July 8, 2021.

Proposal for:

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Protocol 181-002 R3. Clinical proof-of-concept study on rapid immune modulating effects.

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1 Purpose

The goal for this clinical proof-of-concept study is to compare acute immune effects of a novel nutraceutical blend to a placebo. This data is important to verify immune related effects.

2 Background

Unigen, Inc. focuses on identifying and studying the unique bioactive natural products of medicinal botanicals and then developing them into proprietary standardized extracts for use as novel ingredients in cosmeceutical, and nutraceutical products.

Based on the market need for products that support immune health, there is a high interest in science-based natural products with documented rapid effects on the human immune system.

This clinical proof-of-concept study aims at documenting acute effects of consuming a test product through evaluation of immune cell activation, cell trafficking, and cytokine changes to pro- and anti-inflammatory cytokines, antiviral peptides, and restorative growth factors.

Data on immune cell trafficking and surveillance will be collected. The testing will show whether consuming the novel blend leads to a rapid change in the alertness of the immune system to search for and attempt to eliminate microbial invaders, and to collaborate effectively between immune cell types.

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3 Study

3.1 Clinical Study Design

For this clinical study, healthy adults will be tested following an **established placebo-controlled, randomized, double-blinded, cross-over study design.** Specifically, the study design has been used in previous clinical studies on immune modulating products including the yeast-based fermentate Epicor,[i] a bovine colostrum-based peptide- and oligosaccharide-rich extract Immunel,[ii] the algae-based extract for stem cell support StemEnhance,[iii] and an aloe-based folk medicine formulation from Madagascar.[iv] Recently, the NIS Labs' team also published on changes to lymphocyte trafficking, specifically stem cell subsets, using this study design when consuming placebo versus a polyphenol-rich extract from Sea Buckthorn from Tibet.[v]

Below is a simplified diagram illustrating the involvement of each participant, where all study participants will be tested on two different clinic days.

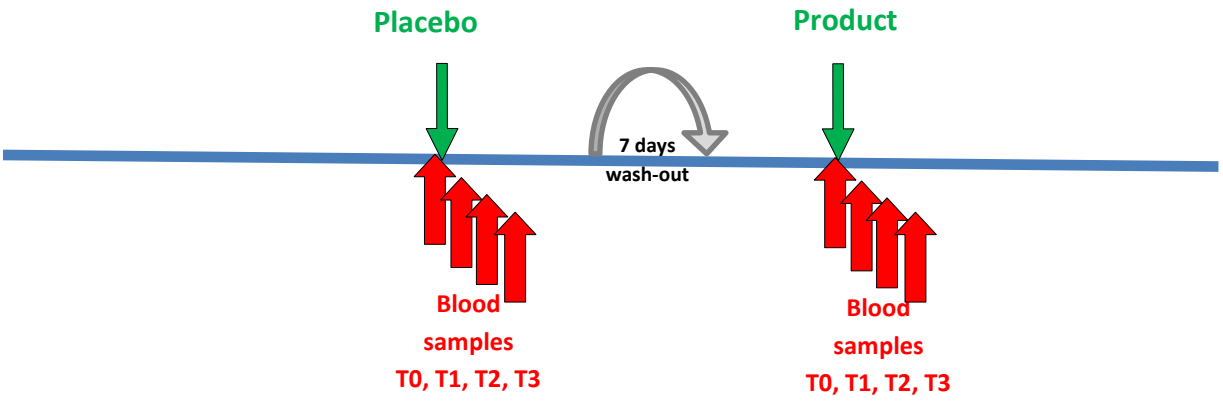

Figure 1. Diagram showing the involvement of each participant. Study participants will be tested on two different clinic days. The sequence of test products shown here is an example only, since the sequence will be randomized.

The shown sequences are examples only; the sequence in which each person will consume active test product or placebo will be randomized.

The test parameters we will be evaluating do not necessarily stay constant, even over a few hours, since they are related to people's metabolism, individual circadian rhythms, and other normal physiological parameters. Therefore, studies of this nature must include a placebo test day, allowing *within-subject* analysis of changes between the test days for each person. This very much strengthens the data analysis from this type of pilot study. In the absence of a

placebo test day, we consider the data inconclusive since changes cannot be interpreted as being related to product intake.

In light of previous data on products such as Epicor[i] and Immunel,[ii] some differences were seen at 1 versus 2 hours, and it is ideal to perform testing at both time points. In addition, data will also be collected after 3 hours.

3.2 Outcome Measures

Primary outcome measure: Immune surveillance. Trafficking and activation of immune cells in vitro.

Figure 2. List of advantages pertaining to outcomes from the study design proposed here. Comparison to a long-term parallel arm cold- and flu-study design.

4 Study Population

4.1 Study Participants

12 healthy people of either gender will be enrolled after IRB-approved, written informed consent. The inclusion/exclusion profile for a study of this nature is not trivial, and each potential study participant is carefully evaluated prior to enrollment. To minimize anticipatory stress and apprehension during initial clinic visits for the study, each study participant must either have participated in previous studies at our facility or must attend a visit where we go through the study procedures, prior to a clinical study day.

4.2 Inclusion Criteria

- Healthy adults;
- Age 18-75 years (inclusive);
- BMI between 18.0 and 34.9 (inclusive);

- Veins easy to see in one or both arms (to allow for the multiple blood draws);
- Willing to comply with study procedures, including:
    - Maintaining a consistent diet and lifestyle routine throughout the study,
    - Consistent habit of bland breakfasts on days of clinic visits,
    - Abstaining from exercising and nutritional supplements on the morning of a study visit,
    - Abstaining from use of coffee, tea, and soft drinks for at least one hour prior to a clinic visit;
    - Abstaining from music, candy, gum, computer/cell phone use, during clinic visits.

4.3 Exclusion Criteria

- Previous major gastrointestinal surgery (absorption of test product may be altered) (minor surgery not a problem, including previous removal of appendix and gall bladder);
- Taking anti-inflammatory medications on a daily basis;
- Currently experiencing intense stressful events/ life changes;
- Currently in intensive athletic training (such as marathon runners);
- Cancer during past 12 months;
- Chemotherapy during past 12 months;
- Currently treated with immune suppressant medication;
- Diagnosed with autoimmune disorders e.g. systemic lupus erythematosus, hemolytic anemia;
- Donation of blood during the study or within the 4 weeks prior to study start;
- Have received a cortisone shot within past 12 weeks;
- Immunization during last month;
- Currently taking antipsychotic hypnotic, or anti-depressant prescription medication;
- Ongoing acute infections (including teeth, sinus, ear, etc);
- Participation in another clinical trial study during this trial, involving an investigational product or lifestyle change;
- An unusual sleep routine (examples: working graveyard shift, irregular routine with frequent late nights, studying, partying);
- Unwilling to maintain a constant intake of supplements over the duration of the study;
- Anxiety about having blood drawn;

- Women of childbearing potential: Pregnant, nursing, or trying to become pregnant;
- Known food allergies related to ingredients in active test product or placebo.

Prescription medication will be evaluated on a case-by-case basis.

4.4 Consumable Test Products

Test products will be provided by study sponsor. Placebo will either be produced by sponsor or NIS Labs, and will consist of rice flour encapsulated in veggie caps.

On each clinic day, immediately after the baseline blood draw, participants will be given a single dose of either the active test product or a placebo, in the presence of the clinic staff. Participants will consume the capsules with water and a few bland soda crackers to stimulate digestive function.

4.1 Study environment

The study of acute changes to levels, activation status, and functionality of immune cells is not trivial. All test parameters undergo circadian changes, and are negatively affected by stress, adrenaline, lack of sleep, and recent illness. The study participants will be instructed to call and reschedule a certain clinic day if they feel that any of these things are reasons to do so.

Also, the study environment is kept controlled for stressors. Cell phones are turned off at entry to clinic. Clinic phones and door chimes are off. Sensory input such as music, coffee/food smells, noises, etc., will be eliminated or kept to an absolute minimum. In order to keep volunteers in a state of mind we refer to as 'perpetually bored', but not falling asleep, they are offered a choice of light reading, crossword puzzles, cards to play solitaire, or DVDs with science lectures.

Upon arrival on the morning of each clinic day, participants will rest quietly for 1 hour prior to baseline blood draw. This resting period is crucial to gain representative baseline data. During this time, questionnaires will be completed to monitor previous meals, snacks, exercise, stressors, and recent sickness. Then a product will be fed to the participants. Two more blood samples will be drawn at 1, 2, and 3 hours after consumption.

4.2 Study Procedures

4.2.1 Explanation of the proposed clinical study procedures

In a clinical trial to monitor immune activating events, we expect a cascade of events, starting by activation of immune cells in the gut, systemic changes to cytokine levels, changes to immune cell trafficking (enhanced immune surveillance), followed by immune surveillance in tissue throughout the body, and possibly re-entry of activated immune cells back into the blood circulation.

Blood samples offer a convenient window into the immune events happening after a product is consumed. We do not have convenient windows into what may happen at the initial gut activation, but we envision this is similar to events in vitro. We do not have windows into tissue and thus cannot monitor downstream events after immune cells migrate from blood into tissue to scavenge for microbial invaders and perform innate and adaptive types of immune responses. Therefore, we mimic this by taking some of the blood samples and challenging the immune cells ex vivo (outside the body) with microbial mimetics.

The testing described in **Section 4.3.1** aims to monitor rapid changes in the types and activation status of immune cells seen in the blood circulation. Increases versus decreases in numbers of immune cells in the blood is a measure of cellular trafficking in and out of the blood stream. We are looking for subtle events, where any systematic changes observed in a majority of the study participants after consuming the same test product suggests immune activating events are induced. This is a good indication that a product has triggered increased immune awareness.

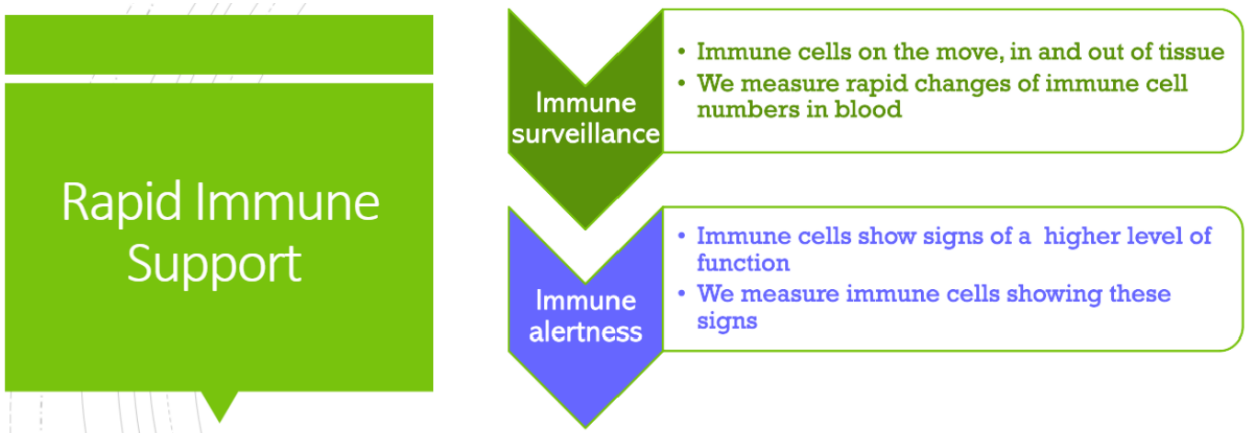

Figure 3. Diagram showing the outcome measures with a general explanation of the test methods.

4.2.2 Blood draws

For each blood draw, we will obtain 1 EDTA vial for a total of 6 mL blood per blood draw. The EDTA blood will be kept refrigerated and staining for flow cytometry will be initiated within 2 hours of drawing the blood.

- A portion of the EDTA blood will be used for the assays described below:
  - **Immune surveillance:**
    - Evaluation of absolute numbers of NK, NKT, T cells, nonNK non-T cells, and monocytes in the blood samples, reflecting changes in immune cell numbers, reflecting immune cell movements in and out of the blood stream (surveillance). The markers include CD3, CD56, and CD57.
    - Immune cell activation status: Evaluation of immune cell activation in vivo: Flow cytometry to evaluate effects on immune cell activation status, using the two activation markers CD25 (the IL-2 receptor) and CD69 (an antigen directly involved in NK cell function).
    - T and B cell subsets and CD45 isoform expression.
    - Gamma/delta T cells and activation status.
  - **Plasma banking:** The remaining EDTA blood will be centrifuged, and plasma will be harvested and aliquoted before freezing at -80oC.
    - The plasma may later be used to test for cytokine profile, reflecting rapid changes to cytokine levels in each study participant.
    - Plasma aliquots will be stored for up to 3 months after the clinical phase is completed, to allow Sponsor to make decisions on such blood tests. After that time, a nominal storage fee will be implemented.

4.3 Testing - Immune cell trafficking and status of alertness

The analysis allows us to detect if consumption of a test product leads to rapid changes in cell numbers in the circulation, and/or activates cells in vivo. Freshly drawn blood samples are used for the testing of changes in immune cell numbers and activation status. The cells from each blood draw are assayed in triplicate.

There will be 3 panels in parallel:

1. NK/T cell panel: CD3, CD56, CD57, CD25, CD69.
2. T/B cell panel with CD45 isoform expression: CD4, CD8, CD19, CD45Ra, CD45R0.
3. Gamma/Delta T cell panel: γδ T Cell Receptor, CD5, CD25, CD56, CD69.

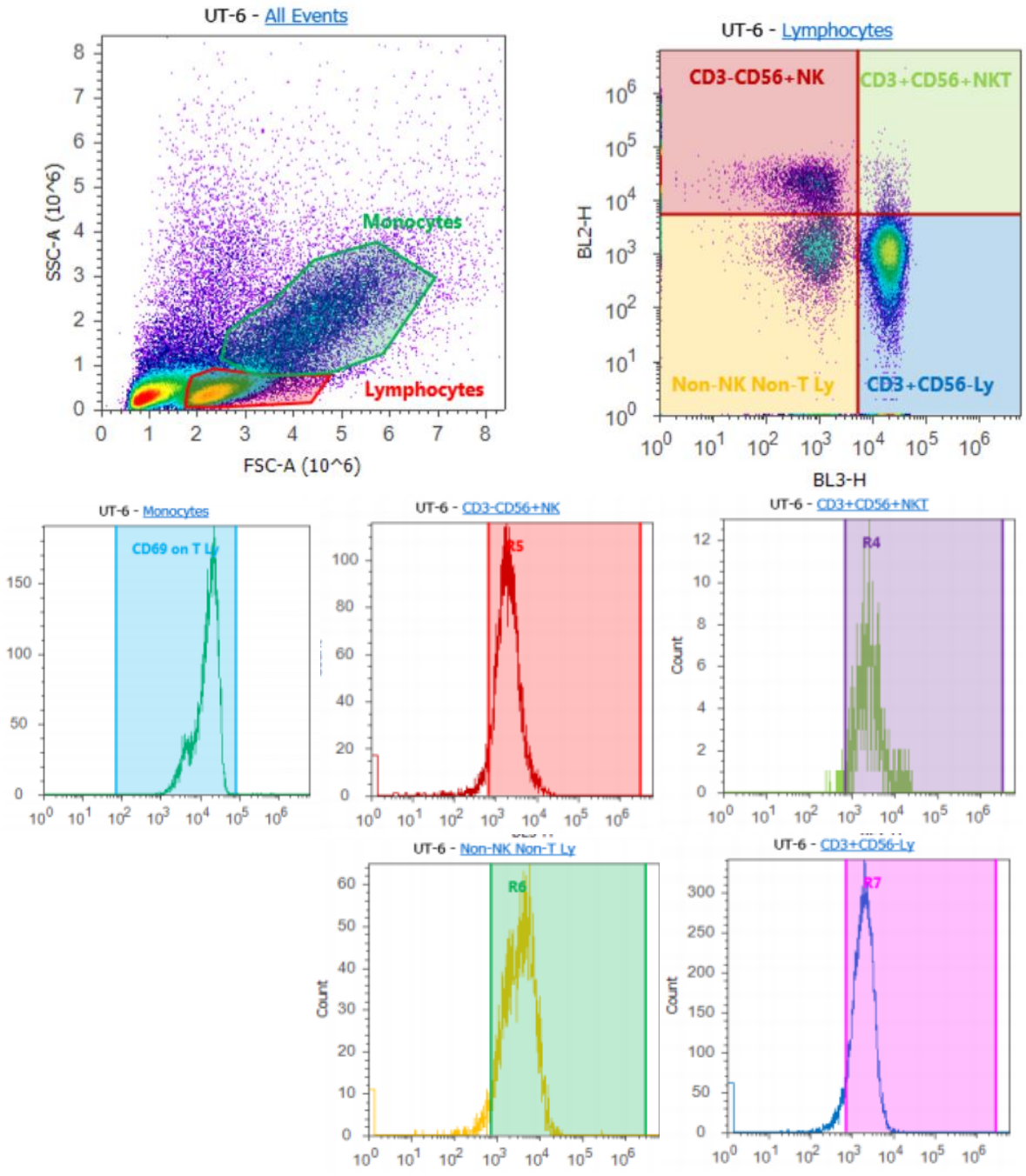

Figure 4. Flow cytometry data showing gates for lymphocytes, monocytes, and the four subsets of lymphocytes, allowing analysis of CD69 expression.

4.3.1 NK/T cell panel

Cells are stained with the T cell marker CD3 and the CD56 and CD57 markers, as well as the two activation markers CD69 and the interleukin-2 receptor CD25. This allows analysis of numbers of the following types of immune cells in the blood circulation at each time point in the study:

- CD3-negative, CD56-positive **NK cells**;
- CD3+ CD56+ **NKT cells**;
- CD3+ CD56- **T lymphocytes**;
- CD3-CD56- **non-NK, non-T lymphocytes**;
- CD3-CD57+ **NK cells**
- CD3- CD56+CD57+ **NK cells**
- **Monocytes** (identified by forward/side scatter profile);

During analysis, expression levels will be determined for the activation molecule **CD69** and growth factor receptor **CD25** on the surface of the cell populations listed above.

Note: Immune surveillance involves the constant recirculation of lymphocyte subsets, including NK and T cells. The trafficking shows a distinct circadian rhythm and is affected by a person's metabolic state. When comparing the acute effects of a consumable immune modulating product on immune surveillance, it is important to have a placebo control test day, to account for a given person's normal circadian changes at the time of the day of testing.

Interpretation: Such rapid changes suggest that consumption of a test product transmits a signal to immune cells to enhance the surveillance of the body for microbial invaders (sending more police patrols on the streets).

4.3.2 T/B cell panel

Cells are stained with the T cell markers CD4 and CD8, the B cell marker CD19, and co-stained with monoclonal antibodies towards CD45Ra and CD45R0 isoforms.[vi] CD45Ra is expressed on naïve T cells and resting B cells. CD45R0 is expressed on memory T cells and recently activated B cells. Cells expressing both isoforms have recently been through an immune activation event. This allows analysis of numbers of the following types of immune cells in the blood circulation at each time point in the study:

- CD4 T lymphocytes
- CD8 T lymphocytes
- CD19 B lymphocytes

Each type of lymphocyte will be analyzed for:

- CD45RA expression
- CD45Ra and CD45R0 co-expression
- CD45R0 expression

4.3.3 Gamma/Delta T cell panel with activation markers

Cells are stained with a monoclonal antibody towards the CD3/ γδ T Cell Receptor,[vii] [viii] and co-stained with CD5. The cells are also stained for CD56 which may be expressed on some γδ T cells.[ix] The 2 activation markers CD69 and CD25 will also be used. This allows analysis of numbers of the following types of immune cells in the blood circulation at each time point in the study:

Add-on panel for numbers of gamma-delta (γδ) T cells (γδTCR+ CD5-):

- CD3/ γδ T Cell Receptor+ CD5- CD56+
- CD3/ γδ T Cell Receptor+ CD5- CD56-
- CD3/ γδ T Cell Receptor+ CD5- CD69+
- CD3/ γδ T Cell Receptor+ CD5- CD25+

5 Budget

The budget for the study described here is **$149,800.**

This budget includes the protocol writing, IRB application and fees, clinical planning, clinic phase, lab tests for the flow cytometry panels, data analysis, and report writing.

Invoicing Schedule:

Down-payment, 70% of previous budget for Protocol version R2	*$85,817.90 (already paid)*
Add-on down-payment for Protocol version R3	*$19,042.10*
Payment upon completion of clinical phase 20%	*$29,960.00*
Payment upon reporting 10%	*$14,980.00*

6 References

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