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Flow Cytometry-Based Single Cell Measurements of Intracellular Cytokine Production by LPS-Activated Murine Macrophage Cells

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The project that I worked on this summer at Sandia National Laboratories CA was part of the Microscale Immune Systems Laboratory (MISL) Grand Challenge, which is aimed at studying host-pathogen interactions with single cell resolution. This is enabled through the development of microfluidic tools that allow for a better understanding of the innate immune response to bacterial pathogens. The study of innate immunity is important because it represents our first line of defense against infection. Since pathogens such as *Franciscella tularensis* and *Yersinia pestis* gain virulence through subversion of the innate immune system, determining their mechanisms of action on a molecular level could lead to improvements in diagnostics and therapeutics.

The key sentry cells of the innate immune system are the macrophages, which recognize, ingest and kill microbes, as well as activate neighboring cells and recruit the adaptive immune response. Macrophages have a number of type I membrane proteins called Toll-like receptors (TLRs) that are used to recognize pathogenic invaders via pathogen-associated molecular patterns (PAMPs). Mammalian leukocytes express at least ten different TLRs, each recognizing a particular PAMP, conferring specificity to this detection system.

The particular Toll-like receptor that we are interested in for the purposes of MISL is TLR4, which recognizes lipopolysaccharides (LPS) from gram negative bacterial cell walls. Binding of the LPS ligand to TLR4 initiates receptor homodimerization, setting in motion a signaling pathway that leads to macrophage activation. Figure 1 depicts a very simplified view of this activation pathway. Briefly, TLR recruits the adapter proteins MyD88 and TRIF,

activating two main branches of the cascade. MyD88 goes on to phosphorylate a number of kinases, including p38, ERK, IKK and JNK. This leads to activation of transcription factors, which translocate to the nucleus to regulate gene expression of various effector molecules. The effector proteins that we are particularly interested in are called cytokines, which serve as inflammatory mediators that signal between cells, broadening and shaping the innate immune response as well as activating the acquired (adaptive) immune response.

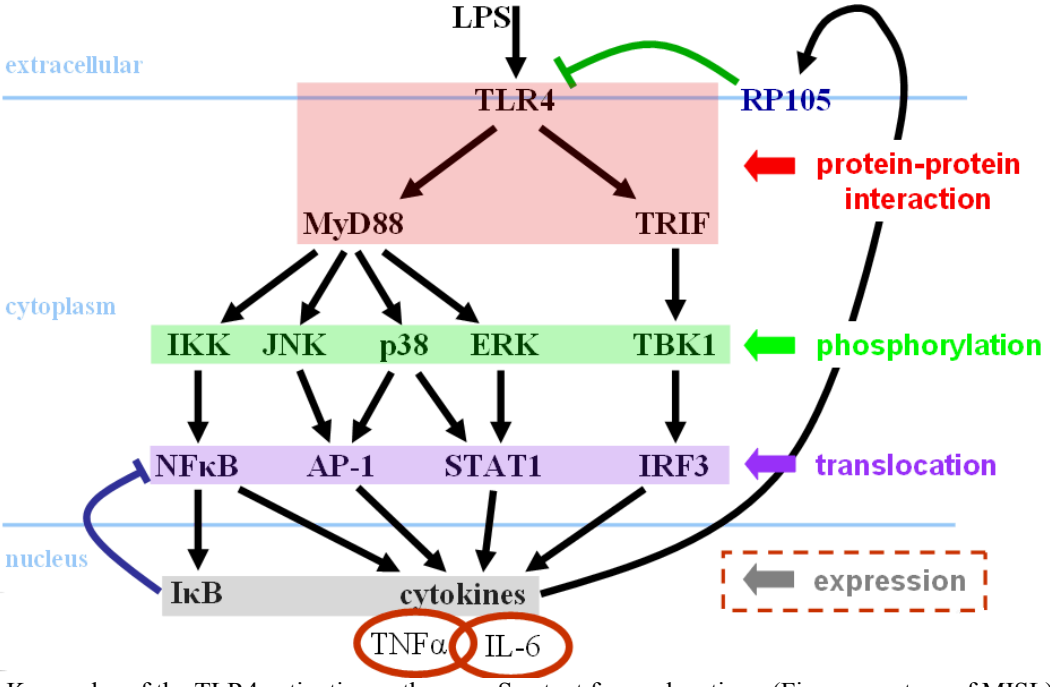

Figure 1. Key nodes of the TLR4 activation pathways. See text for explanation. (Figure courtesy of MISL).

The main goal of my project was to monitor the key effector cytokines tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) on a single-cell level using flow cytometry, following macrophage activation with LPS. We chose to conduct individual cell analyses since pooled populations are heterogeneous with respect to cell cycle or activation states, and thus measuring averaged responses from macroscopic populations can mask underlying microscopic regulatory mechanisms. Flow cytometry is a useful tool because of its ability to measure single cell responses, and does not depend on measuring proteins in the cell supernatant, which can be

dilute and thus difficult for less sensitive assays to detect the proteins. In our studies, we used the Golgi transport inhibitor brefeldin A (bfA) to trap cytokines within each cell. However, since inhibition of the Golgi is no minor action, we studied various controls to ensure that bfA did not have a detrimental effect on the cells. These controls included viability staining with calcein AM and ethidium homodimer, and the examination of downstream signaling, including surface marker expression of RP105 and MTS510, and kinase phosphorylation of ERK and RelA.

Upstream preparation for flow cytometry begins with stimulation of the cells with *Eschericia coli* LPS in the presence or absence of bfA. After various incubation times, cells were fixed and permeabilized to allow for fluorescently-conjugated antibodies to enter the cell and bind the cytokines of interest, and finally run on the flow cytometer. Flow cytometry is a system that forces cells to flow single file past an incident laser of 488nm wavelength. When the light hits the cells, it scatters in different directions. When the directional information from the scattered light is gathered, it provides data on particle size, and thus specific populations can be gated out from uninteresting debris and other cell types. The laser is also used to excite the fluorophores attached to the antibodies, which are in turn bound to the proteins of interest, in this case, TNFα and IL-6. The fluorescence emission is measured and is directly correlated to the amount of cytokine that was produced by the cell due to LPS stimulation. Using such a method, one can determine the effects of various incubation parameters by observing cytokine expression kinetics.

In order to validate our ability to use brefeldin A without negative impact on the cells, we first ran controls to examine viability and downstream protein expression. Following macrophage activation and bfA incubation, viability was measured with a kit that uses calcein

AM to stain live cells by measuring intracellular esterase activity, and uses the ethidium homodimer to stain dead cells by measuring plasma membrane integrity. Viability was determined to be greater than 90% for all incubation time points when measured with flow cytometry using two different channels for the live and dead stains. Images obtained on a fluorescent microscope confirm these results (Figure 2).

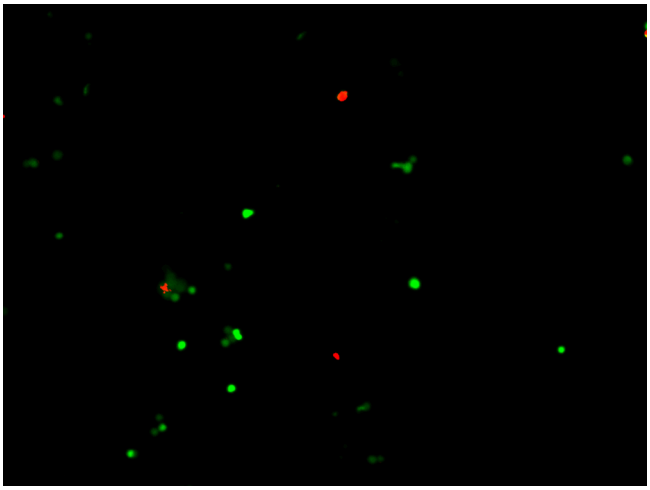

Figure 2. Macrophages incubated with 2uM *E. coli* LPS and 1.5ul/ml bfA for 8 hours, stained with calcein AM (green) and ethidium homodimer (red).

The effect of bfA on surface marker expression was examined next. MTS510 is an antibody to inactive TLR4/MD-2, which is present at the cell surface in unstimulated conditions. Normally, with LPS stimulation, its expression is gradually downregulated. We found that following LPS stimulation, MTS510 was downregulated equally in the presence or absence of bfA, indicating that bfA does not have a pronounced effect on its expression.

RP105 (CD180) is a surface marker that is increasingly expressed only after LPS stimulation, and forming a complex with MD-1, works in concert with TLR4/MD-2 to control LPS signaling. We found that after LPS stimulation, RP105 was upregulated to a slightly lesser extent in the presence of bfA compared to similar samples without bfA. This indicates that over time, bfA could be trapping some of the RP105 molecules within the cells, but that the majority

are still making it to the cell surface. Although it is difficult to tease out the effects of RP105's signaling alone since the cell is responding to many stimuli, both excitatory and inhibitory, the implications of a slightly dampened RP105 response is likely insignificant in terms of overall cell activity.

The final control to determine the effects of bfA on the cell was the measurement of kinase phosphorylation in the TLR4 signaling pathway. The first kinase that we looked at was ERK, also called MAPK. ERK phosphorylation peaked around 30 minutes after LPS stimulation, and was rapidly dephosphorylated to pre-stimulation levels within an hour. This was found to be the case whether bfA was used or not, with the kinetic responses nearly overlapping (Figure 3). This was reinforced by calculating the ratio of obtained mean fluorescence values without bfA to those with bfA. For all time points, this ratio was at or near unity, indicating little to no effect of bfA on ERK phosphorylation. Phospho-ERK kinetics as determined by flow cytometry were also supported by data obtained via western blotting.

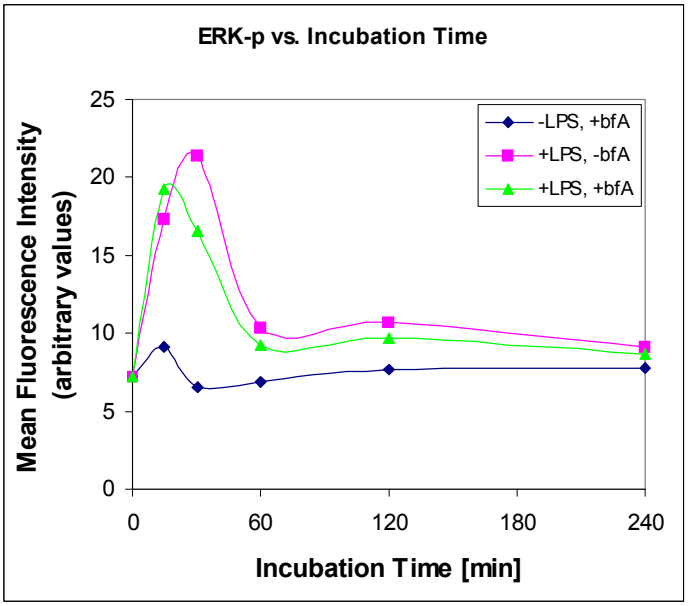

Figure 3. Phospho-ERK kinetics. Blue diamond, -LPS +bfA, pink square, +LPS –bfA, green triangle, +LPS +bfA.

RelA, a subfamily of NF-κB proteins, is another transcription factor that regulates gene

transcription and thus protein expression upon translocation to the nucleus following activation by phosphorylation. As with phospho-ERK, phosphorylated RelA showed an increase 30 minutes following LPS stimulation, and down-modulation within an hour. Kinetics were similar with or without bfA. Additionally, phospho-RelA, in the small time window observed, showed what could possibly be seen as cyclical activity in and out of the nucleus, which is quite typical of nuclear transcription factors.

Having shown that brefeldin A has minimal impact on cell viability, cell surface marker expression and intracellular kinase phosphorylation, we proceeded to use the Golgi transport inhibitor to trap cytokines within the cell. Some methods development and parameter optimization was necessary to maximize cytokine response. The optimal parameters were found to be 1.0ul/ml of bfA added two hours following 2uM *E. coli* LPS addition. Cytokines were measured at two hour-intervals up to 8 hours, and cells were incubated in Teflon-coated culture dishes to prevent cell aggregation in downstream steps. Additionally, a cell concentration of 1 million cells per ml was determined to yield optimal kinetics.

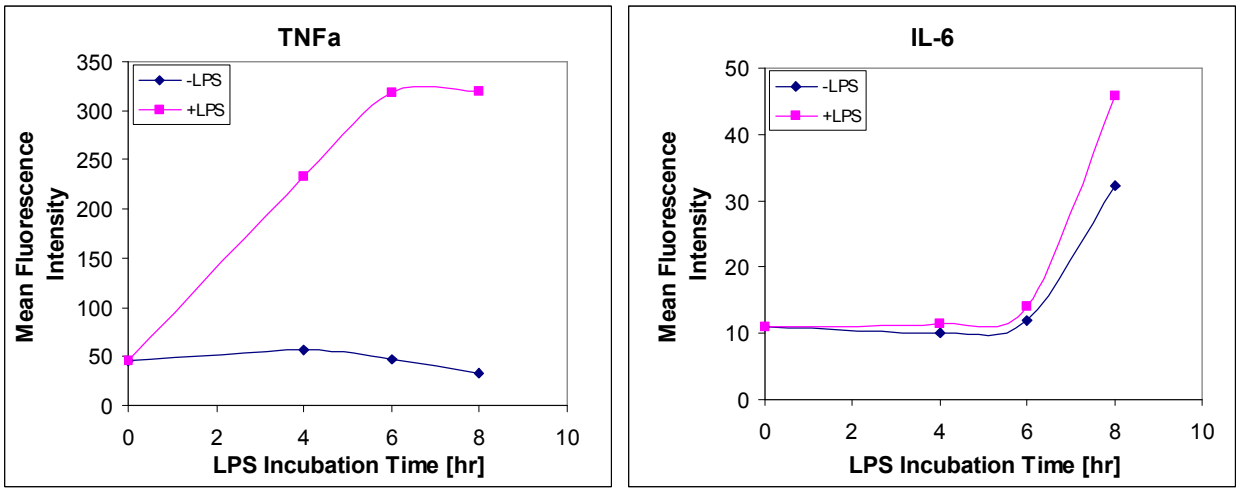

Figure 4. TNFa and IL-6 expression kinetics following incubation with 2uM *E. coli* LPS and 1.5ul/ml bfA. Blue diamond, -LPS, pink square, +LPS.

The kinetic profiles of two cytokines, TNFα and IL-6, were measured. TNFα was seen

to have a more rapid response, increasing by four hours and peaking at six, compared to IL-6, which did not increase until eight hours (Figure 4). Additionally, the magnitude of response for TNFα was much greater than that of IL-6, on the order of hundreds versus tens of units of mean fluorescence intensity. This data obtained with flow cytometry is also supported by that obtained from enzyme-linked immunosorbent assays, or ELISA.

In summary, we demonstrated that the use of the Golgi transport inhibitor brefeldin A to trap cytokines within the cell does not adversely affect the cell in terms of viability, surface marker expression, or downstream protein phosphorylation. When cells were stimulated with *E. coli* LPS in the presence of bfA, single-cell flow cytometry analyses showed increased levels of the cytokines TNFα and IL-6 over time. However, the magnitude of response of TNFα was greater and earlier than that of IL-6, reinforcing the fact that TNFα is a robust marker of macrophage activation.

Studies that we did not get to this summer but that should be included in future experiments include cytometric bead analysis (CBA) to obtain absolute quantitation rather than relative values. CBA also has a multiplexing ability, meaning that it can measure multiple cytokines in a single sample. In these experiments we looked at intracellular cytokines, and so it would be informative to run a parallel experiment to look at the cytoplasmic cytokine secretion in the supernatant while using bfA--in other words, to look at the number of cytokines escaping the bfA trap. Using other concentrations of LPS would enable us to create a dose-response curve and to determine cutoff values that elicit different cytokine responses. Finally, it would be very interesting to examine the cytokine responses to LPS from different pathogens such as *F. tularensis* and *Y. pestis*, which are known to gain virulence by altering different nodes of the TLR4 signaling pathway.

My achievements this summer were numerous. I demonstrated for the first time that the use of brefeldin A does not adversely affect downstream cellular processes, a novel finding that, to our knowledge, is yet unpublished in scientific literature. We hope to publish this data in a paper as soon as results have been repeated for statistical significance. Additionally, my work this summer has confirmed that a single-cell flow cytometric method of measuring cytokines is ready for adaptation to an on-chip microfluidic platform, one of the goals of the MISL Grand Challenge at Sandia. I presented my data at several MISL update meetings throughout the summer, as well as at the Intern Symposium at the conclusion of the summer.

My internship experience this summer positively influenced my academic plans in that it reinforced my intentions to study immunology. My dissertation will most likely study the modulation of cell signaling between different types of immune cells in various infectious states. I was also encouraged this summer that I had made the right decision in switching from a master's to a PhD program, since I enjoy being in the lab and conducting research, and can definitely see myself pursuing research as a career. As far as career influences go this summer, I see national lab work as being in between academia and industry, perhaps more towards academia in the sense that everyone really focuses on empire-building. I have always known that I prefer industry over academia, and working at a large site this summer also reinforced my preference for smaller companies over enormous entities that have a hard time moving forward in any one direction due to ever-increasing expansion into too many peripheral directions, over-obsession with politics and lack of consumer-driven deadlines.

There were no real activities or lectures sponsored specifically for the interns, or if there

were, I was not aware of them. I confess that I was in the lab a lot and was only able to attend one DHS lecture on nuclear weapons (which was extremely interesting), and two talks related to MISL, which did help me to gain a better understanding of my project this summer. The Albuquerque DHS interns also came to our site for a couple of days (I understand there is an exchange every summer, and that the sites swap hosting duties). There were a few talks by Sandians mainly on border protection-related projects, which were poorly given in that it felt like they were trying to sell us on the ideas, and that we were potential customers or investors. They were too general and most of them lacked any sort of data at all.

I attended a workshop this summer that I believe was partly sponsored by DHS, entitled Summer Workshop on Teaching about Terrorism (SWOTT). It was a week-long workshop at Oklahoma University (there are two others at Georgia Tech and University of Maryland), that not only focused on pedagogical aspects, but subtopics of terrorism in general, such as its various definitions, history, psychology, causes, role of women, suicide terrorism, simulations, legal responses, etc. Not only did I come away from it with a better understanding of terrorism as a whole, but I gained tools and resources to equip me in conducting research in such fields. Overall, I felt the takeaway message that most of the speakers brought up was that in reality, the result of most terrorist acts will be of mass disruption, rather than destruction, and that the possibility of CBRN attacks is *extremely* remote in comparison to more conventional methods of violence like explosives or the use of readily available materials that are simple to implement. And though I think continued research and innovation in all aspects of defense, deterrence, prevention and preemption is still important, educating the masses with such a take-home message or equipping them with something like SWOTT would strengthen us on many levels--

afterall, as they say, knowledge is power. The main weapon of terrorists is really fear; if you take that away from them, all they can inflict is potentially tragic inconvenience.