Rolling with cannabinoids: indica-tions of suppressive effects on trained immunity

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Abstract

Certain vaccines, among which Bacillus Calmette-Guérin (BCG), confer protection for not only the specific disease they were meant to protect for, but also other pathogens. The initial stimulus leads to an enhanced immune response by innate immune cells upon exposure to a second unrelated pathogen. This concept, in which innate immunity is able to develop memory through immunometabolic and epigenetic reprogramming, is known as trained immunity, and influencing this has many potential applications. On the one hand, it may improve vaccines, whereas suppression of trained immunity may prove worthwhile in chronic inflammatory conditions. (Endo)cannabinoids are interesting candidates for modulating trained immunity, since they affect many cellular processes, and their receptors are abundantly present in the immune system, especially on monocytes. Therefore, in this research, we investigated the effects of Rimonabant hydrochloride (Rh), a CB1 antagonist, and HU308, a CB2 agonist, on trained immunity mediated through different training-inducing agents. Aside from establishing that Rh and HU308 do not cause cytotoxicity, we investigated the effects of the cannabinoids on cytokine production 24 hours after initial stimulation and after 7 days upon restimulation with lipopolysaccharide. In both training with BCG and β-glucan, the cannabinoids did not affect cytokine production after 24 hours. After 7 days, however, both Rh and HU308 caused a decrease in TNFα and IL-6 levels in cells trained with BCG, but not β-glucan. Immunometabolically, lactate production was not altered in the presence of the cannabinoids in BCG and β-glucan training. Their effects on oxLDL training and tolerization could not be fully established in this research, and need to be further investigated. Future research should focus on including at least two other cannabinoids: a CB1 agonist and a CB2 antagonist, since both receptors are known to have opposite functions. Additionally, more in-depth research should be performed on how these cannabinoids influence trained immunity, such as assessing ROS production, epigenetic reprogramming, and differentiation of stimulated monocytes to M1 or M2 macrophages. Overall, this research elucidated a potential suppressive role for Rh and HU308 on trained immunity, and provides a first look into how cannabinoids affect innate immune training.

Layman's summary

The immune system consists of two main arms, innate and adaptive immunity. Innate immunity is a so-called first line of defense and is non-specific. Adaptive immunity, on the other hand, can protect against specific pathogens, and is able to respond faster and more specific after initial exposure. For that reason, vaccines have always been developed to induce a memory of the pathogen in the adaptive immune system, and provide specific protection against infections. Some vaccines, however, have been observed to also protect against other pathogens than the ones they target. One of them is the Bacillus Calmette-Guérin (BCG) vaccine, which aside from protecting against tuberculosis, caused a general reduction in child mortality in humans. This could be explained by the development of memory in the innate immune system, and it is now known as trained immunity.

Induction of trained immunity causes many changes in cells: they start producing more pro-inflammatory cytokines, small molecules that are messengers of the immune system. In addition, cells also change their metabolic state to be able to create more energy for fighting off pathogens. Lastly, changes happen in the accessibility of DNA, the genetic material of the cell, to be able to more rapidly read genes and produce proteins needed for an immune reaction.

In this study, we investigated the effects of cannabinoids on trained immunity. Cannabinoids are small molecules that occur naturally, but can also be chemically synthesized. They interact with their protein receptors, thereby influencing many cellular processes. These receptors are abundantly present in the immune system, and there is a considerable interplay in processes that affect trained immunity and processes that are influenced by cannabinoids. That is why cannabinoids are interesting candidates for influencing trained immunity, either by enhancing it for increased protection against infections, or by reducing it to potentially mitigate many inflammatory diseases such as cardiovascular disease.

We have found that the cannabinoids we used in this study, Rimonabant hydrochloride and HU308, have suppressive effects on production of cytokines if added during the induction of trained immunity. More research will be carried out in the future to also investigate in-depth how cannabinoids affect the metabolism of immune cells, what is their influence on the accessibility of DNA, and how they affect the transformation of cells from one subtype to another. So far, this research provides the first insight into the interaction between cannabinoids and trained immunity.

Abbreviations

BCG	Bacillus Calmette-Guérin
CB*	Cannabinoid receptor
CNS	Central nervous system
CVD	Cardiovascular disease(s)
ELISA	Enzyme-linked immunosorbent assay
IL*	Interleukin
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
oxLDL	oxidized low-density lipoprotein
OXPHOS	oxidative phosphorylation
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
Rh	Rimonabant hydrochloride
ROS	Reactive oxygen species
TNFα	Tumor necrosis factor α

Introduction

The human immune system consists of two main arms: innate and adaptive immunity. While the former is known to provide protection in a fast, yet non-specific manner, the adaptive arm is able to develop so-called immune memory. After an initial challenge, this part of the immune system can develop memory, causing subsequent challenges to elicit more rapid specific responses. Inducing adaptive immune memory has therefore been the goal of vaccination, thereby inducing protection against the disease of interest.

However, certain vaccines, among which Bacillus Calmette-Guérin (BCG), confer protection for not only the specific disease they were meant to protect for, but also other pathogens. Effectively, it has been observed that the BCG vaccine caused a general reduction in child mortality in humans which could not be explained by only adaptive immunity[1]. This was proven in a study using severe combined immune deficient mice, that had improved survival rates upon *Candida albicans* challenge after vaccination with BCG compared to unvaccinated mice[2]. This concept, in which also innate immunity is able to develop memory, is now known as trained immunity[3].

In the case of BCG, trained immunity causes the reprogramming of cells on several levels. Functionally, upon secondary stimulation, cells shift to expressing a pro-inflammatory cytokine profile, increasing cytokine levels such as tumor necrosis factor α (TNFα) and interleukin (IL) 6 in cell culture supernatants[4]. Production of reactive oxygen species (ROS), an important innate immune effector mechanism, is enhanced as well[4]. In addition, epigenetic changes occur in the context of DNA folding, leading to more open chromatin structures and reduced DNA methylation. Especially, reduced histone 3 lysine 9 trimethylation, and enhanced histone 3 lysine 4 trimethylation are hallmarks of epigenetic reprogramming after induction of innate immune training[2, 5]. Cells also change metabolically upon training, increasing both glycolysis and oxidative phosphorylation (OXPHOS)[5]. Furthermore, proven crucial for BCG-induced trained immunity, is glutamine metabolism, since training was abrogated in the absence of glutamine[5].

When training monocytes with β-glucan, a soluble polysaccharide naturally occurring in for instance yeast and bacteria[6], there are similarities but also differences compared to BCG training. β-glucan training, like BCG training increases cytokine production of TNFα and IL-6, even to a greater extent. Moreover, it leads to distinct cell morphology, which persists upon lipopolysaccharide (LPS) restimulation[4]. Not only pro-inflammatory cytokine production, but also anti-inflammatory cytokines IL-1Ra and IL-10 increase with β-glucan training in cell supernatants[4, 5, 7]. ROS production, however, is not boosted by training monocytes with β-glucan[4]. Epigenetically, there is broad induction of signaling pathways related to innate immunity through H3K4me3 and histone 3 lysine 4 acetylation modifications. This translates to the metabolic effects observed in β-glucan training, since epigenetic signatures have been identified on promoters of genes that affect cell metabolism, such as glycolysis-related genes, mTOR, and HIF1α targets[4]. All of this leads to a shift from OXPHOS to aerobic glycolysis.

Another manner of innate immune training to be discussed here is that with oxidized low-density lipoprotein (oxLDL). Also here, upregulation of pro-inflammatory cytokines including TNFα and IL-6 occurs. Furthermore, oxLDL training results in increased ROS production, and a metabolic shift towards glycolysis with a crucial role for OXPHOS[4, 8]. The fact that oxLDL induces trained immunity has important implications for inflammatory conditions such as cardiovascular disease (CVD). Atherosclerosis is a primary process in

developing CVD. Both macrophages and oxLDL are highly involved in atherosclerosis, and contribute to the chronic inflammatory state which is the foundation of atherosclerosis leading to CVD[8, 9]. Therefore, trained immunity likely plays a crucial role in these processes, and being able to reverse or suppress training may result in slowing down the development of CVD.

(Endo)cannabinoids are interesting candidates for influencing trained immunity. These are metabolites that occur in the human body (endocannabinoids), but can also be naturally produced, or chemically synthesized. Cannabinoids are known to bind to multiple receptors, most importantly cannabinoid receptors (CB) CB1 and CB2, to exert their effects. These receptors are G-protein coupled receptors (GPCRs), and are highly expressed in the central nervous system (CNS). However, especially CB2 is expressed on immune cells, with a central role for monocytes since they express CB1 as well[10-12].

Immunologically, cannabinoids exert numerous effects, such as induction of apoptosis, inhibition of proliferation, suppression of cytokine production, and induction of regulatory T cells. For instance suppression of pro-inflammatory cytokines by CB2 agonist HU308 shows promising potential, since this can decrease overall tissue damage in inflammatory conditions[13, 14]. In monocyte-derived dendritic cells, however, HU308 did not suppress the production of TNFα or IL-6, and CB1 antagonist could rescue their expression even after inhibition through other cannabinoids[15].

Cannabinoids affect multiple cellular processes through many signaling pathways (Figure 1). HU308 for instance, can inhibit signaling pathways including Ras, p38, MAPK, JNK, and AKT through binding to CB2[16]. Furthermore, cannabinoids have immunometabolic effects, such as upregulation of AMPK, thereby suppressing glycolysis and promoting OXPHOS through inhibition of mTOR activity[17].

Also, in CVD, cannabinoids affect the underlying chronic inflammation, and the endocannabinoid system is highly involved in many processes crucial in CVD pathologies[18]. A potent CB1 antagonist reduces vascular inflammation through inhibition of NFκB activation[19]. Overall, there is evidence that stimulation of CB1 is pro-atherogenic, whereas CB1 antagonists and CB2 agonists are anti-atherogenic, underlining the therapeutic potential of cannabinoids for CVD[20].

Altogether, there is a large interplay between trained immunity and cannabinoids. Their paths cross not only functionally, in regards to affecting innate immune processes such as cytokine production, but also immunometabolically, and potentially in many more ways. That macrophages themselves are even producers of endocannabinoids, and upregulate the endocannabinoid system in response to oxLDL, only emphasizes their importance in regulating immunity[21, 22].

Therefore, in this study, to our knowledge for the first time, we investigated the effects of cannabinoids on trained immunity. We trained monocytes isolated from human blood with different training-inducing agents, with or without presence of several cannabinoid modulators, and cannabinoids alone. In addition to assessing the resulting cytokine profiles, we tested the potential cytotoxicity of the cannabinoids, and examined their effects on glucose metabolism after restimulation with LPS. This research, therefore, represents a first look into the application of cannabinoids in trained immunity.

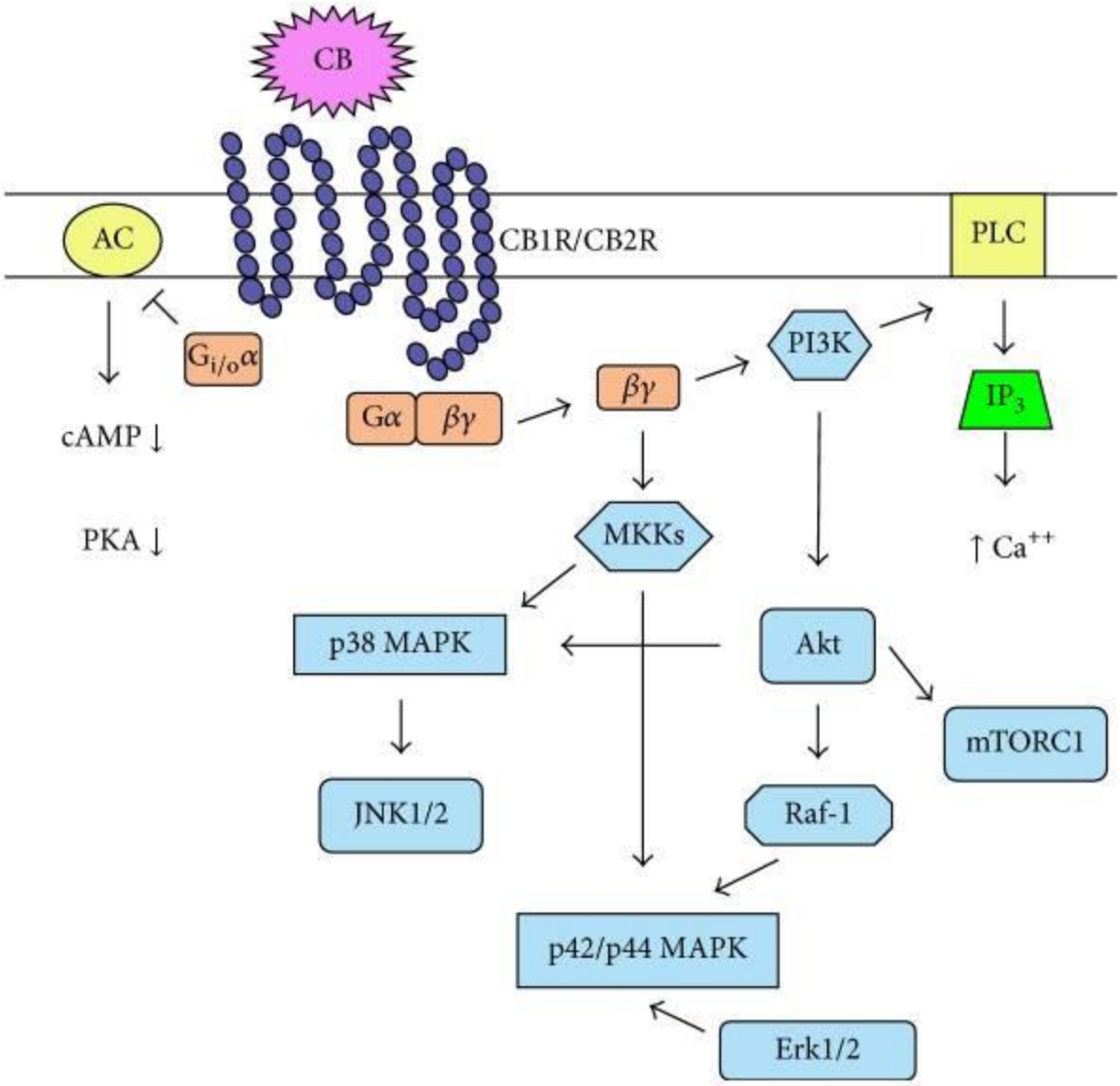

Figure 1: Signaling pathways affected by cannabinoid interaction with their receptors[11].

Results

Stimulation of monocytes with Rimonabant hydrochloride or HU308, either alone or combined with BCG, does not lead to increased cell death after 24 hours

Since the BCG vaccine is most established in inducing trained immunity, we started incubating isolated monocytes with and without BCG, either alone with CB1 antagonist Rimonabant hydrochloride (Rh) or CB2 agonist HU308. After 24 hours of stimulation, however, supernatants were collected to investigate first the cytotoxicity of the compounds. This is to make sure that the compounds themselves, or combined with BCG, do not increase cell death, and that potential stress is the cause for observed altered cytokine profiles rather than a changed immunometabolic state.

To investigate cytotoxicity, 24 hour cell supernatants were collected, with supernatants from lysed cells as a positive control. For both Rh (Figure 2A) and HU308 (Figure 2B), alone or combined with BCG, we observed no major increase in cell death compared to RPMI controls. However, the observed cytotoxicity in RPMI conditions was higher than expected, with around 50% in this experiment *versus* percentages typically observed in this assay around 20-30%. In addition, measured ODs in the positive, lysed cells controls were much lower than usually expected (~0.2 *versus* expected >1.0, data not shown). Therefore, a positive lactate dehydrogenase (LDH) control from the kit was used to investigate whether this would result in higher ODs than the lysed cells control.

Indeed, using the LDH positive control of the kit increased ODs to more expected values (Figure 2C and 2D). It may be needed to optimize the cytotoxicity assay. Nevertheless, either with the lysed cells control or with the LDH positive control, stimulating cells with Rh and HU308 alone or combined with BCG did not result in substantial increases in cell cytotoxicity. Additionally, cell viability was regularly checked throughout experiments using light microscopy.

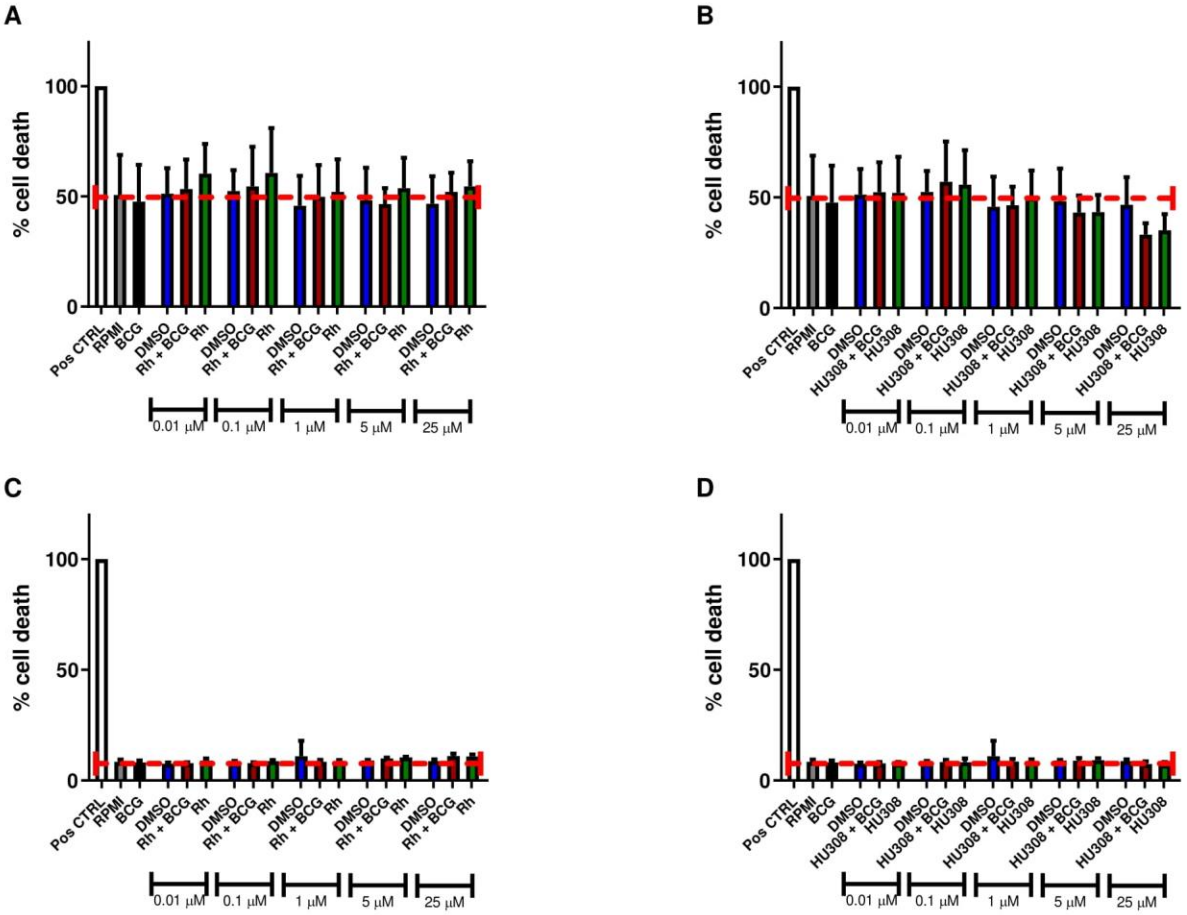

Figure 2: 24 hour-stimulation of monocytes with Rh or HU308 does not lead to increased cell death compared to RPMI controls. *Cell culture supernatant LDH levels using lysed cells as positive control, after 24 hour stimulation with Rh (A) and HU308 (B), or using the kit's LDH positive control after stimulation with Rh (C) and HU308 (D). Shown are percentages with respect to positive controls, as mean ± SD over three different donors. The red dashed lines represent baseline cell death (RPMI).*

Rh and HU308, combined with BCG, decrease TNFα and IL-6 levels after LPS restimulation, but not after initial 24-hour stimulation

After the first 24 hours, treatment of monocytes with Rh or HU308 in any concentration does not lead to a change in TNFα levels (Figure 3A and 3B). For IL-6, however, some donors did show some production when stimulated with BCG + Rh, although this was not significantly different in any concentration when compared to BCG only (Figure 3C). Stimulation with 1 µM HU308 together with BCG did significantly reduce IL-6 levels compared to BCG only, after 24 hours (Figure 3D).

In addition, IL-1Ra levels were analyzed after 24 hours, but no significant changes were observed with either cannabinoid (Figure 4A and 4B). There is a trend of BCG increasing IL-1Ra levels compared to RPMI, but this was not significant, and neither cannabinoid seemed to affect IL-1Ra. Also IL-10 levels after initial stimulation did not show any difference after treatment with Rh (Figure 4C) and HU308 (Figure 4D). Moreover, no IL-10 above detection limit was measured in general, which raises the question of whether the assay sensitivity was adequate.

On day 7, after 24 hours of LPS restimulation, we again assessed cytokine levels in cell supernatants. Overall, BCG significantly increased both TNFα and IL-6 production. The addition of 0.1 µM, 5 µM, and 25µM Rh to BCG stimulation significantly decreased TNFα levels, but Rh alone did not, aside from 5 µM concentrations (Figure 5A). Similar observations were done for stimulation with HU308 + BCG, even in all used HU308 concentrations (Figure 5B). In addition, HU308 suppresses immune responses very well by itself, when compared to its DMSO control. Likewise, IL-6 levels were reduced when stimulating monocytes with BCG + either Rh (Figure 5C) or HU308 (Figure 5D).

Overall, Rh by itself appears to not significantly affect cytokine expression when compared to DMSO controls, in striking contrast to HU308, which suppresses cytokine production greatly. Of note, while observing monocytes using microscopy, especially higher concentrations of both cannabinoids seemed to be lethal to cells after 6 and 7 days. Therefore, the observed decreases in cytokine levels may be because of cell death rather than changes in their immunologic state. Hence we decided not to continue assessing Rh in concentrations of 5 µM and 25 µM, and HU308 in concentrations of 0.1 µM and above.

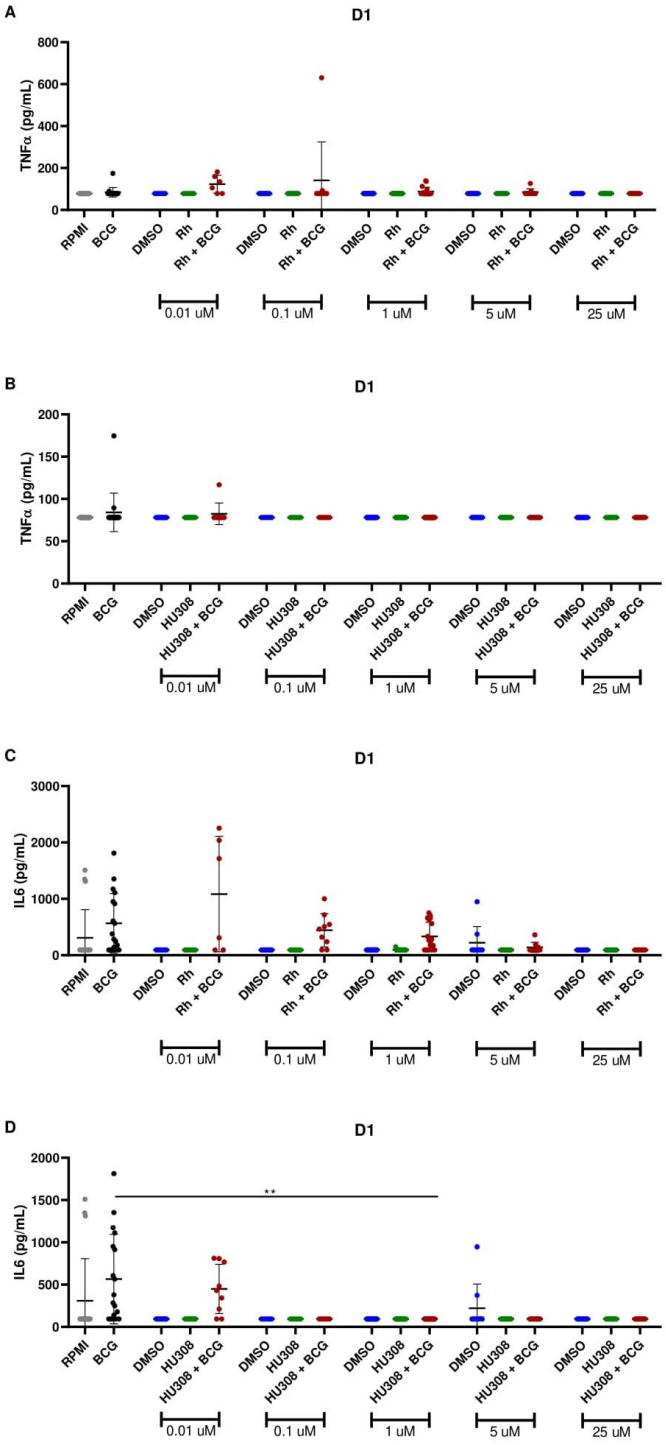

Figure 3: Rh and HU308 do not affect TNFα and IL-6 levels after initial 24 hour stimulation. *Monocytes were incubated with Rh or HU308, either alone or together with BCG. After 24 hours, cytokine levels were assessed in cell culture supernatants. TNFα production was not changed by either Rh (A) or HU308 (B) in any concentration. Rh did also not change IL-6 levels (C), but 1 µM HU308 did (D). Data are shown as mean ± SD, n=9-18, and significance was tested using paired Wilcoxon signed-rank test. ** p<0.005.*

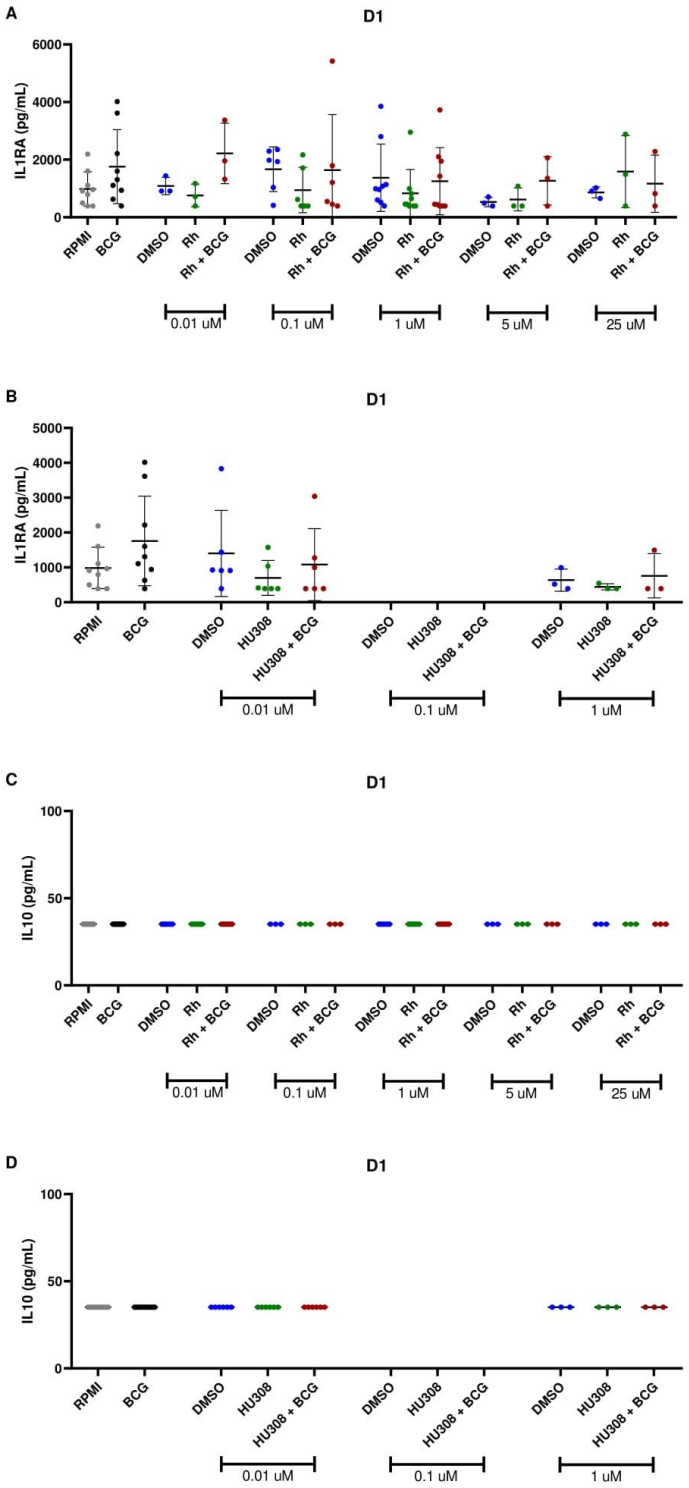

Figure 4: Rh and HU308 do not affect IL-1Ra and IL-10 levels after initial 24-hour stimulation. *Monocytes were incubated with Rh or HU308, either alone or together with BCG. After 24 hours, cytokine levels were assessed in cell culture supernatants. Neither cannabinoid seemed to significantly affect IL-1Ra levels (A & B), and IL-10 did not exceed minimum detection levels in any condition (C & D). Data are shown as mean ± SD, n=9-18, and significance was tested using paired Wilcoxon signed-rank test.*

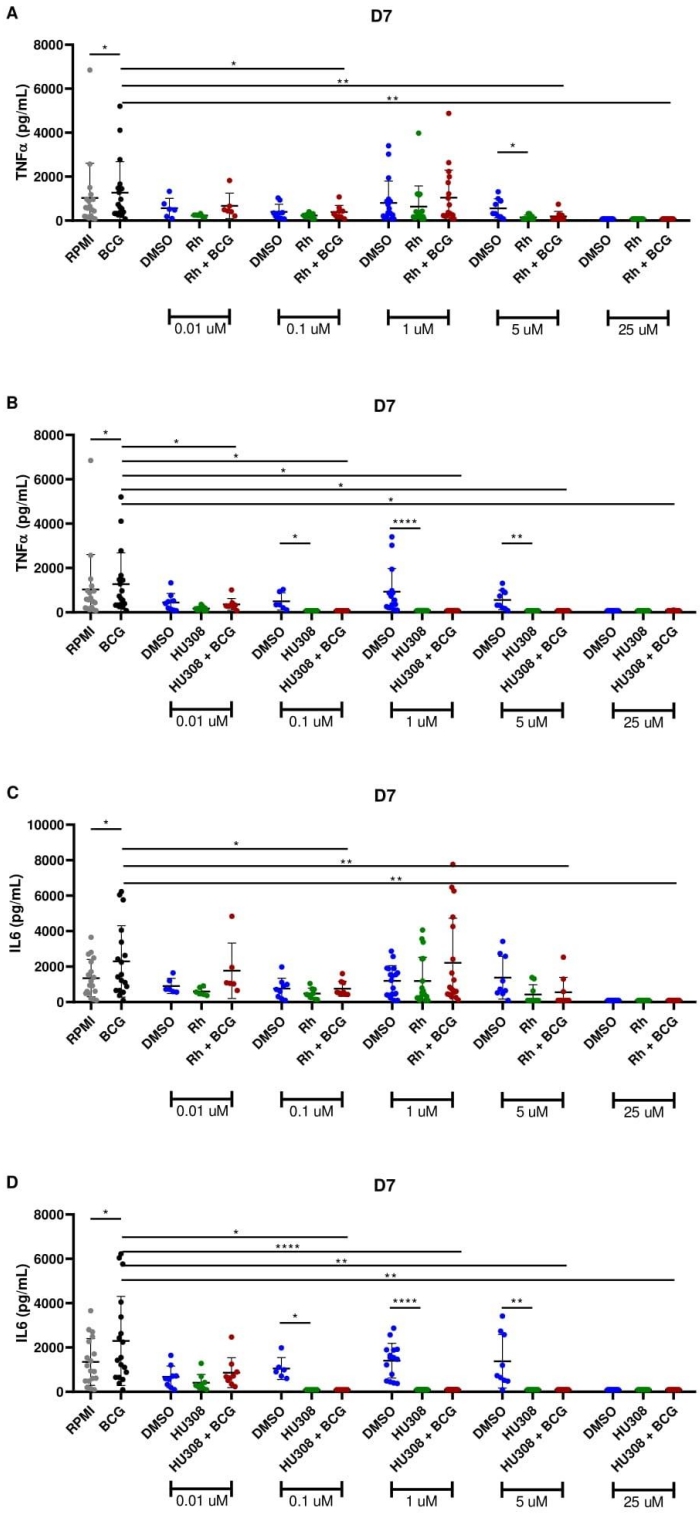

Figure 5: Rh and HU308 decrease TNFα and IL-6 levels after LPS restimulation. *After letting the monocytes rest for five days, they were restimulated using LPS for 24 hours. This decreased TNFα levels in supernatants of cells initially stimulated with Rh + BCG (A), and HU308 + BCG (B). In a similar manner, a reduction was observed for IL-6 when combining Rh with BCG (C) and HU308 with BCG (D). Data are shown as mean ± SD, n=9-18, and significance was tested using paired Wilcoxon signed-rank test. **** p<0.0001, ** p<0.005, * p<0.05.*

Rh and HU308, either alone or with BCG, do not change lactate production in monocytes on day 6

Aside from changes in cytokine levels, BCG training alters the metabolic state of cells. Therefore, we wanted to investigate how Rh and HU308 affect the immunometabolic state of monocytes when used in BCG training. To determine the metabolic state of cells, increased glucose consumption as a result of glycolysis can be used, since this also increases lactate production. This allows for measuring lactate concentrations in supernatants with an Amplex Red fluorometric assay. Here, we only assessed the lower cannabinoid concentrations since the higher ones appeared to induce cell death on day 6. Indeed, as expected, BCG training increased lactate production when compared to RPMI (Figure 6). Neither Rh (Figure 6A), nor HU308 (Figure 6B), however, significantly affected this, both when incubated alone or combined with BCG.

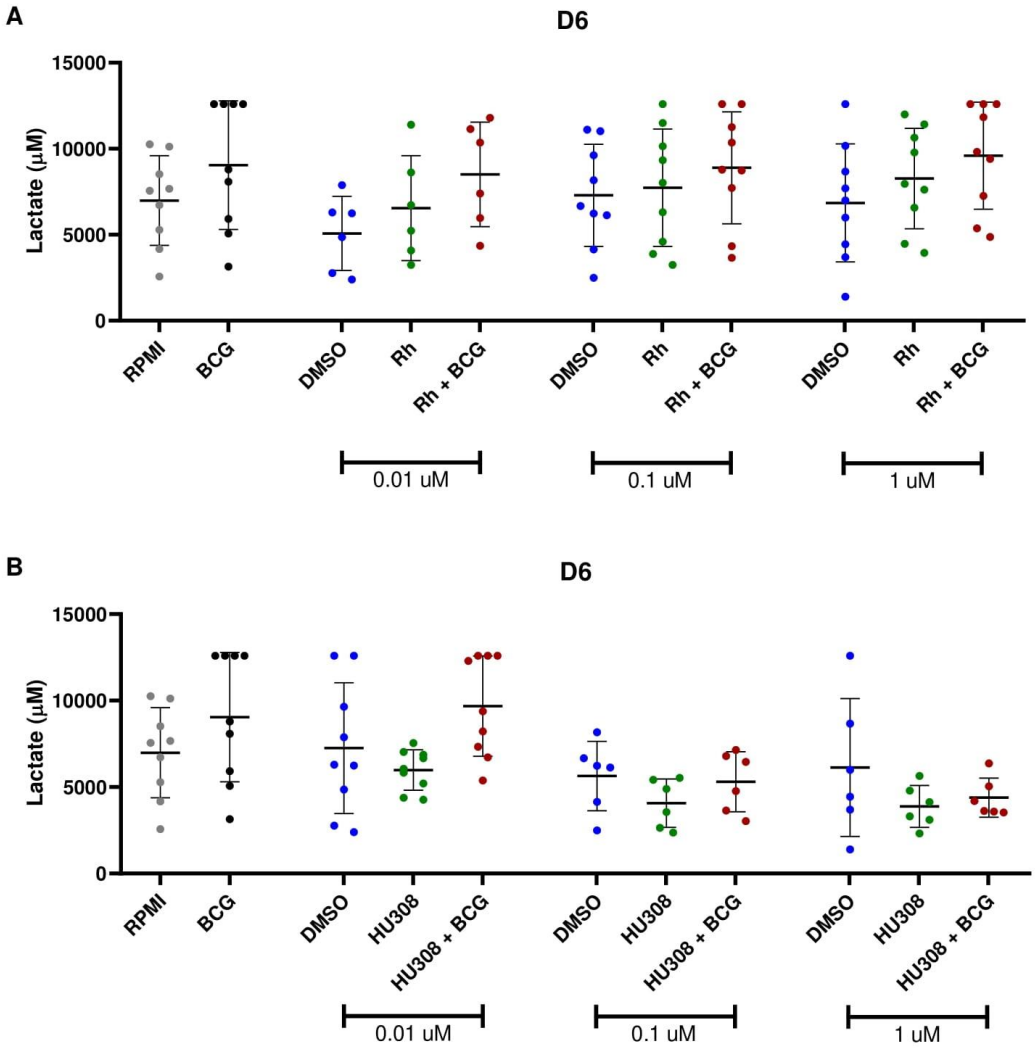

Figure 6: Rh and HU308 do not alter lactate production of monocytes on day 6. *Human monocytes were incubated with Rh or HU308, either alone or with BCG, for 24 hours. Then, cells were washed and rested for 5 days on RPMI. Afterwards, lactate concentrations were measured in these supernatants using a fluorometric assay. Data are shown as mean ± SD, n=9. Significance was tested using paired Wilcoxon signed-rank test.*

Rh and HU308 combined with β-glucan do not significantly affect levels of TNFα, IL-6, IL-1β, IL-1Ra, and IL-10

Another manner of inducing trained immunity, is by stimulating cells with β-glucan. As discussed before, β-glucan training has some similarities with BCG training, but also differences. Therefore, it is worthwhile to investigate how Rh and HU308 affect monocytes trained by β-glucan. In this experiment, we used only 0.01 µM, 0.1 µM, and 1 µM concentration of Rh, and only 0.01 µM for HU308, since we previously observed that higher concentrations resulted in cell death in the long term.

On day 1, like with BCG training, we did not measure any changes in pro-inflammatory cytokines TNFα (Figure 7A), IL-6 (Figure 7B), and IL-1β (Figure 7C). For IL-1Ra (Figure 7D), there were some changes in cytokine levels. Especially β-glucan training increased IL-1Ra levels, though not significant. In addition, the lowest Rh concentration completely abolishes IL-1Ra production, alone and when combined with β-glucan. HU308 alone has a similar effect, although not statistically significant. Finally, day 1 IL-10 levels were measured, yet like in BCG training, no IL-10 could be detected (Figure 7E).

On day 7, after 24 hours of LPS restimulation, we again measured cytokine levels of TNFα, IL-6, and IL-1β. β-glucan did significantly increase TNFα levels compared to RPMI, however, neither cannabinoid alone nor with β-glucan significantly affected TNFα production (Figure 8A). The same goes for IL-6, which was significantly altered in none of the conditions (Figure 8B). However, β-glucan training did show a trend of increasing IL-6 compared to RPMI, indicating that it indeed induced trained immunity. IL-1β levels remained unchanged in all conditions, and the cytokine was not detected beyond minimum detection levels (Figure 8C).

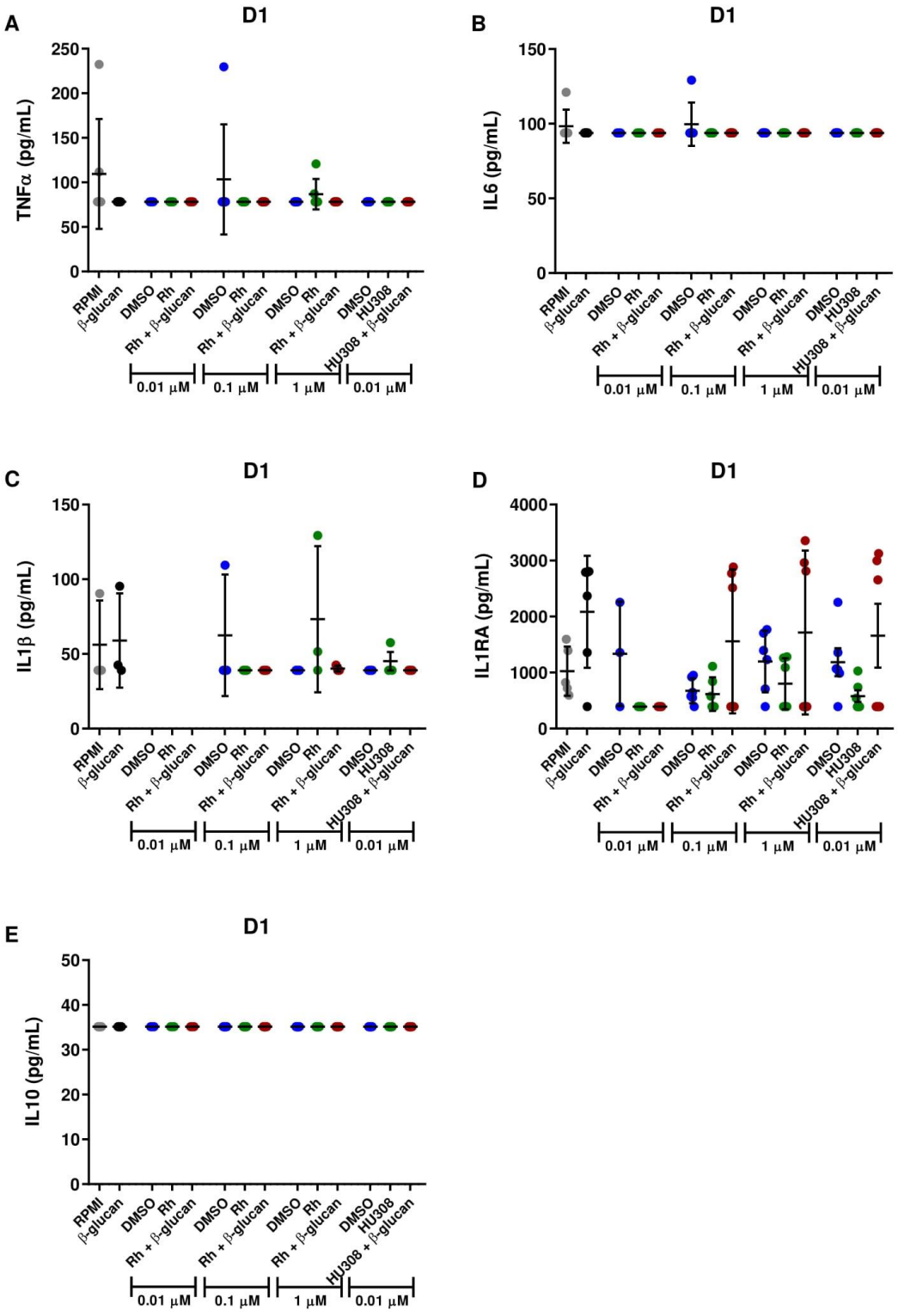

Figure 7: Rh or HU308 alone or combined with β-glucan do not affect cytokine levels after 24 hour-stimulation. *Human monocytes were incubated with Rh and HU308, either with or without β-glucan. After 24 hours, cell culture supernatants were collected and analyzed with ELISA for TNFα (A), IL-6 (B), IL-1β (C), IL-1Ra (D), and IL-10 (E). Data are shown as mean ± SD, n=3-9, and significance was tested using paired Wilcoxon signed-rank test.*

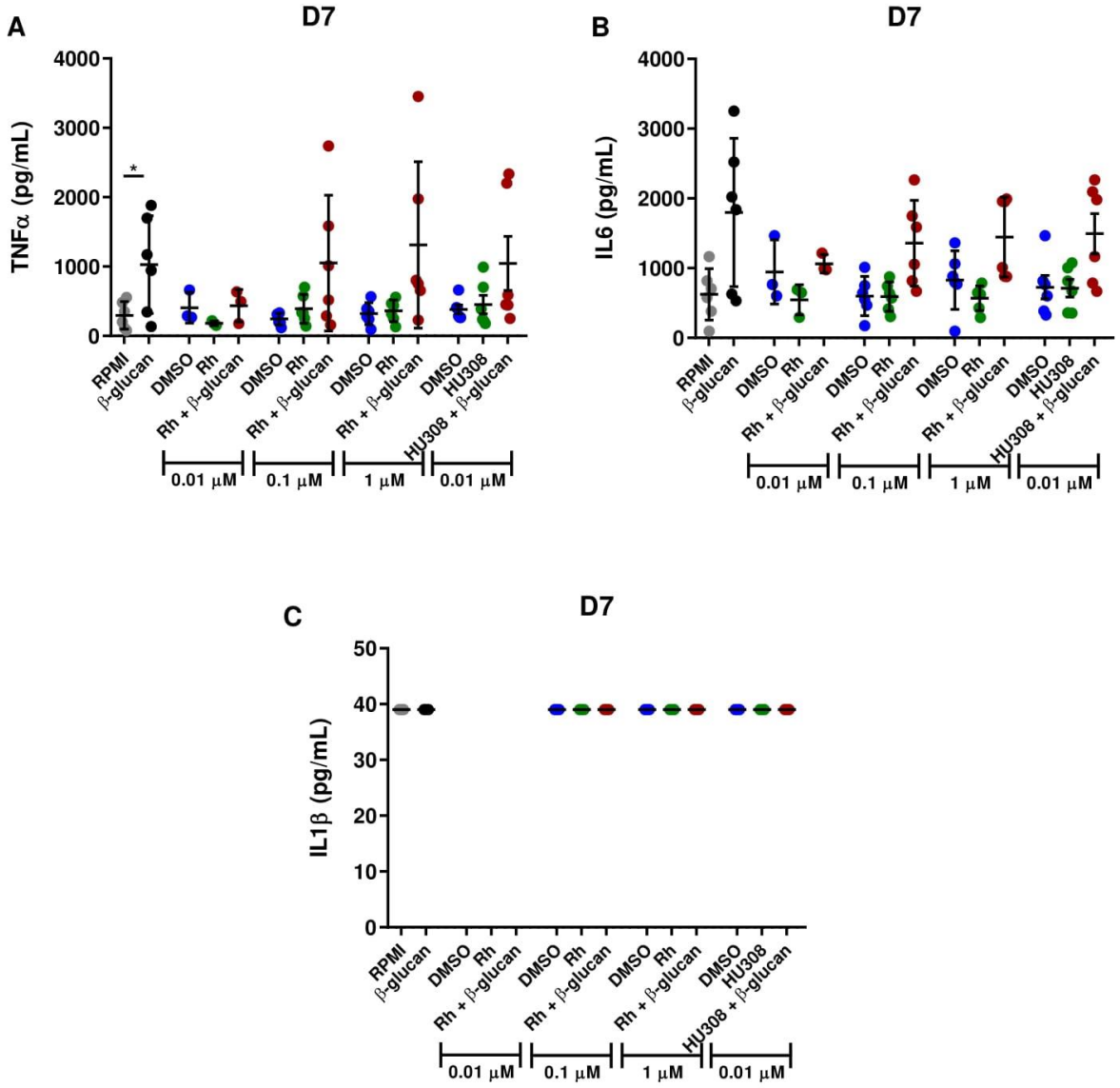

Figure 8: Rh or HU308 alone or with β-glucan do not affect TNFα, IL-6, and IL-1β levels on day 7 of training. *After 5 days of rest, monocytes were restimulated using LPS for 24 hours. Afterwards, cell culture supernatants were analyzed for TNFα (A), IL-6 (B), and IL-1β (C). Although training with β-glucan succeeded, neither cannabinoid affected cytokine levels significantly. Data are shown as mean ± SD, n=3-9, and significance was tested using paired Wilcoxon signed-rank test. * p<0.05.*

HU308, but not Rh, seems to increase lactate production, alone and together with β-glucan

Like BCG training, β-glucan training induces metabolic changes. After stimulation, cells switch from OXPHOS to aerobic glycolysis. To investigate this in our setting, and the effects of Rh and HU308, we performed a lactate assay on day 6 supernatants of β-glucan-trained cells, with and without Rh and HU308 (Figure 9). Although not significantly, β-glucan training did increase glycolysis and thereby lactate production. Rh, however, did not have an effect on this, either alone or with β-glucan.

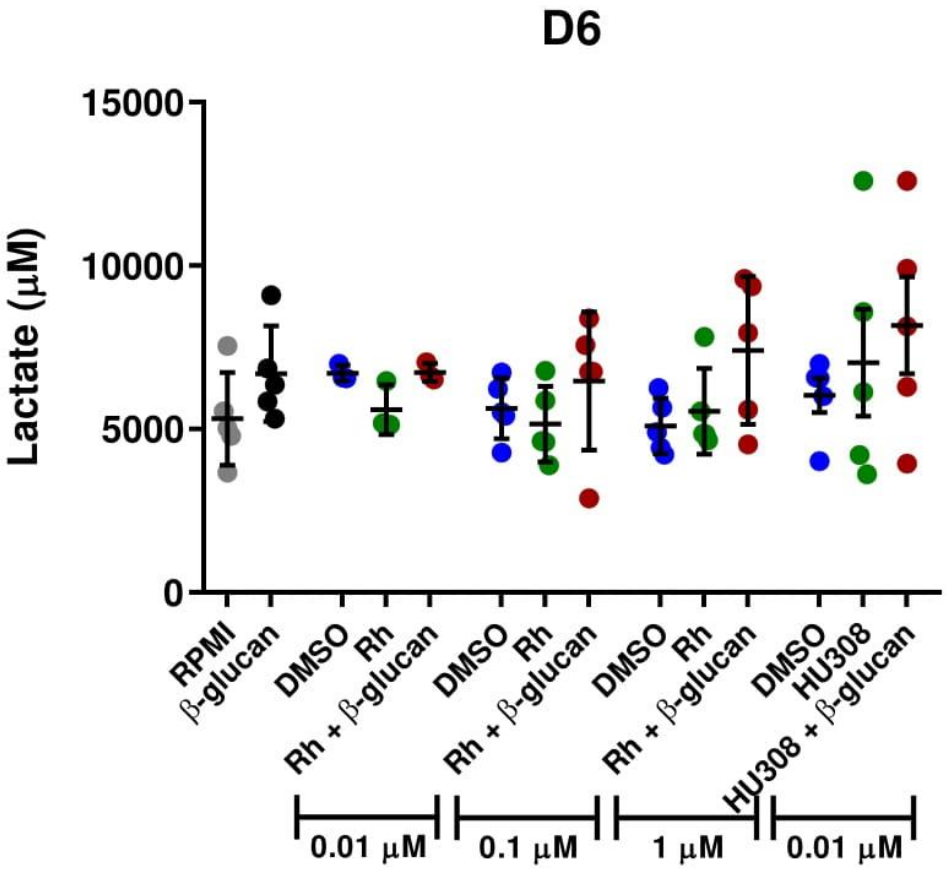

Figure 9: Rh and HU308 do not alter lactate production of β-glucan-trained monocytes on day 6. *After initial stimulation of cells with Rh and HU308 with or without β-glucan, cells were rested for 5 days on RPMI. Supernatants were collected, and lactate levels were analyzed using an Amplex Red-based fluorometric assay. β-glucan training shows a trend in increasing lactate production, although not statistically significant. Data are shown as mean ± SD, n=6, and significance was tested using paired Wilcoxon signed-rank test.*

Rh may inhibit oxLDL training

A final method of inducing trained immunity we investigated was by using oxLDL. oxLDL-induced training has strong links with the underlying inflammatory conditions in CVD. Since, thus far, the cannabinoids we tested seem to decrease cytokine production, they may have implications for reducing inflammation in atherosclerosis and CVD in general. In this experiment, cells were again stimulated for 24 hours with cannabinoids, however now alone or combined with oxLDL. Next, monocytes were washed and rested for 5 days on RPMI, and on day 6 restimulated for 24 hours using LPS. Since no significant effects on cytokine levels have thus far been observed after 24 hours, we only assessed cytokine levels in cell culture supernatants on day 7. Strikingly, oxLDL, instead of increasing, did not change levels of TNFα (Figure 10A) and IL-6 (Figure 10B) compared to RPMI. This indicates that the monocytes were not trained adequately. Both cannabinoids on their own did not have any effects on TNFα and IL-6, however especially 0.1 µM Rh together with oxLDL seems to nearly abolish the production of both cytokines. Overall, none of the conditions reached statistically significant differences, but it must be considered that cells did not get trained with oxLDL and the experiment was performed only with 6 donors. Still, the results suggest that especially the lowest used Rh concentration may inhibit oxLDL training.

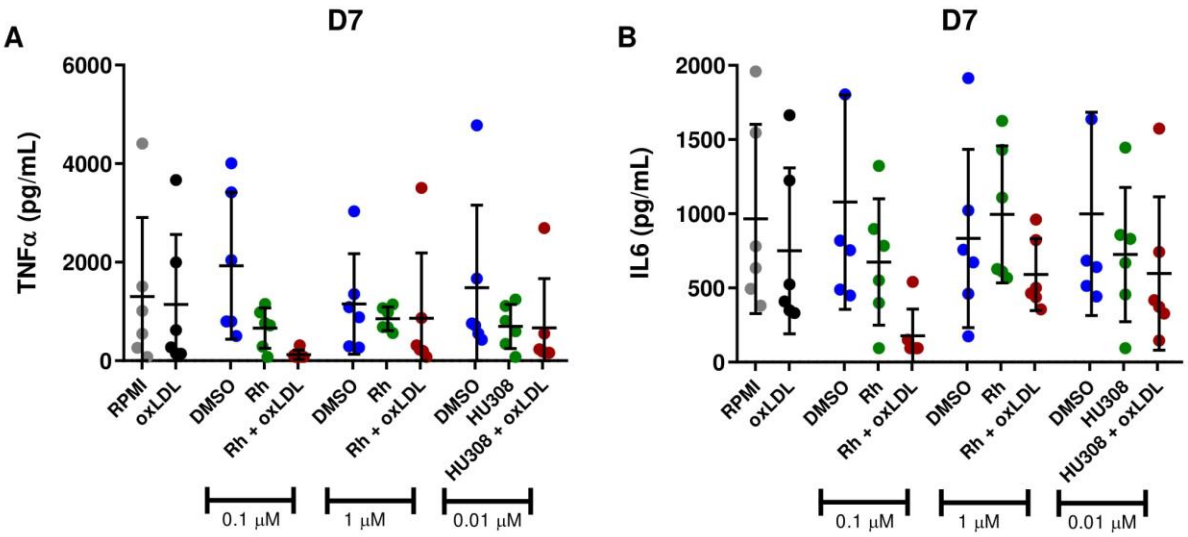

Figure 10: Not HU308, but Rh may inhibit oxLDL training. *Human monocytes were stimulated with Rh and HU308 for 24 hours, either alone or combined with oxLDL. After that, they were washed and rested for 5 days. On day 6, cells were restimulated with LPS, and after 24 hours their supernatants were collected and analyzed using ELISA. Data shown are mean + SD levels of TNFα (A) and IL-6 (B) in day 7 cell culture supernatants, n=6. Significance was tested using paired Wilcoxon signed-rank test.*

Effects of HU308 and Rh on metabolism in oxLDL-trained monocytes remain inconclusive

Even though oxLDL training did not succeed, some trends show that the cannabinoids used may inhibit this type of training when it does occur. Therefore, and also because oxLDL training itself increases glycolysis, lactate levels in day 6 supernatants were assessed (Figure 11). While cytokine levels did not change upon oxLDL stimulation, monocytes do exhibit increased lactate levels compared to RPMI, though not significantly. All cannabinoid concentrations aside from 0.1 µM Rh, do not affect lactate production. Strikingly, 0.1 µM Rh however does increase lactate levels, even to the extent of increasing above oxLDL-trained levels. It is likely, however, that when oxLDL training succeeds, lactate production in the 0.1 µM Rh condition will not exceed oxLDL training conditions anymore. More interesting effects occur when adding either cannabinoid to oxLDL stimulation. That is, already in this experiment, where oxLDL training did not succeed, addition of either cannabinoid brings lactate production back to RPMI only levels. Here, it does not reach statistical significance, but it is likely that when monocytes are trained using oxLDL, both cannabinoids significantly decrease lactate production and thereby immunometabolically inhibit training. Overall, it must be considered that we could only try this with 3 donors so far. Therefore, it is not possible to conclude the effects of Rh and HU308 on oxLDL-induced glycolysis production. Further investigation with higher sample size could reveal if Rh, in inhibiting oxLDL training, interferes with glycolysis.

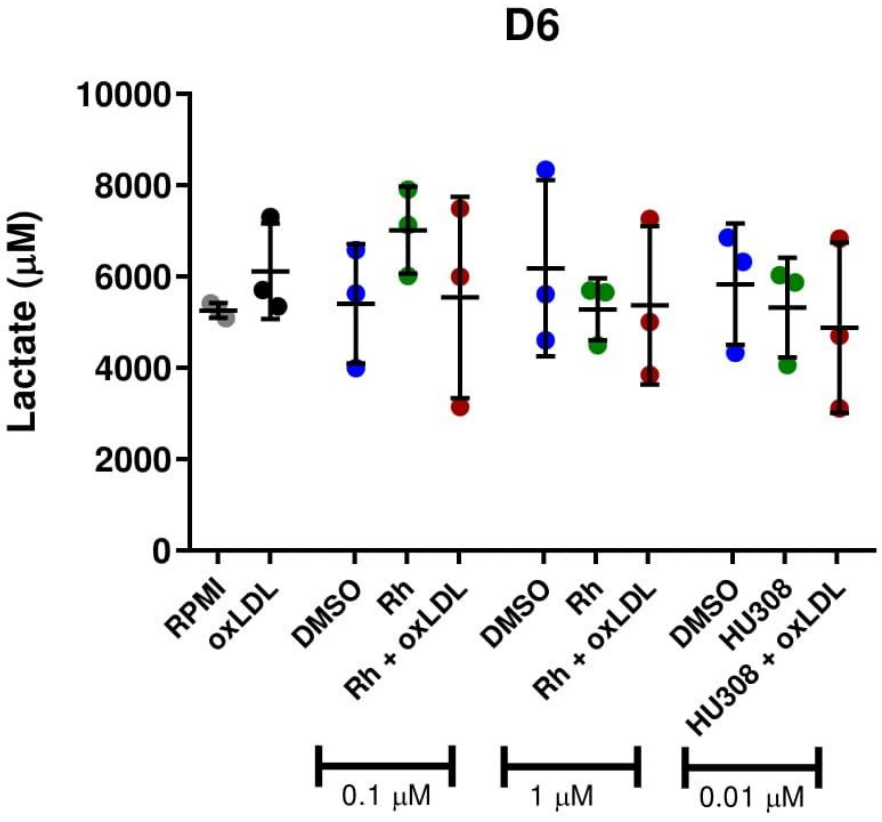

Figure 11: Rh and HU308 may inhibit oxLDL training on a metabolic level by abolishing glycolysis. *Lactate levels in supernatants of 6 days after initial stimulation were assessed using a fluorometric assay. oxLDL training (if successful) seems to increase lactate levels, but when either Rh or HU308 are added, glycolysis is inhibited to levels comparable with RPMI only conditions. Data are shown as mean ± SD, n=3, and significance was tested using paired Wilcoxon signed-rank test.*

Unsuccessful tolerization leads to inconclusive results of potential effects of Rh and HU308 on tolerized monocytes

Finally, since it appears that the cannabinoids we investigated have immunosuppressive effects, we investigated whether they may have an effect on LPS-induced tolerization of monocytes. For this, monocytes were treated as in a training protocol, however instead of a training stimulus, they were incubated with LPS in the first 24 hours to induce tolerance. After this, cell supernatants were collected, and levels of several pro- and anti-inflammatory cytokines were analyzed (Figure 12). We observed no differences in cytokine production after 24 hours of stimulation between RPMI, LPS, Rh or HU308 either alone or with LPS.

Additionally, cytokine levels were measured after restimulation with LPS on day 6 (Figure 13). Whereas TNFα and IL-6 show some decrease in LPS stimulation compared to RPMI, overall, tolerization of cells did not occur. Therefore, no conclusions can be drawn about the effects of Rh and HU308 on tolerization of monocytes in this setting.

In a follow-up experiment, we increased the LPS concentration in the first 24 hours, to induce better tolerization. In 24 hour supernatants, there is a trend of increased production of TNFα, IL-6, IL-1β, and IL-1Ra upon LPS stimulation compared to RPMI, although not significant due to low sample size (Figure 14A-D). The effects of Rh but especially HU308, however, are inconsistent. For IL-10, we did not manage to measure cytokine levels beyond detection levels (Figure 14E). When measuring cytokines again on day 7 after 24 hours of LPS restimulation, we once more observed that cells did not become tolerized, although some tolerization may have occurred as seen in a decrease in IL-6 production (Figure 15A-E). This did not reach statistical significance, however, due to low sample size. Repeating this experiment with more donors, and optimizing the tolerance model further if necessary, will be crucial in being able to draw conclusions on the effects of Rh and HU308 on the tolerization of cells.

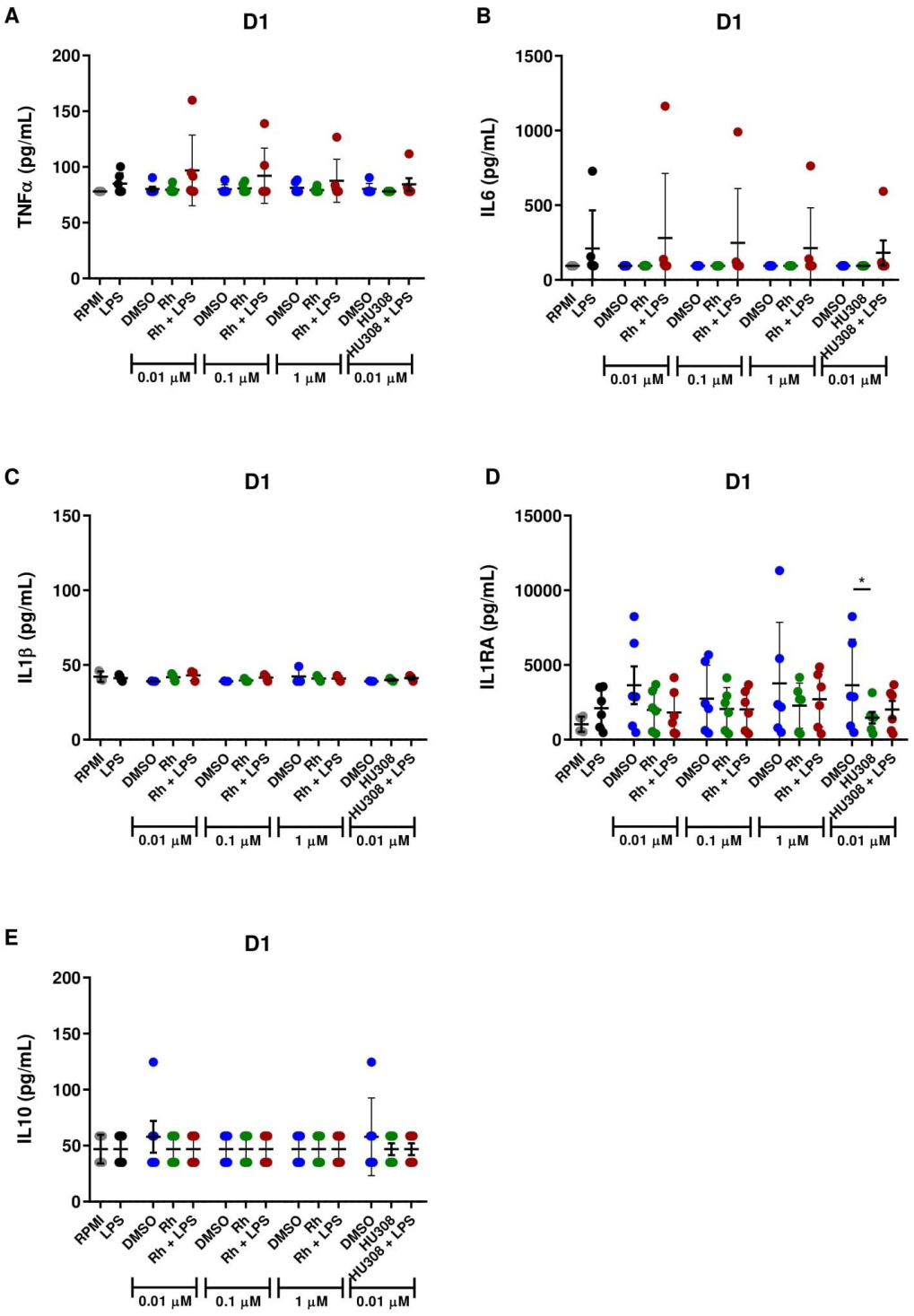

Figure 12: Rh and HU308 do not affect cytokine production after 24 hours of stimulation alone or combined with LPS (1 ng/mL). *Human monocytes were incubated for 24 hours with 1 ng/mL LPS, and Rh or HU308. Afterwards, supernatants were collected to measure cytokine levels using ELISA. No differences are observed for TNFα (A), IL-6 (B), IL-1β (C), and IL-10 (E). LPS stimulation did to increase IL-1Ra (D), although not statistically significant. Data are shown as mean ± SD, n=6, and significance was tested using paired Wilcoxon signed-rank test. * p<0.05.*

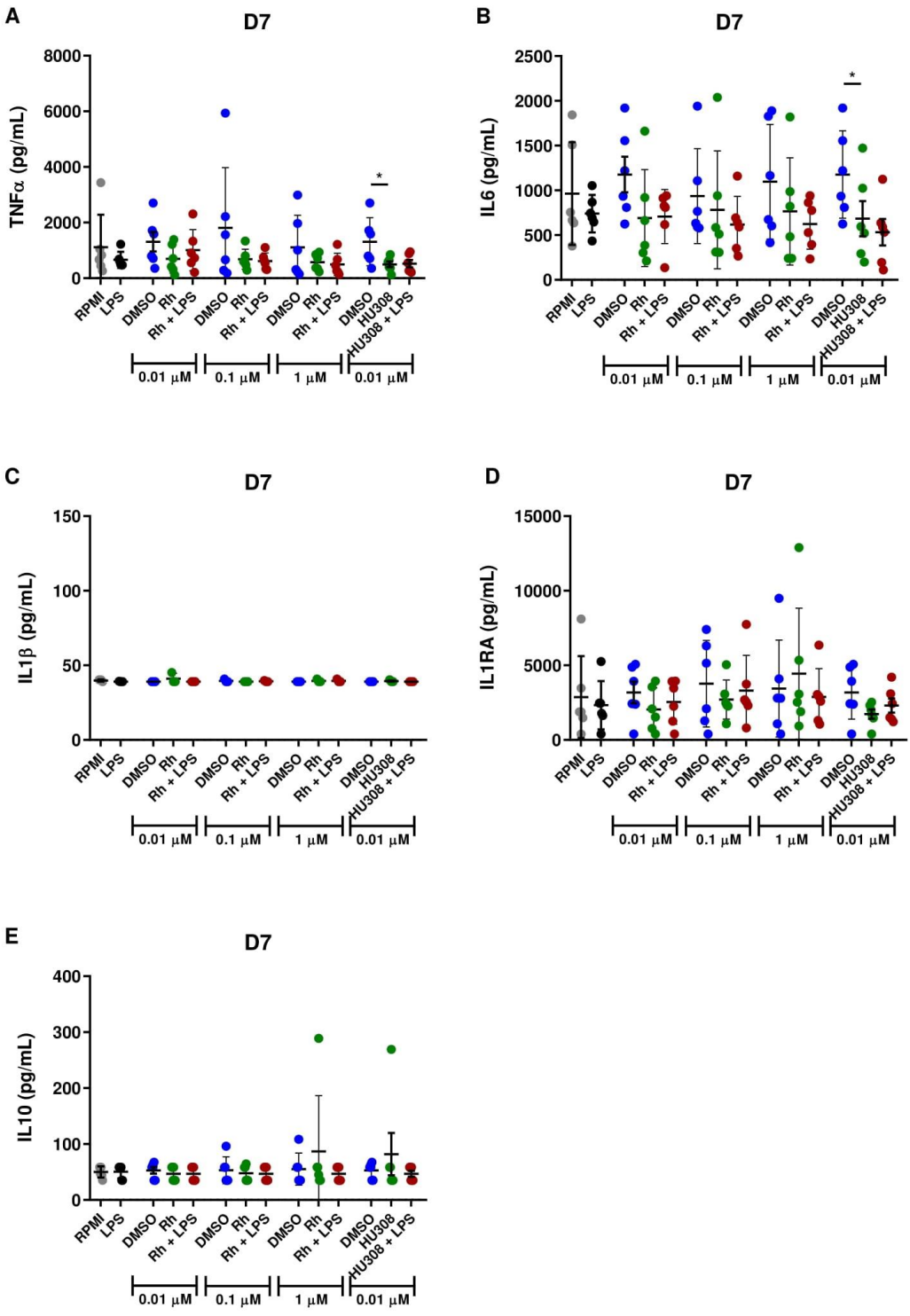

Figure 13: Unsuccessful tolerization of cells prevents observation of potential effects of Rh and HU308 on tolerizing monocytes. *After 24 hours of stimulation with 1 ng/mL LPS and/or Rh or HU308, monocytes were rested in RPMI for 5 days. On day 6, they were restimulated with LPS, and after 24 hours, supernatants were collected for cytokine analysis. Although TNFα (A), IL-6 (B), and IL-1Ra (D) show signs of slight tolerization, this did not occur, and conclusions about the effects of the cannabinoids on it cannot be drawn. Furthermore, we did not detect any IL-1β (C) and IL-10 (E) above minimum detection levels. Data are shown as mean ± SD, n=6, and significance was tested using paired Wilcoxon signed-rank test.*

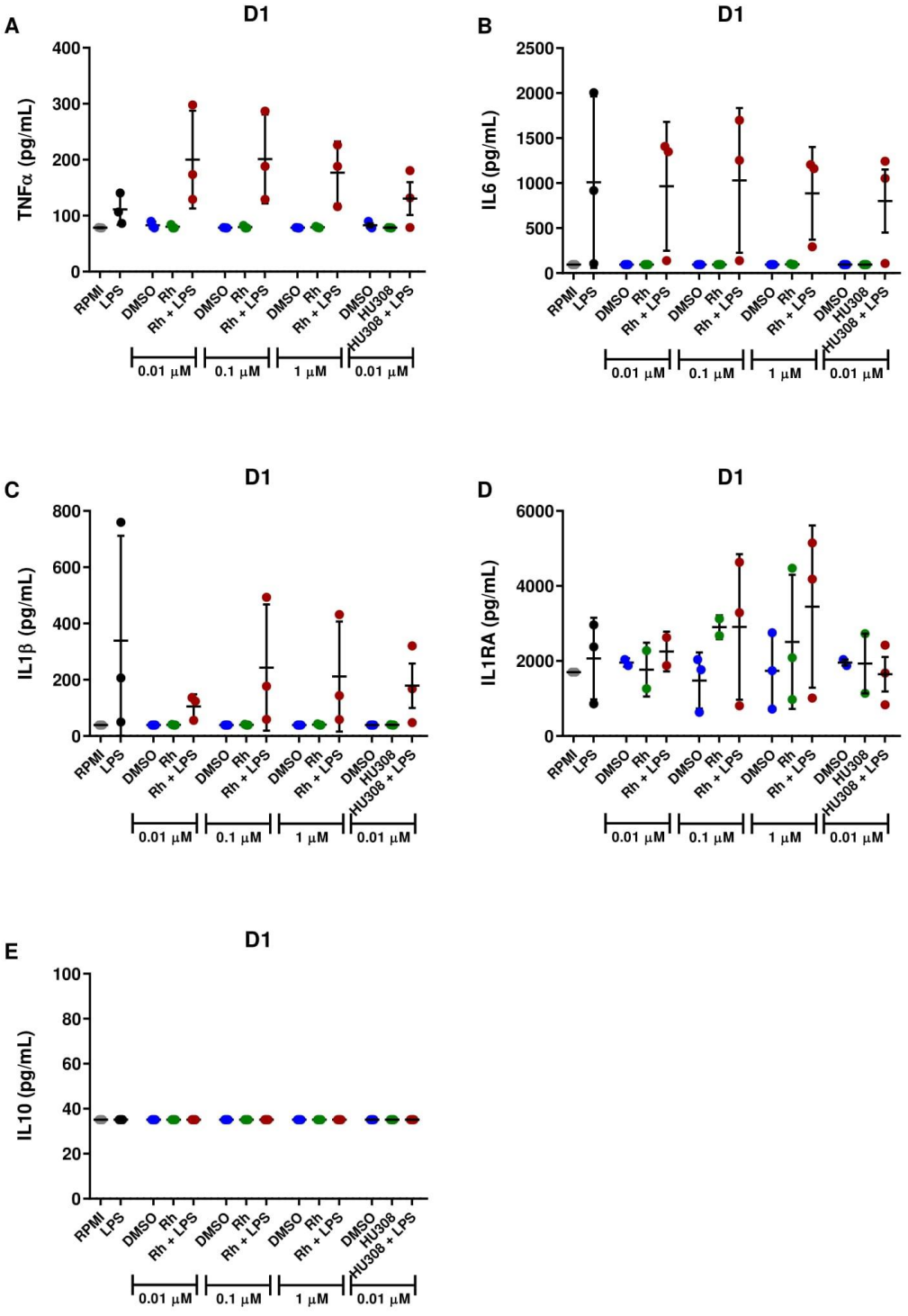

Figure 14: Cytokine production after 24 hours of stimulation with cannabinoids alone or combined with high dose LPS (10 ng/mL). *Human monocytes were stimulated for 24 hours with 10 ng/mL LPS on day 6, either with or without Rh or HU308. After this, supernatants were collected and analyzed using ELISA. TNFα, IL-6, IL-1β, and IL-1Ra (A-D) do show a trend in increased levels after LPS stimulation compared to RPMI. IL-10 (E) levels were not detected beyond minimum detection levels. Data are shown as mean ± SD, n=3, and significance was tested using paired Wilcoxon signed-rank test.*

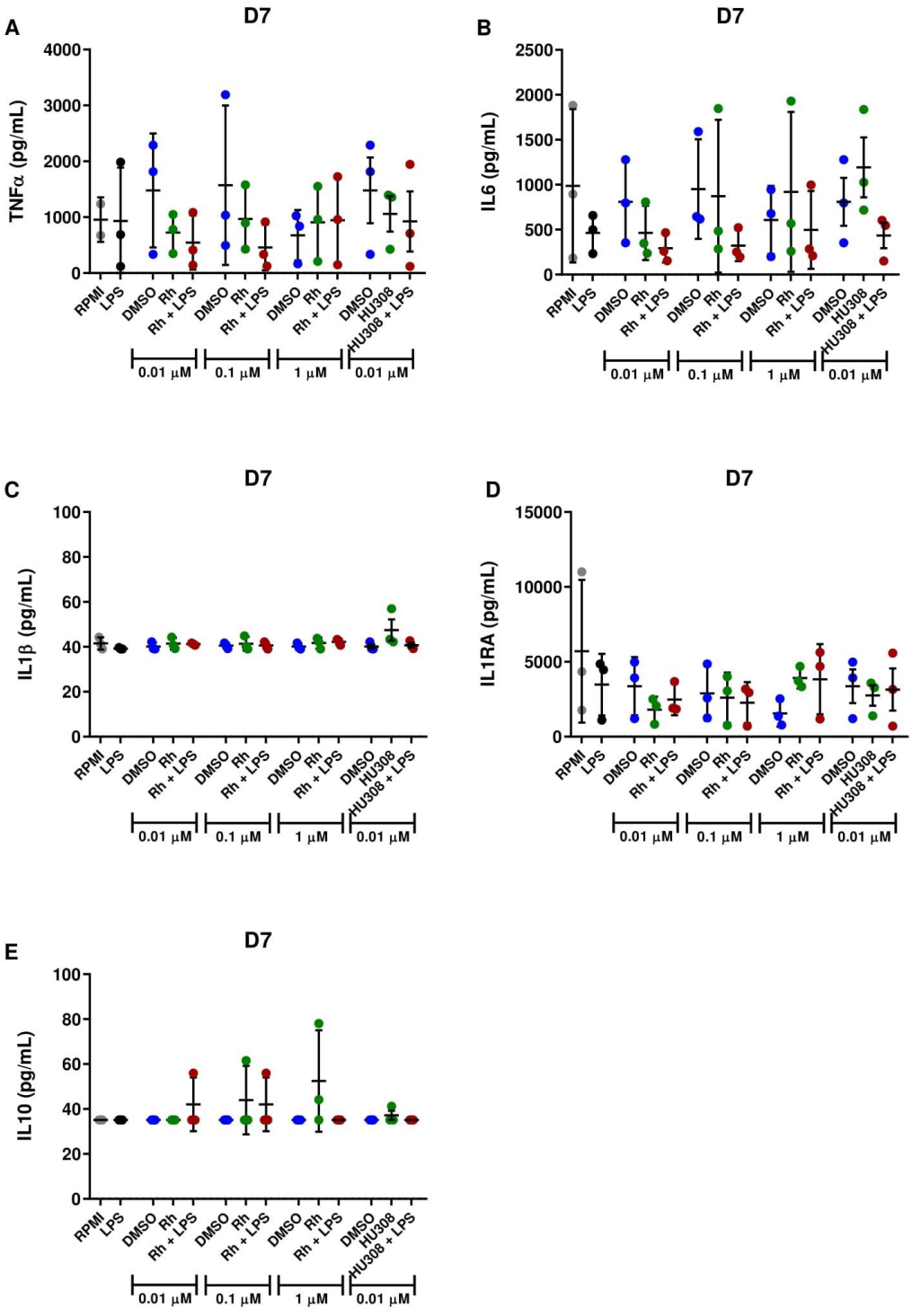

Figure 15: Unsuccessful tolerization of cells prevents observation of potential effects of Rh and HU308 on tolerizing monocytes. *After 24 hours of stimulation with 1 ng/mL LPS and/or Rh or HU308, monocytes were rested on RPMI for 5 days. On day 6, they were restimulated with LPS, and after 24 hours, supernatants were collected for cytokine analysis. TNFα levels were not affected by LPS tolerization (A), and although IL-6 (B), and IL-1Ra (D) show some signs of tolerization, conclusions about the effects of the cannabinoids on it cannot be drawn. Furthermore, we could not detect any IL-1β (C) and IL-10 (E) above detection levels. Data are shown as mean ± SD, n=3, and significance was tested using paired Wilcoxon signed-rank test.*

Discussion

In this research, we investigated, to our knowledge for the first time, the effect of CB1 antagonist Rh and CB2 agonist HU308 on trained immunity. This was assessed on several levels: immunologically as well as metabolically. Their effects were investigated on different programs of trained immunity, since each elicits slightly different responses. In addition, we aimed to analyze the effects of Rh and HU308 on LPS-induced tolerization of monocytes.

Firstly, the possibility of the cannabinoids causing increased cell death after stimulation was excluded with an LDH-based cytotoxicity assay. We found that both Rh and HU308 do not lead to considerable increases in cytotoxicity 24 hours after initial stimulation, compared to RPMI. However, when using manually lysed cells as a positive control, RPMI itself seemed to induce around 50% cell death. A likely cause for this high ratio is a flawed positive control, since 50% cell death does not match with microscopic observations. It may be due to incomplete lysis of cells, yet this possibility was eliminated through observation of lysed cells with light microscopy. We repeated the assay with the positive LDH control delivered with the assay kit as an alternative control. Indeed, this positive control led to much higher ODs of 1.0.

Although the protocol was not optimal, comparing RPMI with the other conditions still allowed us to conclude that the cannabinoids used in this study do not induce cytotoxicity during the first 24 hours of stimulation. In future research, it might be useful to measure cytotoxicity on other days, since we observed that 5 µM and 25 µM Rh, and concentrations of 1 µM HU308 and above did result in cell death on later days in the training protocol. Quantifying this would provide more insight into the long-term cytotoxic effects of Rh and HU308 on human monocytes.

Since the BCG vaccine is an established way of inducing trained immunity and is easily applicable in practice as an already approved vaccine, the next part of this study consisted of observing the immunological effects of Rh and HU308 on BCG-induced trained immunity. After 24 hours of stimulation, no effects of cannabinoids on cytokine levels were observed in any of the used concentrations. However, after LPS restimulation from day 6 to day 7, both Rh and HU308 decreased levels of TNFα and IL-6, compared to RPMI or DMSO controls. A valuable addition as a control in future experiments would be the addition of a BCG + DMSO control.

Furthermore, both cannabinoids significantly suppressed cytokine production when added together with BCG, indicating suppressive effects on trained immunity. These effects, however, did not translate to an altered metabolic state in stimulated cells, as often observed after inducing trained immunity. Increased lactate production is a marker for cells shifting to a glycolytic state. Though not significant, BCG caused an increase in lactate levels on day 6, compared to RPMI, but neither of the cannabinoids had an effect. Thus, although Rh and HU308 potently suppress trained immunity on an immunological level through inhibiting cytokine production, their effects do not change the glycolytic state of cells induced by training. However, other metabolic changes might be mediated by Rh and/or HU308. A manner of investigating this, would be measuring extracellular acidification rate, another readout of glycolysis, and oxygen consumption rate indicating OXPHOS with Seahorse metabolic analyzer.

When inducing trained immunity with β-glucan, effects of cannabinoids differed from BCG-induced training. Cytokine levels were similar 24 hours after stimulation, although 0.01 µM Rh suppressed IL-1Ra production compared to RPMI or its DMSO control. After 7 days, however, β-glucan training did occur, and the addition of 0.01 µM Rh seems to inhibit this, although significantly. In addition, effects were not

consistent throughout the different cannabinoid concentrations, and HU308 did not seem to affect training at all. When investigating lactate production, we observed no effects of Rh and HU308. Apart from future Seahorse analyses, it may be interesting to assess lactate production on a shorter term after initial stimulation, since cannabinoids may affect glycolysis quickly and/or transiently. A limitation of our analyses on the effects of cannabinoids on β-glucan training is that we analyzed only 6 donors, and there is high variability in their response to training and cannabinoids. By including more donors, thereby increasing statistical power, patterns may become more apparent and observed trends can reach statistical significance.

From the experiments carried out with oxLDL as the inducer of training, we could unfortunately not learn much since monocytes did not become trained according to cytokine measurements. In a total of 6 donors, both TNFα and IL-6 levels did not increase on day 7 upon oxLDL stimulation. Therefore, we cannot draw conclusions on the effect of cannabinoids on oxLDL training. There are, however, certain trends visible when stimulating cells with Rh + oxLDL, especially in the lowest Rh concentration. This seems to almost entirely shut down production of TNFα and IL-6. The cannabinoids alone did not affect cytokine levels, indicating that they do not by themselves suppress cytokine production. The lactate assay performed on day 6 also showed no changes with cannabinoids. However, oxLDL itself, seems to increase lactate levels as expected. Yet again, cytokine levels of only 6 donors were analyzed, and the lactate assay was performed on just 3 donors. Therefore, statistical power is much lacking, and more donors should be included to be able to observe trends. Finally, it should also be considered that oxLDL training did not succeed, and the data might change entirely when this does work.

In a final experiment, we investigated the effects of Rh and HU308 on the induction of tolerance. We hypothesized, since thus far, mainly suppressive effects of the used cannabinoids had been observed, that Rh and HU308 may enhance tolerization of cells. However, tolerization with LPS did not succeed, resulting in the inability to conclude the effects of Rh and HU308. Because it seemed that 1 ng/mL LPS was not effective enough, we increased the LPS concentration to 10 ng/mL for initial stimulation in a follow-up experiment to ensure tolerization this time. Unfortunately, again no tolerization occurred, and an investigation is needed on why this consistently failed. A potential explanation may be that the LPS used is no longer effective, and a new LPS batch should be used in subsequent experiments. This is, however, likely not the case, since the same stock of LPS was used for restimulation in training experiments, which did succeed. An possible solution is to increase LPS concentrations for initial stimulation even more, but this is presumably lethal to monocytes and renders us unable to measure cytokine levels on day 7 because most cells will have died. Of note, both tolerization experiments were carried out with 6 and 3 donors, therefore the statistical power is low. Yet, the optimum tolerizing conditions need to be established before including more donors. Only then the effects of Rh and HU308 on the tolerization of monocytes can be analyzed.

Overall, trained immunity is a double-edged sword allowing for many applications in different fields[23-31]. On the one hand, it reduces general mortality caused by infections and seems a valuable way of improving vaccine efficacy. In disease mechanisms, however, it may play a role in maintaining chronic inflammation and exacerbating disease severity. There, finding ways of reversing or suppressing training is worthwhile, leading to the alleviation of disease. Rh and HU308 prove to be interesting candidates in achieving the latter, for this research has shown their suppressive effects on trained immunity.

Rh is a CB1 antagonist, indicating that inhibiting the activity of CB1 has anti-inflammatory effects. This contradicts earlier findings, where CB1 activation instead of inhibition protects against inflammation[10, 32]. In another study, Rh reversed the anti-inflammatory state of cells even in the presence of a CB1 agonist[15].

Indeed, these studies were carried out in different settings and cell types than this research, but it is at least notable that a single cannabinoid can give rise to opposite effects. It is, therefore, crucial to thoroughly investigate the effects of Rh on different immune cells before potential applications. The fact that cannabinoids modulate other receptors beyond CB1 and CB2, and affect many immune functions, may prove it complicated to completely elucidate the mechanisms of action of cannabinoids[12].

Contrary to Rh, HU308 affects CB2 but as an agonist. Since CB1 and CB2 have been found to have opposite effects[33, 34], our observations of HU308 being immunosuppressive were not surprising. However, there are discrepancies in the literature. Where Rajesh *et al*. found HU308 suppressing cytokines like TNFα[13], Angelina *et al*. did not observe any inhibitory effects[15]. In mice, HU308 attenuated inflammasome activation and IL-1β production, but did not affect TNFα and IL-6 levels[35]. Thus, similar to Rh and CB1, HU308 and CB2 seem very variable in their effects. Contradiction also applies to whether cannabinoids promote or attenuate OXPHOS through either activation or inhibition of AMPK[15, 17]. Finally, cannabinoids are found to have apoptotic effects, elucidating yet another mechanism of action[10, 15, 17]. This may also explain our earlier observations of higher concentrations being lethal to cells.

The abovementioned findings in several other studies underline the biggest limitation of our research. Only CB1 antagonist Rh and CB2 agonist HU308 could be investigated due to compound availability. Since CB1 and CB2 appear to have opposite effects, it will be very useful to research how a CB1 agonist or a CB2 antagonist would affect trained immunity. What must also be extensively considered is that aside from the immune system, CB1 and CB2 are abundantly expressed in the central nervous system. This likely has important implications for using cannabinoids to modulate immunity in the clinic. Finding ways to selectively deliver cannabinoids to their targets, thereby reducing off-target adverse effects, will be important eliminating any undesired effects of cannabinoids on the CNS. However, initially, much more *in vitro* and model organism research is needed to step-by-step elucidate the potential of cannabinoids in affecting trained immunity, and the mechanistic basis of their effects.

Several follow-up *in vitro* experiments are already mentioned above. Additionally, BCG training is known to promote ROS production[4]. Therefore, the effects of cannabinoids on this can be measured and will provide insights into another immunometabolic arm affected by cannabinoids. Furthermore, induction of trained immunity leads to epigenetic reprogramming[2, 4, 5]. Performing chromatin-immunoprecipitation, and (single cell) RNA sequencing, can further elucidate how epigenetic signatures of trained cells are altered by the different cannabinoids.

In terms of cytokines, we only tested TNFα, IL-6, IL-1β, IL-1Ra, and IL-10. Other studies, however, have found that activation or inhibition of CB1 and/or CB2 also affected production of cytokines such as IL-1α, IL-2, IL-3, IL-4, IL-5 IL-8, IL-12, IL-13, IL-23 and interferon-γ[10]. Including these readouts in future experimental settings may prove interesting.

Finally, induction of trained immunity differentiates monocytes into macrophages. These can be pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages[7]. Since cannabinoids have shown to influence immunity on several levels, it is likely that their effects skew differentiation towards certain macrophage subtypes. This can be investigated through flow cytometry using markers specifically for M1 and M2 macrophages, such as macrophage mannose receptor for M2 macrophages and CD68 for M1 macrophages[36].

Taken together, this study gives a first insight on the effects of CB1 antagonist Rh, and CB2 agonist HU308 on trained immunity. Since we found profound suppressive effects, they may have important implications

in reducing the underlying inflammatory state of many diseases, among which CVD. In addition, future research might elucidate pro-inflammatory effects of opposites of the used compounds, CB1 agonists and CB2 antagonists. Thus, cannabinoids are versatile and important candidates of influencing trained immunity, and this research forms a foundation for future experiments on their potential.

Methods

Isolation of PBMCs, enrichment for monocytes, and *in vitro* training of human monocytes

Peripheral blood mononuclear cell (PBMC) isolation from buffy coats from healthy donors (Sanquin Blood Bank, Nijmegen), obtained upon informed consent, was carried out using differential density centrifugation over Ficoll-Paque (GE Healthcare), as described by Arts *et al.*[37]. Enrichment for monocytes was performed using Percoll, as described previously by Repnik *et al.*[38]. Cells were resuspended in RPMI 1640 (Dutch Modified) (Gibco) supplemented with 50 µg/mL gentamicin (Thermo Fisher), 1 mM pyruvate (Life Technologies), and 2 mM Glutamax (Life Technologies), and added to a clear flat bottom 96 wells plate (Corning) at a count of 10^5 cells/well. Enrichment for adherent monocytes was performed by incubating cells for 1 hour at 37 °C, 5% CO_2, followed by washing once with warm PBS (Gibco). Then, cells were incubated with RPMI only as negative control, and either 5 µg/mL BCG, 2 µg/mL β-glucan, 10 µg/mL oxLDL, DMSO, Rh or HU308 (in concentrations stated in Table 1), or Rh or HU308 combined with either 5 µg/mL BCG, 2 µg/mL β-glucan, or 10 µg/mL oxLDL. After 24 hours, cell supernatants were collected and stored at -20 °C, monocytes were washed once with warm PBS, and incubated with RPMI culture medium supplemented with 10% human serum. After the 5 days resting period, supernatants were again collected and stored at -20 °C, and monocytes were restimulated with 10 ng/mL LPS. Addition of RPMI instead of LPS served as negative control. Finally, after 24 hours of restimulation, cell culture supernatants were collected and stored at -20 °C.

Tolerance assay

The tolerance assays performed in this study were carried out as described above. However, instead of a training compound (BCG/β-glucan/oxLDL), either alone or combined with cannabinoids, 1 or 10 ng/mL LPS was used.

Cytotoxicity assay

LDH release was measured in 24 hours cell culture supernatants. This assay was performed using CytoTox96 NonRadioactive cytotoxicity assay (Promega). Positive controls included were manually lysed cells, and the positive LDH control as part of the commercial kit. Manually lysed cells were prepared by adding 20 µL of Lysis Buffer to each positive control well, followed by incubation for 45 minutes at 37 °C, 5% CO_2. After that, 24 hours supernatants were transferred into clear flat bottom 96 wells plates (Corning), and in a 1:1 ratio Substrate Mix was added, followed by 30 minutes incubation at room temperature, on a shaker, protected from light. Finally, 50 µL of Stop Solution was added to each well, and absorbance was measured at 490/492 nm using a plate reader (BioTek).

Cytokine detection through ELISA

TNFα, IL-6, IL-1β, IL-10, and IL-1Ra production in cell culture supernatants was measured after 24 hours and 7 days of stimulation using commercial DuoSet ELISA (R&D Systems). Assays were performed according to the manufacturer's instructions. A quick overview of which cytokines were assessed in different training experiments and the compounds (concentrations) used is listed in Table 1.

Table 1: Assessed cytokines on different training agents and compounds used in this study.

Training agent	Compound + concentrations	Cytokines assessed in 24 hour-supernatants	Cytokines assessed in 7 days supernatants
BCG	Rh (1 µM, 5 µM, 25 µM) HU308 (1 µM, 5 µM, 25 µM)	TNFα, IL-6, IL-10, IL-1Ra	TNFα, IL-6
BCG	Rh (0.01 µM, 0.1 µM, 1 µM) HU308 (0.01 µM, 0.1 µM, 1 µM)	TNFα, IL-6, IL-10, IL-1Ra	TNFα, IL-6
β-glucan	Rh (0.01 µM, 0.1 µM, 1 µM) HU308 (0.01 µM)	TNFα, IL-6, IL-1β, IL-10, IL-1Ra	TNFα, IL-6
oxLDL	Rh (0.1 µM, 1 µM) HU308 (0.01 µM)	-	TNFα, IL-6
LPS (tolerance assay)	Rh (0.01 µM, 0.1 µM, 1 µM) HU308 (0.01 µM)	TNFα, IL-6, IL-1β, IL-10, IL-1Ra	TNFα, IL-6, IL-1β, IL-10, IL-1Ra

Lactate assay

Lactate concentrations in cell culture supernatants were measured oin day 6 after using a fluorometric assay. Briefly, day 6 supernatants were diluted 150 times in PBS and transferred to a black 96-wells flat bottom plate (Corning). They were complemented 1:1 with the reaction mix, consisting of 10 mM Amplex Red (Life Technologies), 100 U/mL Lactate oxidase (Sigma), and 10 U/mL horse radish peroxidase (Thermo Scientific), followed by 20 minutes incubation at room temperature, on a shaker, protected from light. Fluorescence was measured at 530/±25 nm and 590/±35 nm using a plate reader (BioTek).

Microscopy

Cell viability and morphology were assessed daily during training experiments using light microscopy. Pictures were taken using Leica LAS software (Leica Microsystems).

Statistical analyses

Data are shown as mean ± SD, and significance is tested using a Wilcoxon matched-pairs signed-rank test. P-values below 0.05 were considered statistically significant. Data analysis was performed with GraphPad Prism 8.0. Briefly, comparisons were made between RPMI controls and BCG/β-glucan/oxLDL training, RPMI controls and DMSO controls, compounds *versus* their DMSO controls, and compounds + BCG/β-glucan/oxLDL *versus* BCG/β-glucan/oxLDL training.

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