Dioxin Inhibits Toll-like Receptor Expression and TLR Activation of TNF-α Production.

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Introduction

2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD), most commonly known as dioxin, is a member of a large family of halogenated aromatic hydrocarbons (HAH) that include polychlorinated and polybrominated biphenyls (PCB and PBB), polychlorinated dibenzofurans and dioxins, (CDF and PCDD) respectively. Many people harbor traces of dioxins and related compounds as a result of exposure to pesticides in their diet or airborne dioxins released by certain type waste incineration. These environmental chemicals have raised much public concern because of their toxicity to the immune system and resulting alterations in host susceptibility to infections.[1-7.] While an increased in susceptibility to infection has been widely reported, the mechanisms by which dioxin increases host susceptibility to bacterial, viral, and parasitic infection are poorly understood.

The Toll-like receptors are a class of evolutionarily conserved transmembrane receptors that are critically involved in host defense.[8] They serve to identify conserved molecular patterns on microbial pathogens, which enables the innate immune system to recognize invading organisms and to induce protective immune responses.[8,9] At present, 11 TLR proteins have been identified in humans.[8] It has been demonstrated that TLR-2 deficient mice are highly susceptible to infections caused Gram-positive organisms where as, TLR-4 null animals are highly susceptible to Gram-negative bacterial infections and exhibit reduced secretion of cytokines.[8]

Common to all TLRs is a complex signaling pathway.[10] The family of TLRs utilizes an adaptor protein, MyD88 in the initial signaling step. MyD88 then recruits members of the IL-1 receptor-associated kinase (IRAK) family, which recruits TRAF6. TRAF6 initiates the cytoplasmic P-38 and ERK-1 signaling molecules, and NF-κB which leads to activation of transcription genes, resulting in expression of pro-inflammatory cytokines.[10]

Currently, little is known about the effect of dioxin on the expression and function of TLRs. In this study we tested the hypothesis that dioxin increases host susceptibility to infection by modulating the expression and function of the Toll-like receptors.

Materials and Methods

Cells: Pro-monocytic, U937 cells, were obtained from ATCC, Manassas, Virginia and were maintained in RPMI-1640 medium supplemented with, 10% Fetal Bovine Serum, 10% Penicillin, Streptomycin and 2mM L-Glutamine.

Cell cultures: U937 cells were cultured in the presence or absence of various concentrations of dioxin (concentration range1-100 ng/ml). At different time intervals (8, 24, 48 hours post exposure) the cells were stained with phycoerythrin (PE) labeled monoclonal antibodies against TLR-2, TLR-4, and CD-14 and isotype control antibody (e-biosciences, San Diego, CA) The Fluorescence signals from labeled cells were analyzed using FACScan (Becton-Dickenson, San Jose, California) . Forward and side scatters were used to gate and exclude cellular debris. Results were recorded for 10,000 cells. Receptor density and percent positive cell were determined. Statistical analysis was performed by the Kolmogorov-Smirnov test using Cell Quest Software System (Becton-Dickinson, Menlo Park, CA). A “D” value of >0.2 is considered statistically significant**.**

Detection of activated P-38, ERK and intracellular TNF: U937 cells were incubated with or without dioxin (1ng/ml, 10ng/ml, 50ng/ml, and 100ng/ml) for six days. Cells were then stimulated with ligands for TLR-2 (PamCys3) and TLR-4 (LPS) for 30 and 60 minuets. Following stimulation, cells were permeabilized and fixed in 2% PFA and stored at -20˚C in methanol until ready to be stained. Cells were stained with FITC labeled monoclonal antibodies against P-38, ERK-1 and isotype control antibody as well as TNF-α and isotype control antibody. The stained cells were

analyzed by FACScan flow cytometer and receptor density and percent positive cells was determined.The Fluorescence signals from labeled cells were analyzed using FACScan (Becton-Dickenson, San Jose, California). Results were recorded for 10,000 cells. Statistical analysis was performed by the Kolmogorov-Smirnov test using Cell Quest Software System (Becton-Dickinson, Menlo Park, CA). A "D" value of >0.2 is considered statistically significant**.**

Results and Discussion

The effect of dioxin on TLR 2 and TLR 4 expression are shown in figure 1 and figure 2. Dioxin, in a concentration dependent manner, inhibited the expression of TLR-2 and TLR-4. Maximal inhibition of TLR expression was observed at a concentration of 50 ng/ml of dioxin.

To determine the exposure time required for dioxin to exert its effect, U937 cells were cultured in the presence or absence of 50ng/ml and 100ng/ml of dioxin for 8, 24 and 48 hours and the expression of TLRs was determined. The results are shown in figures 3 and figure 4. U937 cells exposed to dioxin for a brief period of time (8 hr) showed no effect on the expression of TLR. In contrast cells exposed to dioxin for 24 and 48 hrs showed significant inhibition of TLR expression. These results suggest that the inhibitory effect of dioxin on TLR expression is dependent on the length of exposure.

Upon recognition of their cognate ligands, TLRs activate MAP Kinase signaling pathway which subsequently leads to activation of transcription factors such as NFκB. Transcription factors induce the expression of cytokines, such as TNF-α. In order to determine whether the decreased expression of TLRs was associated with altered TLR signaling and gene expression, we first examined the ability of dioxin treated cells to produce TNF-α in response to TLR ligands. Figure 5 shows that as compared to controls, dioxin treated cell produce decreased levels of TNF-α in response to LPS, a TLR-4 agonist. Similar results were also observed when the dioxin treated cells were activated with peptidoglycan, a TLR-2 agonist. To determine the molecular mechanisms, we examined the effect of dioxin on the activation (phosphorylation) of MAP kinases P-38 and P-ERK1. U937 cells that were exposed to dioxin were stimulated with ligands for TLR-2 (Pam3Cys) and TLR-4 (LPS) for 30 and 60 minuets and activation of MAPK pathway was determined by flow cytometry using antibodies against phospho-P-38 and phospho-P-ERK1. Figure 6 and 7 show that cells exposed to dioxin exhibit decreased activation of P-38 and P-ERK1.

The results presented herein demonstrate that dioxin inhibited the expression of Toll-like receptor 2 and 4 and it is associate with decreased TLR signaling and activation of TNF-α gene expression. Our results suggest that dioxin increases host susceptibility to infections by modulating the expression and function of TLRs.

The TLR mediated signaling cascade converges to activate MAPK and NFκB pathways which induce transcription of cytokine genes which are involved in the immune and inflammatory response. In this study we showed that dioxin treated cells had lower levels of P-38 and P-ERK1 activation than control samples. Moreover, a decreased level of MAP kinase activation was associated with a decreased production of TNF-α, which is a central immunoregulatory molecule.

The mechanisms by which dioxin inhibits the expression of TLR and interferes with MAPK signaling events remains to be determined. Dioxin toxic effects are primarily mediatedby the aryl hydrocarbon receptor (AhR), a ligand-activated basic-helix-loop-helixtranscription factor that governs the expression of distinct sets of genes. Recent evidence shows that the Ah receptor physically interacts with a number of other transcription factors including NF-κB, a key transcription factor that regulates immune cell function and inflammation and modulates their activities. It is likely that dioxin represses TLR expression by inhibiting the function of transcription factor that participate in TLR gene expression.

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