Atherosclerosis & inflammation: Macrophage heterogeneity in focus

Stöger, J.L.

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Chapter 8

Treatment with the immunomodulatory drug glatiramer acetate (Copaxone) does not limit inflammation or atherogenesis in hyperlipidaemic LDLR⁻/⁻ mice

J. Lauran Stöger, Bram Schmitz, Marion J.J. Gijbels, Ine M.J. Wolfs, Victor Lamin, Chantal Pöttgens, M.A. Hoeksema, Erwin Wijnands, Linda Beckers, Menno P.J. de Winther

In preparation
Abstract

Aims & Background

Immunomodulation is a promising strategy for the management of atherosclerotic disease. Glatiramer acetate (GA, Copaxone®, copolymer 1) is a synthetic copolymer of four amino acids that is used for treatment of relapsing-remitting multiple sclerosis. Although its mechanism of action in neurodegenerative disease is yet to be fully elucidated, recent insights point towards a primary immunomodulatory action on antigen presenting cells (monocytes, macrophages, dendritic cells). Although GA has been shown to be efficacious in other chronic inflammatory conditions such as inflammatory bowel disease, it has not yet been evaluated in the context of atherosclerosis development. We hypothesised that GA induces an anti-inflammatory phenotype in macrophages and thus affects inflammation in vivo to reduce atherogenesis.

Methods & Results

We used primary murine macrophages to evaluate whether GA enhances macrophage anti-inflammatory potential. Upon treatment with GA and concurrent TLR4-mediated activation through LPS, bone marrow derived macrophages displayed a dose- and time-dependent increase in expression and production of the anti-inflammatory and atheroprotective cytokine IL-10. This induction was robust as it was able to overcome prior IFNγ stimulation and was found to be inducible through a range of TLR ligands. Moreover, secretion of pro-inflammatory IL-12 was repressed at the highest dose of GA. Although indication of a potent anti-inflammatory response, these observations were offset by enhanced NF-κB-associated production of pro-inflammatory TNF and IL-6. However, we were unable to replicate any of these findings in vivo upon treating high cholesterol fed female LDLR−/− mice with GA for a period of four weeks. In these animals, established atherosclerotic plaques continued to develop similarly to controls under GA-treatment. Blood leukocyte composition and activation in blood and spleen were not affected by GA treatment, except for a small decrease in splenic T-cell levels.

Conclusions

Although GA treatment modulates TLR-induced cytokine responses by primary murine macrophages in vitro, it does not exhibit anti-inflammatory or atheroprotective properties under hyperlipidaemic conditions in an experimental murine model of atherosclerosis.
Introduction

Atherosclerosis is characterised by a chronic inflammatory state that is fuelled by infiltrated leukocytes and other immune mediators \(^1\). Particularly monocyte-derived macrophages are attracted to predilection sites in the arterial vascular tree in response to local low-density lipoprotein retention and thereby constitute the most abundant cell type in nascent atherosclerotic lesions. These cells shape lesion initiation and progression by orchestrating lipid handling, tissue remodelling, phagocytosis and inflammation. As such, these cells tie into many important aspects of atherosclerosis aetiology and its associated clinical sequelae (myocardial infarction, stroke) \(^2,3\). Macrophages, like other mononuclear phagocytes, are associated with profound heterogeneity and plasticity \(^4-6\). The current paradigm recognises several subsets of M1 and M2 macrophages, with respective pro- and anti-inflammatory properties. In atherosclerosis, these and other (contrasting) phenotypes have been identified by virtue of their functional characteristics and expression profiles \(^7-10\). This notion fostered the enticing concept that pharmalogical manipulation of macrophage phenotype and function might hold potential as a therapeutic strategy for prevention and regression of atherosclerosis.

Glatiramer acetate (GA, Copaxone, copolymer 1) is an immunomodulatory drug entailing a synthetic copolymer of four amino acids found in myelin basic protein (glutamic acid, lysine, alanine and tyrosine) \(^11\). GA is FDA-approved for treatment of multiple sclerosis, more specifically reducing relapse rates in its relapsing-remitting form (RRMS) \(^12-14\). Although its exact mechanism of action is yet to be identified, several have been put forward as contributors to its beneficial properties \(^15,16\). Early evidence in both MS and its animal model experimental autoimmune encephalomyelitis (EAE) centred mostly around modulation of T-cell-mediated immunity \(^17-19\). However, these effects could not fully account for disease suppression upon GA treatment in other settings \(^20,21\), suggesting additional mechanism at play. Notably, the GA-induced shift from Th1 to Th2-type immune responses \(^22,23\) was in part found to be secondary to immunomodulation of antigen presenting cells \(^24-26\). Whereas previous studies had shown GA induces anti-inflammatory IL-10 in dendritic cells and peritoneal macrophages \(^27\), Weber et al. demonstrated GA skews monocytes to an anti-inflammatory phenotype characterized by augmented secretion of IL-10 and TGF-β and a decreased production of IL-12 and TNF \(^25\). Accordingly, the current study explores whether 1) GA is able to drive macrophage function towards an anti-inflammatory profile and 2) whether GA can be employed as a means of managing innate immune cell function to impair atherogenesis. We report that GA treatment of bone marrow-derived macrophages enhances levels of both pro- and anti-inflammatory cytokines \textit{in vitro}, while not affecting indices of systemic inflammation in hyperlipidaemic LDLR\(^{-/-}\) mice. Furthermore, a four-week regime of subcutaneous GA administration to these animals did not limit progression of established atherosclerotic plaques.
Chapter 8

Materials and methods

Animals

C57Bl/6 mice were acquired from the animal breeding facility at Maastricht University Medical Center. Female LDLR⁻/⁻ mice (n=18 per treatment group) were acquired from Jackson Laboratories (Bar Harbor, Maine, US) and had been backcrossed to C57Bl6 ten times. The Committee for Animal Welfare of Maastricht University Medical Center approved all study protocols (permit-nr. 2009-156).

Atherosclerosis assessment

LDLR⁻/⁻ mice were fed a high-cholesterol diet (HCD) containing 16% fat, 0,15% cholesterol and no cholate (Western type diet 4021.13, Hope Farms, The Netherlands) for 12 weeks to induce moderate to advanced plaque formation. During the last 4 weeks of high cholesterol feeding, mice received 2,0 mg/day GA. Control animals were administered PBS supplemented with mannitol, a component of Copaxone® (Teva Pharma, Haarlem, the Netherlands). At pre- and post-treatment time points fasting blood samples were collected for analyses as described below. Upon sacrifice, hearts were dissected and frozen in Tissue-Tec (Shandon, Veldhoven, the Netherlands). Ensuing, serial 7µm cryosections were routinely stained with toluidin blue for plaque quantification using Adobe Photoshop software. Moreover, an experienced animal pathologist performed morphometric analysis for lesion composition. On the basis of their morphology, plaques were classified as early, moderate or advanced. Briefly, early lesions consisted of foam cells, but lacked a necrotic core. Moderate lesions displayed a fibrous cap with or without a necrotic core. However, the tunica media mustn’t show macrophage infiltration. Advanced lesions convincingly showed macrophage infiltration into the tunica media and could display elastic lamina degradation as well as more severe necrosis and fibrosis.

Lipid analysis

Total cholesterol and triglyceride levels were determined on plasma and homogenized liver samples using standard enzymatic kits according to manufacturer’s protocols (Sigma-Aldrich, Zwijndrecht, NL).
Flow cytometry analysis

Plasma cytokine levels were measured by flow cytometry using a Cytometric Bead Array kit (BD Biosciences, CA, USA) according to the manufacturer’s instructions. Relative numbers of circulating leukocytes were determined by flow cytometry. T-cells are described as CD3⁺NK1.1⁻, Th-cells as CD3⁺CD4⁺, Treg cells as CD3⁺CD25⁺FoxP3, whereas cytotoxic T-cells were CD3⁺CD8⁺. NK-cells were defined as CD3⁻NK1.1⁻, NKT-cells as CD3lowNK1.1low, B-cells as CD3⁻NK1.1⁻B220⁺, granulocytes as CD3⁻NK1.1⁻B220⁻CD11b⁻Ly6G⁻ and monocytes as CD3⁻ NK1.1⁻B220⁻CD11b⁺Ly6G⁻. Monocytes were further divided in Ly6Chigh, Ly6Clow and Ly6C⁻ populations. We used a PE-Foxp3 staining set (eBioscience, Aachen, Germany) according to manufacturer’s instructions to determine intracellular levels of Foxp3. For assessment of intracellular IFNγ and IL-4 we activated native splenocytes with 1 μg/ml ionomycin and 50ng/ml PMA in the presence of GolgiSTOP (BD Biosciences, CA, USA) for a total of 6 hours at 37°C, 5% CO₂. Subsequently, cells were incubated overnight with appropriate antibodies. An overview of applied antibodies is presented in supplementary Table S1. All flow cytometry measurements were performed using a FACSCanto II with FACSDiva software (both BD Biosciences, CA, USA)

Primary macrophage culture

Bone marrow cells were isolated from the hind limbs of wildtype and IL-10R1-deficient mice and cultured in RPMI-1640 (Life Technologies, Bleiswijk, the Netherlands) with addition of 10% heat-inactivated fetal calf serum (Bodinco, Alkmaar, the Netherlands), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Gibco Invitrogen, Breda, the Netherlands) and 15% L929-conditioned medium (LCM) for 8–9 days to generate bone marrow-derived macrophages (BMM), as described previously. BMM were seeded at 3.5 x 10⁵ cells/well in bacteriologic plastic 24-well plates (Greiner Bio-One, Alphen a/d Rijn, NL) and incubated with 10ng/ml of recombinant murine IL-10 (R&D Systems Inc., Abingdon, UK) as indicated. BMM were activated with 10 ng/ml LPS for 3, 8 or 24 hours where designated.

Gene expression

RNA was isolated from BMM and liver samples using the High Pure RNA Isolation Kit (Roche, Woerden, the Netherlands). With regard to RNA isolation from aortic arches we used the RNeasy mini column kit (Qiagen, Venlo, the Netherlands). 500ng total RNA was reverse transcribed using iScript cDNA Synthesis Kit (BioRad, Veenendaal, the Netherlands). Quantitative PCR (qPCR) was performed using 10ng cDNA, 300nM of each primer, and sensiMix SYBR Hi-ROX (Bioline, Brussels, Belgium) in a total volume of 20μl.
levels were corrected for cyclophilin A as reference gene. Primer sequences are available upon request.

**Cytokine secretion**

IL-10, IL-12, IL-6 and TNF secretion by BMM was quantified in supernatants after LPS exposure and/or GA treatment using corresponding ELISA kits (Invitrogen, Bleiswijk, NL) according to manufacturer’s protocols.

Table S1. Overview of antibodies used for flow cytometry

<table>
<thead>
<tr>
<th>antibody</th>
<th>manufacturer</th>
<th>no.</th>
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<tbody>
<tr>
<td>CD11b PE-Cy7</td>
<td>BD</td>
<td>552858</td>
</tr>
<tr>
<td>Ly6G PE</td>
<td>BD</td>
<td>551461</td>
</tr>
<tr>
<td>Ly6C APC</td>
<td>Miltenyi</td>
<td>130-093-126</td>
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<tr>
<td>CD3e FITC</td>
<td>eBioscience</td>
<td>11-0031-82</td>
</tr>
<tr>
<td>CD4 PerCp</td>
<td>BD</td>
<td>553052</td>
</tr>
<tr>
<td>CD8a eFLU0450</td>
<td>eBioscience</td>
<td>48-0081-82</td>
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<td>CD25 APC</td>
<td>eBioscience</td>
<td>17-0251-82</td>
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<td>B220 APC-eFLU0780</td>
<td>eBioscience</td>
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<td>B220 PE Cy7</td>
<td>eBioscience</td>
<td>25-0452-82</td>
</tr>
<tr>
<td>NK1.1 PerCp-Cy5.5</td>
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<td>45-5941-80</td>
</tr>
<tr>
<td>IL-4 PE</td>
<td>BD</td>
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<tr>
<td>IFNγ PE-Cy7</td>
<td>BD</td>
<td>557649</td>
</tr>
<tr>
<td>Fox P3-PE</td>
<td>eBioscience</td>
<td>12-5775-82</td>
</tr>
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**Luciferase activity**

RAW 264.7 cells stably transfected with the 3x-kB-luc plasmid were with 250 µg/ml GA and 10 ng/ml LPS. Cells were lysed using cell culture lysis buffer for 20 min, after which 10 ml cell lysate was added to 50 ml luciferin. Luciferase activity was measured using the Lumac Biocounter M1500 luminometer.

**Statistics**

Data are presented as mean ± the standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism (GraphPad Software Inc.). Groups were compared using (Welch-corrected) two-tailed, non-paired t-tests. Significance was accepted at the level of p<0.05. *, ** and *** indicate p<0.05, 0.01 and 0.001, respectively.
Results

**GA treatment modulates cytokine production by LPS-activated primary murine macrophages.**

To investigate whether GA induces an anti-inflammatory phenotype in murine macrophages, we first evaluated whether exposure of bone marrow-derived macrophages (BMM) to GA led to induction of IL-10. Although incubation with GA alone had no effect on IL-10 levels (data not shown), combining GA treatment with LPS activation significantly augmented IL-10 secretion and gene expression in a dose- and time-dependent manner (Fig.1A-C). In line with these findings, GA reduced IL-12 production by BMM, but only at the highest concentration (Fig.1D). IL-12 gene expression remained unaffected (Fig.1E). Consequently, the balance between IL-12 and IL-10 strongly favoured an anti-inflammatory environment with increasing GA doses (Fig.1F). In an effort to test the robustness of GA-mediated IL-10 enhancement, we provided an M1 stimulus in the form of IFNγ prior to incubation with GA and LPS. IFNγ abolished IL-10 secretion in LPS-treated samples, but increasing doses of GA provided significantly more IL-10 production (Fig.1G). As IL-10 signalling can create an autocrine positive feedback loop, we investigated if the effects described above are dependent on IL-10 responsiveness. Hereto, we used BMM lacking the ligand-binding domain of the IL-10R complex (IL-10R1<sup>floxed</sup>-LysMCre). However, whereas IL-10R1 deficiency amplified IL-10 and IL-12 production, GA displayed identical effects on these cytokines. Since TNF and IL-6 are profound pro-inflammatory cytokines that were previously shown to be beneficially modulated by GA in neuroinflammatory disease, we subsequently assessed gene expression and protein secretion for these cytokines in BMM. Contrary to our expectations, GA treatment elicited dose-dependent increases in both expression and production of LPS-induced TNF (Fig.2A, B) and IL-6 (Fig.2C, D). As these inflammatory mediators are largely reliant on LPS-mediated NF-κB signalling for their induction we questioned whether GA influences NF-κB activity. Hereto, we employed RAW264.7-cells, stably transfected with a luciferase reporter-construct as described in the methods section. This revealed that GA enhances LPS-induced activity of the NF-κB promoter (Fig.2E).

Thus, GA alters the inflammatory potential of LPS-treated macrophages through dose- and time-dependent increases in IL-10 expression and production, while pro-inflammatory IL-12 production was diminished. However, significantly enhanced levels of the pro-inflammatory cytokines TNF and IL-6 potentially offset this otherwise anti-inflammatory profile.
deficiency, excluding autocrine IL-10 signalling as a mechanism for IL-10 induction. 100 ng/ml IFNγ for 24 hours impaired induction of IL-10, but M1 activation did not prevent GA-induced augmentation of IL-10. (H, I) Production of IL-10 and IL-12 in response to 24h GA and LPS is increased in IL-10R1 deficiency, excluding autocrine IL-10 signalling as a mechanism for IL-10 induction.

Fig.1. GA skews IL-12/IL-10 balance to an anti-inflammatory profile. BMM were incubated with either 6h (RT-qPCR) or 24h (ELISA) incubation with 10 ng/ml LPS before treatment with various doses of GA (A-C) GA dose- and time-dependently augments IL-10 production by BMM. (D, E) ELISA of GA-treated BMM shows reduced IL-12 production, while not affecting gene expression. (F) Ratio of IL-12 and IL-10 production reveals a stronger anti-inflammatory balance with increasing concentrations of GA. (G) Preceding GA and LPS co-incubation with 100 ng/ml IFNγ for 24 hours impaired induction of IL-10, but M1 activation did not prevent GA-induced augmentation of IL-10. (H, I) Production of IL-10 and IL-12 in response to 24h GA and LPS is increased in IL-10R1 deficiency, excluding autocrine IL-10 signalling as a mechanism for IL-10 induction.
**GA amplifies IL-10 induction in response to multiple TLR-ligands**

To investigate whether GA specifically modulates TLR4-mediated macrophage cytokine responses or acts more promiscuously on pattern-recognition receptors, we incubated BMM with a range of TLR ligands. Poly(I:C) (targeting TLR3); Pam3CSK4 (TLR1 and 2); R848 and R-837 (TLR7 and 8) all activated BMM to secrete IL-10 to some extent, but generally less so than LPS. Moreover, in all stimuli but poly(I:C), GA strengthened significantly upregulated IL-10 responses. Although in poly(I:C) an incremental increase in IL-10 expression was found with GA, this did not reach statistical significance in relation to the control condition (only poly(I:C)). From this, it is clear that GA has the potential to affect a broad range of innate immunity receptors to alter macrophage inflammatory responses.

**GA does not affect atherosclerosis development or indicators of systemic inflammation in hyperlipidaemic LDLR<sup>-/-</sup> mice.**

Next, we studied whether GA treatment can impede the progression of lipid-driven atherosclerotic disease. To this end, female LDLR<sup>-/-</sup> mice received a high cholesterol diet (HCD) for 12 weeks and were administered a daily subcutaneous dose of 2.0mg GA for the last 4 weeks of HCD. Control mice were injected with sterile PBS supplemented with mannose, a constituent of Copaxone®. Table I represents the basal characteristics before (8 weeks HCD) and after treatment (12 weeks HCD). Bodyweight, plasma cholesterol and triglyceride levels were comparable between groups at the pre-treatment time-point. Continued high cholesterol feeding increased plasma lipid levels further. Interestingly, plasma triglyceride content was increased more modestly in GA-treated mice, leaving a significantly lowered TG level compared with control mice at 12 weeks HCD. Bodyweight remained equal throughout the experiment.

Appraisal of atherosclerosis development in the aortic roots of these mice revealed no differences in lesion area between GA- and control-treated animals (431.6 ± 21.4 vs. 435.7 ± 19.7 ×10³ µm²) (Fig.4A). Moreover, routine pathological assessment of plaque morphology showed no distinct effect of GA treatment on collagen deposition, necrotic area, number of foam cells, inflammatory macrophages, monocyte adhesion, granulocytes or adventitial influx (Fig.4B). In line with these observations, classification of plaque severity in early, moderate and advanced lesions displayed a similar subdivision between GA-treated and control groups (Fig.4C).
Fig. 2. Combining various doses of GA with 10ng/ml LPS significantly and dose-dependently upregulated production (24 hours) and expression (6 hours) of pro-inflammatory cytokines TNF (A, B) and IL-6 (C, D). (E) Assessment of NF-κB promoter activity through a luciferase reporter construct, showing significantly increased NF-κB activity with GA and LPS vs. LPS incubation alone.

Fig. 3. GA boosts IL-10 secretion by BMM in response to various TLR-ligands as measured by ELISA. Incubation for 24h with different doses of GA and either 10 ng/ml LPS, 5 μg/ml poly(I:C), 2 μg/ml Pam3CSK4, 10 μg/ml R-848 or 1 μg/ml R-837.
Glatiramer acetate treatment of atherosclerosis in mice

Table I. Basal characteristics of control and GA-treated LDLR\(^{-/-}\) mice at 8 and 12 weeks HCD

<table>
<thead>
<tr>
<th></th>
<th>pre-treatment</th>
<th>post-treatment</th>
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<tbody>
<tr>
<td></td>
<td>control</td>
<td>GA</td>
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<tr>
<td>bodyweight (g)</td>
<td>21.7 ± 0.3</td>
<td>22.3 ± 0.3</td>
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<tr>
<td>plasma cholesterol (mM)</td>
<td>35.77 ± 0.80</td>
<td>34.19 ± 0.79</td>
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<tr>
<td>plasma triglycerides (mM)</td>
<td>1.54 ± 0.10</td>
<td>1.57 ± 0.10</td>
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<tr>
<td>liver cholesterol (μg/μg protein)</td>
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</tr>
<tr>
<td>liver triglycerides (μg/μg protein)</td>
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<td>n/a</td>
</tr>
</tbody>
</table>

\(^a\) denotes p < 0.05 vs. post-treatment control group

Fig. 4. GA treatment of LDLR\(^{-/-}\) mice does not differentially affect atherosclerotic lesion size or severity. (A) Quantification of plaque development in the aortic sinus of GA-treated mice and controls after 12 weeks high cholesterol feeding. (B) Routine assessment of lesion morphology by an experienced animal pathologist. Given are semi-quantitative scoring of plaques for collagen content, necrotic area, foam cell size, number of inflammatory macrophages, monocytes adhering to the endothelium, granulocytes and adventitial influx. (C) Pie diagrams of lesion distribution in GA-treated and control mice into early, moderate or advanced lesions demonstrating comparable plaque severity.
Chapter 8

Table II. Relative blood leukocyte profile in control and GA-treated LDLR⁻/⁻ mice

<table>
<thead>
<tr>
<th>Myeloid cells</th>
<th>control</th>
<th>GA</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes (CD11b⁺ Ly6G⁺)</td>
<td>11.5 ± 1.7</td>
<td>10.9 ± 1.6</td>
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<td>Monocytes (CD11b⁺ Ly6G⁺)</td>
<td>11.2 ± 1.3</td>
<td>9.8 ± 0.8</td>
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<tr>
<td>- Ly6C&lt;sup&gt;high&lt;/sup&gt;</td>
<td>13.4 ± 1.3</td>
<td>13.0 ± 2.6</td>
<td>ns</td>
</tr>
<tr>
<td>- Ly6C&lt;sup&gt;low&lt;/sup&gt;</td>
<td>15.9 ± 1.1</td>
<td>15.9 ± 2.6</td>
<td>ns</td>
</tr>
<tr>
<td>- Ly6C&lt;sup&gt;-&lt;/sup&gt;</td>
<td>71.4 ± 2.1</td>
<td>71.7 ± 4.4</td>
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</table>

<table>
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<tr>
<th>Lymphoid cells</th>
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<th>p-value</th>
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<tr>
<td>T-cells (CD3⁺ NK1.1⁺)</td>
<td>35.9 ± 2.2</td>
<td>37.9 ± 2.2</td>
<td>ns</td>
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<tr>
<td>- Th-cells (CD4⁺)</td>
<td>47.4 ± 0.4</td>
<td>47.3 ± 1.3</td>
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<tr>
<td>- Treg-cells (CD25⁺ FoxP3⁺)</td>
<td>5.9 ± 0.3</td>
<td>6.4 ± 0.2</td>
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<tr>
<td>- Cytotoxic T-cells (CD8⁺)</td>
<td>45.0 ± 0.4</td>
<td>44.5 ± 1.1</td>
<td>ns</td>
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<td>NK-T-cells (CD3&lt;sup&gt;low&lt;/sup&gt; NK1.1&lt;sup&gt;low&lt;/sup&gt;)</td>
<td>4.0 ± 0.2</td>
<td>4.8 ± 0.4</td>
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<td>NK-cells (CD3⁺ NK1.1⁺)</td>
<td>8.4 ± 0.6</td>
<td>7.9 ± 0.5</td>
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<td>B-cells (B220⁺)</td>
<td>29.7 ± 2.1</td>
<td>28.3 ± 2.0</td>
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Table III. Relative splenocyte counts in control and GA-treated LDLR⁻/⁻ mice

<table>
<thead>
<tr>
<th>T-cells (CD3⁺ NK1.1⁺)</th>
<th>control</th>
<th>GA</th>
<th>p-value</th>
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<tr>
<td>26.7 ± 0.6</td>
<td>21.8 ± 0.9</td>
<td>0.0004</td>
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<tr>
<td>- Th-cells (CD4⁺)</td>
<td>52.5 ± 0.6</td>
<td>52.0 ± 1.4</td>
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<tr>
<td>- Th1-cells (IFNy⁺)</td>
<td>7.8 ± 1.2</td>
<td>5.2 ± 0.4</td>
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</tr>
<tr>
<td>- Th2-cells (IL-4⁺)</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>ns</td>
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<tr>
<td>- Treg-cells (CD25⁺ FoxP3⁺)</td>
<td>11.0 ± 0.5</td>
<td>10.8 ± 0.5</td>
<td>ns</td>
</tr>
<tr>
<td>B-cells (B220⁺)</td>
<td>46.4 ± 0.4</td>
<td>44.2 ± 1.6</td>
<td>ns</td>
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</table>

Given that atherogenesis was not differentially affected by GA administration, we questioned whether GA affected indices of systemic inflammation. First, we examined blood leukocyte profiles in control and GA-treated LDLR⁻/⁻ mice through flow cytometry (Table II). Relative counts of both myeloid and lymphoid leukocyte populations were unaltered upon 4 weeks of GA treatment. As shown in Table III, similar observations were made with regard to splenocytes, where only relative T-cell numbers were modestly but significantly decreased in GA-treated LDLR⁻/⁻ mice.
To further assess the inflammatory status of PBS- and GA-injected animals, we quantified cytokine levels in plasma of control and GA-treated animals. This exposed no significant differences in circulating levels of IL-10, IL-12p40, TNF, IL-6, IFNγ, or MCP-1 (Fig.5A). Additionally, peritoneal macrophages collected from GA-treated mice showed equal IL-10 production in comparison to control animals after being subjected to GA and LPS in vitro (Fig.5B). Similarly, we were unable to show enhanced IL-10 gene expression in BMM taken from control and GA-injected LDLR<sup>−/−</sup> mice. Remarkably, IL-10 expression was even significantly reduced in BMM derived from GA-treated animals (Fig.4C).

Fig.5. In vivo GA treatment does not elicit enhanced IL-10 in peritoneal or bone marrow-derived macrophages. (A) Cytokine levels in plasma of GA-treated and control mice as measured by Cytometric Bead Array (CBA) and flow cytometry, revealing no significant difference between both groups. (B) IL-10 production by peritoneal macrophages from GA-treated and control (n=6 per group) LDLR<sup>−/−</sup> mice stimulated with 250 μg/ml GA and 10 ng/ml LPS for 24 hours. Comparable IL-10 induction was seen in both treatment groups. (C) BMM derived from GA-treated and control animals were exposed to 250 μg/ml GA and 10 ng/ml LPS to evaluate IL-10 gene expression.

Discussion

The current study investigated the premise that the immunomodulatory drug glatiramer acetate, approved for the treatment of MS, can induce an anti-inflammatory phenotype in macrophages and inhibit atherosclerosis development in an experimental mouse model. Our findings revealed that GA incubation alongside TLR-ligands (e.g. LPS) enhances the production of both pro- and anti-inflammatory cytokines by cultured macrophages. Yet, upon subcutaneous administration of GA in vivo we could not demonstrate that GA provokes similar, or for that matter any, cytokine responses in HCD-fed LDLR<sup>−/−</sup> mice in vivo. In line with these findings, atherosclerotic plaque size, severity and circulating leukocyte profile in the treatment group were unaltered after daily exposure to GA.
Although we present the first data concerning the effects of GA on bone marrow-derived macrophages, GA has been shown previously to influence both pro- and anti-inflammatory cytokine expression and signalling in several related cell types. Early evidence indicated GA’s ability to inhibit the activation of THP-1 cells by the pro-inflammatory mediator IFNγ. Whereas one study reports treating MS patients with GA enhances both their IFNγ and IL-4 signatures, differential modulation of these cytokines was associated with a favourable clinical outcome in another clinical investigation. Additionally, GA appears to negatively influence pro-inflammatory IL-1-mediated signalling. Regarding the cytokines described here, our observations that GA dose- and time-dependently boosts anti-inflammatory IL-10 levels are in line with several reports that signify IL-10 as a GA-target cytokine in antigen presenting cells. Likewise, reduced secretion of IL-12 has been reported on a number of occasions. Contrasting, the NF-κB-associated upregulation of TNF and IL-6 in response to GA and LPS in our studies are at odds with previous work by others. These conflicting outcomes are likely related to differences in target cell types and pathologic settings, which makes it considerably more challenging to define a common mechanism of action for GA in (neuro)inflammatory disease.

Yet, as IL-12, TNF and IL-6 act as opposing forces to IL-10, this leaves us to consider the net result of the macrophage inflammatory profile associated with GA exposure. While the former pro-inflammatory cytokines are well known to promote atherogenesis, IL-10 is generally believed to be atheroprotective. However, our current data do not provide additional insight into this matter, as indices of cytokine levels were equal between control and GA-treated mice. As we do not have a solid explanation for the observed discrepancy between GA-induced responses in vitro and the lack thereof in vivo, this leaves room for some speculation. We consider dosage, duration of treatment and/or timing of GA administration in relation to the stage of plaque development all to be highly influential factors in this matter. In comparison to others, the dose used in this study should certainly be adequate to exert effects. Moreover, instead of aiming to retard growth of established plaques, a more favourable approach might be to target lesion development from the get-go. Alternatively, the complexity of cell-cell interactions within atherosclerosis might prevent or re-direct the development of cytokine responses as seen in vitro. Along these lines, one could also argue that the conditions for cytokine induction in vitro (e.g. sufficient TLR-stimulation) are not met in the more low-grade type of inflammation associated with atherosclerosis. Finally, in agreement with previous findings by our group, hyperlipidaemia as encountered in our LDLR−/− mice could profoundly alter macrophage responses to GA. Therefore, at least in our current approach, GA treatment of hyperlipidaemic LDLR−/− is unsuccessful in slowing down lesion growth of established plaques.
References


