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Direct effects of heroin and methadone on T cell function

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ABSTRACT

Opioid addiction presents a relevant health challenge, with chronic heroin use linked to detrimental effects on various aspects of physical, mental, and sociological health. Opioid maintenance therapy (OMT), particularly using methadone, is the primary treatment option for heroin addiction. Previous studies using blood samples from current heroin addicts and OMT patients have shown immunomodulatory effects of heroin and methadone on T cell function. However, various additional factors beyond heroin and methadone affect these results, including the consumption of other substances, a stressful lifestyle, comorbid psychological and somatic disorders, as well as additional medications. Therefore, we here investigated the direct effects of heroin and methadone on purified human T cells *in vitro*. Our results reveal that both, heroin and methadone directly suppress T cell activation and proliferation. Strikingly, this inhibitory effect was markedly stronger in the presence of methadone, correlating with a decrease in secretion of pro-inflammatory cytokines. While heroin did not interfere with the *in vitro* differentiation and expansion of regulatory T cells (Tregs), methadone significantly impaired the proliferation of Tregs. Overall, our findings suggest a direct inhibitory impact of both opioids on effector T cell function *in vitro*, with methadone additionally interfering with Treg induction and expansion in contrast to heroin.

1. Introduction

Opioid addiction poses a significant global health problem. Approximately 60 million people were engaged in non-medical, illicit opioid use in 2021, with 31.5 million primarily using opiates, such as heroin [1]. Heroin addiction is associated with detrimental effects on sociological, mental, and physical health, along with an increased susceptibility to infectious diseases. This elevated vulnerability to infections arises from several factors, including consumption of contaminated street heroin, sharing of drug paraphernalia, as well as immunomodulatory effects of heroin itself [2–4]. Opioid maintenance treatment (OMT) is the primary treatment for heroin addiction. OMT with opioid agonists, such as methadone or buprenorphine, has been proven to reduce heroin use and craving and to suppress opiate withdrawal. In addition, there are studies that OMT partially reverses the effects of heroin use on immune cell function [5–7].

There is experimental evidence that heroin use exhibits suppressive properties on several immune cells, including T cells. Sacerdote *et al.* assessed the proliferative activity of immune cells in heroin users and OMT patients by analysing phytohaemagglutinin-induced proliferation of peripheral blood mononuclear cells (PBMCs) [6]. They found a reduction in PBMC proliferation in heroin users but not in OMT patients. Well in line, in a previous study we have shown a decreased proliferative capacity of $CD4^+$ T cells derived from blood of heroin addicts compared to healthy controls and OMT patients after *in vitro* stimulation [7]. Furthermore, our recent findings indicate that heroin addicts exhibit impaired T cell proliferation compared to methadone maintenance therapy (MMT) patients [8]. This suggests a potential restorative effect of methadone on heroin-induced immune modulation.

However, the underlying mechanisms of these immunomodulatory effects remain incompletely understood. Interestingly, we previously demonstrated increased numbers of regulatory T cells (Tregs) in blood of illicit heroin users compared to OMT patients and healthy volunteers [7]. Tregs play a crucial role in maintaining immune homeostasis and preventing excessive immune responses [9]. Nonetheless, an elevation in Treg numbers also correlates with chronic infections and tumour

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progression, as they inhibit effector T cells, rendering them unable to sufficiently combat pathogens and abnormal cells [10,11]. Upon Treg depletion, *in vitro* proliferation of $CD4^+$ T cell from heroin addicts was partially restored, indicating a contribution of these regulatory cells to immunosuppression in illicit heroin users [7]. Interestingly, heroin-assisted treatment (HAT) led to normalization of Treg frequencies to levels found in MMT patients [8], suggesting that increased Treg numbers in illicit heroin users might be attributed to their stressful living conditions and/or other factors rather than to heroin consumption itself. Nevertheless, $CD4^+$ T cells isolated from HAT patients still had an attenuated proliferative potential compared to $CD4^+$ T cells from MMT patients [8]. These results underline the complexity of factors that might affect T cell function in opioid-addicted patients, making it difficult to determine the specific effect of heroin and methadone on T cells from these data.

Besides Tregs, cytokines are pivotal mediators of immune regulation. Although the impact of opioids on cytokine production has been intensively studied, results are contradictory. For instance, Azarang and colleagues showed a decreased secretion of pro-inflammatory interferon-gamma (IFN-γ) and an increase in immunosuppressive interleukin (IL)-10 by *in vitro* stimulated whole blood cells from opioid addicts compared to healthy controls [12]. In contrast, animal studies by Holan *et al.* suggested an augmented IFN-γ, IL-12 and IL-1beta(β) production, while anti-inflammatory cytokines such as IL-4 and IL-10 were reduced in heroin-treated mice in comparison to controls [13]. A clinical trial by Neri *et al.* demonstrated lower plasma levels of tumor necrosis factor-alpha (TNF-α), IL-1β and IL-2 in heroin users, which were normalized or even elevated compared to healthy individuals after 2 years of treatment with methadone, illustrating its restorative potential [14]. In accordance, we recently reported decreased secretion of pro-inflammatory cytokines of *in vitro* stimulated $CD4^+$ T cells from heroin addicts in contrast to MMT patients [8]. Nevertheless, these studies were conducted with either whole blood cells or PBMCs from current heroin users or OMT patients, displaying the long-term effect of these substances on immune responses. Other influencing factors such as contaminations, the use of other drugs or medications, individual life circumstances, as well as psychological or physical illnesses, also contribute to observed effects. Since it has been shown that T cells express opioid receptors, it seems very likely that they can directly respond to heroin and methadone [8,15,16]. However, the direct impact of both substances on T cell function remains elusive. Thus, using selected *in vitro* assays, the present study aims to investigate the direct effect of heroin and methadone on the functional phenotype of human T cells from healthy donors.

2. Material and methods

2.1. Isolation of PBMCs

Buffy coats of healthy adult blood donors were obtained from the Institute for Transfusion Medicine and approved by the internal review board of the University Hospital Essen. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Bicoll. Isolated cells were stored in fetal calf serum (FCS, Gibco) with 20 % dimethyl sulfoxide (DMSO, Roth) in liquid nitrogen until use.

2.2. Stimulation of PBMCs

In order to analyse T cell activation, 5×10^5 PBMCs were seeded into a 96 well flat bottom plate in 200 μL IMDM complete (IMDM (Gibco) supplemented with 10 % FCS and 25 μM β-mercaptoethanol (Roth)). Cells were stimulated with T cell TransAct (Miltenyi Biotech) at the recommended dilution of 1:100 in the presence of 50 μM heroin, 50 μM methadone (both Sigma Aldrich), or the respective vehicle (acetonitrile, Sigma Aldrich and methanol, Roth) for 24 h. The work with heroin and methadone was approved by the Opium Office of the Federal Institute for Drugs and Medical Devices (4562054).

2.3. Isolation of T cells

$CD4^+$ T cells were isolated or enriched from PBMCs by magnetic activated cell sorting (MACS) using the $CD4^+$ T cell isolation kit II (Miltenyi Biotech) according to the manufacturer's protocol. In order to get naïve ($CD25^-$) $CD4^+$ T cells for Treg induction, biotin-labelled anti-CD25 antibody (Miltenyi Biotech) was added to the antibody cocktail. For Treg expansion, MACS-enriched $CD4^+$ T cells were stained with fluorescent-labelled antibodies against CD4 (BD Bioscience), CD25 (Miltenyi Biotech), and CD127 (BD Bioscience) and further isolated by fluorescence activated cell sorting (FACS) using a BD ARIA II cell sorter (BD Biosciences).

2.4. Proliferation and cytokine secretion

MACS-sorted $CD4^+$ T cells were stained with the Cell Proliferation Dye eFluor670 (Thermo Fisher) according to the manufacturer's instructions. 5×10^4 cells in 200 μL IMDM complete were stimulated in a 96 well round bottom plate with TransAct (1:100) in duplicates for 48 and 72 h and treated with 50 μM heroin, 50 μM methadone, acetonitrile, or methanol, respectively. T cell proliferation was assessed as loss of the proliferation dye by flow cytometry using a LSRII and DIVA software (BD Biosciences). Cytokine concentrations in cell culture supernatants were quantified by Luminex technology (R&D Systems) according to the manufacturer's protocol.

2.5. Treg differentiation

For the induction of Tregs, 5×10^4 naïve $CD4^+CD25^-$ T cells were isolated and cultured in duplicates in a 96 well flat bottom plate in 200 μL IMDM complete supplemented with 5 ng/mL TGF-β (R&D Systems), 100 U/mL IL-2 (Gibco), TransAct (1:100) and 50 μM heroin, 50 μM methadone, or vehicles for 6 days. On day 4, cells were split 1:2 into new plates with fresh medium containing 5 ng/mL TGF-β, 100 U/mL IL-2, and heroin, methadone, or vehicles.

2.6. Treg expansion

FACS-sorted Tregs, defined as $CD4^+CD25^+CD127^-$, were seeded in 100 μL IMDM complete containing 500 U/mL IL-2, TransAct (1:100) and 50 μM heroin, 50 μM methadone, or vehicles in a 96 well round bottom plate. On day 1, 100 μL fresh medium with 500 U/mL IL-2 and 50 μM heroin or methadone was added to the culture. After 4 days, cells were harvested and seeded in a new 96 well flat bottom plate in 200 μL medium with 500 U/mL IL-2 and 50 μM heroin, methadone, acetonitrile, or methanol. On day 7, cells were harvested, counted, and analysed for their Treg phenotype by flow cytometry.

2.7. Flow cytometry

For flow cytometric analyses, cells were stained with fluorochrome-labelled anti-CD4, anti-CD127, anti-CD69 (all BD Bioscience), and anti-CD25 (Miltenyi and BD Bioscience). The fixable viability dye eFluor780 (FVD, eBioscience) was used to distinguish living and dead cells. For intracellular staining of Foxp3, cells were washed with phosphate-buffered saline (PBS) and incubated with fluorescent-labelled anti-Foxp3 using the Fixation/Permeabilization Kit (both eBioscience) according to the manufacturer's recommendations. Analyses were performed with a LSR II using FACSDiva software from BD Bioscience.

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism 10 software. To test for Gaussian distribution, D'Agostino-Pearson and

Shapiro-Wilk normality tests were used. If data passed normality testing paired *t*-test was performed, otherwise Wilcoxon test was used. *P*-values of 0.05 or less were considered indicative of statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). All mean values and the respective standard error of the mean (SEM) are reported in the supplement.

3. Results

3.1. Effects of heroin and methadone on T cell activation

A growing body of evidence indicates that heroin possesses

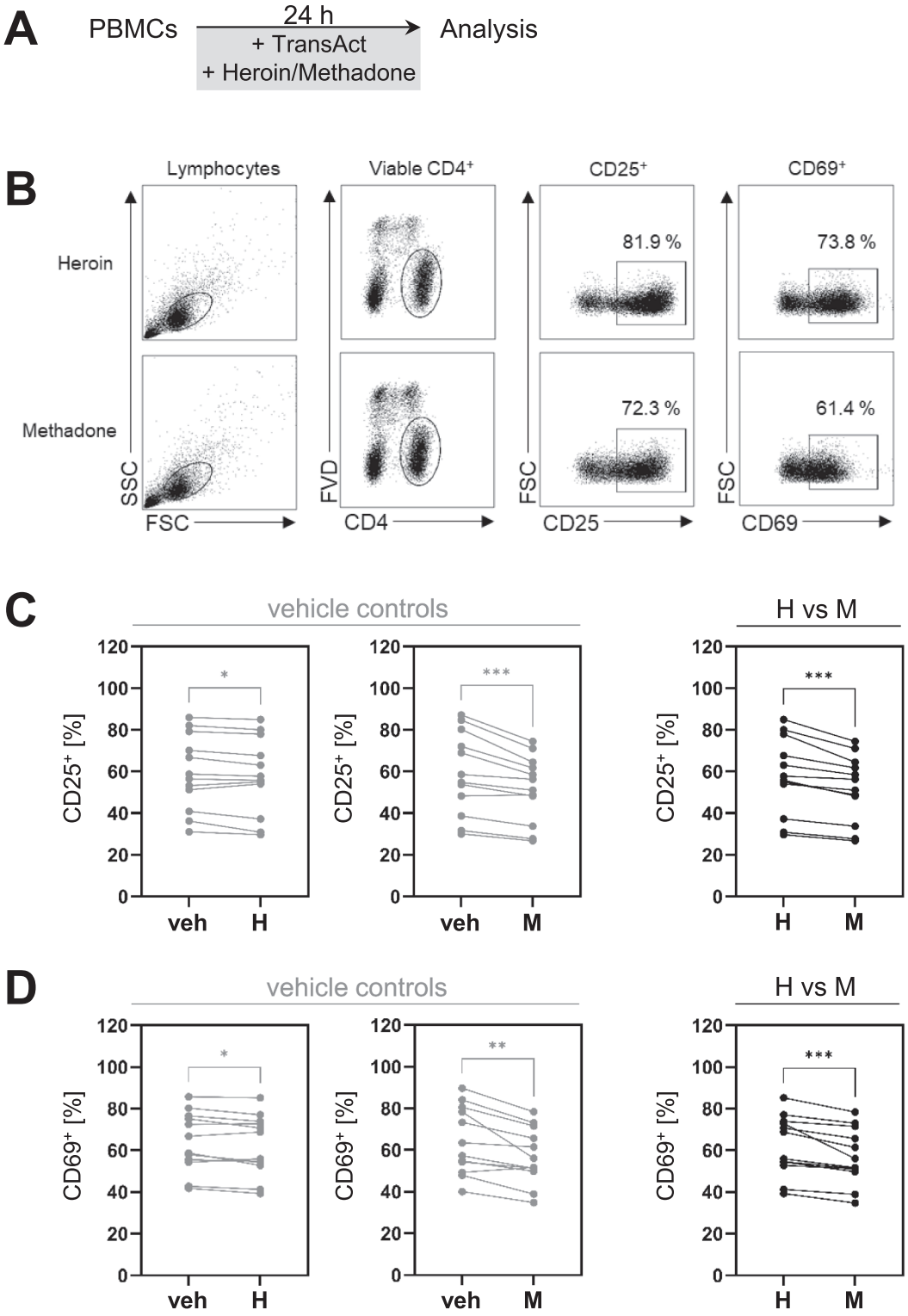

Fig. 1. T cell activation upon heroin and methadone treatment. (A) PBMCs were isolated from blood of healthy donors, stimulated with TransAct beads and either treated with 50 μM heroin (H), methadone (M), or respective vehicle (veh) for 24 h. **(B)** Representative flow cytometry gating strategy for T cell activation. **(C)** Frequencies of CD25- and **(D)** CD69-expressing $CD4^+$ T cells (n = 12). Data from 5 independent experiments are shown. Statistical analyses were performed using paired *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

immunosuppressive properties, while methadone has been demonstrated to partially reverse these effects in OMT programs [6,8]. Here, we aimed to analyse the direct impact of both opioids on T cell activation. To this end, we isolated PBMCs from healthy donors, stimulated them with T cell TransAct beads and either heroin, methadone, or respective vehicles for 24 h (Fig. 1A). T cell activation was determined using flow cytometry by assessing frequencies of CD25- and CD69-expressing $CD4^+$ T cells within the viable lymphocyte population (Fig. 1B). Both, heroin and methadone significantly reduced the expression of activation-associated molecules compared to their respective vehicle controls, with a more pronounced decrease upon methadone treatment (Fig. 1C, D, left). Interestingly, by directly comparing heroin and methadone conditions, cells treated with methadone on average showed significantly lower frequencies of $CD25^+$ (mean: 51.89 ± 4.57 % SEM) and $CD69^+$ $CD4^+$ T cells (56.94 ± 3.89 %) compared to their heroin-treated counterparts with 57.81 ± 5.32 % and 62.23 ± 4.21 %, respectively (Fig. 1C, D, right). Hence, heroin and methadone seem to directly affect T cell activation *in vitro*.

3.2. Reduced $CD4^+$ T cell proliferation in the presence of methadone

In previous studies, we have shown a reduced proliferative capacity for T cells isolated from heroin users compared to OMT and MMT patients, as well as healthy individuals [7,8]. However, it remains elusive whether this observation is due to circumstances of chronic heroin abuse or whether the substance itself directly affects T cell proliferation. Thus,

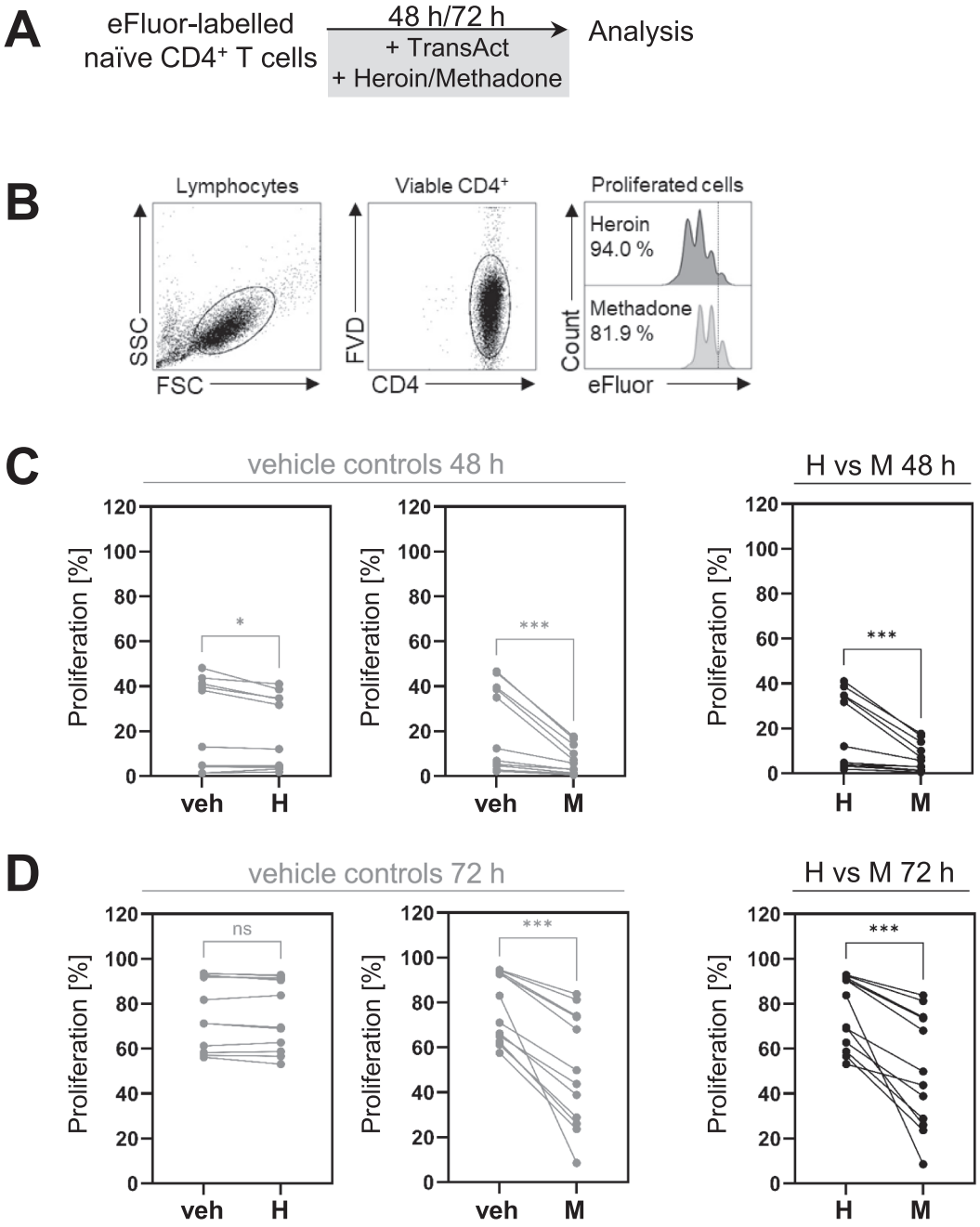

Fig. 2. Proliferation of heroin- and methadone-treated $CD4^+$ T cells. (A) $CD4^+$ T cells were isolated from PBMCs of healthy donors and labelled with eFluor. After 48 and 72 h of stimulation with TransAct beads and heroin (H), methadone (M), or vehicle (veh) treatment, proliferation was analysed. **(B)** T cell proliferation was assessed by flow cytometry, measuring the loss of the proliferation dye eFluor. **(C)** Frequencies of proliferated $CD4^+$ T cells after 48 h and **(D)** 72 h (n = 11–12). Data from 5 independent experiments are shown and statistical analyses were performed using Wilcoxon test (*$p < 0.05$, ***$p < 0.001$).

we next isolated and stimulated CD4$^+$ T cells from healthy donors in the presence of heroin or methadone for 48 and 72 h (Fig. 2A). As control, cells from the same donor were treated with the vehicle solvents acetonitrile and methanol. Proliferation was analysed by flow cytometry as loss of the proliferation dye eFluor670 as exemplary shown in Fig. 2B. After 48 h of stimulation in the presence of both, heroin and methadone, we observed a reduced proliferative activity (mean: 19.10 ± 5.01 % SEM and 7.29 ± 1.92 %) compared to the respective vehicle control (21.84 ± 5.98 % and 21.81 ± 5.73 %), while this decrease was again more prominent for methadone (Fig. 2C, left). Interestingly, after 72 h

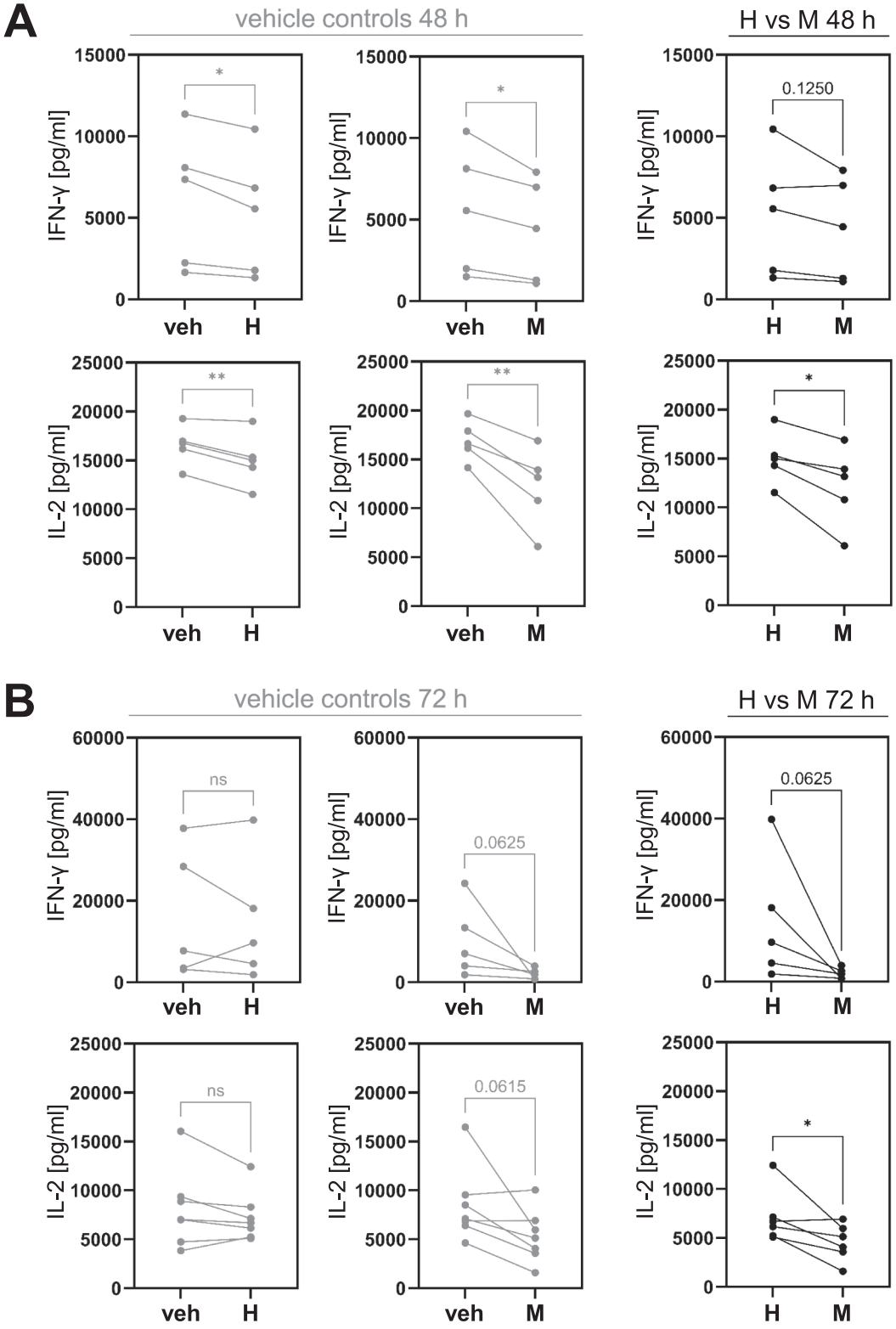

Fig. 3. Cytokine secretion upon heroin and methadone treatment. (A) Cell culture supernatants of stimulated and heroin- (H), methadone- (M), or vehicle- (veh) treated CD4$^+$ T cells (Fig. 2) were analysed for IFN-γ and IL-2 concentrations using Luminex technology after 48 h and **(B)** 72 h (n = 5–7). Data from 2 to 3 independent experiments are shown and statistical analyses were performed using Wilcoxon test and paired *t*-test (*$p < 0.05$, **$p < 0.01$).

these differences were only visible between methadone (50.02 ± 7.37 %) *versus* vehicle (77.93 ± 4.40 %), but diminished for heroin (75.97 ± 4.56 %) *versus* vehicle control (76.73 ± 4.58 %, Fig. 2D, left). A highly significant reduction in T cell proliferation induced by methadone was also detected when directly comparing heroin and methadone conditions after both, 48 and 72 h (Fig. 2C, D, right). Collectively, these results indicate a highly inhibitory effect of methadone on T cell proliferation compared to heroin, which only slightly decreases the proliferative capacity of $CD4^+$ T cells.

3.3. Methadone impairs pro-inflammatory cytokine secretion

In order to assess whether the alleviated T cell proliferation is associated with an impaired secretion of pro-inflammatory cytokines, we collected cell culture supernatants from stimulated and heroin-, methadone-, or vehicle-treated $CD4^+$ T cells after 48 and 72 h (Fig. 2A) and analysed them for IFN-γ and IL-2 concentrations using Luminex technology. Well in line with our previous observations, $CD4^+$ T cells either treated with heroin or methadone showed decreased levels of secreted IFN-γ (mean: 5189 ± 1688 pg/mL SEM and 4351 ± 1407 pg/mL) and IL-2 (15029 ± 1194 pg/mL and 12185 ± 1806 pg/mL) compared to respective vehicle controls (IFN-γ: 6143 ± 1841 pg/mL and 5522 ± 1722 pg/mL, IL-2: 16562 ± 915.2 pg/mL and 16901 ± 917.4 pg/mL) after 48 h (Fig. 3A, left). However, by directly comparing both opioid substances, there was a reduction in cytokine secretion in the presence of methadone, which even reached significance for IL-2 (Fig. 3A, right). Similar results were obtained for heroin *versus* methadone conditions after 72 h of stimulation with on average 14818 ± 6838 pg/mL *versus* 2022 ± 603.6 pg/mL IFN-γ and 7295 ± 953.6 pg/mL *versus* 5333 ± 1021 pg/mL IL-2 (Fig. 3B, right). However, in accordance to our findings regarding proliferative activity, the differences between heroin and vehicle control were abolished after 72 h, while tendencies for reduced cytokine secretion upon methadone treatment compared to controls remained (Fig. 3B, left).

3.4. Methadone-treated $CD4^+$ T cells have a lower potential for Treg differentiation

Besides an impairment of effector T cell function, immunosuppression can also be mediated by the induction of Tregs (iTregs) or the expansion of naturally occurring Tregs (nTregs) [9]. Previously, we demonstrated increased Treg frequencies in blood from heroin users compared to healthy controls and OMT patients [7]. As it has been shown that relative Treg numbers were normalized to levels of MMT

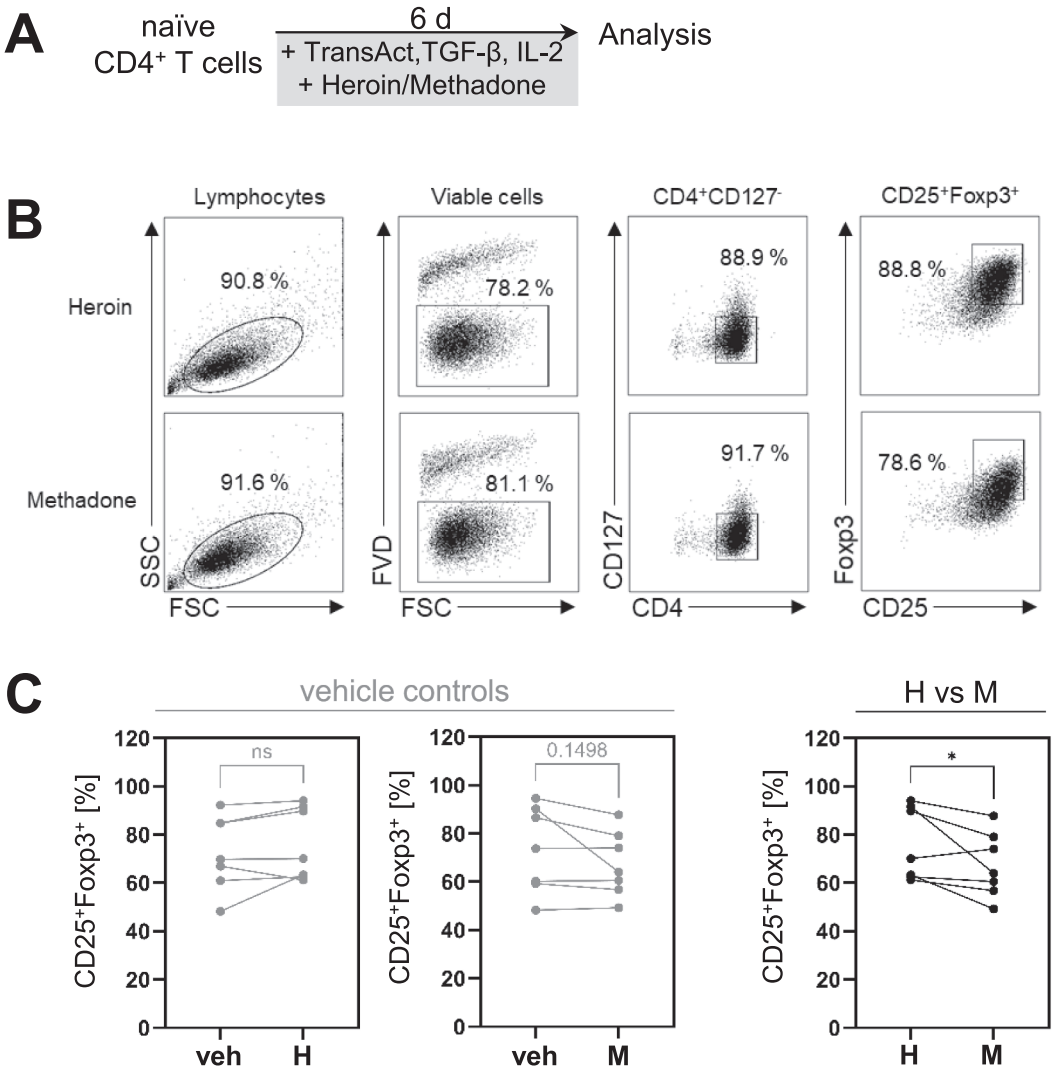

Fig. 4. Treg differentiation of heroin- and methadone-treated $CD4^+$ T cells. (A) Naïve ($CD25^-$) $CD4^+$ T cells were isolated from PBMCs of healthy donors, incubated with TransAct beads, heroin (H), methadone (M), or respective vehicle (veh) and additionally treated with TGF-β and IL-2 for Treg induction for 6 days. **(B)** Treg induction was assessed by Foxp3 and CD25 expression of $CD127^-CD4^+$ T cells by flow cytometry. **(C)** Frequencies of Foxp3- and CD25-expressing $CD127^-CD4^+$ T cells (n = 7). Results from 3 independent experiments are shown. Data were analysed for statistical significance using Wilcoxon test and paired *t*-test (*$p < 0.05$).

participants when heroin was administered in a structured HAT program in contrast to those of illicit heroin users [8], we were interested in the direct effect of heroin and methadone on *in vitro* Treg differentiation. Therefore, we isolated naïve $CD25^{-}CD4^{+}$ T cells from healthy donors and cultured them under Treg-inducing conditions in the presence of either heroin, methadone, or respective vehicle controls for 6 days (Fig. 4A). Afterwards, $CD127^{-}CD4^{+}$ T cells were analysed for Foxp3 and CD25 expression as markers for Treg differentiation (Fig. 4B) [17,18]. While heroin treatment (mean: 76.09 ± 5.67 % SEM) had no impact on Treg differentiation compared to its vehicle control acetonitrile (72.49 ± 5.88 %), methadone slightly decreased frequencies of Foxp3- and CD25-expressing $CD127^{-}CD4^{+}$ T cells (67.33 ± 5.11 %) relative to its vehicle control methanol with on average 73.29 ± 6.73 % (Fig. 4C, left). Interestingly, when comparing heroin *versus* methadone conditions (Fig. 4C, right), methadone treatment significantly reduced the efficacy of Treg induction, suggesting different effects of these opioids on Treg differentiation, at least *in vitro*.

3.5. Methadone inhibits Treg expansion

Next, we were wondering whether the two substances also interfere with Treg expansion. Consequently, we FACS-sorted Tregs defined as $CD25^{+}CD127^{-}CD4^{+}$ from PBMCs of healthy donors and stimulated them with TransAct beads and IL-2, in the presence of heroin, methadone, or the specific vehicle controls for 7 days (Fig. 5A). Exemplary dot plots illustrate that T cells treated with heroin and methadone did not differ regarding frequencies of viable and $CD127^{-}CD25^{+}Foxp3^{+}CD4^{+}$ T cells (Fig. 5B). To determine Treg expansion, cells were counted and absolute Treg numbers were calculated. While there were no differences between heroin (mean: 220250 ± 15337 cells SEM) and its vehicle control (231750 ± 26566 cells), the presence of methadone led to a significant reduction in Treg numbers compared to control conditions from an average of 237750 ± 22533 cells to 11600 ± 18565 cells (Fig. 5C, left). Furthermore, the expansion of Tregs was significantly decreased upon methadone supplementation in direct comparison to heroin (Fig. 5C, right). Taken together, these results suggest that methadone, but not heroin interferes with Treg expansion.

In summary, our results indicate that both, heroin and methadone have a direct inhibitory effect on effector T cell function *in vitro*, with methadone additionally affecting Treg induction and expansion in comparison to heroin.

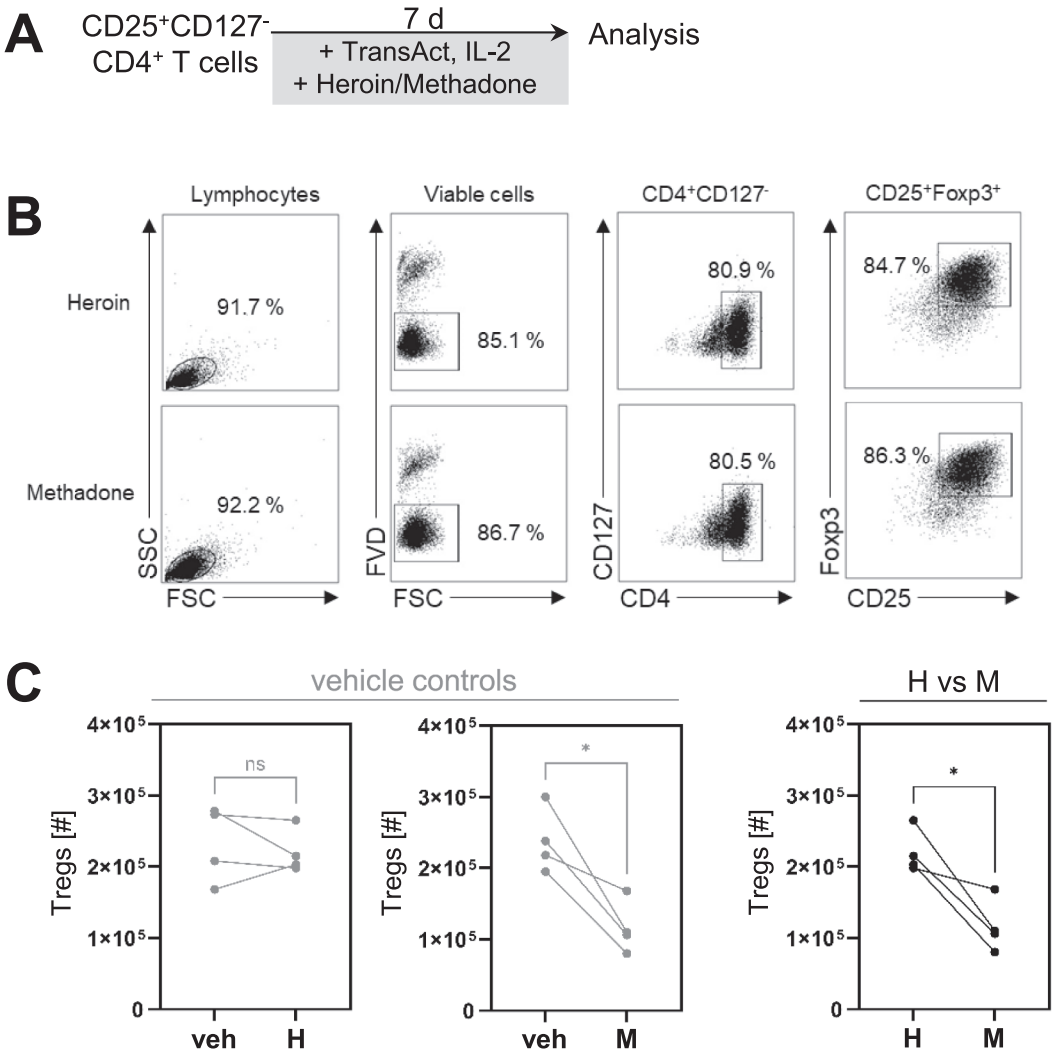

Fig. 5. Treg expansion upon heroin and methadone treatment. (A) For Treg expansion, regulatory ($CD25^{+}CD127^{-}$) $CD4^{+}$ T cells were sorted from PBMCs of healthy donors and cultured in the presence of TransAct beads, IL-2 and heroin (H), methadone (M), or respective vehicle (veh) for 7 days. **(B)** The Treg-like phenotype ($CD127^{-}CD25^{+}Foxp3^{+}$) was validated by flow cytometry after 7 days. **(C)** Treg expansion was determined by cell counting and calculation of absolute numbers (n = 4). Statistical analyses were performed using paired *t*-test (*$p < 0.05$).

4. Discussion

One of the most severe complications of chronic heroin abuse is the impairment of immune function, leading to an increased susceptibility to infections among heroin addicts. Substitution therapy with methadone has been shown to partially reverse the immunosuppressive effects of heroin [6,14,19]. However, most of the studies conducted so far have focused on the analysis of immune cells from individuals with long-term opioid dependence and mainly analysed the whole PBMC population. The direct effect of heroin and methadone on the T cell phenotype and functionality has been scarcely investigated. The fact that T cells do express all three subclasses of opioid receptors (μ, δ, κ) suggests a potential direct immune regulatory role for opioid substances [8,15,16]. Nevertheless, conclusive evidence is currently lacking.

In 1995, Thomas and colleagues described direct immunosuppression of T cells by heroin and methadone *in vitro*. However, they used complete murine spleen cells and pinned down the effect on T cells by evaluating IL-2 and IL-4 concentrations in cell culture supernatants [20]. Here, we show that both, heroin and methadone treatment directly affect *in vitro* activation of human T cells from healthy donors. While heroin only slightly decreased the expression of the activation-associated molecules CD25 and CD69 on $CD4^+$ T cells compared to vehicle-treated cells, the effect of methadone was highly significant, which becomes even more visible by directly comparing heroin *versus* methadone conditions (Fig. 1C and D). In contrast, animal studies showed methadone to be less inhibitory on immune function than morphine [21,22]. Heroin is metabolized to morphine, which is often used to analyse effects of heroin, especially in animal and *in vitro* studies. However, there is evidence suggesting that other metabolites of heroin and heroin itself possess biological activity that might differ from morphine [23]. Well in line with our results, Mazahery *et al.* recently demonstrated a decrease in CD25 and CD69 expression of isolated $CD8^+$ T cells from methadone users compared to healthy controls upon T cell receptor stimulation [24]. However, the same study showed that *ex vivo* stimulation of the μ-opioid receptor without T cell receptor cross-linking moderately decreases CD69 and CD25 expression in healthy control cells, while substantially upregulating $CD45RA^+CD69^+CD25^+$ cells from donors chronically consuming methadone [24]. These observations indicate that immune modulation by opioid substances depends on the duration of exposure and that chronic opioid receptor ligation does not inherently result in T cell immunosuppression, rather it depends on the type of stimulation.

In previous studies, our group demonstrated a decreased proliferation of $CD4^+$ T cells from currently heroin using opioid addicts compared to opioid addicts currently in MMT upon *in vitro* stimulation, accompanied by an impaired secretion of pro-inflammatory cytokines [8]. In contrast, *in vitro*-stimulated and methadone-treated $CD4^+$ T cells from healthy donors exhibited a significantly reduced proliferative activity compared to those treated with heroin (Fig. 2C and D, right). Moreover, methadone treatment markedly decreased T cell proliferation compared to its vehicle control after 48 and 72 h of stimulation, while heroin supplementation only slightly diminished the proliferative capacity after 48 h and showed similar levels in comparison to the solvent control after 72 h (Fig. 2C and D, left). These results seem to be contrary to those obtained by Sacerdote *et al.* and Riß *et al.*, showing that OMT of patients with former heroin abuse normalized lymphocyte proliferation to levels of healthy controls [6,7]. Nevertheless, a suppressive potential of methadone on the proliferative response of *in vitro*-cultured human lymphocytes has also been described previously [25], suggesting that these differences result from direct effects of the substances on T cells *in vitro versus* additional indirect effects in the more complex *in vivo* situation. Moreover, one have to keep in mind that methadone can bind to receptors beyond opioid receptors, including N-methyl-D-aspartate (NMDA) receptors, where it functions as a non-competitive antagonist [26]. NMDA receptors are also expressed by human lymphocytes [27]. Miglio *et al.* demonstrated that NMDA receptor antagonists inhibit phytohemagglutinin-induced proliferation and activation of human T cells [28]. Hence, methadone may modulate T cell function through various receptors and signalling pathways, which require further investigation in future studies.

Well in line with reduced proliferative activity, $CD4^+$ T cells treated with methadone showed a markedly decreased secretion of IFN-γ and IL-2 compared to its vehicle control after 48 h and in tendency after 72 h (Fig. 3A and B, center). In contrast, Chan and colleagues demonstrated elevated levels of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in serum of OMT patients compared to healthy controls [29]. However, they also found a positive correlation between the duration and dosage of methadone therapy and increasing cytokine concentrations, indicating a distinct response based on treatment time and dosing [29]. Furthermore, heroin supplementation resulted in a significant reduction in the secretion of IFN-γ and IL-2 of $CD4^+$ T cells compared to the solvent control after 48 h of stimulation (Fig. 3A, left), in accordance with the findings by Thomas *et al.*, who demonstrated a suppression of IL-2 secretion by *in vitro*-stimulated murine spleen cells in the presence of both, methadone and heroin [20]. However, consistent with the proliferative response (Fig. 2D, left), these differences were diminished after 72 h (Fig. 3B, left). Interestingly, by directly comparing heroin and methadone conditions, we found reduced cytokine concentrations upon methadone supplementation, even reaching a significant level for IL-2 (Fig. 3A and B, right). Conversely, in our previous study, we demonstrated a lower secretion of IFN-γ, IL-6, TNF-α, and IL-2 of stimulated $CD4^+$ T cells isolated from current users of illicit heroin and HAT patients compared to individuals in MMT [8]. This underlines possible differences between the direct effect *in vitro* and the more complex situation in patients, which includes other influencing factors, such as previous heroin abuse by OMT patients and potential parallel use of heroin and methadone. The simultaneous presence of both opioids might strengthen or dampen the individual effects. Moreover, the time between opioid consumption and blood sampling can vary considerably between patients in controlled maintenance programs and heroin users. Consequently, the amount of circulating opioids at the time of sampling, which can influence the study results, is likely to be different. These multifactorial and individual conditions cannot be fully replicated *in vitro*. Nevertheless, it seems that direct treatment with both opioid substances interferes with T cell functionality, with methadone having a more inhibitory effect compared to heroin, at least *in vitro*.

Tregs are cellular mediators of immunosuppression, maintaining the immunologic tolerance to self and foreign antigens in order to prevent immunopathology [9]. Our group provided evidence that opiate addicts currently using illicit heroin have increased Treg frequencies compared to OMT patients and healthy individuals, which likely contribute to immune inhibition in heroin addicts [7]. In accordance, studies by Cornwell *et al.* demonstrated augmented numbers of circulating Tregs in long-term morphine-treated rhesus macaques [30]. However, since patients in structured HAT programs show similar Treg numbers compared to MMT participants, we speculated that heroin consumption itself has no impact on Treg frequencies and that the elevation is rather due to stress and lifestyle conditions and/or possible contaminations of street heroin [8]. Well in line, we here report that treatment with pure heroin has no effect on Treg differentiation and expansion *in vitro* (Fig. 4C and 5C, left). Although OMT had been shown to normalize Treg frequencies to levels of healthy controls [7], we detected a tendency towards decreased induction and significantly reduced expansion of Tregs upon methadone supplementation compared to the vehicle control (Fig. 4C and 5C, center), underlining the importance to differentiate between effects of direct, short-term *in vitro* and long-term chronic *in vivo* exposure. By directly comparing heroin and methadone conditions, we observed significantly lower Treg induction and expansion of methadone-treated cells (Fig. 4C and 5C, right). Hence, one might speculate that normalization of Treg frequencies in OMT patients may result from a decreased induction and expansion of those cells after starting maintenance treatment.

In summary, our study demonstrates that both, heroin and methadone directly modulate human T cell function *in vitro*, with methadone showing a stronger inhibitory effect compared to heroin. It markedly decreased T cell activation, proliferation, and pro-inflammatory cytokine secretion. These results hold relevance beyond the scope of our study, for instance with regard to the pathogenesis of infectious diseases among opioid users. Recent studies have suggested a protective role of OMT against severe COVID-19 infection by reducing inflammation and thereby preventing a cytokine storm, which is a significant cause of mortality during SARS-CoV-2 infection [31]. On the other hand, heroin addicts show a high prevalence for hepatitis C infection and elevated viral loads compared to infected non-heroin users [32]. Hence, immunomodulation by opioid substances is context-dependent and results should be interpreted with caution. Beside alterations of T cell effector functions, our results suggest that the two opioid substances affect Treg induction and expansion differently, with methadone showing a decreased induction and expansion of Tregs *in vitro*, when compared to heroin. However, it is important to differentiate between the direct *in vitro* impact and the complex *in vivo* situation, including the duration of opioid exposure. Further research is warranted to elucidate the mechanisms underlying these effects. Nevertheless, our results contribute to a better understanding of the immune modulating capacity of opioids on T cell function.

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CRediT authorship contribution statement

Anne Ninnemann: Conceptualization, Investigation, Supervision, Visualization, Writing – original draft. **Katharina Hock:** Investigation. **Sina Luppus:** Investigation. **Norbert Scherbaum:** Conceptualization, Writing – review & editing. **Christian Temme:** Resources. **Jan Buer:** Resources, Data discussion. **Astrid M. Westendorf:** Resources, Data discussion, Writing – review & editing. **Wiebke Hansen:** Conceptualization, Supervision, Resources, Writing – review & editing.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2024.112736.

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