



Toxicology Study No. S.0039820-16, November 2017
Toxicology Directorate

Human Cell Line Activation Test of the Novel Energetics 2,6-pyrazinediamine
3,5-dinitro 1 -oxide (LLM-105) and 2,4,6-trinitro-3-bromoanisole (TNBA)

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Good Laboratory Practice Compliance Statement

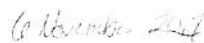
The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations (CFR), Part 792, Good Laboratory Practice Standards, except for the following:

1. The test article characterization (purity) was conducted by the manufacturer and it is not known whether the testing was done in compliance with the above regulation.
2. Due to time constraints, the method of analysis for these compounds could not be validated by the Laboratory Sciences Portfolio (LAB) prior to the study start in compliance with GLP requirements. Because of this the dosing solutions used for all strains were verified after being frozen (at - 80 degrees C) until the method could be validated by the LAB after the study was completed.
3. Due to calibration error, the balance used for verifying pipette function was flagged as "in need of repair". Four years of weight set verification data logs were reviewed and the balance is operable and functioning properly. The balance was continued in use and weight set verification was performed prior to each day's use.
4. Due to error on the part of the PI, the final dilution of the empirical starting stock for both LLM-105 and TNBA were not collected until 24-hours after the test was set up for LLM-105 test #2 and TNBA test #1. As results across all tests were similar, it is expected that the starting concentrations were as expected for these missed collections.
5. At the time the study was conducted, there was not an APHC approved GLP protocol. The method has been validated and a formal report documenting the protocol and local changes has been completed. The current study followed the methodology as described in the formal report and all steps were documented and are found in the archives.

No deviations from the aforementioned regulation affected the quality or integrity of the study or the interpretation of the results.



Emily N. Reinke, Ph.D.
Study Director
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Date

Table of Contents

	<u>Page</u>
1 Summary	1
1.1 Overview.....	1
1.2 Purpose	1
1.3 Conclusions	1
1.4 Recommendations.....	2
2 References	2
3 Authority	2
4 Background	2
5 Materials and Methods	4
5.1 Materials	4
5.1.1 Test Substance.....	4
5.1.2 Cell Line, Chemicals and Reagents	4
5.1.3 Equipment.....	5
5.1.4 Quality Assurance	5
5.2 Methods.....	5
5.2.1 Buffers	5
5.2.2 Tissue Culture.....	5
5.2.3 Reactivity Check.....	6
5.2.4 Range Finding	6
5.2.5 h-CLAT Test.....	7
5.2.6 Antibody Staining and Flow Cytometry	8
5.2.7 Data Analysis	8
5.2.8 Criteria for a Valid Assay	9
6 Results and Discussion	9
6.1 Reactivity Check.....	9
6.2 Range Finding Assay	10
6.3 CD54 and CD86 Expression Following Compound Exposure in THP-1 Cells.....	10
6.4 Criteria for a Valid Assay	11
7 Conclusions	11
8 Recommendations	11

Page

Appendices

A	References	A-1
B	Quality Assurance Statement.....	B-1
C	Archives and Study Personnel	C-1
D	Reagents	D-1
E	Raw Data and Data Analysis.....	E-1

Figures

1	Molecular Structure of the Compounds.....	4
2	Example Results Range Finding PI Assay.....	7
3	Example Dose Response Curve for CD86.....	9

Tables

1	Antibody Concentration	8
2	Results of Reactivity Check.....	10
3	Results of Range Finding	10
4	Results of Compound Analysis	11

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1 Summary

1.1 Overview

The energetic and toxicological properties of 2,6-pyrazinediamine 3,5-dinitro-1 -oxide (LLM-105) and 2,4,6-trinitro-3-bromoanisole (TNBA) are being determined to support an evaluation of LLM-105 and TNBA as replacements for energetics in current use, such as such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and trinitrotoluene (TNT). The following study assessed the skin sensitization potential of LLM-105 and TNBA through the human cell line activation test (h-CLAT), an *in vitro* approach to assess activation of dendritic cells, a critical step in the elicitation of a sensitizing response. Data from this study are used to assist in making environment and health-based decisions regarding the design and selection of formulas and materials for further development of new munition compounds.

1.2 Purpose

The purpose of this study is to provide environmental and occupational health information on new or replacement energetic compounds for military use. This information is critical to the research, development, testing, and evaluation (RDT&E) of munition formulation alternatives. This study addresses, in part, the environmental safety and occupational health (ESOH) requirements outlined in Department of the Army (DA) Regulation 200-1[1]; DA Regulation 40-5 [2]; and DA Regulation 70-1 [3]; Department of Defense Instruction 4715.4 [4]; and Army Environmental Research and Technology Assessment (AERTA requirement PP-3-02-05 [5], Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces. This program is under the direction of the Department of Defense Strategic Environmental Research and Development Program (SERDP).

Research, development, testing, training, and use of substances potentially less hazardous to human health and the environment is vital to the readiness of the U.S. military. Safeguarding the health of Soldiers, civilians, and the environment requires an assessment of alternatives before they are fielded. Continuous assessments begun early in the RDT&E process can save significant time and effort during RDT&E, as well as over the life cycle of the items developed. Residues of pyrotechnics, propellants, explosives, and incendiaries have been found in soil, air, surface, and groundwater samples, creating environmental problems and interfering with training activities.

The Department of Defense is identifying replacements for substances causing environmental and/or occupational risks to health. The purpose of this toxicology study was to examine the skin sensitization potential of LLM-105 and TNBA using the h-CLAT assay, and to conduct the assay consistent with Good Laboratory Practice (GLP) Standard Regulations.

1.3 Conclusions

Both LLM-105 and TNBA were found to elicit positive reactions for both sensitization markers in the THP-1 monocytic leukemia cell line, a dendritic cell surrogate. Both CD54 and CD86 expression levels were increased as a result of 24-hour exposure to LLM-105 and TNBA. Thus, both compounds are skin sensitizers according to the h-CLAT test.

1.4 Recommendations

LLM-105 and TNBA appear to be skin sensitizers upon analysis with the h-CLAT skin sensitizing when combined with Quantitative-Structure Activity Relationship (QSAR) analysis [6]. Further *in vitro* or *in vivo* testing is recommended to more definitively determine the sensitizing potential of these compounds. The h-CLAT is one of many non-animal skin sensitizing tests, and it comprises part of an integrated testing strategy with two other *in vitro* approaches, the DPRA and the LuSens assay [7-11]. A comprehensive assessment of skin sensitization potential requires results from all three assays along with specific *in silico* analysis provides a more robust estimation of skin sensitization than h-CLAT alone [12]. As testing has only occurred with the h-CLAT, it is not yet possible to provide a definitive response as to the sensitization potential. Testing with the direct peptide reactivity assay will be conducted, and the LuSens test is currently under validation by APHC and should be available to complete the *in vitro* tests necessary for analysis. However, the robustness of the h-CLAT assay provides a strong indication that both LLM-105 and TNBA are skin sensitizers and appropriate precautions should be taken when handling the material.

2 References

See Appendix A for list of references

3 Authority

Military Interdepartmental Purchase Request No. W74RDV5291790. This technical report addresses, in part, the environment, safety and occupational health (ESOH) requirements outlined in Department of Defense Instruction (DODI) 4715.4 [13], Department of the Army Regulation (AR) 200-1, Environmental Protection and Enhancement[14]; AR 40-5, Preventive Medicine [15]; and AR 70-1, Army Acquisition Policy [16]; Department of Defense Instruction 4715.4, Pollution Prevention [13]; and Army Environmental Research and Technology Assessment Requirement PP-3-02-05, Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces . It was conducted as part of an on-going effort by Strategic Environmental Research and Development Program.

4 Background

Current regulations require the assessment of human health and environmental effects arising from exposure to substances in soil, surface water, and groundwater. Applied after an item has been fielded, these assessments can reveal the existence of adverse environmental and human health effects that must be addressed, often at substantial cost. It is more efficient to begin the assessment of exposure, effects, and environmental transport of military-related compounds/substances early in the RDT&E process to avoid unnecessary costs, conserve physical resources, and sustain the health of those potentially exposed. The SERDP has been dedicated to finding replacements for substances known to cause environmental and/or occupational risks to health or developing less hazardous new explosives. A goal of this program is to investigate these new compounds with operational and/or environment, safety, and occupational health issues. The candidates under development for high density energetics as part of project WP-2208, GRIMEX: Development of Novel IM Comp B Replacements Based on Green TNT and RDX Replacements, include DNGU, DNP, HK-56, LLM-105, and TNBA.

National defense requires the development of unique energetic compounds to perform specialized mission requirements. These requirements also include the sustainable use of these materials in

the environment, particularly during training operations. The use of RDX (1,3,5-hexahydro-1,3,5-trinitrotriazine) and trinitrotoluene (TNT) in warheads has constrained use of training ranges potentially affecting military readiness. Unexploded ordnance and low-order detonations have become sources of ground water contamination and have affected drinking water resources.

The Centers for Disease Control and Prevention (CDC), Agency for Toxic Substances and Disease Registry (ATSDR) has developed an acute oral minimum risk level (MRL) for RDX of 60 micrograms per kilograms per day ($\mu\text{g/kg-day}$) based on its epileptiform seizure neurotoxicity in humans and rodents [17-20]. The USEPA has derived a chronic reference dose (RfD) of 3 $\mu\text{g/kg-day}$ based prostatic inflammation in rodents. RDX is also classified as a possible carcinogen [21, 22].

TNT is acutely toxic to rats causing ataxia, tremors, and mild convulsions; oral LD_{50} values range from 660 to 1320 mg/kg. The reference dose (RfD) for subchronic and chronic oral exposures of 0.0005 mg/kg-day is based on a LOAEL of 0.5 mg/kg-day for liver effects in dogs. TNT is classified in weight-of-evidence Group C, possible human carcinogen [23, 24].

The Strategic Environmental Research and Development Program is dedicated to finding replacements for RDX and TNT that will reduce or eliminate the health risks from environmental exposure and will reduce adverse ESOH effects; RDX adversely affects the readiness and costs associated with training [25]. To support the development of sustainable, low toxicity materials for use, fast, high-throughput methods are needed to assess relative toxicity of new munition compounds as they are developed. Toxicity tests can be conducted *in vivo* and *in vitro*. *In vitro* methods have the advantage of being relatively inexpensive, high-throughput, and capable of addressing many mechanistic issues at the cellular and molecular level. Specifically for the newly developed materials, the *in vitro* tests are most suitable and effective screening tools, given that often very limited amounts of test substances are available. By identifying ESOH effects early in the acquisition process, unacceptable replacement compounds can be identified. The energetic and toxicological properties of DNGU, DNP, HK-56, LLM-105, and TNBA are being evaluated as potential replacements for TNT and RDX.

The Army Public Health Center, Toxicology Directorate (APHC TOX) was tasked with providing acute toxicity data for DNGU, DNP, HK-56, LLM-105, and TNBA to determine their potential environmental and occupational hazards, which includes skin sensitization. The data from these studies will help in making recommendations for continued development and toxicity testing resulting in appropriate exposure guidance.

LLM-105 (Chemical Abstract Number (CASRN) unknown) and TNBA (CASRN 194486-7-6) are novel energetics under evaluation as replacements for RDX and TNT. Few toxicity data on the compound exist, however, QSAR analysis indicates that both compounds may be strong sensitizers [6]. The Toxicology Directorate of the Army Public Health Center has been tasked with evaluating the skin sensitization potential of LLM-105 and TNBA. Testing in the h-CLAT *in vitro* system constitutes the first evaluation of these compounds *in vitro* skin sensitization methods; multiple test systems are required to confirm results.

The h-CLAT is an *in vitro* approach to analyze dendritic cell activation of test chemicals via the expression of CD54 and CD86 on the cell surface. There are several key steps in the elicitation of a skin sensitizing reaction, including the activation of dendritic cells and the transformation from antigen processing to antigen presenting cells [26]. Multiple cell surface markers are expressed by dendritic cells, with CD54 and CD86 being just two examples. The increase in expression on the

cellular surface of these proteins is measured by flow cytometry as a result of a fluorescent signal on the antibodies which bind to either CD54 or CD86 [27-29]. The criteria for a positive reaction in h-CLAT require either a 2-fold or a 1.5-fold induction of CD54 or CD86 respectively as compared to solvent controls. During a skin sensitizing reaction, activated dendritic cells migrate to the lymph node where the major histocompatibility complexes which they are presenting activated T-cells and T-cell proliferation. Secondary exposure to the chemical will then result in inflammation and an allergic reaction.

5 Materials and Methods

5.1 Materials

5.1.1 Test Substance

Synthesis of LLM-105 (CASRN unregistered) and TNBA (CASRN 194486-7-6) was completed by the Holston Army Ammunition Plant, Kingsport, TN. Full purity analyses for these compounds were not provided by the sponsor, however the sponsor has indicated that purity is estimated to be > 98 percent. The molecular structures of the compounds are shown in Figure 1.

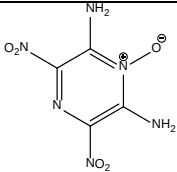
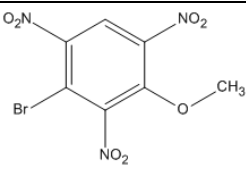
	
2,6-Diamino-3,5-dinitro-2,3-dihydropyrazine-1-oxide (LLM-105)	2,4,6-Trinitro-3-bromoanisole (TNBA)

Figure 1. Molecular Structure of the Compounds

5.1.2 Cell Line, Chemicals and Reagents

The h-CLAT has undergone validation testing within the APHC to verify that the assay performs as expected with APHC equipment when compared to published results [30]. THP-1 cells were acquired from the American Type Tissue Collection (ATCC, Manassas, VA). All tissue culture reagents were acquired from Gibco, a subsidiary of ThermoFisher (Waltham, MA). Cells were cultured in RPMI-1640 containing 10 percent fetal bovine serum, 100 U/mL penicillin, 10 µg/mL streptomycin and 0.05 mM 2-mercaptoethanol. Reagents for flow cytometry were as follows: physiological saline (Sigma-Aldrich, Inc., St. Louis, MO), dimethyl sulfoxide (DMSO, Sigma-Aldrich, Inc.), Dulbecco's phosphate buffered saline without calcium, magnesium or phenol red (Gibco, Inc.), bovine serum albumin fraction V (BSA, Calbiochem, Billerica, MA), globulins Cohn fraction II, II, human (Sigma-Aldrich, Inc.), and propidium iodide (PI, Sigma-Aldrich, Inc.). Control test chemicals were all obtained from Sigma-Aldrich, Inc., to include 2,4-dinitrochlorobenzene (DNCB, CASRN 97-00-7), nickel sulfate (NiSO₄, CASRN 7786-81-4), and lactic acid (LA, CASRN 50-21-5). Antibodies against IgG1 (control) and CD54 were obtained from Dako (Carpinteria, CA) and

antibodies against CD86 (Clone 2331, Fun-1) were obtained from BD Biosciences (San Jose, CA). All antibodies were tagged with the FITC fluorophore. All cells, reagents and chemicals were stored according to manufacturer's instructions. Lot numbers and expirations dates for all reagents used are provided in Appendix D, certificates of analysis are available at the individual manufacturer's websites.

5.1.3 Equipment

The assay reaction was analyzed by flow cytometry utilizing a BD FACSVerse flow cytometer (BD Biosciences)[31].

5.1.4 Quality Assurance

Army Public Health Center policy requires that all experiments and studies conducted by any element of the APHC Directorate of Toxicology will be compliant with the applicable Good Laboratory Practice (GLP) Standard guideline [32]. For this study, the test article dictates that the following GLP guideline applies [33][30].

Code of Federal Regulations (CFR), Title 40: Protection of Environment, Part 792-Good Laboratory Practice Standards.

According to this policy and that these results may be used in regulatory decisions involving the EPA, these h-CLAT test assays were conducted in compliance with GLP standards and followed the appropriate regulatory testing guidelines [34].

In compliance with the GLP requirements, the PHC Quality Systems and Regulatory Compliance Office audited critical phases of this study. A Quality Assurance Statement is provided in Appendix B, which provides the dates of these audits along with the audited phases and the dates that the results of the audits were reported to Management and the Study Director. The additional Quality Assurance/GLP requirement of archives location is provided in Appendix C as well as the names of personnel contributing to the performance of this study.

5.2 Methods

All assay setup was performed according to ECVAM DB-ALM protocol number 158, OECD Guideline [28, 29].

5.2.1 Buffers

FACS buffer was prepared with PBS and 0.1 percent (w/v) BSA the day before use and stored at $+4 \pm 2$ °C. Blocking solution was made up in 1 percent (w/v) globulins in PBS stocks as needed, with stock being used within one week and stored at $+4$ °C. Blocking solution for use on the day of the experiment was diluted to a 0.1 percent solution in FACS buffer immediately prior to use. PI was diluted to 12.5 µg/mL in PBS on the day of the experiment and maintained on ice.

5.2.2 Tissue Culture

Tissue culture media was prepared as described in section 5.1.2 and maintained at $+4 \pm 2^{\circ}\text{C}$. Media was pre-warmed at room temperature prior to use for each cell plating and passage. Cells were maintained at $1.5 \times 10^5 - 8 \times 10^5$ cells/mL and were routinely passaged every 2-3 days. Cells were maintained at 37°C , 5 percent CO_2 . Cells for the assay were in culture for no more than 30 passages or 60 days. Prior to passage or test plating, cell density was determined by counting with the TC-20 automated cell counter (Bio-Rad, Inc., Hercules, CA). Cell viability was determined by Trypan blue staining (Bio-Rad, Inc.). For range finding and h-CLAT testing, cells were plated into 24-well plates at a density of 1×10^6 cells/well in 0.5 mL. For maintenance, cells were plated at $1.5-2.0 \times 10^5$ cells/mL in 25-40 mL media depending on the timing of subsequent tests.

5.2.3 Reactivity Check

Two weeks after cells were thawed; a reactivity check on the batch is carried out utilizing DNCB, NiSO_4 and LA. Each flask of cells was tested separately. DNCB was prepared in 20 mg/mL stock solutions in DMSO and diluted to 2.4 mg/mL in DMSO. Stock solutions of DNCB were maintained at $+4^{\circ}\text{C}$. Serial dilutions of 1:1.2 were carried out for a total of 2 dosing levels and subsequently diluted 1:250 in 0.5 mL media. NiSO_4 was prepared in a 10 mg/mL solution in saline and diluted 1:50 into 0.5 mL media and 1:34.5 into 0.5 mL media. LA was prepared as a 100 mg/mL solution in saline and diluted 1:50 and 1:34.5 into 0.5 mL media. One 1:1.2 dilution was made. DNCB, NiSO_4 and LA were then diluted 1:2 into the 0.5 mL containing 1×10^6 cells. A dead cell well was prepared by diluting 10 μL of 20 mg/mL DNCB (final concentration 0.2 mg/mL) into 1 mL of media containing 1×10^6 cells in a 24-well plate. DMSO and saline control wells were also prepared. The plate was incubated for 24 hours and cells were processed and stained for IgG1, CD54 and CD86 and analyzed by flow cytometry (section 5.2.6).

5.2.4 Range finding

In order to determine appropriate dosing levels, LLM-105 and TNBA were initially prepared in a 25 mg/mL and 500 mg/mL solution in DMSO. Initial range finding concentrations were based on solubility, as determined by the Ames assay [34, 35]. Serial dilutions (1:2) were prepared in DMSO for a total of 8 dilutions. Each dilution was further diluted 1:250 into 0.5 mL media and diluted 1:2 into 0.5 mL media containing 1×10^6 cells in a 24-well plate. Cells from individual flasks were combined prior to plating for the assay. As described in 5.2.3, a DMSO control and dead cell control were prepared. Cells were incubated for 24 hours. Following incubation, each sample was transferred to a 5 mL tube and centrifuged at $200 \times g$ at $+4^{\circ}\text{C}$. Pellets were resuspended in 0.6 mL cold FACS buffer and 0.2 mL transferred to flow cytometry sample tubes. Samples were washed twice in 0.2 mL FACS buffer, resuspended in 0.4 mL FACS buffer and stained with 20 μL of a 12.5 $\mu\text{g/mL}$ PI stock (final concentration 0.625 $\mu\text{g/mL}$). Samples were maintained on ice in the dark and assayed for viability by flow cytometry. The dead cell control and the saline control were used to gate out dead cells stained with PI and the flow cytometer was set to acquire 10,000 live cell hits (PI negative) or 30,000 total hits, whichever was achieved first. Percent viability (ratio of live cells to total acquired cells) was utilized to determine the 75 percent cell viability (CV75) by the following equation (see also Figure 2):

$$\log \text{CV75} = \frac{(75-c) \times \log b - (75 - a) \times \log d}{a - c}$$

where:

a = Percent viability above 75 percent (nearest dose)

b = Dose level of a

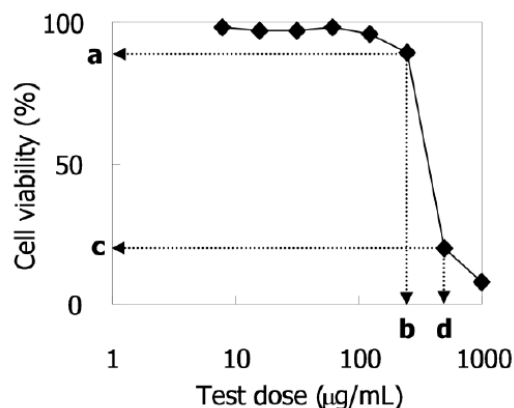
c = Percent viability below 75 percent (nearest dose)

d = Dose level of c

See Figure 1.

The CV75 is the value at which the second highest dose is set for the final test. Additional range finding assays had to be completed for TNBA as it was highly toxic at the initial testing doses.

Figure 2- Example results range finding PI assay*



*ECVAM DB-ALM, *human Cell Line Activation Test (h-CLAT)*, DB-ALM Protocol No. 158. 2015: European Union Reference Laboratory for Alternatives to Animal Testing [28, 29].

The range finding assay was completed a minimum of two times to verify results, if results were similar after two tests, no more testing was completed.

5.2.5 h-CLAT Test

Once the CV75 was determined, a dosing scheme was setup such that the highest dose was 1.2-fold higher than the CV75. LLM-105 and TNBA were weighed and solubilized in DMSO at a concentration 500x of the 1.2 x CV75. The solution was then diluted in a 1.2 serial dilution for a total of 8 concentration levels and each concentration diluted 1:50 in 0.5 mL complete media. This 0.5 mL was then diluted 1:2 into 0.5 mL containing 1×10^6 cells in a 24-well plate. DNCB was prepared from the 20 mg/mL stock by diluting to 2.4 mg/mL in DMSO, serially diluting 1:1.2 for 3 dilutions and then diluting a further 1:250 into media. These were also diluted 1:2 into 0.5 mL media containing 1×10^6 cells. A DMSO control was prepared as was a "dead cell" control containing 10 µL of the 20 mg/mL DMSO stock. Cells were incubated for 24 hours and processed for IgG1, CD54 and CD86 staining and analysis by flow cytometry (section 5.2.6).

At the end of the study, the samples were analyzed by the PHC-Method Development Section of the -Client Services Division, of the Laboratory Sciences Directorate. The final dilution of the

empirical starting stock solution for LLM-105 would theoretically be, for example, 1300 µg/mL. The validated concentrations of the final serial dilution for LLM-105 were: Test 1 – 1220.9 µg/mL; Test 2 – 1148.2 µg/mL. This last concentration was not collected until 24-hours after the test was started, however, degradation was minimal. The validated concentrations of the final serial dilution for TNBA were: Test 1 – 71.9 µg/mL; Test 2 – 201.8 µg/mL. The expected concentration for this dilution was 200 µg/mL. Test 1 for TNBA was not collected until 24-hours after the test was started. The concentrations of the initial concentrates were back-calculated from these verified concentrations and final data was analyzed from both these concentrations and the nominal concentrations. Where no difference was observed in the final EC150 and EC200 predictions, nominal values were reported.

5.2.6 Antibody Staining and Flow Cytometry

Each well was transferred containing cells and treatment or treatment control was transferred to a 5 mL snap-cap tube and collected by centrifugation (250 x g, 5 min, +4 °C) and washed twice in 1 mL cold FACS buffer. Cells were blocked in 0.6 mL 0.1 percent blocking buffer (prepared from the 1 percent stock in FACS buffer) for 15 min. at +4 ± 2 °C. Following blocking, each sample was split into 3 aliquots of 180-200 µL each in a round-bottom 96-well plate. Samples were spun as above and stained with antibodies. See Table 1 for antibody concentrations.

Table 1 – Antibody concentration

	Volume of antibody	Volume of FACS buffer	Total volume of working solution/sample
FITC labeled-mouse IgG1	6 µL	44 µL	50 µL
Anti-CD54 antibody	3 µL	47 µL	50 µL
Anti-CD86 antibody	3 µL	47 µL	50 µL

A master-mix for each antibody was prepared immediately prior to use and added directly to each cell pellet after removal of the blocking buffer. Each plate was gently vortexed to resuspend the cells in the antibody mix and incubated at +4 ± 2 °C in the dark for 30 min. Following the 30-minute incubation, samples were again spun down and washed twice in FACS buffer. Between the first and second wash, samples were transferred to FACS analysis tubes. Samples were maintained on ice throughout the transfer process. Following the final wash, samples were resuspended in 0.4 mL FACS buffer and stained with 20 µL PI. Each sample was gently vortex to mix.

Samples were analyzed by flow cytometry under the following conditions. Acquisition channels were set to read of the appropriate acquisition channel for propidium iodide (PI) and fluorescein isothiocyanate (FITC). The following plots were captured for each sample: 2-dimensional plot of forward and side scatter, 2-dimensional dot plot of FITC vs PI and a histogram plot of both FITC and PI. Live cells were used to determine the correct voltages for the forward scatter and side scatter channels. Dead cells were gated out by PI using the dead cell control and the IgG1 saline control and total acquisition was determined by either 10,000 PI negative hits or 30,000 total hits on the PI channel. For each sample, the geometric mean fluorescence intensity (MFI) was captured for all hits and live/viable cell hits.

From the MFI, the relative fluorescence intensity (RFI) was determined by the following equation:

$$RFI = \frac{\text{MFI of chemical treated cells} - \text{MFI of chemical treated isotype cells}}{\text{MFI of solvent treated cells} - \text{MFI of solvent treated isotype cells}}$$

The cell viability for each concentration was also recorded from the isotype control population.

5.2.7 Data Analysis

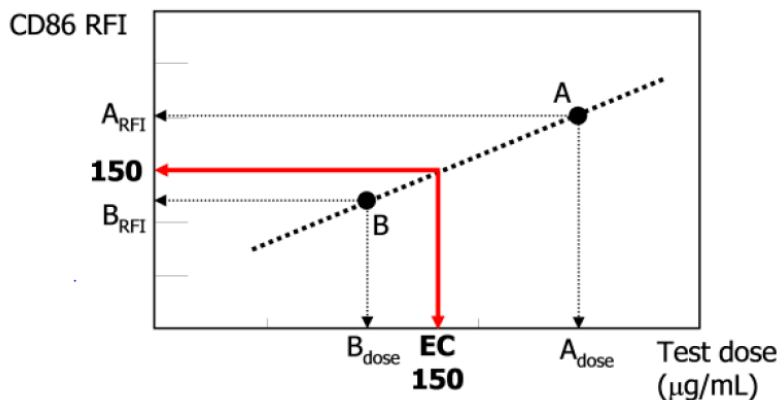
If the RFI for any concentration exceeded the positive criteria (CD54 ≥ 200 and CD86 ≥ 150), the EC200 and EC150 were calculated by the following equation:

$$EC200 (CD54) = B_{dose} + [(200 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})]$$

$$EC150 (CD86) = B_{dose} + [(150 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})]$$

Where A_{dose} , B_{dose} , A_{RFI} and B_{RFI} were determined from the following chart (Figure 3):

Figure 3- Example dose response curve for CD86*



*ECVAM DB-ALM, *human Cell Line Activation Test (h-CLAT)*, DB-ALM Protocol No. 158. 2015: European Union Reference Laboratory for Alternatives to Animal Testing [28, 29].

If the EC200 or EC150 fell below the lowest dose, the values were extrapolated by the following equations.

$$EC200 (CD54) = 2 \exp[\log_2(B_{dose}) + (200 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times [\log_2(A_{dose}) - \log_2(B_{dose})]]$$

$$EC150 (CD86) = 2 \exp[\log_2(B_{dose}) + (150 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times [\log_2(A_{dose}) - \log_2(B_{dose})]]$$

Two independent runs were completed for LLM-105 and TNBA as the data were consistent across both runs. A third run is necessary where the data are different between the first two runs, and a best-of-three decision is made regarding sensitization.

5.2.8 Criteria for a Valid Assay

For a test to be acceptable, the following criteria were met:

- o Cell viability of medium and DMSO controls was more than 90 percent.
- o RFI values for the DNCB control for both CD54 and CD86 exceeded the positive criteria (CD54 ≥ 200 and CD86 ≥ 150).
- o RFI values for the DMSO solvent control did not exceed positive criteria.
- o The MFI ratio of both CD54 and CD86 to isotype control for DMSO and media controls exceeded 105 percent.
- o The cell viability of at least 4 doses was greater than 50 percent.

6 Results and Discussion

6.1 Reactivity Check

The THP-1 cells were checked and verified for reactivity to DNCB, NiSO₄ and lack of reactivity to LA. Cells reacted as expected, with DNCB and NiSO₄ eliciting positive reactions for both CD54 and CD86, while LA was negative in both (Table 2). The cells met criteria for further testing. Results for each flask are listed in the table.

Table 2: Results of Reactivity Check

Test article	Concentration (mg/mL)	Viability (% alive)	RFI (CD86)	RFI (CD54)	Positive (CD86/CD54)
Saline		96.38	100	100	N/N
		92.72	100	100	N/N
DMSO		96.2	100	100	N/N
		93.42	100	100	N/N
DNCB	0.0033	83.64	269.9	383.2	Y/Y
		81.55	287.2	467.63	Y/Y
	0.0040	80.47	313.0	676.9	Y/Y
		81.89	303.5	552.6	Y/Y
	0.0048	92.86*	88.6*	106.7*	N/N*
		78.97	256.7	781.5	Y/Y
NiSO ₄	0.10	74.86	228.7	1880.9	Y/Y
		74.64	183.1	751.1	Y/Y
Lactic Acid	1	96.39	81.03	109.8	N/N
		93.25	64.3	78.2	N/N

*Based on the viability observed and the weight-of-evidence of reactivity in all other DNCB treatments across the flasks, the treatment of DNCB at 0.0048 mg/mL was not applied in flask 1. Due to multiple treatment levels which were reactive and that the same dose in Flask 2 was reactive, the non-treatment does not affect the results of the reactivity check. The study design for this check has added additional dosing concentrations than those required by the OECD guidelines.

6.2 Range finding Assay

Two independent dose finding assays were completed in order to determine the CV75 of LLM-105 in THP-1 cells. The average CV75 between the two assays was 0.008 mg/mL LLM-105 (Table 3). Three independent assays were necessary to determine the CV75 of TNBA. The average CV75 between the two assays which captured the CV75 was 0.0011 mg/mL.

Table 3: Results of Range finding Assays

Compound	Assay	CV75 (mg/mL)	Average (mg/mL)
LLM-105	Assay 1	0.009	0.008
	Assay 2	0.007	
TNBA	Assay 1	Undetermined	0.0011
	Assay 2	0.0009	
	Assay 3	0.0013	

6.3 CD54 and CD86 expression following compound exposure in THP-1 cells

Two independent tests were completed for LLM-105 for both CD54 and CD86. In all runs for both compounds, CD54 and CD86 were positive (Table 4). At the higher dosing levels, the RFI did not exceed positive criteria for TNBA, but this is commonly seen when cell viability is lower, even in the positive controls. At lower viability levels, there is a diffuse labeling of cytoplasmic structures which affects the background levels of stain and negates a positive response. The LLM-105 EC150 range for CD86 was 0.003-0.004 mg/mL and the EC200 range for CD54 was 0.004-0.005 mg/mL. The TNBA EC150 range for CD86 was 0.0002 – 0.0004 mg/mL and the EC200 range for CD54 was 0.0004-0.0006 mg/mL. Data are reported for the nominal concentrations of the compounds due to the fact that for each compound, one concentration was not collected for verification until 24-hours after the dosing solutions were prepared. LLM-105 is a relatively stable compound, and minimal degradation was observed due to this error. Calculated EC150 and EC200 values both by nominal and corrected concentrations were not different for LLM-105. TNBA is highly hygroscopic and exhibits rapid degradation at ambient temperatures when in solution, so the late collection of the dosing solutions for concentration verification did affect the dose curve and final calculated EC150 and EC200 values. However, the second test, TNBA concentrations were effectively the same as nominal concentrations and the EC150 and EC200 values between test 1 and test 2 were similar at nominal concentrations. Therefore, it can be expected that the initial TNBA concentrations were correct, and the noticeable difference between nominal and actual in test 1 is due to the stability of TNBA in solution, not due to erroneous setup of the dosing solutions.

Table 4: Results of Compound Analysis

Compound	CD86 EC150 (mg/mL)	CD54 EC200 (mg/mL)	Positive Control (CD86/CD54)	Positive Test?
LLM-105	0.004	0.004	Y/Y	Yes
	0.003	0.005	Y/Y	Yes
TNBA	0.0004	0.0006	Y/Y	Yes
	0.0002	0.0004	Y/Y	Yes

*The EC200 could not be extrapolated due to the RFI decreasing with each increase in concentration. The test is still considered positive but the EC200 cannot be determined.

6.4 Criteria for Valid Assay

All criteria were met for all the assays.

7 Conclusions

As determined by h-CLAT, LLMN-105 and TNBA are considered positive by the test criteria. QSAR analysis by TOPKAT (BIOVIA, Inc.) predicted that both are potentially strong sensitizers. These data indicate that LLM-105 and TNBA are most likely skin sensitizers, however, further confirmatory testing and analysis are recommended.

8 Recommendations

LLM-105 and TNBA appear to be skin sensitizers upon analysis with the h-CLAT skin sensitizing when combined with QSAR analysis [6]. Further *in vitro* or *in vivo* testing is recommended to more definitively determine the sensitizing potential of these compounds. The h-CLAT is one of many non-animal skin sensitizing tests, and it comprises part of an integrated testing strategy with two other *in vitro* approaches, the DPRA and the LuSens assay [7-11]. A comprehensive assessment of skin sensitization potential requires results from all three assays along with specific *in silico* analysis provides a more robust estimation of skin sensitization than h-CLAT alone [12]. As testing has only occurred with the h-CLAT, it is not yet possible to provide a definitive response as to the sensitization potential. Testing with the direct peptide reactivity assay will be conducted, and the LuSens test is currently under validation by APHC and should be available to complete the *in vitro* tests necessary for analysis. However, the robustness of the h-CLAT assay provides a strong indication that both LLM-105 and TNBA are skin sensitizers and appropriate precautions should be taken when handling the material.

9 Point of Contact

Dr. Emily N. Reinke, the principal investigator, is the point of contact for this project. She may be reached at DSN 584-3980 or commercial 410-436-3980.

Toxicology Study No. S.0039820-16, November 2017

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Prepared by:



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Date

Approved by:



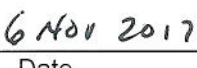
Michael J. Quinn Jr., Ph.D.
Division Chief
Health Effects Division
APHC



Date



Mark S. Johnson, Ph.D., D.A.B.T.
Director, Toxicology
APHC



Date

Appendix A

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Toxicology Study No. S.0039820-16, November 2017

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Appendix B

QUALITY ASSURANCE STATEMENT

For: Toxicology Study No. S.0039820-16, Protocol No. 49-iv17-02-01A,B Human Cell Line Activation Test of the Novel Energetics 2,6-pyrazinediamine 3,5-dinitro-1-oxide (LLM-105) and 2,4,6-trinitro-3-bromoanisole (TNBA) the following critical phases were inspected/audited by the Quality Systems and Regulatory Compliance Office's Quality Assurance Unit:

Pre-Study Inspection

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Draft Type Protocol and Test Article Specific Modification GLP Review	01/10/2017	01/10/2017

In-life inspections

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
In-Vitro Skin Sensitization h-CLAT Assay Validation - PI qualifications and training	01/20/2017	01/24/2017
In-Vitro Skin Sensitization h-CLAT Assay Validation - General Requirements	01/20/2017	01/24/2017
In-Vitro Skin Sensitization h-CLAT Assay Validation - Maintenance and Cal of Equipment	01/20/2017	01/24/2017
In-Vitro Skin Sensitization h-CLAT Assay Validation - Labeling of Reagents	01/20/2017	01/24/2017
In-Vitro Skin Sensitization h-CLAT Assay Validation - Staining and FACS analysis	01/20/2017	01/24/2017
In-Vitro Skin Sensitization h-CLAT Assay - Reagents, Working Solutions and Cell Suspension Storage	02/16/2017	02/19/2017
In-Vitro Skin Sensitization h-CLAT Assay - Preparation of Stock and Working Solutions	02/16/2017	02/19/2017
In-Vitro Skin Sensitization h-CLAT Assay - Cell Suspension and Exposure	02/16/2017	02/19/2017

Post Study Inspection

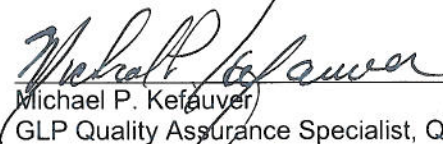
Study Final Report and Raw Data GLP Review	10/13/2017	10/13/2017
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Note 1: The above inspections were conducted to review the procedures for this type of study but may not have been for this specific study.

Note 2: All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table.

Note 3: In addition to the study specific critical phase inspections listed here, general facility and process based inspection not specifically related to this study are done monthly or annually in accordance with QA Standard Operating Procedure.

Note 4: This report has been audited by the Quality Assurance Unit (QSARC), and is considered to be an accurate account of the data generated and of the procedures followed


Michael P. Kefauver
GLP Quality Assurance Specialist, QSARC

6 NOVEMBER 2017
Date

Appendix C

Archives and Study Personnel

C-1. Archives

All raw data, documentation, records, protocols, contributing scientist reports, and a copy of the final report generated as a result of this study will be archived in the storage facilities of the Toxicology Directorate, APHC, for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

Records on the test system will be archived by the Toxicology Directorate for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

The present study used the Toxicology Study No. S.0039820-16

The protocol, raw data, summary data, and the final report pertaining to this study will be physically maintained within Building E-2100, APHC. These data may be scanned to a computer disk. Scanned study files will be stored electronically with the study data in the archive.

Archived SOPs can be found in the Master Control database at APHC. Maintenance and calibration logbooks may be found in Room 1026, Building E-2100, APHC, APG, MD, 21010.

Archivist: Martha Thompson

C-2. Personnel

Management: Mark Johnson, Ph.D., D.A.B.T., Director, Toxicology;
Michael J. Quinn, Ph.D., Program Manager, Health Effects Division (HEF)

Study Director: Emily N. Reinke, Biologist, HERP.

Quality Assurance: Michael P. Kefauver, Chemist, Quality Systems and Regulatory Compliance Office.

Appendix D

Reagents Used

Reagent	Supplier	Product Number	Lot Number	Expiration Date
THP-1	ATCC	TIB-202	62996831	N/A
RPMI-1640	Gibco	22400	1831722	09-17
FBS	Gibco	16140	1851521	11-21
2-Mercaptoethanol	Gibco	21985	1678663	02-18
Penicillin-Streptomycin	Gibco	15140	18535957	10-30-17
Saline	Sigma	S8776	RNBD7305	N/A
DMSO (TC)	Sigma	D2438	RNBD8224	06/17
Globulins	Sigma	G2388	017K7650V	N/A
BSA Fraction V	EMD Chemicals	12660	D00150383	N/A
D-PBS	Gibco	14190	1710578	06-18
Propidium Iodide	Sigma	P4864	MKBR1007V	N/A
CD54 Antibody, ICAM-1 Clone 6.5B5, FITC	Dako	F714301-8	20020092	08-18
CD86 Antibody, Hu Fun-1, FITC	BD	555657	50S1730	10-31-19
IgG1 (mouse), FITC	Dako	X092701-2	20023012	04-18
Flow Cytometer Beads	BD	650622	62638	09-17
Sheath Fluid	BD	342003	0000106241	3-12-18
2,4-dinitrochlorobenzene (DNCB)	Sigma	237329	BCBN7826V	N/A
Nickel Sulfate (NiSO ₄)	Sigma	656895	MKBT0269V	N/A
Lactic Acid (LA)	Sigma	W261106	MKBR4746V	N/A

Appendix E

Raw Data and Analysis

Experiment 1 – LLM-105 Raw Data

Experiment: h-CLAT WP-2208 LLM-105 4-4-17

Statistics					
Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
DMSO Live:All Events	10,000	***	***	100.00	1,034
DMSO Live:Live Cells	9,003	90.03	***	90.03	893
Dead Cells:All Events	10,000	***	***	100.00	1,698
Dead Cells:Live Cells	1,039	10.39	***	10.39	1,888
IgG DMSO:All Events	11,949	***	***	100.00	1,051
IgG DMSO:Live Cells	10,695	89.51	***	89.51	905
IgG DNCB 3.3 ug/mL:All Events	15,164	***	***	100.00	1,349
IgG DNCB 3.3 ug/mL:Live Cells	11,245	74.16	***	74.16	1,161
IgG DNCB 4 ug/mL:All Events	15,139	***	***	100.00	1,363
IgG DNCB 4 ug/mL:Live Cells	11,295	74.61	***	74.61	1,128
IgG DNCB 4.8 ug/mL:All Events	15,240	***	***	100.00	1,313
IgG DNCB 4.8 ug/mL:Live Cells	11,269	73.94	***	73.94	1,112
IgG LLM-105 2.62 ug/mL:All Events	11,729	***	***	100.00	1,535
IgG LLM-105 2.62 ug/mL:Live Cells	10,717	91.37	***	91.37	1,389
IgG LLM-105 3.15 ug/mL:All Events	11,898	***	***	100.00	1,726
IgG LLM-105 3.15 ug/mL:Live Cells	10,791	90.70	***	90.70	1,563
IgG LLM-105 3.7 ug/mL:All Events	12,083	***	***	100.00	1,949
IgG LLM-105 3.7 ug/mL:Live Cells	10,887	90.10	***	90.10	1,769
IgG LLM-105 4.5 ug/mL:All Events	12,443	***	***	100.00	2,229
IgG LLM-105 4.5 ug/mL:Live Cells	10,971	88.17	***	88.17	1,996
IgG LLM-105 5.4 ug/mL:All Events	13,819	***	***	100.00	2,868
IgG LLM-105 5.4 ug/mL:Live Cells	11,633	84.18	***	84.18	2,638
IgG LLM-105 6.5 ug/mL:All Events	14,728	***	***	100.00	3,396
IgG LLM-105 6.5 ug/mL:Live Cells	12,066	81.93	***	81.93	3,125
IgG LLM-105 7.8 ug/mL:All Events	18,061	***	***	100.00	3,708
IgG LLM-105 7.8 ug/mL:Live Cells	12,906	71.46	***	71.46	3,492
IgG LLM-105 9.4 ug/mL:All Events	27,345	***	***	100.00	4,239
IgG LLM-105 9.4 ug/mL:Live Cells	15,003	54.87	***	54.87	4,129
CD86 DMSO :All Events	12,146	***	***	100.00	2,903
CD86 DMSO :Live Cells	10,858	89.40	***	89.40	2,386
CD54 DMSO:All Events	12,021	***	***	100.00	1,361
CD54 DMSO:Live Cells	10,782	89.69	***	89.69	1,204
CD86 DNCB 3.3 ug/mL:All Events	16,089	***	***	100.00	7,175
CD86 DNCB 3.3 ug/mL:Live Cells	11,617	72.20	***	72.20	5,816
CD54 DNCB 3.3 ug/mL:All Events	16,450	***	***	100.00	3,326
CD54 DNCB 3.3 ug/mL:Live Cells	11,567	70.32	***	70.32	3,361
CD86 DNCB 4.0 ug/mL:All Events	13,912	***	***	100.00	6,833
CD86 DNCB 4.0 ug/mL:Live Cells	9,997	71.86	***	71.86	5,523
CD54 DNCB 4.0 ug/mL:All Events	13,905	***	***	100.00	3,401
CD54 DNCB 4.0 ug/mL:Live Cells	10,001	71.92	***	71.92	3,555
CD86 DNCB 4.8 ug/mL:All Events	14,097	***	***	100.00	7,083
CD86 DNCB 4.8 ug/mL:Live Cells	9,987	70.84	***	70.84	5,594
CD54 DNCB 4.8 ug/mL:All Events	14,589	***	***	100.00	3,817
CD54 DNCB 4.8 ug/mL:Live Cells	9,992	68.49	***	68.49	4,055
CD86 LLM-105 2.62 ug/mL:All Events	11,185	***	***	100.00	3,805
CD86 LLM-105 2.62 ug/mL:Live Cells	10,000	89.41	***	89.41	3,220
CD54 LLM-105 2.62 ug/mL:All Events	11,150	***	***	100.00	2,041
CD54 LLM-105 2.62 ug/mL:Live Cells	10,000	89.69	***	89.69	1,831
CD86 LLM-105 3.15 ug/mL:All Events	11,121	***	***	100.00	3,937
CD86 LLM-105 3.15 ug/mL:Live Cells	9,989	89.82	***	89.82	3,358
CD54 LLM-105 3.15 ug/mL:All Events	11,140	***	***	100.00	2,281
CD54 LLM-105 3.15 ug/mL:Live Cells	10,000	89.77	***	89.77	2,031
CD86 LLM-105 3.7 ug/mL:All Events	11,177	***	***	100.00	4,567
CD86 LLM-105 3.7 ug/mL:Live Cells	10,000	89.47	***	89.47	3,900
CD54 LLM-105 3.7 ug/mL:All Events	11,281	***	***	100.00	2,558
CD54 LLM-105 3.7 ug/mL:Live Cells	10,005	88.69	***	88.69	2,319
CD86 LLM-105 4.5 ug/mL:All Events	11,340	***	***	100.00	5,170
CD86 LLM-105 4.5 ug/mL:Live Cells	9,993	88.12	***	88.12	4,364
CD54 LLM-105 4.5 ug/mL:All Events	11,499	***	***	100.00	2,951
CD54 LLM-105 4.5 ug/mL:Live Cells	10,000	86.96	***	86.96	2,665
CD86 LLM-105 5.4 ug/mL:All Events	12,186	***	***	100.00	6,717
CD86 LLM-105 5.4 ug/mL:Live Cells	10,000	82.06	***	82.06	5,648
CD54 LLM-105 5.4 ug/mL :All Events	12,252	***	***	100.00	3,838
CD54 LLM-105 5.4 ug/mL :Live Cells	10,000	81.62	***	81.62	3,504
CD86 LLM-105 6.5 ug/mL:All Events	12,786	***	***	100.00	7,624
CD86 LLM-105 6.5 ug/mL:Live Cells	10,000	78.21	***	78.21	6,335
CD54 LLM-105 6.5 ug/mL:All Events	13,348	***	***	100.00	4,552
CD54 LLM-105 6.5 ug/mL:Live Cells	10,000	74.92	***	74.92	4,268
CD86 LLM-105 7.8 ug/mL:All Events	14,518	***	***	100.00	8,268
CD86 LLM-105 7.8 ug/mL:Live Cells	10,000	68.88	***	68.88	6,834
CD54 LLM-105 7.8 ug/mL:All Events	14,778	***	***	100.00	4,858
CD54 LLM-105 7.8 ug/mL:Live Cells	10,000	67.67	***	67.67	4,739
CD86 LLM-105 9.4 ug/mL:All Events	20,759	***	***	100.00	8,297
CD86 LLM-105 9.4 ug/mL:Live Cells	10,000	48.17	***	48.17	6,932
CD54 LLM-105 9.4 ug/mL:All Events	20,482	***	***	100.00	5,559
CD54 LLM-105 9.4 ug/mL:Live Cells	10,000	48.82	***	48.82	6,012

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Operator: Emily Reinke, Ph.D.

Page 1 of 1

Toxicology Study No. S.0039820-16, November 2017

Experiment 1
Data Analysis

4/4/2017

	Concentration (mg/mL)	Viability (%) (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
DMSO	0	89.51	905	2386	1	100		1204	1	100	
DNCB Control	0.0033	74.16	1161	5816	3.14	314.31		3361	7.36	735.79	
	0.004	74.61	1128	5523	2.97	296.76		3555	8.12	811.71	
	0.0048	73.94	1112	5594	3.03	302.63		4055	9.84	984.28	
LLM-105	0.0026	91.37	1389	3220	1.24	123.63	0.0041	1831	1.48	147.83	0.0041
	0.0031	90.7	1563	3358	1.21	121.20		2031	1.57	156.52	
	0.0038	90.1	1769	3900	1.44	143.89		2319	1.84	183.95	
	0.0045	88.17	1996	4364	1.60	159.89		2665	2.24	223.75	
	0.0054	84.18	2638	5648	2.03	203.24		3504	2.90	289.63	
	0.0065	81.93	3125	6335	2.17	216.75		4268	3.82	382.27	
	0.0078	71.46	3492	6834	2.26	225.66		4739	4.17	417.06	
	0.0094	54.87	4129	6923	1.89	188.66		6012	6.30	629.77	

MDV Concentration Verification corrected values 4/17/17

	Concentration (mg/mL)	Viability (%) (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
DMSO	0	89.51	905	2386	1	100	0.0039	1204	1	100	0.0039
LLM-105	0.0025	91.37	1389	3220	1.24	123.63		1831	1.48	147.83	
	0.0030	90.7	1563	3358	1.21	121.20		2031	1.57	156.52	
	0.0036	90.1	1769	3900	1.44	143.89		2319	1.84	183.95	
	0.0043	88.17	1996	4364	1.60	159.89		2665	2.24	223.75	
	0.0052	84.18	2638	5648	2.03	203.24		3504	2.90	289.63	
	0.0062	81.93	3125	6335	2.17	216.75		4268	3.82	382.27	
	0.0075	71.46	3492	6834	2.26	225.66		4739	4.17	417.06	
	0.0090	54.87	4129	6923	1.89	188.66		6012	6.30	629.77	

Experiment 2 – LLM-105 and TNBA
Raw Data

Experiment: h-CLAT WP-2208 #2 LLM-105 TNBA 4-10-17

Statistics					
Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
DMSO Live Cell Control:All Events	10,000	***	***	100.00	769
DMSO Live Cell Control:Live Cells	9,239	92.39	***	92.39	710
Dead Cells:All Events	5,958	***	***	100.00	1,284
Dead Cells:Live Cells	436	7.32	***	7.32	1,178
IgG DMSO:All Events	10,888	***	***	100.00	780
IgG DMSO:Live Cells	10,000	91.84	***	91.84	722
IgG DNCB 3.3 ug/mL:All Events	12,852	***	***	100.00	1,031
IgG DNCB 3.3 ug/mL:Live Cells	10,004	77.84	***	77.84	913
IgG DNCB 4 ug/mL:All Events	12,716	***	***	100.00	1,000
IgG DNCB 4 ug/mL:Live Cells	10,000	78.64	***	78.64	879
IgG DNCB 4.4 ug/mL:All Events	13,131	***	***	100.00	1,004
IgG DNCB 4.4 ug/mL:Live Cells	10,000	76.16	***	76.16	878
IgG LLM-105 2.62 ug/mL:All Events	11,174	***	***	100.00	1,469
IgG LLM-105 2.62 ug/mL:Live Cells	9,980	89.31	***	89.31	1,347
IgG LLM-105 3.15 ug/mL:All Events	11,059	***	***	100.00	1,564
IgG LLM-105 3.15 ug/mL:Live Cells	10,000	90.42	***	90.42	1,442
IgG LLM-105 3.7 ug/mL:All Events	11,376	***	***	100.00	1,842
IgG LLM-105 3.7 ug/mL:Live Cells	10,000	87.90	***	87.90	1,691
IgG LLM-105 4.5 ug/mL:All Events	11,840	***	***	100.00	2,291
IgG LLM-105 4.5 ug/mL:Live Cells	10,000	84.46	***	84.46	2,088
IgG LLM-106 5.4 ug/mL:All Events	13,151	***	***	100.00	2,958
IgG LLM-106 5.4 ug/mL:Live Cells	10,000	76.04	***	76.04	2,635
IgG LLM-105 6.5 ug/mL:All Events	14,044	***	***	100.00	3,422
IgG LLM-105 6.5 ug/mL:Live Cells	10,000	71.20	***	71.20	3,055
IgG LLM-105 7.8 ug/mL:All Events	18,044	***	***	100.00	4,473
IgG LLM-105 7.8 ug/mL:Live Cells	10,000	55.42	***	55.42	3,899
IgG LLM-105 9.4 ug/mL:All Events	30,000	***	***	100.00	5,510
IgG LLM-105 9.4 ug/mL:Live Cells	8,394	27.98	***	27.98	4,423
IgG TNBA 0.37 ug/mL:All Events	11,530	***	***	100.00	922
IgG TNBA 0.37 ug/mL:Live Cells	10,000	86.73	***	86.73	797
IgG TNBA 0.44 ug/mL:All Events	11,655	***	***	100.00	931
IgG TNBA 0.44 ug/mL:Live Cells	10,000	85.80	***	85.80	817
IgG TNBA 0.53ug/mL:All Events	12,903	***	***	100.00	1,002
IgG TNBA 0.53ug/mL:Live Cells	9,984	77.38	***	77.38	862
IgG TNBA 0.64 ug/mL:All Events	14,894	***	***	100.00	1,130
IgG TNBA 0.64 ug/mL:Live Cells	10,000	67.14	***	67.14	932
IgG TNBA 0.76 ug/mL:All Events	16,451	***	***	100.00	1,193
IgG TNBA 0.76 ug/mL:Live Cells	10,000	60.79	***	60.79	968
IgG TNBA 0.92 ug/mL:All Events	19,328	***	***	100.00	1,212
IgG TNBA 0.92 ug/mL:Live Cells	9,997	51.72	***	51.72	922
IgG TNBA 1.1 ug/mL:All Events	18,861	***	***	100.00	1,199
IgG TNBA 1.1 ug/mL:Live Cells	10,000	53.02	***	53.02	898
IgG TNBA 1.32 ug/mL:All Events	22,660	***	***	100.00	1,300
IgG TNBA 1.32 ug/mL:Live Cells	10,000	44.13	***	44.13	973
CD86 DMSO:All Events	10,912	***	***	100.00	2,001
CD86 DMSO:Live Cells	10,000	91.64	***	91.64	1,798
CD54 DMSO:All Events	11,079	***	***	100.00	1,209
CD54 DMSO:Live Cells	10,000	90.26	***	90.26	1,105
CD86 DNCB 3.3 ug/mL:All Events	13,364	***	***	100.00	6,475
CD86 DNCB 3.3 ug/mL:Live Cells	10,003	74.85	***	74.85	6,163
CD54 DNCB 3.3 ug/mL:All Events	14,044	***	***	100.00	2,322
CD54 DNCB 3.3 ug/mL:Live Cells	10,000	71.20	***	71.20	2,266
CD86 DNCB 4 ug/mL:All Events	13,610	***	***	100.00	6,165
CD86 DNCB 4 ug/mL:Live Cells	9,996	73.45	***	73.45	5,744
CD54 DNCB 4 ug/mL:All Events	14,138	***	***	100.00	2,559
CD54 DNCB 4 ug/mL:Live Cells	9,992	70.67	***	70.67	2,517
CD86 DNCB 4.4 ug/mL:All Events	13,749	***	***	100.00	5,358
CD86 DNCB 4.4 ug/mL:Live Cells	9,995	72.70	***	72.70	4,972
CD54 DNCB 4.4 ug/mL:All Events	14,303	***	***	100.00	2,843
CD54 DNCB 4.4 ug/mL:Live Cells	10,000	69.92	***	69.92	2,923
CD86 LLM-105 2.62 ug/mL:All Events	11,299	***	***	100.00	3,132
CD86 LLM-105 2.62 ug/mL:Live Cells	10,000	88.50	***	88.50	2,789
CD54 LLM-105 2.62 ug/mL:All Events	11,495	***	***	100.00	2,002
CD54 LLM-105 2.62 ug/mL:Live Cells	10,000	86.99	***	86.99	1,847
CD86 LLM-105 3.15 ug/mL:All Events	11,377	***	***	100.00	3,449
CD86 LLM-105 3.15 ug/mL:Live Cells	10,000	87.90	***	87.90	3,049
CD54 LLM-105 3.15 ug/mL:All Events	11,565	***	***	100.00	2,171
CD54 LLM-105 3.15 ug/mL:Live Cells	10,000	86.47	***	86.47	2,001
CD86 LLM-105 3.7 ug/mL:All Events	11,534	***	***	100.00	3,735
CD86 LLM-105 3.7 ug/mL:Live Cells	10,000	86.70	***	86.70	3,329
CD54 LLM-105 3.7 ug/mL:All Events	11,877	***	***	100.00	2,561
CD54 LLM-105 3.7 ug/mL:Live Cells	10,000	84.20	***	84.20	2,331
CD86 LLM-105 4.5 ug/mL:All Events	12,491	***	***	100.00	4,835
CD86 LLM-105 4.5 ug/mL:Live Cells	10,000	80.06	***	80.06	4,246
CD54 LLM-105 4.5 ug/mL:All Events	12,827	***	***	100.00	3,161
CD54 LLM-105 4.5 ug/mL:Live Cells	10,000	77.96	***	77.96	2,829

Experiment: h-CLAT WP-2208 #2 LLM-105 TNBA 4-10-17

Statistics					
Name	Events	% Parent	% Grandparent	% Total	FTTC-A Geo Mean
CD86 LLM-105 5.4 ug/mL:All Events	14,206	***	***	100.00	5,906
CD86 LLM-105 5.4 ug/mL:Live Cells	10,000	70.39	***	70.39	5,007
CD54 LLM-105 5.4 ug/mL:All Events	14,399	***	***	100.00	4,062
CD54 LLM-105 5.4 ug/mL:Live Cells	10,000	69.45	***	69.45	3,554
CD86 LLM-105 6.5 ug/mL:All Events	14,423	***	***	100.00	6,433
CD86 LLM-105 6.5 ug/mL:Live Cells	10,000	69.33	***	69.33	5,440
CD54 LLM-105 6.5 ug/mL:All Events	14,908	***	***	100.00	4,346
CD54 LLM-105 6.5 ug/mL:Live Cells	10,000	67.08	***	67.08	3,949
CD86 LLM-105 7.8 ug/mL:All Events	18,918	***	***	100.00	7,517
CD86 LLM-105 7.8 ug/mL:Live Cells	10,000	52.86	***	52.86	6,265
CD54 LLM-105 7.8 ug/mL:All Events	20,448	***	***	100.00	5,115
CD54 LLM-105 7.8 ug/mL:Live Cells	10,176	49.77	***	49.77	4,833
CD86 LLM-105 9.4 ug/mL:All Events	30,000	***	***	100.00	8,530
CD86 LLM-105 9.4 ug/mL:Live Cells	9,618	32.06	***	32.06	7,354
CD54 LLM-105 9.4 ug/mL:All Events	30,000	***	***	100.00	5,665
CD54 LLM-105 9.4 ug/mL:Live Cells	8,571	28.57	***	28.57	5,740
CD86 TNBA 0.37 ug/mL:All Events	11,546	***	***	100.00	2,782
CD86 TNBA 0.37 ug/mL:Live Cells	10,000	86.61	***	86.61	2,447
CD54 TNBA 0.37 ug/mL:All Events	11,890	***	***	100.00	1,374
CD54 TNBA 0.37 ug/mL:Live Cells	10,000	84.10	***	84.10	1,235
CD86 TNBA 0.44 ug/mL:All Events	11,848	***	***	100.00	2,946
CD86 TNBA 0.44 ug/mL:Live Cells	10,000	84.40	***	84.40	2,585
CD54 TNBA 0.44 ug/mL:All Events	12,033	***	***	100.00	1,417
CD54 TNBA 0.44 ug/mL:Live Cells	10,000	83.10	***	83.10	1,281
CD86 TNBA 0.53 ug/mL:All Events	13,466	***	***	100.00	3,590
CD86 TNBA 0.53 ug/mL:Live Cells	9,990	74.19	***	74.19	2,901
CD54 TNBA 0.53 ug/mL:All Events	13,703	***	***	100.00	1,709
CD54 TNBA 0.53 ug/mL:Live Cells	10,000	72.98	***	72.98	1,589
CD86 TNBA 0.64 ug/mL:All Events	15,069	***	***	100.00	3,924
CD86 TNBA 0.64 ug/mL:Live Cells	10,000	66.36	***	66.36	2,974
CD54 TNBA 0.64 ug/mL:All Events	15,882	***	***	100.00	1,923
CD54 TNBA 0.64 ug/mL:Live Cells	10,000	62.96	***	62.96	1,842
CD86 TNBA 0.76 ug/mL:All Events	17,340	***	***	100.00	4,314
CD86 TNBA 0.76 ug/mL:Live Cells	9,998	57.66	***	57.66	3,067
CD54 TNBA 0.76 ug/mL:All Events	18,576	***	***	100.00	2,102
CD54 TNBA 0.76 ug/mL:Live Cells	10,000	53.83	***	53.83	2,121
CD86 TNBA 0.92 ug/mL:All Events	19,461	***	***	100.00	4,381
CD86 TNBA 0.92 ug/mL:Live Cells	9,991	51.34	***	51.34	2,974
CD54 TNBA 0.92 ug/mL:All Events	21,033	***	***	100.00	1,947
CD54 TNBA 0.92 ug/mL:Live Cells	10,000	47.54	***	47.54	1,847
CD86 TNBA 1.1 ug/mL:All Events	18,524	***	***	100.00	4,264
CD86 TNBA 1.1 ug/mL:Live Cells	10,000	53.98	***	53.98	2,806
CD54 TNBA 1.1 ug/mL:All Events	20,747	***	***	100.00	1,717
CD54 TNBA 1.1 ug/mL:Live Cells	10,000	48.20	***	48.20	1,420
CD86 TNBA 1.32 ug/mL:All Events	24,546	***	***	100.00	4,714
CD86 TNBA 1.32 ug/mL:Live Cells	10,000	40.74	***	40.74	2,607
CD54 TNBA 1.32 ug/mL:All Events	26,902	***	***	100.00	1,837
CD54 TNBA 1.32 ug/mL:Live Cells	10,001	37.18	***	37.18	1,433

Toxicology Study No. S.0039820-16, November 2017

Experiment 2- LLM-15 and TNBA
Data Analysis

4/10/2017

	Concentration (mg/mL)	Viability (%) (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
DMSO	0	91.84	722	1798	1	100		1105	1	100	
DNCB Control	0.0033	77.84	913	6163	4.88	487.92		2266	3.53	353.26	
	0.004	78.64	879	5744	4.52	452.14		2517	4.28	427.68	
	0.0048	76.16	878	4972	3.80	380.48		2923	5.34	533.94	
LLM-105	0.0026	89.31	1347	2789	1.34	134.01	0.0033	1847	1.31	130.55	0.0047
	0.0031	90.42	1442	3049	1.49	149.35		2001	1.46	145.95	
	0.0038	87.9	1691	3329	1.52	152.23		2331	1.67	167.10	
	0.0045	84.46	2088	4246	2.01	200.56		2829	1.93	193.47	
	0.0054	76.04	2635	5007	2.20	220.45		3554	2.40	239.95	
	0.0065	71.2	3055	5440	2.22	221.65		3949	2.33	233.42	
	0.0078	55.42	3899	6265	2.20	219.89		4833	2.44	243.86	
	0.0094	27.98	4423	7354	2.72	272.40		5740	3.44	343.86	
TNBA	0.00037	86.73	797	2447	1.53	153.35		1235	1.14	114.36	0.0006
	0.00044	85.8	817	2585	1.64	164.31		1281	1.21	121.15	
	0.00053	77.38	862	2901	1.89	189.50		1589	1.90	189.82	
	0.00064	67.14	932	2974	1.90	189.78		1842	2.38	237.60	
	0.00077	60.79	968	3067	1.95	195.07		2121	3.01	301.04	
	0.00092	51.72	922	2974	1.91	190.71		1847	2.42	241.51	
	0.00110	53.02	898	2806	1.77	177.32		1420	1.36	136.29	
	0.00133	44.13	973	2607	1.52	151.86		1433	1.20	120.10	

Concentration (ug/mL)	RFI	Log2 Conc	Extrap.	ug/mL
0.37	153.3	-1.43	-1.51	0.35
0.444	164.31	-1.17		

MDV Concentration Verification corrected values 4/17/17*

	Concentration (mg/mL)	Viability % (lgG)	FITC lgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
DMSO	0	91.84	722	1798	1	100		1105	1	100	
LLM-105	0.0023	89.31	1347	2789	1.34	134.01	0.0029	1847	1.31	130.55	0.0041
	0.0028	90.42	1442	3049	1.49	149.35		2001	1.46	145.95	
	0.0033	87.9	1691	3329	1.52	152.23		2331	1.67	167.10	
	0.0040	84.46	2088	4246	2.01	200.56		2829	1.93	193.47	
	0.0048	76.04	2635	5007	2.20	220.45		3554	2.40	239.95	
	0.0057	71.2	3055	5440	2.22	221.65		3949	2.33	233.42	
	0.0069	55.42	3899	6265	2.20	219.89		4833	2.44	243.86	
	0.0082	27.98	4423	7354	2.72	272.40		5740	3.44	343.86	
TNBA	0.00014	86.73	797	2447	1.53	153.35		1235	1.14	114.36	0.0002
	0.00017	85.8	817	2585	1.64	164.31		1281	1.21	121.15	
	0.00020	77.38	862	2901	1.89	189.50		1589	1.90	189.82	
	0.00024	67.14	932	2974	1.90	189.78		1842	2.38	237.60	
	0.00029	60.79	968	3067	1.95	195.07		2121	3.01	301.04	
	0.00035	51.72	922	2974	1.91	190.71		1847	2.42	241.51	
	0.00042	53.02	898	2806	1.77	177.32		1420	1.36	136.29	
	0.00050	44.13	973	2607	1.52	151.86		1433	1.20	120.10	

Concentration (ug/mL)	RFI	Log2 Conc	Extrap.	ug/mL
0.14	153.3	-2.84	-2.92	0.13
0.168	164.31	-2.57		

*Samples for concentration verification were not collected at the time of testing, but 24-hours later. LLM-105 is relatively stable, so there was slight degradation, but not enough to affect the outcome. TNBA does degrade quickly, as noted by visual observation of test solutions and by LS-MDV during method development. The rapid degradation and late collection of test material did affect concentration verification. It is shown here for information purposes only. The second TNBA test with rapid collection of the test solution for concentration verification resulted in expected concentrations. With the similar predictions for EC150 and EC200, it is expected that the first test concentration was also as expected.

Toxicology Study No. S.0039820-16, November 2017

Experiment 3- TNBA Raw Data

Experiment: h-CLAT WP-2208 TNBA #3 4-14-17

Statistics					
Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
DMSO Live cells:All Events	10,000	***	***	100.00	846
DMSO Live cells:Live Cells	9,070	90.70	***	90.70	802
Dead Cells:All Events	10,000	***	***	100.00	1,394
Dead Cells:Live Cells	1,784	17.84	***	17.84	1,439
IgG DMSO:All Events	11,034	***	***	100.00	873
IgG DMSO:Live Cells	9,999	90.62	***	90.62	812
IgG DNCB 3.3 ug/mL:All Events	12,372	***	***	100.00	1,111
IgG DNCB 3.3 ug/mL:Live Cells	9,999	80.82	***	80.82	1,006
IgG DNCB 4 ug/mL:All Events	12,617	***	***	100.00	1,125
IgG DNCB 4 ug/mL:Live Cells	9,978	79.08	***	79.08	1,022
IgG DNCB 4.4 ug/mL:All Events	12,964	***	***	100.00	1,060
IgG DNCB 4.4 ug/mL:Live Cells	9,996	77.11	***	77.11	939
IgG TNBA 0.37 ug/mL:All Events	11,992	***	***	100.00	1,021
IgG TNBA 0.37 ug/mL:Live Cells	9,995	83.35	***	83.35	912
IgG TNBA 0.44 ug/mL:All Events	12,783	***	***	100.00	1,122
IgG TNBA 0.44 ug/mL:Live Cells	9,992	78.17	***	78.17	991
IgG TNBA 0.53 ug/mL:All Events	13,699	***	***	100.00	1,197
IgG TNBA 0.53 ug/mL:Live Cells	10,003	73.02	***	73.02	1,042
IgG TNBA 0.64 ug/mL:All Events	14,983	***	***	100.00	1,256
IgG TNBA 0.64 ug/mL:Live Cells	10,000	66.74	***	66.74	1,079
IgG TNBA 0.76 ug/mL:All Events	17,178	***	***	100.00	1,218
IgG TNBA 0.76 ug/mL:Live Cells	9,989	58.15	***	58.15	1,026
IgG TNBA 0.92 ug/mL:All Events	18,746	***	***	100.00	1,319
IgG TNBA 0.92 ug/mL:Live Cells	10,019	53.45	***	53.45	1,110
IgG TNBA 1.1 ug/mL:All Events	18,608	***	***	100.00	1,407
IgG TNBA 1.1 ug/mL:Live Cells	10,001	53.75	***	53.75	1,120
IgG TNBA 1.32 ug/mL:All Events	18,098	***	***	100.00	1,273
IgG TNBA 1.32 ug/mL:Live Cells	10,028	55.41	***	55.41	1,007
CD86 DMSO:All Events	10,975	***	***	100.00	2,399
CD86 DMSO:Live Cells	10,019	91.29	***	91.29	2,188
CD54 DMSO:All Events	11,047	***	***	100.00	1,378
CD54 DMSO:Live Cells	10,000	90.52	***	90.52	1,265
CD86 DNCB 3.3 ug/mL:All Events	12,723	***	***	100.00	5,792
CD86 DNCB 3.3 ug/mL:Live Cells	9,950	78.20	***	78.20	5,330
CD54 DNCB 3.3 ug/mL:All Events	13,263	***	***	100.00	2,602
CD54 DNCB 3.3 ug/mL:Live Cells	9,969	75.16	***	75.16	2,603
CD86 DNCB 4 ug/mL:All Events	13,601	***	***	100.00	6,107
CD86 DNCB 4 ug/mL:Live Cells	10,000	73.52	***	73.52	5,135
CD54 DNCB 4 ug/mL:All Events	13,583	***	***	100.00	2,717
CD54 DNCB 4 ug/mL:Live Cells	9,969	73.39	***	73.39	2,744
CD86 DNCB 4.4 ug/mL:All Events	***	***	***	***	***
CD86 DNCB 4.4 ug/mL:Live Cells	***	***	***	***	***
CD54 DNCB 4.4 ug/mL:All Events	***	***	***	***	***
CD54 DNCB 4.4 ug/mL:Live Cells	***	***	***	***	***
CD86 TNBA 0.37 ug/mL:All Events	12,131	***	***	100.00	3,998
CD86 TNBA 0.37 ug/mL:Live Cells	10,000	82.43	***	82.43	3,237
CD54 TNBA 0.37 ug/mL:All Events	12,224	***	***	100.00	1,797
CD54 TNBA 0.37 ug/mL:Live Cells	9,991	81.73	***	81.73	1,703
CD86 TNBA 0.44 ug/mL:All Events	13,449	***	***	100.00	4,482
CD86 TNBA 0.44 ug/mL:Live Cells	10,000	74.35	***	74.35	3,398
CD54 TNBA 0.44 ug/mL:All Events	13,577	***	***	100.00	2,166
CD54 TNBA 0.44 ug/mL:Live Cells	10,001	73.66	***	73.66	2,060
CD86 TNBA 0.53 ug/mL:All Events	14,435	***	***	100.00	4,591
CD86 TNBA 0.53 ug/mL:Live Cells	10,011	69.35	***	69.35	3,388
CD54 TNBA 0.53 ug/mL:All Events	14,617	***	***	100.00	2,323
CD54 TNBA 0.53 ug/mL:Live Cells	9,994	68.37	***	68.37	2,299
CD86 TNBA 0.64 ug/mL:All Events	16,064	***	***	100.00	5,408
CD86 TNBA 0.64 ug/mL:Live Cells	9,996	62.23	***	62.23	3,627
CD54 TNBA 0.64 ug/mL:All Events	16,350	***	***	100.00	2,475
CD54 TNBA 0.64 ug/mL:Live Cells	10,000	61.16	***	61.16	2,581
CD86 TNBA 0.76 ug/mL:All Events	19,154	***	***	100.00	5,890
CD86 TNBA 0.76 ug/mL:Live Cells	10,061	52.53	***	52.53	3,571
CD54 TNBA 0.76 ug/mL:All Events	19,784	***	***	100.00	2,230
CD54 TNBA 0.76 ug/mL:Live Cells	9,994	50.52	***	50.52	2,383
CD86 TNBA 0.92 ug/mL:All Events	20,549	***	***	100.00	5,993
CD86 TNBA 0.92 ug/mL:Live Cells	9,998	48.65	***	48.65	3,500
CD54 TNBA 0.92 ug/mL:All Events	21,101	***	***	100.00	2,332
CD54 TNBA 0.92 ug/mL:Live Cells	10,019	47.48	***	47.48	2,458
CD86 TNBA 1.1 ug/mL:All Events	20,751	***	***	100.00	5,790
CD86 TNBA 1.1 ug/mL:Live Cells	10,113	48.74	***	48.74	3,300
CD54 TNBA 1.1 ug/mL:All Events	20,461	***	***	100.00	2,013
CD54 TNBA 1.1 ug/mL:Live Cells	10,042	49.08	***	49.08	1,860
CD86 TNBA 1.32ug/mL:All Events	20,219	***	***	100.00	6,350
CD86 TNBA 1.32ug/mL:Live Cells	9,486	46.92	***	46.92	3,052
CD54 TNBA 1.32 ug/mL:All Events	20,259	***	***	100.00	1,809
CD54 TNBA 1.32 ug/mL:Live Cells	10,006	49.39	***	49.39	1,559

Experiment 3- TNBA

Data Analysis

4/14/2017

	Concentration (mg/mL)	Viability (%) (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
DMSO	0	90.62	812	2188	1	100		1265	1	100	
DNCB Control	0.0033	80.82	1006	5792	3.48	347.82		2603	3.53	352.54	
	0.004	79.08	1022	5135	2.99	298.91		2744	3.80	380.13	
	0.0048	77.11	939	Skip*				Skip*			
TNBA	0.00037	83.35	912	3237	1.69	168.97		1703	1.75	174.61	0.0004
	0.00044	78.17	991	3398	1.75	174.93		2060	2.36	235.98	
	0.00053	73.02	1042	3388	1.70	170.49		2299	2.77	277.48	
	0.00064	66.74	1079	3627	1.85	185.17		2581	3.32	331.57	
	0.00077	58.15	1026	3571	1.85	184.96		2383	3.00	299.56	
	0.00092	53.45	1110	3500	1.74	173.69		2458	2.98	297.57	
	0.00110	53.75	1120	3300	1.58	158.43		1860	1.63	163.36	
	0.00133	55.41	1007	3052	1.49	148.62		1559	1.22	121.85	

*Skipped DNCB #3 as the two lower doses were already positive for both CD86 and CD54, so unnecessary as a positive control

Concentration (ug/mL)	RFI	Log2 Conc	Extrap.	ug/mL
0.37	168.97	-1.43	-2.27	0.21
0.444	174.93	-1.17		

MDV Concentration Verification corrected
values 4/17/17*

	Concentration (mg/mL)	Viability (%) (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
DMSO	0	90.62	812	2188	1	100		1265	1	100	
DNCB Control	0.0033	80.82	1006	5792	3.48	347.82		2603	3.53	352.54	
	0.004	79.08	1022	5135	2.99	298.91		2744	3.80	380.13	
	0.0048	77.11	939	Skip*				Skip*			
TNBA	0.00040	83.35	912	3237	1.69	168.97		1703	1.75	174.61	0.0004
	0.00048	78.17	991	3398	1.75	174.93		2060	2.36	235.98	
	0.00058	73.02	1042	3388	1.70	170.49		2299	2.77	277.48	
	0.00069	66.74	1079	3627	1.85	185.17		2581	3.32	331.57	
	0.00083	58.15	1026	3571	1.85	184.96		2383	3.00	299.56	
	0.00100	53.45	1110	3500	1.74	173.69		2458	2.98	297.57	
	0.00119	53.75	1120	3300	1.58	158.43		1860	1.63	163.36	
	0.00143	55.41	1007	3052	1.49	148.62		1559	1.22	121.85	

*Skipped DNCB #3 as the two lower doses were already positive for both CD86 and CD54, so unnecessary as a positive control

Concentration (ug/mL)	RFI	Log2 Conc	Extrap.	ug/mL
0.4	168.97	-1.32	-2.16	0.22
0.48	174.93	-1.06		