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Conjugated linoleic acid suppresses dendritic cell activation and subsequent Th17 responses

[a1]Eve Draper, [a1]Joseph DeCourcey, [b]Sarah C. Higgins, [a]Mary Canavan, [a]Fiona McEvoy, [a]Mark Lynch, [c]Brian Keogh, [d]Clare Reynolds, [d]Helen M. Roche, [b]Kingston H.G. Mills, [a3]Christine E. Loscher

[a]Immunomodulation Research Group, School of Biotechnology, Dublin City University, Dublin 9, Ireland. [b]Immune Regulation Research Group, School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Ireland. [c]Institute of Molecular Medicine, Trinity College Dublin, Ireland. [d]Nutrigenomics Research Group, UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland.

[1] These authors contributed equally to this manuscript

[2]This work was supported by funds from Science Foundation Ireland and Enterprise Ireland.

[3] Address correspondence and reprint requests to Dr. Christine E. Loscher at the current address: Immunomodulation Group, School of Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail address: christine.loscher@dcu.ie

[4] Abbreviations used in this paper: c9, t11-CLA, cis-9, trans-11 conjugated linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, lauric acid; DC, dendritic cells; and ISRE, interferon stimulated response element.

Abstract

Polyunsaturated fatty acids can modify immune responses, so they may have potential therapeutic effects in inflammatory disorders. We previously demonstrated that the cis-9, trans-11 isomer of the polyunsaturated fatty acid conjugated linoleic acid (CLA) can modulate dendritic cell cytokine productions. Since dendritic cells play a central role in initiating inflammation by directing T helper (Th) cell differentiation, here we examined the effects of CLA on dendritic cell maturation and migration and the subsequent generation of Th cell responses. We examined the effect of CLA *in vitro* on the function of LPS-activated bone marrow-derived dendritic cells and ex vivo using cells from mice with high levels of CLA in their diet. We report that CLA inhibits dendritic cell migration and modulates TLR-induced production of key cytokines involved in Th cell differentiation both *in vitro* and *in vivo*. These changes were accompanied by a significant decrease in expression of MHCII, CD80 and CD86 on the dendritic cell surface. Exposure of dendritic cells to CLA suppressed their ability to promote differentiation of naïve T cells into Th1 and/or Th17 cells *in vitro* and following their adoptive transfer *in vivo*. Furthermore, in a murine model of endotoxin shock, treatment with CLA suppressed LPS-induced induction of circulating IFNγ, IL-12p40 and IL-1β. This is the first study to demonstrate that exposure of antigen-presenting cells to CLA can modulate the subsequent T helper cell response, and the findings may explain some of the beneficial effects of c9, t11-CLA in inflammatory diseases mediated by Th1 and Th17 cells.

Introduction

Conjugated linoleic acid (CLA) is a heterogenous group of positional and geometric isomers of conjugated dienoic derivatives of linoleic acid, an n-6 polyunsaturated fatty acid. The *cis-9, trans-11* CLA isomer (c9, t11-CLA) is the naturally occurring dietary form of CLA and is found in the lipid fraction of meat, milk and dairy products [1]. CLA can suppress pro-inflammatory cytokine production from a variety of cell types [2], and studies in animal models have shown that feeding CLA has beneficial effects on inflammatory-induced growth suppression, endotoxin-induced anorexia and mucosal damage in experimental colitis [3–5] These studies indicate the potential of CLA as an immunomodulatory molecule in the prevention or treatment of inflammatory and immune-mediated diseases. We have previously demonstrated that CLA suppresses dendritic cell (DC) activation, characterised by a decrease in IL-12 and an increase in IL-10 production [6]. Given that DCs play a critical role in directing adaptive immune responses, this study suggested that the effects of CLA on DC function could have consequences for the subsequent activation of T helper cell responses.

Activation of DCs by pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), induces maturation and homing to lymph nodes, where the DC present antigen to naïve T cells. The differentiation of T cells into various subsets is partly determined by the cytokines secreted by DCs and other innate immune cells; IL-12 drives the differentiation of T cells to a Th1 phenotype; IL-23, IL-1 and IL-6 are involved in generating Th17 cells; IL-4 induces Th2 cell differentiation and IL-10 promotes the induction of type I T regulatory cells [7–9]. The pivotal role of these cytokines in directing the development of T helper cell subsets suggests that modulating

their production by DCs may modulate the subsequent adaptive immune response. Indeed, molecules that enhance IL-10 and suppress IL-12 production by DCs have been shown to promote the induction of Th2 and Tr1 cells [10]. Migration of DCs to the lymph node and expression of MHCII molecule and the co-stimulatory molecules, CD40, CD80 and CD86, are also important for T cell differentiation and proliferation [8] and changes in these factors could also alter the ability of a DC to activate naïve T cells.

Th1 cells are considered pro-inflammatory and are highly effective at clearing intracellular pathogens, whereas Th2 cells are associated with the clearance of parasitic infections [8]. A heightened Th1 response is often seen in some chronic inflammatory and autoimmune disorders, including Crohn's disease [11]. However, a more recently described subset of Th cells, Th17 cells, have now also been implicated in the pathogenesis of autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis [7,12]. Th17 cells secrete IL-17, GM-CSF and other inflammatory cytokines [13]. IL-17 functions as a classic effector of innate immunity with actions similar to those of TNF-α or IL-1β [14]. IL-23 serves to expand previously differentiated Th17 cell populations, but IL-6 and transforming growth factor-β (TGF-β) have been shown to induce the differentiation of Th17 cells from naive precursors [15], and IL-1 and TNF-α are also reported to be involved in promoting the Th17 sub-set [16]. Little work has been done to investigate the effects of CLA supplementation on these T-helper phenotypes in disease states. A study investigating the effect of CLA on T-helper cell subsets in healthy individuals found no effect on the immune system of healthy adults. This study identified no differences in proliferation or ratios of specific immune cell phenotypes, however the study did not assess the recently identified T-helper cell

subsets described above. Furthermore, the authors concede that although there is no observed effect on healthy adults, there is no evidence to suggest that there may be beneficial effects on immune-compromised patients which would agree with previous studies in animal models[17]. A study in patients with mild to moderate Crohn's disease, an inflammatory disease of the colon, showed some evidence that CLA supplementation may influence the T-helper phenotypes in immune-compromised patients[18]. This study tentatively shows that subsets of $CD4^+$ T-cells producing IL-17 are reduced over the course of a CLA supplemented diet in these patients. Given that we have previously shown that CLA can modulate antigen presenting cells[6,19], in this study we tested the hypothesis that modulation of DC activation by c9, t11-CLA, has consequences for the subsequent differentiation of Th cell responses.

Materials and Methods

Animals and materials

BALB/c and C57/BL mice were purchased form Harlan (U.K.) and were used at 5-10 weeks of age. Animals were maintained according to the regulations of the European Union and the Irish Department of Health. Spleen cells from OVA transgenic D011.10 mice on a C57/BL background were provided by Prof Kingston Mills (Trinity College, Dublin). c9, t11-CLA was purchased from Alexis Chemicals (Cayman Chemicals), and lauric acid was purchased from Sigma Aldrich (U.K.). Fatty acids were dissolved in sterile DMSO (Sigma-Aldrich) to a stock concentration of 100mM, and stored at in the dark at -20°C. *E. coli* LPS (serotype 127:B8) was purchased from Alexis Biochemicals (U.K.). Lyophilised OVA Peptide (323-339) was purchased from GenScript Corp (NJ,

USA) and reconstituted with sterile water to give a 1mg/ml stock solution which was stored at -20°C. The chemokine CCL19 was purchased from Becton Dickinson (U.K.) and stored at -70°C.

Ethics Statement

All animal procedures were carried out in accordance with Department of Health and Children Ireland regulations and performed under animal license number B100/2799. All animal protocols received ethical approval from the Dublin City University Ethics committee and the Trinity College Dublin Bioresources Ethics Committee.

Isolation and culture of bone marrow-derived DCs

Bone marrow-derived immature DCs (BMDCs) were prepared by culturing bone marrow cells (obtained from the femurs and tibia of BALB/c mice) in RPMI 1640 medium with 5% FCS supplemented with 10% supernatant from a GM-CSF-expressing cell line (J558-GM-CSF). The cells were cultured at 37˚C for 3 days and the supernatant was carefully removed without disturbing the cell monolayer and replaced with fresh medium with 10% GM-CSF cell supernatant. On day 7 of culture, cells were collected, counted and used for assays. For experiments DMSO (vehicle control), c9, t11-CLA (50μM), or in some cases a saturated fatty acid control, lauric acid (50μM), was added to the cells on day 1 of culture and cells were stimulated with LPS on day 7. Supernatants from the experiments were analysed for concentrations of cytokines using commercial DuoSet ELISA kits (R&D Systems), according to the manufacturer's instructions. Cells were washed and used for immunofluorescence analysis. The expression of CD40, CD80,

CD86 and MHCII was assessed using an anti-mouse CD11c (Caltag), and CD40, CD80, CD86 and MHCII (rat IgG2a, BD Biosciences) and appropriately labelled isotype-matched antibodies. After incubation for 30 min at 4ºC, cells were washed and immunofluorescence analysis was performed on a FACsCalibur (BD Biosciences) using CellQuest software.

Chemotaxis assay

BMDCs isolated from BALB/c mice were cultured with fatty acids for 7 days, as described above. Cells were then plated ($1x10^6$ cells/ml) and stimulated with LPS (100ng/ml) for 24 hrs. Following incubation, cells were removed from wells and counted. Transwell® plates (Corning) were used in accordance with the manufacturer's instructions; $3x10^5$ cells were added to the insert well in 100µl of media, and 600µl of media containing the chemokine CCL19 (100ng/ml) was added to the bottom chamber. Plates were incubated at 37ºC for 5 hrs. Cells that had moved to the bottom chamber were collected and resuspended in 4% (v/v) formaldehyde/PBS before being transferred to FACS tubes. Migrated cells were counted for 60 seconds on a FACSCalibur.

Dendritic cell: T cell co-culture

BMDCs isolated from C57/BL mice were grown as previously described in the presence of DMSO or fatty acids until day 7 of culture and activated with ovalbumin (OVA) peptide (323-339; 5 µg/mL) for 24 hrs at 37ºC in 5% CO_2. After 24 hrs, DC were collected and washed twice in sterile PBS/2% FCS and irradiated with 40 Gy (4000 rads) using a gamma irradiator with a Caesium-137 source. Cells were then counted and

resuspended in cRPMI at a final concentration of $2x10^5$ cells/mL. $CD4^+$ T cells were isolated from the spleens of OVA transgenic D011.10 mice by negative selection using the Easysep $CD4^+$ Isolation (Stemcell) and adjusted to a concentration of $2x10^6$ cells/mL. Equal volumes of T cells and DCs were added to a sterile 96-well plate and incubated at 37°C in 5% CO_2 for 5 days. On day 4 of co-culture, plates were centrifugally pulsed and the supernatant was removed and frozen. Fresh medium was added to the cells and on day 7 supernatants were removed and newly harvested OVA-activated DCs ($2x10^5$ cells/mL) were added with recombinant murine IL-2 (10 U/mL; Becton Dickinson). On day 10 supernatants were removed and frozen.

Adoptive transfer

BMDCs isolated from BALB/c mice were cultured in the presence of DMSO, CLA or LA as described above. On day 7 of culture, BMDCs were stimulated with KLH (10μg/mL) or media alone. After 24 hours cells were harvested, washed in PBS and injected into the footpads of recipient BALB/c mice (10^5 cells per footpad in 25μL PBS). Spleens were isolated from recipient mice 7 days-post cell transfer splenocytes were isolated by being pushed through a cell strainer (40μM), erythrocytes lysed, cells washed and resuspended at a concentration of $2x10^6$ cells/ml. 200μL of cells were added to 96-well round-bottom plates pre-coated with media and KLH (2, 10 and 50μg/mL). Supernatant was removed from the plates after 72 hours and assessed for levels of IL-2, IL-17, and IFN-γ using specific immunoassays (R&D DuoSet ELISA kits).

In vitro Th17 T-Cell differentiation

$CD4^+$ T-cells were purified from spleens of BALB/c mice using Easysep $CD4^+$ Isolation (Stemcell). Cells were plated at $1x10^6$/ml in a 24-well plate and stimulated with plate-bound anti-CD3 (5 μg/ml; 145-2C11) plus anti-CD28 (5 μg/ml; 37.51) in the presence of polarising cytokines and neutralising antibodies; 20ng/mL rIL-6, 10ng/mL rIL-1β, 10ng/mL rIL-23, 10μg/mL αIFN-γ (all R&D systems). Cells were re-stimulated in fresh media on day 3. Supernatants were collected on day 6 and assessed for levels of IL-2, IL-17 and IFN-γ using specific immunoassays (R&D DuoSet ELISA kits). Cells were washed and stained for CD4, CD25 and CTLA-4 surface markers (BD Biosciences). Analysis was performed on a FACs ARIA I (BD Biosciences) and analysed using FlowJo software (Tree Star).

Feeding studies

Purified, synthetic linoleic acid (control) and conjugated linoleic acid (c9, t11) oils were supplied by Loders Croklaan, Netherlands, and incorporated into animal feed (1% (w/w)) by Special Diets Services, Essex, UK. Feed was irradiated and checked for purity, and subsequently stored in sealed bags at 4ºC until use. BALB/c mice were purchased at 4-6 weeks of age from Harlan UK Ltd and immediately fed either the control diet or a CLA-incorporated diet for 5 weeks. The feed was changed daily to prevent polyunsaturated fatty acid oxidation. BMDCs were prepared from these mice in the same manner as described above. No fatty acids were added to the DCs during the 7-day culture period.

LPS shock model

BALB/c mice were fed the CLA-supplemented diet or control diet for 5 weeks. Five of the mice in each group received an intravenous injection of 3μg LPS (Alexis Biochemicals) into the tail vein in 100μL sterile PBS. The remaining 5 mice in each diet group served as controls. Mice were left for 6 hrs after which time they were sacrificed and blood collected. The blood was allowed to clot overnight at 4ºC and plasma removed after centrifugation at 12000rpm for 5 minutes. Plasma samples were stored at -20ºC and analysed for the presence of various cytokines using specific immunoassays.

Statistics

One-way analysis of variance (ANOVA) was used to determine significant differences between conditions. When this indicated significance ($p<0.05$), post-hoc Student-Newmann-Keul or Dunnett's multiple comparison test analysis was used to determine which conditions were significantly different from each other. In some experiments, a one tailed unpaired t-test was used. GraphPad Software Inc was used for the statistical tests.

Results

C9, t11-CLA modulates cytokine production by DCs in vitro and ex vivo.

Cytokines produced by activated DCs are key factors in determining the subsequent generation of T helper cell responses. Here we examined the effect of CLA on DC, focusing first on the production of T cell polarizing cytokines. Activation of DCs with LPS resulted in significant production of IL-12p70, IL-12p40, IL-27, IL-23, IL-10, IL-6 and TNFα (Fig. 1). Pre-treatment of the DCs with C9, t11-CLA resulted in significant suppression of IL-12p70, IL-12p40 and IL-27($p<0.05$) and enhanced IL-10 production ($p<0.001$), with no change in IL-23, IL-6 or TNFα. In order to determine whether these changes could also be induced *in vivo*, mice were fed either a CLA-enriched diet or a control diet for 5 weeks and DCs were cultured without any further exposure to CLA. Figure 2A demonstrates that DCs isolated from mice fed on a CLA-enriched diet produced significantly less LPS induced IL-12p40 and IL-12p70 ($p<0.01$ and $p<0.001$ respectively) than DC isolated from mice fed on the control diet. The anti-inflammatory effect of CLA *in vivo* was further confirmed using a murine model of LPS shock. Injection of LPS significantly enhanced serum concentrations of IL-12p40, TNF-α, IFNγ and IL-1β. Mice fed on CLA-enriched diets showed significantly lower concentrations of circulating IL-12p40 and IFNγ than mice fed a control saturated fatty acid diet (Fig. 2B). Furthermore IL-1β was also lower, though not significantly, in the mice fed CLA-enriched diet (Fig 2B).

C9, t11-CLA modulates DC maturation.

Activation of DCs by inflammatory stimuli induces maturation and homing to lymph nodes where they present antigen to naïve T cells. This maturation process is characterised by increased expression of MHC class II molecules as well as co-stimulatory molecules (CD40, CD80, CD86) [6]. In order to determine whether CLA modulated DC maturation we examined its effect on expression of these cell surface markers. We also included a saturated fatty acid control, lauric acid [20]. Immature, unstimulated BMDCs exposed to C9, t11-CLA expressed lower levels of CD80, CD86 and MHCII (Fig. 3, left column). Up-regulation of MHCII, CD40, CD80 and CD86 expression was observed when cells were activated with LPS. Exposure of the DCs to CLA decreased expression of MHCII, CD80 and CD86 (Fig. 3, right column). These changes were not seen in cells treated with the saturated fatty acid control, LA, indeed there was a large difference between the level of expression of cell surface markers comparing LA and CLA-treated cells. Migration of DCs to the lymph node is necessary for their interaction with naïve T cells for the activation of adaptive immunity [21]. Therefore we examined the migration of DCs in response to CCL19, which is secreted by cells within T cell-rich areas of the lymph node. The results shown in Figure 4 demonstrate that activation of DCs with LPS results in enhanced migration to CCL19 ($p<0.001$). Exposure of DCs to CLA significantly decreased DC migration ($p<0.001$). In contrast, culture of LPS-activated DCs with the control saturated fatty acid, LA, further enhanced their migration ($p<0.001$).

CLA-treated DC modulate subsequent T helper cell responses

Given that our data clearly demonstrates that exposure of DCs to CLA *in vitro* or *in vivo* modulates their maturation, we examined whether these changes had consequences for their ability to activate T helper cell responses. DCs were exposed to either CLA or LA during culture, pulsed with OVA peptide and then incubated with splenic $CD4^+$ T cells isolated from OVA D011.10 mice. During these experiments the splenic $CD4^+$ T cells were exposed to two rounds of stimulation with OVA-activated DCs, after which the supernatants were assessed to determine the T cell cytokine production. Incubation of DCs pulsed with OVA peptide resulted in activation of T cells secreting IFNγ, IL-17 and IL-2, with no detectable levels of IL-10. Exposure of DCs to CLA resulted in significantly decreased secretion of IL-17 ($p<0.05$; $p<0.01$) (Fig. 5A), IL-2 ($p<0.001$; $p<0.01$) (Fig. 5B) and IFNγ ($p<0.01$) (Fig. 5C), on day 4 and day 10 respectively. Suppression of these cytokines was not observed in T cells exposed to LA-treated DCs. Given that no fatty acids were added to the co-cultures of DC and T cells, the data suggest that changes in T cell cytokine secretion are a direct result of the changes induced by culture of the DCs with CLA.

CLA-treated DC can modulate T helper cell responses in vivo following adoptive transfer

Given that DC cultured in CLA changed the subsequent T helper cell cytokine profile *in vitro*, we examined whether CLA-modulated DC could modulate T helper cell response *in vivo*. BMDCs were cultured in the presence of DMSO, CLA or LA, pulsed with KLH, washed in PBS, and injected into the footpads of recipient BALB/c mice. Splenocytes from these mice were isolated 7 days later and exposed to a range of concentrations of

KLH *ex vivo*. The data shown in Figure 6 demonstrate that adoptive transfer of CLA-treated DCs can significantly suppress secretion of IL-17 from spleen cells isolated from mice following adoptive transfer (Fig. 6A). Production of IL-2 (Fig. 6B) and IFNγ (Fig. 6C) was also decreased but this was not found to be statistically significant using ANOVA.

CLA can directly suppress activation of Th17 cells

Given the consistent effect of CLA-treated DCs on suppressing IL-17 secretion from T helper cells both *in vitro* and *in vivo*, we next examined if CLA could directly affect the activation of a differentiated Th17 cell population. Isolated $CD4^+$ T cells were stimulated with anti-CD3/CD28 and differentiated into Th17 cells by the addition of rIL-6, rIL-1β, rIL-23, and αIFN-γ to the culture media for 3 days. Supernatants were then removed and differentiated cells were re-stimulated with anti-CD3/28 for a further 3 days until day 6. Figure 7A shows that CLA suppressed the expression of CD25 on Th17 cells on day 6, which was not seen with the saturated fatty acid LA. The expression of CTLA4 was unaltered. The addition of differentiating cytokines to the culture media resulted in a differentiated Th17 population of cells which predominantly secreted IL-17 (Fig 7B). Furthermore, CLA suppressed the production of IL-17 and IL-2 by Th17 cells. There was no effect on IL-4 and IFNγ secretion, and the saturated fatty acid had no observed effect on any of the cytokines.

Discussion

This study demonstrates that c9, t11-CLA inhibits DC migration and modulates the production of key cytokines involved in Th cell differentiation both *in vitro* and *in vivo*. This was accompanied by a significant decrease in expression of MHCII, CD80 and CD86. We demonstrated that exposure of naïve T cells to CLA-treated DC in an *in vitro* co-culture model resulted in suppression of subsequent Th1 and Th17 responses. Furthermore, adoptive transfer of CLA-treated DC resulted in decreased IL-17 production in spleen cells isolated from mice following adoptive transfer. We also demonstrated that CLA had a direct effect on Th17 cells, resulting in a decrease in the expression of CD25 and production of IL-17. Given the reported benefits of dietary fatty acids in inflammatory disease, such as inflammatory bowel disease [22], which are associated with excessive Th1 and Th17 responses, these data provide an explanation for the beneficial effects of CLA.

DCs are considered the most potent antigen presenting cells and they play a critical role in inducing and directing T helper cell responses [23]. We have previously demonstrated that c9, t11-CLA can suppress pro-inflammatory cytokine production following LPS stimulation while enhancing production of IL-10 [6,19]. The present study provides further evidence of the ability of CLA to alter IL-12 production by demonstrating that DCs isolated from CLA-fed mice show decreased production of IL-12p40 and IL-12p70 following stimulation with LPS *ex vivo*. Furthermore, using a murine model of endotoxic shock we demonstrate that IL-12p40, as well as IFNγ and IL-1β, in the circulation following injection of LPS is significantly reduced in mice fed on a diet rich in CLA. IL-12 is an important link between innate and adaptive immunity and

one of its main functions is to direct newly activated T helper cells to a Th1 phenotype [24]. It has been shown to be involved in autoimmune diseases including multiple sclerosis, inflammatory bowel disease and rheumatoid arthritis, and its overproduction is important in inflammatory states such as septic shock [25,26], consequently it represents a potential therapeutic target for the treatment of some inflammatory disorders. We also report that CLA suppresses production of IL-27 in LPS-activated DCs. While IL-27 plays a role in immune regulation, it also plays an important role in polarisation of the immune response by potentiating the early phases of the Th1 response [27].

Along with cytokine production, the ability to migrate to the lymph node and express MHC class II and co-stimulatory molecules is of key importance for activation of Th cells [28]. Here we show that the exposure of DCs to CLA suppressed the migration of the DCs towards the lymphoid chemokine CCL19, which is known to direct maturing DCs to lymph nodes, thereby facilitating their interaction with naïve T cells [23]. This suggests that CLA may suppress the ability of DCs to migrate to the lymph node for the subsequent activation of Th cells. Other dietary fatty acids have been shown to affect cell migration. For example, DHA has been shown to suppress the migration of T regulatory cells [29]. Although not addressed in this study, it may be of benefit to assess the surface expression of the CCL19 ligand receptor CCR7 in order to explain the suppression in the migration of cells following exposure to CLA. We report that CLA specifically suppressed the expression of MHCII, CD80 and CD86 in both resting and LPS-activated DCs. MHCII is essential for the presentation of antigen to the naïve T helper cells. The inhibitory effect of CLA on MHCII suggests that it may affect the ability of DCs to present antigen for T helper cell activation. Previous studies have shown other dietary

fatty acids to modulate expression of MHCII. Specifically, DCs isolated from rats fed an n-3 polyunsaturated fatty acid diet had reduced MHCII expression and consequently could not present antigen to KLH-sensitised responder spleen cells [30] and the ability of activated human monocytes to present antigen to autologous lymphocytes was significantly reduced following culture with n-3 PUFA [31]. The ligation of CD80 and CD86 on antigen presenting cells with CD28 on T cells provides a potent co-stimulatory signal to T cells resulting in proliferation and cytokine production [32]. Our study demonstrates suppression of CD80 and CD86 expression on CLA-treated DCs. This may have consequences for the ability of the DCs to activate T cells. Other dietary PUFA such as DHA have been shown to suppress CD80 in DCs [33]. Furthermore, over-expression of CD80 has been reported in the inflamed tissues of patients suffering from multiple sclerosis, rheumatoid arthritis and inflammatory bowel disease [34,35] and studies have assessed CD80 as a therapeutic target in inflammatory disease [36]. Indeed, blocking CD80 signalling using a CD80-CAP (competitive antagonist peptide) suppressed established inflammation in TNBS-induced colitis [37]. Interestingly, the key cytokines affected by CLA treatment, IL-10 and IL-12, have been reported to have regulatory effects on the expression of cell surface markers on DCs. IL-10 has been shown to inhibit the full maturation of DCs by down-regulating ICAM-1, CD80, and CD86 expression [38] and has the ability to block the translocation of peptide-MHCII complexes to the DC plasma membrane [39]. In contrast, IL-12 can up-regulate MHCII, CD80 and CD86 expression on APC [40]. Therefore the observed changes in IL-10 and IL-12 in CLA-treated DCs may contribute to the altered expression of MHCII, CD80 and CD86 that we observe here.

Given the effects of CLA on cytokine production and expression of cell surface markers which are important for Th cell activation and differentiation, we investigated whether these changes had consequences for subsequent Th cell responses. Our data show that CLA-treated DCs suppressed the production of IFN-γ, IL-17 and IL-2 by $CD4^+$ T cells. IFN-γ defines a Th1 response, and its pro-inflammatory effects at the site of tissue inflammation are well established. One of its major roles is to activate macrophage and DCs, which leads to augmented phagocytosis, increased MHCI and II expression, and induction of IL-12 production [41] . It also has a well-defined role in inflammatory disease, and antibodies against this cytokine have been evaluated for their use in inflammatory disorders with some positive results: for example anti-IFN-γ therapy has proved efficacious in patients with moderate to severe active Crohns disease [42].

CLA-treated DCs also significantly suppressed IL-17 production by subsequently activated Th cells. Furthermore, splenocytes isolated from mice following adoptive transfer of CLA-treated KLH-activated DCs had significantly decreased IL-17 production in response to KLH *ex vivo*. We also demonstrate that IL-17 secretion is significantly downregulated in Th cells that are differentiated into Th17 subset in the presence of CLA. The expression of the T cell activation marker, CD25 was also suppressed in these cells. These data clearly show a significant effect of CLA on Th17 cells. IL-17 is mainly secreted by the recently described Th17 cell subset. Pathogenic Th17 cells have been detected in a number of inflammatory disorders and their presence has been linked to some of the pathology previously attributed to overactive Th1 responses [7,16,43]. IL-17 acts as a potent inflammatory cytokine, coordinating tissue inflammation by inducing the expression of pro-inflammatory cytokines, (such as IL-1, IL-6, IL-8, and TNF-α) which

mediate immune cell infiltration and tissue destruction [9]. IL-17 is frequently detected in the inflamed colon of trinitrobenzenesulfonic acid (TNBS) induced colitic mice, and Zhang and co-workers [43] found that IL-17R knockout mice were significantly protected against TNBS-induced weight loss, IL-6 production, and colonic inflammation. While there is little information on the effects of dietary fatty acids on IL-17, a recent study has shown that DHA can suppress the development of Th17 cells [44]. Furthermore, resolvin E1, a metabolite of EPA, suppressed IL-17 in a model of allergic airway inflammation [45]. Given that many inflammatory diseases are mediated by Th1 and Th17 cells, our findings may explain the beneficial effects of CLA in these diseases. Indeed Bassaganya-Reira *et al*. have recently shown that CLA modulates the immune response in patients with Crohns disease, and this is correlated with a decrease in secretion of IFNγ and IL-17 in peripheral CD4+ T cell [18].

Our study clearly demonstrates that the effects of c9, t11-CLA on DC maturation have consequences for their ability to generate T helper cell responses. It highlights the ability of c9, t11-CLA to suppress cytokines both *in vitro* and *in vivo* that are important for Th1 and Th17 responses. Our findings may explain the beneficial effects of PUFA in inflammatory disease, such as inflammatory bowel disease [46], which are associated with excessive Th1 and Th17 responses.

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Figure legends

Figure 1. CLA modulates cytokine production by murine DCs.

Bone marrow derived DCs were cultured for 7 days with DMSO (vehicle control) or CLA (50μM) and subsequently stimulated with LPS (100ng/ml) for 24 h. The supernatants were recovered and the concentration of IL-12p40, IL-12p70, IL-23p19, IL-27, IL-10, IL-6, and TNF-α were measured using specific immunoassays. The results are mean (± SD) of quadruplicate assays and represent two independent experiments.

***p<0.001, vs DMSO vehicle control determined by one-way ANOVA test

Figure 2. Feeding mice a CLA-enriched diet modulates LPS-induced cytokine production by DCs.

Mice were fed either a 1% (w/w) CLA-enriched diet or a 1% (w/w) linoleic acid-supplemented control diet for 5 weeks. (A) Bone marrow derived DC were cultured for 7 days and stimulated with LPS (100ng/ml) for 24 hours. Supernatants were removed and assessed for concentrations of IL-12p40, IL-12p70, IL-6, and TNF-α using specific immunoassays. Results are means (± SE) of triplicate assays and n=5 per group of mice. **p<0.01, ***p<0.001, using an unpaired t-test to compare the 2 groups. (B) Half the mice from each group (n=5) were injected intravenously with 3μg LPS. Blood was collected 6 hours later and assessed for circulating levels of IL-12p40, IL-12p70, IFN-γ, and IL-1β**.** Results are means (± SEM) of duplicate assays, n=5 mice per group. *p<0.05, **p<0.01, using an unpaired t-test to compare the 2 groups administered with LPS.

Figure 3. CLA modulates the expression of cell surface markers on DCs.

Bone marrow derived DCs were cultured for 7 days with DMSO (vehicle control), CLA (50μM) or LA (50μM) and subsequently stimulated with LPS (100ng/ml). After 24 hours cells were washed and stained with antibodies specific for CD40, CD80, CD86, and MHCII. The first column (left) of histograms represents the unstimulated control immature cells. The second column (right) represents the LPS treated cells. DMSO-treated cells (filled grey histogram) are overlaid with CLA (black line) and LA (broken line) treated cells. Profiles are shown for a single experiment and are representative of 4 experiments.

Figure 4. CLA inhibits the migration of DC towards the chemokine MIP-3β (CCL19).

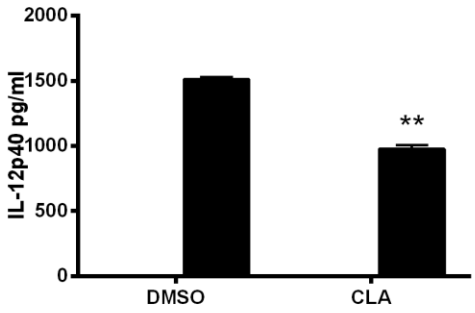
Bone marrow derived DCs were cultured for 7 days with DMSO (vehicle control), CLA (50μM) or LA (50μM) and subsequently stimulated with LPS (100ng/ml) for 24 h. $3x10^5$ cells were placed in the upper chamber of a Transwell plate (8.0μm). Media containing recombinant CCL19 (100ng/ml) was added to the lower chamber and plates were incubated for 5 hours at 37ºC. Media from the bottom well was collected and cells counted for 60 seconds on a FACsCalibur. To serve as a negative control, unstimulated cells were added to the upper chamber of a Transwell plate and media only (containing no CCL19) was added to the bottom chamber. +++ $p<0.001$ using an unpaired t-test between control (without CCL19) and LPS-stimulated DMSO group. ***$p<0.001$ vs DMSO vehicle control determined by one-way ANOVA test

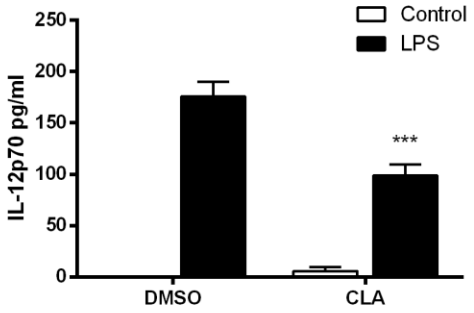
Figure 5. CLA-modulated DC can inhibit subsequent T cell cytokine production. Bone marrow derived DCs were cultured for 7 days with DMSO (control), CLA (50μM), or LA (50μM) and activated with OVA peptide (5μg/ml). After 24 h DCs were irradiated and added ($2x10^5$ cells /ml) to $CD4^+$ T cells ($2x10^6$/ml) purified from the spleens of OVA D011.10 transgenic mice. Supernatants were removed on day 4. On day 7 Fresh PUFA-treated OVA-activated DCs were added along with rIL-2 (10U/ml), and supernatants were again removed on day 10. Supernatants were analysed for concentrations of A) IL-17, B) IL-2 and C) IFN-γ**.** Results are means (± SEM) of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, *p<0.05 vs DMSO vehicle control determined by one-way ANOVA test

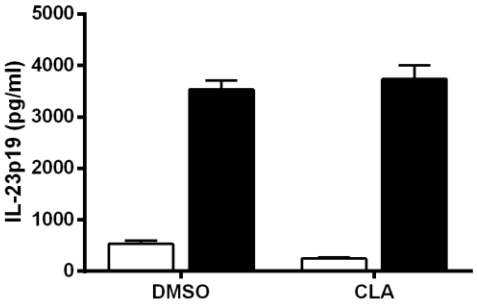
Figure 6. CLA-modulated DCs suppress induction of Th17 responses in vivo. DC were cultured in the presence of DMSO, CLA (50μM) or LA (50μM) for 6 days. On day 6, cells were stimulated with KLH (10μg/ml) or media alone and after a further 24h cells were harvested and injected into the footpads of BALB/c mice (10^5 cells per footpad). After 7 days, spleens were removed and splenocytes adjusted to give $2x10^6$ cells/ml. Cells were added in triplicate to a 96-well plate with increasing concentrations of KLH (2-50μg/ml). Supernatant was removed after 72 hours and levels of A) IL-17, B) IL-2 and C) IFNγ were measured. Results are means ± SE of triplicate assays from 5 separate mice. *p<0.05 vs DMSO vehicle control determined by one-way ANOVA test.

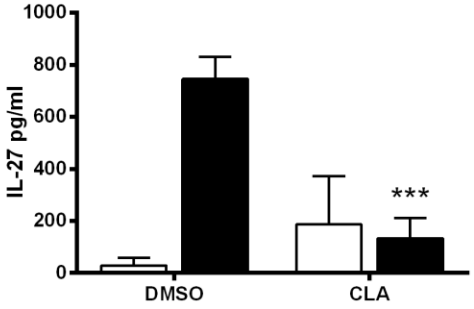
Figure 7. CLA modulates T cell activation and cytokine secretion in Th17 cells.

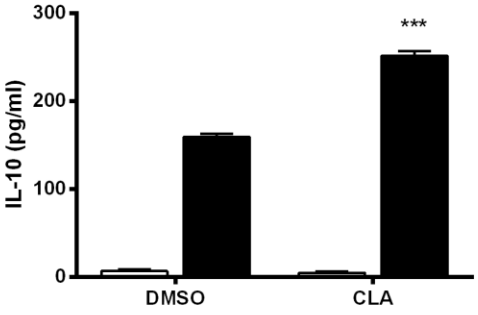
CD4+ T-cells were treated with either DMSO (vehicle control), CLA (50μM) or LA (50μM) and activated with plate-bound anti-CD3 (5 μg/ml; 145-2C11) plus anti-CD28 (5 μg/ml; 37.51) in Th17 polarising conditions for 6 days. (A) The cells were washed and stained with anti-bodies specific to CD4, CD25 and CTLA-4. Profiles are shown for a single experiment and are representative of 3 experiments. (B) The supernatants were recovered and the concentration of IFN-γ, IL-2, IL-4 and IL-17 were measured using specific immunoassays. Results are representative of three experiments. *$p<0.05$, ***$p<0.001$, using an unpaired t-test.

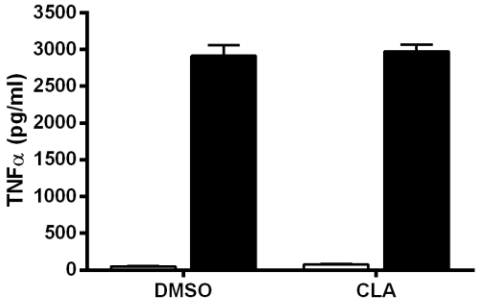


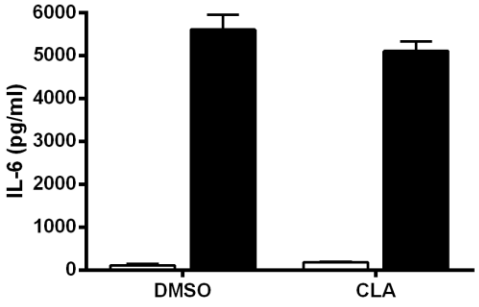


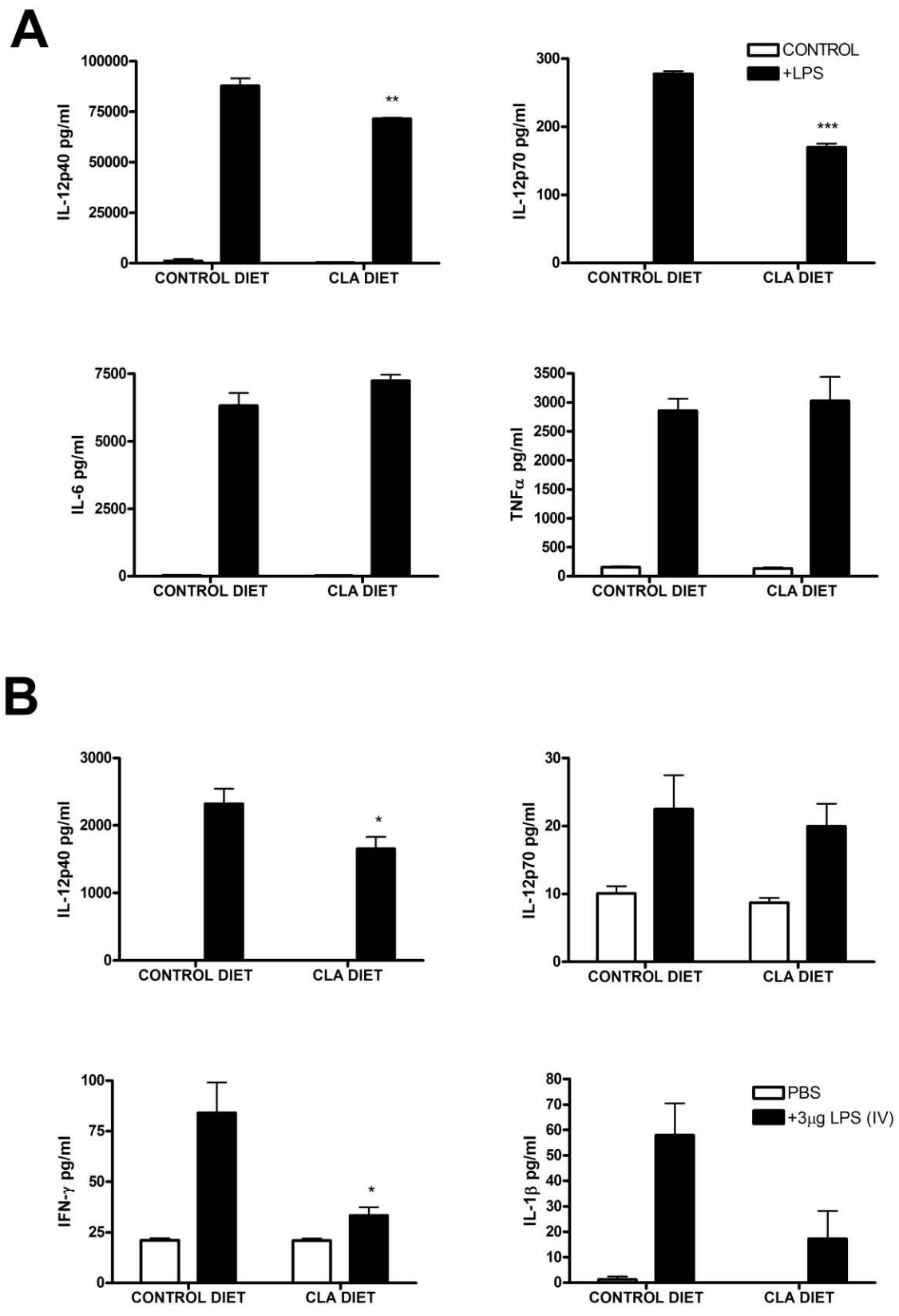


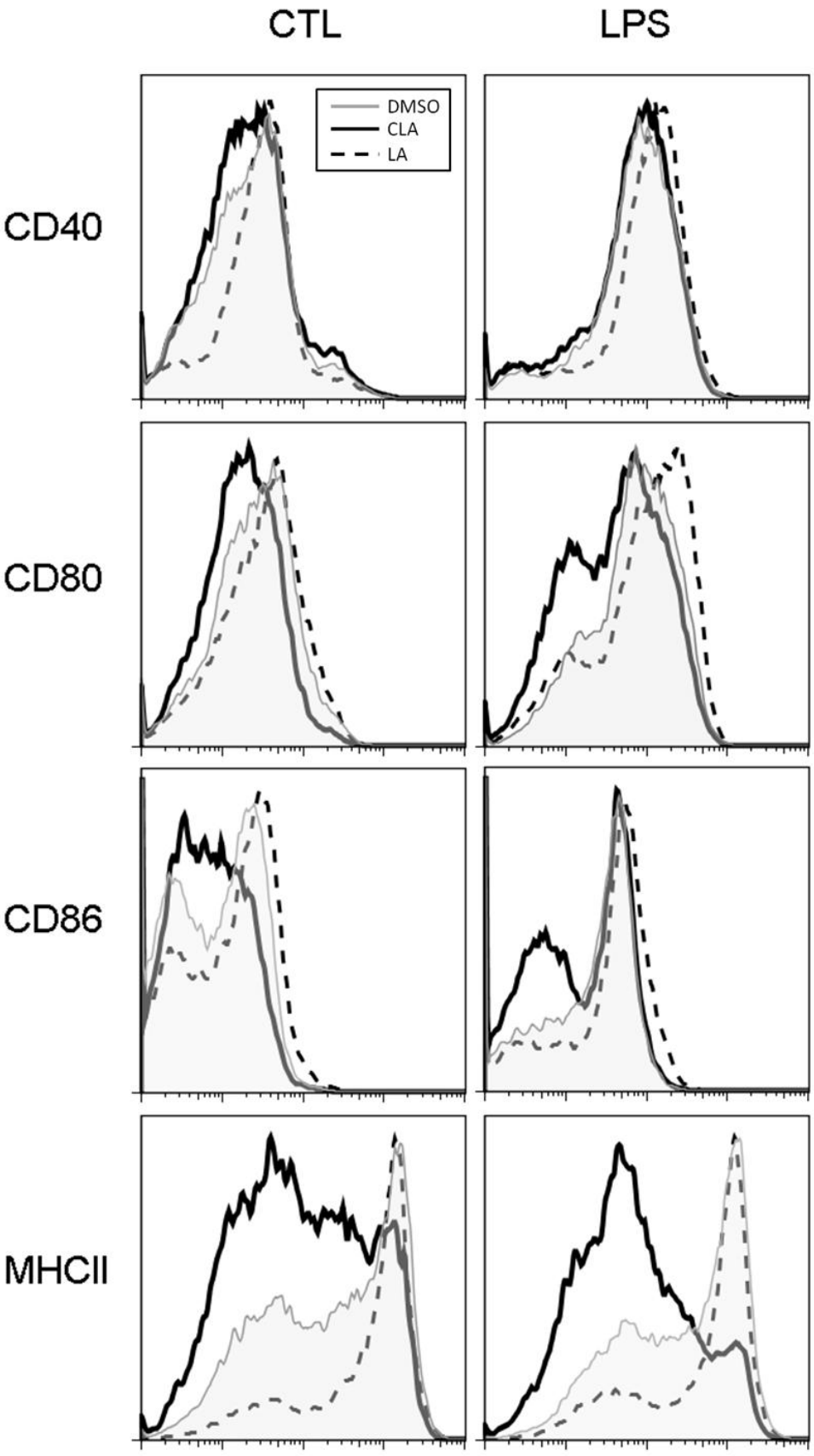


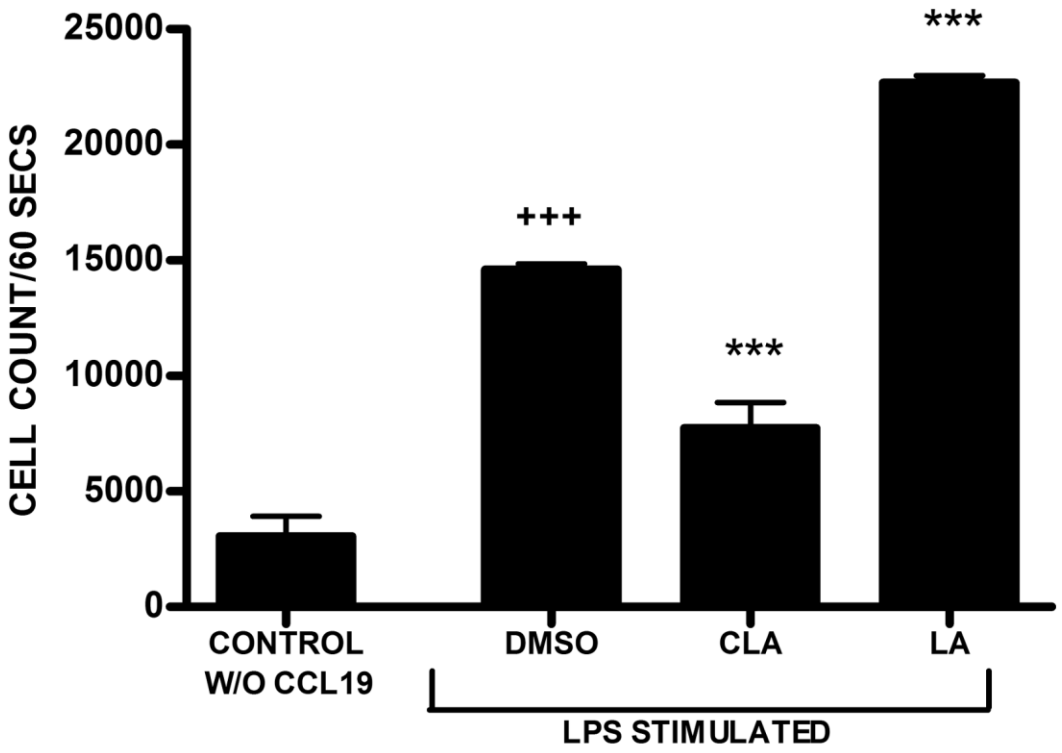


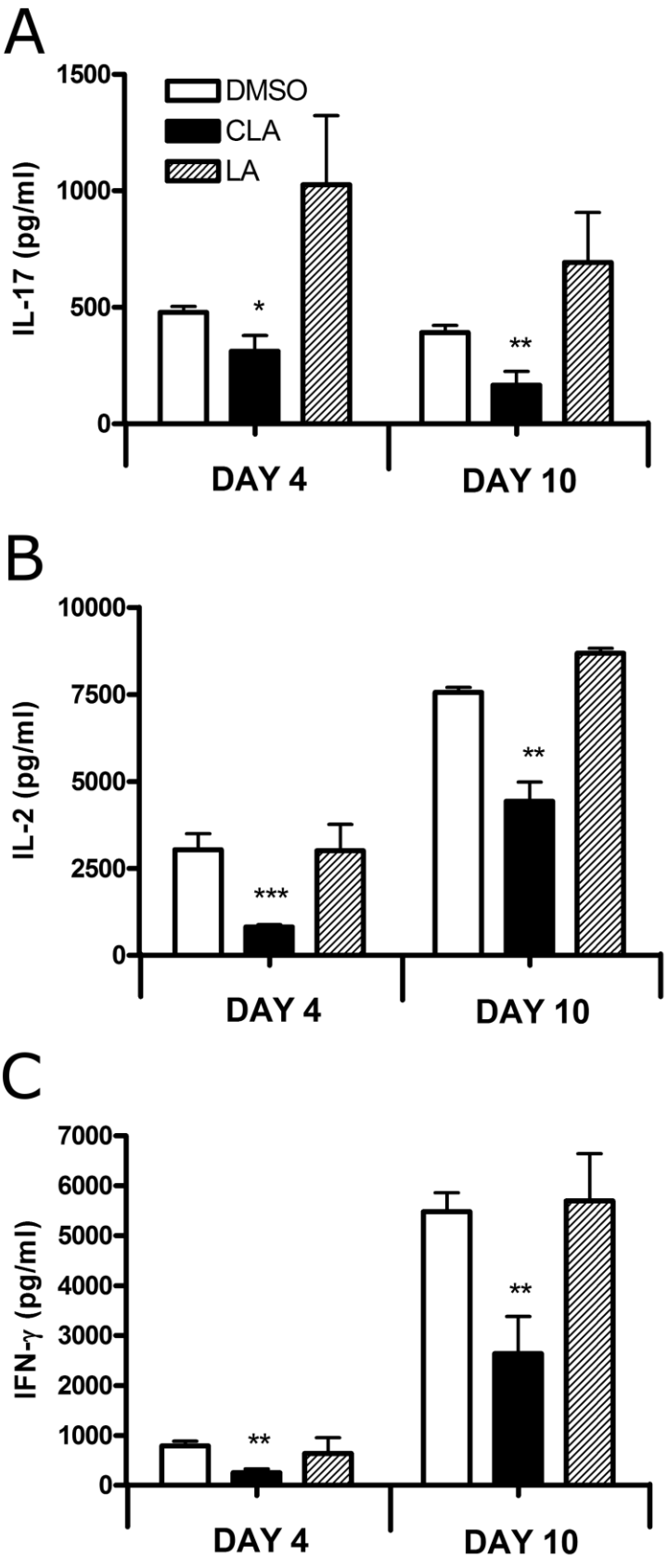


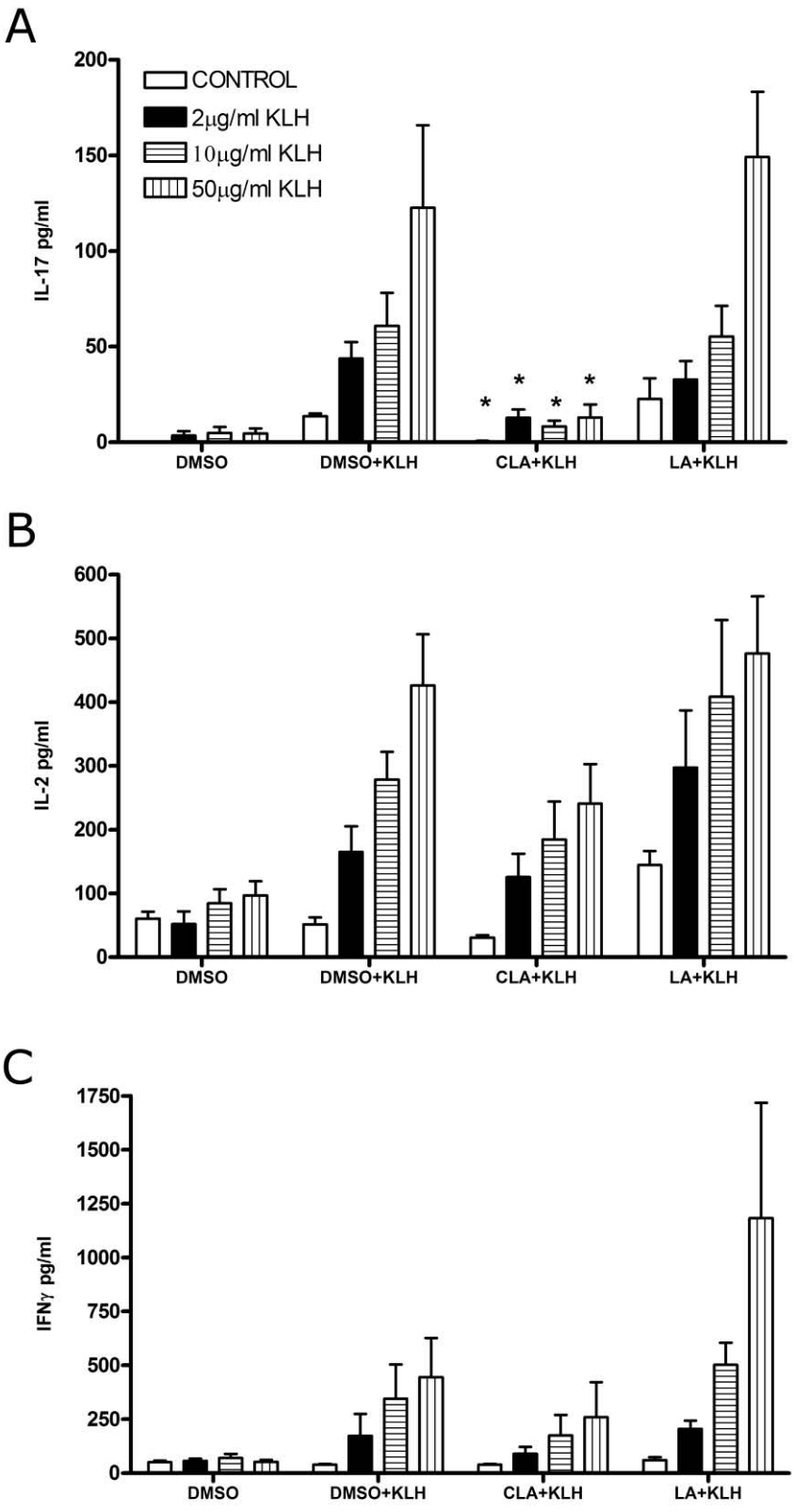












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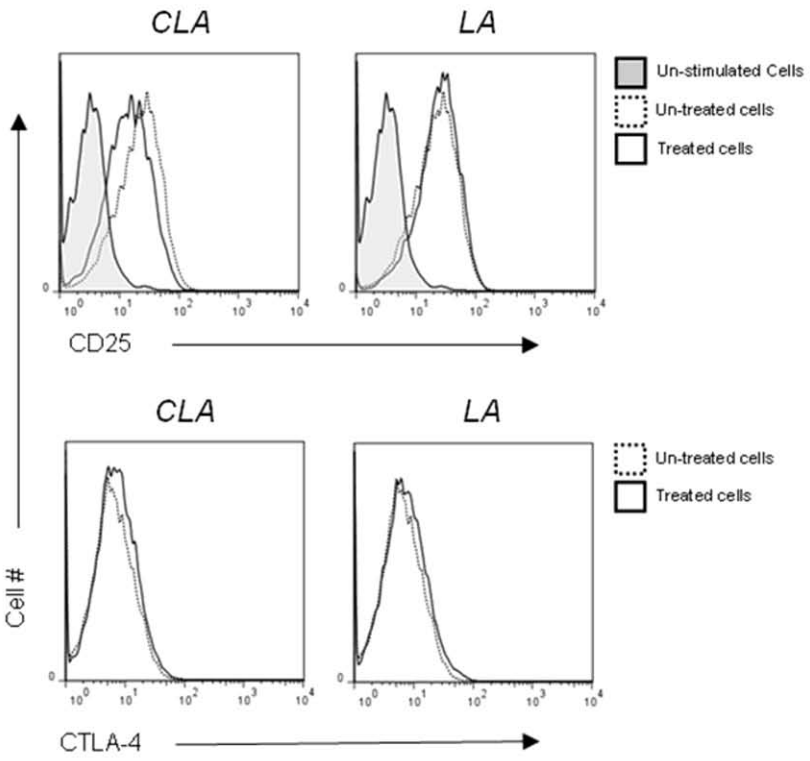

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