Naltrexone inhibits IL-6 and TNFa production in human immune cell subsets following stimulation with ligands for intracellular Toll-like Receptors.

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Conflict of interest statement

The authors declare a potential conflict of interest and state it below

R. A and A.D. are listed as inventors on a patent that describes the use of Naltrexone as a TLR9 antagonist, which has been assigned to the Institute for Cancer Vaccines and Immunotherapy. R.C declares no competing financial interests.

Author contribution statement

A.D and R.A conceived the original idea for the study. R.C and R.A designed the experiments and prepared the manuscript. R.C performed experiments and analysed the data. All authors read and approved the manuscript.

Keywords

Toll-like receptor, Naltrexone, Interleukin-6, Tumour necrosis factor alpha, plasmacytoid dendritic cells, B cells, Monocytes

Abstract

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The opioid antagonist naltrexone hydrochloride has been suggested to be a potential therapy at low dosage for multiple inflammatory conditions and cancers. Little is known about the immune-modulating effects of naltrexone, but an effect on the activity of Toll-like receptor 4 (TLR4) has been reported. We analysed the effects of naltrexone hydrochloride on IL-6 secretion by peripheral blood mononuclear cells in vitro following stimulation with ligands for TLR4 and for the intracellular receptors TLR7, TLR8 and TLR9. Naltrexone did not affect cell viability or induce apoptosis of PBMC. Intracellular staining demonstrated that **naltrexone inhibited production of IL-6 and TNFα by monocyte and plasmacytoid dendritic cell subsets within the PBMC population** following treatment with ligands for TLR7/8 and TLR9 respectively. No effect of cytokine production by PBMC following stimulation of TLR4 was observed. Additionally, naltrexone inhibited IL-6 production in isolated monocytes and B cells after TLR7/8 and 9 stimulation respectively but no effect on IL-6 production in isolated monocytes after TLR4 stimulation was observed. These findings indicate that naltrexone has the potential to modulate the secretion of inflammatory cytokines in response to intracellular TLR activity, supporting the hypothesis that it may have potential for use as an immunomodulator.

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Ethics statements

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The opioid antagonist naltrexone hydrochloride has been suggested to be a potential therapy at low dosage for multiple inflammatory conditions and cancers. Little is known about the immune-modulating effects of naltrexone, but an effect on the activity of Toll-like receptor 4 (TLR4) has been reported. We analysed the effects of naltrexone hydrochloride on IL-6 secretion by peripheral blood mononuclear cells *in vitro* following stimulation with ligands for TLR4 and for the intracellular receptors TLR7, TLR8 and TLR9. Naltrexone did not affect cell viability or induce apoptosis of PBMC. Intracellular staining demonstrated that naltrexone inhibited production of IL-6 and TNFα by monocyte and plasmacytoid dendritic cell subsets within the PBMC population following treatment with ligands for TLR7/8 and TLR9 respectively. No effect of cytokine production by PBMC following stimulation of TLR4 was observed. Additionally, naltrexone inhibited IL-6 production in isolated monocytes and B cells after TLR7/8 and TLR9 stimulation respectively but no effect on IL-6 production in isolated monocytes after TLR4 stimulation was observed. These findings indicate that naltrexone has the potential to modulate the secretion of inflammatory cytokines in response to intracellular TLR activity, supporting the hypothesis that it may have potential for use as an immunomodulator.

Introduction

Naltrexone hydrochloride is an opioid antagonist used commonly in the treatment of opioid and alcohol dependence[1,2]. Naltrexone specifically inhibits the mu and, to a lesser extent, the delta opioid receptors[3], thus preventing the euphoric effects of alcohol or opioid. It has been suggested that treatment with Low Dose Naltrexone (LDN) may be beneficial for a range of inflammatory conditions, including Crohn's disease[4], Multiple Sclerosis (MS)[5] and fibromyalgia[6–8]. Reports also describe therapeutic effects of LDN in treatment for cancers including B cell lymphoma[9] and pancreatic cancer[10,11]. The molecular targets and potential immunomodulatory mechanism(s) of action for naltrexone in inflammatory conditions, however, require further investigation. Studies by Zagon *et al*, indicate that naltrexone can inhibit the non-canonical opioid growth factor receptor (OGFr), resulting in a decrease in cell proliferation[12–14]. Naltrexone and the related opioid antagonist naloxone have also been shown to inhibit the activity of a member of the Toll-like Receptor (TLR) family, TLR4, in an *in vitro* signalling assay and to reverse neuropathic pain in an animal model [15,16].

TLRs recognise conserved molecular patterns and nucleic acids as part of the innate immune response[17]. Eleven members of the TLR family have been described in humans and these vary in their cellular location; TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 and TLR11 are expressed on the cell surface, where they can detect components of extracellular pathogens and some self ligands, whilst TLR3, TLR7, TLR8 and TLR9 are located within endosomes where they respond to the presence of viral, bacterial and self nucleic acids [18]. TLRs also vary in their expression profile between immune cell subsets. For example, B cells express TLR1, TLR6, TLR7, and TLR9[19], whilst monocytes express TLR1, TLR2, TLR4, TLR7 and TLR8[19,20] and plasmacytoid dendritic cells express TLR7 and TLR9[19,21].

56   Although TLR play a key role in the initiation of immune responses to infection,

57   inappropriate TLR activity and/or recognition of self-ligands are associated with

58   inflammatory conditions and autoimmunity[22]. For example, increased expression of TLRs

59   has been observed in peripheral B cells from patients with inflammatory bowel disease [23]

60   while recognition of self-DNA complexes by TLR9 mediates pDC activation in psoriasis[24].

61   TLRs have also been implicated in the tumour microenvironment, with TLR activation linked

62   to angiogenesis, tumour proliferation and immune evasion[25]. Furthermore, some TLR

63   polymorphisms may be associated with development of inflammatory conditions such as

64   Crohn's disease[26,27]. TLRs have, therefore, been investigated as potential therapeutic targets

65   in patients with these diseases[28,29].

66

67   In this study, we sought to investigate the ability of naltrexone hydrochloride to inhibit the

68   effects of TLR-4 signalling in an immune context and to determine whether its inhibitory

69   effects extend to other members of the TLR family. Our results indicate that naltrexone can

70   inhibit production of the inflammatory cytokines IL-6 and TNFα by peripheral blood

71   mononuclear cells (PBMC) following stimulation with known ligands for TLR7, TLR8 and

72   TLR9 but not following stimulation with a TLR4 ligand. Although the interleukin 1 receptor

73   (IL-1R) shares the MyD88 signalling pathway with members of the TLR family, IL-6

74   secretion following IL-1R stimulation was not affected by naltrexone. Our findings also

75   indicate that naltrexone does not affect cell viability or induce apoptosis within the PBMC

76   population.

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Methods

Ethics statement

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University of London Research Ethics Committee (Protocol Approval SGREC15.0006). All

subjects gave written informed consent

87

Cell culture

PBMC were isolated from leukocyte cones (NHS Blood Donor Service) by density

centrifugation over Histopaque (Sigma Aldrich) according to the manufacturer's instructions.

PBMC and resuspended at a concentration of 10^6 PBMC/ml in RPMI-1640 (Sigma Aldrich)

supplemented with 10% fetal bovine serum (Sigma Aldrich), penicillin and streptomycin

(Sigma Aldrich). PBMC viability was assessed using trypan blue dye exclusion using the

BioRad TC20 Automated Cell Counter (BioRad). PMBC with a viability of above 90% were

used in assays. PBMC were plated onto 24 well plates and cultured at standard cell culture

conditions at 37^oC, 5% $CO_2.$

97

Cell stimulation

The following ligands were used to stimulate cells: 1ng/ml LPS-EB Ultrapure, 1µM CPG-

ODN 2395, 1µM R848 and 100ng/ml IL-1 (all Invivogen). For isolated B cell experiments

CD40R was crossed linked using 3µg/ml CD40-L (R&D Biosource) with anti-HA

monoclonal antibody (Sigma Aldrich) and 20ng/ml IL-4 (R&D Biosource). Lyophilized

ligands were resuspended in endotoxin free water as detailed in the manufacturer's

instructions. Ligands were further diluted in RPMI before being added to PBMC at the

concentrations stated. Naltrexone hydrochloride (Sigma Aldrich) was resuspended in

106 endotoxin free water and diluted in RPMI before being added to PBMC at the working

107 concentrations specified.

108

Isolation of CD14+ and CD19+ cells

110 Positive selection of CD14+ and CD19+ was performed by incubating PBMC with MACS

111 CD14+ and CD19+ microbeads in MACS buffer, according to the manufacturer's

112 instructions (Miltenyi Biotec). After incubation of cells and microbeads, cells were washed

113 with MACS buffer, resuspended in MACS buffer and loaded onto a MACS column attached

114 to a magnetic field of a MACS separator. After being washed with MACS buffer three times

115 the column was removed the magnetic field and the CD14+ and CD19+ cells were eluted

116 using MACS buffer (Miltenyi Biotec). Purity of above 90% was confirmed by flow

117 cytometry using CD14 VioBlue mIgG1 antibody and CD20 FITC mIgG1 antibody (Miltenyi

118 Biotec).

119

IL-6 ELISA

121 10^6 PBMC were stimulated with ligands and naltrexone as stated above for 24 hours before

122 cell-free supernatants were collected and IL-6 ELISA was performed using an IL-6 ELISA

123 kit (BD Bioscience) as per manufacturer's instructions. Optical densities were measured

124 using GloMax-Multi+ Microplate with Instinct microplate reader (Promega). Data was then

125 analysed using a 5-parameter sigmoidal curve on Graph Pad Prism Version 7.

126

Intracellular cytokine staining

128 10^6 PBMC were stimulated with TLR-L and naltrexone for 6 hours in the presence of

129 Brefeldin A (eBioscience) for 4 of those hours. After 6 hours PBMC were washed with PBS

130 and cell surface markers were stained using fluorochrome-conjugated monoclonal antibodies.

131 Antibodies used: CD14-VioBlue, mIgG1, clone TUK4, CD1c-VioBright FITC, mIgG2a

132 clone AD5-8E7, CD303 PE-Vio770, mIgG1, clone AC144 (all Milenyi Biotec) and CD19-

133 PE, mIgG1, clone HIB19 (eBioscience) or appropriate isotype. After washing in PBS, PBMC

134 were fixed and permeabilised using BD cell fixation/permeabilization kit. PBMC were then

135 washed in BD perm/wash buffer and stained for IL-6 and TNF-α using TNFα, hIgG1, clone

136 cA2 (Milentyi Biotec) or IL-6 APC, rIgG1, clone MQ2-13A5 (eBioscience) or appropriate

137 isotype. After washing with BD perm/wash buffer PBMC were ran on the BD Canto running

138 BD FACSDiva software and analysed using FlowJo software.

139

140 **Flow cytometry analysis**

141 Unstained PBMC and fluorescence minus one (FMO) controls, in combination with

142 appropriate isotype controls, were used to determine gating. Supplementary figure 3 shows

143 the gating strategy and all flow cytometry data was analysed using FlowJo software. PBMC

144 population was gated based on the size (FSC) and granularity (SSC) of the cells. CD14+ and

145 CD19+ were used to identify monocytes and B cells respectively. Within the CD14- CD19-

146 population myeloid dendritic cells and plasmacytoid dendritic cells were identified by CD1c

147 and CD303 positivity respectively. To determine the expression of the intracellular cytokines,

148 histograms were generated to determine the percentage of subsets that is positive for the

149 marker or cytokine of interest. IL-6 and TNFα positive and negative populations were gated

150 based on FMO in combination with isotype control. Mean fluorescence intensity of TNFα

151 and IL-6 was also determined.

152

153 **Cell viability**

154 1 million PBMC were stimulated with TLR-L and naltrexone for 24 hours before being

155 resuspended in 1x Annexin V binding buffer (eBioscience) and incubated with 5µl Annexin

156 V APC (eBioscience) for 20 minutes. Cells were then washed in 1ml 1x Annexin V binding

157 buffer and resuspended in 200ul 1x Annexin V binding buffer. 5µl 7-AAD was then added

158 and data was collected using the BD Canto. Data was analysed using FlowJo software.

159

160 **Statistics**

161 Data are presented as mean with the standard error of mean and statistical analysis was

162 performed using GraphPad Prism Verison 6.07 for Windows. Data was analysed using a one

163 way ANOVA and Tukey's multiple comparison test. A p value of below 0.05 was deemed to

164 be significant

165

166

167 **Results**

168 **Naltrexone inhibits IL-6 production induced after TLR 7/8 and TLR9 but not TLR4 or**

169 **IL-1R stimulation**

170 It has previously been shown that naltrexone inhibits TLR4 activity both in an *in vitro* assay

171 system and in microglial cells [15,16]. We therefore sought to determine the effect of naltrexone

172 on this and other members of the TLR family in an immune context, focusing on production

173 of IL-6, a key cytokine produced following TLR stimulation. Titrations were performed in

174 order to determine the optimum concentration of TLR-Ls that induce statistically significant

175 IL-6 production in PBMC (supplementary fig.1). PBMC were stimulated with TLR ligands

176 (TLR-Ls) for TLR4 (LPS 1ng/ml), TLR7/8 (R848 1µM) and TLR9 (CpG 1µM) in the

177 presence or absence of naltrexone (1-200µM) and IL-6 production was determined by

178 ELISA. Naltrexone had no effect on IL-6 production following TLR4 stimulation (Fig.1A),

179 however, 200µM naltrexone inhibited IL-6 production following stimulation with ligands for

180 TLR7/8 (Fig.1B, $p<0.05$) and TLR9 (Fig.1C, $p<0.05$) (this data is also presented as dose

181 response curves in supplementary fig.2). As R848 is a ligand for both TLR7 and TLR8 we

182 sought to determine if NTX inhibits IL-6 production after TLR7 (R837 3µg/ml) or TLR8

183 (ssRNA 0.5µg/ml) stimulation. NTX inhibited IL-6 production after both TLR7 and TLR8

184 stimulation in a dose dependent manner, although this did not reach significance

185 (supplementary fig.3) As TLR7, TLR8 and TLR9 signal via the MyD88 pathway whereas

186 TLR4 can signal via both MyD88 dependent and independent pathways [28,30] we hypothesised

187 that naltrexone may affect the MyD88-dependent signalling pathway and that any effects of

188 naltrexone on IL-6 secretion via TLR4 were compensated for by signalling through the

189 MyD88-independent pathway. Stimulation of the IL-1R also results in induction of the

190 MyD88 dependent pathway and the secretion of IL-6. However, when PBMC were

191 stimulated with IL-1 (100ng/ml) in the presence of naltrexone (1-200µM), no effect on IL-6

192 production observed (Fig.1D).

193

194 **Naltrexone inhibits intracellular cytokine production after TLR7/8 and TLR9**

195 **stimulation but not TLR4 stimulation**

196 In order to determine which subset(s) of cells within the PBMC population were effected by

197 NTX, intracellular cytokine staining was performed. In addition to IL-6 production we also

198 examined the effect NTX has on another signature cytokine produced after TLR stimulation,

199 TNF-α. PBMC were stimulated with TLR-L (LPS 1ng/ml, R848 1µM and CpG 1µM) and

200 200µM naltrexone for 6 hours, with the addition of Brefeldin A after 2 hours. PBMC were

201 then stained for cell surface markers, as shown in supplementary fig.4, to identify monocytes

202 (CD14+), B cells (CD19+), myeloid dendritic cells (CD14- CD19- CD1c+, mDCs) and

203 plasmacytoid dendritic cells (CD14- CD19- CD1c- CD303+, pDCs) and for intracellular IL-6

204 or TNFα (Fig.2). Monocytes were identified as a major source of IL-6 following LPS and

205 R848 stimulation (Fig.2A and Fig.2B). In line with our observations from ELISA data

206   described above, naltrexone did not appear to affect IL-6 production by CD14+ cells

207   following LPS stimulation (Fig.2B). A decrease in IL-6 production in monocytes after R848

208   and naltrexone incubation was observed, although this did not reach statistical significance

209   (Fig.2B). Incubation with the TLR9 ligand CpG induced IL-6 production in B cells however,

210   there was not affected by the addition of 200μM naltrexone to cultures (data not shown).

211   Furthermore, at the time point examined no cytokine production was observed in mDC

212   following incubation with LPS, R848 or CpG (data not shown). TNFα was induced following

213   LPS and CpG stimulation in monocytes and pDCs respectively (Fig.2C and Fig.2D). Similar

214   to the results observed for IL-6, naltrexone did not affect TNF-α production following LPS

215   stimulation in monocytes (Fig.2C), whereas naltrexone did inhibit TNF-α production in

216   plasmacytoid dendritic cells following TLR9 stimulation (Fig.2D $p<0.05$).

217

218

219   **NTX inhibits IL-6 production in isolated monocytes and B cells after TLR7/8 and TLR9**

220   **stimulation respectively but has no effect on IL-6 production in isolated monocytes after**

221   **TLR4 stimulation.**

222

223   To further confirm that naltrexone does not inhibit cytokine production after TLR-4

224   stimulation we isolated CD14+ monocytes from PBMC using magnetic bead isolation.

225   Isolated CD14 cells were then stimulated with LPS 1ng/ml and R848 1μM in the presence or

226   absence of naltrexone 200μM for 24 hours. Cell free supernatants were analysed for the

227   presence of IL-6 by ELISA. Similar to the data obtained from intracellular cytokine analysis

228   described above, naltrexone inhibited IL-6 production in monocytes following R848

229   stimulation, but no effect on LPS induced IL-6 production was observed (Fig.3A).

230   Additionally, within the PBMC population, TLR9 is predominately expressed on B cells.

9

231 Therefore, to determine if naltrexone effects IL-6 production in isolated B cells after TLR9

232 stimulation, B cells were stimulated with CpG 1μM in the presence of 200μM naltrexone for

233 24hrs. NTX inhibited IL-6 production after TLR-9 stimulation but not after crosslinking of

234 CD40R and stimulation with IL-4 (Fig.3B)

235

236 **Naltrexone does not affect PBMC viability.**

237 To ensure that the decreases in IL-6 production we observed in the presence of naltrexone

238 were not due to a loss of cell numbers, viability was assessed by trypan blue staining

239 following PBMC incubation with naltrexone (1-200μM) for 24 hours. No change in cell

240 viability was observed (Fig.4A). Additionally, to determine if naltrexone induces apoptosis,

241 annexin V and 7-AAD staining was performed on PBMC following 24 hours incubation with

242 naltrexone and TLR-Ls (Fig.4B). As shown in Fig.4C, there was no evidence to suggest that

243 TLR-Ls or naltrexone incubation induce apoptosis in PBMC at the concentrations tested in

244 this study.

245
246 **Discussion**
247
248 Through their roles as mediators of both innate and adaptive immune functions, TLRs are

249 powerful agents within the immune system. Intracellular TLR have been investigated as

250 potential therapeutic targets for the treatment of inflammatory diseases and cancer[29,31–33].

251 Inhibition of TLR-mediated functions by naltrexone could, therefore, indicate a potential

252 immunomodulatory relevance for this drug in the treatment of inflammatory disease. In this

253 study, we show that naltrexone can inhibit the production of cytokines by PBMC following

254 treatment with ligands for the intracellular receptors TLR7, TLR8 and TLR9. Flow

255 cytometric analysis of individual cell subsets indicated that naltrexone inhibited IL-6

256 production by monocytes in response to TLR 7/8 ligands and TNFα production by pDCs in

257 response to TLR9 ligand. These reductions in cytokine secretion did not appear to result from

258   a loss of cell viability, as no significant effects on cell numbers or expression of apoptotic

259   markers was observed.

260

261   One unexpected finding of this study was that naltrexone did not inhibit cytokine secretion by

262   immune cells following stimulation with LPS, a ligand for TLR4. Previously published work

263   had shown that naltrexone and naloxone can inhibit TLR4 dependent microglial activation,

264   neurodegeneration and nitric oxide production [16,34] and have identified the LPS binding site

265   of the TLR4 co-receptor MD2 as a binding site for the drug [35,36]. Previous studies

266   documented the effect of the purified isomers of naltrexone on TLR4, whereas our study used

267   naltrexone-HCl, a hydrochloride salt commonly prescribed in tablet form to patients. Both

268   isomers have shown to bind MD2 and inhibit TLR4 activity[34,35] in a HEK-293 reporter cell

269   line and rat microglial cells. The (+)-isomer of naltrexone does not act on opioid receptors,

270   which may be beneficial for use in therapies directed at alternative receptors. Further

271   investigations will be necessary to determine the effects of different naltrexone isomers on

272   TLR7, TLR8 and TLR9, which are intracellular and do not associate with MD2.

273

274   Our experiments have shown that naltrexone can inhibit cytokine secretion in response to

275   TLR ligands, although further work will be required to determine the mechanism(s) of action

276   involved. Each of the TLR investigated in the current study (TLR4, TLR7, TLR8 and TLR9)

277   signal through the MyD88 dependent pathway, although TLR4 can also signal via the

278   MyD88 independent TRIF pathway. It could be hypothesized that inhibition of cytokine

279   production following TLR7, TLR8 or TLR9 stimulation results from inhibition of the MyD88

280   pathway, and that the observed lack of TLR4 antagonism in our experiments results from

281   signalling via TRIF pathway, which can induce delayed NFκB activation and resultant IL-6

282   and TNFα production. However, previously published work has suggested that naltrexone

283   inhibits phosphorylation of IRF3, a transcription factor that downstream of TRIF activation[34].

284   Also, our observation that naltrexone did not inhibit cytokine secretion in response to

285   stimulation of the IL-1 receptor, which also signals by the MyD88 pathway would support an

286   interaction upstream of this adaptor protein. Further investigations are required to determine

287   the signalling pathways regulated by naltrexone and how this can account for TLRs effected.

288   Furthermore, intracellular cytokine assays in this study examined the effect of naltrexone on

289   the production of IL-6 and TNFα after six hours incubation. This approach does not provide

290   information of the potential effect of naltrexone on cytokine kinetics. More detailed analyses

291   determining the effect of naltrexone on cytokine production at different time points would be

292   required in order to investigate whether naltrexone may delay cytokine production.

293

294   The reduction of cytokine secretion observed in the presence of naltrexone in our studies did

295   not result from a reduction in cell numbers or a decrease in cell viability, as evidenced by dye

296   exclusion and flow cytometric analysis for markers of apoptosis. This provides further

297   support for our theory that naltrexone can modulate immune cell functions through

298   influencing TLR activity, thus extending the known immune effects of the drug beyond the

299   previously documented inhibition of lymphocyte proliferation *in vitro* and *in vivo* [37,38].

300   However, this study was only performed within the whole PBMC population and therefore it

301   is possible that subtle changes in individual immune cell subsets within the PBMC population

302   would not be detected. Future studies would consider the viability of the individual immune

303   subsets after incubation with naltrexone.

304

305   An ability to modulate TLR activity would provide justification to support the use of

306   naltrexone for the treatment of inflammatory conditions in which these receptors play a

307   pathogenic role. For example, recognition of self-DNA/protein complexes by TLR9 mediates

308   pDC activation in psoriasis, breaking self-immune tolerance[24]. Members of the TLR family,

309   including TLR9 are often ectopically expressed in tumours [39,40], can induce tumour invasion

310   *in vitro*[41], and may be an indicator of poor prognosis *in vivo*. Similarly, expression of TLR9

311   has been found to correlate with the invasive and metastatic potential of pancreatic

312   carcinoma[42].

313

314   Future studies will be required to investigate whether and how naltrexone inhibits TLR-

315   mediated inflammatory effects in other cell types such as mucosal epithelial cells[43], and

316   whether exposure to naltrexone results in upregulation of TLR in a similar manner to that

317   seen for its opioid receptor targets [44,45]. Additionally, whilst this study investigated the effect

318   of naltrexone on IL-6 and TNFα production, further work examining other cytokines, such as

319   IL-12p70, which might be induced after multiple TLR stimulation would provide further

320   insights into the ability of naltrexone to modulate immune subset activity. It will also be

321   important to consider how the potential pleiotropic effects of naltrexone, including inhibition

322   of TLR mediated functions, inhibition of cellular proliferation and other opioid receptor-

323   mediated activity might contribute to its use in the treatment of inflammatory conditions. In

324   this context, it is important to note that previous studies in inflammatory diseases and cancer

325   have adopted a Low Dose Naltrexone regime as opposed to the dosages used in the treatment

326   of opioid and alcohol dependency. Nanomolar but not micromolar doses of naltrexone were

327   previously seen in studies by Liu *et al* to result in upregulation of pro-apoptotic genes,

328   rendering tumor cells more susceptible to chemotherapy [46]. It may, therefore, be necessary to

329   identify suitable dosage regimes to obtain optimal therapeutic effects on individual target

330   pathways in different diseases.

331

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333

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338

Author Contributions

A.D and R.A conceived the original idea for the study. R.C and R.A designed the

experiments and prepared the manuscript. R.C performed experiments and analysed the data.

All authors read and approved the manuscript.

343

Competing financial interests

R. A and A.D. are listed as inventors on a patent that describes the use of Naltrexone as a

TLR9 antagonist, which has been assigned to the Institute for Cancer Vaccines and

Immunotherapy. R.C declares no competing financial interests.

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470   seen with normal doses: Implications for its use in cancer therapy. *Int. J. Oncol.* **49,** 793–802
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472

473   **Figures legends**

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475

476   **Figure 1 – Naltrexone inhibits IL-6 production after TLR7/8 and TLR9 stimulation but**

477   **not after TLR4 or IL-1 stimulation**

478   1×10^6 PBMC were incubated with A) 1ng/ml LPS (TLR4-L), B) 1μM R848 (TLR7/8-L), C)

479   1μM CpG (TLR9-L), D) 100ng/ml IL-1 (IL-1R) in the presence or absence of 1-200μM

480   naltrexone for 24 hours. Cell free supernatants were collected and analysed for IL-6 by

481   ELISA. Data shows the mean, SD values are shown and was analysed using a one way

482   ANOVA and Tukey's multiple comparison test (n=5 TLR-L experiments and n=3 IL-1).

483   *p<0.05, **<0.01

484

485   **Figure 2 - Intracellular cytokine staining for TNFα and IL-6 in monocytes and**

486   **plasmacytoid dendritic cells**

487   1×10^6 PBMC were incubated with either LPS 1ng/ml (A/C), R848 1μM (B) or CpG 1μM (D)

488   and 200μM naltrexone for 6 hours in the presence of brefeldin A for 4 of those hours. After 6

489   hours, PBMC were stained using antibody panel shown in supplementary fig.3 and stained

490   for either intracellular IL-6 or TNF-α. Results show the mean fluorescence intensity (MFI) of

491   IL-6 or TNF-α within that subsets from 5 donors. Histograms are representative of 5

492   independent experiments.

493

494

495

496

497 **Figure 3– NTX inhibits IL-6 production in isolated monocytes and B cells after TLR7/8**

498 **and TLR9 stimulation respectively but has no effect on IL-6 production in isolated**

499 **monocytes after TLR4 stimulation.**

500 A) CD14+ monocytes were isolated from PBMC using magnetic bead isolation. 1×10^5

501 CD14+ cells were incubated with 1ng/ml LPS (TLR4-L) or 1µM R848 (TLR7/8-L), in the

502 presence or absence of 200µM naltrexone for 24 hours. Cell free supernatants were collected

503 and analysed for IL-6 by ELISA.

504 B) CD19+ B cells were isolated from PBMC using magnetic bead isolation. 10^5 B cells were

505 incubated with 1µM CpG or 3µg/ml CD40-L and 20ng/ml IL-4, with or without 200µM

506 naltrexone for 24 hours. IL-6 production was measured in cell free supernatants by ELISA.

507 Data is shows the mean and SD values (n=4).

508

509

510 **Figure 4– TLR-L and naltrexone does not affect the viability of PBMC**

511 A) 1×10^6 PBMC were incubated with 1-200µM naltrexone for 24 hours before percentage

512 viability was assessed using trypan blue exclusion.

513 B/C) 1×10^6 PBMC were incubated with 1ng/ml LPS (TLR4-L), 1µM R848 (TLR7/8-L),

514 1µM CpG (TLR9-L) and 200µM naltrexone for 24 hours. PBMC were incubated with

515 Annexin V and 7-AAD before being analysed by flow cytometry. Fig. 5B shows the gating

516 strategy and Fig.5C show results from 4 donors. AV-7AAD- are viable cells, AV+7AAD- are

517 in early apoptosis and AV+7AAD+ are in late apoptosis.
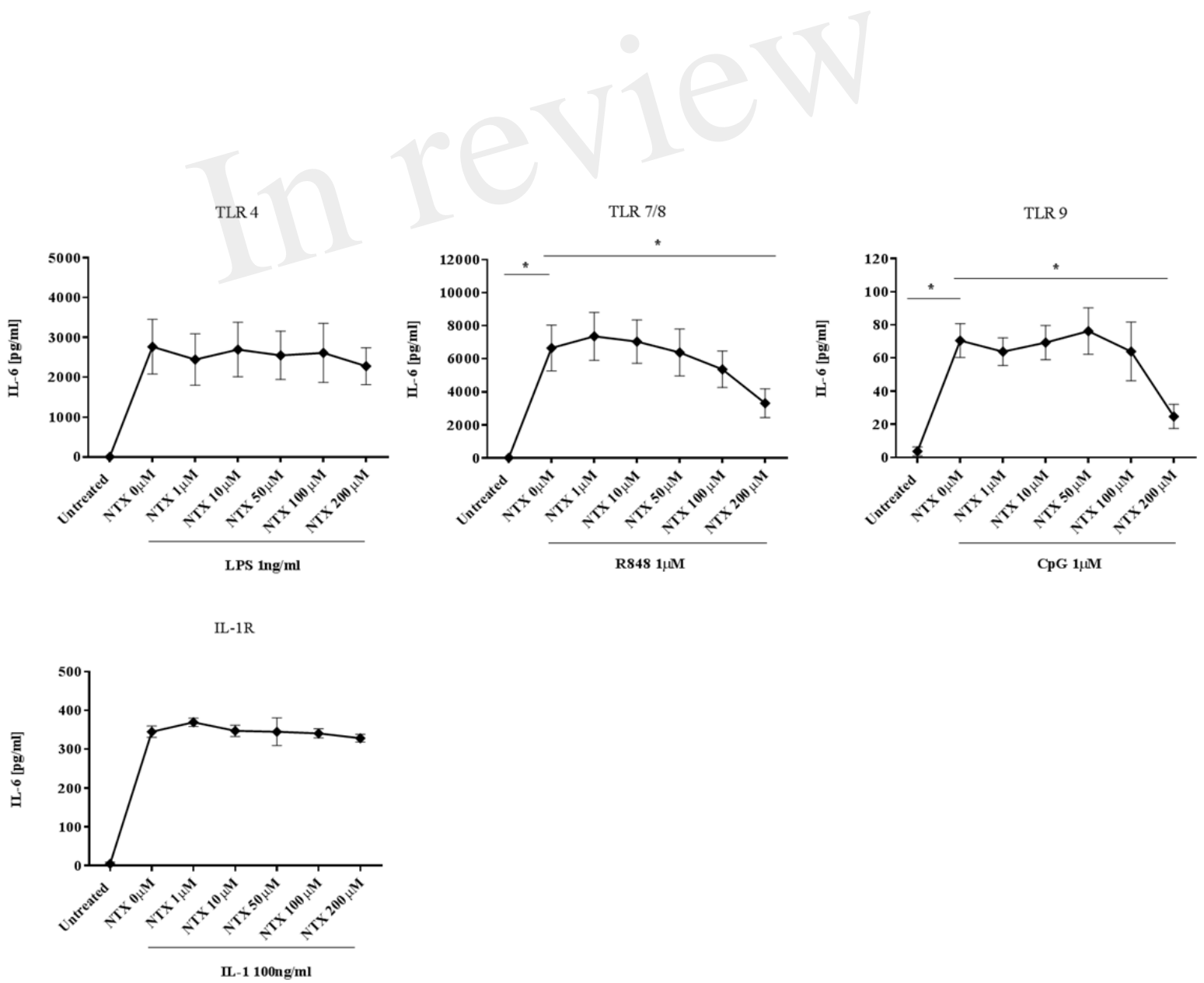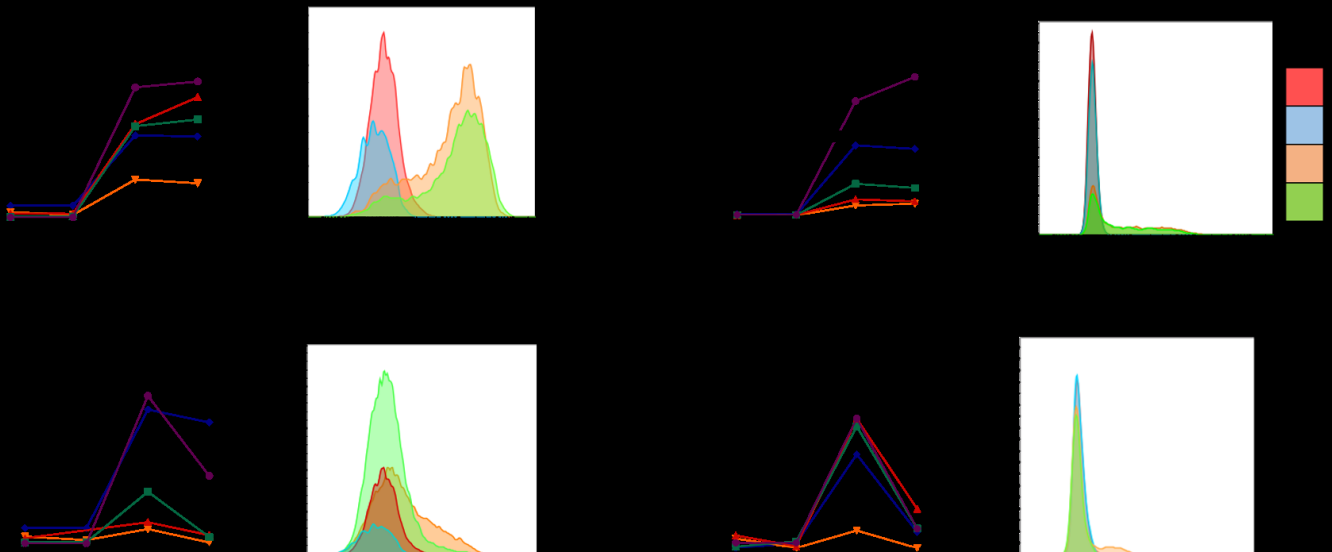
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Figure 1.TIF

Figure 2.TIF

Figure 3.TIF

Figure 4.TIF
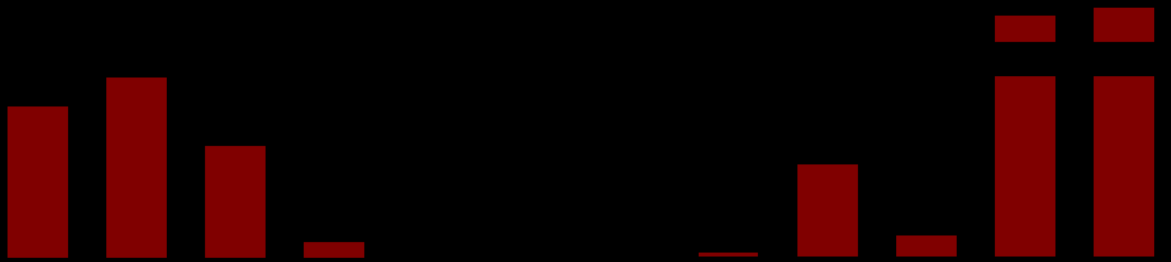

a

% viability

b

7-AAD

AV-7AAD+
3.52

AV+ 7AAD+
19.1

AV- 7AAD-
60.5

AV+ 7AAD-
16.8

Annexin V - APC

c

LPS

%

Untreated
NTX 200 μM
LPS 1ng/ml
LPS 1ng/ml + NTX 200 μM

R848

%

Untreated
NTX 200 μM
R848 1μM
R848 1μM + NTX 200 μM

CpG

%

Untreated
NTX 200 μM
CpG 1μM
CpG 1μM + NTX 200 μM

Late apoptosis
Early apoptosis
Live