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## Immunomodulation and Influenza A Infection

The immune system provides a necessary response to infectious agents but is not always successful in eliminating the threat posed by such organisms. Although some viruses are directly cytopathic and cause cell injury or death others cause cytokine dysregulation resulting in overwhelming inflammatory processes which can be more destructive than the virus itself and lead to death. Such is the case with the Influenza A (H5N1) virus, a purely avian influenza virus which was introduced into the human population of Hong Kong in 1997 causing a case-fatality rate of 33% primarily due to pneumonia as a result of cytokine dysregulation<sup>1,2</sup>. Not only did H5N1/97 cause the upregulation of TNF- $\alpha$  transcription but also the level of its secretion in macrophages<sup>3</sup>. The macrophage production and release of TNF- $\alpha$  induces a cascade of chemotactic cytokines and chemokines resulting in the accumulation of neutrophils in the lungs leading to an acute respiratory distress syndrome and ultimately multiple organ dysfunction. Such cytokines and chemokines include IL-12, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES. In addition to the induction of TNF- $\alpha$ , influenza viruses also cause an increase in other inflammatory cytokines such as IL1 $\alpha$ , IL1 $\beta$ , IL6, IFN- $\gamma$  and IL8-like chemokine (KC) which contribute to the recruitment and activation of virus-specific T-cells and immune response especially in the lungs<sup>4</sup>. These inflammatory cytokines also lead to the activation of inducible isoform of the nitric oxide synthase enzyme (iNOS) which produces nitric oxide (NO) in response to infection<sup>5,6</sup>. Its combination with O<sub>2</sub><sup>-</sup>, an oxygen free radical which is also produced in response to infection, results in the formation of the more reactive peroxynitrite (ONOO<sup>-</sup>) which leads to even greater nitritative stress facilitating viral pathogenesis or virulence. Virulence has also been defined by the ability of certain reassortant viruses to depress the expression of IL-10, an anti-inflammatory cytokine which results in a cytokine imbalance and a more intense inflammatory response (Lipatov AS). And given the fact that a mutation in a non-

structural gene (NS1) in the H5N1/97 virus leads to resistance of the anti-viral effects of TNF- $\alpha$  and the interferons points to the greater virulence of this viral strain <sup>7</sup>.

Although there is a high degree of homology between the human H5N1 HK97 virus and the representative chicken virus, CkHk97, all the genes of the virus causing disease were of avian origin and not the result of reassortment with between the human and avian strains of the virus (Claas EC, 1998). Yet not all Influenza A reassortant viruses cause the same pathogenicity in terms of cytokine and chemokine overproduction (Lipatov AS, 2005). However, the great concern is that humans will become the “mixing vessel” for the two viruses especially since the H5N1 viruses are actively reassorting and crossing interspecies-host barriers leading to a devastating pandemic <sup>8, 9</sup>.

In the immune system, the endocannabinoid system is defined by the presence of endogenous ligands to the CB1 and CB2 cannabinoid receptors, members of the G protein-coupled receptor family which are primarily responsible for attenuating but not preempting an immune response. It has been established that both endogenous and exogenous ligands for the CB2 receptor are immunomodulators leading primarily to a downregulation of Th1 cytokines and chemokines and an upregulation of Th2 cytokines which are antiinflammatory <sup>10</sup>. A study involving both occasional and regular use of smoked marijuana showed that IL-2 was downregulated while IL-10, a Th2 cytokine and TGF- $\beta$ , an immunosuppressive cytokine, were upregulated <sup>11</sup>.

The rank order of expression of CB2 receptors in immune cells is B cells > natural killer cells >> monocytes > PMNs > T8 cells > T4 cells suggests that their increased presence on antigen presenting cells and NK cells plays a significant role in their interaction with lymphocytes <sup>12</sup>. In fact, activation of CB2 receptors on macrophages by a synthetic ligand resulted in the sustained activation of ERK1/2 MAP kinase and enhanced biosynthesis of LPS-induced IL-10 both of which resulted in the inhibition of IL12p40; IL-12 polarizes the immune system toward a Th-1 response especially for protection against intracellular organisms such as viruses <sup>13</sup>.

It has been well documented that cannabinoid receptor ligands result in the reduction of bronchopulmonary inflammation induced by the inhalation of the bacterial endotoxin, LPS <sup>14</sup>. WIN 55,212-2, a non-selective, cannabinoid receptor agonist, and anandamide, an endocannabinoid potently reduced neutrophil recruitment by 59% and 62% respectively. Likewise, the endocannabinoids, the plant derived cannabinoid,  $\Delta^9$ -THC and the aminoalkyl indole derivative, WIN 55,212-2, all decreased the expression of TNF- $\alpha$  in a dose dependent manner. However, this is a concentration and time dependent phenomenon. If the cells were treated simultaneously during the induction period with LPS much higher concentrations of the drug were required to minimally suppress maturation and secretion of TNF- $\alpha$ . Likewise, when macrophages were maintained in the

presence of the drug for 48 hours, considerable amounts of presecretory TNF- $\alpha$  as well as its intermediates remained in treated cells<sup>15</sup>. One mechanism by which THC affects the expression of TNF- $\alpha$  is through inhibition of the conversion of its presecretory 29kDa precursor to the secretory, extracellular mature, 17 kDa protein<sup>16</sup>. When macrophages were pre-treated with the drug as opposed to when the cells were treated at the time of induction with LPS, IFN- $\gamma$  and BSA or FBS significantly lower concentrations were required to suppress the mature, secretory form of TNF- $\alpha$ . In an earlier paper Berdyshev et al. also demonstrated that although ligands did not affect the expression of the p55 TNF- $\alpha$  soluble receptors, they did inhibit the secretion of p75 TNF- $\alpha$  soluble receptors which could enhance the ability to regulate TNF- $\alpha$  activity<sup>17</sup>. Although it was not determined whether the THC inhibition of macrophage derived TNF- $\alpha$  is a CB2 mediated event, there is a strong correlation between the THC inhibition of IFN- $\gamma$  tyrosine phosphorylation of STAT1- $\alpha$  and TNF- $\alpha$  release by macrophages<sup>18,19</sup>. It has recently been established that CB2 receptor agonists inhibit the TNF- $\alpha$  activation of NF- $\kappa$ B, Rho A, the upregulation of ICAM-1, VCAM-1, monocyte endothelial adhesion and transendothelial migration of monocytic THP-1 cells<sup>20</sup>.

The anti-oxidant properties of the cannabinoids have been well described. When compared to cigarette smoke which increased the release of superoxide from macrophages during phagocytosis, marijuana smoke reduced its release by almost half even when compared to the control cells during phagocytosis<sup>21</sup>. These anti-oxidant effects are mediated by a non-receptor mediated pathway and are related to the phenolic structure of the classical cannabinoids since the aminoalkylindole derivative, WIN-55,212-2, did not prevent oxidative cell death in serum-deprived activated cells<sup>22</sup>. One of the pathways suggested by which these effects are obtained is through the inhibition of the redox-sensitive activation of NF- $\kappa$ B which is required for the expression of iNOS and the production of nitric oxide<sup>23</sup>. However, the endogenous ligand, 2-arachidonoyl-glycerol (2-AG), caused an increase in nitrite production in LPS stimulated macrophages<sup>24</sup>.

Research into anti-inflammatory potential of the cannabinoid class of drugs has been directed primarily at diseases mediated by injury, allergens and the immune dysfunction of autoimmune disorders<sup>25</sup>. Allergy and infectious disease processes share many common pathways in the lungs resulting in the influx of inflammatory cells and intraepithelial stored mucosubstances (IM)<sup>26</sup> which are greatly diminished by the treatment of  $\Delta^9$ -THC and cannabiniol in an ovalbumin sensitized mouse model<sup>27</sup>. This effect was mediated by the attenuation of IL-2, IL-5 and IL-13 mRNA expression in a CB2 dependent manner. Others have noted that the inhibition of macrophage migration is CB2 receptor dependent and that THC markedly reduced the expression of CCR2, the receptor for MCP-1, in splenocytes stimulated with TNF- $\alpha$ <sup>28</sup>.

## **Drug Development**

Since it has already been demonstrated that the classical cannabinoid structure acts as a platform for the development of ligands to the CB2 receptor as well as possessing anti-oxidant properties with its phenolic structure, it would be logical to use such a compound in experimental studies with known influenza strains to establish proof of concept. To date, Immugen Pharmaceuticals, Inc. (The Company) has been granted four patents related to a library of compounds which have been shown to possess anti-viral, anti-neoplastic and immunomodulatory properties<sup>29</sup>. The two lead compounds, L759656 and L759633, developed by MerckFrosst in 1996 for which no patents had been applied are now being claimed for their utility in continuation and CIP applications. These are classical cannabinoids, i.e. tricyclic phenolic compounds, which have extremely high affinity to the CB2 receptor at nano molar concentrations<sup>30, 31</sup>. It remains to be determined which non-receptor mediated activities are associated with the compounds. As with other cannabinoids, the drug should be highly soluble in beta-cyclodextrins and could be administered as a nasal aerosol spray or metered dose inhaler to the lungs; both routes would avoid the first pass effects through the liver although it was recently determined that both compounds are metabolically stable after being cultured in human liver microsomes for 120 minutes<sup>32</sup>. The highly lipophilic nature of the drugs would ensure a slow release from fat stores in the body which could result in every other day dosing<sup>33</sup>.

## **Experimental Design**

Since the host response to influenza virus determines the virulence of a particular strain<sup>34</sup> and that the substitution of glutamic acid for aspartic acid at position of 92 of the NS1 molecule is specifically responsible for the virulence of the H5N1 virus<sup>35</sup>, it will be necessary to evaluate the therapeutic potential of the drug in an established animal model, i.e. mouse with an appropriate influenza virus known to cause the observed morbidity and mortality such as the laboratory human virus strain A/ Puerto Rico/8/34 (H1N1), (PR/8), RecPR8-NS (H5N1/97) (Seo SH), the H5N1 human isolate A/Hong Kong/156/97 (HK/156/97) and the avian H5N1 virus A/Chicken/ Hong Kong/YU562/01 (Ck/HK/YU562/01) (Lipatov AS). Assays of the various cytokines and chemokines should also be undertaken. A separate study of the viral infection and treatment of macrophages would also be helpful in understanding further the effects of the drugs. Given the

urgency of the current situation regarding the pandemic basic toxicology studies in mice, dogs and pigs should also be carried out so that an investigational exemption for a new drug (IND) with the FDA prior to testing in human clinical trials can begin as soon as realistically possible.

### **Concluding Remarks**

Although the use of systemic steroids has proven useful for the treatment of a variety of viruses which cause acute airway disease from bronchiolitis to asthma it is probably contraindicated for the treatment of avian influenza<sup>36</sup>. However, unlike steroids, CB2 receptor ligands do not affect innate immunity, but rather modulate the immune response through signaling pathways which help restore homeostasis once a response has been initiated. It is, therefore, possible that the drug could be used prophylactically and therapeutically in communities which are being threatened by H5N1 to blunt a deleterious immune response.

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