SZENT ISTVÁN EGYETEM
ÁLLATORVOS-TUDOMÁNYI DOKTORI ISKOLA

Immunomodulation of pathogen-host interaction

Brief Summary of Doctoral Thesis

Dr. Susan Szathmáry,

Advisor:

Dr. László Stipkovits

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Review of the literature

In the initiation of innate and adaptive immune response Pathogen Recognition Receptors (PRR) play key roles. These receptors recognize the presence of a foreign organism through pathogen associated molecular patterns (PAMPs). These receptors can be found on several cell types. The PRR receptors found on dendritic cells (DC) play an important role in the adaptive immunity. Pathogens through these receptors activate dendritic cells (DCs) and up-regulate the expression of major histocompatibility complex (MHC) molecules and other molecules. The DCs then migrate to the lymphoid tissues. Upon presentation of the antigens to the naive T cells, the T cells differentiate into antigen committed Tcells (Th1, Th2 and T-regulatory). The Th1-Th2 balance is determined by the origin of the DCs, the maturation stimulus and the cytokine environment. These conditions determine how well the host can control the infection.

In this research our goals were to manipulate the immune response. We have used two approaches: removing activators of the PRR receptors or by using the PRR agonists (TLRs 2, 3, 4, 9 and Nod2) as immunomodulators. Affinity purified *M. gallisepticum* antigens were immobilized along with PRR agonist molecules to microparticles in a „pathogen mimicking" particle system. The particles have been tested in *M. gallisepticum* chicken challenge studies and in *in vitro* experiments with dendritic cells.

Own experiments

In **Experiment 1**, we described the removal of a TLR-4 agonist molecule, endotoxin, from solutions by using affinity technology. The endotoxin concentration was measured using LAL assay. We have shown endotoxin binding from water, Pseudomonas supernatant, and salt solutions by spiking the samples with known amounts of endotoxin. We have tested the reusability of the affinity resin by cleaning it with NaOH as well as hot water sanitization. We have also tested any leachables from the affinity resin that could potentially contaminate the solutions. We have also investigated if the resin changed the composition of the salt solution.

In **Experiment 2**, we have described the removal of TLR agonist molecules, such endotoxin (TLR4), peptidoglycan (TLR2/Nod2), lipopeptide (TLR2) and bacterial DNA (TLR9) from blood and plasma using affinity technology. The removal of endotoxin was tested with LAL assay, while we have used monocyte activation assay (TNF-α ELISA) for testing the removal of the other TLR agonist molecules. We have tested that the affinity resin, while removing the TLR agonist molecules, does not negatively affect the blood in terms of coagulation, complement activation, hemolysis and cell counts.

In **Experiment 3**, we have described mycoplasma capture from solutions, such as serum used in cell culture, by affinity technology. We have used serologically and biochemically different mycoplasmas and affinity resins that are specific for different lipid and carbohydrate moieties on the mycoplasma membranes. The resin efficiently removed 10^3-10^5 mycoplasmas from serum without effecting its composition or growth promoting potential.

In **Experiment 4**, we have described a synergistic effect of TLR agonist molecules, such as peptidoglycan (TLR-2/Nod2) and bacterial DNA (TLR-9) on the stimulation of the innate immune response. Monocyte culture-based activation assay for TNF-α and Tissue factor levels (ELISA) were used to demonstrate t such effect. Our results show when we added bacterial DNS ant a concentration of 5-15 μg to peptidoglycan (0.3-1 μg) they synergistically induce 10-15 times more TNF-α and Tissue Factor production.

In **Experiment 5**, we have described the preparation of pathogen mimicking microparticles. As a carrier we have used natural polysacchrarides particles in the size range of a bacteria (1-5 μm). On the surface of these particles we immobilized immunoaffinity purified of *M. gallisepticum* antigens and biochemical modified antigens (Endoglycosidase H digestion, Concanavalin A adsorption, periodate oxidization). In addition, we have immobilized PRR (pathogen recognition receptor) agonists, such as TLR2, TLR3, TLR4, TLR9 and Nod2 agonists to the microparticles. These microparticles were used *in vivo* (Experiment 6) and *in vitro* (Experiment 7) studies.

In **Experiment 6**, we have used a *M. gallisepticum* challenge model in chickens to test the effect of "pathogen mimicking" microparticles. We have set up groups of 3 days old chickens (10 chickens per group) that were treated with different composition of microparticles orally 14 days prior to the challenge or after the challenge. The chickens were challenged with *M. gallisepticum* R_{low}, a highly pathogenic strain of *M. gallisepticum*. Fourteen days after the challenge, the chickens were euthanized and examined for pathological lesions. Samples from different organs were taken for culture for *M. gallisepticum* and for histopathology. In the challenge

experiment, the particles alone did not give any protection. The PRR agonists without antigen gave about 30% protection. The *M. gallisepticum* and *M. gallinarum* membranes gave worse results than the control. The immunoaffinity-purified antigens in 50μg dose gave 30-40% protection while adding the PRR agonist molecules increased the protection to 70-80%. When we modified the different post-transcriptional modifications on the antigen by ConA, EndoH or periodate and immobilized PRR agonists the protection reached 90-100% and it was dose dependent. The results were the same if we treated the chickens prior or after the challenge.

In **Experiment 7**, we have used the pathogen mimicking microparticles to study their effect *in vitro* with PBMC and dentritic cells. We have induced monocytes with Il-4 and GM-CSF to obtain dentritic cells. We measured TNF-α and IL-10 induction using ELISA. We have also labeled the microparticles with fluorescein and used flow cytometry to show interaction with dentritic cells. We have shown that the immunomodulatory microparticles induce changes that are hallmarks of dendritic cells maturation, such as increase in the expression of MHCII molecules and CD86 molecules. These were assayed by flow cytometry. When cells were stimulated with microparticles containing ConA modified antigen, it induced the production of 30 pg TNF-α és 15 pg IL-10. However, when we added PRR agonist along with the ConA treated antigen it induced 150 pg TNF-α and 7 pg IL-10 production. The ratio of TNF-α and IL-10 was the highest in the ConA treated antigen combined with PRR agonist. This confirmed our results from the in vivo challenge experiments where this composition of microparticles gave the best protection.

Discussions

In the first part of this research, we showed that upon the removal of bacterial compounds, such as endotoxin, peptidoglycan, bacterial DNS by affinity methods the level of inflammatory cytokines, such as TNF-α and Tissue Factor is reduced in the blood and plasma. This could have a beneficial affect on the innate immune system. The use of particle-based affinity resins is a novel approach to address these problems.

In the second part, we have showed that a pathogen mimicking microparticle, by using PRR agonists and M. gallisepticum antigens, is useful to prevent pathological lesions and colonization induced by *M. gallisepticum* infection or successfully treat already infected animals. Our pathogen mimicking microparticles represents a novel approach that has not been described in the scientific literature.

Several different particle-based systems have been used experimentally as carrier for different antigens. Chitosan, poly-DL-lactic acid, or polyacryl starch or dextrane micro particles have previously been described as a drug carrier system. ISCOM particles and liposomes have been used as prototype vaccines. The limitations of the particles prepared by precipitation are that the conditions for the precipitation can differ for different antigens. The particle must break down before the antigen is released. The incorporation of different antigens and PRR agonists for the surface is not feasible. In case of polyacrylamide particles, the biocompatibility is a potential problem along with the fact that the breakdown of the particles in the dentritic cells has not been demonstrated.

Our pathogen mimicking microparticles are made of natural polysaccharide of polymers. This provides excellent

biocompatibility and they are readily biodegradable. The immobilization chemistry allows the immobilization of several antigens along with different combinations of PRR agonist molecules in a controlled and highly reproducible fashion. The covalent linkage between the antigen and the particles assures that the same antigen-processing cells take up both the PRR agonist and the antigen.

The biological effects of the mycoplasma mimicking microparticles were demonstrated both *in vivo* and *in vitro* with dendrtitic cells.

Research achievements

Our achievements can be summarized as the following:

We have demonstrated successful removal of TLR-4 agonist molecule, endotoxin from solutions using a novel affinity technology.

We have demonstrated efficient removal of TLR-2, TLR-4, TLR-9 and Nod2 agonist molecules, such as peptidoglycan, endotoxin, lipoprotein and bacterial DNA from blood and plasma using a novel affinity technology without negatively effecting cell counts or coagulation.

We were the first to demonstrate mycoplasma capture from solutions, such as serum used in cell culture, by a novel method using affinity chromatography without affecting the growth supporting properties or the composition of the serum.

We have demonstrated synergistic effect of peptidoglycan and bacterial DNA on the production of TNF-α and Tissue Factor in a monocyte activation assay. This is a new observation that has not been described in the literature.

We have prepared novel pathogen mimicking microparticle compositions using *M. gallisepticum* immunoaffinity purified antigens and different combinations of PRR (TLR 3,4,9 and TLR 4,9-Nod2) agonists. These immunomodulatory microparticles were tested in a *M. gallisepticum* challenge model in chickens and shown reduction of pathological lesions and colonization of inner organs due to mycoplasma.

We have demonstrated the effect of the different post-transcriptional modifications of the mycoplasmal antigens that

participate in the pathomechanism of *M. gallisepticum* infection. We have demonstrated that by removing these post-transcriptional mofications using Endoglycosidase H, Concanavalin A, periodate and deacylation, we have achieved protective immunity. We demonstrated that we have achieved protective immunity not just preventatively, but also therapeutically, post-infection.

We have demonstrated the effect of these immunomodulatory microparticles *in vitro* with PBMC and dentritic cells. We have demonstrated that the different combination of PRR agonists induced the production of different levels of cytokines in cell culture.

List of related publications

Articles

Amoureux, M.C., Hegyi, E., Le, D., Grandics, P., Tong, H., **Szathmary, S**. (2004) A new method for removing endotoxin from plasma using hemocompatible affinity chromatography technology, applicable for extracorporeal treatment of septic patients. J. Endotoxin Res. 10:85-95.

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Abstracts published in journals

Amoureux, M,C., Rajapakse, N., Hegyi, E., Le, D., **Szathmary, S**., Tong, H., George, N. and Grandics, P. (2003) Endotoxin Removal From Human Plasma And Blood By A Hemocompatible, Affinity-Based Extracorporeal Technology For The Treatment Of Sepsis. J. Am.Soc.Nephrology, American Society of Nephrology Meeting, San Diego. Oral presentation

Amoureux, M.C., Rajapakse, N., Hegyi, E., Le, D., **Szathmary, S**., Grandics, P. (2004) Bacterial toxins removal from human plasma and blood by a hemocompatible, affinity-based extracorporeal technology for the treatment of sepsis. J. Am.Soc.Nephrology. American Society of Nephrology Meeting, St Louis. Oral Presentation

Grandics, P., Amoureux, M.C., Rajapakse, N., Hegyi, E., Tong, H., George, N. and **Szathmary, S**., (2003) Endotoxin-Free Dialysate Using DialGuard™ J.Am.Soc.Nephrology. American Society of Nephrology Meeting, San Diego. Oral Presentation

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Proceedings

Stipkovits, L., Biro, J., **Szathmary, S**., Klein, U. (2004) Sensitivity testing of mycoplasma pathogens to antimicrobials. Proceeding of IOM, Athens, USA. Poster

Szabo, I., **Szathmary, S**., Stipkovits, L. (2004) Detection of maternal antibodies to M. hyopneumoniae in swine of farrowing-fattening type of farm. Proceeding of 18th Congress of IPDS. Hamburg. Poster

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