 expression on subsets of other myeloid cell types including DC and macrophages [126-129]. In addition, CD11c, a mouse DC marker, was unexpectedly found expressed on alveolar macrophages [130, 131], a finding that led to revisions of previous studies defining all CD11c+ cell in the lung as DC. Strong autofluorescence of macrophages further complicated the already difficult task of characterizing lung cell populations. To characterize cell types involved in ARDS/ALI, we must develop a reliable method to identify different lung cell types. Previously in our lab, Lin et al utilized the combination of Ly6G, CD11c, MHC class II, CD11b and Gr-1 as surface makers to effectively identify multiple cell types in the lung in both influenza infection models [33] and in an MCP-
SPC transgenic model [33, 132], which mimics constitutive lung inflammation by constitutively expressing a high level of CCL2 under the control of the SPC promoter. Utilizing Gr-1 and CD11b markers further identified inflammatory subsets of monocytes, DCs and macrophages recruited by CCL2 [17], as we will discuss in detail in the following sections. The identities of all these cell types were further confirmed by morphology, expression of co-stimulation markers and cytokines, and their ability to stimulate naïve T cells [33], all of which supported the reliability of our flow-cytometric strategy to characterize leukocyte types in the lung. In conclusion, we now have a reliable system for characterizing leukocyte populations in the lung, particularly for monocytes, DCs and macrophages.

In addition to myeloid cells, lymphocytes (B cell, T cell, and NK cells) may also be involved in lung immune pathology during ARDS/ALI. Among all lymphocyte types, γδT cells and NK cells are the cell types that are likely to cause lung immune pathology during ARDS/ALI since all other lymphocytes are responsible for adaptive immune response and require at least 4-5 days to establish detectable effects. It is known that T cells recruit macrophages and neutrophils during inflammation [133, 134]. Furthermore, γδT cells were reported to modulate inflammatory response during infectious (Nocardia asteroids) and non-infectious (ozone exposure) lung in jury [135] while other evidence suggested pathogenic effects of T cell in the lungs [136, 137]. In a systemic LPS challenge model, NK cells were found to be critical for inflammatory responses during lethal
septic shock in mice [138], although their role in the lung is not clear. Our study focuses on myeloid cell types, but our data (Section 6) support the possibility that lymphocytes, particularly $\gamma$δT or NKT cells, may be involved in ARDS/ALI while further evidence is needed to confirm any pathologic roles.

1.4 Monocyte-derived inflammatory cells and lung immune pathology

Monocytes are the precursors of DC and macrophages. Recent studies of monocyte identified two major monocyte subsets with distinct fates and functions. Constitutive monocytes, which are CX3CR1$^{hi}$ but CCR2, migrate to peripheral tissues under steady state and differentiate into resident DC and macrophages (alveolar macrophages in the lung), whereas inflammatory monocytes, which are CX3CR1$^{lo}$ and CCR2$^+$, respond to inflammatory signals and differentiate into inflammatory DC and exudative macrophages in inflamed tissues (Figure 28) [17, 33, 139-142]. Previous work in our lab characterized inflammatory cells in the lungs of MCP1-SPC mice [33, 132] and also identified a pathologic role of CCR2$^+$ inflammatory monocytes during influenza infection [33]. These inflammatory monocytes/monocyte-derived cells play key roles in causing lung inflammation, and uncontrolled inflammation is the key factor for lung immune pathology in both influenza infection and ARDS/ALI. Therefore, we sought to characterize the accumulation of these cell types during ARDS/ALI, and to determine their roles in immunopathology during ARDS/ALI.
Constitutive monocyte (Top): bone marrow monocytes that express CX3CR1 (fractokine receptor) constitutively responding to CX3CR1 ligands and migrate to peripheral tissue where they differentiate into residential macrophages and DC. Inflammatory monocytes (bottom): a subset of monocytes gains CCR2 expression and responds to CCR2 ligands secreted at sites of inflammation. These CCR2+ monocytes then differentiate into exudative macrophages and inflammatory DC, which express high level of surface markers CD11b and Gr-1. This figure is adapted from and based on the review of Gordon et al.[139] and Shortman et al.[142].
2. Materials and methods

2.1 Mice

All animal experiments were conducted in accordance with National Institutes of Health guidelines and protocols approved by the Animal Care and Use Committee at Duke University. All mice used for experiments were between 8 and 14 wk old.

2.1.1 Source of mice

WT C57/BL6 mice were purchased from Charles River Laboratory (Wilmington, MA). CCR2-deficient mice [143] were purchased from the Jackson Laboratory (Bar Harbor, ME). CX3CR1\textsuperscript{GFP/GFP} mice [17, 144] were purchased from the Jackson Laboratory and crossed with WT mice to produce CX3CR1\textsuperscript{+/GFP} mice. Neutrophil deficient MCL-Fl mice [145] were provided by Dr. Dzhagalov in Dr. You-Wen He’s lab at Duke University.

2.1.2 LPS treatment on mice

For flow analysis, mice were anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg) i.p. and then treated intranasally with 12.5 ug LPS per mouse (50 μl of 250 ug/ml LPS in PBS or PBS control). For morbidity testing, 100ug/mouse LPS was used (50 μl of 2mg/ml sub-lethal dosage) and body weight was recorded at 24 hr intervals. For mortality tests, 1 mg/ml LPS were used (50 μl of 20 mg/ml lethal dosage) and mice were monitored twice a day.
2.2 BAL fluid and cell harvest

2.2.1 Bronchioalveolar lavage (BAL) and lung parenchyma cell isolation

BAL cells were collected as described previously [33, 146]. Briefly, tracheas of euthanized mice were cannulated with an 18-gauge angiocath connected to a 1-ml syringe and the lungs were flushed with 0.6~0.8 ml of PBS five times. BAL cells were washed once with PBS. To obtain lung parenchymal cells, lungs were perfused with 3 ml of HBSS, incubated at 37°C for 30 min, minced, dissociated through a 70-μm mesh strainer, and centrifuged at 450 x g at room temperature for 20 min over a 17% metrizamide (Accurate Chemical & Scientific) cushion. Low-density cells were collected, washed in FACS buffer (PBS with 0.5% BSA, 10 mM HEPES, 10 mM EDTA), and subjected to Ab staining.

2.2.2 BAL fluid harvest

As described in Section 2.2.1, except that each mouse lung was flushed with PBS to a final volume of 4 ml. BAL cells were pelleted by 700g centrifugation and the supernatant were collected as BAL fluid and subjected to LDH or cytokine assays as described below (Sections 2.4.1 and 2.6).
2.3 Flow cytometry

2.3.1 Flow cytometric staining for myeloid cells

Lung parenchymal or BAL cells were stained in HBSS containing 10 mM EDTA, 10 mM HEPES, 5% FBS, 5% normal mouse serum, 5% normal rat serum, and 1% Fc block (eBioscience) at 4°C for 30 min; washed three times; and then analyzed using a BD LSRII flow cytometer. To characterize myeloid cell types, we used the following Abs: anti-I-A/I-E FITC, anti-Ly6G PE, anti-Gr-1 allophycocyanin, anti-CD11b allophycocyanin/Cy7 (all BD Pharmingen); and, anti-CD11c PE-Cy5.5 (eBioscience). To characterize their co-stimulatory molecule expressions we used anti-CD80 PE, anti-CD86 PE, anti-CD40 PE, (all BD Pharmingen) Abs..

2.3.2 Flow cytometry staining for lymphoid cells

Lung parenchymal or BAL cells were stained as above in Section 2.3.1. To characterize lymphoid cell types we used the following Abs: anti-NK1.1 FITC, anti-CD4 PE, anti-CD8 allophycocyanin (all BD Pharmingen) and anti-CD19 PE-Cy5.5 (BioLegend)

2.3.3 Flow cytometry gating and analysis

Initial gating on forward scatter area (FSC-A) versus height (FSC-H) was used to remove doublets. The events were further gated on forward scatter (FSC) versus side scatter (SSC) for living cell size. Ly6G+ cells in living cell gates were identified as neutrophils. Ly6G-negative cells were further analyzed for CD11c and I-A/I-E (MHC
class II) expression (Figures 29 and 31) to identify macrophages (CD11c\(^{hi}\), I-A/I-E\(^{+}\)), DC (CD11c\(^{+}\), I-A/I-E\(^{hi}\)), double negative cells indicative of monocytes (CD11c, I-A/I-E\(^{-}\)), and double-intermediate (DI; CD11c\(^{int}\), I-A/I-E\(^{int}\)) cells indicative of monocytes in the process of differentiating into macrophages or DCs. Within double-negative, macrophage and DC gates, cells were analyzed for Gr-1 and CD11b expression to distinguish inflammatory and non-inflammatory cell types.

**2.4 Lung injury indices**

**2.4.1 LDH assay for cytotoxicity**

48 hr after 12.5 ug/mouse LPS treatment (Section 2.1.2), mice were euthanized and 4 ml of BAL fluid from each mouse were harvested as described above (2.2.1). LDH assay for 80ul of undiluted BAL fluid were performed with LDH-cytotoxicity kit (Sigma) as described in the manufacturer’s instructions. Optical absorbance of BAL fluid from PBS-treated control mice were assayed simultaneously and used to normalize that of LPS-treated mice BAL fluids. For each group, 6 mice were assayed in triplicate.

**2.4.2 Lung wet weight/ body weight ratios**

WT or CCR2-deficient mice were treated with 100 ug/mouse of LPS in PBS or PBS-alone control as in Section 2.1.2. On day 2, mice were euthanized by isoflurane and weighed on a scale (Mettler PM400) to 10 mg accuracy. Mice lungs were then harvested and weighed using an electronic analytical balance (Mettler ToledoAB54-S) to 1mg
accuracy. (Sample number: n=5 for WT mice, n=6 for CCR2/- mice, and n=2 for PBS treatment)

2.5 Morbidity and mortality test

2.5.1 Mouse body weight loss

WT or CCR2-deficient mice were treated with 100 ug/mouse LPS in PBS or PBS-alone control as in Section 2.1.2. Mouse body weights were recorded at 24 hr interval to 10mg accuracy.

2.5.2 Mouse survival test

WT or CCR2-deficient mice were treated with 1mg/mouse LPS in PBS or PBS-alone control as in Section 2.1.2. Mice were monitored over a 4-12 hr interval. Dead mice were removed and recorded immediately.

2.6 Cytokine expression

After 12.5 ug/mouse intranasal LPS-treatment (Section 2.1.2), mouse BAL fluid was harvested as described above (2.2.2.). The BAL fluids were then analyzed in ELISA assays for IL-6 (BD), TNF-α and IL-17A (both R&D system kits) expression in triplicates as described in the manufacturer’s instructions. The lowest standard concentrations are 15.6 pg/ml for IL-6 and TNF-α and 10.9 pg/ml for IL-17A. The expression of IL-23p19 and IL17a were also measured by quantitative real-time PCR (Bio-Rad). Total lung cells,
metrizamide-gradient lung cells (enriched DC faction), or FACS sorted cells were lysed with Trizol (Invitrogen) and total RNA was harvested with RNAeasy kits (Qiagen). RNA was reverse-transcribed into cDNA by Superscript II (Invitrogen) and subjected to quantitative real-time PCR assay (BioRad). A real-time quantitative PCR analysis was performed using a SYBR® Green PCR Master Mix (BioRad) and the MyiQ5 detector system (BioRad) by the following parameters: After an initiation incubation at 50°C for 2 min and at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec, annealing at 65 C for 30 sec, and extension at 72°C for 45 sec. The cytokine gene expression was compared to the expression of GAPDH by $2^{-\Delta(Ct)}$ and normalized by the expression under unstimulated conditions in WT mice. The primer pairs we used are:

**IL-23p19**

sense 5'-TGG CTG TGC CTA GGA GTA GCA-3',

antisense 5'-TTC ATC CTC TTC TCT TCT TAG TAG ATT CATA-3'

**IL-17A**

sense 5'-GCT CCA GAA GGC CCT CAGA-3',

antisense 5'-CTT TCC CTC CGC ATT GACA-3'

**GAPDH**

sense 5'- AGT ATG ACT CCA CTC ACG GCAA-3',

ntisense 5'-TCT CGC TCC TGG AAG ATG GT-3'
2.7 Statistics

All numerical data are presented as mean ± SD. The comparison between survival curves is performed by log rank test in Prism software. The test is equivalent to the Mantel-Haenszel test. All the other data are analyzed by ANOVA or unpaired Student’s t tests using Prism software, as indicated in the figure legends.
3. Inflammatory monocytes and monocyte-derived cells are recruited to the lung during LPS-induced ALI

3.1 Introduction

Previous studies suggested that, during the acute phase of ARDS, or LPS-induced acute lung injury, neutrophil and alveolar macrophage numbers are increased in BAL and correlate with the induction of proinflammatory cytokines and chemokines [16, 99, 113, 147], suggesting their potential pathological roles in lung damage. However, cells types other than neutrophils and macrophages have not yet been well-characterized. In addition, previous studies only characterized BAL cells and BAL fluid, not lung parenchyma cell types. Here, we sought to identify cell types in the lung during LPS-induced ALI. We hypothesize that CCR2+ inflammatory cells are recruited to the lung during LPS-induced ALI and are responsible for lung immune pathology.

3.2 Results

3.2.1 Monocytes recruitment to the lung during ALI

To characterize lung cell populations during LPS-induced lung injury and their roles in lung immune pathology, we employed the previously established flow cytometric analysis to identify CCR2+ inflammatory cells [33]. Mice were intranasally treated with LPS, and myeloid cell populations in the lung parenchyma at days 0, 1, 2, 3
and 4 were enriched by gradient, followed by flow cytometric analysis to identify individual cell types. To identify monocytes and monocyte-derived cells, we first analyzed LPS-treated CX3CR1^{GFP+} mice, which have constant green fluorescent protein (GFP) expression in both monocytes and monocyte-derived cells (CX3CR1\(^+\)) [17]. As shown in Figure 29, we found a recruitment of monocytes (CX3CR1\(^+\)/CD11c\(^-\)/MHC II\(^-\)) and monocyte-derived DC (CD11c\(^+\)/MHC II hi/CX3CR1\(^+\)) to the lungs after LPS exposure. Consistent with their origin from inflammatory monocytes in blood, these newly recruited cells also express CD11b and Gr-1.

We detected expression on these inflammatory cells of the co-stimulatory molecules CD40, CD80, and CD86 at baseline and these molecules were further up-regulated by LPS exposure (Figure 30.). The expression of co-stimulatory molecules further confirmed that these cells are DC/macrophages are being activated upon LPS-treatment.
Figure 29: Characterizing monocyte and monocyte-derived cells during LPS-induced ALI in CX3CR1-GFP

Lung cells from PBS- (left) or LPS- (right) treated mice were analyzed by flow cytometry to identify DC (CD11c+, MHC class II $^+$), macrophage (MAC; CD11c $^{hi}$, MHC class II $^+$), and monocyte (Mono; CD11c$^-$/ MHC class II $^-$). Monocytes that are in the process of differentiating into DC or macrophages are identified as “double intermediate” cells (CD11c$^{int}$, MHC class II$^{int}$). Each cell type was further characterized by their expression of CD11b and Gr-1 for inflammatory subsets. Monocyte lineage was identified by the expression of CX3CR1-GFP.
Figure 30: Expression of co-stimulation markers by inflammatory monocytes, monocyte-derived cells (DC and ExMac), and alveolar macrophages (AM).

Mouse lung cells at 72 hours after LPS treatment were harvested and subjected to flow cytometry analysis. Each cell type was gated as described in Figure 28. Histograms indicate CD80, CD86 or CD40 staining on cells from LPS treated mice (black line), PBS treated mice (blue), or IgG control staining (gray)
3.2.2 Characterization of CCR2-dependent cell recruitment during ALI

Inflammatory monocytes express CCR2 and migrate to peripheral tissues, which express CCR2 ligands during inflammation [139]. In Section 3.2.1, we found that inflammatory monocytes are recruited to the lung during LPS-induced ALI. The majority of recruited monocytes express the same inflammatory markers (CD11b+, and Gr-1+) that we previously identified on inflammatory monocytes and monocyte-derived cells. However, in the LPS model, a requirement for CCR2 has not been determined. To further confirm that these monocytic populations truly require CCR2 for entry into the lung, we applied our established flow cytometric strategy [33] to analyze lung cell populations in CCR2−/− mice in comparison to those in wild-type mice. Mice were treated intranasally with LPS and myeloid cell populations in the lung parenchyma at days 0, 1, 2, 3 and 4 were analyzed by flow cytometry to identify individual cell types. In wild-type mice, we found that LPS treatment increased the numbers of inflammatory monocytes and DC over the period from day 1 to day 4. (Figures 31 and 32). Inflammatory monocytes and DC peaked at day 2 and day 3, respectively. Surprisingly, there was only slight increase in exudative macrophage (ExMAC) numbers (Figures 32 and 32), differing from what we saw with influenza infection or the SPC-MCP mice. As expected, constitutive monocyte (CX3CR1+/CCR2−) numbers remained unchanged throughout the 4-day period analyzed. Alveolar macrophage numbers, on the other hand, remained low during the first three days and then increased greatly around day 3-
4, consistent with the understanding that alveolar macrophages proliferate at damaged tissues and help resolve inflammation and repair damaged tissues [139].

In comparison to WT mice, CCR2<sup>−/−</sup> mice display a reduced accumulation of inflammatory monocytes and moDC (Figures 31 and 32), suggesting that the recruitment of inflammatory monocytes and subsequent development of moDC is truly dependent on CCR2 ligand activity [17, 33]. As expected, the constitutive monocyte population remained at a constant level since their recruitment is thought to be CCR2-independent (Figure 32). The recruitment of exudative macrophages is also impaired, but because the cell number was originally low in the WT mice, this impairment is not statistically significant. Alveolar macrophage numbers in CCR2<sup>−/−</sup> mice are comparable to those in WT mice over the period from day 0-4 (Figure 32), which is expected since they do not express CCR2. We applied the same flow-cytometry strategy to BAL cells and observed similar CCR2-dependency for recruitment (data not shown). In conclusion, these data suggest that, during LPS-induced ALI, CCR2<sup>+</sup> inflammatory monocytes are recruited to the lung and differentiate into inflammatory DCs and exudative macrophages, whereas the recruitment of constitutive monocyte sand alveolar macrophages is CCR2-independent. Additionally, the number of alveolar macrophages increases in the beginning (day 4) of the resolving phase of LPS-induced ALI.
At day 0, 1, 2, 3 and 4, lung cells from LPS-treated mice were analyzed by flow cytometry to identify neutrophil (Ly6G+), monocytes, DC and macrophage (Ly6G-negative non-PMN cells followed by analysis as in Figure 28). Inflammatory monocyte, DC, and ExMac were identified by their expression of CD11b and Gr-1. Lung cells from CCR2-/- mice was also analyzed in comparison to WT mice (day 2 as a representative). Numbers indicate percentage of their parental gate. The absolute number of each cell types were calculated and plotted in Figure 32. One representative data for each time point is shown here.)

Figure 31: Characterizing cell types during LPS-induced ALI
Figure 32: Cell numbers during LPS-induced ALI

Murine lung cells were analyzed as in Figure 31. Total live cell numbers per lung were counted by hemacytometer for calculation of absolute numbers for each cell type. Each time point includes 1-5 mice. (filled square: WT mice; empty square: CCR2-/- mice)
3.2.3 Activation of alveolar macrophage in LPS-induce ALI

Macrophages in an inflamed lung are composed of two different populations. Exudative macrophages (ExMAC) are derived from newly-recruited inflammatory monocytes while alveolar macrophages (AM) are lung residential cells that can be activated and proliferate in response to inflammation. During influenza infection, we were able to identify ExMAC by their expression of CD11b and Gr-1, whereas alveolar macrophage remain CD11b negative throughout the infection [33]. Lung macrophage sub-populations have not been previously studied during LPS-induced ALI. To improve characterization of the model, we analyzed the macrophage sub-populations in LPS-treated WT and CCR2−/− mice by flow cytometry as described in Section 3.2.2. Surprisingly, we found a different pattern than we observed in influenza infection. During LPS-induced ALI, we found a significant increase of CD11b expression on alveolar macrophages (Figures 31 and 33). This up-regulation appears to be a direct effect of LPS since in vitro LPS-stimulation of purified alveolar macrophage also up-regulates CD11b expression independent of co-culture with other cell types (Figure 33). Unlike CD11b, the expression of Gr-1 was not up-regulated in alveolar macrophages, allowing us to separate alveolar and exudative macrophages upon LPS-treatment. Interestingly, the expression level of CD11c is slightly decreased after LPS stimulation (Figure 32, right), although we do not know if it is due to the down-regulation of CD11c on AM or selection of CD11cint cells. However, this change of CD11c expression level
may lead to potentials problem for identifying AM in total lung cell analysis, since the CD11c signal on AM may move down to similar level of that on DCs. Therefore, MHC class II staining, is critical and must be carefully performed to separate DC and macrophages. To summarize the effect of LPS in macrophage populations, we found that exudative macrophage number is only somewhat increased in a CCR2-dependent manner (Figures 31 and 32), whereas alveolar macrophages are directly activated by LPS, independent of CCR2. These data suggest that even though similar monocyte and monocyte-derived cells were found in both influenza infection and LPS-induced ALI, the cell type profiles in the lungs differ from the two models, and therefore the pathogenic roles of these cells may not be not as similar as originally expected.
AM harvested from naïve WT BAL was cultured in vitro with control medium (top) or with 250μg/ml LPS (bottom) for 48 hours. Live cells were gated (left) and analyzed for Gr-1, CD11b, (center) CD11c, and MHC class II (right) expression.
3.2.3 Secondary effects of CCR2+ cells on lymphocyte recruitment

With the exception of a subset of memory T cells, lymphocytes do not express CCR2 and therefore do not respond to CCR2 ligands secreted into inflamed tissues. However, macrophages and DC are capable of inducing the recruitment or proliferation of other cell types by secreting cytokine/chemokines. To determine if there are indirect effects of CCR2+ cells in the recruitment or proliferation of other cell types during ALI, we characterized B cell, CD4+ T cell, CD8+ T cell, and NK cell numbers in CCR2+/− and WT mice by flow cytometry. From day 0 to day 3, the numbers of recruited T, B, or NK cells are very low in both WT and CCR2+/− mice. During days 0-3, lymphocyte numbers in LPS-treated WT mice are slightly higher than those in CCR2+/− or untreated mice, but all observed numbers are very low and considered in the range of background (>2x 10^5 cells per lung for T and NK cells, approximately 5x 10^5 for B cells.). However, at day 4, we found that B and T lymphocytes (both CD4+ and CD8+) populations start to increase in WT mice, but not CCR2+/− mice. NK cell numbers remain low in both strains of mice at day 4. This result suggests that CCR2+ inflammatory cells do not have significant effects on lymphocyte recruitment or proliferation at an early stage (days 0-3), but may have positive effects on T and B cell recruitment/proliferation by day4.
Figure 34: Characterization of lymphocyte populations during LPS-induced ALI

1, 2, 3, or 4 days after LPS (or PBS) treatment, lung cells from WT or CCR2-/- mice were subjected to flow cytometry analysis to identify CD4+ cells, CD8+ T cells, B cells (CD19+) or NK cells (NK1.1 +). Numbers indicate the absolute cell numbers per lung from 3-5 mice at each time point.
3.3 Discussion

T and B cells are critical in adaptive immunity, but often not during the acute phase of inflammation. For adaptive immunity in mouse, it takes at least 4-5 days to begin any detectable cell recruitment or proliferation, and often these events peak by day 7-8. Thus, their numbers are often too low to have significant effects at the early stage of lung inflammation (day 0-3). The fact that we did not detect a significant increase of T and B cells during LPS-induced ALI is not surprising. Since the number of inflammatory monocytes and DCs peak at day 2 and 3, respectively, the effects of CCR2⁺ monocytes on lymphocyte recruitment, if any, should be later than day 3. Consistent with this, we detected an increase of T and B cell numbers in day 4 in WT mice, but not CCR2⁻ mice. In addition, previous studies suggested that the role of B cell and conventional αβ T cell in the acute phase (day 0-3) of ARDS/ALI is not significant [98, 148]. Thus, whether CCR2⁺ monocytes are capable of recruiting lymphocytes or not should be inconsequential for the acute pathogenic phase of ARDS/ALI. NK cells, as a defense line of innate immunity, have been suggested to mediate the possible effects in ARDS/ALI, but are yet to be proved significant in the pathology. Unlike conventional T and B cells that may contribute to the pathogenesis of ARDS/ALI in later stage, NK cell’s contribution to pathogenesis, if any, is expected to be at acute phase (day 0-3) but not in later stages (after day4). There are no previous studies suggesting that monocytes/DC/macrophages have direct effects on NK cell activation or proliferation.
The fact that there are no significant differences in NK cell numbers between WT and CCR2\(^{-}\) further supports our model of monocytes and monocyte-derived cells as the predominant mediators of pathogenic effects in ARDS/ALI.
4. CCR2-dependent cytokine production in LPS-induced ALI

4.1 Introduction

Having identified the recruitment of CCR2+ monocytes and monocyte-derived cells in the lungs during LPS-induced ALI, we sought to investigate their roles in lung injury. Production of pro-inflammatory cytokines is one of the most important events during the acute phase of inflammation. Thus, we decided to investigate the cytokines that may be responsible for the pathogenesis of LPS-induced ALI. Monocytes, DC, and macrophages are all capable of producing IL-6 and TNF-α, two important pro-inflammatory cytokines, thus they were our first targets to investigate.

4.2 Results

To determine the cytokine levels in the lung during ALI, we harvested BAL fluid from LPS-treated WT or CCR2−/− mice. Comparing BAL fluid with the presence (WT) and absence (CCR2−/−) of CCR2+ cells, we found that LPS-induced the production of IL-6 and TNF-α in WT mice but not in CCR2−/− mice, suggesting that CCR2+ cells are required for the induction of IL-6 and TNF-α (Figure 35), the two pro-inflammatory cytokines reported to be secreted by DC and macrophages. We did not detect TNF-α and IL-6 after day 3 (data not shown). In addition, DC and macrophages may also have indirect effects that induce cytokine production from other cell types. Therefore, we also
analyzed BAL fluid for IFN-γ and IL-10 levels, two cytokines that are secreted by T cells. We did not detect any IFN-γ in the BAL fluid during days 0-4, and only detected a constitutively low level of IL-10. Neither of these two cytokines varied in abundance between WT and CCR2−/− mice (Figure 35).

![Graphs of cytokine levels in BAL fluids](image)

**Figure 35: IL-6, TNF-α, and IL-10 level in BAL fluids during LPS-induced ALI.**

24, 48, 72 and 96 hours after intranasal LPS treatment, mice BAL fluid was harvested and subject to ELISA assays for TNF-α, IL-6, and IL-10. (n=5, assayed in triplicates)
4.3 Discussion

We do not have evidence to prove that CCR2+ monocytes and monocyte-derived cells are the direct source of TNF-α and IL-6, but previous studies [149-153] and the results here support this supposition. TNF-α is known to have pathogenic effects following tissue damage and inflammation [149, 154, 155], whereas IL-6 can induce serious local inflammation events and also fever. Production of both cytokines is severely impaired in CCR2−/− mice. Based on these findings we propose a possible pathogenic mechanism of LPS-induced ALI as described in Figure 36.
Figure 36: Proposed model for the direct effects of CCR2+ cells inducing lung immune pathology.

In this model, inflammatory monocytes are recruited to the lungs by CCR2 ligands. After arriving, they derive into inflammatory DCs or exudative macrophages, which secret TNF-α and IL-6. These two cytokines in turn act on other cell types, such as epithelial or smooth muscle cells, to produce more inflammatory signals or reactive oxygen species, leading to lung tissue damage. Neutrophils and alveolar macrophages do not express CCR2 but also contribute to lung damage independently.
5. Role of CCR2$^+$ cells in lung injury, morbidity, and mortality during LPS-induced ALI

5.1 Introduction

We have identified that CCR2$^+$ monocytes and monocyte-derived DC/Macrophages accumulate in the lung during LPS-induced ALI. We also determined their effects on the recruitment of lymphocytes and production of pro-inflammatory cytokines. Here, we sought to determine the role of CCR2$^+$ cells in causing lung immune pathology, which in turn may lead to morbidity and mortality during LPS-induced ALI.

5.2 Results

5.2.1 CCR2$^{-/-}$ mice lungs are less damaged during LPS-induced ALI

Based on their reported pathogenic roles in other systems, we hypothesized that CCR2$^+$ monocyte-derived cells cause immune pathology during LPS-induced ALI. To test this hypothesis, we measured the following indices of lung immune pathology: (1) BAL fluid LDH levels and, (2) lung wet weight/body weight ratio. We found that upon LPS-treatment, BAL fluid LDH levels increased about 6-fold for WT mice, but only 2-fold for CCR2$^{-/-}$ mice (Figure 37), indicating less tissue damage in the lungs in the absence of CCR2$^+$ cells. Similarly, LPS-treated WT mice have lung wet weight/body weight ratios of ~1.7, whereas CCR2$^{-/-}$ mice have ratios of only 1.3 (Figure 37), indicating a less severe lung edema in the absence of CCR2$^+$ cells ($p = 0.0237$). In conclusion, our...
data support an important role of CCR2+ cells in causing lung immune pathology during LPS-induced ALI.

Figure 37: CCR2-/− mice displayed less long damage than WT mice.

(a) WT and CCR2-/− mice were treated with LPS intranasally. BAL fluids were harvested 48 hours after the treatment, and subject to LDH activity assays for cytotoxicity. (n=7, assayed in triplicates) (b) 48 hours after LPS treatment, mice weight was recorded to the accuracy of. Their lungs were then harvested and weighted to the accuracy of 10mg. (n=6)
5.2.2 CCR2\(^{-/-}\) mice have reduced morbidity and mortality during LPS-induced ALI

Having determined that CCR2\(^+\) cells are responsible for causing lung damage during ALI, we sought to determine if this lung damage would lead to morbidity and mortality. We intranasally treated both WT and CCR2\(^{-/-}\) mice with sub-lethal (100\(\mu\)g/mouse) or lethal (1mg/mouse) doses of LPS, followed by daily recording of body weight or survival, respectively. We found that, by day 10, only 30% of WT mice survived LPS-induced ALI at the lethal dosage, whereas 60% of CCR2\(^{-/-}\) mice survived (Figure 38a). All surviving mice regained their body weight to original levels and thus were assumed fully recovered from ALI. For body weight loss, WT mice lost almost 30% of their original weight, slowly regained their weight on day 5, and recovered to their original weight at around day 9 (Figure 38b). In contrast, CCR2\(^{-/-}\) mice only lost about 20% of their weight, quickly re-gained their weight on day 3, and recovered to their original level on day 6. In conclusion, during ALI, mice survived better, lost less weight, and recovered more quickly in the absence of CCR2\(^+\) cells, suggesting that CCR2\(^+\) cells contribute to the morbidity and mortality during LPS-induced ALI.
Figure 38: CCR2-/- mice displayed less morbidity and mortality during LPS-induced ALI.

WT or CCR2-/- mice were intranasally treated with 100 ug/mouse LPS for body weight loss test (a) or 1mg/mouse for mortality test (b). Survival rate and mice body weight were recorded daily. (n=20~23)
5.3 Discussion

In humans, ARDS/ALI has a very high mortality rate (30-70%) [100, 104]. Even after acute inflammation resolves, patients may still develop chronic obstructive pulmonary disease (COPD) or other pulmonary diseases. Here, in our mouse ALI model, we showed that by eliminating CCR2+ cells, lung injury, morbidity and mortality are greatly decreased. These results suggest that CCR2+ cells are promising targets for developing an effective treatment of human ARDS/ALI. Currently there are several CCR2 antagonists being tested in clinical trials [156]. Although they are not designed to treat ARDS/ALI, they may serve as effective treatments for ARDS/ALI in the future.

We have preliminary data suggesting that neutrophils may be less important than CCR2+ monocytes in morbidity during LPS-induced ALI. Neutrophil-deficient mice (Mcl-FL [145]) were treated with LPS for body weight loss as described above. We found no difference in morbidity between neutrophil-deficient and WT mice. However, a flow cytometry analysis to verify the deficiency of neutrophils in these mice revealed that only ~50% neutrophils were depleted in the lungs of these mice. Therefore, a more powerful neutrophil depletion method is needed to clarify the importance of neutrophils in ALI.
6. Impaired neutrophil recruitment during ALI in CCR2\(^{-}\) mice

6.1 Introduction

Neutrophils are reported to be one of the critical cell types that cause lung immune pathology during ARDS/ALI. Although the importance of neutrophils in ARDS pathogenesis is somewhat controversial [98, 107, 108], the elevated number of neutrophils during the acute phase and their ability to secrete pro-inflammatory cytokines and reactive oxygen species (ROS) strongly suggests a role in causing tissue damage. Neutrophils are recruited to sites of inflammation by IL-8 (IL-8 in human, KC in mice). In the lungs, epithelial cells are the major source of IL-8 during ARDS/ALI. Since neutrophils do not express CCR2, they are not expected to be affected by blocking CCL2/CCR2 interactions or by the knockout of CCR2 gene. Moreover, there have been no studies reporting that monocytes/DC/macrophages secrete IL-8 (or KC in mouse), further supporting the idea that neutrophil recruitment is independent of these cell types. However, we do not know if there are indirect effects of CCR2\(^{+}\) cells on the recruitment of neutrophils. DC and macrophages are capable of activating other leukocytes and therefore initiate a series of immune responses that may eventually affect neutrophils. Two potential cell types that may mediate this indirect effect are NKT cells and \(\gamma\delta\) T cells. In certain infections and autoimmune diseases, NKT cells and \(\gamma\delta\) T cells are reported to secrete IL-17a [157-160], which stimulates other cell types to secrete IL-8.
and thereby leads to the recruitment of neutrophils. The induction of IL-17a, however, is controlled by IL-23, a cytokine that has been reported to have inflammatory effects and be secreted by DC and macrophages [161]. Combining all these findings, we proposed a model in which CCR2+ cells indirectly affect neutrophil recruitment/proliferation during LPS-induced ALI. To test this hypothetical model, we first determined whether neutrophil recruitment is impaired in the absence of CCR2+ cells. Provided CCR2+ cells are required, we would then determine IL-17 and IL-23 levels in the mouse lungs during ALI.

### 6.2 Results

#### 6.2.1 Impaired neutrophil recruitment in the absence of CCR2+ cells.

Previous studies utilized Gr-1 as the surface marker to identify neutrophils in flow cytometry. With its high affinity, Gr-1 antibody has been a powerful tool to study neutrophil biology, however, it is now known that Gr-1 Ab stains both Ly6G on neutrophils, and Ly6C on subsets of monocytes/DC/macrophages. Thus, a more discerning flow cytometric strategy is required when studying the interaction of neutrophils and other myeloid cell types. Similar to previous studies in our lab [33], here we used Ly6G as the marker for neutrophils. Among the non-neutrophil populations, we used Gr-1 as the marker for inflammatory subsets of monocytes, DC, and macrophages. As described in Chapter 2, we compared cell numbers in WT and
CCR2−/− mice in parallel from day 0 to day 4 during LPS-induced ALI. Surprisingly, we found a significantly smaller number of neutrophils in CCR2−/− mice, compared to that in WT mice (Figure 39a). As described in Chapter 2, the only cells that both express CCR2 and are found in lesser numbers in CCR2−/− mice are the CCR2-expressing inflammatory monocyte and their derivatives (DC/ExMAC). Since neutrophils do not express CCR2, the previous result suggests that these monocytic CCR2+ cells have indirect effects on neutrophil recruitment.

**6.2.2 Impaired IL-17 and IL-23 production in the absence of CCR2+ cells.**

Having demonstrated that CCR2+ cells have indirect effects on neutrophil numbers, we then sought to understand the underlying mechanisms. As proposed in our model, IL-17 and IL-23 are two potential cytokines involved in this postulated indirect effect. Therefore, we first determined if the production of these two cytokines is CCR2-dependent. We harvested BAL fluid from CCR2−/− and WT mice, measured the IL-17a and IL-23 levels by ELISA, and compared cytokine abundance between the mouse strains. We found that, in day 1 and day 2, LPS induces IL-17a production in the lung, but this induction is significantly impaired in CCR2−/− mice (Figure 39b). As for IL-23, we were not able to detect any IL-23p19 protein in BAL fluid by ELISA, presumably due to limited assay sensitivity. Instead, we determined IL-23p19 mRNA levels in total lung cells. Since we found relatively reduced IL-17 production on day 1 and day 2, and IL-23...
is upstream of IL-17 induction; IL-23 induction, if any, is expected to occur prior to day 1. We measured relative IL-23p19 mRNA (corrected to GAPDH mRNA with the signal normalized to PBS mock-treated mice) in total lung cells, and found a significantly diminished up-regulation of IL-23p19 in CCR2−/− mice at 1.5 and 3.0 hours following LPS-treatment (Figure 39c). We also enriched DC/macrophage cell fractions by metrizamide gradient prior to mRNA measurement and also found loss of IL-23p19 expression in the enriched cells from CCR2−/− mice (Figure 39d). In conclusion, we found that LPS induces lung IL-17a abundance within 48 hours of LPS treatment. LPS also induces IL-23 expression in the lung as early as 1.5 hours after LPS-treatment. Both inductions, however, require the presence of CCR2+ cells.
Figure 39: Effects of CCR2+ cells on neutrophil recruitment, IL-17 and IL-23

(a) Lung cells of WT or CCR2/- mice were harvested at day 0, 1, 2, 3, or 4, and subjected to flow cytometry analysis to identify neutrophils (Ly6G+) numbers. (b) 1 or 2 days after LPS- or PBS- treatment, mouse BAL fluid was collected from WT or CCR2/- mice and subjected to ELISA assay for IL-17a protein concentration. (n=9) (c) 1.5 hr or 3.0 hr after LPS-treatment, total lung cells were harvested from WT or CCR2/- mice and mRNA was extracted for reverse-transcriptional real-time PCR reactions to identify mRNA level of IL-23p19. (n=3 for each set) (d) As in figure c, but lung cells were harvested 24 hr instead. DC and other myeloid cells were enriched by metrizamide gradient prior to mRNA extraction. (n=3)
6.3 Discussion

We found that, during LPS-induced ALI, recruitment of neutrophils and production of IL-17 and IL-23 is highly dependent on CCR2+ cells. Together with the established role of IL-17 and IL-23 in contributing to neutrophil recruitment, we propose a model of how CCR2+ cells indirectly mediate the recruitment of neutrophils (Figure 40).

![Diagram of inflammation/tissue damage](image)

**Figure 40: Model for CCR2+ monocyte regulating neutrophil recruitment**

Inflammatory monocytes are recruited to the lung and differentiate into DC and ExMAC. DC and ExMAC that secrete IL-23, which in turn activates γδT cells or NKT cells to secrete IL-17. IL-17 induces the production of IL-8 by epithelial or smooth muscle cells, which in turn induce neutrophil migration to the lung. In addition, IL-17 also induces neutrophil granulopoiesis (not show here).
In our model, inflammatory monocytes are recruited to sites of inflammation by CCR2 ligands, migrate through damaged epithelial barriers and differentiate into inflammatory DC or exudative macrophages. These two cell types then secrete IL-23, which activates a yet identified cell type (possibly $\gamma\delta$ T cells or NKT cells) to secrete IL-17a. IL-17a in the lung has a well-established role in activating smooth muscle and epithelia cells to secrete IL-8 [162, 163], which in turn recruits neutrophils to the lung. Our data support this model but additional experiments could provide further support. First, the cellular source of IL-17a has not been identified. In the lung, $\gamma\delta$ T cell and NKT cells have been reported to secrete IL-17a upon microbe infection or sterile inflammation [157, 159, 160]. Since adaptive immunity requires at least 4-5 days for induction of detectable cytokine levels, the fact that a significant induction of IL-17a in the BAL fluid within 24 hours of LPS-treatment rules out the possibility of Th17 cells to account for the IL-17a protein we detected. In addition, our LPS-induced ALI model does not include any model antigen, and is thus unlikely to activate an adaptive immune response. We plan to identify the cell types that secrete IL-17a by intracellular flow cytometry, and will narrow down the possible cellular candidates by measuring IL-17a level in T cell-deficient mice. Second, although DC and macrophages have well-established capacity to produce IL-23, we lack direct evidence that CCR2$^+$ cells secrete IL-23 in our model. We do, however, plan to sort individual cell types by flow cytometry, and measure their IL-23 expression by real-time PCR. This experiment will provide a clearer answer of the
cellular source of IL-23 during ALI. It is worth noting that IL-8 and CCR2 are often coincidently expressed during inflammation, and play essential roles in tissue inflammation or pathogenesis [164]. The mechanism of how these two molecules interact with each other, however, is not clear. The model we propose here may provide an explanation for these unsolved questions.
7. Conclusion

The study suggests that, during LPS-induced ALI, CCR2+ monocytes are recruited to the lung, and differentiate into inflammatory DC and exudative macrophages. These CCR2+ cells play critical roles in lung inflammation and tissue damage, which in turn lead to morbidity and mortality. As summarized in Figure 41, the CCR2+ cells directly mediate lung tissue damage and induce further inflammation by secreting TNF-α and IL-6. In addition, they also indirectly induce neutrophil recruitment through an IL-23/Il-17/IL-8 axis, possibly mediated by γδT cells and/or NKT cells and epithelial cells. The fact that inflammatory monocytes, DC, and macrophages have both direct and indirect effects on lung tissue injury supports their critical roles during ARDS/ALI. We have not performed an adoptive transfer experiment in which donor CCR2+ monocytes could be tested for their ability to cause severe lung damage in recipients’ lungs; such experiment that would confirm the critical pathogenic role of CCR2+ inflammatory cells during ALI. Our studies strongly suggest that blocking CCR2 is potentially an effective treatment for ARDS/ALI patients. Fortunately, several CCR2 antagonist drugs are currently in clinical trials for other diseases [156]. Currently available treatments for ARDS/ALI have very limited efficacy and an effective treatment is highly sought after [112]. Based on the pathogenic roles we find here, it may be worth considering these drugs for the treatment for ARDS/ALI, or any other inflammatory
disorders that might be caused by CCR2+ inflammatory monocytes and their derivative cells, moDC and ExMAC.

In steady states (left), monocyte (Mono) and neutrophils (PMN) come and leave the lung with blood streams, while residential alveolar macrophages in the lung act as scavenger for inhaled harmless materials. Upon inflammation (right), inflammatory monocytes are recruited to the lung by CCR2 ligands, and migrate through damaged epithelial barriers. These cells then differentiate into inflammatory DC and ExMAC, which secrete IL-6, TNF-α to induced further inflammation and cause tissue damage. DC and ExMAC also secrete IL-23, which in turn induces IL-17 production of γδ T or NKT cells. IL-17 stimulates epithelial and smooth muscle cells to secrete IL-8 (KC in mouse), which in turn recruits neutrophils to the lung. Neutrophils secrete reactive oxygen species to clear pathogens, but also damage lung tissues. Alveolar macrophages appear to be directly activated by LPS and contribute to lung pathogenesis independently. (Dashed arrow and dashed cells: not yet proved in LPS-induced ALI)

Figure 41: Summary of our findings and proposed model
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Biography

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