Study: The Effects of OMT on the Expression of Immune Cell Biomarkers

Study Protocol

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The Effects of Osteopathic Manipulation on the expression patterns of immune cell biomarkers

1. Specific Aims:

This proposal intends to study the effects of osteopathic manipulative treatment (OMT) on the biomarker expression patterns in human immune cells, by using the novel protein subcellular localization (PSL) microarray technology developed in Dr. Wayne Zhou's (PI) lab. The PSL microarray is the third generation of biochips (after DNA microarray, protein microarray) that can determine both the expression and subcellular localization protein profiling in high throughput screening format allowing for rapid identification of thousands of proteins. For the current study, we propose to use the PSL technology to determine the expression patterns of 60-400 cell surface proteins/biomarkers in human peripheral blood mononuclear cells (PBMC) of study participants with a history of low back pain, before and after OMT. We propose to identify and validate those proteins whose expression levels have been changed in most participants after OMT. From this study, we shall develop a platform to study the effects of different kinds of OMT techniques on participants with various types of pain conditions, and uncover the important immune cells that are affected by OMT techniques, therefore to uncover the molecular mechanisms of OMT.

Here are the specific aims for the current proposal:

> Specific Aim 1: Determine the expression patterns of 60 surface biomarkers for human immune cells in PBMC of the participants, before and after OMT.
>
> Specific Aim 2: Identify those specific proteins whose expression patterns have changed the greatest number of participants after OMT.

2. Research Strategy

2-1. Significance: Within the osteopathic profession most research on the effect of OMT on the immune system has involved lymphatic pump techniques. These techniques involve rhythmic compressions applied to the chest or abdomen, or through the lower extremities to maximize pressure gradients between the thorax and abdomen (page 632, 1). Studies performed in the 1920's and 30's found increases in serum leukocytes (2-3) and antibody titers (4) with splenic lymphatic pump techniques. Contemporary studies have detected changes in serum immune cell concentrations and inflammatory markers in response to lymphatic pump techniques. Mesina et al (5) measured blood cell counts following pectoral traction and splenic pump techniques and found significantly increased basophil counts in the 15-60 minute period post-treatment. However, there was individual variability with one subject demonstrating a significant increase persisting 4 hours post-treatment. Noll et al (6) found a decrease in serum platelet counts after participating nursing home residents received a single 6 minute OMT treatment consisting of lymphatic and myofascial techniques. Walkowski et al (7) found increases in serum cytokines, including IL-6, with a single 7-minute lymphatic treatment consisting of thoracic, splenic, and hepatic pump techniques. Licciardone et al (8) found several cytokines that were significantly associated with low back pain. Of these inflammatory markers, tumor necrosis factor concentrations significantly decreased after 12 weeks with 6 OMT sessions. These changes in serum immunologic factors

may be due to the OMT techniques' effect on the central lymphatic system. Lymphatic pump techniques have been shown to transiently increase central lymphatic flow, lymph leukocyte concentrations, and IL-6 concentrations (9-13).

Changes in lymphatic markers have been seen with other types of manual techniques. Soft tissue massage has been shown to significantly increase thymic and splenic T cell number in mice (14), increase serum natural killer cell and lymphocyte numbers in women with early stage breast cancer (15), and increase natural killer cell cytotoxicity in stable preterm infants (16). Serum from participants receiving spinal manipulation (HVLA) has been shown to significantly increase *in-vitro* immunoglobulin G and IL-2 synthesis in peripheral blood mononuclear cells (17-18). Spinal manipulation has also been shown to modify serum IL-6 levels (19) and decrease lymphocyte activation (20). The proposed study will expand upon the biomarker evidence base by investigating the effects of OMT on the biomarker expression patterns in serum immune cells using the PSL (protein subcellular localization) microarray technology.

The PSL (protein subcellular localization) microarray technology is the novel technology developed in Dr. Zhou's lab. It provides a unique approach to simultaneously determine subcellular localization and expression profiles of potentially thousands of proteins from one experiment. The technology has been already been used to identify the differences in protein expression between normal and cancer breast cell lines (21-23). The technology has also identified proteins with unique subcellular localization patterns between normal and cancer cells with breast origin and prostate origin (21-23).

2-2. Innovation: We propose to study the effects of OMT on serum immune cells using a system biology approach through the use of novel PSL microarray technology. No studies have yet investigated the effects of OMT using this technology, which may be able to detect changes in biomarker expression on immune cells even in the absence of cell concentration changes. We propose to use 40 participants for the experiments, 20 in the OMT group, and 20 in the control group. This number of participants should give us adequate statistical power to detect the difference in biomarker expression change. In addition, this proposal could provide a platform for future studies investigating the effects of different OMT techniques, and therefore to understand the molecular mechanism for OMT.

2-3. Preliminary data:

2-3a. Summary of the PSL microarray technology and its application on biomarker identification (Ref). The PSL microarray technology, also known as DAMA staining technology, is a novel technology that has combined the power of immunohistochemical staining and the parallel analysis of antibody microarrays (2123). It is equivalent to carry out 1000 immunostaining experiments from single immunostaining experiment. To our knowledge, this is the unique technology that can determine both the subcellular localization and expression profiles of thousands of proteins in high-throughput format.

Instead of capturing ligands on the array support like DNA microarray, PSL microarrays deliver multiple antibodies to their antigens in a position-addressable manner (Fig. 1). In this method, cells grown to the desired states on the cover slip, are fixed and permeabilized with standard methods (step 1). An array of antibodies on a membrane is then placed on the top of the cell with the antibodies facing the cells and incubated for an hour. During the incubation, antibodies dissociate from the array membrane and bind to their respective antigens in the cells without significant lateral diffusion (step 2). Afterward, the array membrane is removed, leaving the bound antibodies attached to the cellular antigens *in situ* (step 3). Enzyme-labeled secondary antibodies are then used to detect the bound primary antibodies on the cells (step 4). This way, the expression levels of multiple antigens can be determined simultaneously.

When fluorophore-conjugated secondary antibodies, instead of enzyme-labeled secondary antibodies, are used to detect the bound antibodies (step 4, Fig. 1), both the expression level and cellular localizations of multiple antigens can be determined using the fluorescent or confocal microscopes.

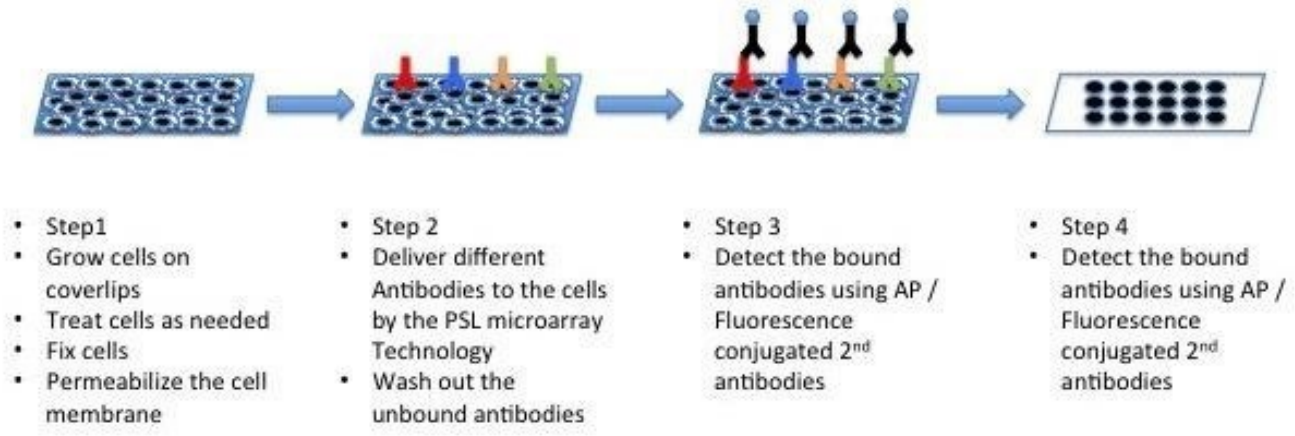


Fig. 1. Schematic representation of the PSL microarray technology

Identification of potential biomarkers for breast cancer by using the PSL microarray technology. (21) Dr. Zhou's lab has developed the PSL microarray technique and demonstrated its application on identifying the differentially expressed proteins between normal and cancer breast cell lines (4). For this purpose, protein expression profiles of ten different cell lines from human mammary glands were obtained using the PSL microarray technology and subjected to intensity integration and data analysis. Ten proteins were identified by DAMAPEP, (DAMA protein expression profiling, a software developed at his lab to retrieve, normalize, scale and analyze the intensity data), to have differential expression levels in cancer cells versus normal cells. Five proteins, RAIDD, Rb p107, Rb p130, SRF and Tyk2, were identified to have higher expression levels in cancer cells, and were confirmed by western blotting analysis (21).

Identification of potential biomarkers with different subcellular localizations for breast cancer and prostate cancer, by using PSL microarray technology (22-23). Dr. Zhou's lab has developed the PSL microarray technology that provides a new approach to the global analysis of protein subcellular localization (SCL) profiling in fixed cells. His lab has developed and optimized this technology, generated ChipView, a program for management and analysis of molecular image database, and utilized the technique to identify

proteins with unique SCL in breast cancer cell lines. The subcellular profile images of 360 proteins in a normal breast cells are shown in Fig 2a, and the subcellular localization profiles of the 360 proteins in two benign and seven carcinoma cells are shown in Fig 2b. From data analysis, a protein, Cyclin B1, was identified and validated with different SCLs between normal and cancer cell lines (22). Similar research has been carried out in prostate cancer and normal cell lines (23).

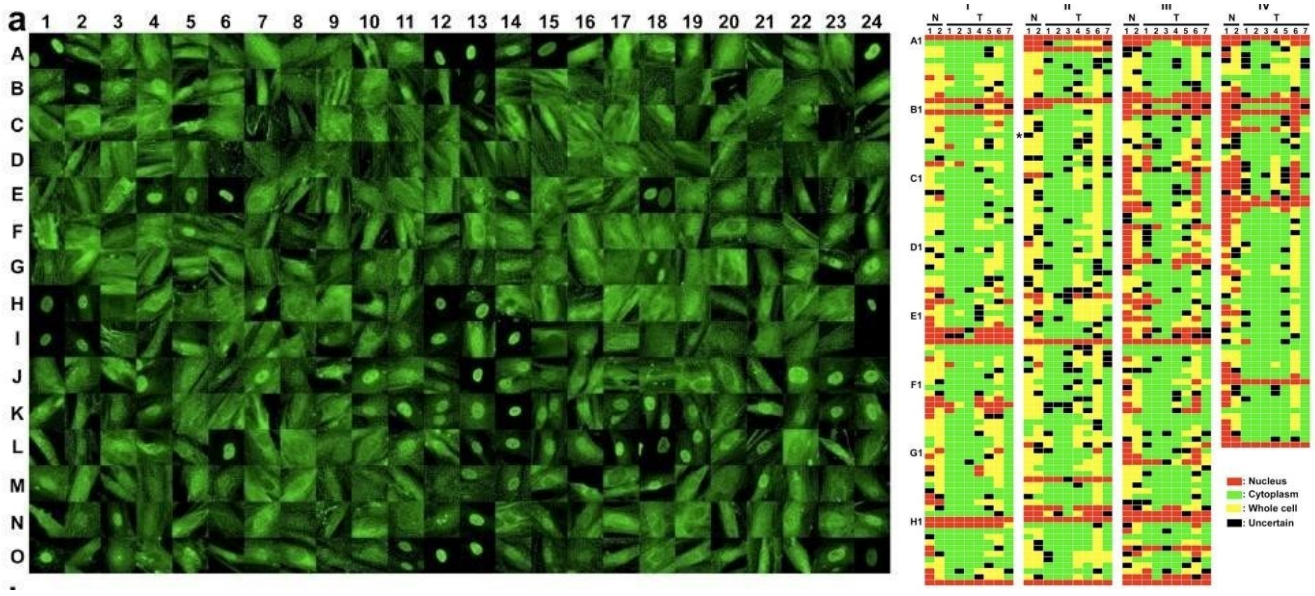


Fig 2. (A, left) Summary of representative molecular images of 360 proteins in one normal breast cell, as determined by the DAMA staining technology (22).
Each image represents the image of the corresponding protein in the tested sample. Images shown were taken with 63x objective lens. (B, right) Subcellular localization (SCL) profiles of 360 proteins in two benign and seven carcinoma derived cells, presented with a custom-defined color code scheme. Red indicates a protein is localized to the nuclei, green indicates a protein is localized to the cytoplasm, yellow indicates a protein is localized both to nuclei and in cytoplasm, and black represents an uncertain SCL for the protein.
Each column represents the SCL codes of the proteins in the same cell line, and each row represents the SCL codes of the same protein in different cell line samples.

2-3b. Summary of the funded study on "identify novel biomarkers for sDILI (severe Drug Induced Liver Injury) by using novel PSL microarray technology (was called DAMA staining technology). This grant proposal was funded by the Warner / Fermaturo research grant. Dr. Zhou's lab has studied the effects of different compounds on the expression profiles of 23 different Cytochrome P450s in HepG2 cells.

His lab has obtained 9 different compounds, 3 sDILI compounds (Ketoconazole, Troglitazone, Flutamide) and 6 non-sDILI compounds (Erythromycin freebase, Erythromycin estolate, SDS, Budesonide, Nicardipine, Olmesartan). The expression profiles of 23 different cytochrome P450s (CYPs) in the HepG2 cells treated with those compounds and controls are obtained and summarized in Fig 3. Unfortunately, no differentially expression changes for any P450 subtype proteins were observed between cells treated with different concentration (40 M and 80 M) of sDILI compounds and non-sDILI compounds.

Treatment of flutamide has been reported to increase the expression of liver cytochrome P450 1A2 (24). Therefore, his lab has determined the expression profiles of 23 different cytochrome P450s in the HepG2 cells treated with different concentration of Flutamide, (0 - 250 M) (Fig 4). No increase of cytochrome P451A2 was observed either.

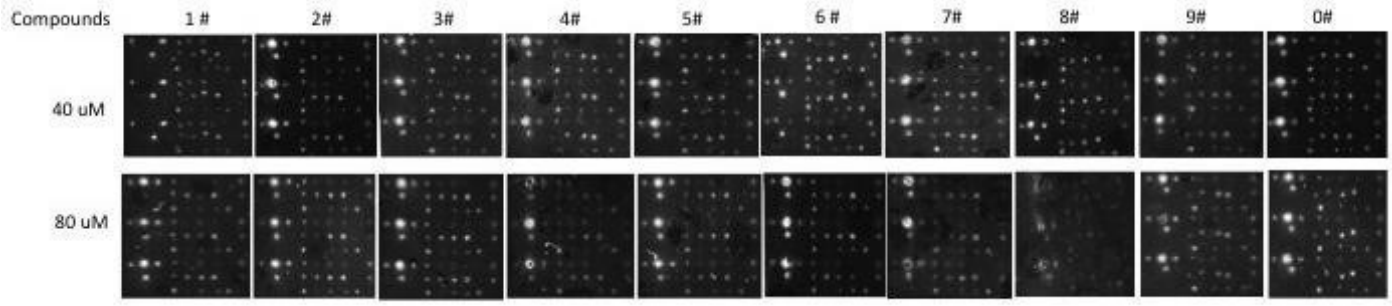


Fig. 3. The expression profiles of 23 CYPs in HepG2 cells treated with different compounds and control (#0). The expression profiles were determined in triplets.

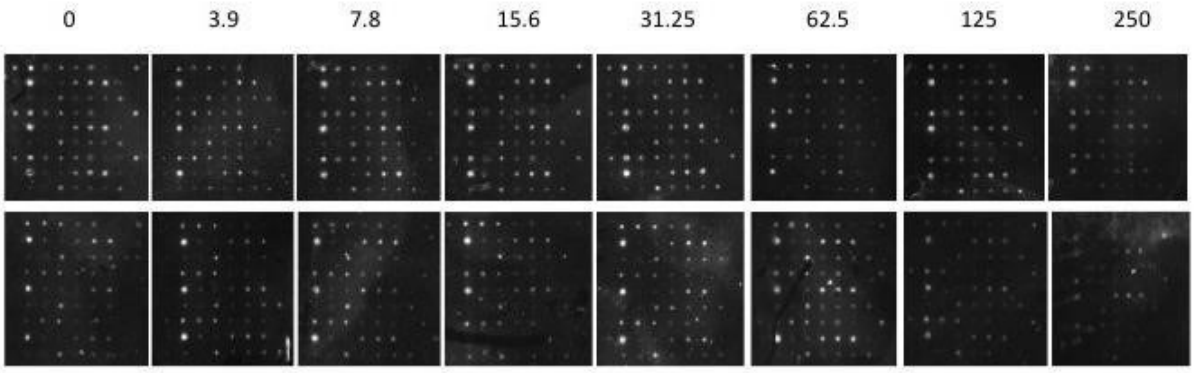


Fig. 4. The expression profiles of 23 CYPs in HepG2 cells treated with different concentration of Flutamide. The expression profiles were determined in triplets, and each experiment was repeated at least twice.

2-4. Approach:

Specific Aim 1. Determine the expression pattern of 60 surface biomarkers for human immune cells in PBMC of participants, before and after OMT.

Experimental design: This prospective, randomized, controlled trial is proposed to investigate the expression patterns of ~ 60 cell surface proteins in human immune cells, in the peripheral blood mononuclear cells (PBMC) of each participant, before and after intervention – OMT or seated control. This study will utilize participants with a history of low back pain (LBP) because several studies have demonstrated that patients with LBP have a significantly greater incidence and severity of somatic dysfunction than people without LBP (12, 26-27). Somatic dysfunction is the clinical indication of OMT, therefore participants with more somatic dysfunction may have a greater response to OMT than participants with less somatic dysfunction.

The study will be conducted over the course of one year. Forty men and women ages 20 to 65 years with at least one or more episodes of LBP in the past two weeks will be randomly assigned into one of two groups – control or OMT groups. Twenty participants will be assigned to each group. The study will begin recruitment in July 2017 pending institutional review board approval. Participants will be recruited from the surrounding Kirksville

area. Individuals who cannot tolerate OMT or have coagulation disorders will be excluded. Potential participants will be excluded from the study if they have had nonsteroidal antiinflammatory medication in the 48 hours prior to the study or if they have had corticosteroids in the 2 weeks prior to the study. Participants will be randomly assigned into two groups, control or OMT using a random number generator. Males and females will be randomized separately to ensure equal distribution into the two study groups. Demographics including sex, age, and body mass index (BMI) will be collected on all participants. All participants will receive two blood draws: first blood draw prior immediately prior to intervention and second blood draw following the intervention.

OMT intervention: The OMT treatment will begin with a brief physical examination to identify somatic dysfunction that the treating physician judges to be relevant to the individual participant's low back pain. OMT will be performed to improve the somatic dysfunctions that the treating physicians finds most relevant. The types of OMT techniques used will include muscle energy, articular, or high velocity-low amplitude (HVLA) as indicated by the physical findings and will be at the discretion of the treating physician. Additional techniques such as still, counter strain, facilitated positional release, balanced ligamentous tension, and cranial techniques may also be used at the discretion of the treating physician. The treatment will conclude with 2 minutes of pedal lymphatic pumping technique. The total treatment time will not to exceed 20 minutes. To encourage participation in the proposed study, participants who are assigned to the control group will have an opportunity to receive OMT after the second blood draw.

Control protocol: The control group will wait in another room for approximately 30 minutes.

PBMC cell isolation. PBMC cells will be isolated and prepared according to the company protocol which is summarized as below. Venous blood will be collected via venipuncture into the Vacutainer CPT tube (BD Biosciences). CPT tubes will be centrifuged at 1800 x g for 20 minutes at room temperature. After centrifugation, CPT tubes will be brought to a biological safety cabinet and carefully opened. The PBMC cells, which are in the layer just under the plasma (Figure 5), will be transferred to a 15 ml conical tube. The conical tubes will be centrifuged at 250 x g for seven minutes at room temperature. The PBMC cells will be re-suspend at 5 x 10^6 viable lymphocytes per ml in PBS buffer.

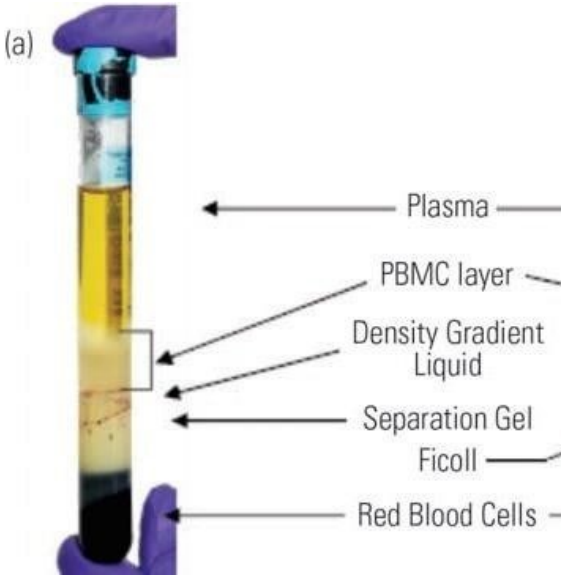


Fig 5. Typical PBMC layers after centrifugation in BD Vacutainer CPT tube

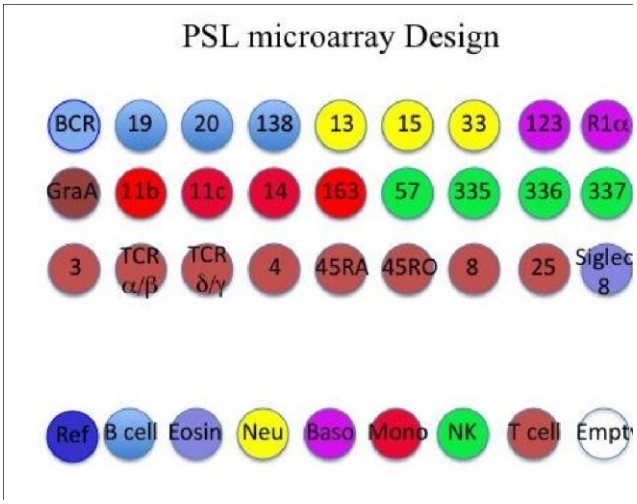


Fig 6 Diagram for the PSL microarray. Different color corresponds to different lymphocytes, # corresponding to CD#

Coverslip preparation. 100μl of the suspended PBMC cells will be added to a poly-lysine coated coverslip (22 mm x 22 mm), and dried for 10-20 minutes at room temperature. The cells attached to the coverslips will be washed three times in PBS, and fixed with 4% paraformaldehyde in PBS. The detail protocol would be modified and optimized in the experiments.

Antibody selection and microarray preparation. The goal of this project is to purchase ~60 antibodies, which are the surface biomarkers for different human immune cells. We shall determine the expression patterns of the 60 proteins in the PBMC cells from the participants, before and after the OMT treatment by using the PSL microarray. The proteins with differentially expressed level after the OMT treatment will be identified from data mining and statistical analysis.

We plan to first purchase 30 antibodies, which are the biomarkers for different human lymphocytes. The selected antibodies and their major expression in different lymphocytes are summarized in Table 1. The PSL microarrays containing those antibodies will be made according the design in figure 6 by using the purchased antibodies and the nanoplotter at ATSU, and will be used for the proposed research. Most of the 30 proteins shown in Table 1 are expressed in 1-2 kinds of different lymphocyte cells. An increase or decrease in expression of those proteins could be related to the increase or decrease of the specific types of lymphocytes, which could provide potential mechanism for OMT on immune system activity.

The second set of 30 antibodies will be selected from the ~150 surface proteins summarized in the Appendix (with their expression patterns in human immune cells) and purchased later pending the findings from the analysis of the first 30 antibodies. The samples will be saved and stored in liquid nitrogen and retested for the second set of 30 antibodies.

The long-term plan. The long-term plan of the proposal is develop a research methodology for study the effect of OMT on immune system activity. The goal of the current proposal is to generate preliminary data, so that we could apply for the NIH grant to determine the expression patterns of 150-200 cell surface biomarkers for human immune cells in the PBMC cells of the participants, before and after OMT. For this purpose, a grant application based on the preliminary data generated from the current proposal will be submitted to NIH or AOA. Additional antibodies, as those listed in the Appendix, will be purchased if funded. The microarray containing 150-200 antibodies will be made similar to those used in the publication (23), and used for the longterm research.

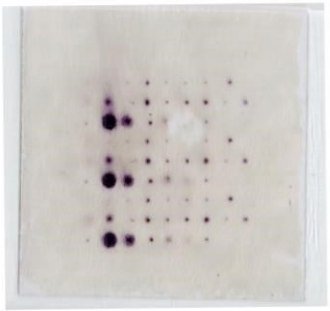
Table 1. Summary of Antibodies used for the research and their expression in immune cells

	Markers	B Cells	Myeloid and mast cells	IK ell	s T Cells

Cell Types	Antibody	Plasma CEo B Cells	lls sinophil Neutrophil Basophil Monocy	ce IK ell	s T cells CD4 T Cell CD4 memory CD8 CD8 T cell T Cells T Cell T cell
Total B cell	BCR CD19 CD20 CD138	X X X X			
Eosinophil	CD9 CD32 Siglec -8	X	X X X X		X
Neutrophil	CD13 CD15 CD33		X X X X X		X
Basophil	CD123 Fc□ R1□		X X X X		
Monocyte	CD11b CD11c CD14 CD163	X X X X	X X X X	X X	X X
NK cell	CD57 CD335 CD336			X X X	

	CD337			X							
T cell	CD3				X	X	X	X	X	X	
	TCR □□□				X		X	X	X	X	
	TCR □□□				X	X					
CD4	CD4		X				X	X			
	CD45RA	X	X				X				
	CR45RO	X	X					X			
CD8	CD8			X					X	X	
	CD25		X					X			
	Granzyme A		X							X	

Determination of the expression profiles of the lymphocyte biomarkers in the PBMC cells. We propose to use similar approach as the published one (21) (also was summarized in the preliminary studies section B3) to determine the expression profiles of the 60 lymphocyte biomarkers in the PBMC cells, before and after the OMT. In summary, the PSL microarray containing the biomarkers will be put on top of the fixed PBMC cells, and incubated for 2 hours at the room temperature. The antibodies will be delivered to the targeted PBMC cells without diffusion. The sample will be washed three times in PBS (phosphate-buffered saline) to remove the unbound antibodies. The bound antibodies will be detected by alkaline phosphatase-conjugated secondary antibodies, followed with 1-step NBT/BCIP (nitro blue tetrazolium/5-bromo- 4-chloro-3-indolyl phosphate) substrate (Pierce). Images of protein expression profiles will be scanned by Brother MFC-7365DN scanner, and processed by Photoshop for intensity integration. The expression profile of 23 Cytochrome P450 in HepG2 cells is as shown in Figure 8. The expression profile of the lymphocyte biomarkers in the PBMC samples of the participates would be similar to those (Fig 8)



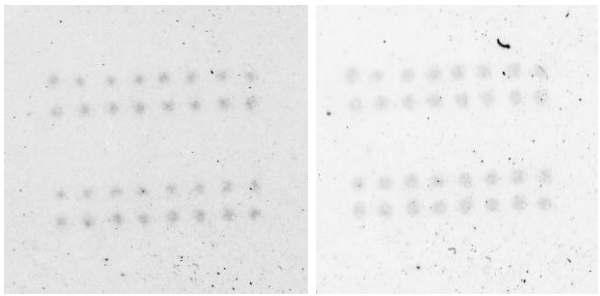


Figure 9. The bound antibodies (Actin, row 1/3; GADPH row 2/4) detected by with DyLight 680 (right) and DyLight 800 (left) triplicate.

Figure 8. The expression profile of 23 CYPs in HepG2 cells are shown in antibodies

The biomarker expression profiles in the PBMC cells can also be detected by the fluorescence-conjugated secondary antibodies. A protocol has been developed to detect the bound antibodies at 680nm and 800nm, by using the DyLight-680 conjugated secondary antibody and the DyLight-800 conjugated antibody (Rockland Antibody), respectively (Figure 9). The images show the bound antibodies for Actin (row 1 and 3), and for GADPH (row 2 and 4) on HTB129 cells, a breast cancer cell, obtained by Odyssey Imaging Systems. Both methods will be used to analyze biomarker expression patterns in the proposed study to identify the more effective method for future experiments.

Specific Aim 2. Identify those specific proteins whose expression patterns have changed in the greatest number of participants after OMT.

Sample Size. To estimate the sample size needed to detect a mean difference of 0.5 in ratio of intensity (After/Before) between the OMT and in the control group, standard deviations of intensity (After/Before) in the two groups were estimated using PSL microarray staining, by using the data similar to the images shown in Figure 4. The data integration was carried out for each image by using the ScanAlyze in Image J, the intensity integration programs for DNA and protein microarray analysis. As summarized in Table 2, the mean ratio in the Control group is 1.32 with standard deviation of 0.34 and mean ratio in the Intervention group is 1.42 with standard deviation of 0.32 (28). Those data have been used to calculate the sample size needed for the proposed study as described below.

Table 2 Summary data from PSL Microarray Analysis

Group	# Obs	Mean	Std Dev	Median
Control	4	1.32	0.34	1.20
Intervention	6	1.42	0.32	1.43

Group sample size calculation. Sample size calculations based on the data summarized in Table 2 reveal that recruiting a total 22 participants, 11 in the OMT group and 11 in the control group, will allow us to detect a difference of 0.5 in intensity (After/Before) in the two groups with a power of 0.92 at a significance level of 0.05, using two sample *t*-tests with unequal variances. We propose to use 40 patients for the experiments, 20 in the OMT group, and 20 in the control group. Therefore, we should have an adequately powered study.

Data Extraction and data analysis. The expression profiles of 60 biomarkers in the PBMC samples obtained by the PSL microarray staining, will be extracted by the program ScanAlyze of ImageJ, from the images similar to the above figures. The ratio change of every biomarker protein in PBMC samples, before and after the OMT will be analyzed by DAMAPEP, a software developed at the PI's lab to retrieve, normalize, and scale the data from

the exported intensity integration files. The mean change in intensity (After/Before) of biomarker expression for each of the 60 biomarkers will be compared between the OMT and control group using two sample *t*-test and biomarkers whose expression has changed significantly between the two groups will be identified. If needed, modification on published data analysis protocol and software in the paper (21) will be carried out for the project in order to identify the proteins with statistically meaningful expression change between the OMT group and the control group. The comparison would eliminate and normalize the expression change of biomarkers due to fluctuation and personal variation, and identify those differentially changed biomarkers due to the OMT.

Potential Limitations: The purposed study is investigating the impact of OMT on immune cell biomarkers. OMT can be accomplished with a wide variety of techniques. Most studies investigating the effect of OMT on immune cells use lymphatic pump OMT techniques. Other types of OMT techniques may not have the same effect. The purposed study will use a wide variety of OMT techniques consistent with what a patient with low back pain would receive in a clinical setting and conclude the treatment with a lymphatic pump OMT technique. Therefore, the current design will not allow for conclusions about the effect of any specific type of technique. Future studies will investigate the effect of different types of OMT techniques on the immune cell biomarkers.

Another potential limitation is that the method could be not sensitive enough to detect changes in biomarkers after 30 minutes post OMT. If no changes are seen during the initial investigation we will modify the protocol to increase the sensitivity of the technology or try different OMT techniques.

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4. PROTECTION OF HUMAN SUBJECTS

4.1 DETAILED DESCRIPTION OF THE PROPOSED INVOLVEMENT OF HUMAN SUBJECTS

The proposed study will involve 40 participants receiving two venous blood draws; one blood draw immediately before and one immediately after the intervention. Twenty participants will receive OMT for up to twenty minutes as part of the protocol with the remaining 20 participants receiving OMT if desired.

4.2 SOURCES OF RESEARCH MATERIAL

a. Demographic information and past medical history. Basic demographic information and past medical history including frequency of low back pain will be collected from participants.

b. Surface protein expression of 60 cell surface proteins in human immune cells, in the peripheral blood mononuclear cells (PBMC) of each participant will be analyzed from peripheral blood before and after intervention.

4.3 RECRUITMENT AND INFORMED CONSENT

Participants will be recruited from the surrounding Kirksville area. A research investigator or their assistant will review the study with a potential participant, determine the participant's eligibility, and obtain informed consent using an informed consent document that will be reviewed and approved by the Institutional Review Board for ATSU-Kirksville pending study funding. The study will be registered with ClinicalTrials.gov.

4.4 POTENTIAL RISKS

4.4.1 PHYSICAL RISKS

The physical risks to the participant include the OMT and venous blood draws. The participant may feel some post-treatment soreness from the OMT and they may experience bruising from the blood draws. 4.4.2 PSYCHOLOGICAL RISKS

There are no psychological risks to the participant from the OMT or blood draws.

4.4.3 SOCIAL RISKS

There are no social risks to the participant from the OMT or blood draws. The health information obtained during the study will be kept in a locked file cabinet and information used in this study will be de-identified prior to data analysis and results dissemination.

4.4.4 ECONOMIC RISKS

There are no economic risks to the participant from the OMT or blood draws.

4.4.5 LEGAL RISKS

There are no legal risks to the participant from the OMT or blood draws.

4.5 ALTERNATIVE PROCEDURES OR THERAPIES

The participant may choose not to participate in the study.

4.6 PROCEDURES FOR PROTECTING AGAINST RISKS

Any residual soreness that a participant experiences from the OMT or blood draws is likely to be insignificant, though the participant may contact the Dr Karen Snider for options regarding care. The results of this research may be published; however, neither participants' names nor identities will be revealed, and records will remain confidential. Confidentiality will be maintained by using coded identification numbers on all forms. All consent forms and study data will be kept and treated as personal medical records. Only the investigators and study personnel will have access to this information. As required by Federal law, all personal health information will be maintained in accordance with the Health Insurance and Portability and Accountability Act (HIPAA) to ensure privacy.

4.7 RISKS AND BENEFITS TO SUBJECTS

4.7.1 POSSIBLE BENEFITS TO SUBJECT

There are minimal risks to the participants. The minimal risks to the participants are reasonable compared to the knowledge to be gained from the proposed study and the benefit to the osteopathic profession. The participants may benefit from the OMT with regards to their low back pain, though treatment for low back pain is not an intended outcome.

4.7.2 POSSIBLE BENEFITS TO SOCIETY

The benefit to society will be the information obtained regarding the possible effects of OMT on immune biomarker expression. This knowledge can be integrated into the training of future osteopathic physicians.

5. DATA SAFETY MONITORING PLAN

Not applicable.

6 INCLUSION OF WOMEN AND MINORITIES

Women and minorities are encouraged to participate in the proposed study.

7. TARGETED/PLANNED ENROLLMENT TABLE

Time	# patients
Q4, 2017	10
Q2, 2018	30
Total	40

The first set of 10 participants samples will be saved and retested with the second set of 30 antibodies.

8. INCLUSION OF CHILDREN

Children are excluded from this study.

9. VERTEBRATE ANIMALS

Not applicable

10. SELECT AGENT RESEARCH

Not applicable

11. MULTIPLE PD/PI LEADERSHIP PLAN

Not applicable

12. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

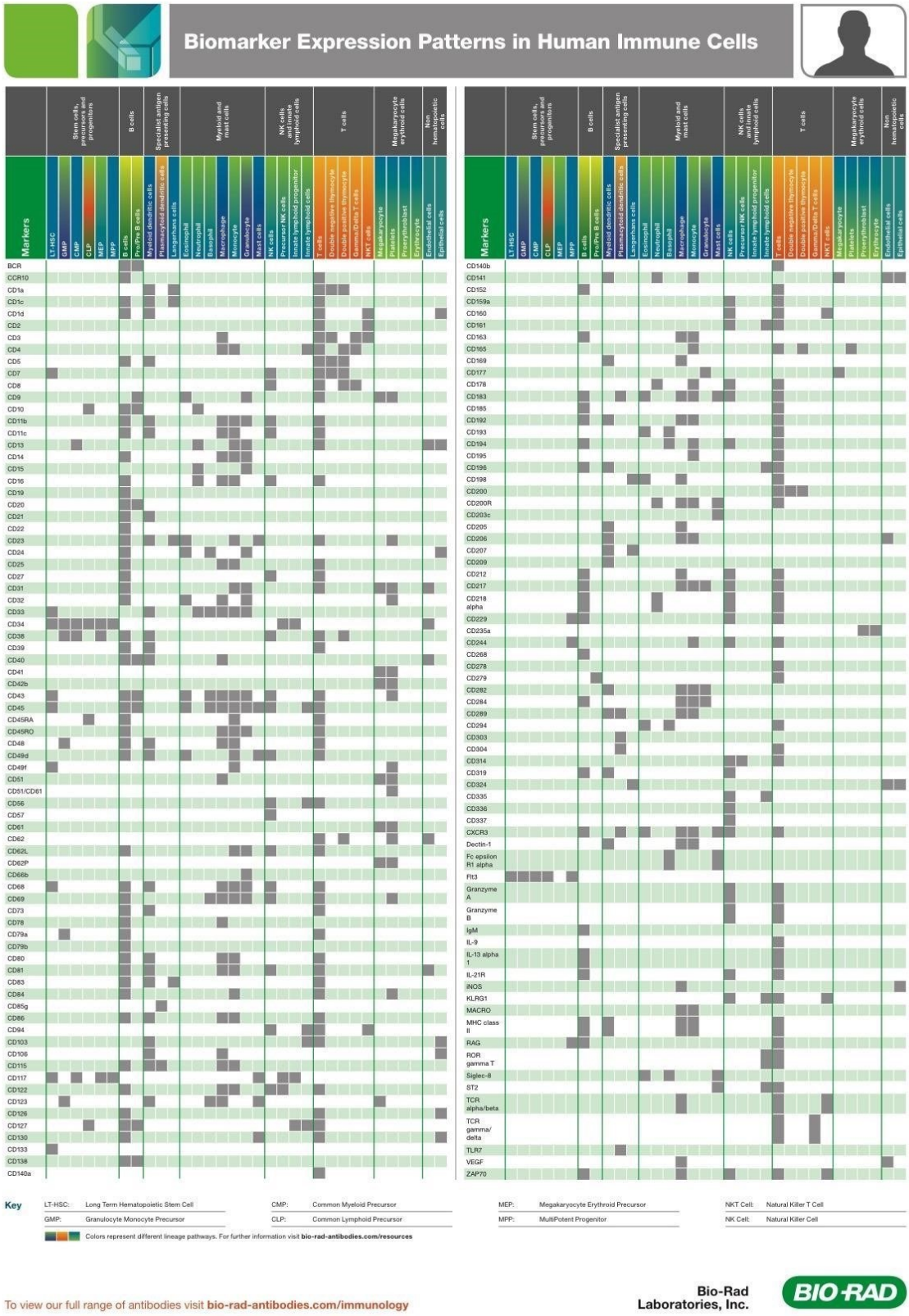
Not applicable

13. LETTERS OF SUPPORT (E.G., CONSULTANTS)

Not applicable

14. RESOURCE SHARING PLAN

The research findings from this study are expected to be published in JAOA. Travel funds for presentation of the study findings have been requested to attend OMED in 2018.


Biomarker Expression Patterns in Human Immune Cells
Markers
Stem cells, primitive and progenitors
B cells
Specialist antigen processing cells
Myeloid and mast cells
NK cells and innate lymphoid cells
T cells
Megakaryocyte erythroid cells
Non hematopoietic cells
LT-HSC
GMP
CMP
CLP
MEP
MPP
B cells
Pro/Pre B cells
Myeloid dendritic cells
Plasmacytoid dendritic cells
Langerhans cells
Eosinophil
Neutrophil
Basophil
Macrophage
Monocyte
Granulocyte
Mast cells
NK cells
Precursor NK cells
Innate lymphoid progenitor/ ILC precursor
Innate lymphoid cells
T cells
Double negative thymocyte
Double positive thymocyte
Gamma/Delta T cells
NKT cells
Megakaryocyte
Platelets
Proerythroblast
Erythrocyte
Endothelial cells
Epithelial cells
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CCR10
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CD1c
CD1d
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CXCR3
Dectin-1
Fc epsilon R1 alpha
Flt3
Granzyme A
Granzyme B
IgM
IL-9
IL-13 alpha 1
IL-21R
iNOS
KLRG1
MACRO
MHC class II
RAG
ROR gamma T
Siglec-8
ST2
TCR alpha/beta
TCR gamma/ delta
TLR7
VEGF
ZAP70
Key
LT-HSC: Long Term Hematopoietic Stem Cell
GMP: Granulocyte Monocyte Precursor
CMP: Common Myeloid Precursor
CLP: Common Lymphoid Precursor
MEP: Megakaryocyte Erythroid Precursor
MPP: MultiPotent Progenitor
NKT Cell: Natural Killer T Cell
NK Cell: Natural Killer Cell
Colors represent different lineage pathways. For further information visit bio-rad-antibodies.com/resources
To view our full range of antibodies visit bio-rad-antibodies.com/immunology
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Appendix