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Immunotoxicological Study of Depleted Uranium

A Dissertation

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Doctor of Philosophy

Degree

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Bin Wan

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DEDICATION

This dissertation is dedicated to my parents who have tremendously supported me and to my wife and my son for their love and understanding throughout these years spent in the Graduate School.

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Abstract

The purpose of this study was to examine toxicity of depleted uranium (DU) to the immune system and the gene expression profile of DU-exposed immune cells including peritoneal macrophages, primary CD4⁺ T-cells and T Cell Hybridoma (TCH) PLP1 5B6. Flow cytometry analysis of annexin-V and Propidium Iodide (PI) binding revealed that DU causes death in those cells at various concentrations. The non-cytotoxic concentrations of DU determined in macrophages, primary CD4⁺ T-cells, and T cells hybridoma are 50µM, 100 µM, and 500 µM, respectively. Findings also revealed that DU-exposed macrophages are able to promote CD4⁺ T-cell proliferation in a concentration dependent manner.

In addition, mouse cytokine cDNA array and a confirmative method (Quantitative RT-PCR or Northern blot) were used to study the gene expression profile of DU-exposed cells. The results demonstrate that DU can modulate macrophage gene expressions such as NF-kBp65, Midkine, c-jun, and IL-10 that are related to multiple signal transduction pathways, suggesting possible involvement of DU in immune or inflammatory response, cancer development, and chronic disease. Array analysis of DU-exposed primary CD4⁺ T-cells reveals that DU up-regulates gene expression of a number of cytokines such as IL-5, Midkine, indicating the activities of DU in inducing cancer development, promoting eosinophil-related inflammatory diseases, and encouraging Th2 polarization. Furthermore, the possible involvement of DU in cancer development was demonstrated in TCH using microarray analysis. However, no Th2 cytokine was up-regulated in TCH after DU exposure.

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PART 1
LITERATURE REVIEW

Depleted Uranium

Uranium is a natural element that is present in soil (with an average concentration of 3 ppm in U.S.), rocks, surfaces and underground water, air, plants, and animals. As a result, it occurs in trace amounts in many foods and in drinking water. In nature, uranium exists in the +2, +3, +4, +5, and +6 valence states, but mostly in the hexavalent form and associates with oxygen as the uranyl ion, UO_2^{2+} . There are three major natural uranium isotopes including U^{234} , U^{235} , and U^{238} , with specific activities 6,200 $\mu\text{Ci/g}$, 2.2 $\mu\text{Ci/g}$, and 0.33 $\mu\text{Ci/g}$ and half-life times of 2.4×10^5 years, 7.0×10^8 years, 4.5×10^9 years, respectively. All these isotopes are α -particle emitters, and because the α -particle penetrates only 25-80 μm in tissues, uranium is primarily an internal radiation hazard. Of these isotopes, only U^{235} is valuable for production of electric power, and has been enriched in producing nuclear fuel [Priest, 2001]. The byproduct of this enrichment process is depleted uranium (DU), which contains about 70% less U^{235} (a decrease from 0.71% to 0.3%) and 80% less U^{234} than does natural uranium. Therefore, the radioactivity of DU is much reduced compared with natural uranium. It has been considered that DU radiation exposure presents a negligible hazard [Fisenne and Welford, 1986; Priest, 2001], thus the major hazard rendered by DU comes from its chemical activities. Although the specific activities of all isotopic mixtures of uranium (depleted, enriched, and natural) differ in great degrees, their chemical actions/behaviors are identical. Uranium is also categorized as a heavy metal due to its high density, which is approximately 1.7 times the density of lead (18.9g/cm³ vs. 11.35g/cm³) [McClain et al., 2001].

Environmental contamination of Uranium

The release of uranium into the environmental compartments such as water, soil, air, presents a serious threat to human and ecological health in many parts of world and the environmental pollution raises great concerns about the consequences [Hass et al., 1998]. Uranium has been widely used in many commercial and military industries, e.g. fuel for nuclear power plants, ceramic glazes, light bulbs, photographic chemicals, chemical catalyst, radiation shielding, and munitions [Murray et al., 2002]. Therefore, uranium is readily redistributed into air, water, and soil by these anthropogenic processes. The three industrial processes responsible for most of this redistribution are operations associated with the nuclear fuel cycle including the mining, milling, and processing of uranium ore or uranium end-products, the production of phosphate fertilizers, and the inappropriate disposal of uranium mine tailing. The natural processes that may contribute to uranium redistributions include wind resuspension of uranium dust, water erosion and volcanic eruption that can move the rock and soil containing uranium element. Sometimes both anthropogenic and natural processes contribute to the uranium contamination. For example, a higher background concentration of uranium in rock may cause local high uranium levels, but human mining activities may cause serious contamination of sediments in water reservoirs by disturbing or increasing the release of uranium from rock [Stastny et al., 2002].

In a study on uranium leaching from glass and ceramic items in which uranium was used as a coloring agent, the resulting leachate exhibited a maximum concentration of 30µg U/liter from glass items and compared to 300,000µg U/liter from ceramic items

[Landa and Councell, 1992]. Elevated uranium levels have been reported in at least 54 of 1,517 current or former EPA National Priorities List (NPL) hazardous waste sites [HazDat, 1999]. The redistribution of uranium from uranium mining, milling, production operations, and radioactive waste disposal sites into surface water and underground water has been documented [Yang and Edwards, 1984; Swanson, 1985; Cottrell et al., 1981]. As examples, uranium levels in groundwater and surface water ranged from 1,500 to 16,000 pCi/L, or 2.2 to 23.4 $\mu\text{g/g}$ in Uravan site and in the tailing pond of the United Nuclear site, the uranium concentrations were 3,900pCi/L, or 5.7 $\mu\text{g/g}$ [HazDat, 1998]. The accident of the tailings pond dam in 1979 released ~93 million gallons of tailings liquid into the Rio Puerto [EPA, 1988]. A survey on 130 sites in Canada showed that the mean average uranium concentration in drinking water was 0.4 $\mu\text{g/liter}$ during 1990-1995 [OMEE, 1996]. Up to 700 $\mu\text{g/liter}$ of uranium has been reported in private water supplies in Canada [Moss et al., 1983].

The uranium contamination was evident in soil and sediment samples too. HazDat's [1998] study showed that 30 of the 51 sites have elevated uranium concentrations in soil samples and 16 of 51 in sediment samples with big variations. In some case, the soil uranium concentrations were as high as 24,000 $\mu\text{g/g}$. Another example is the United States Radium Corporation site (New Jersey), uranium concentrations ranged from 90 to 12000 pCi g^{-1} (~130-18,000 $\mu\text{g/g}$); for the Monticello site (Utah), uranium levels were reported to range from 1 to 24,000 pCi g^{-1} (~1.5-36,000 $\mu\text{g/g}$) [HazDat, 1998]. In Russia, after thirty years of mining and milling activities of the Priargunsky Mining Chemical Production Company, the uranium concentration in

adjacent steppe soils has been enriched by a factor of 600 [Gongalsky, 2003]. Sediment-bound uranium concentrations in a southeastern wetland system were reported to be as high as 1065 mg/Kg [Punshon et al., 2003(b)].

Uranium contamination also comes from military use. DU was first deployed in military armaments during the Gulf War in Iraq, where tons of DU has been released into the environment. In the Gulf War, several hundred US military personnel were exposed to DU particles in explosions or were injured by DU fragments [Murray et al., 2002; Domingo, 2001; Durakovic, 1999]. Uranium contamination has also been found in Russia, Germany, and Yugoslavia etc. Without exceptions, the extensive contamination has been identified either as the results from intensive uranium industrial activities, or, in Yugoslavia, from military action, mainly due to the usage of DU weapons [Hamilton, 2001; Durakovic, 1999; Meinrath et al., 2003 ; McDiarmid et al., 2000].

DU environmental transportation

The threat of environmental pollution from the release and dispersion of uranium into the biosphere has stimulated intensive research focusing on the geochemical properties of uranium and the interaction of uranium and dissolved species with biological systems. Numerous studies have been generated focusing on uranium release, transportation, immobilization, remobilization, and bio-concentrating as well as bioaccumulation [Hass et al., 1998; Danesi, 2003; Martin, 2003; Thomas, 2000(a)(b)]. The environmental mobility of uranium depends on its physicochemical properties and a variety of environmental or geochemical factors including ground, surface and soil water composition, mineral surfaces, and biological systems etc. [Hass et al., 1998]. In a study

of selected Kosovo soil samples where DU ammunition was fired in 1999, Danesi and his coworkers [2003] reported that most of DU particles were $<5\text{ }\mu\text{m}$ in diameter and more than 50% of the particles had a diameter $<1.5\text{ }\mu\text{m}$, indicating a potential environmental and health effect of the DU contamination through resuspension and inhalation of those particles. A modeling study on the dispersion of uranium aerosol after impact and combustion of depleted uranium munitions indicated that most uranium deposition occurs very close to the release point. The deposition rate a few meters near the release point is more than ten times higher than that at a couple of kilometers from the release point. Various atmospheric stabilities, heights of release, and wind speeds may influence the release/deposition significantly [Mitsakou et al., 2003]. In contrast to impact aerosols, the aerosol dust from uranium mines may be washed out by rain and potentially dispersed far away from the release point(s) through water flux. By studying rainwater samples in northern Australia, Martin [2003] reported that below-cloud washout of dust from the nearby Ranger uranium mine constitutes a large portion of the measured uranium activity in those samples. The first one or two millimeters of rainfall decrease the dust radionuclides concentrations by one half or more [Martin, 2003].

Generally speaking, a drier climate and active weather causes a lower deposition rate of uranium dust than wetter and stable climate does. In Thomas's [2000(b)] study, higher deposition rates were found for uranium at the site closest to mill and ore dust sources, but uranium dry deposition was reported to be more important than rainfall for uranium transportation in the Key Lake uranium mill operations in Northern Saskatchewan, Canada.

In addition, resuspension of uranium from soil onto plant surfaces may result in off-site transport by allowing uranium to enter food chain movement [Punshon et al., 2003(b)]. Uranium associated with small particles or natural dusts is more accessible for direct plant or animal uptake than that occurring with larger particulates [Thomas, 2000(b)].

In water system, especially estuary ecosystems, uranium dissolution plays a major role in releasing the uranium from contamination sources, such as industrial activities, and in fixation of uranium on particulate materials. Tidal activity may play an important role in the transport and homogenization along the estuary too [Bolivar, 2000]. Consequently, under the influences of these natural processes, the routine operation of nuclear re-processing plants, especially in the past and future reactor accidents, can result in an enrichment of DU particles in most types of sediments [Hamilton, 2001].

Bioavailability

Uranium in the environment undergoes complicated geochemical and biological reactions that result in reduction, immobilization, or redistribution. Physicochemical conditions, such as pH, presence of salt ions or organic matters, determine the availability of uranium. In natural soils, uranium is present primarily in the +6 oxidation state as the uranyl (UO_2^{2+}) cation; under acidic and reducing conditions, UO_2^{2+} is the predominant species. Hydroxide complexes such as UO_2OH^+ and phosphate complexes such as UO_2HPO_4 occur under near neutral conditions, while carbonate complexes such as UO_2CO_3 predominate under alkaline conditions [Ebbs et al., 1998]. In sulfate rich, acid

and near-neutral solutions, uranium (VI) may be absorbed on goethite or on schwertmannite in perchlorate solutions [Walter et al., 2003].

In water-sediment systems of the Savannah River site, aqueous uranium concentrations were found highly correlated to dissolved organic carbon (DOC), and ranged from 29 to 410µg/L, indicating that uranium availability is determined by naturally occurring organic carbon [Sowder et al., 2003]. Decomposition of organic matter may result in uranium fixation [Gurban et al., 2003]. In a shallow aquifer contaminated by uranium mill tailings, nitrate reduction was found to have a negative impact on biological uranium (VI) reduction [Elias et al., 2003]. In high-salinity subsurface sediments, acetate can stimulate the removal of uranium (VI) from the groundwater by *Pseudomonas* and *Desulfosporosinus* species [Nevin et al., 2003].

In addition to geochemical and microbial reduction of uranium, plants play an essential role in up-taking and transferring uranium into the bio-sphere. A number of plants have been reported to be able to trap uranium from water or soil spheres, including lichen (*Peltigera membranacea*) [Hass et al., 1998], carrot (*Daucus carota*) as a host for fungus (*Arbuscular mycorrhizal*) [Rufyikiri et al., 2003], peas (*Pisum sativum*) [Ebbs et al., 1998], understory flora (*Discanthelium* sp. and *Andropogon* sp.) [Punshon et al., 2003(a)], grass-pasture [Vera et al., 2003], sunflower (*Helianthus annuus*) and Indian mustard (*Brassica juncea*) [Shahandeh et al., 2002(a)(b)], respectively. Of these plants, lichens have the highest capability of accumulating uranium with the highest reported concentration, ~42,000ppm [Hass et al., 1998].

The major organs involved in uptaking uranium are roots and/or fungus hyphae, leaves, and shoots [Rufyikiri et al., 2003; Shahandeh et al., 2002(a)(b)]. Soil properties

that determine chemical forms of uranium also dictate phytoaccumulation of uranium [Ebbs et al., 1998]. Other fractions that will influence the formation of uranium complexes include carbonate, manganese, iron, and organic [Shahandeh et al., 2002(a)].

Ecological effects of DU contamination

Animals living near uranium mining and milling sites also tend to have elevated concentrations of uranium possibly due to food chain transportation or direct deposition and absorption of uranium. The uranium concentrations in vegetation and animal samples collected near operating Key Lake uranium tailing and milling sites in Northern Saskatchewan, Canada, were significantly higher than those from a control site. As examples, the concentrations (wet weight) of small mammal samples ranged from 6-130ppb, bird samples ranged from <100-200ppb, vegetation samples ranged from <100-3100 $\mu\text{g Kg}^{-1}$ [Thomas, 2000(a)].

The accumulation of uranium in water or the bio-sphere has been reported to cause toxic effects on local organisms. Markich and his coworkers [2003] demonstrated that the valve movement of freshwater bivalves (*Velesunio angasi*) was affected by sub-lethal uranium concentrations. Uranium also causes toxic effects on alga [Charles et al., 2002; Franklin et al., 2000]. Water hardness (Ca and/or Mg) and pH conditions significantly influence the toxicity of uranium due to the competition between uranium and other ions (e.g. Ca, Mg, and H^+) for binding site [Charles et al., 2002; Franklin et al., 2000].

In highly contaminated sites, some organisms tend to concentrate uranium. For example, at a uranium mine operating for 30 years in south-eastern Siberia, Russia,

uranium has been highly enriched in adjacent areas. As a result, ground beetles (*Poecilus gebleri*, *Poecilus fortipes*, *Blaps rugosa*, and *Nicrophorus investigator*) concentrated a large amount of uranium in their bodies [Gongalsky, 2003]. Gongalsky's [2003] study reported that the uranium concentrations of ground beetles in contaminated sites were ~2-40.5 times (highest concentration ~8.1mg kg⁻¹) higher compared to those in control sites. As a consequence, the soil macroinvertebrates abundance, biodiversity, and communities were adversely affected in uranium contaminated sites. This study provided strong evidence that the uranium contamination has severe negative effects on the soil food web [Gongalsky, 2003]. A similar study conducted at a former uranium mill site in southeastern Utah reported approximately the same levels of uranium in aquatic macroinvertebrates (highest concentration 8.5 µg/g) and mean uranium concentrations in macroinvertebrates near the mill site were more than 10 times higher compared to those in reference sites [Peterson et al., 2002].

In aquatic vertebrates, such as lake whitefish (*Coregonus clupeaformis*), fed with 100-10,000µg U/g dietary uranium for 10, 30, and 100 days, uranium accumulations were observed in gill, scales, bone, liver, kidney, and intestine, the highest concentrations and gross pathologies occurred in all exposed fishes by day 100 including tubular necrosis, inflammation, and hemorrhaging in renal organ, and focal hepatocyte necrosis and alternations of bile ductile epithelium [Cooley, 2000(a)(b)]. A recent report on the induction of immune cell apoptosis by immunotoxicants (i.e. azathioprine and T-2 toxin) indicates that fish and mammals may respond similarly to immunotoxicant challenges [Gogal, 2000]. Therefore, the uranium immunotoxicological effects may also occur in other terrestrial animals including human beings.

A comprehensive study of the toxicology of uranium using three typical species living in soil (worm, *Eisenia fetida*), water columns (fish, *Brachydanio rerio*), and at the water/sediment interface (clam, *Corbicula fluminea*) implied that all these three species can accumulate uranium to different degrees and are very sensitive to uranium toxicity, e.g. 96-hLC₅₀ for *Brachydanio* was 3.05 mg U/L, *Corbicula* 1872 mg U/L, and *Eisenia* 13.48µg U/cm² [Labrot et al., 1999]. The study implied potential ecotoxicological effects of uranium on terrestrial and aquatic food webs.

Another potential ecological impact of uranium is through the behavior of uranium-reducing geobacteria. Phylogenetic analysis of 16s ribosomal DNA from unincubated and incubated sediments from an inactivated uranium mine revealed that microbial populations were changed from microaerophilic proteobacteria to anaerobic low G+C gram-positive sporeforming bacteria by the incubation [Suzuki et al., 2003]. Provided with sufficient organic substrates, the soil/sediment bacteria community could be stimulated resulting in microbial population shifting, which may alter soil/sediment physicochemical properties or impact native communities directly.

Toxicokinetic and toxicodynamics of uranium in animal models and humans

The chemical toxicity of a uranium compound depends on the nature of the compound and its route of exposure. Absorption of uranium from the gastrointestinal (GI) tract depends on the solubility of the uranium compounds, previous food consumption and the concomitant administration of oxidizing agents, such as iron(III) ion and quinhydrone [Sullivan et al., 1986]. The average human GI absorption of the total

ingested uranium is 1-2% [Wrenn et al., 1985]. The absorption rate of uranium through ingestion or drinking in Sprague-Dawley rats is much lower than in humans. The absorption of a uranium dose of 800mg/Kg of body weight in starved Sprague-Dawley rats is only 0.17%, but can increase to 3.3% when uranium was administered simultaneously with iron(III) ion [Sullivan et al., 1986]. The absorption rate can be as low as 0.06% in Sprague-Dawley rats having free access to drinking water containing 600mg/liter of uranyl nitrate hexahydrate for up to 91 days [Tracy et al., 1992].

Following the absorption, uranium rapidly appears in the blood stream and reacts with endogenous matters forming a variety of complex, non-diffusible such as uranium-albumin, or diffusible such as ionic uranyl hydrogen carbonate complex [Moss, 1985]. Because of their high affinity with phosphate, carboxyl, and hydroxyl groups, uranyl compounds readily combine with proteins and nucleotides to form stable complexes [Moss, 1985]. Clearance from blood is rapid through urine and feces. The urinary excretion rate for uranium is estimated to be 0.04-0.5 $\mu\text{g/day}$ and the fecal excretion rate 1.4-1.8 $\mu\text{g/day}$ [Priest, 2001]. Subsequently uranium accumulates in kidney and the skeleton, as well as other tissues and organs, such as liver and spleen. Deposition of uranium in human tissues follows the rank order: skeleton > liver > kidney with observed ratios of 63:2.8:1 [Kathren et al., 1989]. Mineralized tissues/organs such as skeleton have been suggested as the main storage sites [Wrenn et al., 1985]. The Uranyl ion exchanges with bone but does not enter into the crystal lattice. 80-90% of skeleton deposited uranium is lost within about 1.5 years. Dissolved uranium also localized within the central nervous system, lymph nodes, testes, and spleen of rats, suggesting the potential of uranium in causing health problems at these sites [Pellmar et al., 1999; Domingo,

2001]. In lake whitefish exposed to 100, 1,000, and 10,000 μ g U/g (uranyl acetate dehydrate) in food for 10, 30, and 100 days, the mineralized tissues, such as scale and bone, accumulated high concentrations of uranium in a dose- and time-dependent manner. Also, after 10 days 10,000 μ g U/g exposure, the uranium concentration of intestine was measured as high as 478 μ g U/g [Cooley, 2000(a)(b)].

Toxic metals can have two types of effects: (1) indirect, by adding additional metabolic cost of accumulating, transporting, storing, and excreting the contaminants; and (2) direct, by compromising the cellular membranes and/or specific biochemical pathways [Morgan et al., 1990]. Kidney damage is generally accepted to be the principle toxic effect of ingested DU and much is known about this effect [Domingo, 1995]. Toxicity studies using animals including humans suggested that renal dysfunction is the most characteristic response to DU exposure in either short or long terms [Leggett, 1989; Zamora et al., 1998]. Animal experiments on DU have showed a disturbance in renal function with doses as low as 10 μ g U/kg. Both Na⁺ transport dependent and Na⁺ transport independent ATP utilization and mitochondrial oxidative phosphorylation in the proximal tubule can be inhibited by uranyl [Domingo, 1995; Leggett, 1989]. In addition, low dose of DU showed a transient depression in the rate of erythropoiesis in rat [Giglio et al., 1989].

***In vitro* depleted uranium studies**

Only a few molecular-based DU studies have been reported in the literature. One such experiment used cultured kidney cells, LLC-PK. The cells were exposed to 1mM uranyl acetate for 48 hours resulting in the release of lactate dehydrogenase. Pretreatment

of cells with 1mM uranyl acetate for 24 hours or heat stress (42 degree for 30 min) before 48 hours exposure lessened the release [Furuya et al., 1997]. In another study, exposure to 0.01~0.3 mM of DU resulted in decreased Chinese hamster ovary cells viability, depressed cell cycle kinetics, and increased sister chromatid exchanges, micronuclei, and chromosomal aberrations [Lin et al., 1993]. A report suggested that, at least *in vitro*, DU can cause human cell transformation to a neoplastic phenotype indicating carcinogenic activity of DU [Miller et al., 1998].

Immunotoxic effects of DU

While the literature is full of references involved in studies of the radiation effects of uranium, to date few studies have directly addressed the effect of DU on the immune system. In one such study lymphocytes of uranium fuel enrichment plant workers were examined for chromosomal alterations. Frequencies of chromosome aberrations and sister chromatid exchanges were higher than those in control populations. However, their external radiation exposure was deemed insufficient to account for the observed changes [Martin et al., 1991]. In another genotoxicity study, background frequencies of chromosomal aberrations and sister chromatid exchanges in peripheral blood lymphocytes collected and cultured from DU-exposed veterans were identical to those of non-exposed Gulf War veterans and similar to those noted on other control populations [McDiarmid et al., 2000].

Animal experiments with subcutaneous implantation of DU have indicated the types of cellular changes that occur with DU entry. Microscopic analysis of animal tissue near the site of uranium dioxide subcutaneous injection revealed electron-dense uranium

deposition in the intercellular space. After 24 to 48 hours, macrophages appeared with large phagosomes containing uranium in the cytoplasm. Cellular alterations such as nuclear inclusions, mitochondria alterations, and large intracellular vacuoles were evident. This initial cellular efflux was attributed to a non-specific inflammatory response [De Rey et al., 1984]. The internalization of uranium by macrophages has been evidenced, and the results showed that uranium can induce cell apoptosis as well [Kalinich et al., 2002; Kalinich and McClain, 2001].

The human literature lacks information on adverse immunological or lymphoreticular effects of uranium. Most of the studies were conducted using animal model, *in vitro* cell lines or uranium dust. The presence of systemic lupus erythematosus (SLE)-typical antibodies in quartz dust-exposed uranium miners indicates a potentially higher risk for the development of systemic autoimmune disease [Conrad et al., 1996; 1998]. A study on rabbit alveolar macrophages showed that the uranium mineral dust may induce the abnormal expression and releasing of TNF and IL-6 to cause the proliferation of lung fibroblasts and synthesis of collagen [Zhou et al., 1998]. It has been shown that uranium can induce TNF- α secretion by alveolar macrophages [Gazin et al., 2004]. An epidemiological study revealed a possible association between the exposure of uranium and the lymphatic cancer, but further evidence is needed to determine this relationship [US institute of medicine, 2000]. Acute and chronic uranium poisoning has been reported to cause pathological changes in the testes and thyroid glands, similar to those of an autoimmune process. In addition, autoantibodies to the testes and thyroid glands were also found in the blood of poisoned animals [Malenchenko et al., 1978].

Mammalian immune system and immune cells

Mammalian immune system has been evolving to such a harmonic degree that it can rapidly and efficiently defend the individuals from almost all kinds of challenges. The responsive components in the immune system are cells and molecules, which constitute immunity. Immunity is a reaction to foreign substances, including microorganisms and macromolecules, such as proteins and polysaccharides, without causing a physiological or pathological consequence. Most of the functions of immune system rely on special cells (i.e. immune cells). The immune cells present in circulating blood or lymph and exist in almost all tissues or organs.

Healthy individuals are protected by two kinds of immunities: innate immunity and adaptive immunity. Innate immunity provides the first line of defense against microbes by means of three mechanisms: (1) physical and chemical barriers, such as skin and epithelial surfaces that prevent the entrance of pathogens; (2) blood protein, such as complement systems that facilitate lyses of cells; (3) phagocytic cells, such as macrophages for engulfing foreign materials. Adaptive immunity is a highly evolved system that can be stimulated by infectious agents and provides much higher defensive and specific capability than innate immunity. The major components of adaptive immunity are lymphocytes (i.e. T-lymphocytes and B-lymphocytes), and their products such as cytokines and antibodies. Adaptive immunity can be further categorized into two types: humoral immunity and cell-mediated immunity. Humoral immunity is mediated by blood protein--antibodies, which are the product of B-lymphocytes after stimulation. Cell mediated immunity is mediated by T-lymphocytes [Abbas et al., 1997]. The innate and

adaptive immunities work coordinately and form an integrated defensive system protecting the individuals from pathogenic insult.

T-lymphocytes precursors arise in the bone marrow and then migrate to the thymus where they mature into two distinct subsets of cells in terms of their functions: T-helper cells (Th) and cytolytic/cytotoxic T-cells (CTLs). The principle functions of T-cells are to (1) regulate the immune response to antigens (Th cells) and (2) serve as effector cells (CTLs) to eliminate intracellular microbes. T-cells do not produce antibodies, but their antigen receptors on the cell membrane are structurally similar to antibodies and called T-cell receptors (TCRs). TCRs can only recognize the antigens attached to the major histocompatibility complex (MHC) and expressed on the surfaces of other cells such as macrophages. In response to antigenic stimulation, Th cells secrete protein hormones, called cytokines, which function to stimulate activation and proliferation of themselves and other immune cells, including B-cells and macrophages. Therefore, Th-cells play a vital role in immune response regulation through cytokines. Unlike Th-cells, CTLs play an essential role in cytotoxic processes that lyse cells such as cells infected by viruses or intracellular microbes and produce antigens.

B-lymphocytes are derived from bone marrow in mammals. B-cells are the only cells capable of producing antibodies having a major role and function in humoral immunity. B-cell receptors are membrane-bound “antibodies” that recognize the MHC-antigen complex and initiate B-cell activation and proliferation. This process finally results in the development of effector B cells and leads to antibody productions, such as immunoglobulin (Ig) G, IgE or IgM. In the condition of low antigen concentrations, B-

cells can also function as antigen presenting cells (APCs) for Th-cell activation, but this functionality is minor compared with other APCs (e.g. macrophages or dendritic cells).

Macrophages, found in most organs and connective tissues, are foremost among the cell types involved in the innate immune response and have a wide range of important functions in relation to inflammatory responses against foreign substances, including (1) phagocytosis of smaller cells such as bacteria and infected host cells, (2) direct or antibody directed killing of these cells, and (3) interaction with the adaptive immune system in the areas of antigen-processing and presenting, and (4) cytokine production [Burke and Lewis, 2002; Servet-Delprat et al., 2002]. Macrophages are able to search for antigens and will engulf and destroy them by the action of lysosomal enzymes. The broken fragments are then placed on their outer surface where eventually a Th-cell will bind and secrete lymphokines to activate B-cells leading to antibody release. Macrophages themselves produce cytokines, such as IL-1, IL-10, IL-12, and IFN- γ , that activate Th-cells and regulate the immune response [Stout and Suttles, 1997; Banchereau and Steinman, 1998; Lalani et al., 1997]. Macrophages express a variety of receptors that mediate the reorganization and phagocytosis of cells and subsequent generation of inflammation to eradicate the infection [Ying and Teo, 2003].

Another set of cells, called dendritic cells (DCs). Compared to DCs, macrophages are more abundant and more uniformly distributed throughout the body. It is widely accepted that macrophages are precursors of most DCs [Mills et al., 2000; Banchereau and Steinman, 1998]. The full repertoire of functions of DCs are still unknown, but most appear to be involved in antigen presentation [Lipscomb and Masten, 2002; Fong et al., 2000; Syme et al., 2001]. Therefore, DCs have been termed professional APCs. While

macrophages and DCs share in antigen presentation and cytokine production in common, a significant difference is that mature DCs lose phagocytic ability, while macrophages maintain the ability until dead [Granucci et al., 2003].

In addition to lymphocytes and mononuclear phagocytes, additional blood leukocytes in the immune system are granulocytes including basophils, eosinophils, and neutrophils. These cells are effector cells in inflammatory responses, and can be attracted by cytokines and chemokines to the infection sites. The major players in the immune system including T-cell, B-cell, and macrophage work coordinately to regulate and perform the eradication of antigens and maintain health in individuals. The interactions between macrophages (from innate and adaptive immunities) and lymphocytes (from adaptive immunity) stimulate each other's function and provide an important amplification mechanism for adaptive immunity.

The Th1/Th2 paradigm

In cell-mediated immunity, T-lymphocytes function in activating macrophages and lysing virus-infected cells and tumor cells. T-lymphocytes can be categorized and functionally divided into $CD4^+$ (T-helper lymphocytes) and $CD8^+$ (Cytotoxic T-lymphocytes) cells by the type of antigen receptors and a small number of accessory markers on their cell surface. Naïve $CD4^+$ T-cells can be further differentiated into T-helper 1 (Th1) and T-helper 2 (Th2) cells, each producing its own set of cytokines and mediating distinct effector functions [Mosmann et al., 1986; Abbas et al., 1996]. Th1 cells can produce pro-inflammatory cytokines, such as interferon-gamma ($IFN-\gamma$), that activate macrophages and induce delayed type hypersensitivity (DTH) responses. Th2

cells can secrete high levels of IL-4, IL-5, and IL-13, which stimulate the production of Mast cells, eosinophiles, and IgE antibodies and regulate cell-mediated immunity [Mosmann et al., 1989]. Both Th1 and Th2 cells produce several proteins such as IL-3, TNF- α and Granulocyte-Macrophage Colony-Stimulating Factor [Mosmann and Sad, 1996]. Mouse Th2 cells also secrete IL-10, which is normally expressed in both Th1 and Th2 cells in humans [Sallusto et al., 1998].

The decision of the pathways pre-Th cell differentiation will follow is dependent on the cytokine environment. For example, under the influence of IL-12, naïve T-cells enter the Th1 path, while IL-4 promotes Th2 pathway. After the stimulation, Th1 or Th2 cells reciprocally inhibit each other to reinforce the response. Under the influence of IL-12, and Th2 cell differentiation is inhibited. Whereas IL-4 is necessary for Th2 differentiation and inhibits the development of IFN- γ -producing cells [Hsieh et al., 1993]. The Th2 cytokine IL-4 and Th1 cytokine, IFN- γ , show antagonistic activities. In addition, IL-10 inhibits the production of IFN- γ and other Th1 cytokines by interfering with antigen presentation by macrophages [Paul et al., 1994]. Once an imbalance between Th1 and Th2 has established, the antigen may be presented directly or indirectly for activation of different effector cells (e.g. macrophages, neutrophils, cytotoxic T-cells) for Th1 responses and eosinophils, mast cells for the Th2 response (Figure 1). (Figure 1 can be found in appendix at the end of the part.)

Th1/Th2 paradigm-related diseases

Studies showed that the complex balance between Th1 and Th2 cells can be disturbed by a variety of factors including heavy metals [Pelletier et al., 1986; Krocova et

al., 2000]. It has been postulated that deviation of the T-cell population to Type 1 phenotype fosters T-cell-mediated autoimmunity and inflammation. Studies on experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis and diabetes mellitus have suggested the important role of Th1 polarity in the development of these diseases [Kuchroo et al., 2002]. In contrast, Th2 cytokines have been speculated to be protective in Th1-derived pathological auto-reactive responses. However, a Th2 polarity was also found associated with some autoimmune diseases, such as systemic lupus erythematosus (SLE). In addition, shift to a Th2 population has been found in allergic diseases such as atopic asthma, Omenn's syndrome [Mosmann and Sad, 1996]. The high prevalence of asthma and allergic bronchopulmonary aspergillosis, both of which are Th2 mediated diseases, suggests that Th2 may play an important role in inducing inflammation of the airway as well.

It is well known that some heavy metals may induce a Th2-shift while some others preferentially induce Th1 responses. Mercury chloride has been shown to promote a Th2-shift leading to autoimmune disease [Bagenstose et al., 1999]. The mechanism is due to the induced production of degranulation antibodies (IgE) by Th2-type cytokines [Pelletier et al., 1986]. *In Vitro* studies have reported that treatment of macrophages with lead results in the production of Th1 cytokines and suppression of Th2 cytokines while cadmium enhances the Th2 cytokine regulatory pathway [Krocova et al., 2000].

Apoptosis and necrosis

In an individual, cell death is as important as cell production/proliferation. There are two ways of cell death, apoptosis and necrosis. To maintain a constant cell number,

remove unwanted cells or damaged or infected cells, metazoans use a conserved physiological cell death process termed ‘apoptosis’. In contrast, under general stress (e.g. physical, chemical environmental changes), cells can be damaged and lose whole cellular integrity, and is termed ‘necrosis’. These two modes of cell death differ fundamentally in their morphology, biochemistry, and biological relevance. Depending on the nature of stimulus, a cell may follow either one of these two modes leading to death. Necrosis is a non-specific mode of cell death and can be characterized by cell and mitochondria swelling, resulting in disruption of the cell membrane and cell lysis. Apoptosis is a programmed cell death, which is regulated by gene expression [Opferman and Korsmeyer, 2003] and can be characterized as chromatin condensation and margination, cell shrinkage, membrane blebbing (formation of blebs on the cell membrane), and formation of apoptotic bodies. Apoptotic cells undergo enzymatic internucleosomal DNA destruction and then are rapidly cleared by phagocytosis, either by neighboring cells or professional phagocytes such as macrophages [Vaux, 2002].

Cell death and immunotoxicity

Apoptosis plays a role not only in tissue and organ development, but also in regulation of the immune response. Generation of immunity is highly dependent on proliferation and differentiation of immune-competent cells, which is regulated by cytokines and cell-cell interactions. In the immune system, both cell number and function are essential determinants of the output of an immune response. Reducing the number of immune-competent cells through uncontrolled or massive cell death after xenobiotic treatment may lead to serious adverse effects such as immunosuppression [Pallardy et al.,

1999; Byung et al., 1997]. Furthermore, lymphocyte apoptosis can potentially lead to an altered immune response by several mechanisms including [Rodenburg et al., 2000; Li et al., 2001]:

1. Defect elimination of auto-reactive lymphocytes.
2. Leakage/translocation of cell contents leading to exposure of intracellular materials such as phosphatidylserine.
3. Modification of auto-antigens, or serum protein binding to the apoptotic cells, which are recognized by auto-antibodies [Casciola-Rosen et al., 1994] and eventually develop autoimmune diseases.

Low numbers of apoptotic cells are removed predominantly by macrophages, the process of which is accompanied by an anti-inflammatory response. If in larger number, apoptotic cells or dead cells may be digested by professional antigen presenting cells (APCs), the resulting fragments are presented in MHC complex and invoke a strong cytolytic response, creating a proinflammatory environment [Li et al., 2001]. Subsequently, a response against the auto-antigens may be initiated and then epitopes spread to unmodified antigens causing the development of autoimmune diseases [Rodenburg et al., 2000]. Therefore, studying the degree/mode of cell death induced by xenobiotic treatment provides a means to assess the nature of its immunotoxicity.

Assessment of cell death

Recent advancements have been made in the assessment of cell death. These techniques include the colony-formation assay, the vital dye exclusion test, and flow cytometry analysis, which allows rapid, individual analysis of large numbers of cells. The

most commonly used method is flow cytometry analysis. The principles for the method is that during the apoptosis process, cells lose phospholipid asymmetry resulting in the translocation of phosphatidylserine from inner leaflet to outer leaflet of the plasma membrane. The exposed phosphatidylserine becomes available for annexin-V-FITC binding, which permits the identification of apoptotic cells in a flow cytometer by analyzing the FITC fluorescence. Additionally, due to the difference of membrane integrity of apoptotic and necrotic cells, Propidium Iodide (PI) can be used to stain necrotic cells while viable cells exclude PI and cannot be stained. Therefore, annexin-V-FITC/PI analysis readily discriminates these two cell death modes from each other [Bertho et al., 2000; Sigma Annexin-V-FITC apoptosis detection kit manual].

Gene regulation at transcriptional and protein levels

The DNA microarray techniques allows the study of hundreds of thousands genes simultaneously. In the field of toxicology, larger scale gene expression analysis provides new insights for better understanding some key issues, such as the mode of action, the molecular mechanism of toxic effects. Due to its high throughput capability, the microarray technique has been extensively used in toxicological studies for gene expression profile analysis by examining the abundance of RNA extracts, assuming that the expression of individual proteins is proportional to the levels of their corresponding mRNAs. However, due to protein translation, degradation and post-transcriptional modifications, changes at the mRNA level are not necessarily reflecting the changes at protein level. Furthermore, such protein processing steps may be critical for a protein's function. It has been suggested that because of post-translational modifications such as

phosphorylation/dephosphorylation, methylation, and glycosylation, the 100,000 human genes may actually produce 10 to 20 million distinct proteins [Service, 2000]. Cellular functions, such as communication, metabolism and building cellular structures, are directly dependent on proteins instead of mRNA. Therefore, analysis of alterations at RNA levels is insufficient for examining or predicting the output of toxicant challenges. With the advancement of proteomic techniques, gene expression studies on the protein level using functional proteomics will definitely provide invaluable complementary information for microarray studies [Banks et al., 2000].

Appendix

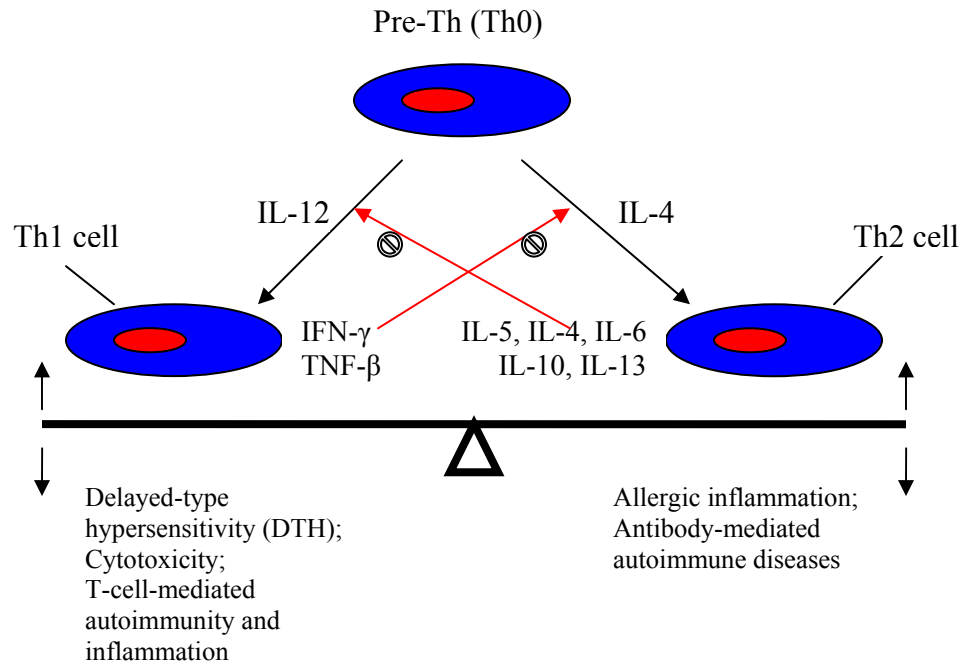


Figure 1: T-cell development and the reciprocal inhibition of Th1 and Th2 cells. Under the influence of a variety of factors including cytokines IL-4 and IL-12, naïve T-cells (Th0) can mature into one of two phenotypes, Th1 or Th2, which then inhibit each other. The dynamic balance between Th1 and Th2 cells is associated with the development of abnormal immune responses/diseases.

PART 2

RATIONAL AND SPECIFIC AIMS

Rational

Concerns have arisen about environmental and health effects of DU due to its wide use in industry and military. DU can enter the body by inhalation, contamination of wounds, ingestion, and releasing of some embedded DU shrapnel [McClain et al., 2001]. Exposure to radiation from DU metal presents a negligible hazard and the primary harm rendered by DU comes from its chemical activity [Priest, 2001; Fisenne and Welford, 1986]. Studies have reported that exposure to DU or DU-related industrial dust causes respiratory diseases and cancers or tissue injuries. Histopathological changes were observed in liver, thyroid, and kidney [Gilman et al., 1998]. The detection of systemic lupus erythematosus-typical antibodies in quartz dust-exposed uranium miners indicates a potentially higher risk for the development of systemic autoimmune disease [Conrad et al., 1996; 1998]. Acute and chronic uranium poisoning have been reported to cause the pathological changes in the testes and thyroid glands, similar to those of an autoimmune process. Autoantibodies to the testes and thyroid glands were also found to circulate in the blood of poisoned animals [Malenchenko et al., 1978]. Several *in vitro* studies suggest that uranium exposure can cause several toxic effects on immune cells (e.g. macrophages) including alteration of cytokine production and cell morphological changes, even cell death. Some of the changes are associated with immune responses [De Rey et al., 1984; Kalinich et al., 2002; Kalinich and McClain, 2001]. After the gulf war, in which several hundred military personnel were exposed to DU to various degrees, it was hypothesized that the gulf war syndrome may be explained as a DU induced systemic shift in cytokine balance from a Th1 towards a Th2 profile [Rook and Zumla, 1997;

Skowera et al., 2004]. Interestingly, the Gulf War Syndrome (GWS) is clinically similar to autoimmune diseases. Several years after the gulf war, the GWS still prevalent indicating that the effects maybe persistent after exposure.

While these above mentioned studies present evidence for a DU related toxic effect on the immune system, little is known about the molecular mechanisms of these effects. The present study was designed to gain some understanding of the molecular mechanisms of DU immunotoxicity, as well as to determine the toxicity of DU to immune cells and the influences of DU on the immune functions. There are four major kinds of cells in mammalian immune system, i.e. T-lymphocyte, B-lymphocyte, dendritic cell, and macrophage. Of these cells, B-cells are mainly responsible for antibody production while their other functions such as cytokine production and antigen presentation are minor compared with other cells. T-cells regulate immune responses by cytokine production and play essential roles in cell-mediated immunity. Furthermore, it is widely accepted that macrophages are a precursor of most DCs and share some features with DCs [Mills et al., 2000; Banchereau and Steinman, 1998]. Therefore, this study focused on assessing the toxic effects of DU on T-cells and macrophages, which play major regulatory roles in innate and adaptive immunity, respectively.

Specific Aims

1. Determine the cytotoxicity of DU in primary macrophages, primary CD4⁺ T-cells, and T-Cell Hybridoma (TCH) PLP1 5B6.
2. Assess the accessory cell function of DU-exposed macrophages.

3. Identify the differentially expressed genes in peritoneal adherent macrophages, primary CD4⁺ T-cells, and TCH PLP1 5B6 cells under DU exposure to understand the molecular mechanism(s) of DU toxicity.

PART 3
MATERIALS AND METHODS

Reagents

Uranyl nitrate ($\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) and Sodium nitrate (NaNO_3) were purchased from Mallinckrodt specialty chemicals Co. Lipopolysacchride (LPS) and Concanavalin A (Con A) are products from Sigma Inc.

Mouse Strains

BALB/c and DO11.10 transgenic mice were originally obtained from the Jackson Laboratory and bred and housed under pathogen-free conditions in the Animal Care facility at the University of Tennessee, Knoxville. Six-to-eight week old mice were anesthetized and killed by cervical dislocation, after which cells were obtained.

Isolation of peritoneal macrophages and DU exposure

Thioglycollate (TG) broth (Difco) (TG, 3% wt/vol) was injected intraperitoneally (1ml/mouse) in Balb/c mice. Four days later, the peritoneal cavity was aseptically lavaged with 10ml ice-cold RPMI-1640 (Gibco) containing 1U/ml sterile heparin to obtain peritoneal elicited cells (PECs). PECs from 3-4 mice were pooled in each experiment to obtain enough cells for analysis. PECs were plated onto 25cm² Corning cell culture flasks or polystyrene 6-well flat bottom microtiter plates and were incubated at 37°C, 5% CO₂-95% air, and 95% humidity for 4 hours to allow the macrophages to adhere to the surfaces. The surfaces were washed two times with warm Phosphate-Buffered Saline (PBS) to remove all non-adherent cells and the macrophage layer was cultured overnight in complete RPMI-1640 containing 10% low-endotoxin, heat deactivated Fetal Bovine Serum (FBS) (Sigma Chemical Co.), 10⁻⁵M 2-mercaptoethanol,

L-glutamine (20mM), penicillin and streptomycin (100U/ml each). The next day, the macrophage layer was washed twice with warm PBS and lifted using a cell scraper. Cells were then washed (200g, 10min, 4°C) twice, stained with Trypan Blue exclusive dye and counted. After that, the cells were adjusted to a concentration of 1×10^6 cells/ml in complete RPMI-1640 (cRPMI-1640) and exposed to uranyl nitrate ($\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) at various concentrations (0-200 μM) in 25cm² flasks (for RNA isolation) or 6-well plates (for flow cytometry analysis). Simultaneously, an aliquot of the cells was used to determine the purity of macrophages using Phycoerythrin (PE)-conjugated monoclonal antibodies (mAb) against CD11b (BD Pharmingen), a surface glycoprotein found on mature macrophages. Cells were washed with cold PBS and 0.4 μg of antibody was added and incubated for 30 minutes on ice, followed by a PBS wash. The samples were then subjected to flow cytometer analysis and 15,000~30,000 events were read. Fresh cells from new living animals will be purified every time the assays were repeated.

Purification of splenic CD_4^+ T-cells and DU exposure

Spleens from 3-4 DO11.10 mice were pooled together and a single cell suspension was obtained by mechanic disintegration. After erythrocyte lysis (ammonium chloride), CD_4^+ T-cells were negatively isolated using the Magnetic Activated Cell sorting (MACs) method according to the manufacture's protocols (Miltenyi Biotec.). In brief, splenic cells were stained with a cocktail of biotin-conjugated monoclonal antibodies against CD8a(Ly-2) (rat IgG2a), CD11b (Mac-1) (rat IgG2b), CD45R (B220) (rat IgG2a), DX5 (rat IgM) and Ter-119 (rat IgG2b) for 10 minutes at 4°C. Then the mixture was incubated with Anti-Biotin MicroBeads for 15 minutes at 4°C. After dilution

of the cell solution, the cells were centrifuged and re-suspended in degassed PBS buffer at a density 1×10^8 cells/500 μ l. The cell suspension was passed through an LS magnetic separation column and subsequently washed three times with 3 ml degassed PBS buffer to collect CD4⁺ T-cells. The negatively selected cells were centrifuged and re-suspended in cRPMI-1640 media at density 1×10^6 cells/ml. An aliquot of the cells were stained with PE conjugated anti-mouse CD4 antibodies to assess the CD4⁺ T-cell purity. Cells were washed with cold PBS and 0.4 μ g of antibody was added and incubated for 30 minutes on ice, followed by a PBS wash. The samples were then subjected to flow cytometer analysis and 15,000~30,000 events were read.

The cells density was then adjusted to 1×10^6 cells/ml and the DU exposure was performed in an anti-CD3 coated 96-well plate with various concentrations (0-500 μ M) of DU and 1mM NaNO₃ or 1 μ g/ml Staurosporine (positive control) for 24 and 48 hours before RNA isolation or flow cytometry analysis. Fresh cells from new living animals will be purified every time the assays were repeated.

T-cell hybridoma PLP1 5B6

T-cell hybridoma (TCH) 5B6 (Th1 type) is an immortalized murine T-cell line. This cell line recognizes proteolipid protein (PLP)-1 peptide 139-151 in association with MHC class II I-A^s [Legge et al., 1997]. Cells were typically grown in 25-cm² flasks or polystyrene 6-well cell culture plates and maintained in humidified incubator at 37 °C and 5% CO₂-95% air. The suggested cell culture media (DMEM, Dulbeccos Modified Eagle Medium) were used to accommodate the cell growth requirements and contains 10% FBS, 50 μ g/ml gentamycine sulfate, 2mM L-glutamine, 50 μ M 2-Mercaptoethanol,

0.11mg/ml sodium pyruvate, and $1\times$ essential and non-essential amino acids. The media was replenished every other day, and cells were washed and seeded for DU treatment in 6-well plate.

Lymphoproliferation assay

T-lymphocyte proliferation assay was used to estimate the macrophage function; the assay was performed in triplicate. A 200 μ l of the suspension of elicited peritoneal macrophages (1×10^6 cells/ml) was placed into wells of 96-well microtitre plate (Costar), allowed to adhere for 4 hours, washed and cRPMI-1640 media containing various DU concentrations (10, 50, 100, 200, 500, and 1000 μ M), or an equal amount of medium with no DU was added. Media containing 1mM of NaNO₃ was set as a NO₃⁻ control. After 2 hours incubation, the cells were washed three times with cRPMI-1640, then 200 μ l of the purified CD4⁺ T-cell suspension (1×10^6 cells/ml with 5 μ g/ml Con A) was added to each well containing treated adherent macrophages according to Krocova [2000]. Simultaneously, non-T-cell controls were set up by replacing the T-cells with same amount of culture medium. The plate was incubated at 37°C, 5% CO₂-95% air, and 95% humidity for 48 hours. Then, 10 μ l of MTT (3-(4,5)-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) solution (5mg/ml) was added to each well and the plate was allowed to incubate for an additional 4 hours. At the end of the incubation, 100 μ l of acidic isopropanol (0.04M HCl in absolute isopropanol) was added and mixed to dissolve the converted dye, formazan. The absorbance data were recorded by spectrophotometer at 562nm.

Cell staining and flow cytometry analysis

The cell death analysis using flow cytometry was performed in triplicate. Treatment of cells was conducted according to the protocol provided with the annexin-V-Fluorescein (annexin-V-FITC) Apoptosis Detection Kit (Sigma Inc.). The cells were treated with different concentrations uranyl nitrate solution at cell density of 1×10^6 cells/ml. After the treatment, cells were harvested and washed once with PBS. Cells were then resuspended in 1x binding buffer at a cell density of approximately 1×10^6 cells/ml. 500ul of the cell suspension was stained with 5 μ l of annexin-V-FITC and 10 μ l of propidium iodide in a 12 $\times$ 75mm test tube for 10 minutes at room temperature, protected from light. Cells were analyzed using flow cytometry FACScan (BD bioscience) by counting 30,000 events. The software package WinMDI version 2.8 was used to analyze the flow cytometry data. A SSC vs. FSC plot was used to distinguish debris and cells for setting the analysis gate. Two-tail T test were performed to test the significance of differences between treatments using Excel (Microsoft), or alternatively, histogram plot analysis was performed using CellQuest software (BD bioscience).

RNA isolation

Total RNA preparation for microarray analysis was performed on pooled cells from 3-4 mice (in case of primary cells), and was repeated 3-4 times for 3-4 microarray replicate experiments. Cells were treated with various concentrations of DU in media at 37°C, 5% CO₂-95% air, and 95% humidity. Isolation of total RNA using TRIzol reagent was conducted according to the manufacture's protocol (GibcoBRL). In brief, 1ml of TRIzol reagent was applied directly onto a 10cm² monolayer to lyse the cells. The

homogenized samples were incubated at room temperature for 5 minutes before extraction with chloroform. Phase separation was achieved by placing the samples at room temperature for 5 minutes, followed by centrifugation at 12,000×g and 4°C for 15 minutes. The aqueous phase was mixed with 0.5 ml cold isopropyl alcohol per 1 ml TRIzol reagent. Samples were incubated at room temperature for 10 min, then centrifuged at 12,000× g and 4°C for 10 min. The RNA pellet was washed once with 1 ml of cold 75% ethanol, then centrifuge at 7,500× g and 4°C for 8 min, after which the pellet was air dried for 5 minutes, and then resuspended in nuclease-free water. The total RNA samples were quantified using a Beckman DU-640B spectrophotometer (Beckman Instruments, Inc.). The integrity of RNA samples and genomic DNA contamination were assessed by agarose gel electrophoresis. Total RNA samples were treated with RNase-free DNase I to remove residual genomic DNA.

cDNA labeling and array hybridization

³³P-labeled cDNA probes were synthesized according to the protocol provided with the StripAble™ cDNA Synthesis and Removal Kit (Ambion Inc.). The Panorama™ mouse cytokine gene array consisting of 514 different cytokine-related cDNAs printed as PCR products onto charged nylon membranes was used. The detailed description and a list of genes included on the array can be found on Sigma Genosys's website (http://www.sigma-genosys.com/epp_mammalia_mc_generalinfo.asp). Briefly, for each sample, 2 µg of total RNA was annealed to a mixture of mouse cytokine gene cDNA labeling primers, by incubation at 65°C for 5 minutes. The cDNA synthesis was performed in 20-µl reactions containing 1× RT reaction buffer (50mM Tris pH8.3, 75mM

KCl, 3mM MgCl₂), 5mM DTT, 1× dNTP solution containing modified nucleotide for synthesis of strippable probes, 200 units MMLV-RT and 20μCi [³³P-dATP] (3000Ci/mmol, 10mCi/ml; ICN) with incubation at 42°C for 2 hours. Unincorporated nucleotides were removed from the [³³P]-cDNA products using NucTrap® probe purification columns (Stratagene). The specific activities of labeled cDNAs were determined by scintillation counting (Packard Inc.). EDTA was added to bring the final concentration to 10mM and the labeled cDNAs were then heat denatured at 95°C for 5 minutes. Arrays were prehybridized with ULTRArray hybridization buffer (Ambion, Inc.) in roller bottles in a hybridization oven (Fishery scientific) at 65°C for at least 1 hour. The pre-hybridization buffer was discarded and then denatured cDNA mixture and fresh hybridization buffer were incubated for overnight with rolling rate at 6 rpm, 55°C. The arrays were then washed extensively at 50°C under both low and high stringency conditions (2×SSC, 0.5% SDS in low stringency wash solution, 0.5×SSC, 0.5% SDS in high stringency wash solution) for 2×30 min. The membranes were air dried briefly, sealed in a clear plastic bag and exposed to Kodak low energy storage phosphor screens HD (Molecular dynamics) for 2-3 days.

Immediately after imaging, the membranes were stripped for reuse according to the protocol provided by StripAble™ cDNA Synthesis and Removal Kit (Ambion Inc.). Briefly, at room temperature, 1× Probe Degradation Buffer was added into the roller bottle with the membranes and incubated at room temperature for 5 minutes. The Probe Degradation Buffer was discarded and the membranes were incubated in 1×Blot Reconstitution Buffer containing 0.2%SDS for 20 min at 68°C. Typically, over 95% of the labeled cDNAs will be removed from the membranes with this treatment.

Quantification and statistical comparison of gene expression profiles

The exposed storage phosphor screen was scanned at 50-micron resolution on a Storm 840 PhosphorImager (Molecular Dynamics). The image files were analyzed using ArrayVisionTM software version 6.0 (Imaging Research), and the numerical output was exported in Microsoft Excel format to ArrayStat Version 1.0 (Image Research) for statistical analysis. Blots were normalized to the mean values of the entire array with background subtraction. The data were then transformed logarithmically, and a Z-test for two independent conditions was performed on 3-4 sets of repeated individual experimental data according to Benjamini and Hochberg [1995]. Differentially expressed genes were identified on the basis of the significance level.

Quantitative real time Reverse Transcription PCR (QRT-PCR) analysis

QRT-PCR was used as a verification method for gene expression. Real time-PCR was performed in triplicate in a DNA Engine Opticon system (MJ research, Inc.) using SYBR Green I as the detection format (Qiagen). Each assay was performed in triplicate. After first strand cDNA synthesis in reverse transcription reaction, PCR amplification was carried out in a total volume of 25 µl containing 1× SYBR Green I and ROX, 1× PCR Buffer, 1× dNTP mix, HotStarTaq DNA Polymerase, 0.3µM each primer, and 1 µl of cDNA solution. The reactions were incubated at 95°C for 15 min and then cycled 40 times under the following steps: 94°C for 15 s, 50–60°C for 30 s, 72°C for 30 s. At the end of the PCR reactions, the temperature was increased from 55°C to 99°C at a rate of 1 °C/10 s, and the fluorescence was measured at every 1°C, with a 10 seconds hold between reads to construct the melting curve. A non-template control (NTC) was

performed for both the putative differentially expressed and reference genes. After melting curve analysis, the reactions were incubated at 72°C for an additional 7 min before agarose gel electrophoresis of the PCR products. Both melting curve data and gel images of the PCR products were used to assess the specificity of the QRT-PCR assay.

At the end of the PCR, all QRT-PCR data were stored automatically by Opticon software for further analysis. During PCR cycling, some initial cycles were considered as baseline by software and virtually the fluorescence intensity changes were not too much. When PCR reaction went through a certain number of cycles and the fluorescence intensity become detectable, the cycle number is termed threshold cycle number (Ct). Ct always occurs in the exponential phase of the PCR reaction and is inversely proportional to the initial number of template molecules in the sample. The software automatically determined the baseline and Ct values. For both reference gene and interested gene, standard curve was constructed and the slope was determined to calculate the PCR efficiency according to Pfaffl [2001; 2002]. The gene expression level (Ct value) was normalized with the GAPDH (glyceraldehydes-3-phosphate dehydrogenase) house keeping gene, and then the normalized values of DU-treated cells were divided by those of control cells to obtain the relative expression ratio [Pfaffl, 2001; Pfaffl et al., 2002].

Northern blot analysis

Northern blot was also used to confirm the results from cDNA array analysis. 20 microgram of total RNA per lane were denatured in 2.2 M formaldehyde and electrophoresed in a 1.5% agarose-formaldehyde gel using MOPS/EDTA as the running buffer. The RNA was blotted by capillary transfer onto a nylon membrane and cross-

linked by exposure to UV radiation according to the protocol provided by TurboBlotter rapid downward transfer systems (Schleicher & Schuell Inc.). The templates used for synthesizing probes were amplified from total RNA by RT-PCR. The fragments (~200-300 bps in size) were gel purified using GeneClean gel purification Kit and sequenced. PCR-generated fragments labeled with [³²P-dATP] were purified by NucTrap purification columns. Hybridization was carried out at 42°C in ULTRAhyb hybridization buffer (Ambion, Inc.) for overnight in rolling bottles. After washing at 42°C twice for 15 minutes in low-stringency condition, twice for 15 minutes in high stringency condition, the blots were exposed to a low energy storage phosphor screens HD for 2 days. The screen was scanned using storm 840 phosphorimager at 50-micron resolution; signal quantification was performed using ImageQuant 5.0 software (Molecular Dynamics).

PART 4

TOXICOLOGICAL EFFECTS OF DEPLETED URANIUM ON PERITONEAL ADHERENT MACROPHAGES

Abstract

Due to macrophages' active phagocytic activity and ubiquitous presence throughout the body, they are one of the first cells involved in the body's response to uranium exposure and play a vital role in both innate and adaptive immunities. In this study, the toxicological effects of depleted uranium (DU)-uranyl nitrate on mouse peritoneal adherent macrophages were investigated.

The results showed that DU caused cytotoxic effects on macrophages in a time and concentration dependent manner. Macrophages treated with 200 μ M of DU exhibited a significant ($P<0.05$) increase in cell apoptosis after as little as 4 hours, and the cell apoptosis also occurred after 24 hours of treatment with 100 μ M DU. Macrophage apoptosis was accompanied with cell necrosis. The morphological changes revealed by microscopy in apoptotic cells were the formation of apoptotic bodies, chromatin condensation and margination, cell shrinkage, and membrane blebbing. The accessory cell function of macrophages has been evaluated using a lympho-proliferation assay. Pretreatment of macrophages with DU for 2 hours significantly enhanced their ability to induce the proliferation of CD4⁺ T-lymphocyte, indicating altered immune functions of macrophages under DU exposure.

The PanoramaTM mouse cytokine gene array, which contains 514 different cytokine-related cDNAs printed as PCR products onto charged nylon membranes, was used to study the gene expression profiles of macrophages under DU exposure. The microarray results identified 24 up-regulated genes and 5 down-regulated genes in macrophages under non-cytotoxic DU exposure. The induced genes were: NF-kBp65,

LTBP-2, Wnt-8b, Midkine, c-jun, NF-kB inducing kinase, BDNF, SOCS-1, c-myc, NSG1/p21, IL-10, stat-1, IL-12Rb1, CIS3, BMP-9, SMAD7, Toll-like R2, CT-1, EphA3, MMP-13, BMP-11, GDNF, Intergrin-b7 and MMP-8 with ratios ranging from 1.6 to 3.9. The repressed genes were Toll-like R6, Cox-2, VCAM-1, FLIPL and SARP-1 with relatively constant ratios, 0.5-0.6. The induction of six selected genes was further confirmed using quantitative RT-PCR. Some of the differentially displayed genes were related to signal transduction pathways or cytokine production suggesting possible involvement of DU in cancer development, immune or inflammatory responses, and chronic diseases.

Introduction

Uranium is a naturally occurring element that is present in soil, rocks, surface and underground water, air, plant, and animals. As a result, it occurs in trace amounts in many foods and in drinking water. The extensive use of uranium in military and industrial applications has provoked a great concern for the possible environmental and health effects of uranium exposure. Uranium has been used in many aspects, e.g. fuel for nuclear power plant, ceramic glazes, light bulbs, photographic chemicals, chemical catalyst, radiation shielding and munitions. Therefore, uranium can be redistributed into air, water, and soil by anthropogenic processes. Elevated levels of uranium have been identified in at least 54 of 1,517 current or former EPA NPL hazardous waste sites [HazDat, 1999]. Contamination of surface water and underground water by effluents from uranium mining, milling, and production operations has been documented as well [Yang and Edwards, 1984; Swanson, 1985]. DU was first deployed in military

armaments during gulf war, tons of DU have been released to environment and several hundreds US military personnel have been exposed to DU particles in explosions or been injured by DU fragments.

Studies of DU toxic effects on specific organs or tissues, and some more epidemiological studies revealed that exposure to DU-related industrial dust causes respiratory diseases and cancers or tissue injuries, the mechanisms of these pathological changes are still mystery. Histopathological changes were observed mainly in rat liver, thyroid, and kidney [Gilman et al., 1998]. Much information was known for kidney damage, but the effects maybe the secondary consequences of the interaction between metals and immuno-competent cells. Thus, although the observed toxic response is on the kidneys, the effect may be presumably due to the immunotoxicity of metals [Priest, 2001].

Macrophages are ubiquitously distributed mononuclear phagocytes responsible for a variety of homeostatic, immunological, and inflammatory processes. They play a pivotal role in the immune system, functioning as phagocytic cells, antigen presenting cells etc. [Abbas et al., 1997]. Their wide tissue distribution makes these cells well suited to provide an immediate defense against foreign elements prior to leukocyte immigration. Macrophages are capable of engulfing foreign materials such as dust in lung tissue, or microbes and participate in both innate and adaptive immunities. The molecule(s) engulfed by macrophages can be processed and presented on the cell surface in the context of MHC-I (for stimulating cytolytic T-cells) or MHC-II (for stimulating helper T-cells) molecules and act as a stimulus to lymphocytes. This accessory cell function of macrophages is essential for the induction of T-cell-dependent immune response. In

addition, macrophage themselves produce cytokines such as IL-1, IL-10, IL-12, IFN- γ etc. that can activate Th cells and regulate immune responses [Stout and Suttles, 1997; Banchereau and Steinman, 1998; Lalani et al., 1997]. Macrophages also express a variety of receptors that mediate the reorganization and phagocytosis of cells and subsequent generation of inflammation to eradicate infection [Ying and Teo, 2003]. Heavy metals were able to modulate the macrophages functions and cytokine productions *in Vitro* [Krocova et al., 2000; Gazin et al., 2002]. It has been shown that macrophages can actively uptake DU particles [Kalinich et al., 2002], which could lead to much higher intracellular DU concentrations in macrophages, therefore these cells are more readily subject to DU effects.

Previous work showed that DU has toxic effects on the immune system of mammals [Pellmar et al., 1999]. As a heavy metal, DU may exert its toxicity by affecting the functions of immune cells after interfering with the signal transduction pathway as well as the production of cytokines [Gazin et al., 2002]. Only a few studies have addressed the effects DU has on immune cells, but they did not seriously attempt to elucidate the overall molecular mechanisms involved, which would be invaluable for selecting therapeutic targets of DU-related diseases and better understanding the risk of environmental DU exposure.

The development of high throughput microarray technology allows gene expression studies on thousands of gene simultaneously, exponentially increasing the information in the field of toxicogenomics. Recent applications of this technique in immunology include the monitoring of changes in the expression caused by inflammation [Heller et al., 1997], cytokines [Panelli et al., 2002; Der et al., 1998] and bacterial

infections [Ragno et al., 2001], or comparisons between cell populations [Chtanova et al., 2001]. Unfortunately, there is still a lack of the toxicogenomic study on DU exposed immune cells in spite of the observed heavy metal toxicity of DU on immune system, the molecular mechanisms of its immune toxicity are mostly unknown.

In this study, we investigated the cytotoxicity of DU on peritoneal adherent macrophages and its effect on accessory cell function of macrophages using lymphoproliferation analysis. Most importantly, a series of cytokine genes have been identified as differentially expressed in these cells under the DU exposure.

Results

Purity of macrophages and CD4⁺ T-cells

PE-conjugated anti-mouse cd11b and anti-mouse CD4 antibodies were used to stain and detect the purity of macrophages and T-cells, respectively. The quadrant data showed that the purities of macrophages and CD4⁺ T-cells are > 95% after purification (Figure 1). (All tables and figures in appendix at the end of the part)

Lymphoproliferation assay

Concanavalin A (Con A) has been used to activate T-cells for many years. The likely mechanism is that Con A indirectly crosslink the T-cell receptor (TCR) and send the activation signals. Activation of T-cells is also dependent on the presence of non-T-cells that function as accessory cells, which provide additional and essential costimulatory signals for T-cell proliferation. [Coligan et al., 1991] In this assay, ability of macrophage functioning as accessory cells to stimulate lymphocyte proliferation was

tested. Macrophages were pretreated with different concentrations of DU ranging from 10 to 1000 μ M and then T-cell proliferation responses to the Con A (5 μ g/ml) with the help of these macrophages were monitored by MTT analysis (all data can be found in appendix at the end of the part). Exposure to DU with concentrations >200 μ M significantly enhanced the functionality of macrophages as shown by increased T-cell proliferation under the induction of Con A. Lower concentrations of DU treatment such as 10, 50, and 100 μ M did not change the T-cell proliferation with O.D.(562nm) values similar to those of the controls. However, 200, 500 and 1000 μ M of DU treatment of macrophages significantly ($P < 0.05$) enhanced T-cell proliferation with the O.D. measurements of T-cells increasing from 0.56 (control) to 0.76, 0.86 and 0.87, respectively. In addition, 1mM NaNO₃ treatment did not influence the measurement indicating that the alteration of T-cell proliferation caused by uranyl nitrate treatment is due to uranyl ions (UO₂²⁺) but not nitrate ions (NO₃⁻). The samples without addition of T-cells showed similar readings (white bars in figure 2.) indicating the viable macrophages numbers were equal in all the samples after the DU treatment. Therefore, it is safe to preclude the possibility that the alteration of T-cell proliferation are caused by unequal macrophage cells.

Macrophage apoptosis under DU exposure

Induction of apoptosis of macrophages was determined by flow cytometry analysis of Annexin-V-FITC/Propidium iodide (PI)-labeled cells. Annexin-V protein can bind, by a calcium-dependent process, to phosphatidylserine (PS) presented on the surface of cells undergoing apoptosis [Bertho et al., 2000]. PS is normally sequestered on the inner leaflet of plasma membrane. However, during apoptosis, membrane

phospholipid asymmetry is lost and PS is exposed to the outer leaflet, where it can interact with annexin-V. Cells that stain positively for Annexin-V and negatively for PI are considered as cells in early stage of apoptosis, while that stain positively for both indicators are either in late stage of apoptosis or they are undergoing necrotic cell death.

The treatment of macrophages with DU, uranyl nitrate, resulted in apoptotic cell death. As can be seen in figure 3, treatment with 20, 50, and 100 μ M DU for 4 hours did not cause significant increase of Annexin-V staining. The same result was observed for cells treated with 20 and 50 μ M DU for 24 hours; however, there was a significant increase of Annexin-V positive cells after treatment with 200 μ M DU for 4 hours and 24 hours ($P < 0.05$), with the percentages of 4.5% and 30.5%, respectively, while the percentage of Annexin-V positive cells in control groups are 3.5% and 6.7%, respectively. 100 μ M DU exposures for 24 hours also led to much higher percentage of annexin-V positive cells, 12.27%, at $P < 0.05$. The results indicated that the amount of phosphatidylserine in the plasma membrane outer leaflet, or cell apoptosis, increases with increasing DU concentration and over time as shown in figure 3C.

Morphological changes of macrophage under DU exposure

Morphological changes occur as cells undergo apoptotic death that can be characterized as chromatin condensation and margination, cell shrinkage, membrane blebbing, and formation of apoptotic bodies. The morphological changes of macrophages treated with 100 μ M DU for 24 h were investigated using bright-field microscopy and atomic force microscopy and are shown in figure 4. Compared to control cells, which have smooth-round shape and apparent nucleus structure (panel A and C), the treated

cells undergo shrinkage, losing nuclear and membrane structure, indicative apoptosis. The formations of apoptotic bodies are visible in panels B and D.

Cytokine gene expression profiles of macrophages upon DU exposure

To determine a non-cytotoxic DU concentration, the percentage of necrotic cells and cells in late apoptotic stage after 24 hour exposure were measured using flow cytometry analysis. The percentage of dead cells (PI⁺ and Annexin-V-FITC⁺) was significantly increased from 8.2% (control) to 12.4% and 46.2% under 100 and 200 μ M DU exposures, respectively, $P < 0.05$ (Figure 5). Lower concentrations, such as 20 and 50 μ M, did not cause any significant change in dead cell percentage. Therefore, the DU concentration for microarray experiments was set at 50 μ M.

Uranium, as a heavy metal, is known to modulate production of cytokines in macrophage cells lines [Gazin et al., 2002]. In the present study, peritoneal adherent macrophages were treated with or without 50 μ M DU in 25cm² flasks for 24 h. Total RNA isolation was performed immediately after removing the culture media. Cell cytokine gene expression was measured by microarray. Table 1 lists the methods and parameters for Z-tests performed on three replicate data. The statistical analysis was based on two independent conditions: control (PIM_1A) and 24hrs 50 μ M DU exposure (PIM_1B). The data normalization was performed on the mean intensity values of whole array. The minimum No of replicate was set as 2 in Table 1 meaning that for any particular gene, if there is only one valid observation present within a condition, that datum was disregarded for analysis. The outliers were detected by examining standardized residuals automatically by ArrayStat software. Curve fit random error

estimate method was employed for proportional model with offset. The effective alpha value for statistical tests was set at 0.05. Table 1 shows there were 29 (6% of all analyzed genes) genes that were differentially regulated upon 50 μ M DU exposure. Of these 29 genes, 24 (5%) were up-regulated and 5 (1%) were down-regulated. Figure 6 displays the representative arrays of cytokine gene expression of macrophages with 50 μ M DU or without DU exposure. Some of the differentially displayed gene spots were indicated with arrows in those arrays based upon statistically analysis described in Material and Method section (Figure 6). The average normalized expression values for these differentially displayed genes were plotted in Figure 7. Toll-like R6, VACM-1, Cox-2, FLIPL, and SARP-1 were down-regulated by almost the same ratios (0.5~0.6) with similar *p* values, while the ratios for up-regulated genes range from 1.6 to 3.9 with more various *p* values ranging from 5.8E-08 to 0.05 (Table 2). The gene functions involved include signal transduction (8 genes), Interleukin and Interleukin receptor (2 genes), cytokine and cytokine receptor (1 gene), apoptosis related (4 genes), Neurotrophic group (3 genes), TGF-beta family (2 genes), cell surface protein (2 genes), protease or related factor (2 genes). Binding protein, developmental factors, Eph family, adhesion molecule and Integrin all have 1 gene been differentially regulated. The results implied the possible involvement of multiple pathways in the toxic effects of DU.

Quantitative RT-PCR assay for differential gene expression confirmation

In order to confirm the results obtained from microarray analysis, quantitative RT-PCR (QRT-PCR) was performed on 6 randomly selected subset of differentially displayed genes identified by microarray analysis. The intention of these experiments was

to demonstrate that the results of microarray analysis could be confirmed by an independent method. QRT-PCR was performed in triplication on Midkine, IL-10, Stat-1, c-jun, BMP-11 and Toll-like R6 compared to the consistent expression of GAPDH. The primers for the selected genes and GAPDH were shown in Table 3, the amplicon sizes range from 119 to 240bp. The reason GAPDH was chosen as the reference gene is because GAPDH gene shows no change in expression between samples, the average value of the ratios of all 8 house keeping genes as suggested previously was very close to that of GAPDH alone, ranging 0.9~1.1.

Firstly, just like the microarray analysis, the integrity of RNA was confirmed in a 1.5% formaldehyde denaturing agarose gel (Figure 8A); the brightness ratios of 28 s and 18 s ribosomal RNA bands in both control (C) and DU-treated samples (T) were ~ 2:1, no genomic DNA contamination was observed on the gel. After that, 2 µg of total RNA were used to synthesize the cDNA in a 33 µl reverse transcription reaction. Then the real-time PCR (using 1 µl of cDNA solution) was set up together with standard curves. For constructing standard curves of both reference and interested genes, a total RNA sample from another experiment was used, and 2 µl of cDNA solution and a series of dilutions of the cDNA solution were used as template for real-time PCR. The PCR was performed in triplication with a no-template control (NTC) for each gene. In figure 8B, the Midkine and GAPDH amplification plots are shown; the average GAPDH Ct values were very similar between control (Ct = 20.97) and DU-treated samples (Ct = 21.66), whereas the average Midkine Ct values for control and DU-treated samples were 27.90 and 27.09, respectively. At the end of PCR reaction, a melting curve analysis was performed. In figure 8C, the specific Midkine T_m peak (86 °C) was observed while the NTC curve has a

primer-dimer T_m peak at 81 °C, which was well separated from that of Midkine curve. In figure 8D, GAPDH T_m peak (82 °C) and NTC primer-dimer T_m peak (73°C) were observed. Also the PCR products were analyzed by gel electrophoresis to estimate the specificity. In figure 8E, both control and DU-treated cells express GAPDH (240bp) and Midkine (230bp). The gel image showed very specific bands for these PCR products and NTC showed no visible band at the corresponding size, as expected, which confirmed the QRT-PCR specificity observed in melting curve plots. The standard curves for both Midkine and GAPDH were constructed to obtain the slopes for PCR efficiency determination [Pfaffl, 2001; Pfaffl et al., 2002]. In figure 8F, the slopes of GAPDH and Midkine standard curves were determined as -3.2672 and -2.7583 with both R^2 values >0.99. The ratio was calculated in Table 4 with a mathematical model referred [Pfaffl, 2001; Pfaffl et al., 2002].

The differential gene expression ratios of c-jun, BMP-11, Stat-1, IL-10, and Toll-like R6 were also determined (data not shown) and compared with those determined by microarray analysis (Table 5). The ratios of all genes except for IL-10 were accordance with those determined by microarray analysis, fitting into the 95% confidence intervals. QRT-PCR assay results in much higher ratio when measuring IL-10 expression.

Discussion

The extensive use of uranium in military and industries has provoked the concern of the possible environmental and health effects of DU exposure. DU can enter the body by inhalation, contamination of wounds, and releasing of some embedded shrapnel, which removal may cause excessive tissue damage [McClain et al., 2001]. Macrophages

play vital roles in both innate and adaptive immunities. Due to its active phagocytosis activity and ubiquitous presence throughout the body, macrophages are one of the first cells involved in the body response to DU exposure. Therefore it is important to know what type of influences DU may have on macrophages. To address this question, primary macrophages from mouse peritoneum were used to study the DU cytotoxicity and its effects on the immune function of macrophages. Most importantly, gene expression profile and differentially expressed genes were identified, which may aid in deriving the molecular mechanisms of DU toxicity on the immune system and understanding the risk of DU exposure.

The first question asked was whether or not DU induces macrophage death and at what level this toxic effect occurs. Once DU enters the cells, the acid phosphatases in the phagolysosomal vesicle of macrophages transform soluble DU into an insoluble form [Berry et al., 1997] and thus inhibit the release of DU. Accumulation of DU has been observed in various macrophage cell lines [Gazin et al., 2004; Kalinich et al., 2002]. With the accumulation of DU in the intracellular space, the cell functions may be altered and cell death occurs.

In this study, flow cytometry analysis of annexin-V binding was employed to study the apoptosis. The results showed that apoptosis occurred after 4 hours with 200 μ M DU and 24 hours with 100 μ M DU treatments. A 50 μ M of DU concentration was determined as non-cytotoxic during 24 hours exposure. These results are similar to that of a previous study using a macrophage cell line, J774, and uranyl chloride as the testing materials. [Kalinich et al., 2002]. The non-cytotoxic concentrations of DU were

comparable in experiments using human osteoblast cells [Miller et al., 1998] and macrophages.

Time and concentration dependent responses were also evidenced in this study; whether the responses were due to accumulation of DU or because of the extended exposure time is unknown. Internalization of DU has been observed previously [Kalinich et al., 2002]. Thus, it is likely that both of these mechanisms contribute to the cytotoxicity. Associated with apoptosis are morphological changes including the formation of apoptotic bodies, chromatin condensation and margination, cell shrinkage, membrane blebbing that were observed using both light and atomic force microscopes. Cell death consists of two major ways: apoptosis and necrosis. In this study, twenty-four hours DU exposure induces both apoptosis and necrosis at 100 and 200 μ M concentrations (Figure 3C and 5), indicating apoptosis and necrosis concurred in macrophages under DU exposure.

In the immune system, apoptosis plays roles not only in tissue and organ development, but also in regulation of the immune response. Both cell number and functions are essential determinants of the output of an immune response. Reducing the number of immune-competent cells through uncontrolled or massive cell death after xenobiotic treatment may lead to serious adverse effects such as immunosuppressions [Pallardy et al., 1999; Byung et al., 1997]. Low numbers of apoptotic cells are removed predominantly by macrophages, which is accompanied by an anti-inflammatory response. In case of larger number of apoptotic cells or inefficient clearance of the dead cells, the released cellular content might become available for professional antigen presenting cells (APCs), presented in the context of MHC-I or MHC-II molecules and invoke a strong

cytolytic response, leading to a proinflammatory context. Subsequently, response against the auto-antigens is initiated and epitope spread to unmodified antigens causing autoimmune diseases [Rodenburg et al., 2000].

This presents the second question, i.e. how DU affects the immune function of macrophages. It is known that several heavy metals, such as mercury and lead, can induce pathological immune manifestation [Jiang and Moller, 1995; Lee et al., 2001; Shen et al., 2001; Pollard and Landberg, 2001; Krocova et al., 2000]. Most of these are activators of immune system causing hyperactivity. The altered immune function can lead to hypersensitivity or autoimmune diseases [Druet, 1995]. In contrast, some heavy metals compromise the immune system, leading to immunosuppression [Krocova et al., 2000].

In addition to the phagocytosis, another important function of macrophages is the antigen presenting or the ability for providing the co-stimulatory signals for activation of lymphocytes, especially T lymphocytes [Burke and Lewis, 2002; Servet-Delprat et al., 2002]. In the presence of Con A, *in vitro* T lymphocyte activation requires accessory cells for co-stimulatory signals, such as IL-1 [Pollard and Landberg, 2001]. In this study, the ability of DU exposed macrophages to function as accessory cells was assessed by measuring the CD4⁺ T lymphocyte proliferation. The results indicated that higher concentrations (200~1000µM) of DU exposure were able to activate murine CD4⁺ T-cells *in vitro*, leading to significant T-cell proliferation. This response was concentration dependent. Compared with other heavy metals such as lead, which induced macrophage to enhance lymphocyte proliferation at a concentration range of 12~120 µM [Krocova et al., 2000], tendency of DU in lymphocyte activation is similar but under higher

concentrations ($>200\ \mu\text{M}$). A spectrum of cytokines has been reported to become differentially regulated upon heavy metal exposure [Lee et al., 2001; Shen et al., 2001; Krocova et al., 2000]. Most of these are related to signal transduction and interleukins that may modulate immune functions. The enhancement of lymphocyte proliferation by lead was due to the suppression of nitric oxides productions (NO), which are known to regulate the proliferation response of lymphocytes. [Krocova et al., 2000]. However, treatment for short time periods or at low concentrations of DU results in a small number of dead cells that are broken down or phagocytosed by surviving cells in the culture [Kalinich et al., 2002], which may also result in alteration of availability of co-stimulatory molecules or MHC molecules. The results in this study demonstrated that short time, high concentration of DU exposure is able to perturb immune function rapidly.

Previous studies on heavy metals indicated the extensive involvement of cytokine regulations in the cytotoxicity and immune modulation activities [Lee et al., 2001; Shen et al., 2001; Krocova et al., 2000;]. It is essential to know the global changes of cytokine gene expression in macrophages when exposed to DU, that information would be invaluable to illustrate the molecular mechanisms for DU toxicity and provide important information on DU-related diseases. In this study, we used mouse cytokine gene array to study the gene expression of cytokines that are involved in signal transduction pathways and immune responses.

In our array there were 514 genes related to signal transduction, interleukin and receptors, apoptosis, cell surface proteins, neurotropic factors and development et al. Eight house keeping genes were included for normalization purpose, several negative and positive control were also spotted for monitoring the array hybridization. We investigated

the effects of 50 μ M DU in the differential gene expression profiles in cultured peritoneal adherent macrophages. Our data showed that the up-regulated genes were those of NF-kBp65, LTBP-2, Wnt-8b, Midkine, c-jun, NF-k B inducing kinase, BDNF, SOCS-1, c-myc, NSG1/p21, IL-10, stat-1, IL-12Rb1, CIS3, BMP-9, SMAD7, Toll-like R2, CT-1, EphA3, MMP-13, BMP-11, GDNF, Intergrin-b7, and MMP-8; down-regulated genes are Toll-like R6, Cox-2, VCAM-1, FLIPL, and SARP-1. The differential expression of 6 randomly selected genes was confirmed by quantitative RT-PCR with ratios similar to those determined in microarray analysis (table 5). The IL-10 expression ratio was higher in QRT-PCR assay than in array analysis, though the up-regulation tendencies from the two assays were the same. Part of the reason is due to its much higher sensitivity of RT-PCR in transcripts detection [Pfaffl et al., 2002] and similar results have been documented for other genes in previous work [Jung et al.,1995].

Additionally, as a positive controls, macrophages were stimulated with LPS (1 μ g/ml) for 24 hours and several highly induced genes, such as IL-1 α , IL-1 β , IL-6, cox-2, GRO α have been successfully identified using the mouse cytokine array analysis (data not shown). Induction of these genes by LPS has been well documented by others using similar conditions [Suzuki et al., 2000; Kraatz et al.,1999]. Agreement with this positive control induction profile suggests that the microarray results are reliable.

The most highly expressed gene was NF- κ B p65 in our cDNA array analysis. p65, also termed RelA, is a subunit of NF- κ B complex. NF- κ B may consist of homo- or heterodimer of different subunits [Zandi and Karin, 1999]. The most abundant form of NF- κ B is composed of p50 or p52 and p65, which contains transactivation domains necessary for gene induction [Tak and Firestein, 2001]. The NF- κ B family of

transcription factors not only are key regulators of genes involved in immune and inflammatory reactions [Li et al., 2001; Tak and Firestein, 2001], but also involved in many aspects of cell growth, differentiation and proliferation via the induction of certain growth and transcription factors (e.g. c-myc, ras, p53). They bridge a critical gap between various forms of cellular stresses and subsequent cellular responses—gene regulation and expression. Activation of NF- κ B protects cells from undergoing apoptosis in response to cellular stress.

NF- κ B subunits are frequently up-regulated in various conditions. A number of stress factors have been demonstrated to rapidly activate NF- κ B in a variety of cells, such as human intestinal lamina propria mononuclear cells (LPMC), thymocytes, Neurons and monocytes [Monteleone et al., 2004; Li et al., 2001; Wooten, 1999]. NF- κ B activation plays an anti-apoptotic role. In the absence of p65, massive apoptotic death of some embryonic cells, such as hepatocytes occurred [Wang et al., 1998]. This study shows that NF- κ B is associated with enhanced transcription of collagenase 13 or MMP-13 gene and c-myc is consistent with other reports [Tak and Firestein, 2001]. Due to its involvement in a wide variety of cellular activities (e.g. inflammation, apoptosis, transformation, oncogenesis), NF- κ B could be considered as a coordinating element in the body's response to stress, injury and especially in the pathways of the immune responses.

NF- κ B is an important nuclear factor controlling TNF- α transcription in macrophages. Inhibition of NF- κ B would abolish the secretion of TNF- α by macrophages under DU exposure [Gazin et al., 2002]. A variety of human inflammatory diseases are associated with activation of NF- κ B. Studies on those diseases, such as Rheumatoid arthritis (RA) and asthma, have localized p65 and p50 in nuclei of

mononuclear cells with greater DNA binding activities in RA compared to controls [Han et al., 1998]. The connection between activation of NF-kB and inflammation lies in the fact that NF-kB regulates genes encoding pro-inflammatory cytokines [Baldwin, 2001]. In necrotic cells, the inflammatory gene expression triggered by intracellular content from different cells also depends on Toll-like receptor 2 [Li et al., 2001]. In this study, DU induced activation of Toll-like R2 has been detected, indicating that DU exposure could increase the risk of invoking inflammatory response. However, some studies have indicated that NF-kB may also mitigate the inflammatory response by inducing the expression of anti-inflammatory genes and induction of leukocyte apoptosis [Bierhaus and Nawroth, 2003]. Co-regulation of NF-kB and IL-10 has been reported [Xu and Shu, 2002]. Bierhaus's work on the interaction between NF-kB and membrane receptors indicated that once the positive feedback between NF-kB and the trans-membrane receptor, RAGE, has been established, perpetual activation of NF-kB eventually converts cellular stress into cellular dysfunction leading to chronic diseases [Bierhaus and Nawroth, 2003]. Activation of NF-kB requires degradation of I-kB with the help of I-kB kinases (IKKs), the activity of which depends on binding with NF-kB inducing kinase (NIK) [Wooten, 1999; Chen et al., 2001]. The activation of NIK as shown in this study confirm that the NF-kB signal pathway was adopted by macrophages under the DU exposure.

Therefore, the likely scenario of non-cytotoxic DU exposure might be as that DU induces NIK activity leading to the up-regulation of NF-kB (by increasing the p65 subunit level) in macrophages and these signal transductions merely constitute the first step for cells counteracting the stress that may or may not initiate cell apoptosis and

inflammatory response. The elevated level of NF- κ B further activates expression of a variety of cytokine genes, such as c-myc and MMP-13, and enhances cell proliferation, inducing immune responses or cause cancers. Actually, the DU tumorigenic activity has been reported in a study that exposed osteoblast cells to 10 μ M of DU [Miller et al., 1998.].

Interestingly, the co-regulation of NF- κ B (p65) and IL-10 was also observed in this study. Murine IL-10, also termed cytokine synthesis inhibitory factor (CSIF), can be produced by T-cells, B-cells, macrophages, and a variety of other cell types. IL-10 is also a pleiotropic cytokine that can act on T-cell, B-cells, and macrophages with a diverse array of actions, which differ depending on cell type, nature of stimulus and the cellular microenvironment. [Malefyt et al., 1991; Lalani et al., 1997]. IL-10 was reported as an anti-inflammatory cytokine that can inhibit the production of IL-1 (α and β), IL-6, and TNF- α , and IFN- γ et al. at the transcriptional level [Malefyt et al., 1991; Chomarat et al., 1993] and thus inhibit Th1 cell development in mice. However, although IL-10 may create a microenvironment for Th2 cell development [Mosmann and Sad, 1996], macrophage-specific overexpression of IL-10 predominantly acts in an autocrine/paracrine manner, resulting in chronically deactivated macrophages that manifest an impaired ability to control pathogens [Lang et al., 2002].

It has been reported that IL-10 may play a vital regulatory role in a variety of diseases, such as experimental autoimmune encephalomyelitis (EAE), multiple sclerosis, Asthma, polymyositis and dermatomyositis et al. [Bettelli et al., 2003; Lalani et al., 1997], some of which are involved Th2 inflammation [Maganan et al., 2000]. IL-10 not only promotes various diseases but also mediate the transition from acute to chronic disease

states [Sadler et al., 2003]. More interestingly, elevated blood IL-10 concentration has been detected in symptomatic gulf war veterans who were potentially exposed to DU in battlefield [Zhang et al., 1999; Skowera et al., 2004]. The data show that DU may contribute to an increase in IL-10 concentration through the action on macrophages that can actively engage and engulf the DU particle.

The expression of the neurotrophic factor—Midkine has been highly induced under the exposure of DU. Midkine is of interest to us due to its important roles in development, oncogenesis, inflammation, and tissues remodeling [Muramatsu, 2002]. Midkine is a basic, low molecular-weight protein that is expressed in vertebrates from human to zebrafish and promotes cell survival, migration of inflammatory cells such as neutrophils, and macrophages, and neurite growth [Sato et al., 2001]. High induction of Midkine expression has been reported in various human tumors, such as cancers of the gastrointestinal tract, kidney, lung and mammary gland, neuroblastomas et al [Sakitani et al., 1999]. Midkine are thought to enhance tumor progression by promoting tumor cell growth, survival, and migration and angiogenic activity. The midkine level often increases in the early stage of cancer progression thus might be used as a tumor marker [Muramatsu, 2002].

It is well known that midkine is able to induce histamine release rapidly in a dose-dependent manner [Mazurek et al., 1986]. The released histamine is a key mediator of allergic inflammation by accumulating inflammatory cells in the peritoneum of mice [He et al., 2004; He et al., 1997; Cairns et al., 1996.]. Therefore, Midkine may also play a role in inflammation. Furthermore, histamine has been reported to induce IL-10 production in human macrophages too [Sirois et al., 2000]. This may partially explain the co-induction

of Midkine and IL-10 in our dataset. Midkine activation in microglia has been reported associated with the progression of experimental autoimmune encephalomyelitis (EAE) suggesting a contributing role of Midkine in the development of EAE [Liu et al., 1998].

Our data showed an approximately 3 fold induction of Midkine expression in macrophages upon DU exposure, suggesting that DU may be carcinogenic through the activation of Midkine which has been shown to prevent cell death. Furthermore, the potentials of DU in inducing both inflammatory reactions and EAE have been indicated, but the determination depends on overall cytokine context and thus requires careful discrimination. Previous studies have reported Midkine expression in skeletal muscle cells epithelium, thyroid and kidney cells [Garver et al., 1993; Hu et al., 2002]. This study demonstrated that under DU exposure, macrophages are able to produce high level of Midkine too. Because macrophages present ubiquitously throughout the body (e.g. microglial cells in the central nerve system, Kupffer cells in liver, alveolar macrophages in pulmonary airways), DU exposure may lead to unexpected local toxic effect(s) on tissues or organs.

These data indicated that at non-cytotoxic concentrations, DU modulates macrophage responses or functions by differentially affecting the signaling pathways in peritoneal adherent macrophages, causing a complex of toxic effects. The *in vitro* system used in this study maybe helpful in identifying molecular targets for the toxic actions of DU and the elucidation of the molecular mechanisms for DU-related diseases. Based on the microarray results, a putative cascade pathway for DU toxicity on macrophages is indicated in figure 9.

Appendix

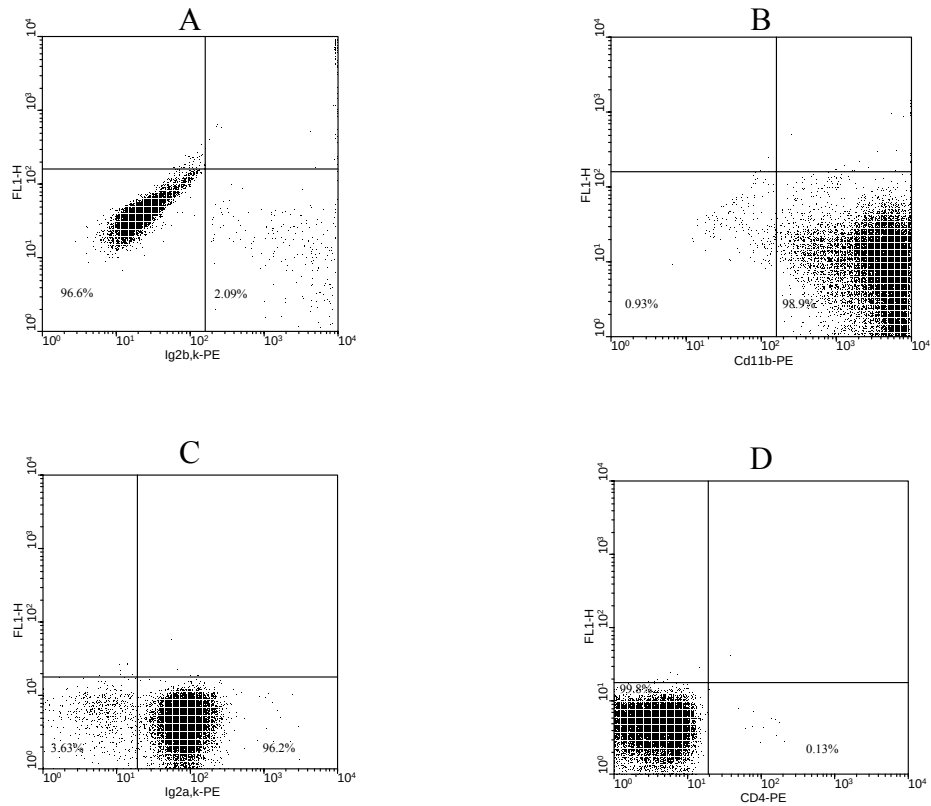


Figure 1: Flow cytometry dotplots for macrophage and CD4⁺ T-cell staining. A, isotype control (Ig2b,κ) for anti-Cd11b mAb; B, anti-Cd11b mAb staining of macrophages; C, isotype control (Ig2a,κ) for anti-CD4 mAb; D, anti-CD4 mAb staining of primary T-cells. The purity of macrophages and CD4⁺ T-cells are both >95%.

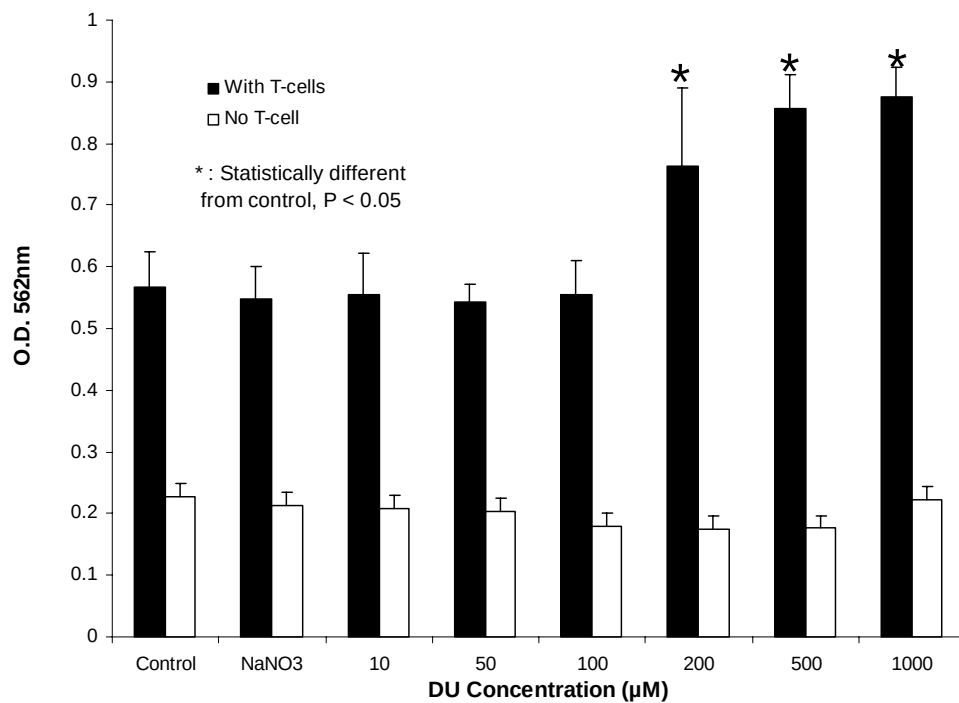
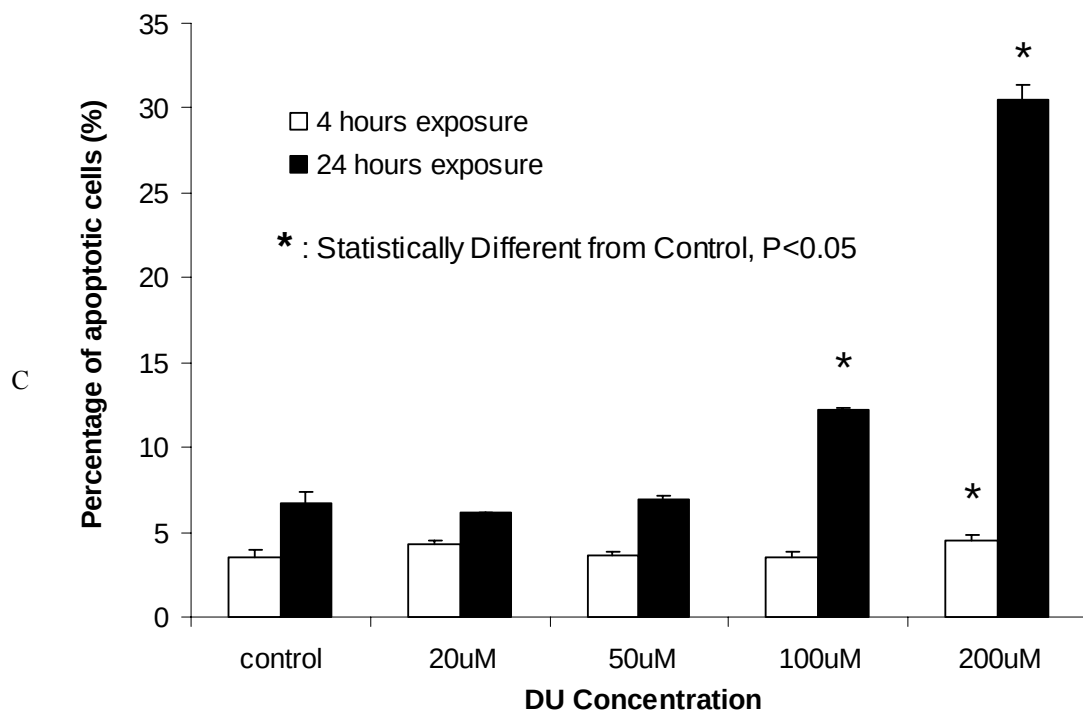
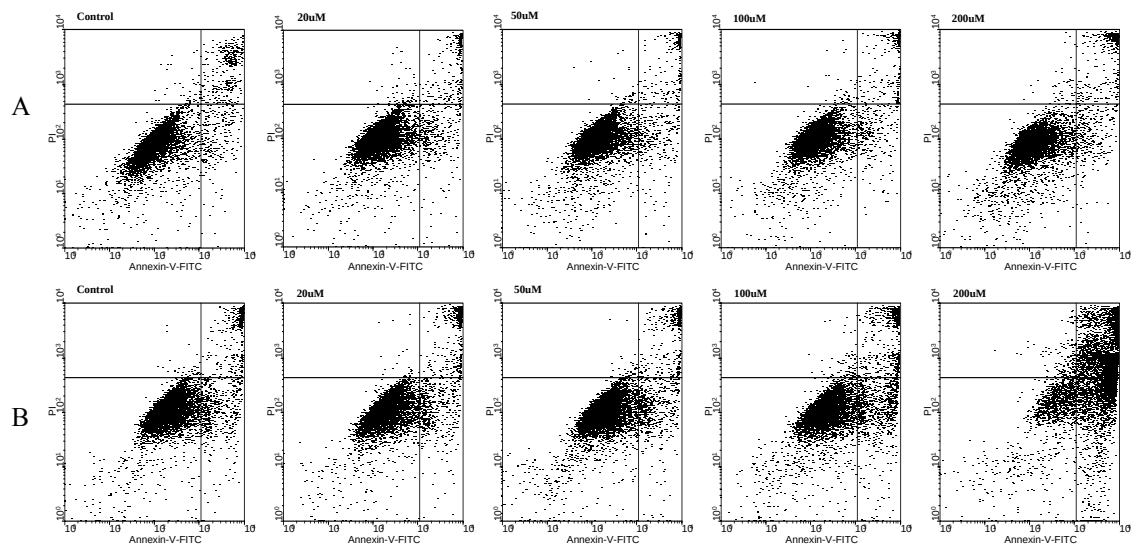


Figure 2: Effect of DU on the accessory cell function of peritoneal adherent macrophages. Adherent peritoneal macrophages were treated with DU in given concentration for 2 hours, and washed. Then the macrophages were co-cultured with non-treated CD4⁺ T-cells for additional 48 hours. The effect of DU on lymphocytes (CD4⁺ T-cells) proliferation was determined by MTT assay which indirectly reflects the accessory cell function of macrophages. Results are expressed in O.D. values read at 562nm wavelength. White bars stand for measurements of samples without T-cell addition, black bars stand for the measurements of samples with equal number of T-cells addition. Asterisks (*) indicate the difference from control is statistically significant (P<0.05). The error bars represent the standard deviation from triplicate results.

Figure 3: Apoptosis of peritoneal adherent macrophages under DU exposure. Macrophages were treated with 0(control), 20, 50, 100, and 200 μ M DU for 4 h (A) or 24 h (B). Data are presented in C as percentage of cells with annexin-V binding (apoptotic cells) at various times post-treatment and are the mean of triplicate experiments. Error bars represent standard deviation. Asterisks indicates that the difference from controls is statistically significant ($P < 0.05$). Panel A and B are representative dotplots for triplicate experiments.



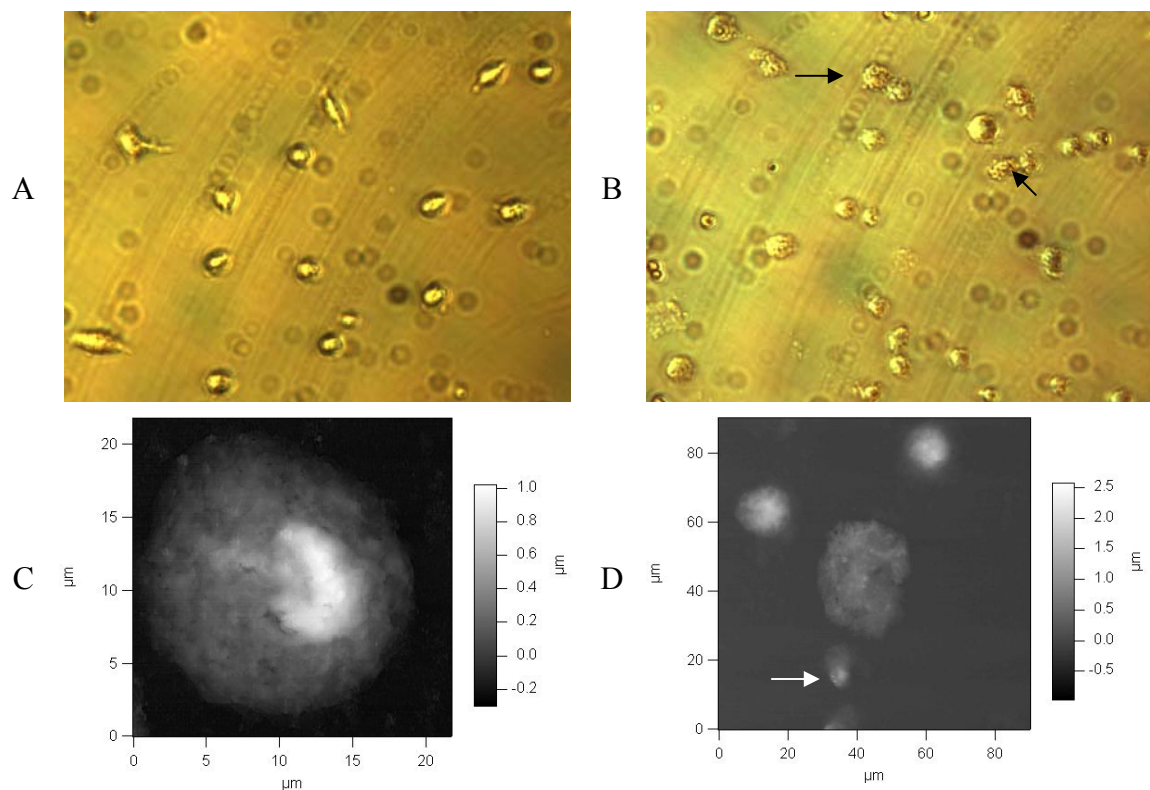


Figure 4: Representative bright-field and atomic force photomicrographs of macrophages with or without DU exposure. The cells were cultured in medium without or with 100 μ M DU-uranyl nitrate for 24h then processed for microscopy. Panels A and B are bright-field images of control and DU treated cells, respectively. Panels C and D are atomic force microscopy images of single control and DU treated cells, respectively. The arrows in panel B indicated cells undergoing apoptosis after 24 h 100 μ M DU exposure. The arrow in panel D indicates the formation of an apoptotic body.

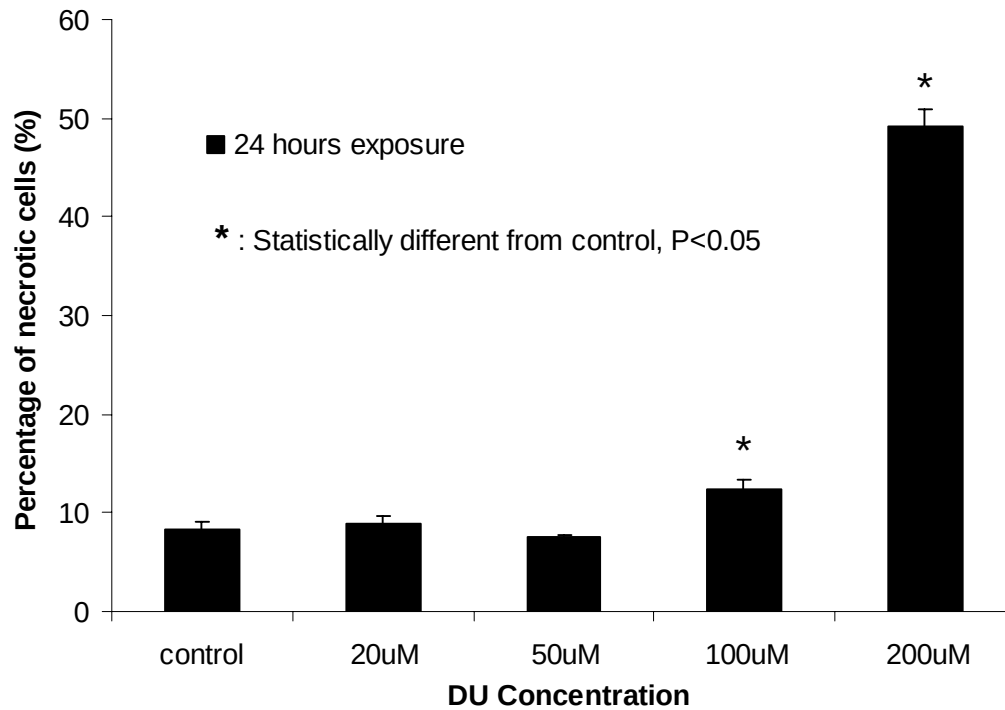


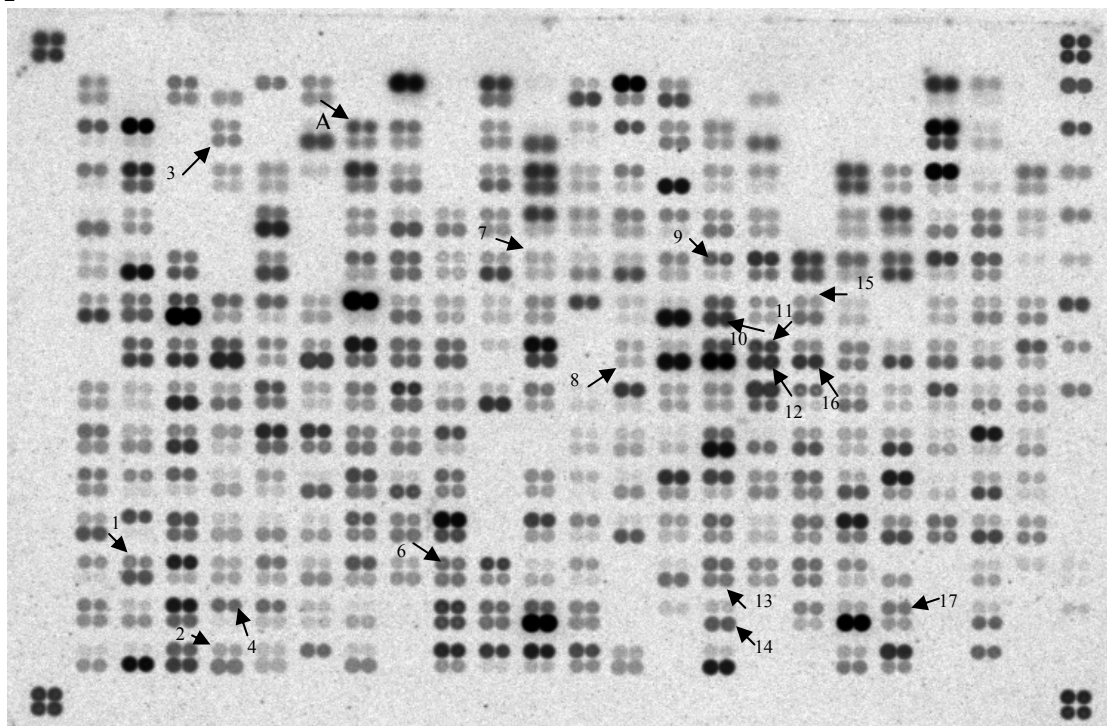
Figure 5: Necrosis of macrophages under DU exposure. The cells were treated with 0, 20, 50, 100, and 200 μ M DU for 24 h and the percentages of unviable cells (Annexin-V-FITC⁺ and PI⁺) were determined by flow cytometry analysis. Asterisks indicate that the difference from control is statistically significant, $P < 0.05$. Error bars represent standard deviation from triplicate.

Table 1:
Summary of statistical analysis of macrophage microarray data

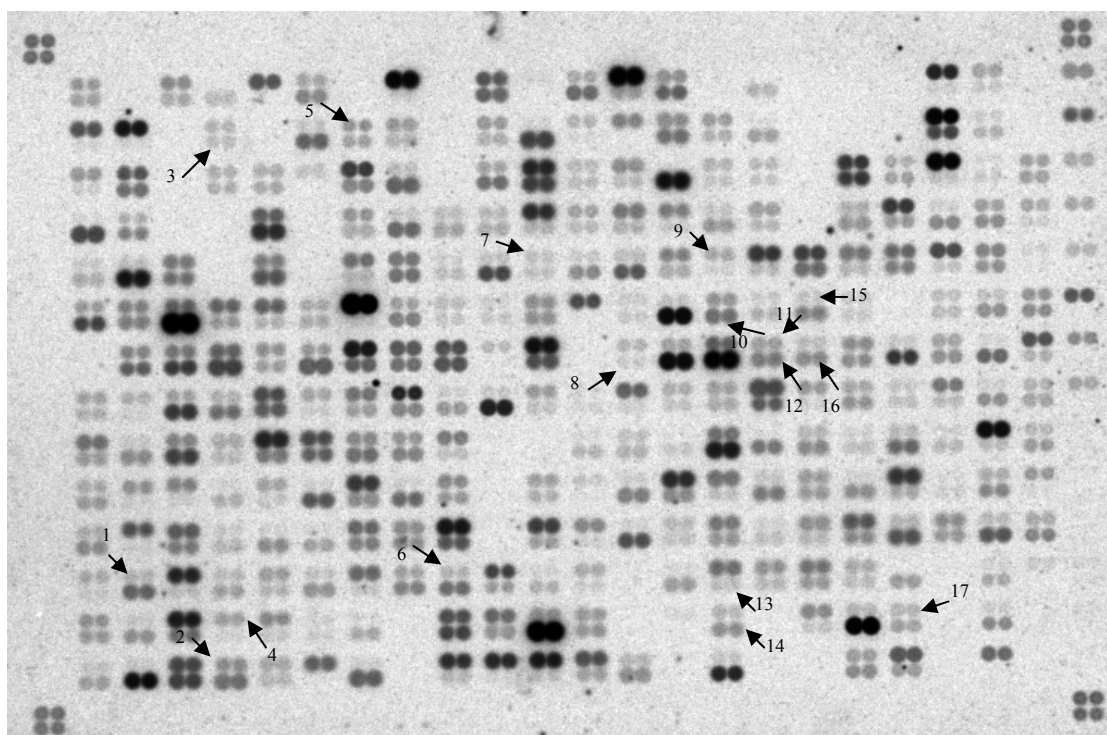
z-test for Two Independent Conditions	
Conditions:	PIM_1.A : MQ DU - control (none exposure) PIM_1.B : MQ DU - 24hrs 50uM DU exposure
Input Parameters	
Test:	z-test
Normalization (Center):	By Mean (Non-Iterative)
Nominal Alpha:	0.05
Multiple Test Correction:	No Correction
PIM_1.A	
Source:	A - control (none exposure)
Model Selection:	Manual
Model Selected:	Proportional Model with Offsets
Outlier Detection:	Yes
Threshold Selection:	Automatic
Threshold Selected:	± 3.00 MADs
Minimum No. of Replicates:	2
Conditions:	Independent
Random Error Estimation Method:	Pooled - Curve Fit
PIM_1.B	
Source:	B - 24hrs 50uM DU exposure
Model Selection:	Manual
Model Selected:	Proportional Model with Offsets
Outlier Detection:	Yes
Threshold Selection:	Automatic
Threshold Selected:	± 3.06 MADs
Minimum No. of Replicates:	2
Conditions:	Independent
Random Error Estimation Method:	Pooled - Curve Fit
Output Summary	
Effective Alpha:	0.05
Confidence Interval Alpha:	0.05
Significant:	29 (6%)
PIM_1.A > PIM_1.B	5 (1%)
PIM_1.A < PIM_1.B	24 (5%)
Not significant:	523 (94%)

Figure 6: Macrophage mouse cytokine gene array hybridization images. Membranes were hybridized with P³³-labelled cDNA probes from control (B) and DU-exposed cells (A). Panel A and B are representative microarray images from three experiments. The arrows in panel A and B indicated the differentially expressed genes identified by statistical analysis at $P < 0.05$ (the arrows were labeled numerically). The arrays are representative of three replicate experiments. The selected gene names indicated by numbers in the order from 1 to 17 are: NF-k B inducing kinase, Toll-like R6, LTBP-2, Toll-like R2, CT-1, Wnt-8b, EphA-3, IL-10, c-jun, c-myc, SOCS-1, Stat-1, NF-kBp65, NSG1/p21, BDNF, Midkine, and BMP-9. Of these indicated genes, Toll-like R6 was down-regulated. Other differentially displayed genes can be found in figure 7.

A



B



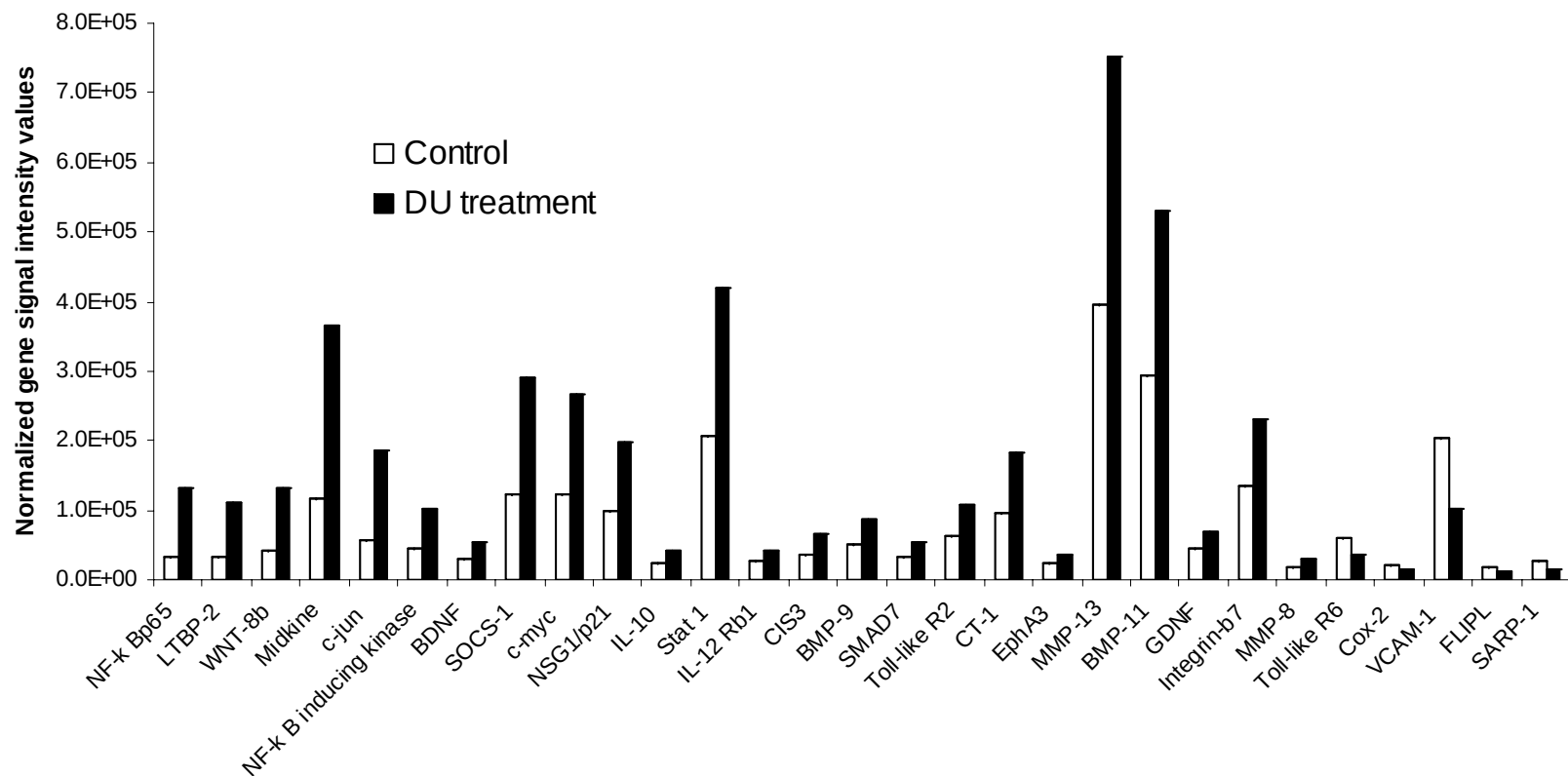


Figure 7: The normalized cDNA array signal intensities of differentially displayed genes in macrophages. Data from three parallel experiments were first normalized across replicates, within conditions. Then the data were further normalized across the conditions using a statistical Z-test. 29 genes have been identified as differentially expressed under DU exposure, of which 5 were down-regulated and 24 were up-regulated ($P < 0.05$).

Table 2: Expression ratios of significantly regulated genes in DU-exposed peritoneal adherent macrophages as determined by microarray analysis

Gene information ^a			Z-test ^b	Ratios ^c	Upper confidence interval ^d	Lower confidence interval ^d
Genbank	Gene names	Gene group				
NM_009045	NF-k Bp65	Signal transduction	5.8E-08	3.9	6.3	2.4
AF004874	LTBP-2	Binding protein	1.8E-06	3.2	5.3	2.0
NM_011720	WNT-8b	Developmental factors	9.3E-06	3.2	5.4	1.9
NM_010784	Midkine	Neurotrophic group	1.5E-05	3.1	5.2	1.9
NM_010591	c-jun	Signal transduction	7.1E-05	3.2	5.8	1.8
NM_016896	NF-k B inducing kinase	Apoptosis-related	1.5E-03	2.4	4.0	1.4
NM_007540	BDNF	Neurotrophic group	2.4E-03	1.7	2.5	1.2
NM_009896	SOCS-1	Signal transduction	2.8E-03	2.4	4.2	1.4
NM_010849	c-myc	Signal transduction	7.2E-03	2.2	3.8	1.2
NM_010942	NSG1/p21	Signal transduction	7.8E-03	2.0	3.3	1.2
NM_010548	IL-10	Interleukin	8.2E-03	1.7	2.5	1.2
NM_009283	Stat 1	Signal transduction	9.2E-03	2.0	3.4	1.2
NM_008353	IL-12 Rb1	Interleukin receptor	1.1E-02	1.6	2.4	1.1
NM_007707	CIS3	Signal transduction	1.1E-02	1.8	2.8	1.1
AF188286	BMP-9	TGF-beta family	1.4E-02	1.8	2.7	1.1
NM_008543	SMAD7	Signal transduction	1.9E-02	1.6	2.5	1.1

Table 2: Continued

Gene information ^a			Z-test ^b	Ratios ^c	Upper confidence interval ^d	Lower confidence interval ^d
Genbank	Gene names	Gene group				
AF185284	Toll-like R2	Cell surface protein	1.9E-02	1.8	2.8	1.1
NM_007795	CT-1	Cytokine and receptors	1.9E-02	1.9	3.4	1.1
M68513	EphA3	Eph family	2.1E-02	1.6	2.3	1.1
NM_008607	MMP-13	Protease or related factor	2.2E-02	1.9	3.3	1.1
AF092734	BMP-11	TGF-beta family	3.2E-02	1.8	3.1	1.0
D49921	GDNF	Neurotrophic group	3.5E-02	1.6	2.4	1.0
M95632	Integrin-b7	Intergrin	3.9E-02	1.7	2.9	1.0
NM_008611	MMP-8	Protease or related factor	4.9E-02	1.6	2.5	1.0
NM_011604	Toll-like R6	Cell surface protein	2.7E-02	0.6	0.9	0.4
NM_011198	Cox-2	Apoptosis-related	2.3E-02	0.6	0.9	0.4
NM_011693	VCAM-1	Adhesion molecule	1.2E-02	0.5	0.9	0.3
NM_009805	FLIPL	Apoptosis-related	1.1E-02	0.6	0.9	0.4
NM_009144	SARP-1	Apoptosis-related	7.3E-04	0.6	0.8	0.4

Legend to table 2

Note: The genes that were down-regulated in DU-treated peritoneal adherent macrophages are displayed with a grey background and genes that were up-regulated are displayed with a white background.

^a The Genbank accession number and gene names for each gene; the gene groups that each gene belongs to are included.

^b The two-tailed Z-tests p values for the comparison of replicate samples for each gene between control and DU.

^c The ratio expression values for the average expression values of each gene between DU and control. i.e. ratio = intensity value from DU treated cells divided by that from control cells.

^d Confidence intervals determined at $p < 0.05$.

Table 3: Primer sequences used in Quantitative Reverse Transcriptional PCR (QRT-PCR) for differentially expressed genes in macrophages

Targets	Sequences^b	Amplicon Size (bp)
IL-10	F: 5'-CAT GGG TCT TGG GAA GAG AA-3' R: 5'-CAT TCC CAG AGG AAT TGC AT-3'	194
Midkine	F: 5'-ACC GAG GCT TCT TCC TTC TC-3' R: 5'-GGC TCC AAA TTC CTT CTT CC-3'	230
BMP-11	F: 5'-TTC ATG GAG CTT CGA GTC CT-3' R: 5'-AGC ATG TTG ATT GGG GAC AT-3'	299
c-jun	F: 5'-TGA GAA CTT GAC TGG TTG CG-3' R: 5'-AAA GTC CAT CGT TCT GGT CG-3'	222
Stat-1	F: 5'-TGG TGA AAT TGC AAG AGC TG-3' R: 5'-TGT GTG CGT ACC CAA GAT GT-3'	119
Toll-like R6	F: 5'-ACA CAA TCG GTT GCA AAA CA-3' R: 5'-GGA AAG TCA GCT TCG TCA GG-3'	128
GAPDH ^a	F: 5'-TGA TGA CAT CAA GAA GGT GGT GAA G-3' R: 5'-TCC T TG GAG GCC ATG TAG GCC AT-3'	240

^a Sequences referred from Lee et al., 2000.

^b F: Forward Primer; R: Reverse Primer. Primer sequences are designed using Primer 3 internet based software (http://www.broad.mit.edu/cgi-bin/primer/primer3.cgi/results_from_primer3).

Figure 8: QRT-PCR analysis of GAPDH and Midkine relative mRNA levels in control and DU treated macrophages. (A) Electrophoresis of 2 µg of total RNA in a denaturing 1.5% agarose formaldehyde gel; the ratio between the brightness of 28s and 18s rRNA bands was in the correct range of 2:1. M, Millennium RNA marker (Ambion, Inc.); C, control RNA sample; T, RNA from DU-Treated cells. (B) Representative amplification plot for GAPDH and Midkine in control and treatment samples; broken line indicated a cycle threshold (Ct) value. (C) Representative melting curves for Midkine. Red line indicated PCR product with template addition, Green line indicated non-template control (NTC); Note that NTC product did not produce any curve peak meaning there is no amplification. (D) Representative melting curves for GAPDH. Red line indicated PCR product with template addition, Green line indicated non-template control (NTC); (E) Electrophoresis of PCR products in a 1% agarose gel; the 240bp band corresponds to GAPDH and ~230bp band to Midkine amplified from control and DU-treated cells. (F) Standard curves for GAPDH and Midkine; the linear regression equations were given in the panel for each gene.

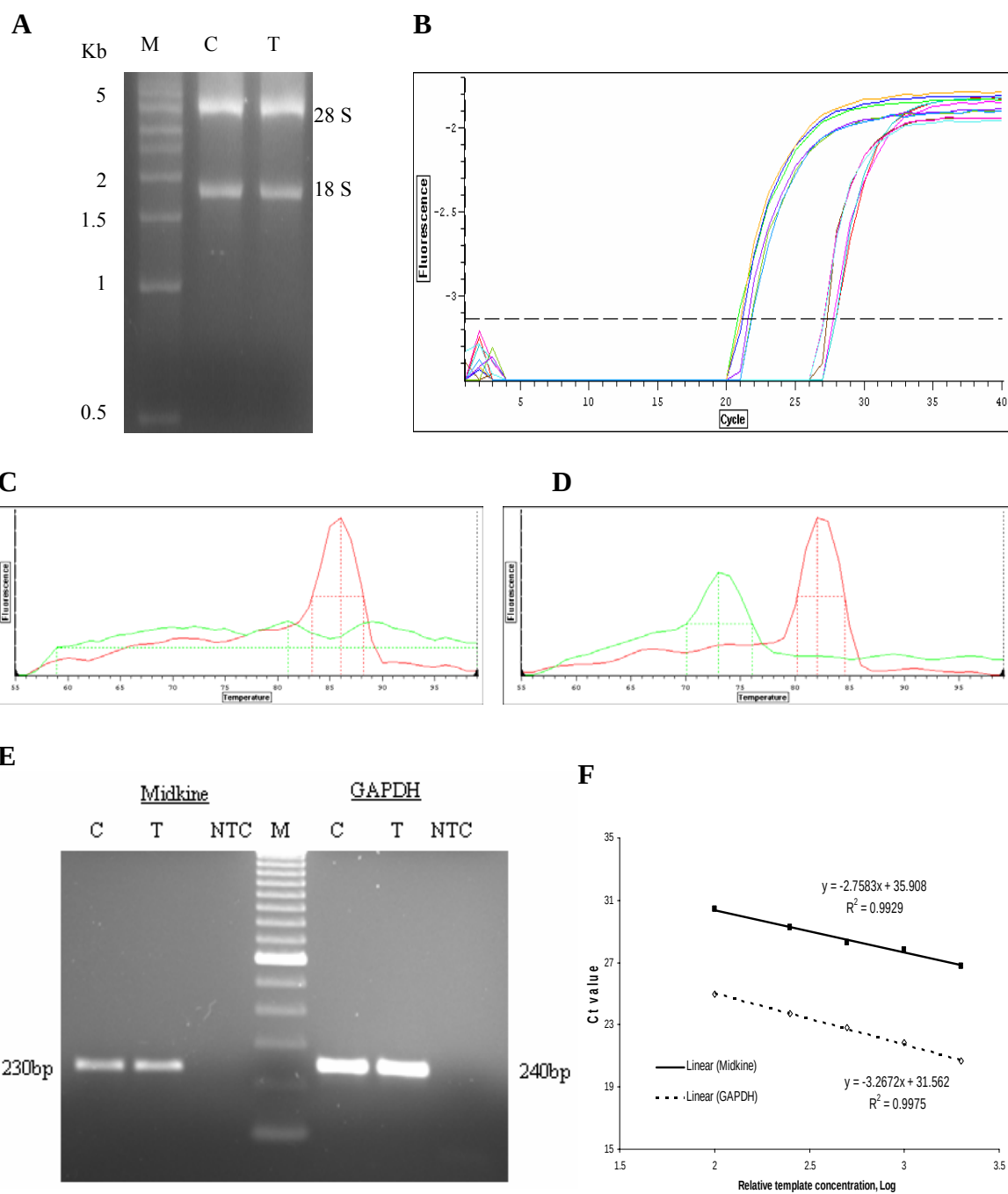


Table 4: PCR efficiency and gene expression ratio of Midkine and GAPDH

Genes	Slopes	PCR efficiencies (E)^a	Ratio (T/C)^b
Midkine	-2.7583	2.304317	3.2
GAPDH	-3.2672	2.023357	--

^a PCR efficiency (E) = $10^{-1/\text{slope}}$. [Pfaffl, 2001; Pfaffl et al., 2002]

^b Ratio (T/C) = $\frac{\text{Power}(E_{\text{target gene}}, \Delta C_{\text{t target gene}}(\text{control-sample}))}{\text{Power}(E_{\text{reference gene}}, \Delta C_{\text{t reference gene}}(\text{control-sample}))}$; $E_{\text{target gene}}$, PCR efficiency of target gene; $E_{\text{reference}}$, PCR efficiency of reference gene; $\Delta C_{\text{t target gene}}(\text{control-sample})$, the difference of target gene Ct values between control (C) and DU-treated samples (T); $\Delta C_{\text{t reference gene}}(\text{control-sample})$, the difference of reference gene Ct values between control and DU-treated samples [Pfaffl, 2001; Pfaffl et al., 2002].

**Table 5: Comparison of the gene expression ratios in macrophages
determined by microarray and QRT-PCR analysis**

Gene Names	Array Ratios	QRT-PCT Ratio	Array Ratio Confidence Intervals
Midkine	3.1	3.2	5.2~1.9
c-jun	3.2	1.9	5.8~1.8
BMP-11	1.8	1.6	3.1~1.0
Stat-1	2.0	2.2	3.4~1.2
IL-10	1.7	6.9	2.5~1.2
Toll-like R6	0.6	0.6	0.9~0.4

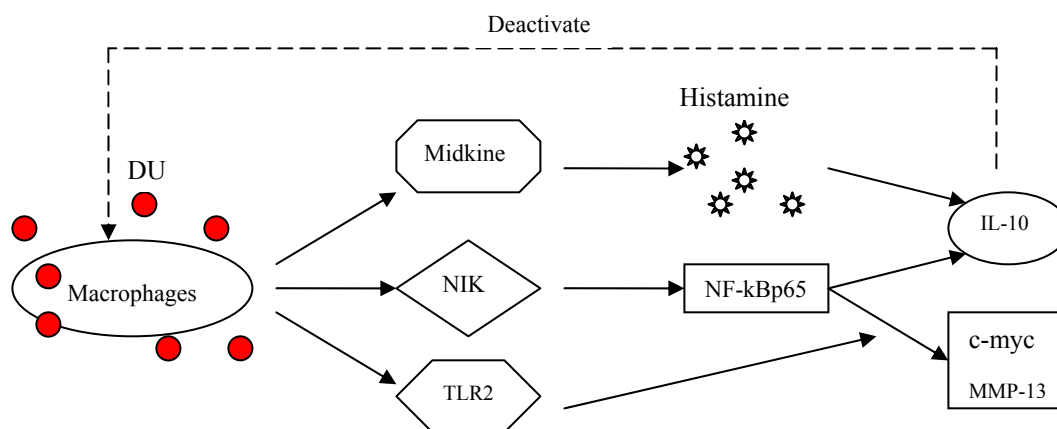


Figure 9: Putative pathways for DU toxic effects on macrophages. NIK: NF-kB p65 inducing kinase; TLR2: Toll-like R2; The red circles represent DU.

PART 5

**TOXICOLOGICAL EFFECTS OF DEPLETED URANIUM
ON PRIMARY CD4⁺ T-CELLS**

Abstract

In this study, the toxic effects of depleted uranium-uranyl nitrate on mouse CD4⁺ T-cells were investigated. Mouse cytokine gene array were used to study the gene expression in CD4⁺ T-cells upon DU exposure; flow cytometry and annexin-V/PI staining analysis were employed to study the cell death.

Results showed that DU caused cytotoxic effect on T-cells when concentrations were >500 μ M during the period of 24 and 48 hours exposure. T-cells undergoing apoptosis were also accompanied with cell necrosis. The microarray experiments have identified 4 up-regulated genes and 10 down-regulated genes in CD4⁺ T-cells under non-cytotoxic DU exposure, those induced genes were IL-5, Midkine, TECK/CCL25 and VEGF-A with ratios range from 1.8 to 4.2, and *P* values less than 0.001. The down-regulated genes were EphA3, CCR-4, LIF, GDF-7, EBF, CD27/TNFRSF7, SLIT-3, Neurotactin/CX3CL1, SMAD1, and A1. These genes were involved in several functions including developmental factor, chemokine and receptors, signal transduction, TGF-beta and TNF superfamilies et al. with ratios ranging from 0.4 to 0.7. The altered expressions of four selected genes were confirmed using quantitative RT-PCR. The functions of the differentially expressed genes suggested potential activities of DU in inducing cancer development, promoting eosinophil-related inflammatory diseases, and encouraging Th2 polarization.

Introduction

In mammalian immune system, T-cells play a vital role in adaptive immunity. CD4⁺ T-cells, or T-helper (Th) cells, are of special importance in that they are regulatory cells determining the nature and output of an immune response. Depending on the stimulation, Th cells further differentiate to either Th1 (T-helper cell type 1) or Th2 (T-helper cell type 2). The Th phenotypes are characterized by the cytokines they produced. The major lymphokines secreted by Th1 cells are tumor-necrosis factor beta (TNF- β), and interferon-gamma (IFN- γ), while Th2 cells secrete high levels of interleukin-4 (IL-4), IL-5, and IL-13 [Mosmann and Sad, 1996]. Due to the different biological functions of these cytokines, Th1 and Th2 cells have diverged functions. There is also a reciprocal inhibition mechanism for enhancing Th1 or Th2 differentiation. The complex balance between Th1 and Th2 cells can be disturbed by a variety of factors including heavy metals, drugs, and cytokines. It has been postulated that deviation of T-cell population to Type 1 phenotype fosters T-cell-mediated autoimmunity and inflammation. By contrast, Th2 cytokines have been speculated to be protective in Th1-derived pathological auto-reactive responses and cause allergic responses. However, Th2 deviation was also characterized in some autoimmune diseases [Mosmann and Sad, 1996].

Previous works showed that DU has toxic effects on mammalian immune system [Pellmar et al., 1999]. As a heavy metal, DU may exert its toxicity by affecting the functions of immune cells after interfering with the signal transduction pathway as well as the production of cytokines [Gazin et al., 2002]. In regard to Gulf War, in which several hundred military personal were exposed to DU to various degrees, it has been hypothesized that the GWS is due to a systemic shift in cytokine balance towards a Th2

profiles [Rook and Zumla, 1997; Skowera et al., 2004]. Elevated blood IL-10 concentration or higher number of IL-10 producing CD4⁺ T-cell has been detected in symptomatic gulf war veterans who were potentially exposed to DU in battlefield [Zhang et al., 1999; Skowera et al., 2004]. Studies on DU exposed primary T-cells are scarce in spite of the observed heavy metal toxicity of DU on immune system and the possible effects on Th1/Th2 profile. The molecular mechanisms of DU immunotoxicity are mostly unknown.

The information on the gene expression profile of DU-exposed primary CD4⁺ T-cells will be invaluable for selecting therapeutic targets of DU-related diseases and better understanding the risk of environmental DU exposure. In this study, we investigated the DU cytotoxicity on primary CD4⁺ T-cells under anti-cd3 stimulation. The differentially displayed genes have been identified in those cells under DU exposure. One hypothesis tested here is that DU exposure would cause Th2 shifting.

Results

CD4⁺ T-cell apoptosis under DU exposure

Induction of apoptosis was determined using flow cytometry of Annexin-V-FITC/Propidium iodide (PI)-labeled CD4⁺ T-cells. A SSC vs. FSC plot was used to set the analysis gate as can be seen in figure 1 (All tables and figures in the Appendix at the end of the part). The cells outside of the gate were considered as debris and were excluded for analysis.

The treatment of CD4⁺ T-cells with DU, uranyl nitrate, resulted in apoptotic as well as necrotic cell death. As can be seen in figure 2, treatment with 1, 10, and 100μM

DU for 24 hours did not cause significant increase of neither Annexin-V staining or PI staining, indicating that these treatments did not induce cell apoptosis or necrosis. However, there was a significant increase of apoptotic and necrotic cells after treatment with 500 μ M DU for 24 hours ($P<0.01$), with percentages of 64.5% and 15.3% respectively, while the apoptotic and necrotic cell percentages of negative controls were 3.5% and 1.5%, respectively. As expected, the positive controls had a much higher percentage of necrotic cells, whereas the percentages of necrotic and apoptotic cells in 1mM NaNO₃ group were not different from those of negative control. The results indicated that the amounts of apoptotic cells and necrotic cells increase when exposed to 500 μ M DU for 24 hours. The results of NaNO₃ treatment indicated that these changes were not due to NO₃⁻ ion. Similar results were also observed after 48 hours exposure (Figure 3).

Cytokine gene expression profiles of macrophages upon DU exposure

The non-cytotoxic DU concentrations, as determined above, were < 500 μ M. Therefore, in the present study CD4⁺ T-cells were treated with or without 100 μ M DU in an anti-CD3 coated 96-well plate for 24 h. Total RNA isolation was performed after collecting the cells in the medium, and cell cytokine gene expression was measured by microarray. Table 1 lists the methods and parameters for Z-tests performed on three replicate data sets. Statistical analysis was based on two independent conditions: control (PIM_1C) and 24hrs 100 μ M DU exposure (PIM_1D). Data normalization was performed based on the mean intensity values of the entire array. For any particular gene, if only one valid observation was present within a condition, that datum was disregarded

for analysis. The outliers were detected by examining standardized residuals with the ArrayStat software. A curve fit random error estimate method was employed for proportional model with offset [ArrayStat Manual].

The nominal alpha value for statistical tests was set at 0.05, while after false positive error rate correction the effective alpha value was 0.00147. Table 1 shows there were 14 (2.9% of all analyzed genes) genes that were differentially regulated upon 100 μ M DU exposure. Of these 14 genes, 4 (0.9%) were up-regulated and 10 (2%) were down-regulated. Figure 4 displays a representative array of T-cell cytokine gene expression with 100 μ M DU or without DU exposure. Differentially displayed genes are indicated with arrows based upon the statistical analysis described in the Material and Method section. The average normalized expression values for these differentially displayed genes were plotted in figure 5.

IL-5, Midkine, TECK/CCL25 and VEGF-A were up-regulated by ratios range from 1.8 to 4.2, with *P* values less than 0.001. A greater number of genes were down-regulated under DU exposure, which means that the T-cell physiological functions were shut down to some degree upon DU exposure. These genes were: EphA3, CCR-4, LIF, GDF-7, EBF, CD27/TNFRSF7, SLIT-3, Neurotactin/CX3CL1, SMAD1, and A1. The down-regulated genes were involved in several functions including developmental factor, chemokine and receptors, signal transduction, TGF-beta and TNF superfamilies et al with ratios ranging from 0.4 to 0.7 and *P*-values ranging from 0.0012 to 4E-08 (Table 2).

Quantitative RT-PCR assay for differential gene expression confirmation

In order to confirm the results obtained from microarray analysis, quantitative RT-PCR (QRT-PCR) was performed on a selected subset (Mostly up-regulated ones) of differentially displayed genes identified by microarray analysis. QRT-PCR was performed in triplicate on three up-regulated genes: Midkine, IL-5, TECK/CCL25 and one down-regulated gene, CD27/TNFRSF7, compared to the consistent expression of GAPDH. The primers for the selected genes and GAPDH were shown as in table 3, the amplicon sizes range from 105 to 240bp. GAPDH was chosen as the reference gene is because GAPDH gene shows no change in expression between samples and the average value of the ratios of all 8 house keeping genes was very close to that of GAPDH alone.

Firstly, the integrity of RNA was confirmed in a 1.5% formaldehyde denaturing agarose gel (Figure 6A); the brightness ratios of 28 s and 18 s ribosomal RNA bands in both control (C) and DU-treated cells (T) were $\sim 2:1$, no genomic DNA contamination was observed on the gel. 2 μg of total RNA were used to synthesize the cDNA in a reverse transcription reaction. For constructing standard curves of both reference and interested genes, a total RNA sample from another experiment was used. 1 μl of cDNA solution and a series of dilutions of the cDNA solution were used as template for real-time PCR. The PCR was performed in triplicate with a no-template control (NTC) for each gene. In table 4, the IL-5 and GAPDH Ct values are shown; GAPDH average Ct values were very similar between control (Ct = 19.78) and DU-treated samples (Ct = 19.64), whereas the IL-5 average Ct values for control and DU-treated samples were 31.49 and 30.02, respectively. At the end of the PCR reaction, a melting curve analysis was performed. In figure 6C, the specific IL-5 T_m peak (76 °C) was observed while the

NTC curve did not form an obvious primer-dimer T_m peak, which means the PCR reactions specifically amplify IL-5. In figure 6D, the GAPDH T_m peak (82 °C) and NTC primer-dimer T_m peak (74°C) were observed although the signal strengths of the NTC T_m peak was much lower than that of GAPDH. Again the PCR products were analyzed by gel electrophoresis to estimate the specificity. In figure 6E, both control and DU-treated cells express GAPDH (240bp) and IL-5 (105bp). The gel image showed single band for each PCR product and NTC showed no visible band at the corresponding size, as expected, which confirmed the results observed in melting curve plots. The standard curves for both IL-5 and GAPDH were constructed to obtain the slopes for PCR efficiency determination [Pfaff, 2001; Pfaffl et al., 2002]. In figure 6B, the slopes of the GAPDH and IL-5 standard curves were determined as -4.5245 and -2.7421 with both R^2 values >0.96. The IL-5 expression ratio between control and DU-treated samples was calculated as 3.2 (Table 4) according to a mathematical model described by Pfaffl [Pfaffl, 2001; Pfaffl et al., 2002].

The differential gene expression ratios of Midkine, CD27/TNFRSF7, and TECK/CCL25 were also determined (data not shown) and compared with those of microarray analysis (Table 5). The tendencies of the ratios of all genes were mostly in accordance with those determined by microarray analysis. QRT-PCR assay gives higher ratio when measuring IL-5 expression, and lower ratio when it comes to TECK/CCL25.

Discussion

In mammalian immune system, T-cells play essential roles in adaptive immunity especially in cell-mediated immunity. The principle functions of T-cells are to regulate

all immune responses to protein antigens and to serve as effector cells for the elimination of intracellular microbes. T-helper (Th) cells are of special importance in that they are regulatory cells that determine the nature and output of an immune response. Depending on the stimulation, Th cells further differentiate to either Th1 or Th2. There is a reciprocal inhibition mechanism for enhancing Th1 or Th2 differentiation. The complex balance between Th1 and Th2 cells can be disturbed by a variety of factors including heavy metals, drugs, and cytokines. Deviation of T-cell population to Type 1 phenotype fosters T-cell-mediated autoimmunity and inflammation. By contrast, Th2 cells have been speculated to be protective in Th1-derived pathological auto-reactive responses and cause allergic responses and some autoimmune diseases [Harber et al., 2000; Mosmann and Sad, 1996]. During the last decade, the increasing use of DU in weapon on the battlefield has raised concerns as to its health consequences. It has been postulated that DU exposure may contribute to the GWS, and epidemiological studies had revealed that the GWS patients tend to have higher percentage of Th2 subset cells [Rook and Zumla, 1997; Skowera et al., 2004]. Here we have investigated the cytotoxicity of DU on CD4⁺ T-cells and the influence DU has on gene expression. This work may help to resolve the hypothesis of DU-induced Th2 shifting as regarded in gulf war syndrome.

In the immune system, apoptosis plays roles not only in tissue and organ development, but also in the regulation of the immune response. Both cell number and function are essential determinants of the output of an immune response. Reducing the number of immune-competent cells through uncontrolled or massive cell death after xenobiotic treatment may lead to serious adverse effects such as immunosuppression. [Pallardy et al., 1999; Yoo Byung Sun et al., 1997] Alternatively, in case of an increase in

the number of dead cells or inefficient clearance of the dead cells, the released cellular content may become antigens and be presented in the context of MHC-I or MHC-II molecules of antigen presenting cells, invoking a strong cytolytic response. Subsequently, response against auto-antigens may be initiated and epitopes spread to unmodified antigens causing autoimmune diseases [Rodenburg et al., 2000].

In this study, we employed flow cytometry analysis of the cell membrane symmetry to study the CD4⁺ T-cell apoptosis and necrosis upon DU exposure. Results showed that apoptosis and necrosis occurred simultaneously after 24 hours when T-cells were exposed to 500 μ M DU; below 500 μ M apoptosis and necrosis were not observed. Unlike macrophages, which can actively engulf DU particles [Kalinich et al., 2002], CD4⁺ T-cells do not exhibit phagocytosis activity, therefore, they are more resistant to DU exposure. The DU cytotoxicity study on peritoneal adherent macrophages also showed that the non-cytotoxic concentration (Part 4) of macrophages was about 2-fold lower than that of T-cells (50 μ M vs.100 μ M). Furthermore, a time dependent response was not observed in this study since both 24 and 48 hours exposure resulted in the same apoptotic and necrotic responses. Compared with other heavy metals, such as mercury, DU was much less toxic to CD4⁺ T-cells. Whereas lead and vanadium have approximately the same toxicity as DU has on those cells [Shen et al., 2001].

Previous studies indicated that some heavy metals (e.g. vanadium and cadmium) modulate T-lymphocyte responses by exerting their effects on the signal transduction pathway in CD4⁺ T-cells [Lee et al., 2001]. The extensive involvement of cytokine production in heavy metal immune modulation activities have also been reported [Shen et al., 2001; Krocova et al., 2000]. However, studies on DU toxicological effects scarcely

addressed the large scale gene expression on immune cells, and the molecular mechanisms of the DU immunotoxicity were mostly unknown.

As a part of our efforts, the hypothesis that DU may induce Th2 shifting was tested in this study using a mouse cytokine gene array. The gene expression of cytokines that are involved in signal transduction pathways and immune responses were globally studied as well. This array is composed of 514 genes related to signal transduction, interleukin and receptors, apoptosis, cell surface proteins, neurotropic factors and developmental factors et al. In CD4⁺ T-cells exposed to 100μM DU, the up-regulated genes were: Midkine, IL-5, TECK/CCL25, and VEGF-A, whereas the numbers of down-regulated genes were: EphA3, CCR-4, LIF, GDF-7, EBF, CD27/TNFRSF7, SLIT-3, Neurotactin/CX3CL1, SMAD1, and A1. The number of down-regulated genes was much more than that of up-regulated ones (10 genes vs. 4 genes), which may indicate that, to some degree, the physiological functions of CD4⁺ T-cells were shut down. The differential expression of four selected genes was confirmed by quantitative RT-PCR (Table 5), indicating that the results of gene expression array analysis are reliable.

A 3 fold induction of midkine expression in CD4⁺ T-cells was observed upon non-cytotoxic DU exposure. It is interesting to note that DU could induce Midkine expression in both primary macrophages (in Part 4) and CD4⁺ T-cells, which not only indicates a common mechanism of DU immunotoxicity and also suggests a possible biological marker for DU exposure. The highly induced Midkine (Mdk) is a neurotrophic factor that has important roles in development, oncogenesis, inflammation, and tissue remodeling [Muramatsu, 2002]. Midkine is a basic, low molecular-weight protein that is expressed in vertebrates from zebrafish to human and promotes cell survival, migration

of neuron, neutrophils, and macrophages, and neurite growth [Sato et al., 2001]. High induction of Midkine expression has been reported in various human tumors, such as cancers of the gastrointestinal tract, kidney, lung and mammary gland, neuroblastomas et al [Sakitani et al., 1999]. Midkine are thought to enhance tumor progression by promoting tumor cell growth, survival, migration and angiogenic activity. The midkine level often increases in the early stage of cancer progression and thus, might be used as a tumor marker. Midkine may also plays a role in inflammation. It is known that midkine is able to induce histamine release from rat peritoneal mast cells with a rapid response in a dose-dependent manner [Mazurek et al., 1986]. The released histamine is a key mediator of allergic inflammation by the accumulation of inflammatory cells in the peritoneum of mouse [He et al., 2004; He et al., 1997; Cairns et al., 1996.]. Furthermore, histamine was reported to induce IL-10 production [Sirois et al., 2000], thus potentially creating a microenvironment for Th2 cell differentiation.

Our results suggest that DU may be carcinogenic through the activation of Midkine, which also has the potential to induce allergic inflammation. Previous studies have reported that midkine expression is restricted in skeletal muscle cells epithelium, thyroid and kidney cells [Garver et al., 1993; Hu et al., 2002]. This study indicated that Midkine expression is inducible in other types of immune cells (such as primary CD4⁺ T-cells) under DU exposure.

Our microarray data also showed an approximately 2-fold induction of IL-5 expression upon DU exposure. IL-5 is a cytokine predominately secreted by CD4⁺ T-cells. After activation or maturation of naïve T-cells, IL-5 becomes the signature cytokine of Th2 cells, which also produce cytokines such as IL-4, IL-10 and IL-13 [Cousins et al.,

2002; Mazzearella et al., 2000]. Therefore, up-regulation of IL-5 expression in our experiments indicates a Th2 differentiation tendency after DU exposure. The co-induction of other Th2 cytokines (e.g. IL-4) with IL-5 was not observed in this study. This is not uncommon since other heavy metals, such as mercury, were also found able to induce autoimmunity by initiating Th2 shifting, but not necessarily with IL-4 production [Bagenstose et al., 1999]. The down-regulation of IL-5 in CD4⁺ T-cells, in some case such as pulmonary tuberculosis, occurs without any change of IL-4 gene expression [Lai et al., 1997], indicating the loose link between IL-4 and IL-5 expression. Th2 cytokines encourage antibody production, especially IgE responses, and enhance eosinophil proliferation and function. Thus they often are found in association with strong antibody (e.g. autoimmune diseases) and allergic responses [Harber et al., 2000; Mosmann and Sad, 1996].

Of the cytokines produced by Th2 cells, IL-5 is responsible for eosinophil growth, differentiation, activation, and survival of eosinophils and priming eosinophils to respond to chemoattractants such as eotaxin, an eosinophil-selective chemokine in allergic inflammation [Mishra et al., 2002; Saito et al., 2002]. In asthma, a dramatic number of eosinophils have been found to accumulate at the site of antigen challenges [Temple et al., 2001]. This is also a common medical problem in patients with diverse diseases, including gastroesophageal reflux, eosinophilic esophagitis, eosinophilic gastroenteritis and parasitic infections as well as pulmonary eosinophilia [Mishra et al., 2002; Matsumoto et al., 2003]. It is well documented that IL-5 plays a major role in the development of these diseases. The induction of IL-5 expression in CD4⁺ T-cells under the DU exposure suggests that non-cytotoxic concentrations of DU may be able to

promote Th2 shifting and cause allergic responses. In the long run, DU exposure may eventually result in autoimmune diseases.

This study demonstrated that DU is cytotoxic to CD4⁺ T-cells and is able to modulate T-helper cell differentiation by regulating Th cell cytokine expression. The *in vitro* system used in this study is suitable for identifying molecular targets of DU toxicity and revealing the potential molecular mechanisms of the immune modulation ability of DU.

Appendix

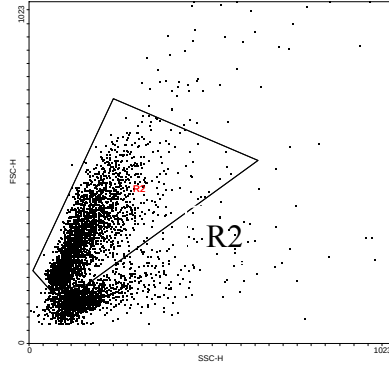
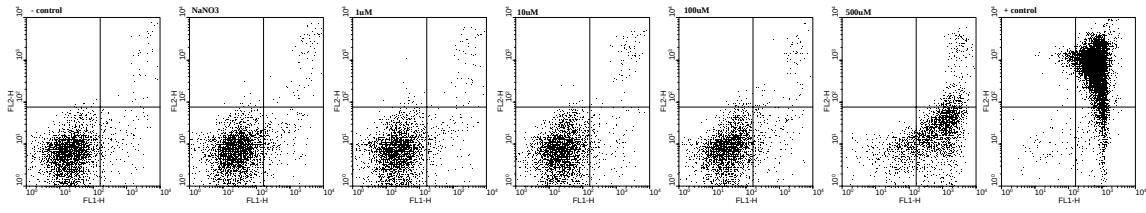


Figure 1: Flow cytometry FSC vs. SSC plot of $CD4^{+}$ T-cells and the analysis gate. The analysis gate was the polygon indicated as R2. The cells outside of the gate were excluded from analysis.

Figure 2: Apoptosis and necrosis of CD4⁺ T-cells under 24 hours DU exposure. Quadrant analysis of the flow cytometry plot of Annexin-V/PI binding to control and DU-uranyl nitrate treated CD4⁺ T-cells. CD4⁺ T-cells were treated with 0 (negative control), 1, 10, 100, 500μM DU and 1mM NaNO₃ or 1μg/ml Staurosporine (positive control) for 24 hours. In panel A, the x-axis of dotplots, FL1-H, represents Annexin-V binding and the y-axis, FL2-H, represents PI binding. The flow cytometry dotplot data are presented in panel B as percentage of cells binding Annexin-V (apoptotic cells, Annexin-V⁺/PI⁻) and PI (necrotic cells, Annexin-V⁺/PI⁺) under various treatments and are the mean of triplicates. Error bars represent standard deviation. Asterisk indicate that the difference from negative control is statistically significant ($P<0.01$). Panel A shows representative dotplots from triplicates.

A



B

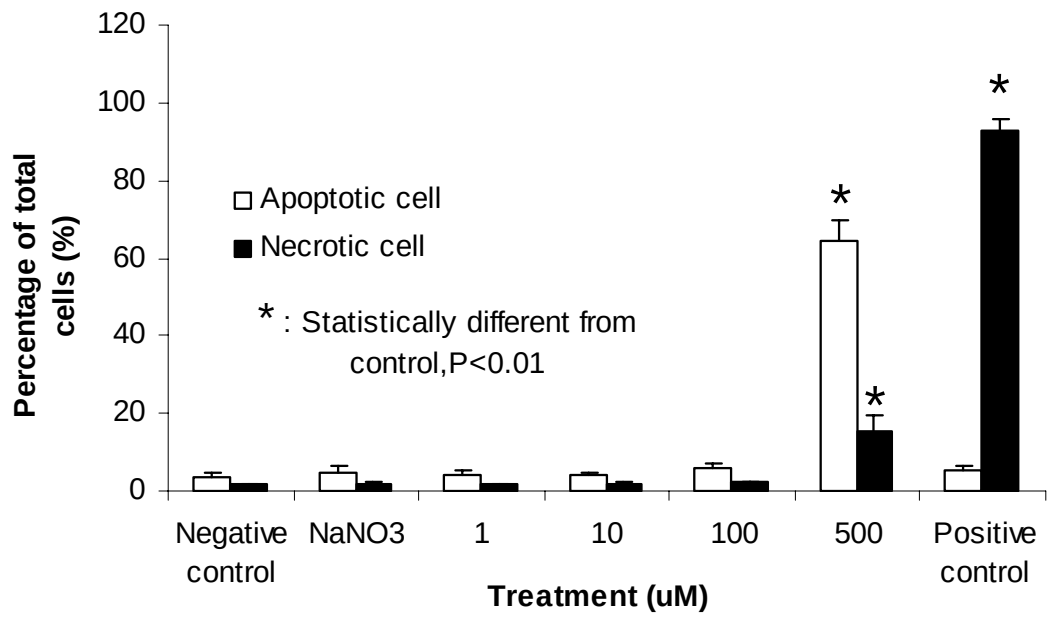
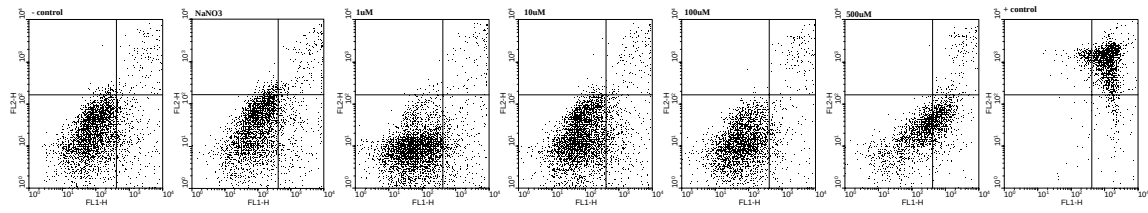


Figure 3: Apoptosis and necrosis of CD4⁺ T-cells under 48 hours DU exposure. Quadrant analysis of the flow cytometry plot of Annexin-V/PI binding to control and DU-uranyl nitrate treated CD4⁺ T-cells. CD4⁺ T-cells were treated with 0 (negative control), 1, 10, 100, 500µM DU and 1mM NaNO₃ or 1µg/ml Staurosporine (positive control) for 48 hours. Panel A shows representative dotplots from triplicates for each treatment, the x-axis of dotplots, FL1-H, represents Annexin-V binding and the y-axis, FL2-H, represents PI binding. The flow cytometry dotplot data are presented in panel B as percentage of cells binding annexin-V (apoptotic cells, annexin-V⁺/PI⁻) and PI (necrotic cells, annexin-V⁺/PI⁺) under various treatments and are the mean of triplicates.

A



B

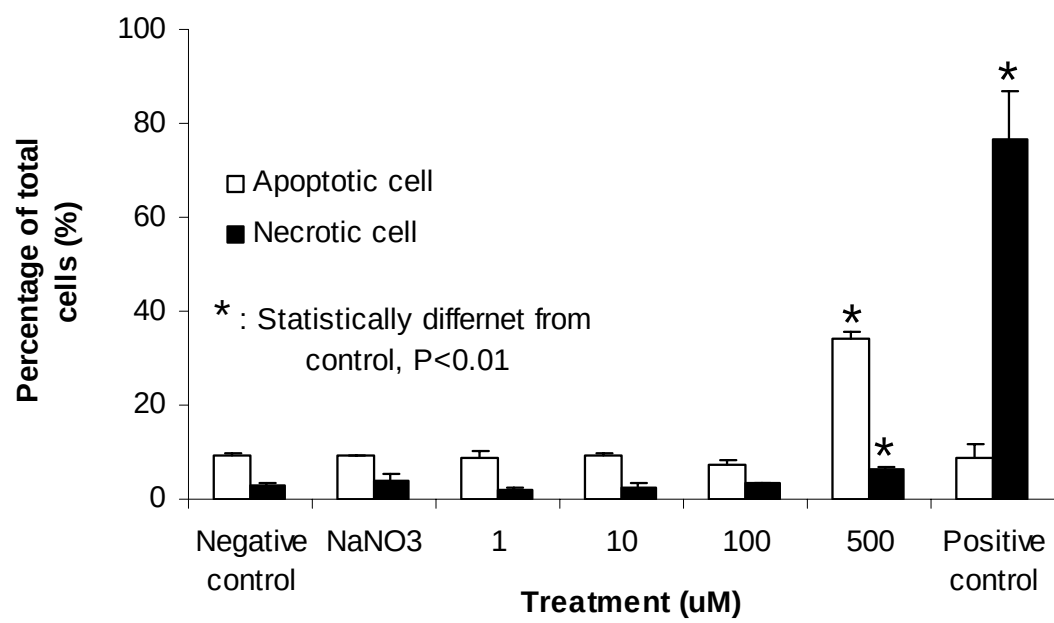
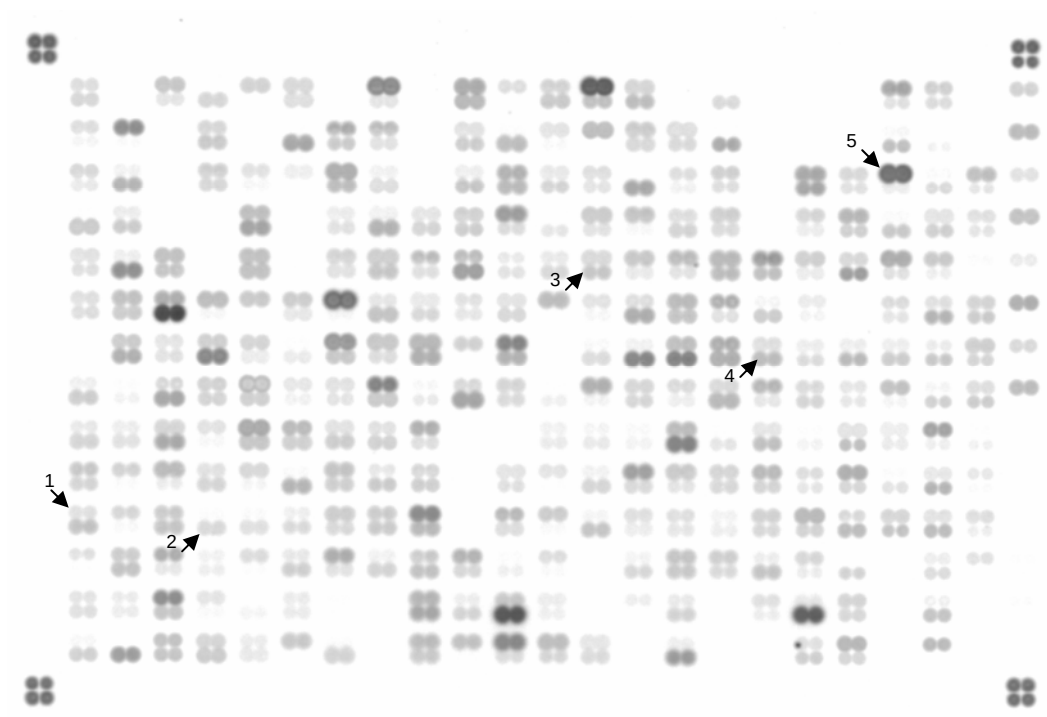


Table 1: Summary of statistical analysis of CD4⁺ T-cells microarray data

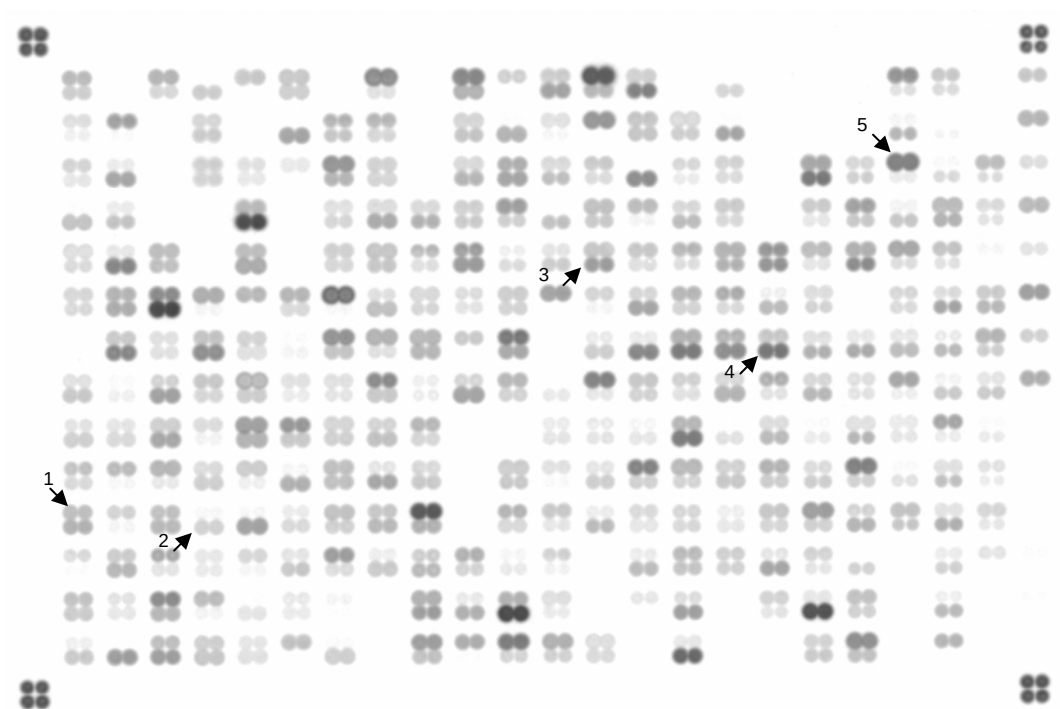
z-test for Two Independent Conditions	
Conditions:	PIM_1.C : Primary T triplication - Control PIM_1.D : Primary T triplication - 100uM 24 hrs exposure
Input Parameters	
Test:	z-test
Normalization (Center):	By Mean (Non-Iterative)
Nominal Alpha:	0.05
Multiple Test Correction:	False Discovery Rate
PIM_1.C	
Source:	C - Control
Model Selection:	Manual
Model Selected:	Proportional Model with Offsets
Outlier Detection:	Yes
Threshold Selection:	Automatic
Threshold Selected:	± 2.94 MADs
Minimum No. of Replicates:	2
Conditions:	Independent
Random Error Estimation Method: Pooled - Curve Fit	
PIM_1.D	
Source:	D - 100uM 24 hrs exposure
Model Selection:	Manual
Model Selected:	Proportional Model with Offsets
Outlier Detection:	Yes
Threshold Selection:	Automatic
Threshold Selected:	± 2.56 MADs
Minimum No. of Replicates:	2
Conditions:	Independent
Random Error Estimation Method: Pooled - Curve Fit	
Output Summary	
Effective Alpha:	0.00147874
Confidence Interval Alpha:	0.05
Significant:	14 (2.9%)
PIM_1.C > PIM_1.D	10 (2%)
PIM_1.C < PIM_1.D	4 (0.9%)
Not significant:	525 (95%)
Not analyzed:	12 (2%)

Figure 4: CD4⁺ T-cells mouse cytokine gene array hybridization images. Membranes were hybridized with P³³-labelled cDNA probes from control (A) and DU-exposed cells (B). The arrows in panel A and B indicate the differentially displayed genes identified by statistical analysis at $P < 0.00147$. The array images are representative of three replicate experiments. The selected gene names indicated by numbers in the order from 1 to 5 are: VEGF-A, TECK/CCL25, IL-5, Midkine and GDF-7. Other differentially displayed gene names can be found in figure 6 and table 2.

A



B



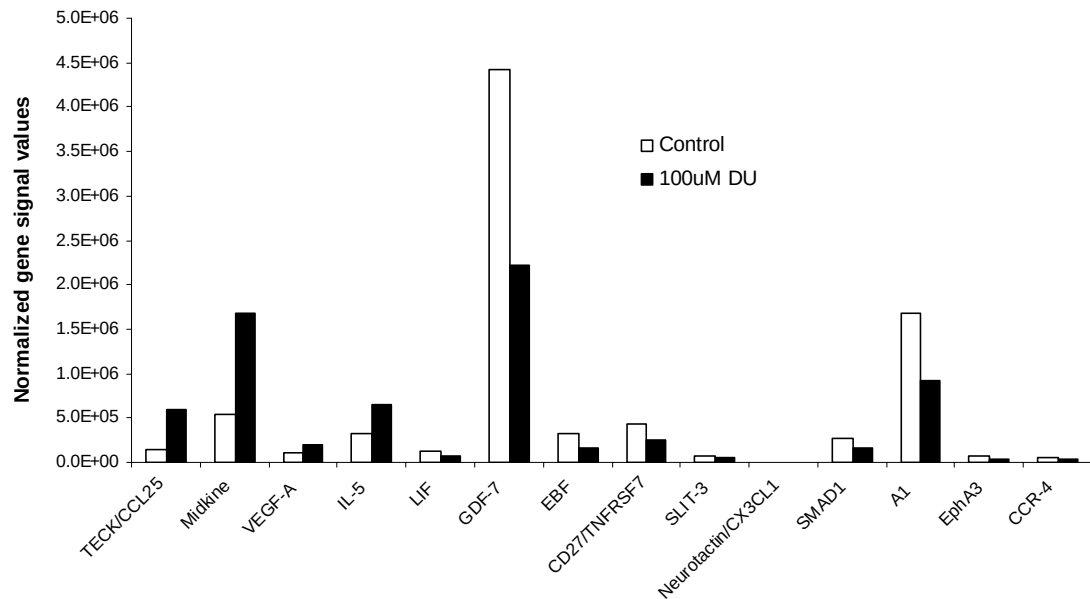


Figure 5: The normalized cDNA array signal intensities of differentially displayed genes in primary CD4⁺ T-cells. Data from three independent experiments were first normalized across replicates, within conditions. The data were then normalized across conditions using a Z-test. 14 genes were identified differentially displayed under DU exposure, of which 10 were down-regulated and 4 were up-regulated ($P < 0.00147$).

Table 2:
Expression ratios of significantly regulated genes in DU-exposed CD4⁺ T-cells as determined by microarray analysis

Gene information ^a			Z-test ^b	Ratios ^c	Upper confidence interval ^d	Lower confidence interval ^d
Genbank	Gene names	Gene group				
NM_009138	TECK/CCL25	Chemokine	1.5E-14	4.2	6.2	2.9
NM_010784	Midkine	Neurotrophic group	2.9E-07	3.0	4.7	1.9
NM_010558	IL-5	Interleukin	9.5E-04	1.9	2.9	1.3
NM_009505	VEGF-A	Angiogenic factor	2.5E-05	1.8	2.2	1.4
M68513	EphA3	Eph family	4.0E-08	0.5	0.6	0.4
NM_009916	CCR-4	Chemokine receptor	1.8E-05	0.6	0.8	0.5
NM_008501	LIF	Cytokine and receptors	3.0E-05	0.6	0.8	0.4
U08339	GDF-7	TGF-beta superfamily	2.4E-04	0.5	0.7	0.3
NM_007897	EBF	Signal transduction	3.7E-04	0.5	0.7	0.4
L24495	CD27/TNFRSF7	TNF superfamily	6.4E-04	0.6	0.8	0.4
AF088902	SLIT-3	Developmental factor	9.2E-04	0.7	0.8	0.5
NM_009142	Neurotactin/CX3CL1	Chemokine	1.2E-03	0.4	0.7	0.2
NM_008539	SMAD1	Signal transduction	1.3E-03	0.6	0.8	0.5
L16462	A1	Apoptosis-related	1.5E-03	0.6	0.8	0.4

Legend to Table 2

Note: The genes that were down-regulated in DU-treated CD4⁺ T-cells are displayed with a grey background and genes that were up-regulated are displayed with a white background.

^a The Genbank accession number and gene names for each gene; The gene groups that each gene belongs to are included.

^b The two-tailed Z-tests *p* values for the comparison of replicate samples for each gene between control and DU groups.

^c The ratio expression values for the average expression values of each gene between DU and control. i.e. ratio = intensity value from DU treated cells divided by that from control cells.

^d Confidence intervals determined at $p < 0.05$

Table 3: Primer sequences used in QRT-PCR for selected differentially expressed genes in CD4⁺ T-cells

Targets	Sequences^b	Amplicon Size (bp)
IL-5	F: 5'-GTC CCT ACT CAT AAA AAT CAC CA -3' R: 5'-GAA TAG CAT TTC CAC AGT ACC C -3'	105
Midkine	F: 5'-ACC GAG GCT TCT TCC TTC TC-3' R: 5'-GGC TCC AAA TTC CTT CTT CC-3'	230
TECK/ CCL25	F: 5'-TGG AGA ACC CCAACA GTA CAA -3' R: 5'-TTC AGC AGA CTC CTC CCA TC -3'	137
CD27/ TNFRSF7	F: 5'-TTT GAC CTC CTC TGG CAT CT -3' R: 5'-TCA CGG CAG TCA GTC CTT C -3'	126
GAPDH ^a	F: 5'-TGA TGA CAT CAA GAA GGT GGT GAA G-3' R: 5'-TCC T TG GAG GCC ATG TAG GCC AT-3'	240

^a Sequences referred from [Lee et al., 2000](#).

^b F: Forward Primer; R: Reverse Primer. Primer sequences are designed using Primer 3 internet based software (http://www.broad.mit.edu/cgi-bin/primer/primer3.cgi/results_from_primer3).

Figure 6: QRT-PCR analysis of GAPDH and IL-5 relative mRNA levels in control and DU-treated CD4⁺ T-cells. (A) Electrophoresis of 2 µg of total RNA in a denaturing 1.5% agarose formaldehyde gel; the ratio between the brightness of 28s and 18s rRNA bands was in the correct range of 2:1. M, Millennium RNA marker (Ambion, Inc.); C, control RNA sample; T, RNA from DU-Treated cells. (B) Standard curves for GAPDH and IL-5; the linear regression equations were given in the panel for each gene. (C) Representative melting curves for IL-5. The red line indicates the PCR product with template addition; the green line indicates non-template control (NTC); Note that NTC product did not produce an obvious curve peak indicating that no amplification occurred. (D) Representative melting curves for GAPDH. The red line indicates the PCR product with template addition; The green line indicated a non-template control (NTC); (E) Electrophoresis of PCR products in a 1% agarose gel; the 240bp band corresponds to GAPDH and 105bp band to IL-5 amplified from control (C) and DU-treated (T) cells.

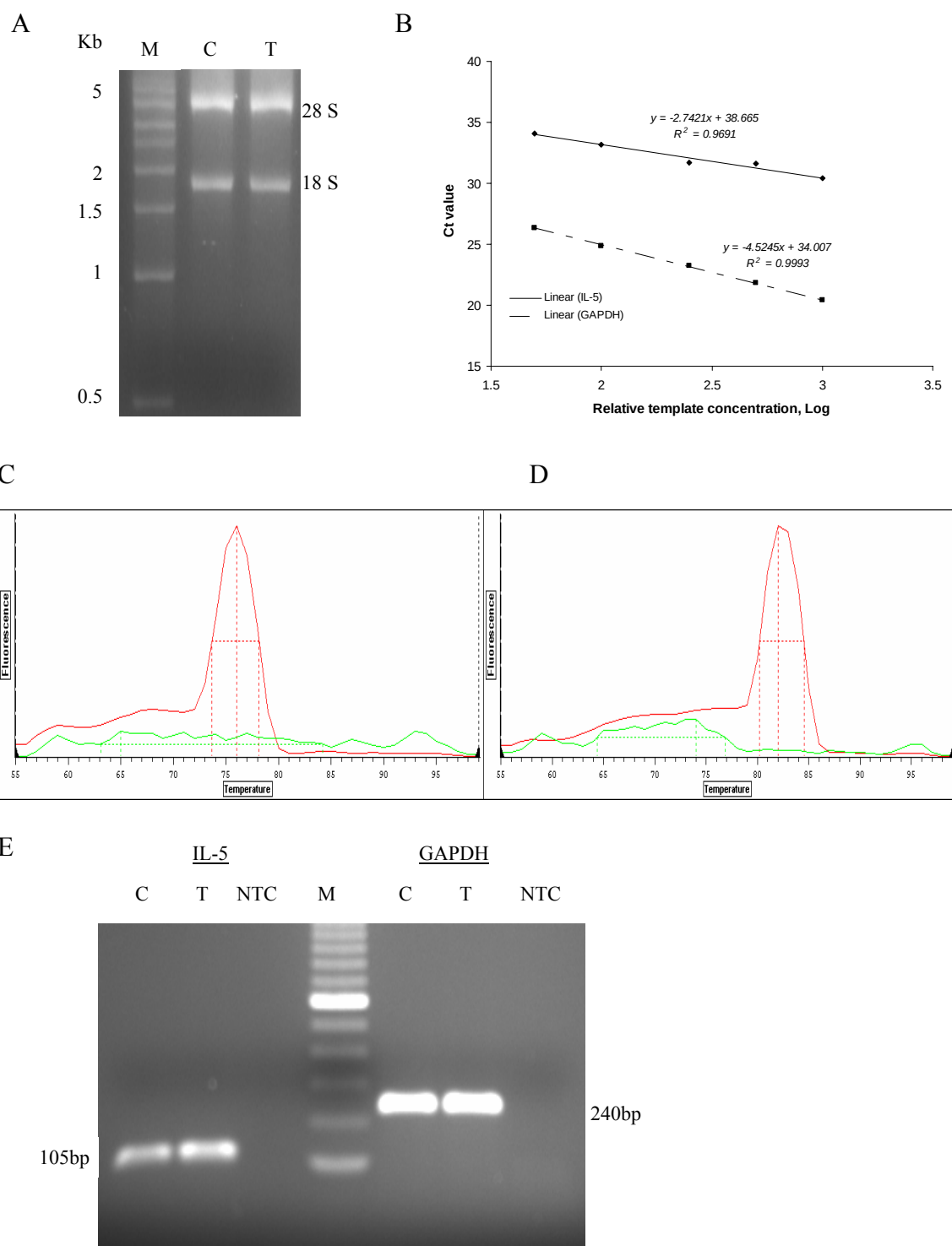


Table 4: PCR efficiency and gene expression ratio of IL-5 and GAPDH

Genes	Ct values		Standard Curve Slopes	PCR Efficiency (E) ^a	Ratio(T/C) ^b
	Control	DU- Treatment			
IL-5	32.04	29.87	-2.7421	2.31	3.20
	31.07	30.23			
	31.37	29.97			
Average	31.49	30.02			
GAPDH	19.19	19.18	-4.5245	1.66	--
	20.01	19.65			
	20.13	20.10			
Average	19.78	19.64			

^a PCR efficiency (E) = $10^{-1/\text{slope}}$. [Pfaffl, 2001; Pfaffl et al., 2002]

^b Ratio (T/C) = $\text{Power}(E_{\text{target gene}}, \Delta C_{\text{t target gene}}(\text{control-sample})) \div \text{Power}(E_{\text{reference gene}}, \Delta C_{\text{t reference gene}}(\text{control-sample}))$; $E_{\text{target gene}}$, PCR efficiency of target gene; $E_{\text{reference}}$, PCR efficiency of reference gene; $\Delta C_{\text{t target gene}}(\text{control-sample})$, the difference of target gene Ct values between control (C) and DU-treated samples (T); $\Delta C_{\text{t reference gene}}(\text{control-sample})$, the difference of reference gene Ct values between control and DU-treated samples [Pfaffl, 2001; Pfaffl et al., 2002].

Table 5: Comparison of the differential gene expression ratios in CD4⁺ T-cells determined by microarray and QRT-PCR analysis

Gene Names	Array Ratios	QRT-PCT Ratios	Array Ratio Confidence Intervals
Midkine	3.0	1.9	4.7~1.9
TECK/CCL25	4.2	1.4	6.2~2.9
IL-5	1.9	3.2	2.9~1.3
CD27/TNFRSF7	0.6	0.7	0.8~0.4

PART 6

IMMUNOTOXICOLOGICAL EFFECTS OF DEPLETED URANIUM ON T CELL HYBRIDOMA TCH PLP1 5B6

Abstract

Previous experiments indicated that DU induces Th2 cytokine expression during primary CD4⁺ T-cell activation and differentiation (Part 4 and 5). To study the toxic effect of DU on Th1 cells and the gene expression pattern of the cells exposed to DU, a Th1 type hybridoma cell line was chosen and cells were exposed to DU for 4 and 24 hours. The results showed that TCH cells treated with 1000μM of DU exhibited cell apoptosis after as little as 4 hours, and the apoptosis also occurred after 24 hours of treatment with 500μM DU. The microarray results identified 18 genes that were up-regulated in TCH cells upon non-cytotoxic DU exposure and no genes were down-regulated. The induced genes were MMP-13, WNT-5a, TIMP-3, Integrin-αL, IL-11, M-CSF, TP1, IGF-I, Lymphotoxin, WNT-16, UCP2, Axl, CCR-7, PIN, BMP-11, Jak-2, BMP-7, and Ret, with induction ratios ranging from 2.6 to 11.6. The gene expression was confirmed using northern blots. Some of these genes were related to proteases, cytokines, and signal transduction, indicating possible involvement of DU in cancer development.

Introduction

Upon activation, naïve CD4⁺ T-cells can differentiate into Th1 and Th2 cells, each producing its own set of cytokines and mediating distinct effector functions [Mosmann et al., 1986; Abbas et al., 1996]. The complex balance between Th1 and Th2 cells can be disturbed by a variety of factors including heavy metals [Pelletier et al., 1986; Krocova et al., 2000]. Previous experiments revealed that DU can boost Th2

cytokine (IL-5) expression, indicating a systemic Th2 shift during naïve CD4⁺ T-cell differentiation.

To determine the effect of DU on Th1 cells, a T-hybridoma cell line TCH PLP1 5B6 was used in this study. These cells are immortalized murine T-cells that have been primarily used for studying experimental autoimmune encephalomyelitis (EAE) because they are autoreactive [Kuchroo et al., 2002]. The cells have specific T-cell receptors (TCR) that recognize proteolipid protein (PLP)-1 peptide 139-151 in association with MHC class II [Legge et al., 1997]. When activated, the cells can induce EAE in the central nerve system (CNS). In addition, during the progress of EAE, Th1 cytokines predominate in the inflammatory EAE lesions in the CNS [Kuchroo et al., 2002], suggesting the Th1 commitment of the cells. Without PLP1 139-151 peptide stimulation, the cells remain in the resting state and can be considered as Th1 memory cells, which when re-activated, form Th1 memory effector cells [Dutton et al., 1998].

Previous experiments have indicated that DU is able to interfere with cytokine production and signal transduction in peritoneal macrophages and primary CD4⁺ T-cells. It is hypothesized here that upon exposure to DU, many of the TCH cells may also be affected in cell communication and cytokine production. In this study, the toxicity of DU to TCH cells has been studied and differentially expressed genes in DU-exposed TCH cells have been identified.

Results

Apoptosis and necrosis of TCH upon DU exposure

In this study, annexin-V and PI binding have been employed to assess the ability of DU to induce cell apoptosis, as well as necrosis, in TCH cells. Annexin-V-FITC positive cells are those undergoing apoptosis, while PI positive cells are in a necrotic state. As shown in figure 1 (Panel A and B) (All tables and figures in appendix at the end of the part), 1000uM DU exposure for 4 hours causes peak shifting from left to right in both Annexin-V and PI binding histograms as compared with control samples, indicating the occurrence of both apoptosis and necrosis in TCH cells. The corresponding geometric means of all treatments and shows approximately a 2~3 folds of differences between controls and 1000uM DU group after 4 hours exposure ([Table 1](#)). Four hours of 200 and 500uM DU exposure do not cause an obvious change in either the peak positions or geometric means of their fluorescence intensities ([Table 1](#)), indicating that neither apoptosis nor necrosis occurred. After 24 hours, 500uM DU starts to induce apoptosis in TCH cells as indicated in figure 1C. Only 1000uM DU causes necrosis ([Figure 1D](#)). A comparison of the peaks from control and sodium nitrate treated cells at both 4 hours and 24 hours time points shows a very close fit, indicating that the contribution of the nitrate group from uranyl nitrate in inducing either apoptosis or necrosis is negligible ([Figure 1](#)).

Gene expression analysis

To identify any molecular link between DU exposure and cytokine gene expression in TCH cells, the cells were treated with or without 500uM DU for 4 hours. Total RNA isolation was performed immediately after removing the culture media, and

cell cytokine gene expression was measured using microarrays. Table 2 lists the methods and parameters for the Z-tests performed on four replicate data sets. The statistical analysis was based on two independent conditions: control (PIM_1.A) and 500 μ M DU exposure (PIM_1.B). The data normalization was performed on the mean intensity values of whole array. For any particular gene, if there are fewer than 3 valid observations present within a condition, that datum was disregarded for analysis. The outliers were detected by examining standardized residuals automatically using ArrayStat software package. A curve fit random error estimate method was employed using proportional model with offsets and the nominal alpha value was set at 0.05. Table 3 lists all 18 (3.5% of all genes) genes that were up-regulated upon 500 μ M DU exposure. These genes are MMP-13, WNT-5a, TIMP-3, Integrin- α L, IL-11, M-CSF, TP1, IGF-I, Lymphotactin, WNT-16, UCP2, Axl, CCR-7, PIN, BMP-11, Jak-2, BMP-7, and Ret. The ratio was determined as the fold increase in DU-treated cells over that in controls. The ratios of these genes range from 2.6 to 11.6. The gene functions involved in response to DU exposure include signal transduction, cytokine or receptor production, neurotrophic factors, protease production, and the TGF-beta superfamily. No genes were down-regulated. The average normalized expression values for the differentially expressed genes are plotted in figure 2.

Northern blots

To confirm the microarray results, northern blots analysis was performed on five genes that were determined as to be differentially expressed in the microarray experiments. TCH cells were exposed to 200 and 500 μ M of DU for 4 hours, then total

RNA was isolated and processed for northern blot analysis. The primer sequences for 300bp amplicons are listed in [Table 4](#). The presence of rRNA bands in formaldehyde denaturing gel images were used to normalize the amount of total RNA. The results showed that the expression of BMP-11, IGF-I, Integrin- α L, UCP-2 and Wnt-5a in TCH cells were induced when exposed to 200 and 500uM DU for 4 hours ([Figure 3](#)). The ratios determined in northern blot analysis range from 1.7 to 2.9 ([Table 5](#)), which are lower than those from microarray analysis.

Discussion

It has been shown that DU is capable of causing cell apoptosis and necrosis in primary CD4⁺ T-cells (Part 5). At non-cytotoxic concentrations, DU interferes with expression of a number of cytokines including Th2 cytokines. In this study, a Th1 type hybridoma cell line (TCH PLP1 5B6) [[Kuchroo et al., 2002](#)] was used to evaluate the toxicity of DU and the capability of DU in inducing a Th2 shift in those cells. The cells were exposed to various concentrations of DU for 4 and 24 hours. Whether cells undergo apoptosis or necrosis is dependent on the concentration of DU. At lower DU concentration (500uM), cells become apoptotic, whereas at higher concentration (1000uM) cells undergo necrosis ([Figure 1](#)). Compared with the concentrations that cause cytotoxicity in primary CD4⁺ T-cells and peritoneal macrophages in 24 hours, the concentrations required elicit cytotoxic effects in TCH cells are much higher. The reason may be due to their lack of the phagocytosis activity that macrophages possess and their immortalized phenotype that protects cells from cell death. Due to their resistance to DU toxicity, TCH cell death may possibly be attributed to the nitrate ions present at high DU

concentrations. To rule out this possibility, a sodium nitrate control was used to evaluate the impact of nitrate ions. The results showed no difference between the nitrate ion controls and negative controls in cell apoptosis and necrosis (Figure 1), indicating that the nitrate ion do not influence the toxicity of DU on TCH cells.

Based on the microarray analysis, no Th2 cytokines were up-regulated in TCH cells upon DU exposure, indicating that this Th1 type cell line can not be induced into a Th2 phenotype by DU exposure. Therefore, DU induces a Th2 shift in primary CD4⁺ T-cells possibly by promoting the differentiation of naïve T-cells into Th2 type, but not by inducing Th1 cells to express Th2 cytokines. Surprisingly, no genes were down-regulated after DU exposure. This results may be explained partially because of the resting status of TCH cells [Kuchroo et al., 2002] so that most of cytokine genes expressions are normally shut down or kept in low level. That probably is the case since only 20 genes showed detectable expression levels in the microarray experiments and 90% of them (18 genes) were up-regulated to a high degree.

MMP-13 belongs to a group of proteases that are responsible for degrading collagen macromolecules [Hibbs et al., 1987], such as the extracellular matrix (ECM). The MMP group is comprised of several members including MMP-1, MMP-2, MMP-13 that have a high functional redundancy. Ablation of a particular MMP will induce the expression of other MMPs to compensate. Inflammatory cells have been reported to influence the remodeling of the extracellular matrix directly by MMPs and a variety of biological active agents, such as TNF- α , IL-6, TGF- β and IL-1 modulate the synthesis of MMPs [Solis-Herruzo et al., 1999; Edwards et al., 1987]. Deregulation of the production of MMPs are presumed to cause the tissue alterations observed in stasis dermatitis

[Herouy et al., 2001]. The presence of MMP mRNA has been found associated with cell lesions in diseases such as rheumatoid arthritis, cancer and acute myocardial infarction [Ye, 2000]. For tumor cells to metastasize, they produce MMPs to break away from neighboring cells to penetrate the surrounding stroma. The up-regulation of MMP-13 in TCH cells indicates that DU exposure may cause cell lesions, degradation of the extracellular matrix and facilitate tumor cell spreading.

IGF-I (insulin-like growth factor-I) is well recognized as a mitogen that promotes cell cycle progression, cell survival, and inhibition of apoptosis [Raile et al., 2003]. IGF-I expression has been observed in macrophages and salivary acinar cells. IGF-I does not only promote macrophage proliferation but also prevents apoptosis by suppressing the activity of Capase-3 [Limesand et al., 2003; O'Donnell et al., 2002]. In this study, increased gene expression of IGF-I in DU-exposed TCH cells was also evidenced, demonstrating that non-cytotoxic DU exhibits anti-apoptosis activity. Due to the endocrine mode of action of IGF-I, DU may exert its effects on other cells such as macrophages and endothelial cells that are far from the exposure site.

Recently, several studies have suggested that elevated circulating IGF-I levels are associated with an increased risk of cancer [Sandhu et al., 2004]. IGF-I is able to up-regulate the expression of vascular endothelial growth factor (VEGF) by way of the paracrine pathway in thyroid cancer and plays a key role in the pathophysiology of several neoplasias [Poulaki et al., 2003]. Induction of IGF-I in TCH cells after DU exposure indicates a potential carcinogenic activity of DU. This statement is supported by the occurrence of the expression of other up-regulated genes in the microarray

experiments, such as Axl (Table 3), the expression of which is implicated in human thyroid tumorigenesis [Ito et al., 1999].

Understanding patterns of expressed genes is expected to provide insight into complex regulatory networks and will most probably lead to the identification of genes relevant to new biological processes, or implicated in the development of disease [Vandesompele et al., 2002]. The induction of many other genes identified in this study such as IL-11, Wnt-5a, and PIN suggests that multiple pathways are involved in DU toxicity. Theoretically, if the genes belong to a common pathway, co-regulation of these genes is expected and may reveal some links between genes and within these pathways. However, the experimental conditions such as cell lines, testing concentrations and exposure time may also exert profound effects. The TCH cell line used in this study is an immortalized Th1 cell line that possesses cancer cell characteristics. This cell line may respond differently to DU exposure compared to primary or undifferentiated cells. However, at least two genes, MMP-13 and BMP-11, are also induced in macrophages upon DU exposure, indicating a consistent influence of DU on protease expressions. TCH cells represent a highly differentiated Th1 cell population and the results presented here maybe more relevant to the toxic effects of DU on memory Th1 cells *in vivo*.

Appendix

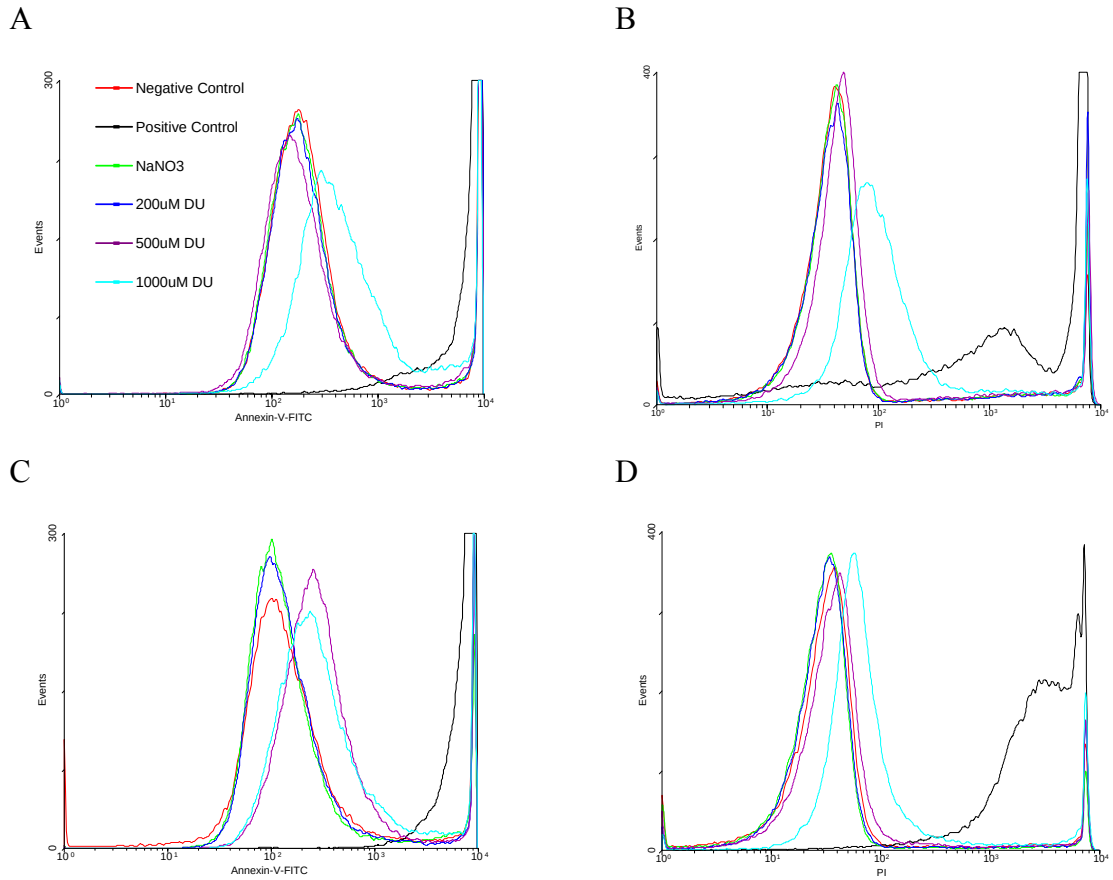


Figure 1: Histogram analysis of Annexin-V and PI binding to TCH cells. The cells were treated with 0 (negative control), 200, 500, 1000 μ M Uranyl nitrate, 2mM sodium nitrate or 1 μ g/ml Straurosporine (positive control). Peak shifting from left to right indicates tendency of induction of apoptosis or necrosis upon DU exposure. (A) Annexin-V-FITC staining of TCH cells after 4 hours exposure; (B) PI staining of TCH cells after 4 hours exposure; (C) Annexin-V-FITC staining of TCH cells after 24 hours exposure; (D) PI staining of TCH cells after 24 hours exposure. Different treatments are indicated with different color lines.

Table 1: Geometric means of fluorescence intensities for annexin-v-FITC and PI staining in TCH cells under DU exposure

	4 hours Exposure				24 hours Exposure			
	Annexin-V-FITC (Apoptosis)		PI (Necrosis)		Annexin-V-FITC (Apoptosis)		PI (Necrosis)	
Treatment	GMean ^a	C.V ^b	GMean	C.V	GMean	C.V	GMean	C.V
Negative Control	297.9	24.7	56.5	40.1	173.5	30.2	38.9	40.8
Staurosporine	7093.5	7.88	805.8	35.4	7597.5	4.8	2348.9	15.4
NaNO3	314.2	25.7	62.5	41.0	170.4	25.1	33.3	37.5
200uM	346.7	26.8	70.8	44.2	182.1	25.4	38.8	39.4
500uM	301.7	27.8	78.6	39.6	351.7	20.1	47.0	38.4
1000uM	623.2	21.3	154.7	30.6	383.7	21.9	91.5	32.4

^a Gmean: Geometric mean

^b C.V: Co-efficiency of variation.

Table 2:
Summary of statistical analysis of TCH microarray data

PIT_1 : z-test for Two Independent Conditions	
Conditions:	PIM_1.A : 4 - Control PIM_1.B : 4 – 500uM DU Exposure
Input Parameters	
Test:	z-test
Normalization (Center):	By Mean (Non-Iterative)
Nominal Alpha:	0.05
Multiple Test Correction:	No Correction
PIM_1.A	
Source:	A - Condition 1
Model Selection:	Manual
Model Selected:	Proportional Model with Offsets
Outlier Detection:	Yes
Threshold Selection:	Automatic
Threshold Selected:	± 2.13 MADs
Minimum No. of Replicates:	4
Conditions:	Independent
Random Error Estimation Method: Pooled - Curve Fit	
PIM_1.B	
Source:	B - Condition 2
Model Selection:	Manual
Model Selected:	Proportional Model with Offsets
Outlier Detection:	Yes
Threshold Selection:	Automatic
Threshold Selected:	± 2.69 MADs
Minimum No. of Replicates:	4
Conditions:	Independent
Random Error Estimation Method: Pooled - Curve Fit	
Output Summary	
Effective Alpha:	0.05
Confidence Interval Alpha:	0.05
Significant:	18 (3.5%)
PIM_1.A > PIM_1.B	0 (0.00%)
PIM_1.A < PIM_1.B	18 (3.5%)
Not significant:	2 (0.4%)
Not analyzed:	495 (96%)

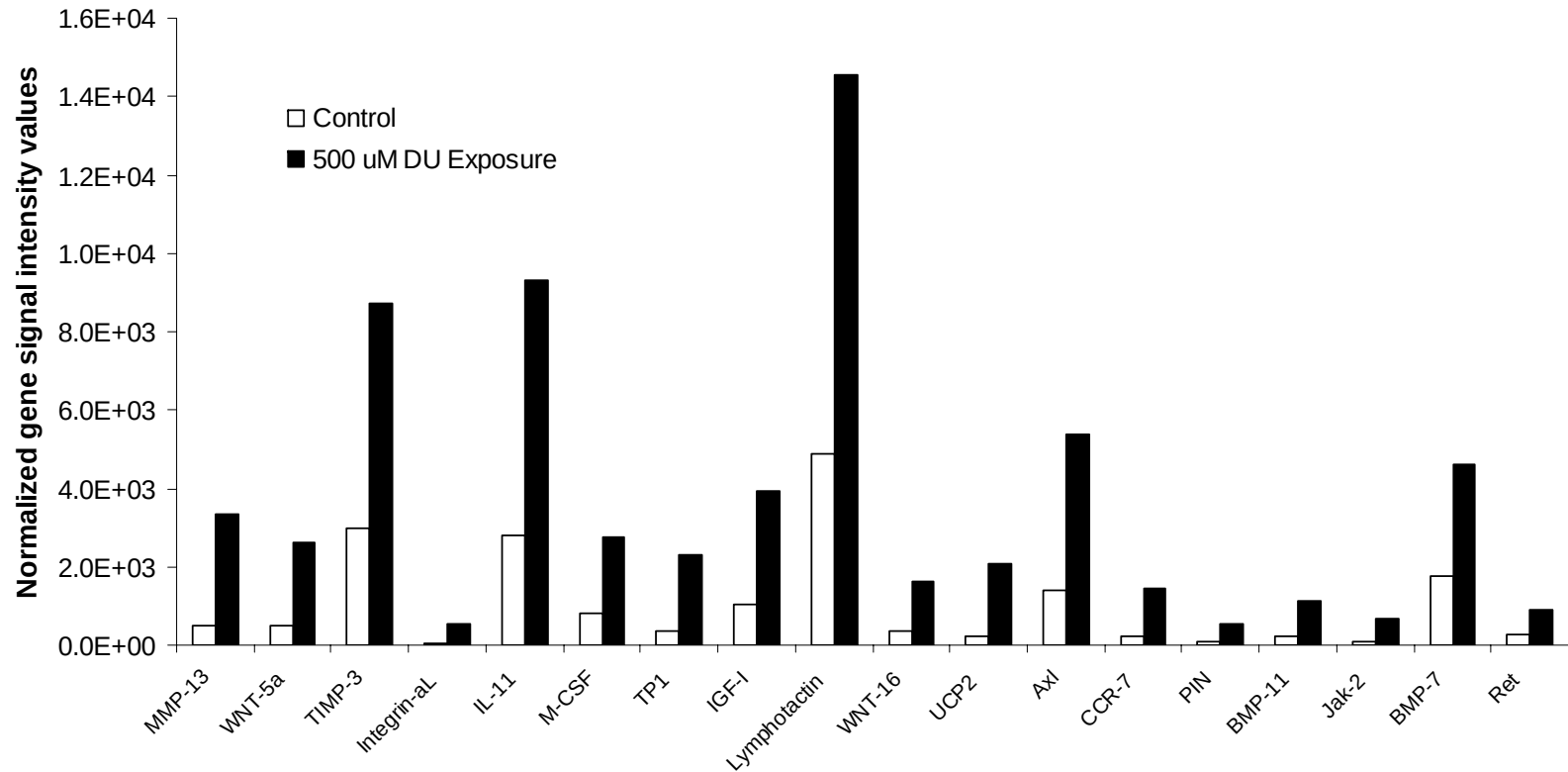


Figure 2: The normalized cDNA array signal intensities of differentially displayed genes in TCH cells. Data from four parallel experiments were first normalized across replicates, within conditions. Then the data were further normalized across the conditions using a statistical Z-test. 18 genes have been identified as up-regulated under DU exposure ($P < 0.05$). No gene was down-regulated.

Table 3:**Expression ratios of significantly regulated genes in DU-exposed TCH cells as determined by microarray analysis**

Gene information ^a			Z-test ^b	Ratios ^c	Upper confidence interval ^d	Lower confidence interval ^d
Genbank	Gene names	Gene group				
NM_008607	MMP-13	Protease or Related Factor	2.2E-16	6.7	10.5	4.2
NM_009524	WNT-5a	Developmental factor	9.0E-12	5.0	8.1	3.2
NM_011595	TIMP-3	Protease or Related Factor	1.8E-09	2.9	4.1	2.0
M60778	Integrin- α L	Integrin	1.1E-07	11.6	28.6	4.7
NM_008350	IL-11	Interleukin	1.8E-07	3.3	5.2	2.1
NM_007778	M-CSF	Cytokines and Receptor	1.8E-07	3.3	5.3	2.1
NM_009351	TP1	Telomerase Related	4.4E-07	6.5	13.6	3.1
NM_010512	IGF-I	Cytokines and Receptor	7.3E-07	3.8	6.5	2.2
NM_0008510	Lymphotactin	Chemokine	5.2E-06	3.0	4.8	1.9
AF172064	WNT-16	Developmental factor	6.5E-05	4.5	9.5	2.1
NM_011671	UCP2	Weight Regulation	8.4E-05	8.6	25.1	2.9
NM_009465	Axl	Cytokines and Receptor	9.3E-05	3.8	7.5	1.9
NM_007719	CCR-7	Chemokine Receptor	1.5E-03	5.9	17.6	2.0
AA041883	PIN	NO Metabolism	6.7E-03	6.4	24.8	1.7
AF082348	BMP-11	TGF- β Superfamily	6.9E-03	5.0	16.2	1.5
NM_008413	Jak-2	Signal Transduction	1.1E-02	6.1	24.5	1.5
NM_007557	BMP-7	TGF- β Superfamily	1.1E-02	2.6	5.4	1.2
NM_009050	Ret	Neurotrophic Group	2.2E-02	3.1	8.4	1.2

Legend to table 3

- ^a The Genbank accession number and gene names for each gene; the gene groups that each gene belongs to are included.
- ^b The two-tailed Z -tests p values for the comparison of replicate samples for each gene between control and DU.
- ^c The ratio expression values for the average expression values of each gene between DU and control. i.e. ratio = intensity value from DU treated cells divided by that from control cells.
- ^d Confidence intervals determined at $p < 0.05$.

Table 4: Primer sequences used in northern blot analysis for selected genes in TCH cells

Targets	Sequences^a
BMP-11	F: 5'-TTC ATG GAG CTT CGA GTC CT-3' R: 5'-AGC ATG TTG ATT GGG GAC AT-3'
IGF-I	F: 5'-CCC CAC TGA AGC CTA CAA AA-3' R: 5'-GGG AGG CTC CTC CTA CAT TC-3'
Intergrin-aL	F: 5'-CTG CTT GGA CTT CCA CTT CC-3' R: 5'-TTG CTC AGT GTC CAC TCC AC-3'
UCP-2	F: 5'-GCC ACT CAC TTC TGC CTT C-3' R: 5'-AGC ATG GAG AGG CTC AGA AA-3'
Wnt-5a	F: 5'-ACC TGG CAG ATG TAG CCT GT-3' R: 5'-TCC CCT CTG AGT CTT GTT G-3'

^a F: Forward Primer; R: Reverse Primer. All primer sequences are designed using Primer 3 internet based software (http://www.broad.mit.edu/cgi-bin/primer/primer3.cgi/results_from_primer3).

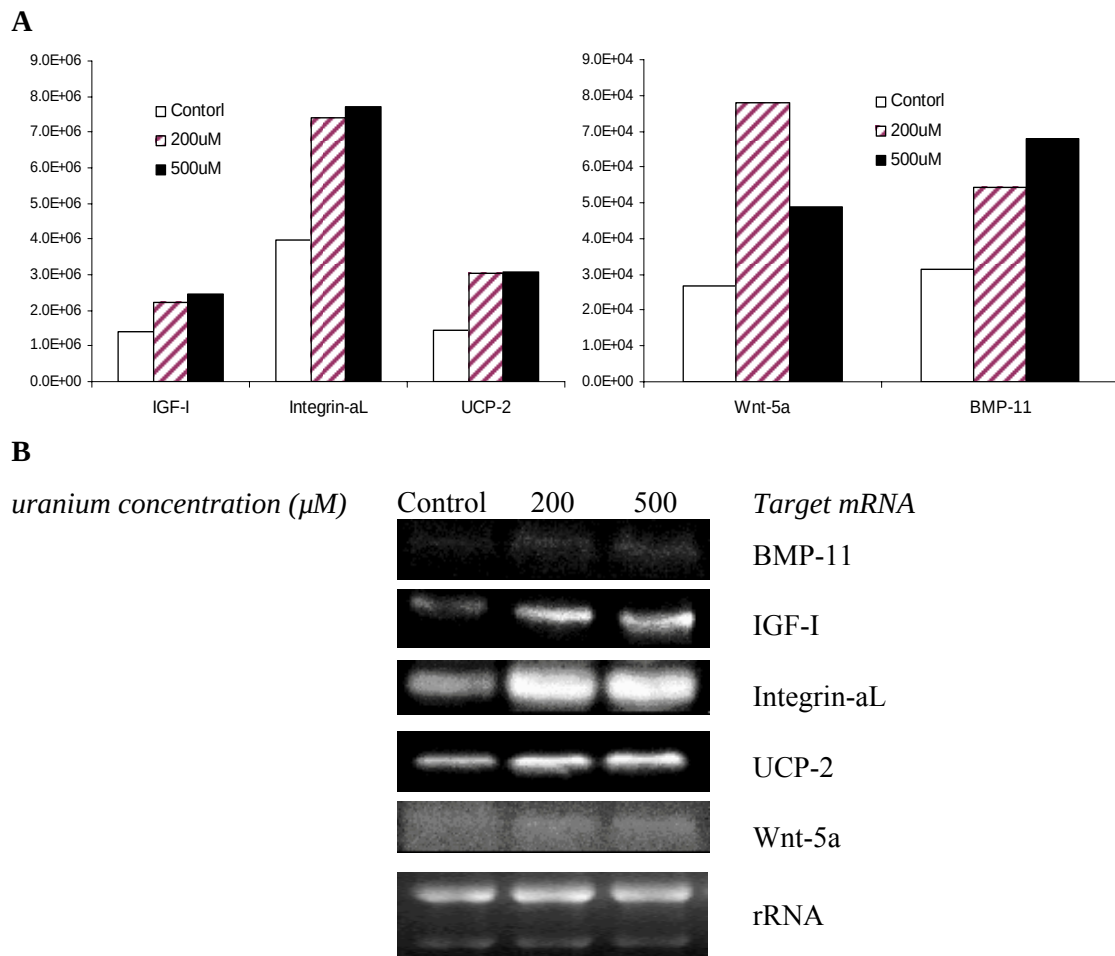


Figure 3: Northern blot analysis of the expression of BMP-11, IGF-I, Integrin-aL, UCP-2, and Wnt-5a in TCH cells under 200 and 500 μM DU exposures. (A) The phosphor signal intensities for IGF-I, Integrin-aL, UCP-2, Wnt-5a, and BMP-11 in TCH cells exposed to 200 and 500uM DU for 4 hours. (B) The northern blot hybridization images of the five genes. rRNA bands in the formaldehyde gel images were analyzed for controlling total RNA loading.

**Table 5: Gene expression ratios determined by
northern blot analysis**

Gene names	Ratio^a	
	200μM	500μM
BMP-11	1.7	2.1
IGF-I	1.6	1.7
Intergrin-aL	1.87	1.9
UCP-2	2.1	2.1
Wnt-5a	2.9	1.8

^a Ratio was determined as the fold increase of intensity value from DU treated cells over that from control cells.

PART 7

CONCLUSIONS AND PERSPECTIVES

The contamination caused by the extensive use of DU in industrial and military applications has provoked concern about the environmental consequences and potential human health problems. Although toxic effects of DU on immune system have been implicated in previous studies, the molecular mechanisms of these effects are still mostly unknown. The first part of this study demonstrates the effect DU has in modulating macrophage function as well as causing cell death. Short-term, high concentrations of DU exposure increase the ability of macrophages to activate T-lymphocyte proliferation, indicating an altered macrophage accessory cell function. Macrophage cell apoptosis occurs with cell necrosis upon DU exposure. Chromatin condensation and margination, membrane blebbing, and formation of apoptotic bodies have been evidenced microscopically. At non-cytotoxic concentrations, DU modulates macrophage gene expression to interfere with cytokine production and signal transduction. For the first time, a large scale gene expression profile has been generated for DU-exposed macrophages. The results show that DU can up-regulate a number of cytokines such as IL-10, which causes Th2 cell differentiation and in the long run, can deactivate macrophages causing impaired immune function. We also observed the up-regulation of NF-kBp65 and Midkine, suggesting a possible role for DU in cancer development and immune responses.

The second part of this study was designed to assess the toxic effects of DU on primary CD4⁺ T-cells. Upon stimulation, naïve cells further differentiate to either Th1 cells or Th2 cells and a dynamic balance is established between these two subsets of cells. However, the complex balance between Th1 and Th2 cells can be disturbed by a variety of factors including heavy metals and cytokines. A shift in the T-cell population

to the Type 1 phenotype fosters T-cell-mediated autoimmunity and inflammation. In contrast, Th2 cytokines have been proposed to have a protective effect in Th1-derived pathologies and directly cause allergic responses or some autoimmune diseases [Mosmann and Sad, 1996]. Our results demonstrate that DU preferentially enhances Th2 differentiation by inducing IL-5, which activates eosinophils and causes allergic inflammation [Mishra et al., 2002; Saito et al., 2002]. The up-regulation of Midkine was also observed, which suggests a possible carcinogenic activity of DU to immune cells. In addition, the cytotoxicity of DU has been evaluated. The results show that primary CD4⁺ T-cells are more resistant to DU toxicity than macrophages and that cell apoptosis occurs at higher concentrations than those in macrophages.

Finally, the toxic effects of DU on a Th1 hybridoma cell line (TCH PLP1 5B6) were studied. The cytotoxic study shows that TCH cells are more resistant to DU exposure than primary CD4⁺ T-cells. Cell apoptosis took place at a concentration of 500μM after 24 hours exposure. The gene expression profile of DU-exposed TCH cells did not show Th2 cytokine up-regulation. However, protease-related genes such as MMP-13, which degrades extracellular matrix [Hibbs et al., 1987] and facilitates the spreading of tumor cells, were up-regulated. Other up-regulated genes such as IGF-I, Axl, and BMP-11 are involved in cancer development, indicating a possible DU carcinogenic involvement. In conclusion, this study provides DU toxicity information on three types of immune cells and suggests several molecular mechanisms for DU immunotoxicity.

In this study, the DNA microarray technique was used to study the toxic effects of DU on immune cell function. However, changes at the mRNA level are not necessarily proportional to changes at the protein level because of differences in rates of protein

translation and degradation. Furthermore, nucleotide screens are unable to provide information on the post-translational modifications of a protein [Yanicek and Perkel, 2003]. Information obtained from expression arrays would ideally be complemented by analysis of alterations in protein levels, post-translational modification, and function [Banks et al., 2000].

The roles of DU in the induction of immune response, altering immune function, boosting Th2 differentiation and potentially in cancer progression have been demonstrated in cell culture. However, an *in vivo* study on the toxic effect of DU on immune system may be more physiologically relevant and represents actual exposure scenario, and therefore should be a priority for future study.

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