A preliminary evaluation of the effects of opioids on innate and adaptive human *in vitro* immune function

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Abstract

Background: Studies have demonstrated that whereas some opioids have little effect on immunity (e.g. buprenorphine), others can be immunosuppressive (e.g. morphine) or immunostimulatory (e.g. tramadol). However, a variety of approaches have been used and the findings are variable. We hypothesised that opioids have differential effects on immunity via direct actions on neutrophils, monocytes, NK and T cells and this is the first study to systematically evaluate the influence of eight opioids on neutrophil and monocyte phagocytosis and oxidative burst responses, natural killer (NK) cell cytotoxicity and T cell responsiveness *in vitro*.

Methods: Peripheral blood was obtained from healthy volunteers and the effects of clinically relevant concentrations of morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine on phagocytosis and oxidative burst responses were determined using whole blood flow cytometry. The influence of opioids on the capacity of resting and IL-2 stimulated isolated peripheral blood mononuclear cells (PBMCs) to kill NK cell-sensitive K562 cells, and the responsiveness of PBMC sub-populations to IL-2 and polyclonal stimulation were also evaluated.

Results: Methadone, oxycodone and diamorphine inhibited the production of IL-6 by IL-2 stimulated PBMCs. None of the opioids influenced the other measured immune parameters, although there was a trend for morphine, tramadol, fentanyl and buprenorphine to inhibit phagocytosis and oxidative burst responses to *E.coli*.

Conclusions: Preliminary studies using standardised *in vitro* methodologies have demonstrated that some therapeutic opioids suppress IL-6 production. Although this

might potentially suppress bacterial defence mechanisms, it would have little direct effect on anti-cancer immunity. These findings should be confirmed in larger *in vitro* and clinical studies.

Keywords: opioids; phagocytosis; oxidative burst; NK cell cytotoxicity; T cells; immunoregulation

1. Introduction

The commonly prescribed opioids in palliative care include morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine [1, 2] and these exhibit multisystem effects due to their interactions with receptors that are targeted by the endogenous opioid system. The reported presence of opioid receptors on activated T lymphocyte, monocyte, and granulocyte populations [3] suggests that opioids might also influence immune function.

As the immune system plays a key role in anti-tumour immunity and protection against infection, both of which are important in the non-terminal patient receiving palliative care, its impairment might have an influence on the clinical progress of patients that require pain relief. This concept appears to be clinically relevant, as opioids have been associated with an increased risk and severity of infection [4, 5]. Although previous *in vitro* and *in vivo* studies have demonstrated that opioids can have differential effects on the immune system, these have used different methodologies and the conclusions are variable. Notwithstanding this, the general consensus is that some opioids (eg, buprenorphine) seem to have no effects on immune function, whereas others tend to be immunosuppressive (eg, morphine) or immunostimulatory (eg, tramadol) [2, 6, 7]. This is likely due to a combination of direct effects on immune cells and, *in vivo*, via indirect effects that involve centrally-mediated mechanisms and the systemic production and release of immunomodulatory mediators [8-10]. *In vitro* experiments indicate that the immunoregulatory effects of morphine are mediated via interactions with μ opioid receptors (MORs) on immune cells [3].

Opioid-immune system interactions are complex and their differential immunological consequences depend on the molecular structure of the opioids, their receptor binding profiles and non-opioid mediated effects such as serotonin re-uptake inhibition in the case of tramadol [11]. The discovery that morphine, fentanyl, buprenorphine, methadone and oxycodone are Toll-like receptor 4 (TLR4) agonists suggests that they might also directly influence innate immune responsiveness [12], probably via a MOR mediated mechanism [13]. It has also recently been demonstrated that chronic administration of morphine can abrogate the natural tolerance to the proinflammatory effects of lipopolysaccharide due to effects on TLR4 [14].

Opioids also have indirect effects on immune cells *in vivo* via centrally-produced mediators such as immunosuppressive glucocorticoids that are released by the hypothalamic-pituitary-adrenal (HPA) axis, or the release of immunosuppressive amines from the sympathetic nervous system (SNS) which innervates lymphoid organs [10].

The choice of opioids that are prescribed in clinical practice could therefore be critical in patients with cancer or infection, in whom impaired immunity could significantly influence their clinical course. Although therapeutic opioids predominantly exert their analgesic effects via the MOR pathway, their analgesic and side-effects differences are mediated through variations in drug-receptor interactions. We therefore hypothesised that opioids also have differential effects on immunity via direct actions on neutrophils, monocytes, NK and T cells. This study therefore used standardised and consistent methodologies to systematically assess

the *in vitro* effects of eight therapeutic opioids on multiple aspects of immune function.

2. Methods

2.1. Ethics

Ethical approval for this study was obtained from the University of Sheffield Medical School Research Ethics Committee (Reference Number: SMBRER102).

2.2. Blood samples

Peripheral blood from healthy volunteers aged 25-33 years old was collected into lithium heparin Vacutainers® (BD Biosciences, Oxford, UK) following written informed consent. All blood samples were taken in the morning or early afternoon. At least three subjects were used to screen for the effects of each opioid on the immune function parameters and the number of experimental replicates was increased if effects were suggested.

2.3. Opioids

Morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine were obtained from the Pharmacy at the Royal Hallamshire Hospital, Sheffield, UK. Each opioid was diluted with RPMI 1640 medium containing 10% v/v fetal bovine serum (FBS; Invitrogen Ltd., Paisley, UK), 100 μg/ml streptomycin and 100 U/ml penicillin or phosphate buffered saline (PBS) immediately prior to each experiment. Concentrations of the opioids used in the

current study (Table 1) were reflective of *in vivo* levels that have been reported in volunteer and clinical studies [15-22].

Although diamorphine and codeine are prodrugs of morphine, and some other opioids have effects mediated by their metabolites, these opioids were included to produce a baseline of the direct immune effects of the (pro)drugs, including potential non-MOR effects. For example, tramadol was included because its analgesic effect depends on the active metabolite, the molecule may also exert immune effects via serotoninergic pathways [6, 23]. Furthermore, diamorphine has been shown to have direct effects on exon 11 variants of MOR-1 receptor [24].

Opioid	**Final concentrations used in this study (ng/ml)**			
Morphine	20	100	500	2,500
Tramadol	80	400	2,000	10,000
Fentanyl	0.8	4	20	100
Buprenorphine	0.8	4	20	100
Methadone	20	100	500	2,500
Oxycodone	4	20	100	500
Diamorphine	10	50	250	1,250
Codeine	16	80	400	2,000

Table 1: Opioid concentrations used in this study.

The opioid concentrations used in this study relate to those that are found clinically, with the maximal clinical concentration falling in the middle of the concentrations used, with 5x dilutions above and below the clinical maximum plasma concentration. In the NK cell cytotoxicity assay, the central two concentrations were used. In the phagocytosis and oxidative burst assay the lowest concentration was used in addition to these. In the T cell and cytokine assays all concentrations were used (with the

addition of a supra-maximal concentration, 10x that of the top concentration, for the T cell assays).

2.4. Influence of opioids on neutrophil and monocyte function

The influence of opioids on neutrophil and monocyte phagocytic and oxidative burst responses was determined by whole blood flow cytometry using PHAGOBURST® and BURSTTEST® kits (both ORPEGEN Pharma GmbH, Heidleberg, Germany) according to the supplier's recommended protocols. For these assays, heparinized whole blood (50μl) was incubated for 60 min at 37°C with 50μl of opioid or PBS. For the evaluation of phagocytosis, samples were then incubated with 10μl FITC-labelled opsonised *E.coli* (bacteria/ml) for 10 min at 37°C (4°C as a control). Opioids were not washed out. Following quenching (to exclude fluorescent signals from adhered rather than internalized bacteria), erythrocytes were lysed, samples washed and the proportion of neutrophils and monocytes that had phagocytosed FITC-*E.coli* and the total amount of *E.coli* that had been phagocytosed (on the basis of the median fluorescent intensity, MFI) were analysed using a BD Biosciences FACSCalibur™ flow cytometer and BD Biosciences CELLQuest™ acquisition and analysis software. For the oxidative burst reaction, opioid-treated samples and controls were incubated for 10 min at 37°C with 10μl opsonised, unconjugated *E.coli* (bacteria/ml), 8.1μM phorbol myristate acetate (PMA), 5μM N-formyl-methionine-leucine-phenylalanine (fMLP) or washing solution (negative control). Dihydrorhodamine 123 substrate solution (10μl) was then added, and the samples were incubated at 37°C for a further 10 min. Opioids were not washed out. Erythrocytes were lysed, samples washed and the proportion of cells

undergoing the oxidative burst reaction and the intensity of this response were analysed. For both assays, data on a minimum of 5,000 events (cells) in the monocyte region and more than 10,000 cells in the neutrophil region were collected. The reproducibility of these assays (coefficient of variation) is 0.1% for the proportion of neutrophils undergoing the oxidative burst reaction to *E. coli*; 4.8% for the MFI of this response; 1.1% for the proportion of monocytes undergoing the oxidative burst reaction to *E. coli*; and 6.5% for the MFI of this response (ORPEGEN Pharma GmbH).

2.5. Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were used for the natural killer (NK) cell and T cell assays. PBMCs were separated from whole blood by density gradient centrifugation (300xg for 30 minutes at room temperature, no brake) using Nycoprep 1.077 (Axis-Shield POCAS, Oslo, Norway). As platelet-derived secretory products can inhibit NK cell cytotoxicity responses, platelets were removed by layering 10 ml of Nycoprep 1.068 (comprising 4 parts Nycoprep 1.077 and 1 part PBS) under 10 ml of the PBMC interface acquired during the first centrifugation and samples were centrifuged at 400xg for 15 min at room temperature (no brake) [25]. The supernatant was removed and the cell pellet was washed and re-suspended in supplemented RPMI 1640 medium, as detailed above (viable cells/ml).

2.6. Influence of opioids on natural killer (NK) activation and function

The effect of opioids on the capacity of PBMCs to kill NK cell-sensitive K562

human erythroleukemic target cells (Health Protection Agency Culture Collection, Porton Down, UK) was determined using flow cytometry, as described previously [25]. Briefly, PBMCs (cells/well) were stimulated with 100 U/ml (50 ng/ml) recombinant human IL-2 (Miltenyi Biotec) in the presence or absence of different opioid concentrations for 3 days (37°C, 5% v/v , 100% humidity) in 96-well microtiter plates. PBMCs were harvested, washed (to remove IL-2 and opioids) and incubated with MitoTracker™ Green (MTG; Invitrogen Ltd., Paisley, UK) labelled K562 target cells (effector:target (E:T) ratios of 100:1, 50:1, 25:1 and 12.5:1) in 12x75 mm polycarbonate tubes for 3 h at 37°C. The viability stain propidium iodide (PI; Sigma-Aldrich, Gillingham, UK) was added to the samples 10 min prior to analysis and the proportions of viable (MTG^+PI^-) and non-viable (MTG^+PI^+) K562 target cells were determined using a BD™ LSRII flow cytometer (BD Biosciences) [25].

For each E:T cell ratio, NK cell cytotoxicity was represented by the proportion of non-viable K562 cells (number of MTG^+PI^+ K562 cells/total number of MTG^+ labelled K562 cellsx100) following corrections being made for background target cell death. The area under the cytotoxic curve (AUCC), a composite calculation of cytotoxicity which incorporates all four E:T ratios [26], was also determined as we have previously shown that this increases the reproducibility of the assay [25].

2.7. Influence of opioids on the activation of peripheral blood mononuclear cells

The influence of opioids on the ability of PBMC sub-populations to be activated by IL-2 (100 U/ml) and anti-CD3/anti-CD28 monoclonal antibody (mAb) coated beads (Suppression Inspector, 1:1 bead:cell ratio, Miltenyi Biotec Ltd, Bisley, UK)

was determined by flow cytometry. For this, PBMCs were incubated with opioids or PBS and anti-CD3/anti-CD28 mAb coated beads for 3 days at 37°C, 5% v/v and 100% humidity. Cells were harvested and the supernatants stored at -80°C for subsequent analysis of IL-1β, IL-6, IL-8, IL-10, IL-17A, IFN-γ and TNF-α levels using Cytometric Bead Arrays (CBAs) and a BD FACSArray™ (both BD Biosciences).

The expression of the activation markers CD25 and CD69 by $CD4^+$ and $CD8^+$ T cell subpopulations and NK cells following stimulation in the presence and absence of opioids were determined using a BD™ LSRII flow cytometer and BD Biosciences FACSDiva™ software. For the flow cytometric analysis, PBMCs (1.) were incubated with 0.5µl mouse serum for 15 min at room temperature, after which they were incubated with Pacific Blue™-conjugated anti-CD4 (clone RPA-T4, BioLegend), Alexa Fluor™ 700-conjugated anti-CD8a (clone RPA-T8, BioLegend), FITC-conjugated anti-CD16/CD56 (clones LNK16, MEM-188, AbD Serotec), PE-conjugated anti-CD25 (clone MEM-181, AbD Serotec) and anti-CD69 (clone FN50, BioLegend) mAbs for 30 min at 4°C. Cells were washed and kept on ice prior to flow cytometry. The viability stain 7-Aminoactinomycin D (1µl/100µl cell suspension, Cambridge Biosciences, Cambridge, UK) was added 5-10 min before analysis.

For the analysis, cells were initially identified on the basis of their size and granularity. Doublets and dead cells were excluded and the proportion of $CD4^+$ T cells, $CD8^+$ T cells and NK cells expressing CD25 and CD69, and the intensity of expression were determined. Unstained and cells incubated with non-reactive isotype-matched reagents served as controls.

2.8. Statistical analysis

Data were analysed in Microsoft Excel 2010 and Predictive Analytics SoftWare (PASW) version 18.0 using a paired 2-tailed Student *t*-test (*P value < 0.05) and are presented as mean ± standard error of the mean (SEM), or as individual experiments, as indicated.

3. RESULTS

3.1 Effect of opioids on neutrophil and monocyte phagocytosis and oxidative burst responses

None of the opioids inhibited phagocytosis, although there was a non-significant trend for morphine, tramadol, fentanyl and buprenorphine to inhibit the intensity of the phagocytic response (Figure 1).

None of the opioids had any effect on the proportion of neutrophils that underwent the oxidative burst responses by fMLP, PMA and *E. coli*, although there was a non-significant trend for responses to *E. coli* to be inhibited and PMA to be enhanced, by morphine, tramadol, fentanyl and buprenorphine (Figure 2). These data suggest that the capacity of opioids to influence the recruitment of cells into oxidative burst and its intensity is dependent on the inducing stimuli. The effects of opioids were cell type-dependent, as the 'effects profile' for monocytes differed from that which was observed for neutrophils (Figure 3). It is therefore possible that these opioids have differential effects on various aspects of immunity.

3.2. Effect of opioids on $CD16^{+}CD56^{+}$ natural killer (NK) cells

The incubation of PBMCs for 3 days with IL-2 (100 U/ml) enhanced the ability of NK cells to kill K562 target cells and this was unaffected by any of the opioids tested (Figure 4). IL-2 increased CD69, but not CD25 expression on NK cells and neither of these markers were influenced by any of the opioids (data not shown). Incubation of PBMCs with anti-CD3/anti-CD28 mAb coated beads for 3 days also increased the proportion of NK cells that expressed CD69 and, to a greater extent, the intensity of CD69, but not CD25 expression (data not shown). The expression of CD69 and CD25 was not affected by any of the opioids (data not shown).

3.3. Effect of opioids on T cell activation and cytokine secretion

Opioids had no consistent effect on the activation status (CD25, CD69 expression) of $CD4^+$ and $CD8^+$ T cells following a 3-day incubation of PBMCs with anti-CD3/anti-CD28 mAb coated beads. This remained the case if opioids were added at the same time, 1 day after or 1 day before the anti-CD3/anti-CD28 mAb coated beads, or if platelets were present in the PBMC preparations (data not shown, representative graph - Figure 5). Anti-CD3/anti-CD28 mAb coated beads had little effect on cytokine secretion and none of the opioids tested had an influence on this (data not shown).

The stimulation of PBMCs with IL-2 increased the production of IL-1β, IL-6, IL-8, IL-17A, IL-10, IFN-γ and TNF-α. The only consistent statistically significant effect was that methadone, oxycodone and diamorphine decreased IL-6 production from IL-2 stimulated PBMCs (Figure 6).

4. Discussion and Conclusions

Opioids exert actions on most biological systems and have differing physicochemical properties which can elicit a range of pharmacodynamic effects. This is the first systematic analysis of the *in vitro* effects of eight commonly used therapeutic opioids at clinically relevant concentrations across a standardised profile of tests that reflect innate and adaptive immune potential.

4.1. Neutrophil and monocyte phagocytosis

Previous studies using various methodologies have evaluated some therapeutic opioids *in vitro* and *in vivo* and have yielded a range of effects on phagocytosis. Morphine has generally inhibited phagocytosis [27-32], whereas tramadol [31], fentanyl [33] and methadone [32, 34] generally had no effect on phagocytosis. Although none of the opioids in the current study had any statistically significant effects, there was a trend for morphine, tramadol, fentanyl and buprenorphine to inhibit neutrophil and monocyte phagocytosis.

4.2. Neutrophil and monocyte oxidative burst reaction

Previous *in vitro* and *in vivo* studies have generally shown morphine [30, 32] and methadone [30, 34] to inhibit oxidative burst, whereas fentanyl has been reported to have no effect on neutrophil oxidative burst responses in healthy volunteers [16]. Although no opioid tested had a consistent effect on oxidative burst responses in the current study, there was a trend for morphine, tramadol, fentanyl, buprenorphine and methadone to inhibit oxidative burst responses.

4.3. Natural killer cell cytotoxicity

The previous literature using differing methodologies is often conflicting, however in general morphine [35], fentanyl [7, 36], methadone [37] and diamorphine [38] decrease NK cell cytotoxicity, whereas buprenorphine has no effect [36] and tramadol enhances it [6, 39]. In the current study, none of the opioids had any effect on the activation status or cytotoxicity of unstimulated and stimulated human NK cells in PBMC preparations. This suggests that the reported *in vivo* manifestations of opioids on NK cells are mediated indirectly through central pathways.

4.4. Effect of opioids on IL-2 and anti-CD3/anti-CD28 mAb-induced T cell activation and cytokine secretion

This is the first *in vitro* study to have assessed the effect of opioids on the activation status of human T cells on the basis of CD25 and CD69 expression. Anti-CD3/anti-CD28 mAb stimulation activated $CD4^+$ and $CD8^+$ T cells, which was not affected by any of the opioids. Previous *in vitro* studies, using proliferation or cytolytic assays, are generally consistent with the current study, in that clinically relevant concentrations of morphine, methadone and diamorphine had no effect [40-42].

Cytokine secretion from PBMCs was used as another measure of lymphocyte activation. There are several heterogeneous studies relating to the effects of opioids on cytokine secretion in the literature. In general, morphine has been reported to decrease IL-1β, IL-2, IL-4, IL-6, IL-12 and IFN-γ production [9, 27, 43], but to have mixed effects on IL-10 and TNF levels [9, 43]. Fentanyl has been demonstrated to

decrease IL-2 and IFN-γ production, whereas buprenorphine has been reported to have no effect [36]. Codeine has been shown to increase IL-8 and TNF-α production [8]. In post-operative studies, morphine has been reported to inhibit IL-2 production, which was unaffected by fentanyl and either unaffected or increased by tramadol [44, 45].

The only significant effect which was been observed in the current study was that methadone, oxycodone and diamorphine significantly decreased IL-6 secretion from IL-2 stimulated PBMCs. IL-6 has a range of pro- and anti-inflammatory effects and its levels correlate with the acute phase reactant C-Reactive Protein [46]. IL-6 also influences lipid and bone metabolism [47]. Any inhibition of IL-6 production could therefore have multiple downstream effects on several organ systems.

The findings of this systematic evaluation indicate that further studies are needed with larger numbers of subjects to evaluate the effects of morphine, tramadol, fentanyl, buprenorphine and methadone on neutrophil and monocyte phagocytosis and oxidative burst. As NK and T cells are likely to be modified by indirect effects of opioids, these also need further evaluation in *in vivo* models. However, all of these aspects of immune defence need to be investigated in human clinical models with disease outcomes as endpoints in order to better inform clinical practice.

4.6. Limitations of present study

The use of *in vitro* models meant that only the direct effects of the opioids were measured on immune cells. However, clinically used opioids also have indirect

effects via the SNS and HPA axis, which may modulate or negate these direct effects in whole animals. The sample size was small, as the primary aim of this study was to screen for immune effects of many opioids and a larger sample may have shown greater differences between opioids. Thus caution is needed as to not over-interpret the results. However, retrospective power and sample size calculations have been performed for those assays for which opioids potentially showed an effect and some of these calculations have a power greater than 80% with a sample size compatible with that performed. Only healthy volunteers were included and although the immune cells were activated *in vitro* this might not truly reflect effects that could be present on cells from different patient groups, especially those with immune activation or suppression from other biological or therapeutic causes. Further confirmatory studies should therefore employ larger samples, using the same opioids in all subjects and using a test-retest method to look for variations *within* individuals as well as between subjects.

4.7. Conclusion

Opioids are currently prescribed for pain on the basis of their reported efficacy, their clinically recognisable toxicities and economic factors. The findings of this small, but systematic healthy volunteer preliminary investigation indicate that opioids might differentially influence IL-6 production by a direct effect on the cells, with methadone, oxycodone and diamorphine being suppressive. There might also be direct effects of morphine, tramadol, fentanyl, buprenorphine and methadone on neutrophil and monocyte phagocytosis and oxidative burst, which warrant further

investigation with more subjects. However, further investigation in human clinical models with disease outcomes as endpoints is paramount.

Despite the potential negative impact of certain opioids on aspects of immune function *in vitro*, optimal pain control remains the key clinical goal, as pain itself might be immunosuppressive [6, 48]. In keeping with the move towards personalised medicine, including for pain management [49], with more comprehensive clinical data, we could more rationally select the opioid to administer to an individual patient with cancer, in order to optimise pain control without potentially negatively impacting on their immune function. However, with currently available data, opioid choice should principally be on efficacy and more clinically overt toxicities in the individual patient.

Author Contributions

JB, SHA, AGP conception and design, JB and GF acquisition of data and analysis, JB and AGP interpretation of data; JB drafted the article, with revisions from all authors. All authors discussed the results, commented on the manuscript and approved the final version to be published.

JB is the guarantor.

None of the authors declare a conflict of Interest

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Figure legends:

Figure 1 Effect of morphine, tramadol, fentanyl, buprenorphine and oxycodone on neutrophil and monocyte phagocytosis. The proportion of neutrophils and monocytes phagocytosing FITC-*E.coli* (**left panel**) and the intensity of the phagocytic response (median channel of fluorescent intensity, MFI; **right panel**). Data are means ± SEM from 5 subjects (n=4 for monocytes at 20 and 100 ng/ml of morphine) and are presented as absolute differences from the no opioid control. No opioid (baseline) values (mean ± SEM) are included at the top of the graphs. No statistically significant differences between the responsiveness of opioid-treated and untreated cells were detected (paired Student *t*-test)

Figure 2 Effect of morphine, tramadol, fentanyl, buprenorphine and oxycodone on neutrophil oxidative burst responses. The proportion of neutrophils undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells, median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. Data are means ± SEM from 5 subjects and are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Statistically significant differences between the responsiveness of opioid-treated and untreated cells is indicated (* = P<0.05, paired Student *t*-test)

Figure 3 Effect of morphine, tramadol, fentanyl, buprenorphine and oxycodone on monocyte oxidative burst responses. The proportion of monocytes undergoing the oxidative burst reaction (**left panel**) and the intensity of this response in these positive cells, median channel of fluorescent intensity (MFI, **right panel**), to fMLP, PMA and *E.coli*. Data are means ± SEM from 5 subjects and are presented as absolute differences from the no opioid control. No opioid control (baseline) values (mean ± SEM) are included at the top of the graphs. Statistically significant differences between the responsiveness of opioid-treated and untreated cells is indicated (* = P<0.05, paired Student *t*-test)

Figure 4 Representative figure illustrating the effect of morphine on IL-2 induced NK cell cytotoxicity of K562 target cells. Isolated PBMCs, without platelets, were incubated for 3 days with IL-2 and opioid. Controls comprised unstimulated and IL-2 stimulated cells cultured in the absence of opioid. The adjusted area under the cytotoxic curve (AUCC) was calculated using the formula presented in the methods. Values for the adjusted AUCC for each of the experimental conditions are indicated in the legend. No statistical difference in the NK cell cytotoxicity of untreated and morphine-treated PBMCs was observed (paired Student *t*-test). Data are means ± SEM of 3 subjects

Figure 5 Representative figure illustrating the effect of morphine on CD69 expression by $CD4^+$ and $CD8^+$ T cells. The proportion (%) of $CD4^+$ and $CD8^+$ cells expressing CD69 (**upper panel**) and the intensity of CD69 expression on positive cells (median channel of fluorescent intensity, MFI; **lower panel**). PBMCs were incubated with the indicated concentrations of morphine for 3 days prior to assessing CD69 expression. All cells, other than the unstimulated control, were activated with anti-CD3/anti-CD28 mAb coated beads for 3 days, added at the same time as the opioid. No statistical difference between the responsiveness of opioid-treated and untreated anti-CD3/anti-CD28 mAb stimulated cells was observed (paired Student t-test). Data are means ± SEM from 5 subjects

Figure 6 Effect of morphine, methadone, oxycodone and diamorphine on IL-6 secretion by IL-2 stimulated cells. Effect of methadone, oxycodone and diamorphine on IL-6 production by IL-2 (**left Y-axis, blue**) and Miltenyi anti-CD3/anti-CD28 mAb coated bead (**right Y-axis, red**) stimulated PBMCs. IL-6 concentrations are presented on the Y-axes, in pg/ml. Unstimulated (unstim) and stimulated (stim) controls without opioid are included. **IL-6 levels in IL-2 stimulated PBMCs were decreased by methadone at all concentrations, 4, 20 and 500 ng/ml of oxycodone, and 10, 50 and 1250 ng/ml of diamorphine decreased levels** ($P<0.05$; paired Student *t*-test). Data are means ± SEM from a minimum of 3 subjects