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Deficient Tumor Necrosis Factor-α Production in Lipoarabinomannan Activated Macrophages from Toll-like Receptor-4 Deficient Mice: Implication for Mycobacterial Susceptibility[1]

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ABSTRACT

Mice with a point mutation of toll-like receptor-4 (TLR-4) (C3H/HeJ) are hypo-responsive to LPS and more susceptible to *mycobacterial infections* than their control wild type (C3H/OuJ). We have previously shown that TLR-4-deficient mice produced NO in response to the mycobacterial product, ara-lipoarabinomannan (LAM), in the presence of either Interferon-beta (IFN-β) or Interferon-gamma (IFN-γ), with a dose response curve that produced levels of NO almost as high as those observed in C3H/OuJ mice at high concentrations of ara-LAM plus either IFN-β or -γ. We now report that tumor necrosis factor-alpha (TNF-α), an important cytokine for intracellular killing of mycobacteria, remains deficient in these C3H/HeJ mice compared to C3H/OuJ mice even at a high concentration of ara-LAM with either IFN-γ or IFN-β. In addition, TNF-α was further down regulated by taurine chloramine (Tau-Cl) in C3H/OuJ mice. The low level of TNF-α produced in the TLR-4-deficient (C3H/HeJ) mice was also further down regulated by Tau-Cl. These findings implicate the TLR-4 as an additional candidate locus for mycobacterial susceptibility, and provide a pathway for better understanding the molecular basis of this locus in the immunopathogenesis of mycobacterial infection.

RÉSUMÉ

Les souris consanguines mutantes de souche C3H/HeJ présentent une mutation ponctuelle du récepteur N°4 analogue au récepteur « Toll » (TLR-4), ne répondent pas bien à un test endotoxémique au lipopolysaccharide (LPS) et sont plus susceptibles à une infection mycobactérienne que leurs homologues témoins (C3H/OuJ). Nous avons auparavant montré que les souris déficientes en TLR-4 étaient capables de produire du monoxyde d'azote (NO),

en présence soit d'interféron β (IFN-β), soit d'interféron γ (IFN-γ), lorsque stimulées par l'extrait de mycobactéries de type ara-lipoarabinomannane (LAM), avec une courbe dose-réponse montrant des niveaux de production de NO presque aussi élevés que les souris C3H/OuJ à haute concentration de ara-LAM avec soit de l'IFN-β, soit de l'IFN-γ. Nous rapportons ici que la production de facteur α de nécrose tumorale (TNF-α), une cytokine importante pour la destruction intracellulaire des mycobactéries, est déficiente chez cette souche de souris C3H/HeJ, lorsque comparée à celle de la souche C3H/OuJ, même à des concentrations élevées de ara-LAM avec soit de l'IFN- β, soit de l'IFN-γ. De plus, TNF-α étant inhibé par la taurine chloramine (Tau-Cl) chez les souris C3H/OuJ, le faible niveau de TNF-α produit par les souris déficientes en récepteur TLR-4 (C3H/HeJ) était encore plus inhibé par Tau-Cl. Ces résultats confirment TLR-4 comme un candidat pour un locus additionnel de susceptibilité aux mycobactéries et montrent une piste permettant de mieux comprendre les bases moléculaires de ce locus dans l'immuno-pathogénie des infections à mycobactéries.

RESUMEN

Los ratones con una mutación en punto para el receptor toll-like 4 (TLR-4) (C3H/HeJ) son hipo-respondedores al LPS y más susceptibles a las infecciones micobacterianas que sus contrapartes no mutadas (C3H/OuJ). En experimentos previos hemos demostrado que los ratones deficientes en TLR-4 producen NO en respuesta a la ara-lipoarabinomanana (ara-LAM) bacteriana, en presencia de interferón beta (IFN-β) o interferón gamma (IFN-γ), con una curva de dosis-respuesta similar a la observada en los ratones C3H/OuJ estimulados con una alta concentración de ara-LAM más IFN-β o IFN-γ. Ahora reportamos que el factor de necrosis tumoral alfa (TNF-α), una citocina importante para la muerte intracelular de las micobacterias, permanece deficiente en los animales C3H/HeJ en comparación con los ratones C3H/OuJ, aún en presencia de una alta concentración de ara-LAM e IFN-β o IFN-γ. La cloramina de la taurina (Tau-Cl) redujo considerablemente la expresión de TNFα en los ratones C3H/OuJ, y suprimió todavía más, el bajo nivel de esta citocina en los ratones deficientes en TLR-4 (C3H/HeJ). Estos resultados implican a TLR-4 como un candidato de un locus de susceptibilidad adicional a la infección por micobacterias.

TNF-α production is known to be important in killing intracellular microbes such as mycobacteria ([2, 4, 5, 8, 9, 13, 34, 44]). Indeed, TNF-α receptor knock-out mice are significantly more susceptible to mycobacterial challenge ([4]). While almost all cell types can produce TNF-α in response to appropriate stimuli, the macrophage is among the highest producers of TNF-α ([34]). The major stimuli for macrophage TNF-α production are LPS or the mycobacterial equivalent lipoarabinomannan (LAM) ([46]). Indeed, the IL-12 and IFN-γ pathway appears to be the dominant protective pathway against mycobacteria and bacteria in general ([7]), whereas IFN-α or β are dominant for viral infection. Toll-like receptors, including 10 human homologs, have been described ([29, 30, 32]). The toll receptors are recognized as being the initial recognition of pathogen associated molecular pattern (PAMPS) and initiate subsequent signal transduction that determine the ultimate fate of infection into either tuberculoid (TH1) or lepromatous (TH2) leprosy ([29, 30, 32]). The adapter proteins or other signaling molecules that de-termine the TH1 or TH2 fate, including the possibility of additional endogenous ligands, are currently an area of investigative research.

We have hypothesized that taurine, a sulfur containing amino acid present in animals, but present in plants, which lack an adaptive immune system, plays a pivotal role in the TH1-TH2 decision. Taurine is essential to the development and survival of mammalian cells and is the most abundant amino acid in leukocytes (20–50 mM) ([40]). Taurine protects tissues from damage caused from the inflammatory response in a variety of models. Our previous studies have shown that Tau-Cl, formed from taurine and halide-dependent myeloperoxidase ([17, 25]), inhibits production of NO, TNF-α, and prostaglandin E_2 (PGE^2) by macrophages activated by LPS and IFN-γ ([38, 40, 41, 43]). In addition, production of NO and TNF-α in a LAM-activated macrophage cell line was also suppressed by Tau-Cl ([46]). Our previous data show that taurine can protect against oxidant-induced injury through the formation of taurine chloramine (Tau-Cl)

produced by myeloperoxidase from activated polymorphonuclear leukocytes (PMNs) and taurine ([45]). In addition, Tau-Cl down regulates the production of NO and TNF-α in both LPS and LAM activated macrophage cell lines ([40, 46]).

In the current study, we report a deficiency of TNF-α production in response to mycobacterial ara-LAM with both IFN-γ and β in TLR-4 deficient mice (C3H/HeJ). This finding has implications for the role of TLR-4 deficiency in human leprosy, tuberculosis, and opportunistic mycobacterial infections.

MATERIALS AND METHODS

Animals. C3H/OuJ (LPS-high responder, *lps^n*) and C3H/HeJ (LPS-low responder, *lps^d*, TLR-4 deficient) female mice (8–12 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.). Animals were kept with free access to food and water.

Reagents. Ara-LAM (*Mycobacterium* sp. Rapid growing or *Mycobacterium tuberculosis* H37Ra) was kindly provided by Dr. John T. Belisle (Colorado State University, Fort Collins, CO, U.S.A.). Recombinant murine IFN-γ, RPMI 1640, HBSS without Ca^{2+} and Mg^{2+}, fetal calf serum (FCS), penicillin, streptomycin, and glutamine were purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). IFN-β was obtained from Lee Biomolecular Research Laboratories, Inc. (San Diego, CA, U.S.A.). The endotoxin content was measured by Limulus test kits (Biowhittaker, Inc.; Walkersville, MD, U.S.A.; and Associates of Cape Cod, Inc., Woods Hole, MA, U.S.A.). Dulbecco's minimal essential medium (DMEM) and taurine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and sodium hypochlorite (NaOCl) was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Tau-Cl was synthesized and monitored in our laboratory as previously described ([40]).

Cell culture preparation. Murine peritoneal exudate cells were collected from the abdominal cavity with Ca^{++}, Mg^{++} free HBSS 4 days after 1 ml of an intraperitoneal injection of 5% thioglycollate broth (Difco, MI). The NO assay was conducted using complete medium: DMEM, 10% FCS, 2 mM glutamine, penicillin, and streptomycin without phenol red. Cells were washed with complete medium and 2 × 10^5 cells/well were used in 96 well plates (Corning Glass Works, Corning, NY, U.S.A.). After overnight adherence in 5% CO_2, nonadherent cells were removed. Differential staining using Diff-Quik (American Scientific Products, McGraw Hill, Illinois, U.S.A.) confirmed the remaining cells were macrophages. Stimulators included either LPS (10 μgm/ml) or LAM (10 μgm/ml) in combination with rIFN-γ (50 U/ml) or rIFN-β (1000 U/ml). After 24 hr, 50 μl of supernatant was removed from each well for TNF-α determination.

TNF-α measurement by ELISA. TNF-α secretion was measured by ELISA as described previously ([38, 41]). Briefly, 50 μl of hamster anti-mouse TNF-α monoclonal antibody (R&D Systems, Minneapolis, MN, U.S.A.), diluted 1:500 in carbonate buffer (pH 9.6), was incubated in a 96 well ELISA plate (Thermolab Inc., Chantilly, VA, U.S.A.) overnight at 4°C. After washing the plate, 2% BSA in PBS-tween 20 (T-PBS, 0.1 M PBS with 0.05% between) was added and the plate was incubated at 37°C for 2 hr. The plate was washed with T-PBS and 50 μl of either various concentrations of standard murine rTNF-α or diluted samples were added to the plates. After incubation at 4°C overnight and washing with T-PBS, 50 μl of rabbit anti-mouse TNF-α polyclonal antibody, diluted 1:500 in T-PBS was added and the plate was incubated at 37°C for 1.5 hr. After washing, the plate was incubated with 50 μl of goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Zymed Laboratories, San Francisco, CA, U.S.A.) at 1:5000 dilution for 1 hr. The color was developed with 0-phenylenediamine dihydrochloride and H2O2 in citrate buffer (pH 5.0). The plate was read at 490 nm by MR 600 microplate reader (Thermolab Inc.). The concentration of TNF-α released into the medium was calculated by using murine rTNF-α as a standard.

Nitrite measurement. Nitrite (NO_2^-) concentration was measured in 100 μl aliquots of conditioned medium from the 96 well plates by incubating with an equal volume of Greiss reagent (1% sulfanilamide +0.1% napthylethylene diamine dihydrochloride +2.5% phosphoric acid) at room temperature for 10 min in a microplate as previously described ([39, 40]).
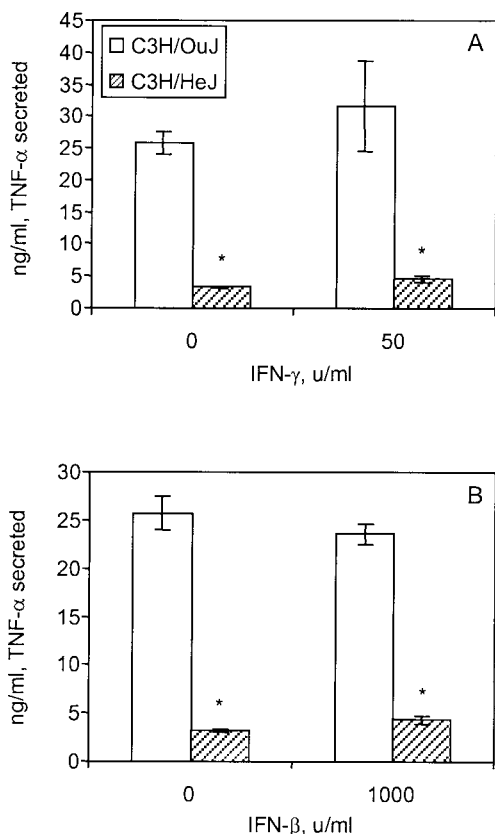
FIG. 1. TNF-α production induced by LPS (10 μg/ml) plus IFN-γ (**A**) and IFN-β (**B**) in peritoneal macrophages from high-responder mice (C3H/OuJ, □) and low-responder mice (C3H/HeJ, ▨). The control value in C3H/OuJ was 0.2–2 ng/ml and in C3H/HeJ was 0 ng/ml. Data is mean ± S.D. of triplicate samples. This experiment is representative of three independent experiments. Thioglycollate-elicited peritoneal macrophages were activated for 24 hours.

FIG. 2. TNF-α production induced by LAM (10 μg/ml) plus IFN-γ (**A**) and IFN-β (**B**) in peritoneal macrophages from high-responder mice (C3H/OuJ, □) and low-responder mice (C3H/HeJ, ▨). The control value in C3H/OuJ was 0.2–2 ng/ml and in C3H/HeJ was 0 ng/ml. Data is mean ± S.D. of triplicate samples. This experiment is representative of three independent experiments. Thioglycollate-elicited peritoneal macrophages were activated for 24 hours. TNF-α was measured using ELISA.

Statistical analysis. Differences between groups were determined by Student's unpaired *t*-test and analysis of variance techniques. To control for multiple group comparisons, the Scheffe pairwise method was employed. Both statistical tests resulted in the same group differences at the level of p <0.05.

RESULTS

TNF-α was measured by ELISA in the supernatant of LPS plus IFN-γ and -β stimulated peritoneal exudate macrophages in both C3H/OuJ and C3H/HeJ mice. Significantly lower (p <0.001) levels of TNF-α were detected from the macrophages of toll receptor deficient C3H/HeJ mice when activated with LPS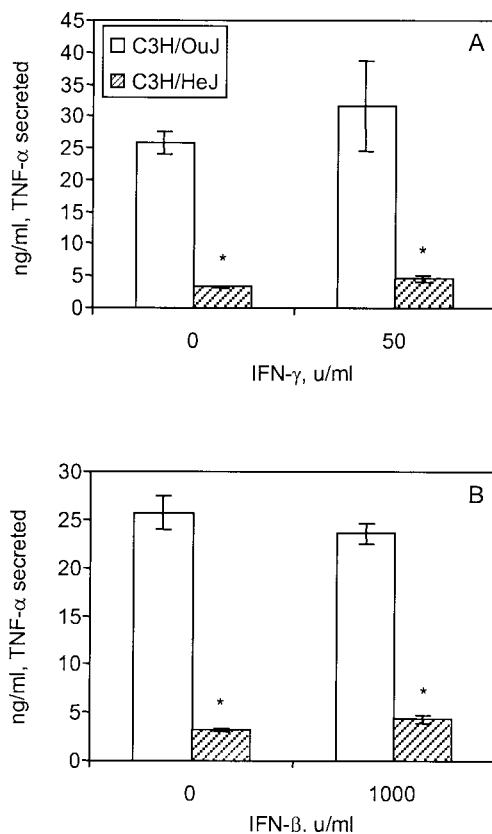 or LAM at all concentrations of both IFN-γ and IFN-β (Figs. 1A, B and 2A, B). IFN-β and IFN-γ did not augment the production of TNF-α in either LAM or LPS activated macrophages from either C3H/OuJ or C3H/HeJ mice (Figs. 1 and 2). However, as a control, parallel experiments demonstrated that IFN-β or IFN-γ augmented production of NO in LAM and LPS activated macrophages in either strain (see reference 39). IFN-β or IFN-γ alone (without LPS or LAM) did not stimulate production of TNF-α in either strain of mice (data not shown). Parallel experiments demonstrated that NO was produced in response to IFN-β or IFN-γ alone (C3H/HeJ, 0–2 nmoles/well and C3H/OuJ, 0.5–4 nmoles/well).

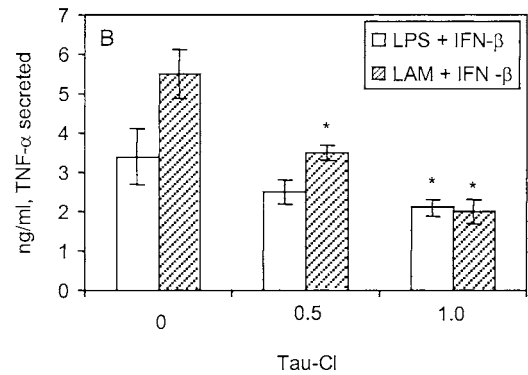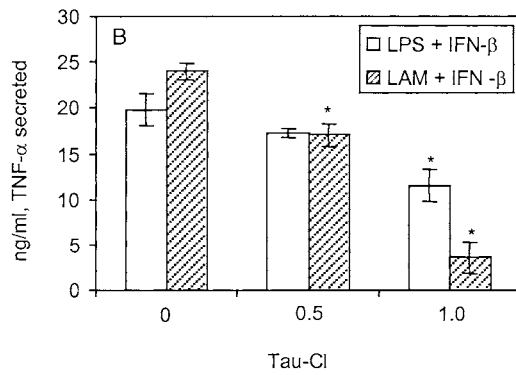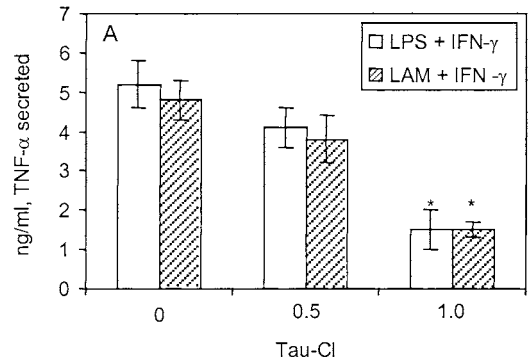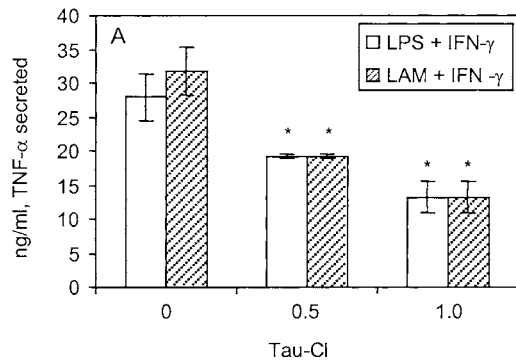FIG. 3.   Inhibition of TNF-α production by Tau-Cl in activated peritoneal macrophages from high-responder macrophages. (**A**) Macrophages were activated by LPS (10 µg/ml) or LAM (10 µg/ml) plus IFN-γ (50 U/ml) or (**B**) macrophages were activated by LPS (10 µg/ml) or LAM (10 µg/ml) plus IFN-β (1000 U/ml). The data are represented as mean ± S.D. of triplicate samples. This experiment is representative of three independent experiments.

FIG. 4.   Inhibition of TNF-α production by Tau-Cl in activated peritoneal macrophages from low-responder macrophages. (**A**) Macrophages were activated by LPS (10 µg/ml) or LAM (10 µg/ml) plus IFN-γ (50 U/ml). (**B**) Macrophages were activated by LPS (10 µg/ml) or LAM (10 µg/ml) plus IFN-β (1000 U/ml). The data are represented as mean ± S.D. of triplicate samples. This experiment is representative of three independent experiments.

Addition of Tau-Cl (1.0 mM) significantly reduced ($p < 0.05$) TNF-α production in the LPS and LAM activated macrophages of both the high responder C3H/OuJ and low responder C3H/HeJ mice. At 0.5 mM, Tau-Cl significantly decreased TNF-α with LAM and either IFN-γ or -β activated macrophages in both strains of mice, but did not change TNF-α production in C3H/HeJ macrophages activated with LPS and IFN-γ.

DISCUSSION

Our data show a reduced ability of peritoneal exudate cells of C3H/HeJ, TLR-4-deficient mice to produce TNF-α in response to both LPS and ara-LAM regardless of whether or not IFN-γ or IFN-β were added. These experiments were performed with simultaneous activation of macrophages with IFN-γ or -β plus LPS or LAM. Our data do not show macrophage augmentation with IFN-γ or IFN-β plus LPS or LAM. Other protocols, which pretreat macrophages with IFN-γ or -β prior to LPS or LAM activation, do show a synergistic effect. These differences are likely due to simultaneous addition of IFNs as opposed to step-wise priming.

The reduction of TNF-α in TLR-4-deficient mice was greater than our previously reported reduction in nitric oxide (NO) ([39]). Previous studies have shown the TLR-2 receptor to be an important determinant of susceptibility to mycobacteria

([23, 27, 53]). As C3H/HeJ mice have a point mutation of TLR-4 and are known to have a higher lethality and greater susceptibility to challenge with mycobacteria ([18, 26]), TLR-4 is also likely to play a role in mycobacterial susceptibility. Our finding of a diminished production of TNF-α in response to ara-LAM, even in the presence of IFN-γ or IFN-β, indicates a possible mechanism for increased levels of mycobacterial susceptibility in TLR-4-deficient (C3H/HeJ) mice. This is in keeping with studies of Netterman, *et al.* ([36]) who showed defective TNF-α synthesis in TLR-4-deficient mouse macrophages activated with a secondary cross-linking antibody. Means, *et al.* ([28]) showed that live *M. tuberculosis* (Mtb) activated macrophages via both TLR-2 and TLR-4. Subsequently, Heldwein, *et al.* ([18]) found that the induction of TNF-α secretion by Mtb-stimulated macrophages is mediated by these TLR proteins, although predominately by TLR-4, in keeping with our findings.

Other recent studies implicate toll-like receptors in mammalian mycobacterial diseases ([11, 12, 51]). Toshchakov, *et al.* ([52]) showed that TLR-4 mediates IFN-β induced Stat1/alpha/beta gene expression in macrophages in a TIRAP (toll- interleukin 1 receptor domain containing adapter protein) dependent manner. Kobayashi, *et al.* ([22]) have recently identified a caspase recruitment domain that is downstream of both TLR2 and TLR4 and is also required for optimal T-cell receptor signaling. Finally, Supajatura, *et al.* ([48]) showed that mast cells that have both TRL-2 and TLR-4 produced less TNF-α and 1L-1β in response to the TLR-2 against peptidoglycan, even though TNF-α and 1L-1β were the predominant cytokines produced by mast cells with LPS stimulation via TLR-4 ([49]). In our previously published study ([46]) all reagents such as IFN-β and IFN-γ stimulated strong NO production, but in these studies they elicited only low levels of TNF-α shown in the present study.

The resulting TNF-α deficiency in TLR-4 deficient macrophages could play a role at the level of mycobactericidal activity, since TNF-α is known to be important in killing and retarding the growth of mycobacteria ([5, 24]). TNF-α is also important in initiating macrophage apoptosis ([21, 33]) which is known to be important and beneficial to the host for both mycobacterial and salmonella infections ([57]). We have previously shown that supernatants of mitogen- and antigen-activated leukocyte cultures from tuberculoid but not lepromatous patients inhibit the growth of *Legionella pneumophila* ([19]). Yoshida, *et al.* ([56]) have shown the Tlr 4 locus to be the dominant locus controlling natural resistance of murine macrophages to *L. pneumophila.*

In the afferent limb of the immune response, TLR-4 and TLR-2 have been found predominantly on the dendritic cell type (DC1) ([20]), the antigen-presenting cell most necessary for the mycobacterial protective TH1 response ([14, 28, 31, 32, 34, 35, 37, 47, 54]). While the above discussion may explain some of the mechanisms that could predispose C3H/HeJ, TLR-4-deficient mice to mycobacterial infections, a similar defect in humans could have an even greater effect on mycobaterial susceptibility. TNF-α plays a major role in mycobactericidal activity in human macrophages, and significant controversy exists concerning the role of NO in intracellular-killing in human macrophages ([10]). This study has identified a defect in TNF-α production in response to the non-pathogenic ara-LAM. Future studies should take advantage of the availability of additional cell wall, membrane, and cytosolic components from pathogenic mycobacteria.

Our previously published data demonstrated that Tau-Cl significantly inhibited NO production when macrophages from C3H/HeJ and C3H/OuJ were activated with IFN-γ or IFN-β in addition to LPS and LAM ([39]). We have also shown that Tau-Cl inhibits iNOS at the transcriptional level, but inhibits TNF-α more modestly at a post-translational level ([41]). We have hypothesized that Tau-Cl, a product of taurine, hypochlorous acid, and myeloperoxidase ([55]) may be a mechanism to protect against tissue destruction during mycobacterial infection ([46]). The biological significance of Tau-C1 may be inhibition of oxidant-induced injury by inhibiting production of proinflammatory cytokines ([15, 16]). Taurine is in murine and human leukocytes at mM concentrations, whereas other free amino acids are in the μM level ([40]). The role of taurine in inflammation of both innate and acquired immunity may be far-reaching. Recent studies indicate that Tau-C1 inhibits the

NF-κB pathway which is also regulated via Toll-like receptors, and is known to be important in host pathogen interactions ([3, 50]).

Evidence indicates the human homologue of C3H/HeJ, TLR-4 deficient mice is quite common, on the order of 3.3% to 7.9% of the population, as defined by hyporesponsiveness to inhaled LPS ([1]). While the identified mutations in humans are in the extracellular domain of TLR-4 rather than the intracellular domain as described in the C3H/HeJ mice ([42]), the TLR-4 in addition to TLR-2 should be considered a candidate susceptibility-locus for future studies in leprosy, tuberculosis, and atypical mycobacterial infections. Genetic advances in understanding complex diseases and the recent discovery of 12 rare amino acid variants in the human TLR- 4 ([6, 47]) should allow testing the role of this receptor in mycobacterial diseases.

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