

Gamma-Tocotrienol, A Radiation Countermeasure, Reverses Proteomic Changes In Serum Following Total-Body Gamma Irradiation In Mice

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Research Article

Keywords: Biomarkers, countermeasure, gamma-tocotrienol, mice, proteomics, radioprotection, serum

Posted Date: December 2nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1096765/v1>

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Version of Record: A version of this preprint was published at Scientific Reports on March 1st, 2022. See the published version at <https://doi.org/10.1038/s41598-022-07266-5>.

Abstract

Radiological incidents or terrorist attacks would likely expose civilians and military personnel to high doses of ionizing radiation, leading to the development of acute radiation syndrome (ARS). While the effects and symptoms of high dose radiation exposure have been established, prophylactic treatments and methods for determining the extent of exposure remain limited. We examined the effectiveness of prophylactic administration of a developmental radiation countermeasure, γ -tocotrienol (GT3), in a total-body irradiation (TBI) mouse model. CD2F1 mice received GT3 24 h prior to 11 Gy cobalt-60 gamma-irradiation, a supralethal dose for this strain. Two-dimensional differential in-gel electrophoresis was followed by mass spectrometry and Ingenuity Pathway Analysis (IPA). Analysis revealed a change in expression of 18 proteins in response to TBI, and these changes were reversed with prophylactic treatment of GT3. IPA revealed a network of associated proteins involved in cellular movement, immune cell trafficking, and inflammatory response. Of particular interest, significant expression changes in beta-2-glycoprotein 1, alpha-1-acid glycoprotein 1, alpha-2-macroglobulin, complement C3, mannose-binding protein C, and major urinary protein 6 were noted after TBI and reversed with GT3 treatment. This study reports the network and specific serum proteins which could be translated as biomarkers of both radiation injury and protection by radiation countermeasures.

1. Introduction

Intended or unintended exposure to high dose radiation exposure can result in devastating health consequences¹. Hence, radiation medical countermeasures are an urgent and unmet need². Acute radiation syndrome (ARS) occurs in humans following total-body or partial-body exposures to ionizing radiation with doses above 1 Gy at a high dose rate. ARS includes the hematopoietic (H-ARS; 2 – 6 Gy), gastrointestinal (G-ARS; 6 – 8 Gy), and the neurovascular (>8 Gy) sub-syndromes³. The neurovascular sub-syndrome is considered untreatable even with full supportive care and countermeasures⁴. Individuals exposed to radiation doses resulting in H-ARS and GI-ARS can respond to radiation countermeasures. Therefore, radiation countermeasures are being developed for these two sub-syndromes of ARS⁵.

Over the last six decades, efforts have been made to develop medical radiation countermeasures for ARS. However, in the U.S., only four agents are approved by the Food and Drug Administration (FDA) for H-ARS^{6–16}. All four agents are radiomitigators for use *after* radiation exposure. To date, there is no approved radioprotector that can be used *prior* to radiation exposure for protection from the harmful effects of radiation exposure. Many natural products have been evaluated for the prevention and treatment of diseases and compared to their synthetic counterparts in preclinical and clinical studies^{17,18}. Vitamin E is a family of antioxidant agents which regulate peroxidation reactions and scavenge free-radicals^{19,20}. Vitamin E has eight different isoforms belonging to two subgroups: four saturated tocopherols (α , β , γ , and δ) and four unsaturated tocotrienols. These eight agents are collectively known as tocopherols. Several *in vitro* studies have demonstrated that tocotrienols are mechanistically stronger antioxidants than tocopherols^{18,21–26}.

γ -Tocotrienol (GT3) is an antioxidant and an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, and a component of vitamin E^{27,28}. It is under advanced development as a radioprotective countermeasure that can be used for prophylaxis²⁹. GT3 has been demonstrated in several studies to increase survival against lethal doses of radiation in murine model when administered prior to total-body irradiation, and its dose reduction factor has been estimated to be 1.29³⁰. GT3 has also been shown to accelerate hematopoietic recovery in murine as

well as nonhuman primate model³⁰. GT3 treatment has led to increased bone marrow hematopoietic progenitors in CD2F1 mice exposed to gamma-radiation³¹. Furthermore, GT3 induced high levels of some cytokines and growth factors, particularly, granulocyte colony-stimulating factor (G-CSF). The administration of G-CSF antibody abolished its radioprotective efficacy³². In a previously published study, GT3 and another tocol, α -tocopherol succinate, were found to reverse jejunum protein expression changes in an H-ARS model³³. Furthermore, GT3 has been shown to have radioprotective activity in nonhuman primates when administered 24 h prior to 5.8 Gy and 6.5 Gy TBI³⁴.

Biomarkers may be used to measure the absorbed radiation dose and the drug dose conversion from animal models to humans. Thus, it is critical to identify, validate, and quantify biomarkers^{35,36}. Several countermeasures are under advanced development following the US FDA Animal Rule^{36,37}. Hence, it is vital to identify biomarkers for countermeasure efficacy that can be helpful to convert drug doses from animal model studies to those that are applicable when used in clinical settings³⁷⁻³⁹.

The differential protein expression in serum samples collected from mice treated with GT3 and exposed to total-body γ -radiation were analyzed. The serum samples were used for two-dimensional differential in-gel electrophoresis (2D-DIGE) coupled with mass spectrometry and advanced bioinformatics tools. Our objective for this study was to understand the specific biological pathways and networks regulating the radiation response and the molecular mechanism(s) underlying GT3 radioprotection. We observed changes in the expression of 18 proteins in response to irradiation, and these changes were reversed as a result of prophylaxis with GT3. Such proteins could be translated as biomarkers for radiation injury and GT3 efficacy and warrant further studies.

2. Results

The purpose of this study was to report the comprehensive changes in mouse serum protein expression by GT3, a promising radiation countermeasure. The molecular mechanism of this radioprotective agent was also investigated by considering the pathways and networks involved. The mice were prophylactically treated with GT3 or vehicle 24 h prior to supralethal TBI of 11 Gy. This TBI dose was selected because it is known to lead to gastrointestinal injury (GI-ARS). After 24 h post-irradiation, serum samples were collected. The 2D-DIGE method was employed to investigate changes in protein expression. Spot detection software was used to select 100 – 200 spots with isoelectric points ranging between pI 4 and 9 and molecular weights ranging from 10 to 150 kDa for trypsin digestion from the approximately 2,500 proteins of interest from each gel (Fig. 1). The analyses identified proteins using MALDI-TOF/TOF and the MASCOT search engine (Fig. 2).

The gel analysis software identified 132 unique spots, of which 78 protein identities were determined. Of this cohort, 53 spots of these proteins exhibited a 1.5-fold or greater change in expression when comparing the serum samples of unirradiated animals to those from the TBI groups. The 53 spots represented 23 unique proteins because there were multiple instances of the same protein, likely the result of potential post-translational modifications. From these 23 proteins, 19 of the appearances reflected either a consistent increase or decrease in expression in response to TBI with respect to the unirradiated control group. Finally, 18 of these 19 proteins reflected an expression reversal when compared to the prophylactic GT3-treated group.

There were 18 proteins detected in the serum samples of the mice that received TBI and prophylactic treatment with GT3 that showed a marked change in expression which was reversed compared to non-prophylactic treatment with GT3, indicating a potentially protective effect of GT3 treatment (Fig. 3). Protein expression ratio changes are

presented with a color scale to indicate increase (red) or decrease (green). One protein, beta-2-glycoprotein 1, showed a decrease in expression while the remaining 17 proteins exhibited an increase in expression in response to TBI with respect to the unirradiated group. With GT3 prophylactic treatment, beta-2-glycoprotein 1 showed an increase in expression while the remaining 17 proteins exhibited a decrease in expression, reversing the initial observation and indicating a treatment specific effect.

The profile of serum proteins showed a change in expression in response to TBI with respect to the unirradiated group and then a corresponding reversal with GT3 prophylactic treatment were offered to the IPA software program to determine potential pathways and a chart a network based upon previously reported protein relationships (Fig. 4). The network suggested by IPA identified a number of potential relationships particularly associated with immune response involving interleukins, transcription factors, receptors, and apoptosis-related proteins.

3. Discussion

Proteomic analysis has proven to be an essential tool for dissecting the mechanisms contributing to survival following exposure to ionizing radiation⁴⁰. While tocols have shown the potential for radioprotection against ARS in nonhuman primate studies, the underlying mechanisms and pathways of protection are not well understood^{30,41}. The present study was designed to probe the serum proteins circulating following TBI and contribute new evidence of the specific proteins and networks involved in the radioprotection of GT3 treatment.

A total of 18 proteins found in the serum were determined to show a change in expression following TBI which was reversed with GT3 prophylactic treatment. Only one protein, beta-2-glycoprotein 1, showed a decrease in expression with TBI compared to the unirradiated group. Beta-2-glycoprotein 1, also known as ApoH, is a phospholipid binding protein which has anti-oxidant properties⁴². While the present study found beta-2-glycoprotein 1 declined with 11 Gy exposure, interestingly, Rithidech *et al.* found an increase in expression with a sub-lethal exposure of 3 Gy at 48 h⁴³. Of course, it is not surprising that the proteomic profile is temporally dynamic and dose dependent, making the case for further investigation into time course and irradiation dose to enhance our understanding of the proteomic dynamics which can inform biomarker identification.

The remaining 17 proteins identified showed an increase in expression following 11 Gy radiation. Nine of these proteins were also found to be elevated in expression by Rithidech *et al.* at 48 h following 3 Gy irradiation including alpha-1-acid glycoprotein 1, alpha-2-macroglobulin, clusterin, complement C3, hemopexin, haptoglobin, kininogen-1, mannose-binding protein C, and major urinary protein 6⁴³. While we found alpha-1-antitrypsin 1-1 to be elevated at 24 h, Rithidech *et al.* and colleagues found expression decreased after one week with respect to the unirradiated control⁴³.

Fu *et al.* found serum amyloid A, interleukin (IL)-22, urokinase, resistin, and IL-6 to be good predictors of mortality in a mouse model⁴⁴. This study did not distinguish between the specific forms of serum amyloid A, but our study also revealed changes in serum amyloid A-2. While our study did not uncover the other proteins identified by Fu *et al.*, it is likely that low abundant interleukins would be less likely to be detected by the 2-D gel method. Another recent study observing long term exposure to low-dose radiation found interleukin IL-5, IL-12p40, P-selectin, and serum amyloid A1 to be good predictors of low-dose radiation exposure with biodosimetric potential⁴⁵.

The proteins identified in our study can be grouped into binding proteins, inflammatory response proteins, coagulation proteins/factors, protease inhibitors, and proteins involved in metabolism and growth. The binding

proteins include beta-2-glycoprotein 1, hemopexin, haptoglobin, mannose-binding protein C, and major urinary protein 6. It should be noted that these binding proteins also play a role in inflammatory response⁴³.

The inflammatory response proteins identified include alpha-1-acid glycoprotein 1, alpha-1-antitrypsin 1-1, inter alpha-trypsin inhibitor, heavy chain 4, serum amyloid A-2 protein, and haptoglobin. Interestingly, both alpha-1-acid glycoprotein 1 and serum amyloid A-2 protein have recently been reported as potential biomarkers of radiation injury, and these protein expression levels were reversed in our study with prophylactic GT3 treatment⁴⁶. Seven of the proteins identified in our study can be classified as coagulation-related proteins. These members include alpha-2-macroglobulin, serum albumin, beta-2-glycoprotein 1, clusterin, hemopexin, haptoglobin, and kininogen-1. Of particular note is alpha-2-macroglobulin, which was recently reported as a potential radioprotectant in the esophagus in patients receiving thoracic radiotherapy⁴⁷. Further evidence of radioprotection was previously reported in a rat model where the animals received 6.7 Gy X-ray TBI⁴⁸⁻⁵⁰.

Another group of proteins, the protease inhibitors, appeared to actively respond to irradiation injury, which was reversed with GT3 treatment. This group includes alpha-1-acid glycoprotein 1, alpha-1-antitrypsin 1-1, alpha-2-macroglobulin, and kininogen-1. It is important to note that we have demonstrated earlier that GT3 also restores gamma-irradiation-induced perturbations of miRNA (miR-30a-5p, miR-126-5p and miR-375) as well as metabolites (carnitine, propionylcarnitine, xanthine, and creatine) in a rhesus macaque nonhuman primate model^{51,52}.

Other proteins detected fall under the metabolism and growth category. These include epidermal growth factor receptor, leucine-rich alpha-2-glycoprotein precursor, and keratin, type II cytoskeletal 73. Finally, we can classify two of the proteins identified as members of the complement system. These two proteins include complement factor B and complement C3.

It should be noted that there are study design limitations of this proteomic analysis. While the 2-D DIGE/MS approach resolves the multitude of data points, the limited throughput does not easily allow for evaluating multiple variables. Our results indicate further studies of variable irradiation dose, GT3 dose, and time points are warranted. Moreover, additional biological replicates can enhance the statistical significance of these findings. Additionally, we decided not to remove abundant serum proteins for this study, but this added step may be helpful in resolving proteomic changes of less prominent proteins.

Many of the proteins detected in the present study are known to be glycosylated. These proteins include alpha-1-acid glycoprotein 1, alpha-1-antitrypsin 1-1, alpha-2-macroglobulin, beta-2-glycoprotein 1, kininogen-1, hemopexin, and haptoglobin. While this study does not report on the glycosylation status of the serum proteins identified, this may be an interesting area of further inquiry. Changes in sialylation of glycoproteins following TBI and serum N-glycome following local irradiation exposure in mouse serum have been reported^{53,54}. Site-specific glycosylation of seven major plasma proteins in response to partial-body irradiation therapy in cancer patients was recently reported⁵⁵. This group included beta-2-glycoprotein 1, a protein found in the present study to show a reversible change in expression with GT3 treatment. However, the extent of the expression and time course exhibited variability between subjects. Clerc and collaborators have highlighted how N-glycosylation of human plasma proteins can significantly alter protein structure and function⁵⁶. Furthermore, these changes can be considered consequences of disease state and can potentially serve as more precise biomarkers of disease. Five of the proteins identified in the current study found to be increased in expression with radiation exposure are major plasma glycoproteins including alpha-1-acid glycoprotein 1, alpha-1-antitrypsin 1-1, alpha-2-macroglobulin, beta-2-

glycoprotein 1, kininogen-1, hemopexin, and haptoglobin. Therefore, we postulate that post-translational modifications, such as glycosylation may have utility in identifying more precise biomarkers of radiation exposure.

4. Material And Methods

4.1. Animals and animal care

We procured six to eight-week-old male CD2F1 mice from Harlan Laboratories, Inc. (Indianapolis, IN, USA). Mice were kept in quarantine for one week, and microbiological examination confirmed the absence of *Pseudomonas aeruginosa*³³. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility, eight per cage, in rooms maintained at 21±2°C, relative humidity of 50±10%, with a 12 h light/dark cycle, and 10 – 15 cycles of fresh air per hour. Certified rodent rations (Teklad Rodent Diet, Harlan Laboratories, Inc.) and acidified water (HCl, pH = 2.5 – 2.8) were provided to the mice *ad libitum*. The study was performed according to the *Guide for the Care and Use of Laboratory Animals* of the Institute of Laboratory Animal Resources, National Research Council, U.S. National Academy of Sciences⁵⁷. All animal procedures were completed according to a protocol (P-2011-04-002) approved by the Institutional Animal Care and Use Committee of the Armed Forces Radiobiology Research Institute/Uniformed Services University of the Health Sciences. This study is reported in accordance with ARRIVE guidelines.

4.2. Experimental groups

There were three groups: untreated and sham irradiated, vehicle-treated and irradiated, and GT3-treated and irradiated. There were eight mice in each treatment group for serum sample collection and proteomic analysis.

4.3. Irradiation of mice

Irradiation boxes consisted of compartmentalized Plexiglas boxes designed to hold eight mice. Mice were exposed to a bilateral, midline dose of 11 Gy ⁶⁰Co γ-radiation at a dose rate of 0.6 Gy/min, as described earlier⁵⁸. Radiation dosimetry was established primarily on the alanine/EPR (electron paramagnetic resonance) system, currently accepted as one of the most accurate methods for relatively high radiation doses and was used for intercomparisons between national metrology institutions. The calibration curves (spectrometer e-Scan, Bruker Biospin, Inc., Madison, WI, USA) used in dose measurements are based on standard alanine calibration sets procured from the U.S. National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA)^{59,60}. The alanine dosimeters obtained from NIST had been calibrated in terms of absorbed dose to water using the US national standard radiation sources. Identical alanine dosimeters were placed midline within mouse phantoms (Plexiglas 1" diameter, 3" length) and irradiated for predefined periods of time. Measurement of their EPR signals using the calibration curve constructed with alanine dosimeters from NIST-provided dose rates to water in the core bodies of mice. A small correction was subsequently applied for the difference in mass energy absorption coefficients between water and soft tissue.

4.4. Drug preparation and administration

Yasoo Health, Inc. (Johnson City, TN, USA) supplied GT3 formulation in 5% Tween-80 in saline. GT3 formulation was adjusted to a final concentration for injection of 200 mg/kg in 0.1 ml total volume. Equal volumes of olive oil were administered as the vehicle (olive oil in 5% Tween-80 in saline)³³. A Luer-Lock syringe with a 23 G needle was used for all drug administrations. Subcutaneous injections were administered at the nape of the neck 24 h before irradiation.

4.5. Blood collection and serum separation

Blood was collected from anesthetized (Isoflurane, Abbott Laboratories, Chicago, IL, USA) mice via the inferior vena cava with a 23-gauge needle. After collection, blood was transferred to CapiJect serum separator tubes (3T-MG; Terumo Medical Corp., Elkton, MD, USA), allowed to clot for 30 min, and centrifuged at 400 g for 10 min. The serum was collected and stored at -70 °C until analysis as described earlier ³⁷.

4.6. Protein identification

To quantify changes in serum protein expression, Cy dyes (Cy3 or Cy5) were added to serum samples for labeling. Both eight vehicle control and GT3-treated serum samples were combined and labeled with Cy2, as previously described ³³. The 2D-DIGE separation method was employed in triplicate ⁶¹. The Amersham Biosciences 2D-gel system (Amersham Biosciences, Piscataway, NJ, USA) was used to analyze the 2D-gels, and images were captured with the Typhoon TRIO and analyzed by ImageQuantTL software (GE Healthcare, Chicago, IL, USA). Decyder software version 6.5 (GE Healthcare) in combination with Ettan Spot picker (Amersham Biosciences) was used to select between 150 and 200 protein spots per gel for identification.

Digestion of the gel spots was performed in sequencing-grade modified trypsin protease at 37 °C before desalting and incorporation into the α -cyano-4-hydroxy-cinnamic acid matrix, which was then spotted onto a MALDI plate. The Applied Biosystems Proteomic Analyzer Spectrometer was used for matrix assisted laser desorption/ionization time of flight tandem mass spectrometry (MALDI-TOF/TOF) combined with peptide fingerprinting mass mapping, and peptide fragmentation mapping yielded specific protein identification. A GPS Explorer workstation equipped with MASCOT search engine (Matrix Science, Boston, MA, USA) was used to resolve peptide mass and associated fragmentation spectra from a primary sequence database.

Pathway and network analyses were prepared using IPA software (Qiagen, Redwood City, CA, USA) and were based on statistical significance as well as functional and biological relevance of the detected proteins. The IPA software was directed to focus on proteins that have the potential to appear in mouse serum for the purpose of more relevant pathway and network analyses.

4.7. Statistical analysis

A Student's *t*-test was employed for all statistical analyses and a threshold of 1.5-fold difference was set to determine a significant change in protein expression. Each data point is the result of three biological replicates with a *p* value of less than 0.05. A protein score confidence interval percentage or ion confidence interval percentage of greater than 95% was considered significant for the MALDI-TOF/TOF analysis.

Declarations

Author Contributions:

V.K.S. designed the study, V.K.S., S.Y.W., and O.O.F. performed the in vivo study, E.R. and V.A.R. performed proteomic data analysis, E.R., V.A.R., and V.K.S. drafted the manuscript, E.R., V.A.R., and V.K.S. revised the manuscript. All authors reviewed and approved the manuscript.

Funding:

This study was supported by an intramural grant (11615) from the Armed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences awarded to VKS.

Acknowledgments:

The opinions or assertions contained herein are the private views of the authors and are not necessarily those of the Uniformed Services University of the Health Sciences and the Department of Defense or the US Food and Drug Administration and the Department of Health and Human Services. Organizations, commercial products and trade names referenced do not imply endorsement by the United States Government. We are grateful to Ms. Alana Carpenter for editing the manuscript.

Conflicts of Interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Data Availability:

All data generated or analyzed during this study are included in this article.

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Figures

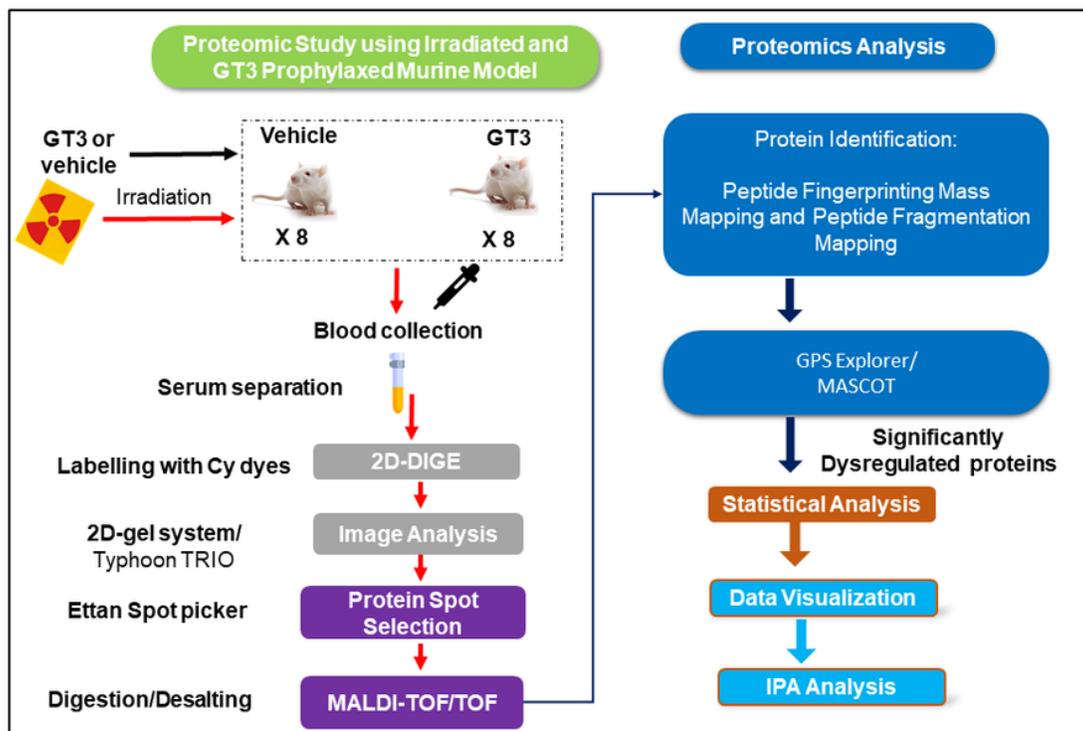


Figure 1

Study outline. Schematic of the animal study, sample preparation, and subsequent data analysis.

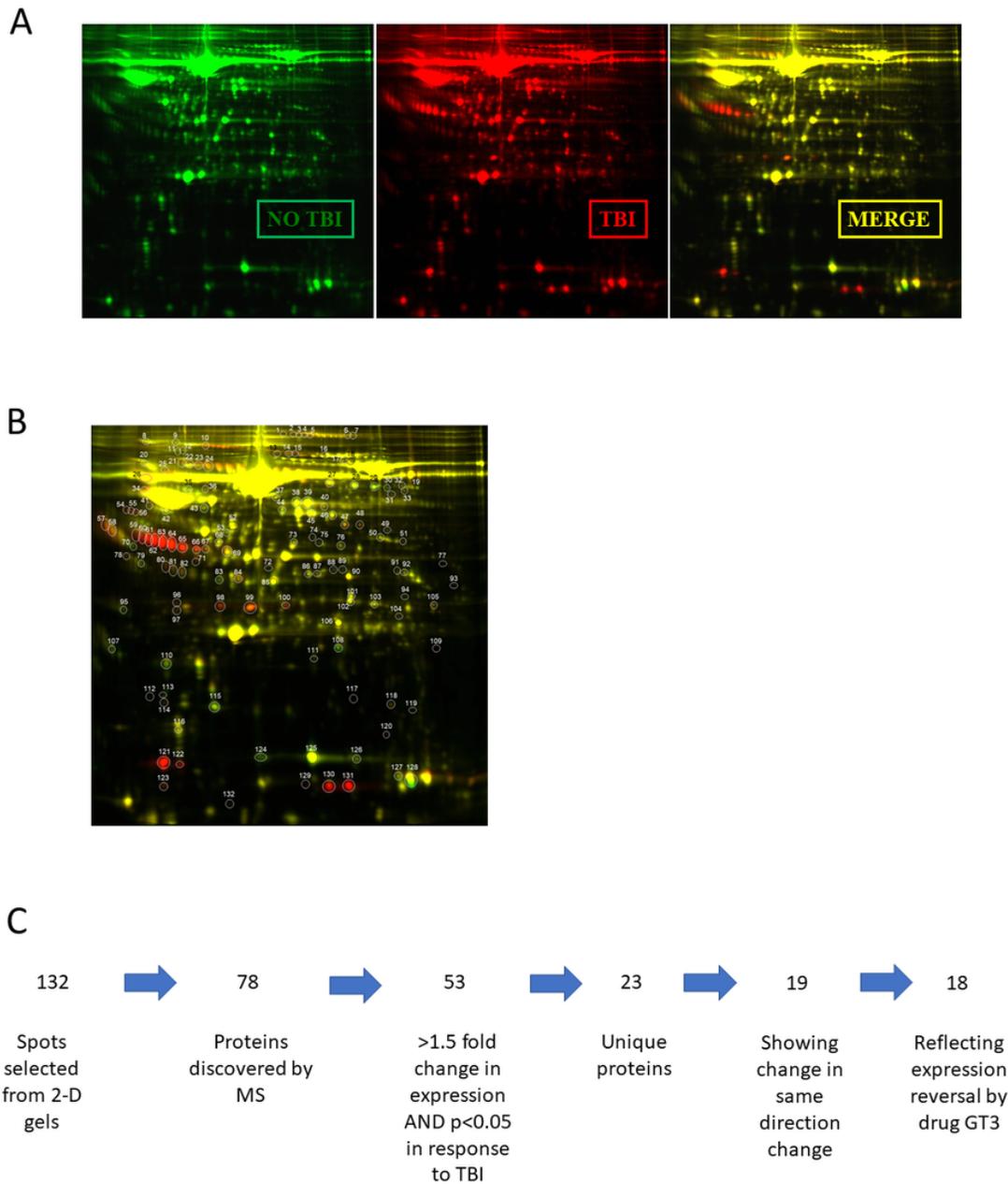


Figure 2

Detection and selection process for identifying proteins for proteomic analysis. A) Lysine groups of serum proteins from three experimental samples were directly labeled with cyanine CyDye DIGE Fluor and run on same gel for 2D analysis. B) Individual protein spots detected by selection software. C) Proteins identified by MS were screened for change in expression, statistical significance, consistency in expression change, and uniqueness.

UniProt Name	Protein	TBI : No TBI	p-value	TBI : GT3	p-value
A1AG1_MOUSE	Alpha-1-acid glycoprotein 1	8.8	2.60E-12	-5.9	3.10E-11
A1AT1_MOUSE	Alpha-1-antitrypsin 1-1	2.0	4.50E-06	-2.3	2.80E-10
A2M_MOUSE	Alpha-2-macroglobulin	1.5	1.20E-05	-3.0	3.60E-11
ALBU_MOUSE	Serum albumin	2.7	4.00E-04	-3.9	7.50E-09
APOH_MOUSE	Beta-2-glycoprotein 1	-2.1	1.60E-12	2.6	3.20E-10
CFAB_MOUSE	Complement factor B	1.8	3.50E-08	-2.3	6.40E-10
CLUS_MOUSE	Clusterin	1.7	1.20E-10	-1.9	8.00E-10
CO3_MOUSE	Complement C3	2.0	5.60E-04	-1.6	5.60E-04
EGFR_MOUSE	Epidermal growth factor receptor	2.0	2.70E-08	-1.6	8.70E-08
Q91XL1_MOUSE	Leucine-rich alpha-2-glycoprotein precursor	4.1	1.10E-06	-3.5	1.30E-09
HEMO_MOUSE	Hemopexin	1.9	2.40E-06	-7.7	1.50E-11
HPT_MOUSE	Haptoglobin	3.5	1.60E-10	-2.7	2.80E-10
ITI4_MOUSE	Inter alpha-trypsin inhibitor, heavy chain 4	1.8	1.10E-04	-2.0	5.00E-08
K2C73_MOUSE	Keratin, type II cytoskeletal 73	4.3	2.80E-06	-2.3	5.00E-06
KNG1_MOUSE	Kininogen-1	1.6	2.10E-03	-3.0	8.00E-10
MBL2_MOUSE	Mannose-binding protein C	1.8	5.80E-06	-1.8	8.00E-09
MJP6_MOUSE	Major urinary protein 6	1.7	4.20E-06	-2.3	7.80E-09
SAA2_MOUSE	Serum amyloid A-2 protein	19.5	3.40E-14	-1.9	4.10E-07

Figure 3

Serum proteins reflecting a change in expression in response to TBI and reversal with prophylactic treatment of GT3. Protein expression ratio changes are presented with a color scale to indicate increase (red) or decrease (green).

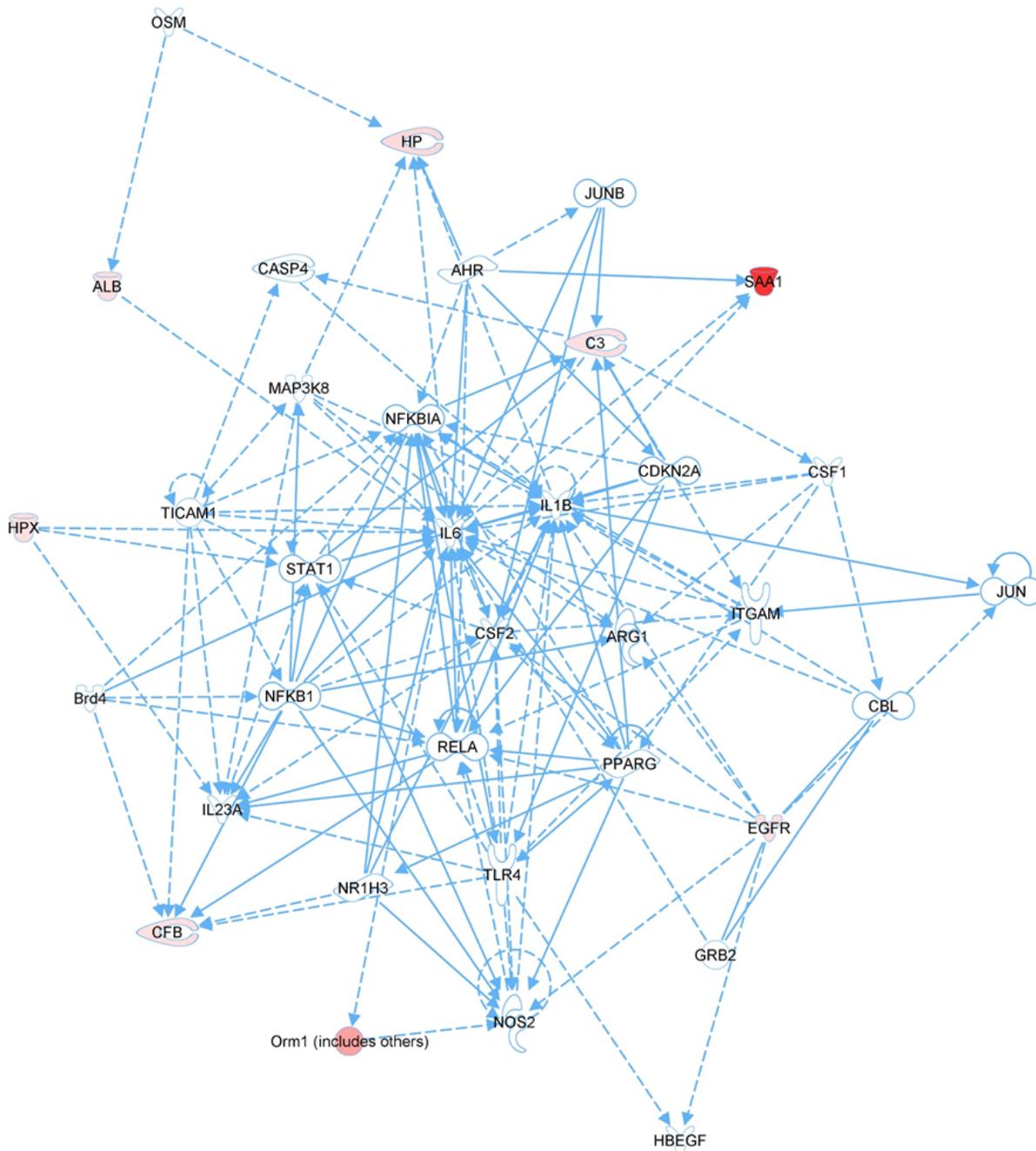


Figure 4

Network revealing relationships of serum proteins showing change in expression in response to TBI and reversal with prophylactic treatment of GT3. Ingenuity Pathway Analysis revealed proteins associated with cellular movement, immune cell trafficking, and inflammatory response. Proteins with red-fill backgrounds signify that these were down-regulated in response to TBI relative to no TBI. Proteins with no color-fill background were not identified in the MS analysis, but have a known relationship to proteins in the network.