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Dysregulation of the CD4\(^+\) T cells lineage differentiation in dyslipidemic patients and impact of lipoprotein-apheresis treatment: A case study

Papin J\(^1\); Brennand A\(^1\); Arbore G\(^2\); Hohenstein B\(^3\); Kamvissi V\(^4\); Kemper C\(^5\) and Bornstein SR\(^6\)

\(^1\), Department of Endocrinology and Diabetes, Division of Diabetes and Nutritional Sciences, King’s College London, London SE5 9NU, UK.

\(^2\), MRC Centre for Transplantation, Division of Transplant Immunology and Mucosal Biology, King’s College London, London SE1 9RT, UK.

\(^3\), Nephrological Center Villingen-Schwenningen, Albert-Schweitzer-Str. 6, 78052, Villingen-Schwenningen, Germany; Faculty of Medicine Carl Gustav Carus, Fetscherstraße 74, 01307, Dresden, Germany.

\(^4\), Department of Medicine III, Technische Universität Dresden, Dresden, Germany; Department of Endocrinology and Diabetes, Division of Diabetes and Nutritional Sciences, King’s College London, London SE1 1UL, UK.

\(^5\), MRC Centre for Transplantation, Division of Transplant Immunology and Mucosal Biology, King’s College London, London SE1 9RT, UK; Laboratory of Molecular Immunology and Immunology Center, National Heart, Lung, and Blood Institute, Bethesda, MD 20892, USA.

\(^6\), Department of Internal Medicine III, Technische Universität Dresden, Dresden, Germany; Department of Endocrinology and Diabetes, Division of Diabetes and Nutritional Sciences, King’s College London, London SE5 9NU, UK.

Corresponding author: Julien PAPIN, The Rayne Institute, Division of Diabetes and Nutritional Sciences, King’s College London, 123 Coldharbour Lane, SE5 9NU London; Electronic address: julien.papin@kcl.ac.uk

ABSTRACT

Background and aim: Lipoprotein-apheresis (LA) is a therapeutic approach used against severe forms of dyslipidemia in patients who are non-responders or intolerant to pharmacological treatments. However, little is known about the potential pleiotropic effects of LA, particularly regarding the immune system and its regulation. Thus, in an attempt to analyse the potential effects of dyslipidemia and LA on the regulation of CD4\(^+\) T cells activation and lineage differentiation, we compared the CD4\(^+\) T cells cytokines secretion profiles of dyslipidemic patients before and after LA with the profiles observed in healthy donors.

Methods: CD4\(^+\) T cells were isolated from 5 LA patients and 5 healthy donors and activated with anti-CD3 or anti-CD3 + anti-CD46 antibodies. The supernatants were collected after 36h incubation and levels of secreted cytokines analysed by flow cytometry.
**Results:** Our results revealed a deep remodelling of CD4⁺ T cells cytokines secretion patterns in dyslipidemic patients compared to healthy donors, as reflected by a 15 times higher IFN-γ secretion rate after CD3 + CD46 co-activation in dyslipidemic patients after LA compared to healthy subjects and 8 times higher after CD3 activation alone ($p=0.0187$ and $p=0.0118$ respectively). Moreover, we demonstrated that LA itself also modifies the phenotype and activation pattern of CD4⁺ T-cells in dyslipidemic patients.

**Conclusion:** These observations could be of fundamental importance in the improvement of LA columns/systems engineering and in developing new therapeutic approaches regarding dyslipidemia and associated pathologies such as atherosclerosis and type 2 diabetes.

**Keywords:** dyslipidemia; lipoprotein-apheresis; cytokines; innate and adaptive immunity; CD4⁺ T cells

**INTRODUCTION**

Patients with severe forms of dyslipidemia are subject to atherosclerosis and subsequent coronary ischemic events and thus require an intensive, effective, continuous, and personalized form of treatment [1]. Therefore, patients who are poor- or non-responders, or intolerant to pharmacological treatments require alternative therapeutic approach such as LA. In Germany, LA is currently representing the treatment of choice for patients not reaching the target levels of LDL-C under a maximum cholesterol lowering treatment approach such as PCSK9 inhibitors, and patients suffering from elevated lipoprotein(a) (Lp(a)). In other countries, LA is only performed to a very limited extent, mainly in patients with homozygous familial hypercholesterolemia (HoFH).

The extracorporeal treatment is highly effective, but may exert additional effects by triggering an immunologic response via blood–biomaterial interactions. Recent investigations confirmed that LA not only show acute lipid-lowering and cholesterol-lowering effects, but also efficacy in reducing several plasmatic pro-inflammatory cytokines. On the other hand, there is evidence that the complement system, postulated to be of pathogenic importance for development of atherosclerotic lesions [2 [2-10]], gets activated by LA treatment due to bio-incompatibility and thus increases the
amount of systemic complement activation products [3]. However, there is also data suggesting that surfaces might bind complement components thereby reducing complement activation during the LA process [4]. These non-lipid-lowering effects can be defined as pleiotropic or pleiotropic-equivalent and may be both pro- and anti-inflammatory. The net result of these reactions is important to the patient [5] and measures to attenuate the inflammatory responses elicited by the extracorporeal treatment should be taken to obtain optimal bio-compatibility.

The presence of T cells in human atherosclerotic plaques has been described in 1985 [6]. A few years later, MHC class II and IFN-γ were found in lesions, and antibodies to oxidized low-density lipoprotein (LDL) were described [7]. Today, the concept that atherosclerosis is an inflammatory disease is no longer controversial [8], and investigations on how this inflammation is regulated have emerged. In this regard, T cells are of particular interest, both because of their secretion of mediators that influence plaque development and because their activity depends on the triggering of specific antigens that are found within the disease site. T cells are regulated by soluble and membrane-bound molecules from many cell types and, in turn, they act on most other cells. This network of cell-to-cell interactions affects the development of many inflammatory and autoimmune diseases.

In this context, we decided to investigate the potential effects of dyslipidemia and LA on the regulation of CD4⁺ T cells activation and induction in dyslipidemic patients, with a particular regard on the pro- / anti-inflammatory secreted cytokines balance.

MATERIAL AND METHODS

Patients and ethics

The study was reviewed by the local ethics committee and all subjects gave written informed consent. In a pilot approach, a total number of 5 patients undergoing stable weekly LA treatment at the Extracorporeal Treatment and Apheresis Center of the University Hospital Carl Gustav Carus in Dresden were investigated. To perform the analyses, heparinized blood was collected immediately
prior to and following a single LA session. Samples were sent to King’s College London for further analysis. All findings were compared to an age- and gender-matched healthy cohort.

Reagents

Cell-stimulating mAbs were purified from a specific hybridoma (anti-CD3; OKT-3) or generated in-house (anti-CD46; TRA-2-10) [9].

T cell isolation and activation

PBMCs were separated to CD4+ T cells using the MACS human CD4+ Positive T Cell Isolation Kit (Miltenyi Biotech Ltd, Bisley, UK) according to manufacturer’s instructions. Purity of isolated lymphocyte fraction was typically >97%. CD4+ T cells were activated by 36 h incubation at 37°C in complete RPMI medium in 48-well culture plates (2.5 – 3.0 x 10^5 cells/well) coated with mAbs to CD3 or CD3 + CD46 (2.0 μg/ml PBS each) and addition of 25 U/ml rhIL-2.

Cytokine measurements

Cytokine production and secretion by CD4+ T cells was measured using the Th1/Th2/Th17 Cytokine Bead Array kit (560484, BD Biosciences) according to manufacturer’s instructions and a LSR Fortessa Cytometer (BD Biosciences).

Statistics

Numerical data are presented with mean and standard error mean (SEM). Levels before LA versus after LA or versus healthy donors were compared by unpaired t tests. All tests were two tailed, and results with p<0.05 were considered statistically significant (*). Analyses were performed using PRISM 6 (Graph Pad Software Inc, La Jolla, CA).

RESULTS

Dyslipidemia and LA have an important impact on the release of pro-inflammatory markers by activated CD4+ T cells
Among pro-inflammatory cytokines secreted by activated CD4^{+} T cells, we found IFN-γ to be significantly over secreted in all the conditions of activation in patients with dyslipidemia before and after LA compared to healthy donors. IFN-γ secretion rate after CD3 + CD46 co-activation was 15 times higher in dyslipidemic patients after LA compared to healthy subjects and 8 times higher after CD3 activation alone (FIG 1A). We also observed a 4 times increase in IFN-γ secretion by CD4^{+} T cells after CD3 and CD3 + CD46 activation in dyslipidemic patients before LA. These results suggest that dyslipidemia is directly correlated with an important increase in IFN-γ secretion by CD3 and CD3 + CD46 activated T cells and that this effect is amplified by LA.

Even if not statistically significant, we observed a clear tendency for IL-2 to be over secreted in CD4^{+} T cells from dyslipidemic patients compared to healthy donors, mostly after CD3 + CD46 co-activation (FIG 1B). As expected, the levels of IL-2 were barely detectable in non-activated and CD3 activated cells, but were slightly increased in cells from healthy donors after CD3 + CD46 activation. Surprisingly, the levels measured in cells from dyslipidemic patients before and after LA were respectively 30 and 45 times more elevated compared to healthy donors.

Finally, we observed that IL-17A secretion induced by CD3 and CD3 + CD46 activation in CD4^{+} T cells is increased in dyslipidemic patient before and after LA compared to healthy donors (FIG 1C). As expected, IL-17A was not secreted either by non-activated or activated CD4^{+} T cells in healthy donors but surprisingly, we observed an increase of IL-17A secretion by cells isolated from dyslipidemic patients after CD3 activation. This effect was slightly, but not significantly amplified by LA. Interestingly, after CD3 + CD46 activation, the IL-17A secretion rates doubled compared to CD3 activation alone.

In all the conditions of activation, TNF-α secretion rates were slightly higher in the LA patients compared to healthy donors, but the difference did not reach a statistical significance. Therefore, our results demonstrate that LA does not induce a significant increase in TNF-α secretion by activated CD4^{+} T cells (FIG 1D). However, it is interesting to observe that when compared to healthy donors,
the rates of TNF-α secretion in dyslipidemic patients are slightly increased before LA and go back to nearly normal values after treatment (FIG 1D), potentially unravelling an influence of LA on TNF-α secretion pathways in activated CD4⁺ T cells.

IL-6 secretion patterns were analysed in our experimental model and unexpectedly, our results suggest that CD4⁺ T cells from patients with dyslipidemia treated with LA have lower IL-6 secretion rates than healthy donors either when non-activated, but also after CD3 and CD3 + CD46 activation. Furthermore, there was a decrease of IL-6 secretion from cells isolated from LA patients after treatment (FIG 2A). It has to be noted that concerning the IL-6 secretion patterns from our 5 dyslipidemic patients, 2 groups could be clearly distinguished (FIG 2B) with an opposite effect of LA inducing either a moderate to high decrease in IL-6 secretion for 3 patients (left panel) and a slight or moderate increase for the other 2 (right panel).

Dyslipidemia and LA impact the release of anti-inflammatory cytokines IL-10 and IL-4

While not significant in this limited cohort, differences seem to develop in IL-10 secretion between cells from dyslipidemic and healthy donors after CD3 and CD3 + CD46 activation. After CD3 activation, CD4⁺ T cells from patients before LA secreted 4 times more IL-10 than cells from healthy donors and this increase was amplified to 5 times in the same patients after LA (FIG 3A). As expected, CD3 + CD46 activation increased IL-10 secretion in healthy donors as well as in dyslipidemic patients but surprisingly, this augmentation was more marked in dyslipidemic patients after LA, with values 6 times higher than in healthy donors (FIG 3A).

In this setting, IL-4 secretion from activated CD4⁺ T cells was only slightly increased in LA patients either before or after treatment when compared to healthy donors for which IL-4 secretion was null in all the conditions as expected (FIG 3B). Nevertheless, regarding the very low amounts of IL-4 secreted in our experimental conditions, we can interrogate about the physiological relevance of this observation.

DISCUSSION
In this pilot study, we compared the cytokines secretion profiles from CD4\(^+\) T cells isolated from 5 healthy donors and from 5 dyslipidemic patients before and after LA. The secretion profiles were analysed after CD3 (TCR alone) and CD3 + CD46 co-activation, with a particular regard on the pro-/anti-inflammatory cytokines balance and on the NLRP3 inflammasome activation, since a complement-NLRP3 axis in CD4\(^+\) T cells has been recently described as a novel potential therapeutic target for the modulation of Th1 activity in autoimmunity and infection in humans [10].

**Dyslipidemia promotes alterations in CD3 + CD46 co-activation mechanisms in CD4\(^+\) T cells**

In humans, CD46 is a widely distributed transmembrane glycoprotein that inhibits complement activation on host cells and CD46 engagement was shown to induce low IFN-γ, and high IL-10 in naïve CD3-activated CD4\(^+\) T cells, a phenotype shared with Tr1 cells. Therefore, CD46 plays an important role in the regulation of adaptive immunity and its deficiency is associated with chronic inflammation and autoimmune diseases [11]. Surprisingly, in LA patients, CD46 co-activation of CD4\(^+\) T cells induced an important increase in IFN-γ secretion. This effect could be due to a dysfunction in the CD46-dependant signalling pathways in activated CD4\(^+\) T cells and could induce their incapacity to switch from a Th1 to a Tr1 phenotype, thus promoting a deleterious inflammatory state in patients with dyslipidemia. More importantly, this effect tends to be amplified by LA (p=0.0643). In parallel, IL-2 secretion rates were significantly increased after CD46 co-activation in LA patients compared to healthy donors and this effect also tends to be amplified by the treatment. This could be related to deleterious biomaterial interactions which promote activation of signalling pathways inducing pro-inflammatory cytokines secretion in CD4\(^+\) T cells.

**Pro-inflammatory cytokines secretion by activated CD4\(^+\) T cells is globally increased in dyslipidemic patients and LA tends to amplify this effect**

The pro-inflammatory cytokines investigated herein included IFN-γ, IL-2, IL17A, TNF-α and IL-6.

The principal Th1 cytokine, IFN-γ, is produced by most T cells, including CD4\(^+\), in the human atherosclerotic plaque [7]. IFN-γ potentiates most stages that lead to inflammation in atherosclerosis...
but surprisingly, a study demonstrated that LDLR−/− mice transplanted with bone marrow from IFN-γ−/− mice exhibited larger atherosclerotic lesions than mice that received bone marrow from IFN-γ+/+ mice [13], implicating a protective role of IFN-γ which also possesses certain potentially anti-atherosclerotic properties in vitro [12]. Thus, interpretation on the global in vivo effects of LA on IFN-γ secretion by CD4+ T cells is complex.

IL-2 is produced mainly by activated Th1 cells and is an important regulator of the immune response and homeostasis. It also plays a central role in downregulating immune responses and its absence results in severe autoimmunity [14]. Interestingly, recent studies have indicated that a major function of the IL-2/IL-2 receptor system lies in directing development and function of T regulatory cells (Tregs) [15]. In atherosclerotic diseases, high levels of IL-2 have been detected in atheromatous plaques [16] but despite this evidence, the role of IL-2 in the pathogenesis and/or in the progression of various atherosclerotic diseases has not yet been determined. Our study suggest that the high levels of IL-2 associated with dyslipidemia could be partially explained by an over secretion of this cytokine by CD46 co-activated CD4+ T cells. The fact that LA could trigger this effect might, again, reflect alterations of the CD46-mediated signalling pathways in these cells after LA, resulting in their incapacity to regulate the transition from innate to adaptive immunity (even in the presence of high IL-2 concentrations), therefore promoting pro-atherosclerotic effects and decreasing the efficacy of the treatment.

We also observed a tendency for IL-17A secretion to be increased in LA patients compared to healthy donors, this effect being more marked after CD46 co-activation. This result might reflect the tendency of a CD4+ T cells pool to differentiate into Th17 lymphocytes in dyslipidemic patients. This is in accordance with previous observations by other groups which reported the presence of Th17 cells in human atherosclerotic lesions [17]. The role of IL-17A in atherosclerosis is still under debate, however the overall data indicate that enhanced IL-17A production associated with increased IL-10 (regulatory Th17) and reduced IFN-γ will most probably limit lesion development and promote
plaque stability. In contrast, dual production of IL-17A and IFN-γ will most likely promote lesion progression and instability [18]. Intriguingly, recent evidences showed that both Th17 cells and immunosuppressive Tregs derived from naïve CD4+ cells and can be reciprocally induced, contingent upon the presence of either IL-6 or IL-2, respectively, in the presence of TGF [19]. Furthermore, it has been shown that Tregs exhibit “plasticity” and can become IL-17--producing cells after treatment with IL-6 both in vitro [20] and in vivo [21]. Therefore, considering our results and the increased secretion of IL-17A, IFN-γ and IL-10 together with the slight decrease in IL-6 secretion in CD46 co-activated CD4+ T cells from dyslipidemic patients, interpretation of the interplay between these cytokines, the resulting net effect on T cells differentiation and impact on atherosclerosis development is highly hazardous. We can however, speculate on the existence of a Th17 pool of T cells resulting from the IL-6 stimulated dedifferentiation of Tregs that would promote inflammation in dyslipidemic patients. This hypothesis is in range with previous results clearly associating high levels of circulating IL-6 with dyslipidemia and atherosclerosis development [22].

TNF-α is produced by Th1 cells as well as macrophages and other cell types. TNF-α is a pro-inflammatory cytokine and promotes several autoimmune diseases. In addition, TNF-α inhibits lipoprotein lipase, leading to hypertriglyceridemia and reduced fatty acid oxidation, and stimulates production of oxygen and nitrogen radicals [23]. Here, we observed a slight increase of TNF-α secretion by activated and co-activated CD4+ T cells in LA patients before treatment which is in line with previous observations. Interestingly, following LA, patients presented a level of secretion comparable to healthy donors, suggesting that LA could be responsible for the removal of TNF-α secretion stimulators, therefore reducing its pro-inflammatory and pro-atherogenic effects in dyslipidemic patients.

We did not observe any significant changes in IL-6 secretion by activated CD4+ T cells in our conditions, but a tendency for a decrease in LA patients before and after treatment compared to controls. It is surprising since elevated circulating IL-6 rates have been clearly associated with
dyslipidemia and the progression of atherosclerosis. On the other hand, several types of immune cells secrete IL-6, including Th2 lymphocytes. Moreover, IL-17A acts on a variety of cells to induce the production of cytokines, including IL-6 and TNF-α [24]. Therefore, this result tends to suggest that CD4$^+$ T cells are not involved in the increased circulating IL-6 rates observed in patients with dyslipidemia. However, high levels of IL-6 in dyslipidemic patients could be responsible for the dysregulation of CD4$^+$ T cells activation and induction.

**Anti-inflammatory cytokine IL-10 secretion by CD4$^+$ T cells tends to increase after CD46 co-activation and LA amplifies this effect**

We also measured IL-10, a cytokine that is mainly produced by Th2 lymphocytes, and to a lesser extent by monocytes, macrophages and activated T lymphocytes. IL-10 inhibits the production of chemokines by Th1 lymphocytes (IFN-γ, TNF-α, IL-2 and IL-3) and macrophages (IL-1, IL-6, IL-8, GM-CSF, TNF-α). In opposition with IL-6, IL-10 gene transfer can significantly reduce the atherosclerotic plaque area and the macrophage infiltrated area and decrease apoptosis of cells from the lipid core, thereby reducing the risk of plaque rupture [25]. A study by Mallat et al. also demonstrated other relevant proof of the anti-inflammatory and immunosuppressive activity of IL-10 [26]. Nevertheless, it is still unclear whether high plasma levels of IL-10 are to be considered as a marker of anti-inflammatory patterns (with a reduced likelihood of developing atherosclerosis), or conversely whether they are counter regulatory consequences related to primitive pro-inflammatory patterns [27]. The latter is more likely to contribute to the effects of dyslipidemia and LA on IL-10 secretion pattern after CD46 co-activation observed in this pilot study. Actually, another major source of IL-10 is CD4$^+$ Treg cells, thus the observed increase in IL-10 secretion in dyslipidemic patient’s co-activated T cells could reflect an attempt of these cells to switch from IFN-γ to IL-10 secretion, meaning from a Th1 to a Treg phenotype. However, the high levels of IFN-γ observed in the same condition suggest that this transition, essential for the regulation of innate and adaptive immune response does not occur in our model. This hypothesis, again, is in line with potential alterations in the CD46-mediated
signalling events in dyslipidemic CD4+ T cells after CD46 co-activation. Interestingly, it has been demonstrated that IL-10-mediated suppression of Th1 cells is achieved not only through IL-10 produced extrinsically, but also through a negative feedback loop that induces “intrinsic” IL-10 expression in cells also expressing IFN-γ during Th1 lineage differentiation. Moreover, it was reported that CD46 and IL-2-induced switching is defective in rheumatoid arthritis (RA) patients. T cells from patients with RA fail to shut down IFN-γ production upon CD46-activation, and lack the IFN-γ IL-10+ cell subpopulation. As a consequence of this intrinsic cell defect, differentiating Th1 cells produce up to 20 times more IFN-γ compared to T cells from healthy individuals [28]. In addition, and in contrast to T cells from healthy individuals, the IFN-γ IL-10+ T cells from RA patients lack suppressor functions. Thus, we postulate that a similar mechanism could occur in dyslipidemic patients and that this particular phenotype is further modulated by intrinsic effects of LA.

*Complement-NLRP3 inflammasome axis could be involved in the particular cytokines secretion pattern in CD4+ T cells from LA patients before and after treatment*

It has been recently demonstrated that certain LA systems are associated with an elevated circulating rate of alternative complement products C3a and Bb [29]. The complement system has recently begun to attract interest as a therapeutic target since complement components might enhance production of IL-1b [30] which is critically involved in several inflammatory diseases [30]. However, it is unknown whether, or how, complement and IL-1b are linked. One possibility is that the interaction of complement system with TLRs on phagocytes enhances the activation of the NLRP3 inflammasome and promotes maturation of the pro-inflammatory cytokines IL-1b and IL-18 [31]. This theory brings a new perspective to the search for therapeutic targets in diseases characterized by abnormal complement and IL-1b activity, including dyslipidemia and atherosclerosis progression [32].

On the other hand, it is well established that CD4+ T cells depend on the activation of T cell–expressed complement receptors binding C3 activation fragments for normal Th1 induction and contraction [28, 32]. Indeed, C3- and CD46-deficient patients suffer from recurrent infections and
have severely reduced Th1 responses. Conversely, uncontrolled intracellular C3 activation (or dysregulated CD46 engagement) in T cells contributes to hyperactive Th1 responses observed in autoimmunity [28, 31-33]. Recently, Arbore et al. demonstrated the existence of a similar mechanism involving C5 and C5a receptors (C5aR) in human CD4⁺ T cells, thus establishing the existence of a crucial link between intracellular complement products synthesis and the regulation of innate and adaptive immunity through the regulation of IFN-γ secretion by CD4⁺ T cells. Surprisingly, they further observed an enrichment of transcripts associated with inflammasome activation, including NLRP3 and IL-1b, in CD4⁺ T cells co-activated with CD3 and CD46. Finally, their results demonstrated that the regulated cross-talk between intracellularly activated complement components (the “complosome”) and the NLRP3 inflammasome is fundamental to human Th1 induction and regulation, suggesting that the complement-NLRP3 axis in T cells may represent a novel therapeutic target for the modulation of Th1 activity in autoimmunity and infection.

We did not analyse the expression of complement components or NLRP3 in our model, neither the secretion of IL-1b. Therefore, we can only hypothesize on the role of the complement and complement-NLRP3 axis in the effects of dyslipidemia and LA on CD4⁺ T cells cytokines secretion pattern. However, it is not unreasonable to think that this major newly described complement-inflammasome crosstalk could be of fundamental interest in targeting pro-inflammatory components associated with dyslipidemia and the development of atherosclerosis.

CONCLUSION

Using anti-CD3 antibodies alone or a combination of anti-CD3 + anti-CD46 antibodies for activation, we observed a deep remodelling of CD4⁺ T cells cytokines secretion patterns in LA patients compared to healthy donors. These alterations include higher IFN-γ, IL-2, IL-17A and IL-10 secretion rates after CD46 co-activation. This suggest a defect in the CD46 co-activated CD4⁺ T cells lineage differentiation and the incapacity of these cells to acquire a full regulatory phenotype (Treg), therefore maintaining a Th1 response and a pro-inflammatory environment, which in turn promotes dyslipidemia and
Atherosclerotic diseases. These results also raise questions on the lineage differentiation of CD4$^+$ T cells and on the role of the NLRP3 inflammasome and complement system in LA patients. Moreover, we demonstrated that LA itself also modifies CD4$^+$ T cells phenotype and activation in LA patients: LA tended to amplify the over secretion of pro-inflammatory cytokines but had minor impact on anti-inflammatory cytokines.

While our results are not sufficient to clearly establish a beneficial or unfavourable role of LA on immunity, it seems that this technique tends to increase the effects already induced by dyslipidemia on CD4$^+$ T cells activation pattern. It is reasonable to believe that behind its LDL-lowering effect, LA could also provide a supportive modulation system for circulating cytokines in dyslipidemic patients and improve the efficiency of the treatment. However, more extensive evaluations are necessary to clearly define the role of LA in this context.

**Conflict of interest:**

**JP, AB and SRB:** King’s College London supports research in Professor Bornstein’s group.

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Figure 1: CD4+ T cells pro-inflammatory cytokines secretion in dyslipidemic patients is altered and this effect tends to be amplified by LDL-a.

Isolated CD4+ T cells from dyslipidemic and healthy donors were activated with TCR alone (CD3), co-activated with CD3 and CD46 antibodies or maintained non-activated for 36h, then the concentrations of secreted cytokines were measured by cytometry.
Figure 2: Isolated CD4⁺ T cells IL-6 secretion profiles

A: Isolated CD4⁺ T cells from dyslipidemic and healthy donors were activated with TCR alone (CD3), co-activated with CD3 and CD46 antibodies or maintained non-activated for 36h, then the concentrations of secreted IL-6 were measured by cytometry. B: A segregation of 2 different sub-populations between dyslipidemic patients was observed regarding LDL-a effects on IL-6 secretion.
Figure 3: *Isolated CD4\(^+\) T cells anti-inflammatory cytokines secretion profile in dyslipidemic patients.*

Isolated CD4\(^+\) T cells from dyslipidemic and healthy donors were activated with TCR alone (CD3), co-activated with CD3 and CD46 antibodies or maintained non-activated for 36h, then the concentrations of secreted cytokines were measured by cytometry.