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

Rachel Cant1*, Angus G. Dalgleish1, Rachel L. Allen1

1St George's, University of London, United Kingdom

Submitted to Journal:
Frontiers in Immunology

Specialty Section:
Antigen Presenting Cell Biology

Article type:
Original Research Article

Manuscript ID:
249153

Received on:
16 Dec 2016

Revised on:
16 Jun 2017

Frontiers website link:
www.frontiersin.org
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

Word count: 197

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 TNFa 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.

Funding statement

This study was funded by the Institute for Cancer Vaccines and Immunotherapy (Registered Charity 1080343).

Ethics statements

(Author is required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: Yes

Please provide the complete ethics statement for your manuscript. Note that the statement will be directly added to the manuscript file for peer-review, and should include the following information:

- Full name of the ethics committee that approved the study
- Consent procedure used for human participants or for animal owners
- Any additional considerations of the study in cases where vulnerable populations were involved, for example minors, persons with disabilities or endangered animal species

As per the Frontiers authors guidelines, you are required to use the following format for statements involving human subjects:

This study was carried out in accordance with the recommendations of 'name of guidelines, name of committee' with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the 'name of committee'.

For statements involving animal subjects, please use:

This study was carried out in accordance with the recommendations of 'name of guidelines, name of committee'. The protocol
was approved by the 'name of committee'.

If the study was exempt from one or more of the above requirements, please provide a statement with the reason for the exemption(s).
Ensure that your statement is phrased in a complete way, with clear and concise sentences.
This study was carried out in accordance with the recommendations of St George's, University of London Research Ethics Committee (Protocol Approval SGREC15.0006). All subjects gave written informed consent.
Naltrexone inhibits IL-6 and TNFα production in human immune cell subsets following stimulation with ligands for intracellular Toll-like Receptors.

Rachel Cant¹*, Angus G. Dalgleish¹, Rachel L. Allen¹

¹St George’s, University of London, Institute for Infection and Immunity, London, SW17 0RE, United Kingdom.
* corresponding author email cant.rachel@gmail.com

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

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\(^19\)\(^,\)\(^21\).
Although TLR play a key role in the initiation of immune responses to infection, inappropriate TLR activity and/or recognition of self-ligands are associated with inflammatory conditions and autoimmunity\textsuperscript{22}. For example, increased expression of TLRs has been observed in peripheral B cells from patients with inflammatory bowel disease\textsuperscript{23} while recognition of self-DNA complexes by TLR9 mediates pDC activation in psoriasis\textsuperscript{24}. TLRs have also been implicated in the tumour microenvironment, with TLR activation linked to angiogenesis, tumour proliferation and immune evasion\textsuperscript{25}. Furthermore, some TLR polymorphisms may be associated with development of inflammatory conditions such as Crohn’s disease\textsuperscript{26,27}. TLRs have, therefore, been investigated as potential therapeutic targets in patients with these diseases\textsuperscript{28,29}.

In this study, we sought to investigate the ability of naltrexone hydrochloride to inhibit the effects of TLR-4 signalling in an immune context and to determine whether its inhibitory effects extend to other members of the TLR family. Our results indicate that naltrexone can inhibit production of the inflammatory cytokines IL-6 and TNFα by peripheral blood mononuclear cells (PBMC) following stimulation with known ligands for TLR7, TLR8 and TLR9 but not following stimulation with a TLR4 ligand. Although the interleukin 1 receptor (IL-1R) shares the MyD88 signalling pathway with members of the TLR family, IL-6 secretion following IL-1R stimulation was not affected by naltrexone. Our findings also indicate that naltrexone does not affect cell viability or induce apoptosis within the PBMC population.
Methods

Ethics statement

This study was carried out in accordance with the recommendations of St George’s, University of London Research Ethics Committee (Protocol Approval SGREC15.0006). All subjects gave written informed consent.

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°C, 5% CO$_2$.

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
endotoxin free water and diluted in RPMI before being added to PBMC at the working concentrations specified.

Isolation of CD14+ and CD19+ cells

Positive selection of CD14+ and CD19+ was performed by incubating PBMC with MACS CD14+ and CD19+ microbeads in MACS buffer, according to the manufacturer’s instructions (Miltenyi Biotec). After incubation of cells and microbeads, cells were washed with MACS buffer, resuspended in MACS buffer and loaded onto a MACS column attached to a magnetic field of a MACS separator. After being washed with MACS buffer three times the column was removed the magnetic field and the CD14+ and CD19+ cells were eluted using MACS buffer (Miltenyi Biotec). Purity of above 90% was confirmed by flow cytometry using CD14 VioBlue mIgG1 antibody and CD20 FITC mIgG1 antibody (Miltenyi Biotec).

IL-6 ELISA

$10^6$ PBMC were stimulated with ligands and naltrexone as stated above for 24 hours before cell-free supernatants were collected and IL-6 ELISA was performed using an IL-6 ELISA kit (BD Bioscience) as per manufacturer’s instructions. Optical densities were measured using GloMax-Multi+ Microplate with Instinct microplate reader (Promega). Data was then analysed using a 5-parameter sigmoidal curve on Graph Pad Prism Version 7.

Intracellular cytokine staining

$10^6$ PBMC were stimulated with TLR-L and naltrexone for 6 hours in the presence of Brefeldin A (eBioscience) for 4 of those hours. After 6 hours PBMC were washed with PBS and cell surface markers were stained using fluorochrome-conjugated monoclonal antibodies.
Antibodies used: CD14-VioBlue, mIgG1, clone TUK4, CD1c-VioBright FITC, mIgG2a clone AD5-8E7, CD303 PE-Vio770, mIgG1, clone AC144 (all Milenyi Biotec) and CD19-PE, mIgG1, clone HIB19 (eBioscience) or appropriate isotype. After washing in PBS, PBMC were fixed and permeabilised using BD cell fixation/permeabilization kit. PBMC were then washed in BD perm/wash buffer and stained for IL-6 and TNF-α using TNFα, hlgG1, clone cA2 (Milentyi Biotec) or IL-6 APC, rlgG1, clone MQ2-13A5 (eBioscience) or appropriate isotype. After washing with BD perm/wash buffer PBMC were ran on the BD Canto running BD FACSDiva software and analysed using FlowJo software.

**Flow cytometry analysis**

Unstained PBMC and fluorescence minus one (FMO) controls, in combination with appropriate isotype controls, were used to determine gating. Supplementary figure 3 shows the gating strategy and all flow cytometry data was analysed using FlowJo software. PBMC population was gated based on the size (FSC) and granularity (SSC) of the cells. CD14+ and CD19+ were used to identify monocytes and B cells respectively. Within the CD14-CD19- population myeloid dendritic cells and plasmacytoid dendritic cells were identified by CD1c and CD303 positivity respectively. To determine the expression of the intracellular cytokines, histograms were generated to determine the percentage of subsets that is positive for the marker or cytokine of interest. IL-6 and TNFα positive and negative populations were gated based on FMO in combination with isotype control. Mean fluorescence intensity of TNFα and IL-6 was also determined.

**Cell viability**

1 million PBMC were stimulated with TLR-L and naltrexone for 24 hours before being resuspended in 1x Annexin V binding buffer (eBioscience) and incubated with 5µl Annexin V.
V APC (eBioscience) for 20 minutes. Cells were then washed in 1ml 1x Annexin V binding buffer and resuspended in 200ul 1x Annexin V binding buffer. 5µl 7-AAD was then added and data was collected using the BD Canto. Data was analysed using FlowJo software.

Statistics
Data are presented as mean with the standard error of mean and statistical analysis was performed using GraphPad Prism Version 6.07 for Windows. Data was analysed using a one way ANOVA and Tukey’s multiple comparison test. A p value of below 0.05 was deemed to be significant.

Results
Naltrexone inhibits IL-6 production induced after TLR 7/8 and TLR9 but not TLR4 or IL-1R stimulation
It has previously been shown that naltrexone inhibits TLR4 activity both in an in vitro assay system and in microglial cells\(^{15,16}\). We therefore sought to determine the effect of naltrexone on this and other members of the TLR family in an immune context, focusing on production of IL-6, a key cytokine produced following TLR stimulation. Titrations were performed in order to determine the optimum concentration of TLR-Ls that induce statistically significant IL-6 production in PBMC (supplementary fig.1). PBMC were stimulated with TLR ligands (TLR-Ls) for TLR4 (LPS 1ng/ml), TLR7/8 (R848 1µM) and TLR9 (CpG 1µM) in the presence or absence of naltrexone (1-200µM) and IL-6 production was determined by ELISA. Naltrexone had no effect on IL-6 production following TLR4 stimulation (Fig.1A), however, 200µM naltrexone inhibited IL-6 production following stimulation with ligands for TLR7/8 (Fig.1B, p<0.05) and TLR9 (Fig.1C, p<0.05) (this data is also presented as dose
response curves in supplementary fig. 2). As R848 is a ligand for both TLR7 and TLR8 we sought to determine if NTX inhibits IL-6 production after TLR7 (R837 3μg/ml) or TLR8 (ssRNA 0.5μg/ml) stimulation. NTX inhibited IL-6 production after both TLR7 and TLR8 stimulation in a dose dependent manner, although this did not reach significance (supplementary fig. 3) As TLR7, TLR8 and TLR9 signal via the MyD88 pathway whereas TLR4 can signal via both MyD88 dependent and independent pathways 28,30 we hypothesised that naltrexone may affect the MyD88-dependent signalling pathway and that any effects of naltrexone on IL-6 secretion via TLR4 were compensated for by signalling through the MyD88-independent pathway. Stimulation of the IL-1R also results in induction of the MyD88 dependent pathway and the secretion of IL-6. However, when PBMC were stimulated with IL-1 (100ng/ml) in the presence of naltrexone (1-200μM), no effect on IL-6 production observed (Fig.1D).

Naltrexone inhibits intracellular cytokine production after TLR7/8 and TLR9 stimulation but not TLR4 stimulation

In order to determine which subset(s) of cells within the PBMC population were effected by NTX, intracellular cytokine staining was performed. In addition to IL-6 production we also examined the effect NTX has on another signature cytokine produced after TLR stimulation, TNF-α. PBMC were stimulated with TLR-L (LPS 1ng/ml, R848 1μM and CpG 1μM) and 200μM naltrexone for 6 hours, with the addition of Brefeldin A after 2 hours. PBMC were then stained for cell surface markers, as shown in supplementary fig.4, to identify monocytes (CD14+), B cells (CD19+), myeloid dendritic cells (CD14- CD19- CD1c+, mDCs) and plasmacytoid dendritic cells (CD14- CD19- CD1c- CD303+, pDCs) and for intracellular IL-6 or TNFα (Fig.2). Monocytes were identified as a major source of IL-6 following LPS and R848 stimulation (Fig.2A and Fig.2B). In line with our observations from ELISA data
described above, naltrexone did not appear to affect IL-6 production by CD14+ cells following LPS stimulation (Fig.2B). A decrease in IL-6 production in monocytes after R848 and naltrexone incubation was observed, although this did not reach statistical significance (Fig.2B). Incubation with the TLR9 ligand CpG induced IL-6 production in B cells however, there was not affected by the addition of 200µM naltrexone to cultures (data not shown). Furthermore, at the time point examined no cytokine production was observed in mDC following incubation with LPS, R848 or CpG (data not shown). TNFα was induced following LPS and CpG stimulation in monocytes and pDCs respectively (Fig.2C and Fig.2D). Similar to the results observed for IL-6, naltrexone did not affect TNF-α production following LPS stimulation in monocytes (Fig.2C), whereas naltrexone did inhibit TNF-α production in plasmacytoid dendritic cells following TLR9 stimulation (Fig.2D p<0.05).

NTX inhibits IL-6 production in isolated monocytes and B cells after TLR7/8 and TLR9 stimulation respectively but has no effect on IL-6 production in isolated monocytes after TLR4 stimulation.

To further confirm that naltrexone does not inhibit cytokine production after TLR-4 stimulation we isolated CD14+ monocytes from PBMC using magnetic bead isolation. Isolated CD14 cells were then stimulated with LPS 1ng/ml and R848 1µM in the presence or absence of naltrexone 200µM for 24 hours. Cell free supernatants were analysed for the presence of IL-6 by ELISA. Similar to the data obtained from intracellular cytokine analysis described above, naltrexone inhibited IL-6 production in monocytes following R848 stimulation, but no effect on LPS induced IL-6 production was observed (Fig.3A). Additionally, within the PBMC population, TLR9 is predominately expressed on B cells.
Therefore, to determine if naltrexone effects IL-6 production in isolated B cells after TLR9 stimulation, B cells were stimulated with CpG 1µM in the presence of 200µM naltrexone for 24 hrs. NTX inhibited IL-6 production after TLR-9 stimulation but not after crosslinking of CD40R and stimulation with IL-4 (Fig.3B).

**Naltrexone does not affect PBMC viability.**

To ensure that the decreases in IL-6 production we observed in the presence of naltrexone were not due to a loss of cell numbers, viability was assessed by trypan blue staining following PBMC incubation with naltrexone (1-200µM) for 24 hours. No change in cell viability was observed (Fig.4A). Additionally, to determine if naltrexone induces apoptosis, annexin V and 7-AAD staining was performed on PBMC following 24 hours incubation with naltrexone and TLR-Ls (Fig.4B). As shown in Fig.4C, there was no evidence to suggest that TLR-Ls or naltrexone incubation induce apoptosis in PBMC at the concentrations tested in this study.

**Discussion**

Through their roles as mediators of both innate and adaptive immune functions, TLRs are powerful agents within the immune system. Intracellular TLR have been investigated as potential therapeutic targets for the treatment of inflammatory diseases and cancer. Inhibition of TLR-mediated functions by naltrexone could, therefore, indicate a potential immunomodulatory relevance for this drug in the treatment of inflammatory disease. In this study, we show that naltrexone can inhibit the production of cytokines by PBMC following treatment with ligands for the intracellular receptors TLR7, TLR8 and TLR9. Flow cytometric analysis of individual cell subsets indicated that naltrexone inhibited IL-6 production by monocytes in response to TLR 7/8 ligands and TNFα production by pDCs in response to TLR9 ligand. These reductions in cytokine secretion did not appear to result from
a loss of cell viability, as no significant effects on cell numbers or expression of apoptotic markers was observed.

One unexpected finding of this study was that naltrexone did not inhibit cytokine secretion by immune cells following stimulation with LPS, a ligand for TLR4. Previously published work had shown that naltrexone and naloxone can inhibit TLR4 dependent microglial activation, neurodegeneration and nitric oxide production\textsuperscript{16,34} and have identified the LPS binding site of the TLR4 co-receptor MD2 as a binding site for the drug \textsuperscript{35,36}. Previous studies documented the effect of the purified isomers of naltrexone on TLR4, whereas our study used naltrexone-HCl, a hydrochloride salt commonly prescribed in tablet form to patients. Both isomers have shown to bind MD2 and inhibit TLR4 activity\textsuperscript{34,35} in a HEK-293 reporter cell line and rat microglial cells. The (+)-isomer of naltrexone does not act on opioid receptors, which may be beneficial for use in therapies directed at alternative receptors. Further investigations will be necessary to determine the effects of different naltrexone isomers on TLR7, TLR8 and TLR9, which are intracellular and do not associate with MD2.

Our experiments have shown that naltrexone can inhibit cytokine secretion in response to TLR ligands, although further work will be required to determine the mechanism(s) of action involved. Each of the TLR investigated in the current study (TLR4, TLR7, TLR8 and TLR9) signal through the MyD88 dependent pathway, although TLR4 can also signal via the MyD88 independent TRIF pathway. It could be hypothesized that inhibition of cytokine production following TLR7, TLR8 or TLR9 stimulation results from inhibition of the MyD88 pathway, and that the observed lack of TLR4 antagonism in our experiments results from signalling via TRIF pathway, which can induce delayed NFκB activation and resultant IL-6 and TNFα production. However, previously published work has suggested that naltrexone
inhibits phosphorylation of IRF3, a transcription factor that downstream of TRIF activation\textsuperscript{34}. Also, our observation that naltrexone did not inhibit cytokine secretion in response to stimulation of the IL-1 receptor, which also signals by the MyD88 pathway would support an interaction upstream of this adaptor protein. Further investigations are required to determine the signalling pathways regulated by naltrexone and how this can account for TLRs effected. Furthermore, intracellular cytokine assays in this study examined the effect of naltrexone on the production of IL-6 and TNF\textalpha after six hours incubation. This approach does not provide information of the potential effect of naltrexone on cytokine kinetics. More detailed analyses determining the effect of naltrexone on cytokine production at different time points would be required in order to investigate whether naltrexone may delay cytokine production.

The reduction of cytokine secretion observed in the presence of naltrexone in our studies did not result from a reduction in cell numbers or a decrease in cell viability, as evidenced by dye exclusion and flow cytometric analysis for markers of apoptosis. This provides further support for our theory that naltrexone can modulate immune cell functions through influencing TLR activity, thus extending the known immune effects of the drug beyond the previously documented inhibition of lymphocyte proliferation \textit{in vitro} and \textit{in vivo} \textsuperscript{37,38}. However, this study was only performed within the whole PBMC population and therefore it is possible that subtle changes in individual immune cell subsets within the PBMC population would not be detected. Future studies would consider the viability of the individual immune subsets after incubation with naltrexone.

An ability to modulate TLR activity would provide justification to support the use of naltrexone for the treatment of inflammatory conditions in which these receptors play a pathogenic role. For example, recognition of self-DNA/protein complexes by TLR9 mediates
pDC activation in psoriasis, breaking self-immune tolerance\textsuperscript{24}. Members of the TLR family, including TLR9 are often ectopically expressed in tumours \textsuperscript{39,40}, can induce tumour invasion \textit{in vitro}\textsuperscript{41}, and may be an indicator of poor prognosis \textit{in vivo}. Similarly, expression of TLR9 has been found to correlate with the invasive and metastatic potential of pancreatic carcinoma\textsuperscript{42}.

Future studies will be required to investigate whether and how naltrexone inhibits TLR-mediated inflammatory effects in other cell types such as mucosal epithelial cells\textsuperscript{43}, and whether exposure to naltrexone results in upregulation of TLR in a similar manner to that seen for its opioid receptor targets \textsuperscript{44,45}. Additionally, whilst this study investigated the effect of naltrexone on IL-6 and TNFα production, further work examining other cytokines, such as IL-12p70, which might be induced after multiple TLR stimulation would provide further insights into the ability of naltrexone to modulate immune subset activity. It will also be important to consider how the potential pleiotropic effects of naltrexone, including inhibition of TLR mediated functions, inhibition of cellular proliferation and other opioid receptor-mediated activity might contribute to its use in the treatment of inflammatory conditions. In this context, it is important to note that previous studies in inflammatory diseases and cancer have adopted a Low Dose Naltrexone regime as opposed to the dosages used in the treatment of opioid and alcohol dependency. Nanomolar but not micromolar doses of naltrexone were previously seen in studies by Liu \textit{et al} to result in upregulation of pro-apoptotic genes, rendering tumor cells more susceptible to chemotherapy \textsuperscript{46}. It may, therefore, be necessary to identify suitable dosage regimes to obtain optimal therapeutic effects on individual target pathways in different diseases.
Acknowledgements

This study was funded by the Institute for Cancer Vaccines and Immunotherapy (Registered Charity 1080343).

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.

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.

References


Figures legends

Figure 1 – Naltrexone inhibits IL-6 production after TLR7/8 and TLR9 stimulation but not after TLR4 or IL-1 stimulation

1x10^6 PBMC were incubated with A) 1ng/ml LPS (TLR4-L), B) 1µM R848 (TLR7/8-L), C) 1µM CpG (TLR9-L), D) 100ng/ml IL-1 (IL-1R) in the presence or absence of 1-200µM naltrexone for 24 hours. Cell free supernatants were collected and analysed for IL-6 by ELISA. Data shows the mean, SD values are shown and was analysed using a one way ANOVA and Tukey’s multiple comparison test (n=5 TLR-L experiments and n=3 IL-1).

*p<0.05, **<0.01

Figure 2 - Intracellular cytokine staining for TNFα and IL-6 in monocytes and plasmacytoid dendritic cells

1x10^6 PBMC were incubated with either LPS 1ng/ml (A/C), R848 1µM (B) or CpG 1µM (D) and 200µM naltrexone for 6 hours in the presence of brefeldin A for 4 of those hours. After 6 hours, PBMC were stained using antibody panel shown in supplementary fig.3 and stained for either intracellular IL-6 or TNF-α. Results show the mean fluorescence intensity (MFI) of IL-6 or TNF-α within that subsets from 5 donors. Histograms are representative of 5 independent experiments.
Figure 3– NTX inhibits IL-6 production in isolated monocytes and B cells after TLR7/8 and TLR9 stimulation respectively but has no effect on IL-6 production in isolated monocytes after TLR4 stimulation.

A) CD14+ monocytes were isolated from PBMC using magnetic bead isolation. 1x10^5 CD14+ cells were incubated with 1ng/ml LPS (TLR4-L) or 1µM R848 (TLR7/8-L), in the presence or absence of 200µM naltrexone for 24 hours. Cell free supernatants were collected and analysed for IL-6 by ELISA.

B) CD19+ B cells were isolated from PBMC using magnetic bead isolation. 10^5 B cells were incubated with 1µM CpG or 3µg/ml CD40-L and 20ng/ml IL-4, with or without 200µM naltrexone for 24 hours. IL-6 production was measured in cell free supernatants by ELISA. Data is shows the mean and SD values (n=4).

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

A) 1x10^6 PBMC were incubated with 1-200µM naltrexone for 24 hours before percentage viability was assessed using trypan blue exclusion.

B/C) 1x10^6 PBMC were incubated with 1ng/ml LPS (TLR4-L), 1µM R848 (TLR7/8-L), 1µM CpG (TLR9-L) and 200µM naltrexone for 24 hours. PBMC were incubated with Annexin V and 7-AAD before being analysed by flow cytometry. Fig. 5B shows the gating strategy and Fig.5C show results from 4 donors. AV-7AAD- are viable cells, AV+7AAD- are in early apoptosis and AV+7AAD+ are in late apoptosis.
In review
In review