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BRIEF CONCLUSIVE REPORT

INFLAMMATION, EXTRACELLULAR MEDIATORS AND EFFECTOR MOLECULES

Immune modulatory effects of progesterone on oxLDL-induced trained immunity in monocytes

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Abstract

Atherosclerotic cardiovascular diseases (CVD) are among the leading causes of death in the world. Monocyte-derived macrophages are key players in the pathophysiology of atherosclerosis. Innate immune memory following exposure of monocytes to atherogenic compounds, such as oxidized low-density lipoproteins (oxLDL), termed trained immunity, can contribute to atherogenesis. The current study aimed to elucidate intracellular mechanisms of oxLDL-induced trained immunity. Using untargeted intracellular metabolomics in isolated human primary monocytes, we show that oxLDL-induced trained immunity results in alterations in the balance of intracellular steroid hormones in monocytes. This was reflected by a decrease in extracellular progesterone concentrations following LPS stimulation. To understand the potential effects of steroid hormones on trained immunity, monocytes were costimulated with oxLDL and the steroid hormones progesterone, hydrocortisone, dexamethasone, β-estradiol, and dihydrotestosterone. Progesterone showed a unique ability to attenuate the enhanced TNFα and IL-6 production following oxLDL-induced trained immunity. Single nucleotide polymorphisms in the nuclear glucocorticoid, progesterone, and mineralocorticoid receptor were shown to correlate with ex vivo oxLDL-induced trained immunity in 243 healthy volunteers. Pharmacologic inhibition experiments revealed that progesterone exerts the suppression of TNFα in trained immunity via the nuclear glucocorticoid and mineralocorticoid receptors. Our data show that progesterone has a unique ability to suppress oxLDL-induced trained immunity. We hypothesize that this effect might contribute to the lower incidence of CVD in premenopausal women.

KEYWORDS
cardiovascular disease, oxLDL, progesterone, steroid hormone, trained immunity

Abbreviations: BCG, Bacillus Calmette-Guérin; CVD, cardiovascular disease; DHT, dihydrotestosterone; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; MSEA, metabolite set enrichment analysis; oxLDL, oxidized LDL; PR, progesterone receptor; SNP, single nucleotide polymorphism.

1 | INTRODUCTION

Cardiovascular diseases (CVD) are among the deadliest diseases globally.[1] Atherosclerosis is the most important disease mechanism leading to CVD and is characterized by a state of chronic low-grade inflammation of the arterial wall. Macrophages are the most abundant immune cells in atherosclerotic plaques and contribute to the initiation, progression, and destabilization of these plaques. A series of recent studies showed that the anti-inflammatory drugs colchicine and canakinumab reduce CVD events, highlighting the important role of inflammation in the pathophysiology of CVD.[2–4]

Macrophages preserve a long-term nonspecific memory of previous encounters with immunologic stimuli, as seen by an augmented response in terms of cytokine production to subsequent immune challenge. This novel phenotype of innate immune adaption termed "trained immunity" is induced in response to a broad range of stimuli, such as β-glucan, the Bacillus Calmette-Guérin (BCG) vaccine, as well as endogenous ligands such as oxidized LDL (oxLDL).[5–8] This new avenue of macrophage activation is being studied for its potential role in chronic low-grade inflammation, a cornerstone of atherosclerotic disease. OxLDL-induced trained immunity is mediated, at least in part, by modulation of cellular metabolism and epigenetic remodeling.[7,9] This maladaptive innate immune reprogramming has been observed in atherosclerosis-prone mice fed a Western-type diet, which persisted even weeks after switching back to a normal diet.[10] Importantly, also in humans with elevated LDL cholesterol concentrations, circulating monocytes have a trained immune phenotype.[11] It is therefore rational to hypothesize that trained immunity contributes to long-term activation of monocytes in patients with atherosclerosis. Indeed, circulating monocytes of patients with established coronary atherosclerosis show an increased cytokine production capacity, with accompanying epigenetic and metabolic characteristics of trained immunity.[12]

Current management of patients with CVD is aimed at risk factor control. However, a considerable number of patients remain inadequately protected under current clinical guidelines, giving rise to a significant residual cardiovascular risk. This urges the identification of novel pharmacologic targets to reduce CVD. With gross metabolic reprogramming of the macrophage serving as an important scaffold for the sustained inflammation seen in atherosclerosis, there is interest in the nature of these metabolic changes for clinical understanding and manipulation. In order to elucidate new immunometabolic processes for the better prediction and management of CVD, we performed an unbiased metabolomics analysis of oxLDL-trained macrophages. Herein, we found that the intracellular balance of steroid hormones was altered by oxLDL-induced trained immunity. After investigating the immune modulatory effects of various key steroid hormones, we identified progesterone as an inhibitor of trained immunity, a mechanism that is partly under the control of the nuclear glucocorticoid and mineralocorticoid receptor (GR and MR).

2 | MATERIALS AND METHODS

2.1 | Reagents

Water-soluble progesterone, β-estradiol, 5α-dihydrotestosterone (DHT) solution in methanol, hydrocortisone, mifepristone, PF-02413873, spironolactone, and *Escherichia coli* LPS (serotype 055:B5) were all obtained from Sigma–Aldrich (St. Louis, MO, USA). LPS was further purified as described previously.[13] Dexamethasone was obtained from Centrafarm (Breda, The Netherlands).

2.2 | Preparation of oxidized-LDL

OxLDL was prepared from LDL as described previously.[14] Briefly, LDL was isolated from serum of healthy subjects via ultracentrifugation. LDL was then oxidized with 20 μM $CuSO_4$ for 16 h in a shaking heat block at 37°C at 600 rpm followed by further dialysis. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). We previously showed that this oxLDL is endotoxin free.[7]

2.3 | PBMC and monocyte isolation

PBMCs and monocytes were isolated from blood from healthy donors after they provided written informed consent (Sanquin Blood Bank, Nijmegen, The Netherlands) as described previously.[15]

2.4 | In vitro training of adherent monocytes

Training of adherent monocytes was performed as described previously.[6] Adherent monocytes were incubated with 200 μl RPMI medium supplemented with 10 μg/ml oxLDL in presence or absence of the various test compounds. In experiments with pharmacologic interference with hormone receptors, compounds were added in combination with oxLDL and progesterone. Medium was supplemented with 10% human pooled serum. Cells were left to rest for 5 days with a medium change on day 3. Cells were restimulated with RPMI medium or 10 ng/ml LPS on day 6. After 24 h, supernatants were collected and stored at −20°C until cytokine measurements.

2.5 | Cytokine, pregnenolone, and progesterone measurements

Production of TNFα, IL-6, pregnenolone, and progesterone in supernatants was measured using the IL-6 and TNF-α DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA), Pregnenolone ELISA kit (Abnova, Taipei, Taiwan) and Progesterone Competitive ELISA Kit

(Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions.

2.6 | Metabolomics analysis

Approximately 1×10^7 monocytes were seeded into 10 cm Petri dishes (Greiner, Alphen aan de Rijn, The Netherlands) ± oxLDL (10 μg/mL) in 10 ml medium volumes for 24 h, washed with warm PBS and incubated in normal culture medium at 37°C, 5% CO_2. Following 5 days in culture, cells were washed 3 times with 75 mM ammonium carbonate adjusted to pH 7.4 with formic acid, and snap-frozen in liquid nitrogen. Intracellular metabolites were extracted with 70% ethanol heated to 70°C, supernatants were collected and stored at −80°C. Untargeted analysis of intracellular metabolites was performed by flow injection-time-of-flight mass spectrometry on an Agilent 6550 QTOF Instrument operated in negative mode 4 GHz, high resolution in a mass to charge (m/z) range of 50–1000, as described previously.[16]

Data were analyzed using the online platform MetaboAnalyst version 4.0. Metabolite set enrichment analysis (MSEA) was performed on a list of mass spectrometry peaks and fold changes derived from the comparison of oxLDL trained to naïve macrophage metabolites.[17,18] Pathway analysis was performed using the manually curated metabolite MFN library original to the mummichog package.

2.7 | Genetic analysis

Genotyping was performed on 267 healthy individuals of Western European ancestry from the 300BCG cohort[19] using the commercially available single nucleotide polymorphism (SNP) chip, Infinium Global Screening Array MD v1.0 from Illumina. Adjacent ex vivo training with oxLDL was carried out in PBMCs collected for each individual. The methods for QTL mapping have been described previously.[20]

2.8 | mRNA extraction and RT-PCR

Monocytes were cultured as described earlier. mRNA was extracted by TRIzol (Life Technologies, Santa Clara, CA, USA) after 24 h and 6 days of oxLDL training, according to the manufacturer's instructions, and cDNA was synthesized using iScript Reverse Transcriptase (Invitrogen, Waltham, MA, USA). Relative expression was determined using the SYBR Green method (Invitrogen) on an Applied Biosciences StepOne PLUS qPCR machine, and the values are expressed as fold increases in mRNA levels, relative to those in nontrained cells, using 18s as a reference gene. Primers are listed in Table 1.

2.9 | Statistics

Cytokine and hormone measurements were performed on cells from 6 donors. Data are presented as means ± SEM. Trained immunity was expressed as the fold change of cytokine production capacity of oxLDL-trained cells relative to the untrained cells, as previously reported.[8,21] In addition, in Figure S1, we provide the absolute cytokine values. Statistical testing was performed by using the Wilcoxon matched-pairs signed rank test using GraphPad Prism 6. p Values below 0.05 were considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | Enrichment of steroid hormone metabolites in oxLDL-trained macrophages

Metabolic reprogramming is a foundational aspect of trained immunity induction. Previous work on monocytes trained with the fungal cell wall component β-glucan and the live attenuated vaccine BCG revealed marked alterations to the metabolism of the macrophages, with noticeable changes in glycolysis, oxidative phosphorylation, and glutamine utilization.[22] In addition, we recently reported that also oxLDL-induced trained immunity is dependent on glycolysis[9] and OXPHOS.[23] In order to identify other metabolic pathways important for oxLDL-trained immunity, we performed untargeted intracellular metabolomics on oxLDL-trained macrophages. MSEA pathway analysis revealed "C21-steroid hormone biosynthesis and metabolism" as the most altered pathway (Figure 1(A), adjusted $p = 0.22$, normalized enrichment score [NES] = −2.03). A NES of −2.03 suggests a lowered presence of intracellular steroid hormones in the trained cells. Additionally, "Androgen and estrogen biosynthesis and metabolism" appeared as the third most altered pathway (p adj = 0.22) with a NES of −1.69 (table with metabolites shown in Table S1 and S2, respectively).

In an effort to validate the finding that steroid hormones are altered in oxLDL-trained macrophages, we identified individual hormones of interest from these pathways. Pregnenolone was modestly decreased in the oxLDL-trained macrophages (data not shown). Pregnenolone is a metabolite that is a precursor for the synthesis of all steroid hormones. Downstream of pregnenolone is progesterone that lays at the crossroads of many steroid hormone metabolic processes, while having well-characterized effects on biologic processes including inflammation.[24] Progesterone was decreased in our dataset. We validated these findings using commercial ELISA kits, by measuring the concentrations of pregnenolone and progesterone in the supernatants of oxLDL-trained macrophages (Figure 1(B)). This was done with and without the stimulation of LPS. Interestingly, extracellular levels of progesterone were lowered following LPS restimulation in the oxLDL-trained macrophages, reminiscent of the MSEA where progesterone was lower in oxLDL-trained macrophages. The concentration of pregnenolone was not altered by oxLDL training nor by LPS restimulation.

The mechanism by which these cells deplete progesterone from the medium is as yet unknown, though it is interesting to speculate that the hormones are depleted in order to facilitate an increase in proinflammatory cytokine production upon immune challenge. There is some evidence that tissue resident macrophages produce some of the

TABLE 1 Forward and reverse primer sequences (5′–3′) for genes used for quantitative real-time PCR

Gene	Forward primer	Reverse primer
18S	GATGGGCGGCGGAAAATAG	GCGTGGATTCTGCATAATGGT
PR (PGR)	GGAAGGGCAGCACAACTA	AAGGAATTGTATTAAGAAGTAA
GR	ATAGCTCTGTTCCAGACTCAACT	TCCTGAAACCTGGTATTGCCT
MR (NR3C2)	CAGCAGTGAAATGGGCAAAG	TCGTACATGCAGGGTAGAGT

(A)

MSEA MFN	Pathway Total	Hits	P-val	P-adj	NES
C21-steroid hormone biosynthesis and metabolism	112	41	0.016	0.2248	-2.026
C5-Branched dibasic acid metabolism	10	5	0.017	0.2248	1.636
Androgen and estrogen biosynthesis and metabolism	95	29	0.019	0.2248	-1.689
Nitrogen metabolism	6	4	0.019	0.2248	1.472
Alanine and Aspartate Metabolism	30	21	0.019	0.2248	1.642
Drug metabolism - cytochrome P450	53	15	0.020	0.2248	-1.924
Ascorbate (Vitamin C) and Aldarate Metabolism	29	19	0.020	0.2248	1.779
Arginine and Proline Metabolism	45	29	0.020	0.2248	1.884
Aminosugars metabolism	69	27	0.023	0.2248	1.677
Tryptophan metabolism	94	40	0.025	0.2248	1.667
Pyrimidine metabolism	70	45	0.026	0.2248	1.746
Blood Group Biosynthesis	44	7	0.032	0.2248	1.642
Glycosphingolipid biosynthesis - neolactoseries	16	7	0.032	0.2248	1.642

(B)

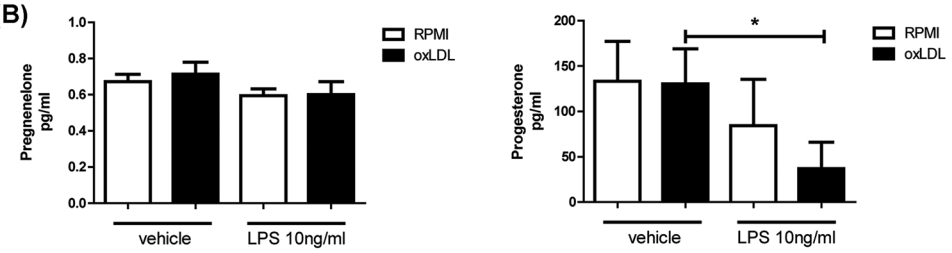

FIGURE 1 oxLDL-induced trained immunity alters the abundance of intracellular and extracellular steroid hormones. Adherent monocytes were trained with oxLDL (10 μg/ml) or RPMI for 24 h, washed with PBS and rested in normal culture medium for 5 days. (A) Untargeted metabolomics analysis was performed on intracellular metabolites. Represented are the results of unannotated gene set enrichment (GSEA) pathway analysis of the identified raw *m/z* score and their respective *p* values (Table shows total pathway hits, hits from uploaded metabolites data, raw *p* values, gamma-adjusted *p* value, and normalized enrichment score (NES); $n = 3$). (B) Following 5 days of resting in normal culture media, trained monocytes were stimulated with LPS (10 ng/ml) or RPMI for 24 h. Levels of pregnenolone and progesterone were determined via ELISA. (Wilcoxon matched pairs signed rank test, $n = 6$)

necessary enzymes needed to metabolize hormones,[25] potentially offering clues to how LPS stimulation results in a decrease in hormone levels.

3.2 | Progesterone but not hydrocortisone, dexamethasone, β-estradiol, or DHT modulate oxLDL-trained immunity in macrophages

Steroid hormones are well characterized as having immune regulatory effects,[24,26] therefore we next aimed to investigate the potential impact of adding various steroid hormones on the training response in monocytes stimulated with oxLDL. Progesterone, hydrocortisone, β-estradiol, and DHT were selected for their central positions in "C21-Steroid hormone biosynthesis and metabolism" and "Androgen and estrogen biosynthesis and metabolism," respectively, as well as their well-described biologic functions. The corticosteroid dexamethasone was also included. Here, adherent monocytes were exposed to RPMI medium or oxLDL combined with progesterone, β-estradiol, DHT, hydrocortisone, or dexamethasone for 24 h. Following a 5-day wash-out period, cytokine production was determined in response to 24 h LPS restimulation (Figures 2(A)–2(E) and Figure S1 for absolute cytokine values).

Progesterone at a concentration of 100 pg/ml significantly lowered TNFα production following oxLDL training, compared with the RPMI control condition (Figure 2(A)). Hydrocortisone also decreased TNFα production in oxLDL-trained macrophages, although not significantly ($p = 0.0625$; Figure 2(B)). Progesterone and hydrocortisone significantly reduced IL-6 production at concentrations of 100 and 1000 pg/ml, respectively. Although dexamethasone reduced cytokine production capacity in general in both trained and untrained cells, it did not specifically interfere with the trained immune response (i.e., did not

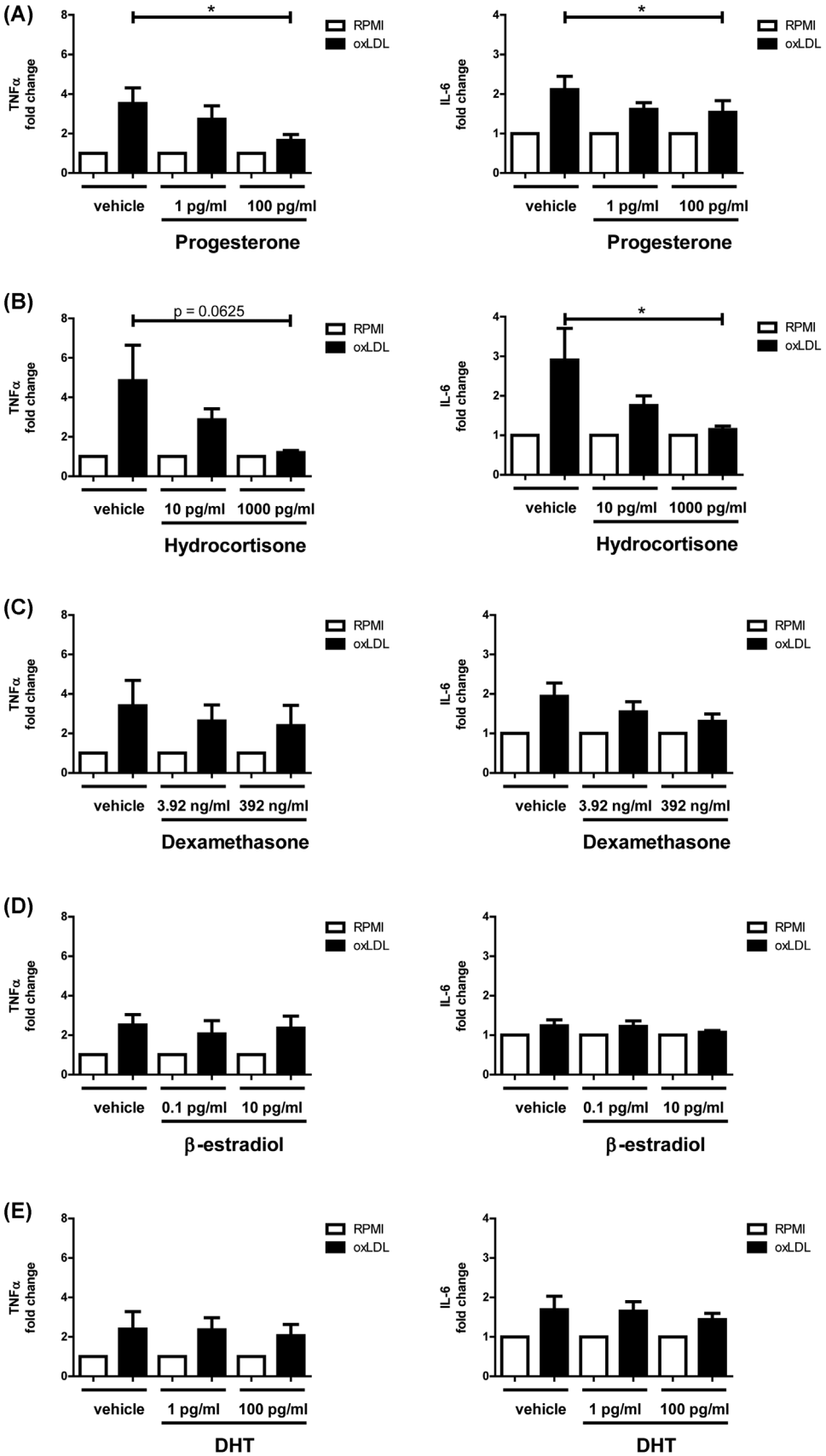

FIGURE 2 Progesterone inhibits oxLDL-induced trained immunity. Monocytes were trained with oxLDL (10 μg/ml) or RPMI for 24 h ± (A) progesterone, (B) cortisol, (C) dexamethasone, (D) β-estradiol, or (E) DHT. Monocytes were then washed with PBS, rested for 5 days in culture media, and subsequently stimulated with LPS (10 ng/ml) for 24 h. Levels of TNFα and IL-6 were measured in supernatants via ELISA. (Wilcoxon matched pairs signed rank test, $n = 6$, $^*p < 0.05$)

affect the fold change of cytokine production in the trained cells). No meaningful reduction in either TNFα or IL-6 production was observed for β-estradiol or DHT (Figures 2(C)–2(E)). For β-estradiol and DHT, previous data from de Bree et al.[27] have demonstrated the inability of these sex hormones to interfere with trained immunity induced by BCG.

It is well known that premenopausal women are less likely than men to suffer from CVD as a result of atherosclerosis.[28] This risk increases for postmenopausal women and may even exceed that of men.[29] Estrogen, the primary female sex hormone, is commonly considered to be responsible for the lowered risk of atherosclerosis in premenopausal women, due to its cardioprotective effects.[30] Following menopause, in addition to a reduction in the circulating estrogen concentration, there is a parallel decrease in the production of progesterone by the ovaries. However, research on the effect of progesterone levels on the risk for developing CVD is scarce and most studies on sex differences in CVD focus on the effects of estrogen on atherosclerosis, rather than those of progesterone. This lack of knowledge is present despite clear immune-modulatory effects of progesterone interfering with signaling induced through pattern-recognition receptors.[31–33] We now show that progesterone is able to ameliorate oxLDL-induced trained immunity, which is thought to contribute to the incessant atherosclerotic vascular wall inflammation in CVD. We therefore hypothesize that this effect of progesterone might contribute to the lower incidence of CVD in premenopausal women, and the increase in CVD risk following menopause.

To better understand how progesterone modulates trained immunity, we performed a series of additional studies making use of genetic variation in progesterone-related genes, and pharmacologic inhibitors of steroid hormone receptors.

3.3 | SNPs in the hormone receptors and enzymes involved in steroidogenesis correlate with ex vivo oxLDL-induced trained immunity in PBMCs

In order to gain more insight into the importance of steroid hormones in modulating oxLDL-trained immunity, we investigated how SNPs (in a window of ±250 kb) in genes encoding for receptors and enzymes in the steroid metabolism pathway correlate with the ex vivo training capability of adherent PBMCs from 243 healthy volunteers, which were included in the 300BCG cohort of the Human Functional Genomic Study (www.humanfunctionalgenomics.org). In all these individuals, isolated PBMCs were exposed to oxLDL ex vivo according to the trained immunity protocol, and the fold change in TNFα and IL-6 production after restimulation with LPS for 24 h on day 6 was measured and used as a measure for trained immunity. By using this approach in the 300BCG and other cohorts, we have previously identified important roles for enzymes involved in glycolysis and oxidative phosphorylation in oxLDL-induced trained immunity.

We investigated genes encoding for the steroid hormone receptors as well as genes relevant for steroid metabolism (Figure 3(A)). We observed many SNPs strongly associated with an augmented cytokine response ($p < 0.05$). The most strongly correlated SNPs were found in the estrogen receptor *ERS1* (rs2296254, $p = 0.0004$) for TNFα and the *GR* (rs246608, $p = 0.0013$) for IL-6. There was also a relatively strong association between a SNP in *GR* with TNFα responses (rs246432, $p = 0.0225$), as well as SNPs for the nuclear progesterone receptor (PR) (*PGR*) for both TNFα and IL-6 production capacity (rs660541, $p = 0.0013$; and rs1943758, $p = 0.0036$, respectively). SNPs around the MR (*NR3C2*) show nominal significance for TNFα (rs1529935, $p = 0.0254$) and IL-6 (rs10032250, $p = 0.0108$). SNPs in the membrane PR *PAQR8* further showed strong correlations with TNFα and IL-6 production (rs12210492, $p = 0.0032$ and rs1266823, $p = 0.0073$, respectively).

To explore the expression of the GR, MR, and PR, we measured the expression levels with RT-PCR. We could demonstrate expression of the GR, and MR, but not of the PR. This is in accordance with a lack of expression data of the PR in human whole blood and monocytes/macrophages on the online GTEX platform (www.gtexportal.org) as well as the The Human Protein Atlas database (www.proteinatlas.org). Expression of the GR showed a trend toward being elevated following 24 h of oxLDL stimulation (Figure 3(C)); however, GR transcription was unaltered on day 6. Similarly, the MR (NR3C2) expression was unaltered by oxLDL treatment at both time points.

SNPs in genes relating to steroid metabolic processes correlated less strongly with cytokine production after oxLDL-induced trained immunity. However, there was a correlation between a SNP in hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2 (*HSD3B2*) and TNFα (rs4659181, $p = 0.0011$). This gene encodes an enzyme that is responsible for the conversion of pregnenolone into progesterone. Another SNP in the HMG-CoA reductase (*HMGCR*) enzyme that is responsible for the synthesis of cholesterol, which serves as the backbone of all steroid hormones, was correlated with IL-6 production (rs149280707, $p = 0.0029$). *HMGCR* is an important rate limiting enzymes in the mevalonate pathway, which has previously been shown by our group to play an important role in trained immunity.[34]

These findings underscore our in vitro findings that the steroid hormones are relevant for oxLDL-induced trained immunity in human monocytes.

3.4 | Nuclear hormone receptors are responsible for the immune modulatory effects of progesterone on oxLDL-induced trained immunity

Pharmacologic interference of progesterone binding to its cognate receptors provides important evidence on the pathways through which progesterone leads to an inhibition of oxLDL-induced trained immunity. Although SNPs around *ESR1* correlated strongly with TNFα and IL-6 production following oxLDL-induced trained immunity, we have demonstrated that β-estradiol does not modulate oxLDL-induced trained immunity. To date, there are no reports of progesterone exerting any effects through the ESR1. Additionally, there are no commercially available inhibitors for the membrane PRs, and downstream

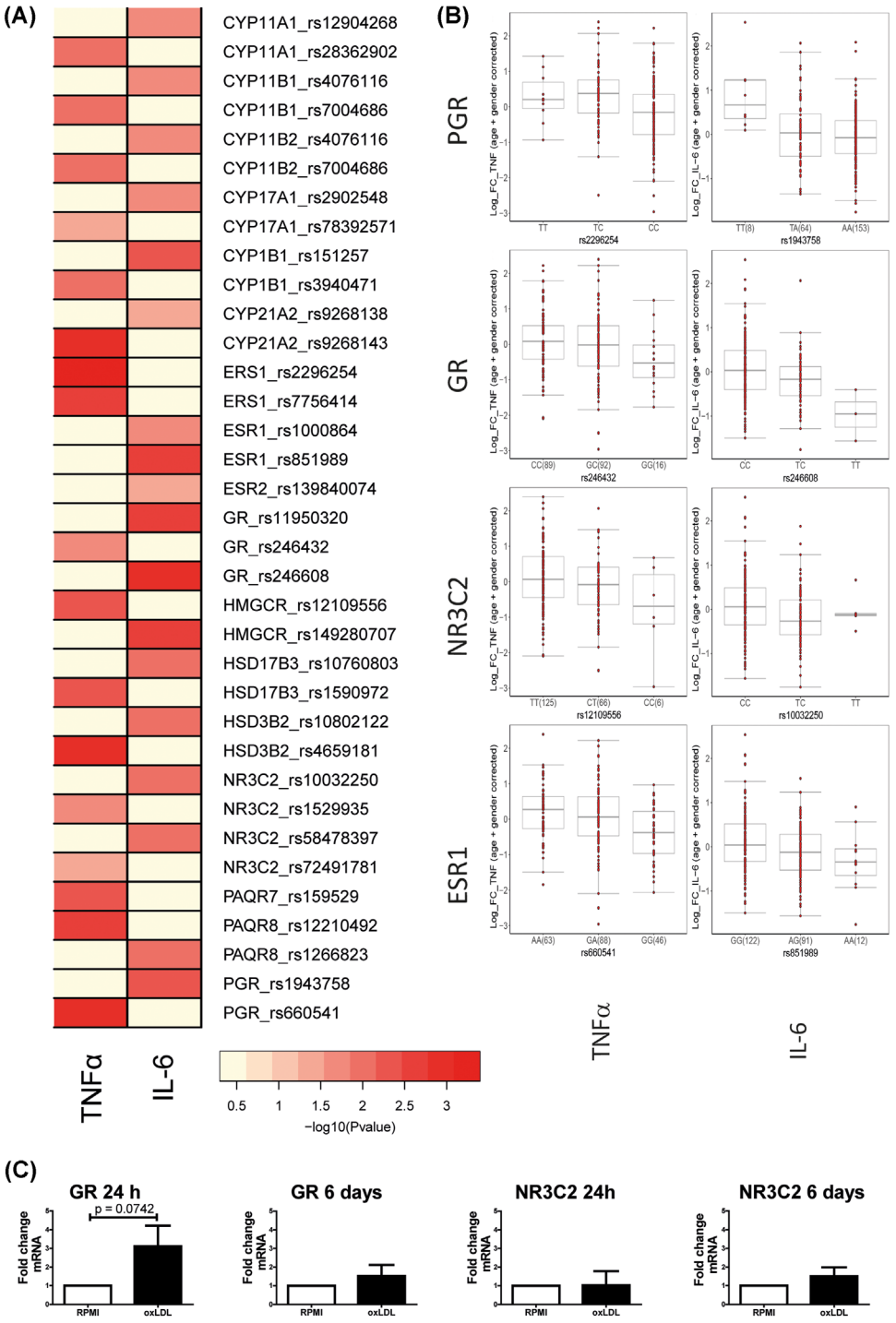

FIGURE 3 SNPs around the steroidogenesis genes correlate with cytokine production after ex vivo training with oxLDL. (A) Heatmap highlighting SNPs in the steroidogenesis pathways that correlate with levels of TNFα and IL-6 following ex vivo training with oxLDL (10 μg/ml) and restimulation with LPS (10 ng/ml). The color legend for the heatmap indicates the range of p values from QTL mapping. (B) Boxplots showing the genotype-stratified cytokine levels for the most strongly correlated SNPS in the PGR, GR, NR3C2, and ESR loci for TNFα and IL-6, respectively ($n = 215$ healthy volunteers for TNFα, $n = 228$ healthy volunteers for IL-6)

targets of these G protein-coupled receptors are ubiquitous to other cellular processes, which have already been implicated in having other, nonrelated roles in trained immunity.[8,35] With the unclear role that ESR1 and membrane PRs may play in macrophage responses toward progesterone, we decided to focus on the contributions of the nuclear receptors to the progesterone-mediated suppression of oxLDL training, which we have shown to be expressed by these cells (Figure 3(C)).

To this end, the nuclear PR and GR antagonist mifepristone was added in conjunction with oxLDL and progesterone costimulation. Mifepristone restored oxLDL training in the presence of progesterone

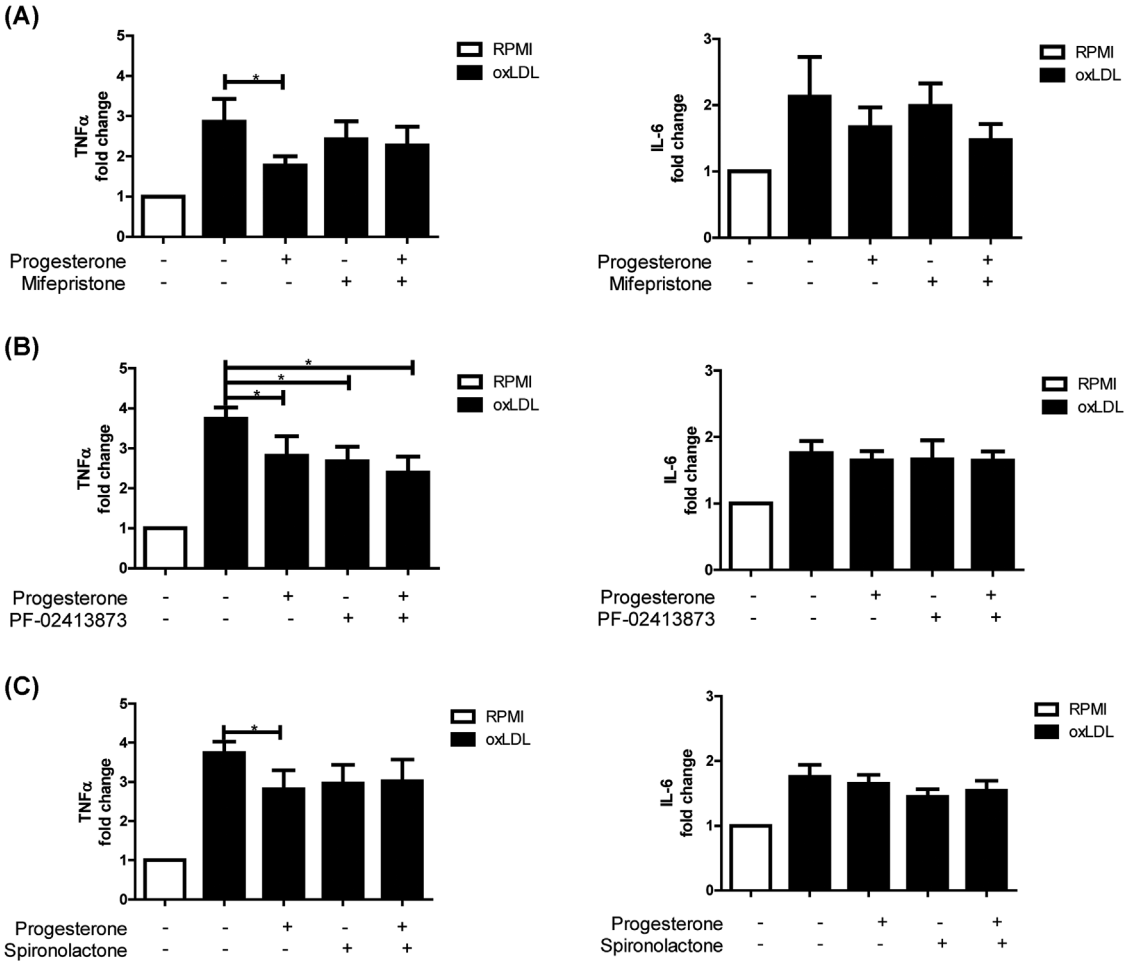

FIGURE 4 Nuclear progesterone, glucocorticoid, and mineralocorticoid receptors are important for the inhibition of oxLDL training by the steroid hormone progesterone. Monocytes were trained with oxLDL (10 μg/ml) or RPMI for 24 h ± progesterone in the presence of either (A) the combined PR and GR antagonist mifepristone (1 μM), (B) the selective PR antagonist PF-02413873 (150 nM), or (C) the MR antagonist spironolactone (1 μM). Monocytes were then washed with PBS, rested for 5 days in culture media, and subsequently stimulated with LPS (10 ng/ml) for 24 h. Levels of TNFα and IL-6 were measured in supernatants via ELISA. (Wilcoxon matched pairs signed rank test, $n = 6$, $^*p < 0.05$)

for TNFα, while having no restorative effect on IL-6 production (Figure 4(A)). To further differentiate between the PR and GR, the selective PR antagonist PF-02413873 was used. This PGR antagonist could not prevent the inhibiting effect of progesterone on oxLDL-induced trained immunity (Figure 4(B)). Accordingly, we could not detect any PGR mRNA expression in oxLDL-treated (24 h) and -trained (6 days) macrophages. These data suggest that the effects of mifepristone are largely conferred via the GR. Progesterone is also known to bind to the MR, which has recently been demonstrated to play a role in induction of trained immunity in monocytes stimulated with aldosterone.[36] Pharmacologic inhibition of the MR with spironolactone resulted in a small restoration of TNFα cytokine production upon the addition of progesterone, so that progesterone in the presence of spironolactone did not significantly lower TNFα production in oxLDL-trained cells (Figure 4(C)). This finding suggests that, in addition to the GR, the MR is also involved in the inhibiting effect of progesterone on trained immunity.

Progesterone confers at least part of its inhibitory functions via the nuclear GR and MR, related pathways that are well studied in interfering with immune responses. The GR, once bound to progesterone, translocates into the nucleus where it interferes with the binding of NF-κB and AP-1 to their cognate gene targets.[37] Additionally, the GR acts as a transcription factor in its own right, driving the expression of many immune modulatory genes. Wang et al.[38] have demonstrated that addition of the synthetic glucocorticoid triamcinolone acetonide in a set-up reminiscent of trained immunity, with a 24 h treatment followed by a 5 day wash out period, resulted in broad changes of the chromatin architecture which lead to the increased transcription of a number of anti-inflammatory genes.

In summary, oxLDL induces a long-term proinflammatory response in macrophages in vitro,[7] and this long-term inflammation is believed to contribute to the pathogenesis of atherosclerosis and CVD.[12] The present study shows that progesterone specifically interferes with the enhanced cytokine production capacity in oxLDL-trained

macrophages. These effects are shown to be under the influence of the GR and the MR. Given the accumulating evidence that trained immunity contributes to the pathophysiology of atherosclerosis, we hypothesize that this interfering effect of progesterone might contribute to the lower CVD risk in premenopausal women, who have higher progesterone concentrations, but this needs to be further substantiated in future studies.

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DISCLOSURE

The authors declare no conflicts of interest.

AUTHORSHIP

L.G. and N.P.R. conceived and designed the experiments. L.G., D.E.V., C.D.C.C.H, V.M., L.C.B. S.J.C.F.M.M., V.A.C.M.K, V.P.M., S.T.K., and J.H.P. performed the experiments. L.G. and D.E.V. analyzed the results. L.G. and D.E.V. wrote the manuscript. L.A.B.J., M.G.N., and N.P.R. critically read the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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