# Environmental Hazard *in Vitro* Biomarker Discovery Tools

**MAJ Kelly M. Halverson[1], Ph.D., Dr. John A. Lewis[1], Dr. David A. Jackson[1], Dr. Jack Chen[2], Mr. William Dennis[1], Ms. Linda Brennan[1], Ms. Christine Baer[3], Mr. Matthew Permenter[3], Dr. Teresa Krakauer[4]**

[1] U.S. Army Center for Environmental Health Research
568 Doughten Drive, Fort Detrick, MD 21702, USA

[2] Advanced Biomedical Computing Center, SAIC-Frederick
Bldg. 430, Miller Street, Frederick, MD 21702, USA

[3] SAIC, U.S. Army Center for Environmental Health Research
568 Doughten Drive, Ft Detrick, MD 21702, USA

[4] U.S. Army Medical Research Institute of Infectious Diseases
1425 Porter Street, Fort Detrick, MD 21702, USA

## 1.0 Summary

Individuals may respond to environmental hazards quite differently due to numerous factors, including proximity to the toxicant, the individual's size, health, past exposures, and heredity. Therefore, exposure monitoring must take these variations in individual response into account. While environmental sampling equipment provides an important indication of a toxicant's presence, it does not monitor individual responses. The development of biomarkers of exposure, effect, and susceptibility (1) is a priority at the U.S. Department of Defense Health Affairs in the area of occupational environmental health surveillance (OEHS). Not only do biomarkers offer the opportunity to quantify toxic exposures, but also to identify possible future adverse health effects by regular or contingent testing before, during, and after deployments. In an effort to rapidly advance biomarker discovery the U.S. Army Center for Environmental Health Research (USACEHR) is working to develop new methods combining multiple approaches. This report demonstrates the feasibility of incorporating *in vitro* cellular models with label free quantitative proteomic screening technologies, functional genomics and an enzyme-linked immunosorbent assay (ELISA) as biomarker discovery tools. This multi-tiered approach advances not only the knowledge about the effects of trinitrotoluene (TNT) and dinitrobenzene (DNB), but may also identify future biomarkers of exposure and/or effect.

## 2.0 Introduction

The diversity and complexity of global missions that our Soldiers, Sailors, Airmen and Marines undertake today has increased the need for adequate Force Health Protection from potential occupational health risks. Exposure of our military personnel to Toxic Industrial Chemicals (TICs), Toxic Industrial Materials (TIMs), and Military Relevant Chemicals (MRCs) can cause adverse health effects and subsequently affect mission readiness. However, there are few tools for monitoring and diagnosing TIC/TIM/MRC exposures and effects. As an initial step in assessing exposures and/or effects, the U.S. Army Center for Environmental Health Research, in collaboration with the U.S. Army Medical Research Institute of Infectious Diseases, has developed an *in vitro* biomarker discovery model. Biomarkers, which are defined as

Halverson, K.M.; Lewis, J.A.; Jackson, D.A.; Chen, J.; Dennis, W.; Brennan, L.; Baer, C.; Permenter, M.; Krakauer, T. (2007) Environmental Hazard *in Vitro* Biomarker Discovery Tools. In *Defence against the Effects of Chemical Hazards: Toxicology, Diagnosis and Medical Countermeasures* (pp. 25-1 – 25-10). Meeting Proceedings RTO-MP-HFM-149, Paper 25. Neuilly-sur-Seine, France: RTO. Available from: http://www.rto.nato.int.

physiological or biochemical changes indicative of exposure and/or effect to a toxicant, are tools that can be used as an important component of a robust occupational environmental health surveillance program.

Currently two cell systems, human peripheral blood mononuclear cells (PBMC) and the human hepatocellular carcinoma-derived cell line (HepG2) are being evaluated for their responses to environmental toxicant exposures. The PBMC are an ideal choice for an *in vitro* biomarker discovery tool since they are a component of an easily accessible biofluid (blood), which is an important biomarker requirement. Whereas, the HepG2 are an established hepatotoxicity model and the liver is the primary organ of detoxification. In order to develop potential biomarkers of exposure and/or effect from these cellular models, we have utilized and integrated three distinct technologies, evaluated the subsequent data, and assessed the validity of the responses. The technologies utilized were quantitative proteomics using ion intensity, functional genomics with Affymetrix® microarrays, and enzyme-linked immunosorbent assays (ELISA) which determines the proinflammatory cytokine and chemokine responses of the human PBMC.

The results of the cellular models exposure to toxicants such as TNT and DNB will be presented. Evaluation of the data generated from a 24 hour PBMC exposure to TNT and DNB identified proteins with at least a 1.5 fold quantitative difference when the exposed vs. unexposed cells were compared. Interestingly, the cytokine and chemokine responses of the human PBMC suggest that both toxicants may be immunosuppressants. The human liver-derived HepG2 cells were also exposed to TNT and DNB and protein and mRNA were prepared from these samples for proteomic and genomic analysis. These data demonstrated that proteins and nucleic acid changes were detectable after a 24 hour toxicant exposure in the HepG2 cellular model.

This study presents the application of advanced proteomic and functional genomic screening methods combined with *in vitro* human cellular models. The data presented will demonstrate the potential for advancing environmental hazard biomarker discovery research to improve occupational environmental health surveillance for our Soldiers, Sailors, Airmen and Marines.

## 3.0 Methods

The methods described here advanced the *in vitro* biomarker discovery project at USACEHR. One of the key requirements for a clinically useful biomarker in humans is that it should be easily accessible, such as in serum, plasma, urine or saliva. For this reason, human PBMC are an attractive choice for exposure response investigations. Additionally, in an effort to identify markers indicative of general toxicity, HepG2 cells were also exposed to varying concentrations of TNT and DNB to investigate cellular responses for potential future monitoring. The proposed *in vitro* strategy takes a three tiered approach to characterizing new biomarkers, discovering novel protein and mRNA biomarkers and assessing cytokine/chemokine expression levels. All will be discussed here.

### 3.1 Peripheral Blood Mononuclear Cell Exposures

Human PBMC were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from randomly selected, healthy donors. PBMC were cultured in 6-well plates at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at a concentration of $10^6$ cells/mL. Cells were either quiescent or stimulated with Staphylococcal Enterotoxin B (SEB) (300 ng/mL) immediately after addition of either TNT or DNB. The concentrations of both TNT and DNB were set at 5, 50, and 500 times the Military Exposure Guideline (MEG) concentrations. This equates to 0.3, 3.0, and 30 mg/L for TNT, and 0.15, 1.5, and 15 mg/L for DNB. Controls included both quiescent and SEB stimulated PBMC in FCS

supplemented DMEM. Following a 22-24 hr exposure, the medium was removed and centrifuged to remove suspended cells. The supernatants were analyzed to determine concentrations of TNF-α, IL-6, IL-2, IFN-γ, MCP-1, MIP-1α, and MIP-1ß by enzyme-linked immunosorbent assay (ELISA) with cytokine- or chemokine-specific antibodies, as previously described (2, 3). Human recombinant cytokines and chemokines (20 to 1,000 pg/ml) were used as calibration standards for each plate. The detection limit for these assays was 20 pg/mL.

After removal of the supernatants, the attached cells were harvested, pooled with the suspended cell pellet obtained after centrifugation of the supernatants, washed twice with Phosphate Buffered Saline (PBS), and lysed using the QIAshredder and Qiagen RNeasy Kit (Qiagen Inc., Valencia, CA). The RNA and protein were separately isolated from the lysate according to the manufacturer's recommendations. The isolated protein was digested with trypsin, and the resulting peptides were analyzed by liquid chromatography/ mass spectrometry ($LC/MS^E$) on a Waters quadrupole time-of-flight (Q-TOF) Premier mass spectrometer coupled to a NanoAcquity ultra performance liquid chromatography (UPLC) system (Waters Corp., Milford, MA).

### 3.2 HepG2 Hepatoma Cell Exposure

HepG2 cells, passage 76, were obtained from the American Type Culture Collection (Manassas, VA) and maintained in T75 flasks at 37°C in DMEM (500 mL) with 4.5 g/L glucose, 2 mM L-glutamine supplemented with 10% Fetal Bovine Serum (FBS), 20 mM HEPES Buffer, and 100 units Penicillin and 100 µg Streptomycin.

HepG2 cells were seeded in 6-well plates, fresh complete DMEM culture media was added, and the plates were incubated for 48-72 hr until a confluent monolayer was obtained. Prior to toxicant exposure, the complete medium was removed by aspiration and replaced with serum-free, antibiotic-free DMEM with 4.5 g/L glucose and 2 mM L-glutamine. The cells were incubated for at least 1 hr at 37°C to ensure cell stability prior to toxicant exposure. The culture medium was again aspirated and replaced with serum-free, antibiotic-free DMEM with 4.5 g/L glucose and 2 mM L-glutamine without (controls) or with 0.3, 3.0, and 30 mg/L TNT, or 0.15, 1.5, and 15 mg/L DNB. After exposing the cells for 22-24 hr, they were washed twice with PBS, harvested, and lysed using the RNeasy Kit. The RNA and proteins were isolated from the lysate according to manufacturer instructions. RNA quality and quantity were determined by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). After tryptic digestion of the isolated protein, the resulting peptides were analyzed by $LC/MS^E$ on a Waters Q-TOF Premier mass spectrometer coupled to a NanoAcquity UPLC system.

### 3.3 Mass Spectrometry

Following tryptic digestion, the peptides were separated with reverse phase chromatography using a Waters NanoAcquity UPLC coupled to a Waters Q-TOF Premier Mass Spectrometer.

| Table 1. Time Table Program for the Analytical Separation of TNT and DNB Peptides | | | |
|---|---|---|---|
| NanoAcquity Pump Program | | | |
| Time (minutes) | Solvent | | Flow Rate µL/min |
| | 0.1% Formic acid in Water | 0.1% Formic acid in Acetonitrile | |
| | Percentage of Composition | | |
| 0 | 99 | 1 | 1.2 |
| 1 | 99 | 1 | 1.2 |
| 101 | 70 | 30 | 1.2 |
| 105 | 50 | 50 | 1.2 |
| 106 | 85 | 15 | 1.2 |
| 111 | 85 | 15 | 1.2 |
| 112 | 99 | 1 | 1.2 |
| Stop time 140 minutes | 99 | 1 | 1.2 |

The peptides for the TNT and DNB exposures were trapped using a Waters Symmetry C18 180 µm X 20 mm, 5 µm particle size column using a 5 µL/min flow rate for 6 minutes using 0.1% formic acid. The analytical separations for DNB and TNT were performed using a Waters NanoAcquity UPLC column BEH 130 C18 100 µm X 100 mm, 1.7 µm particle size. The gradient table for this separation is shown in Table 1. The injection volume was 9.9 µL and the analytical column was maintained at 35°C.

Peptides were detected using a Waters Q-TOF Premier. The Q-TOF premier is a quadrupole, orthogonal acceleration time-of-flight tandem mass spectrometer. The peptides were ionized using electrospray ionization in positive ion mode. Data was collected over the 50-1900 m/z range. Data for the high and low energy scans were collected for 0.8 seconds. An external lock mass was used for calibrating mass accuracy. A lockmass reading was taken every 30 seconds using a 1 second scan.

### 3.4 Database Searches

Raw data was extracted and database searches were performed using Protein Lynx Global Server 2.2 (PLGS2.2; Waters Corporation, Milford, MA). The database used was created from the entire National

Center for Biotechnology Information (NCBI) human Reference Sequence (RefSeq) protein database (www.ncbi.nlm.nih.gov) supplemented with possible contaminating proteins, including human keratins, bovine serum albumin (BSA), and trypsin. Random peptide sequences greater than or equal in number to the RefSeq data were added to the database as a control for false positives.

### 3.5 Microarray Analysis

Changes in mRNA levels due to toxicant exposure were determined using the Affymetrix® GeneChip® Human Genome U133 Plus 2.0 Array. This array allows analysis of the expression level of 38,500 human genes and over 47,000 transcripts and variants. Target preparation and labeling of ~ 3 µg of total RNA was completed using Affymetrix's® GeneChip® One-Cycle Target Labeling and Control Reagents following the manufacturers instructions. A one way ANOVA using JMP® software (SAS Inc., Cary, NC) was used to test the results and the reported results have a p-value of < 0.5 and a > 2.0 fold quantitative difference when comparing exposed to unexposed samples. Data mining was performed using Partek Pro® 6.0 statistical analysis and data visualization software (Partek, Inc, St. Louis, MO). Biological pathway analysis was performed using MetaCore® software (GeneGo Inc., St. Joseph, MI).

## 4.0 Results

### 4.1 PBMC proinflammatory cytokine and chemokine response

The PBMC exposures examine the possible immunosuppressive effects of toxicants by stimulating PBMC simultaneously with a superantigen, SEB. SEB stimulates PBMC by binding to MHC class II and T-cell-receptors resulting in production of inflammatory cytokines. The results of cytokine and chemokine measurements in the supernatant of SEB plus toxicant exposed PBMC are shown in figures two and three. For both the TNT (Fig. 1) and the DNB (Fig. 2) exposures, we assayed the concentrations of the cytokines TNFα, IL-6, IFNγ, IL-2, MCP-1, MIP-1α and MIP-1β by ELISA.

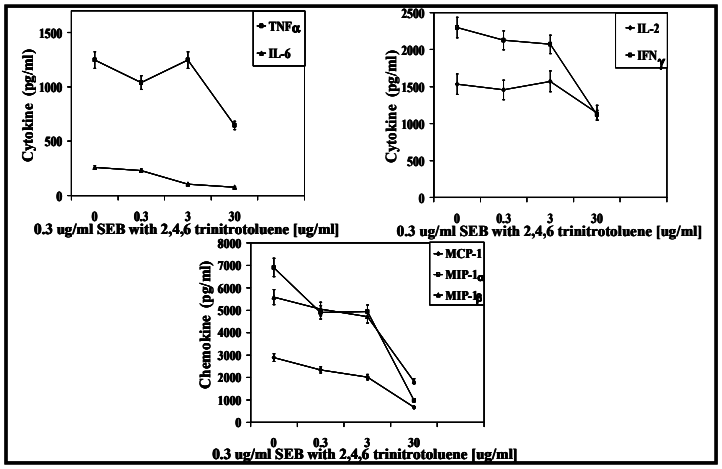


**Fig. 1** – SEB-stimulated PBMC proinflammatory response following exposure to TNT. PBMC from one donor stimulated with 0.3 µg/mL SEB were exposed to 0.0, 0.3, 3.0, and 30 mg/L TNT for 22-24 hr.

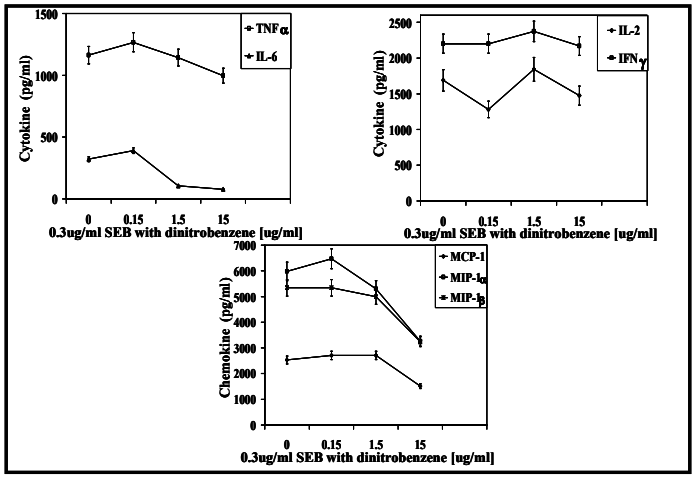


**Fig. 2** – SEB-stimulated PBMC proinflammatory response following exposure to DNB. PBMC from one donor stimulated with 0.3 μg/mL SEB were exposed to 0.0, 0.15, 1.5, and 15 mg/L DNB for 22-24 hr.

Since neither TNT nor DNB stimulated PBMC to produce proinflammatory cytokines and chemokines, PBMC were stimulated with SEB to investigate the effects of toxicant exposure on activated cells. The results of TNT and DNB exposure on SEB-stimulated PBMC cytokine and chemokine responses are shown in Fig. 1 and Fig. 2 respectively. Each assayed cytokine appears to decrease in concentration with increasing toxicant concentration. This is most apparent for the chemokines. This could suggest that both TNT and DNB inhibit the PBMC proinflammatory response.

### 4.2 PBMC TNT Exposure Protein Analysis

Preliminary analysis of the peptide identifications obtained from the mass spectrometry data from at least three separate replicates resulted in the identification 225 intra-cellular proteins from the TNT exposed PBMC. 62 of these proteins were identified in all replicates from each of the four exposure conditions. An abundance change of at least 1.5 fold following exposure was seen in four proteins. There were four proteins identified which were found only in samples treated with either 50 or 500x the TNT MEG. A comparison of the control sample to the high TNT dose identified two proteins that were not found in TNT treated cells.

### 4.3 HepG2 Hepatoma mRNA Analysis

Analysis of the microarray data identified 544 genes with a greater than two-fold change in expression level due to TNT exposure when a comparison to the control is made. Analysis of the DNB exposure data resulted in the identification of 343 genes. In order to reduce the data set complexity and identify changes to both TNT and DNB, a comparison of the two gene lists was performed using Partek, generating the Venn diagram shown in Figure 3. As seen in Figure 3, 32 genes are common to both TNT and DNB exposure. From this, a list of genes was generated also using Partek (*data not shown*), in order to further compare TNT and DNB exposure.

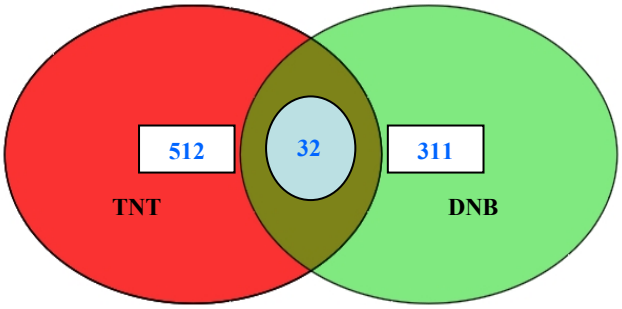


**Fig. 3** - Comparison of gene lists generated from Affymetrix microarray analysis

A challenge in microarray analysis is assigning biological context to gene expression differences in a dataset and in this case due to an *in vitro* exposure to an environmental hazard. In order to accomplish this we used the process and pathway mapping software MetaCore®. This tool identified the top 10 processes affected by the 32 common genes with expression differences due to TNT and DNB exposure (Figure 4). The generated list did not identify any genes specific to any particular dose. In fact, most were found to either increase or decrease in correlation with the doses. As can be seen from the list, signaling pathways and pro-inflammatory response processes seem to be affected by this common gene list.

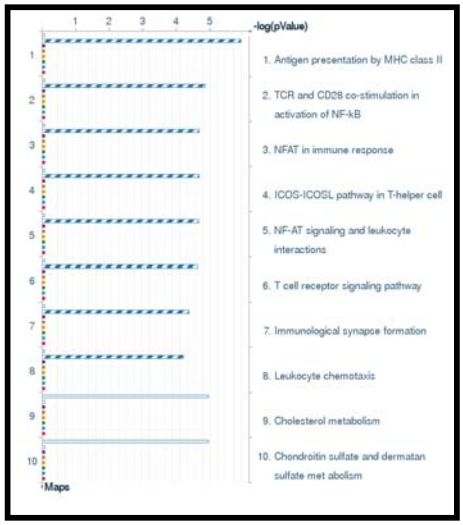


**Fig. 4** – MetaCore top 10 GeneGo processess affected by the 32 identified genes with quantifiable differences in both TNT and DNB

Figure 5 depicts the multiple pathways and networks that interact with the MHC class II antigen presentation pathway appearing in the process list. Furthermore, this detailed map provides additional biological context to the expression differences identified in the initial data analysis and also shows the dose response of the MHC Class II expression. The dose responses are depicted with the thermometers next to the MHC Class II symbol. Thermometers 1-3 represent the increasing TNT doses and 4-6 represent the increasing DNB doses.

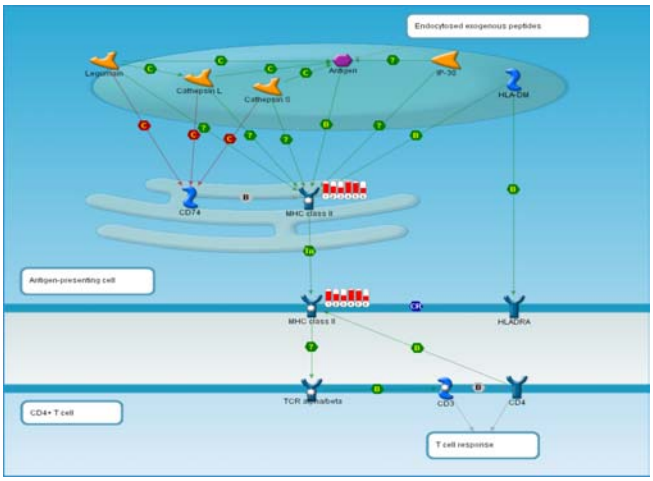


**Fig. 5** – Metacore mapping of antigen presentation by MHC class II receptor

## 5.0 Conclusions

The data presented here-in strongly support the use of a multi-tiered *in vitro* approach to advance biomarker discovery. This approach takes advantage of the strengths of each technique and minimizes the weaknesses by applying biological context to the lists of both mRNA and protein differences identified following exposure to an environmental toxicant. The incorporation of cellular models with quantitative proteomics, functional genomics and pro-inflammatory response patterns have the potential to aid in the identification of biomarkers indicative of environmental toxicant exposure and the possible effects of these exposures on the warfighter. In addition, we show that quantitative proteomics using ion intensity and functional genomics using a whole human genome microarray are feasible methods for identification and quantification of protein and mRNA differences in exposed versus unexposed cells. Lastly, our preliminary results suggest that both TNT and DNB may suppress SEB-stimulated cytokines and chemokines, and that these toxicants may act as immunosuppressants and affect the pro-inflammatory pathways of other cells as suggested by HepG2 data.

## 6.0 Acknowledgements

The research described was sponsored in part by the Department of Defense 2006 and 2007 In-House Laboratory Independent Research (ILIR) Program administered through the U.S. Army Medical Research Institute of Chemical Defense (USACEHR 2007 ILIR: *In vitro* Cell Model for Identification and Characterization of Biomarkers of Exposure and/or Effect to TICs/TIMs/ MRCs) and the Defense Threat Reduction Agency (Research Plan, DTRA #D_X007_04_RD_B). We would like to thank Lieutenant Colonel Matthew J. Schofield, Dr. William H. van der Schalie, and Dr. Thomas P. Gargan II for their comments and review of this manuscript.

## 7.0 References

1. **ASD (HA) Memorandum**, Subject: Enhanced Science and Technology Support for Occupational and Environmental Health Surveillance (OEHS), June 2003.

2. **Krakauer, T.** 1994. Inhibition of toxic shock syndrome toxin-induced cytokine production and T cell activation by interleukin 10, interleukin 4, and dexamethasone. J. Infect. Dis. **172:**988-992.
3. **Krakauer, T.** 1999. Induction of CC chemokines in human peripheral blood mononuclear cells by staphylococcal exotoxins and its prevention by pentoxifylline. J. Leukoc. Biol. **66:**158-164.

**8.0 Disclaimers**

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army. Citations of commercial organizations or trade names do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.