Methods:

Chemicals and Test Materials

Nickel sulfate hexahydrate ($NiSO_4$, N72-3) in powder form was purchased from ThermoFisher Scientific (Fair Lawn, NJ). Dosing solutions were prepared using mineral oil (ThermoFisher Scientific; Fair Lawn, NJ) as a delivery vehicle.

Humanized TLR-4 Mouse Model

All mice used in this study were generated from an in-house breeding colony. The transgenic mouse model was originally developed in 2010 by Hajjar et al. for use in bacterial infectivity models (36). The humanized TLR-4 (hTLR-4) strain was established on a C57BL/6 background and is characterized by the expression of human sequence-specific TLR-4 proteins by innate immune cells. The strain was preserved by Jackson Laboratory (B6.Cg-Tg(LY96)7020Haj Tg(TLR4)5271Haj/J; stock no: 031051) and in 2018, a limited number of hTLR-4 mice became available for purchase from the vendor. Female mice bearing heterozygous expression of the hTLR-4 gene were purchased from this colony, along with wild-type C57BL/6 males (Jackson Laboratory, Bar Harbor, ME), to serve as the primary breeding pairs from which the in-house hTLR-4 mouse colony was derived.

Mice were mated at 6-10 weeks of age using standard procedures described in an existing breeding protocol approved for use in the National Institute for Occupational Safety and Health (NIOSH) Animal Facility (17-009). Several mating cycles were required to obtain an adequate number of age-matched animals for use in the studies. Pups were weaned at 5 weeks postnatal for all litters. At this time point, tail biopsies were taken from each pup and sent for genotypic analysis to Transnetyx, Inc. (Cordova, TN) to determine hTLR-4 status. In an effort to maximize colony output associated with the breeding colony, mice of both genders and genotypes were incorporated into the allergy studies.

Mice were housed 4 per cage (maximum) in polycarbonate ventilated cages with HEPA-filtered air according to genotype and gender. Mice were provided food (Harlan Teklad Rodent Diet 7913) and water ad libitum in a controlled humidity/temperature environment with a 12 h light/dark cycle in the AAALAC International-approved NIOSH Animal Facility until 10 weeks of age. All procedures in the subsequent studies comply with the ethical standards set forth by Animal Welfare Act and the Office of Laboratory Animal Welfare (OLAW). The studies were approved by the CDC/NIOSH Health Effects Laboratory Division Institutional Animal Care and Use Committee in accordance with approved animal protocols (13-SA-M-022, 18-001).

Local Lymph Node Assay (LLNA)

The LLNA study was performed in accordance with established protocols and minor modifications previously described by Anderson et al. (2007) (41, 42). Three dosing solutions containing $NiSO_4$ were prepared at concentrations of 2.5, 5.0, and 10% (w/v) in mineral oil. As shown in figure 1A, mice (n = 3-4 per group) were exposed topically to vehicle control (mineral oil) or$NiSO_4$ on the dorsal sides of both ears (25 µl dosing solution per ear) for three consecutive days (days 1, 2, and 3). Following two days of rest, on day 6, mice were injected intravenously via the lateral tail vein with 20 µCi tritiated ([^{3}H])-thymidine (Dupont NEN, specific activity 2 Ci/mmol). Five hours after the [^{3}H]-thymidine injection, mice were euthanized via CO_2 asphyxiation. The left and right auricular lymph nodes were excised from each mouse and processed between frosted microscope slides to yield a single cell suspension in phosphate-buffered saline (PBS). Cells were washed, resuspended in 5% trichloroacetic acid (TCA), incubated overnight at 4°C, then analyzed using a Packard Tri-Carb 2500TR liquid scintillation counter. Stimulation indices (SI) were then calculated by dividing the mean disintegrations per minute (DPM) for each test group by the mean DPM for the corresponding vehicle control group. Dermal sensitization in the LLNA is defined by an SI value of >3.

Assessment of Additional Immune Parameters Following Dermal Exposure to Nickel

In order to better characterize the immune responses associated with nickel sensitization in the hTLR-4 mouse model, another study was performed without radioactivity-based endpoints. As shown in figure 1B, hTLR-4 positive female and male mice were incorporated into the study (n = 5-10 per group). Mice were exposed to either vehicle control or 10% $NiSO_4$ using identical procedures as those employed for dosing in the LLNA study. After three consecutive days of exposure (days 1, 2, and 3), mice were rested for six days and euthanized on day 10 by CO_2 asphyxiation. Directly following euthanasia, the body cavity was opened, and blood was drawn from the abdominal aorta. For each animal, 100 µL of whole blood was dispensed into EDTA-coated tubes and placed on a roller until analyses were performed. The remaining fraction was collected into a serum separator tube. The auricular lymph nodes (right and left) were collected from each animal and placed in sterile phosphate-buffered saline (PBS) until processing. The spleen was harvested from each animal, weighed, and placed in sterile PBS until processing.

Phenotypic Analysis of Lymphoid Tissues

Following harvest, lymph nodes and spleens were processed between frosted microscope slides to yield single cell suspensions in sterile PBS. The total number of lymph node cells was evaluated for each animal using a Coulter Multisizer II (Coulter Electronics; Hialeah, FL). Phenotypic analysis was then performed on lymph node and spleen cells by flow cytometry.

For each sample, approximately 500,000 cells were plated and suspended in staining buffer (PBS with 1% bovine serum albumin and 0.1% sodium azide) containing Fc receptor blocking anti-mouse CD16/32 (BD Biosciences). Cells were incubated for 5 minutes, washed, and resuspended in staining buffer containing fluorochrome-conjugated antibodies. Lymph node and spleen cells were stained with a panel of fluorochrome-labeled antibodies for cell surface markers for lymphocyte subset differentiation. CD2-BV605, CD3-APC, CD4-FITC, CD8-PE, CD44-APC-R700, CD45-PerCP, CD45R(B220)-PE-Cy7, and CD86-BV421 were used to discriminate between populations of CD4+ T-lymphocytes, CD8+ T-lymphocytes, B-lymphocytes, and natural killer (NK) cells, as well as determine the corresponding activation state. Cells were incubated for 30 min, washed, and fixed in 100 µl Cytofix Buffer (BD Biosciences).

Compensation controls were prepared using corresponding cell types stained with a single fluorophore. For each sample, 100,000 events were recorded on an LSR II flow cytometer (BD Biosciences, San Diego, CA). In all analysis, doublet exclusion was performed and cellular populations were gated using FSC-A x SSC-A parameters prior to subsequent analysis. All data analysis was performed using FlowJo 7.6.5. Software (TreeStar Inc., Ashland, OR).

Circulating Leukocyte Cellular Differentials

The 100 µL aliquot of whole blood retained from each animal was used for differential analysis of circulating leukocyte populations using an IDEXX ProCyte Dx Hematology Analyzer (IDEXX Laboratories; Westbrook, ME). Total leukocyte number was determined for each sample, and leukocyte subsets were differentiated to yield absolute number of lymphocytes, monocytes, neutrophils, eosinophils, and basophils.

Serum Protein Analysis

The remaining volume of whole blood collected from each animal was aliquoted into a serum separation tube and centrifuged for 10 minutes at 3000 rpm to yield serum and stored at -20°C until analysis.

To quantify circulating total immunoglobulin (Ig)E levels, serum was diluted 1:10 and assessed by enzyme-linked immunosorbent assay (ELISA) using the Mouse IgE ELISA kit (Innovative Research; Novi, MI) according to manufacturer instructions. Upon completion of the assay, the samples were allowed to develop for 30 minutes and absorbance was determined at 450 nm using a Spectramax Vmax plate reader (Molecular Devices). The concentration of IgE in each sample was interpolated from a standard curve using multipoint analysis.

Concentrations of various cytokines were also assessed in the serum of mice. Cytokines were quantified using a Milliplex MAP Kit magnetic bead panel (EMD Millipore Corporation, Billerica, MA) and analyzed on a Luminex 200 system (Luminex Corporation, Austin, TX). The specific analytes of interest included a panel of T-helper (Th) type 1-associated cytokines (IL-2, IL-12, interferon [INF]-g) and several major Th2-related cytokines (IL-4, IL-5, IL-13). In addition, IL-6 and IL-10 levels were also assessed.