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The intravenous injection of oxidized LDL- or Apolipoprotein B100 – Coupled splenocytes promotes Th1 polarization in wildtype and Apolipoprotein E – Deficient mice

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ABSTRACT

Background: Th1 responses in atherosclerosis are mainly associated with the aggravation of atherosclerotic plaques, whereas Th2 responses lead to a less pronounced disease in mouse models. The fixation of antigens on cells by means of ethylene carbodiimide (ECDI), and subsequent injection of these antigen-coupled splenocytes (Ag-SP) to induce tolerance against the attached antigens, has been successfully used to treat murine type 1 diabetes or encephalomyelitis in. We analyzed this approach in a mouse model for atherosclerosis.

Methods and results: OTII-transgenic mice that were treated with a single dose of 5×10^7 OVA-coupled splenocytes (OVA-SP), had decreased splenocyte proliferation, and lower IFNγ production in vitro upon antigen recall. However, in vivo CD4 cell activation was increased.

To try lipoprotein-derived, "atherosclerosis-associated" antigens, we first tested human oxidized LDL. In wild type mice, an increase of IFNγ production upon in vitro recall was detected in the oxLDL-SP group. In Apolipoprotein E – deficient (ApoE−/−) mice that received oxLDL-SP every 5 weeks for 20 weeks, we did not find any difference of atherosclerotic plaque burden, but again increased IFNγ production.

To overcome xenogenous limitations, we then examined the effects of mouse Apolipoprotein B100 peptides P3 and P6. ApoB100-SP treatment again promoted a more IFNγ pronounced response upon in vitro recall. Flow cytometry analysis of cytokine secreting spleen cells revealed CD4 positive T cells to be mainly the source for IFNγ.

In ApoE−/− mice that were administered ApoB100-SP during 20 weeks, the atherosclerotic plaque burden in aortic roots as well as total aorta was unchanged compared to PBS treated controls. Splenocyte proliferation upon antigen recall was not significantly altered in ApoB100-SP treated ApoE−/− mice.

Conclusion: Although we did not observe a relevant anti-atherosclerotic benefit, the treatment with antigen-coupled splenocytes in its present form already impacts the immune responses and deserves further exploration.

1. Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial vessel wall, with a substantial contribution of both innate and adaptive immune mechanisms [1,2]. T helper cell response type 1 (Th1) inflammation predominates in atherosclerosis and clearly promotes its aggravation, whereas Th2 and regulatory T (Treg) cell responses are associated with a less rapid formation of atherosclerotic plaques [3]. Among the T cells in the lesion area, T cell receptor (TCR) variability is limited, pointing to a defined reservoir of epitopes that might be relevant for the development of atherosclerosis in mice [4].

Recent studies have successfully demonstrated that the treatment of Apolipoprotein E-deficient (ApoE−/−) mice with "atherosclerosis-associated" proteins or peptides, in combination with

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adjuvants such as Freund's adjuvant or Alum, attenuates Th1 inflammation by skewing the T effector cell function to a Th2 a Treg response, and reduces lesion size compared to naive controls [5–7]. Interestingly, the administration of adjuvants only, i.e. without supplemented antigens, also reduces atherosclerosis (to a lesser degree though), and modifies the inflammatory profile likewise [5,7]. However, such approach cannot be translated to the clinic and is associated with strong local and systemic side effects.

To avoid the complications of a too generalized immune modulation through the use of adjuvants, several regimen involving the induction of antigen-specific tolerance have been tested in the context of atherosclerosis. In a recent study by our team, the continuous subcutaneous infusion of Apolipoprotein B100 (ApoB100) peptides via osmotic minipumps at low doses and without adjuvants led to a reduction of atherosclerosis in young male ApoE−/− mice, and abrogated disease progression after onset in older male ApoE−/− mice [8]. In that model the effects were promoted by the induction of Tregs.

Antigen presentation to MHCII-restricted CD4 T cells in the absence of costimulation by antigen-presenting cells (APC) is held responsible for the induction of anergy or the termination of T helper cell function [9–12]. Based on this concept, T cell tolerance towards murine epitopes has been experimentally induced through the treatment of mice with peptides or proteins that were cross-linked with apoptotic cells by ethylene carbodiimide (ECDI) [13]. Also, this approach has been successfully tested for human T lymphocytes in vitro [14]. The proposed mechanisms for these effects include lack of co-stimulation between antigen-coupled splenocytes (Ag-SP) and T cells, and recognition of Ag-SP as “self” by host APC after incorporation and processing [12]. This route for tolerance induction has been successfully investigated in experimental type 1 diabetes mellitus [15], autoimmune encephalomyelitis [16], autoimmune myocarditis [17], or transplant rejection [18] in mice, which all have a strong Th1-driven immunoresponse.

In our study, we hypothesized that the use of Ag-SP containing lipoprotein-derived proteins or peptides might reduce the Th1 driven inflammation in ApoE−/− mice and therefore attenuate or even prevent the development of atherosclerosis.

2. Methods

2.1. Mice

Male C57bl6 wildtype mice were purchased from Janvier Labs. OT II mice (C57BL/6-Tg(TcraTcrb)425Cbn/Crl) were purchased from Charles River Laboratories. Apolipoprotein E-deficient mice were taken from our own colony. Mice received standard chow and water ad libitum. Experiments were conducted according to the French veterinary guidelines and those formulated by the European Community for experimental animal use (L358-86/609EEC), and were approved by the Institut National de la Santé et de la Recherche Médicale.

2.2. Cell culture and proliferation assays

Cells were cultured in RPMI 1640 supplemented with Glutamax, 10% FCS, 0.02 mmol/L.

β-mercaptoethanol and antibiotics (referred to as “*complete*” RPMI).

For cytokine measurements, CD4 T cells were cultured at 1×10^5 cells per well for 48 h. In some experiments, microplates were anti-CD3–coated (10 g/mL), otherwise antigen-recall was performed by adding the antigens to *complete* RPMI.

For proliferation, cells were cultured at 37 °C for 72 h and pulsed with 1 μCi of [^{3}H]thymidine (Amersham) for the last 18 h of culture. Thymidine incorporation was assessed using a TopCount NXT scintillation counter (PerkinElmer).

2.3. Ethylene carbodiimide-coupled cell treatment

Ethylene carbodiimide peptide-coupled splenocyte (peptide-SPs) treatment was carried out described by Niens et al. [19]. Briefly, spleens were removed from female C57bl/6J mice, mechanically minced through 40 μm cell strainers, and the erythrocytes lysed. The splenocytes were incubated with ECDI (150 mg/3.2×10^8 cells, Calbiochem) and indicated protein (Chicken ovalbumin (Sigma), or human oxidized LDL (Clinisciences)) or peptide (Apolipoprotein B100 peptides P3/$ApoB_{3501–3516}$ and P6/$ApoB_{978–993}$ were kindly provided by Dr Harley Tse, La Jolla) on ice for 1 h, hand-shaking every 10 min. The peptide-SPs were washed, centrifuged, filtered to remove cell clumps, and resuspended in PBS. The mice received 50×10^6 SP in 200 mL PBS by intravenous injection. For atherosclerosis experiments, ApoE−/− mice were given multiple treatments at 5-week intervals as indicated.

In an initial series of experiments, the reaction of splenocytes and ECDI was verified to ensure that mostly apoptotic cells were generated, and that the majority of added protein or peptide bound to the cell surface after 1 h incubation on ice. After intravenous injection of CFSE-labeled ECDI splenocytes, these cells were detectable in the spleen, lungs and liver, whereas blood, lymph nodes and kidney were negative (Fig. S1–3). Hence, for cell culture experiments focus was on spleen cells rather than lymph node cells.

2.4. ELISA and ELISpot assays

For ELISA analysis of cytokine production, cells were cultured on CD3-coated microplates (5 μg/mL). IFNγ, IL-4, or IL-10 in the supernatants were measured using specific ELISAs (R&D Systems). ELISpot assays for IFNγ (BD), IL-4 (BD), and IL17 (Mabtech) were performed according to manufacturer's instructions. Cells were incubated for 24 h (IFNγ) or 48 h (IL-4, IL17) in complete RPMI in the presence of Ovalbumin, human oxLDL, or ApoB100 peptides 3 and 6 as indicated.

2.5. Flow cytometry

Splenocytes were labeled with anti − CD3 − PerCp-Cy5.5 (clone 145-2C11, eBioscience), anti - CD4 − FITC or PE-Cy7 (clone RM4-5, eBioscience), and anti − CD69 − PE (clone H1.2F3, eBioscience), and then analyzed by flow cytometry on a Fortessa cytometer (Becton Dickinson). For intracellular cytokine staining, lymphocytes were stimulated in vitro with leukocyte activation cocktail (BD) according to the manufacturer's instructions for 4 h. Surface staining was performed before permeabilization using an intracellular staining kit (eBioscience) and anti-IFNγ − FITC (clone XMG1.2, BD).

2.6. Histology

Hearts were stored in 4% paraformaldehyde at 4 °C Before inclusion in a cutting medium and further storage at −80 °C, hearts were treated with 30% sucrose in phosphate-buffered saline for 24 h. Successive 10-μm transversal sections of aortic sinus were obtained. Lipids were detected using Oil Red O staining.

2.7. Statistical analysis

Data are expressed as mean ± SEM Data were compared and intergroup differences were analyzed with 1-way ANOVA and post hoc Tukey's test. Other data were analyzed by a 2-tailed Student *t*

test. Differences were considered statistically significant when the probability value was ≤0.05.

3. Results

3.1. Ovalbumin-coupled splenocytes (Ova-SP) in OTII-tg mice

To test the impact of Ag-coupled splenocytes (SP) on the T effector cell functions, OTII-TCR transgenic mice with a specific T cell receptor against chicken Ovalbumin (OVA) were injected either PBS-SP, or OVA-SP that treated with either normal or high ECDI concentration during coupling. After 2 weeks, all mice were immunized with complete Freund's adjuvant (CFA) and OVA s.c., and then sacrificed another 2 weeks later. In flow cytometry, CD4 T cell levels were equal (Fig. S4A), but a slight increase of activated CD4/CD69 positive T cells in mice pretreated with OVA-SP with normal ECDI concentration was detectable (Fig. S4B). Proliferation was statistically not different, but seemed increased in OVA-SP pretreated mice (Fig. S4C). IFNγ in the supernates of anti-CD3 and OVA stimulated splenocytes was equally increased, IL-10 levels were lower, but also unchanged between the groups. IL-4 concentration was very low or not detectable (Fig. S4D).

Because CFA/OVA immunization is a very strong immunogenic stimulus in these highly preconditioned mice, the experiment was repeated just with PBS-SP vs OVA-SP pretreatment without CFA immunization. We additionally compared the previously used OVA-concentration during ECDI-coupling with a 10fold higher concentration. Interestingly the number of CD4/CD69 positive T cells was increased (Fig. S5A and B), whereas the proliferative response this time was reduced in these mice (Fig. S5C). In FACS analysis, other subsets of innate or adaptive immunity did not show reproducible trends between the experiments. For cytokine detection, we wanted to extrapolate antigen-induced effects from global activation with anti-CD3. Since cytokine levels in cell culture supernatant are very low without further co-stimulation with anti-CD3 (data not shown), we used ELISpot analysis to detect and enumerate cytokine producing cells.

IFNγ was significantly decreased in both OVA-SP treated groups compared to the ECDI-SP group. This decrease was not significant, but by trend vs PBS treated mice. IL4 and IL17 were not statistically different between all groups (Fig. S5D).

3.2. Human oxLDL-coupled splenocytes in Apolipoprotein E – deficient mice by trend aggravates atherosclerosis

To transfer the model into murine atherosclerosis, we treated male Apolipoprotein E – deficient mice (ApoE−/−) either with oxidized LDL (oxLDL-)-SP, OVA-SP, or PBS, and injected all mice CFA/oxLDL after 2 weeks. The mice were sacrificed another 2 weeks later. Surprisingly, we found no differences in CD4 T cell levels or activation, although oxLDL-SP treated mice produced significantly more IFNγ upon in vitro recall (Fig. 1A–C).

In order to investigate the impact of repetitive applications (once every 5 weeks) of human oxLDL-SP, we injected young ApoE−/− mice for 20 weeks. At sacrifice, IFNγ production was significantly increased after in vitro antigen recall (Fig. 2A), IL-4 production was not different between the groups (Fig. 2B). Lesion size of atherosclerotic plaques in the aortic roots was by trend increased (Fig. 2C).

3.3. Apolipoprotein B100-derived peptides 3 (P3) and 6 (P6)-coupled splenocytes

Next, we sought to determine the role of this treatment approach for atherosclerosis with two highly immunogenic, mouse Apolipoprotein B100 (ApoB100) derived peptides, namely P3 ($ApoB_{3501-3516}$) and P6 ($ApoB_{978-993}$).

C57Bl6J mice received either PBS-SP, ApoB100-SP, or only PBS, before immunization with CFA/ApoB100. The cytokine profile in ELISpot analysis pointed towards a Th1-polarisation in ApoB100-SP pretreated mice (Fig. 3A). FACS analysis of in vitro stimulated cells with the peptides revealed, that CD4 T cells were responsible for the increase of INFγ, which confirmed a Th1 effector cell response (Fig. 3B). On the contrary, CD8 positive cells (another essential source for IFNγ) did not change their secretion profile (Fig. 3C).

3.4. Development of atherosclerosis in male ApoE-deficient mice is unaltered following intravenous treatment with P3/6-SP

To investigate the impact of this approach on atherosclerosis development, we treated male ApoE-deficient mice every 5 weeks with i.v. ApoB100-SP or PBS, for a duration of 20 weeks. As expected from the previous results, IFNγ production was stronger among ApoB100-SP treated mice after antigen-specific in vitro recall (Fig. 4A), whereas IL4 production was similar between the 2 groups (Fig. 4B). Proliferation of splenocytes was not significantly affected, but tended to increase in the ApoB100-SP treated mice (Fig. 4C). Atherosclerotic lesion size in the aortic root did not differ between ApoB100-SP and PBS treated animals (Fig. 4D).

4. Discussion

In our study, we tried to establish a method to induce antigen-specific tolerance in murine atherosclerosis. Therefore we

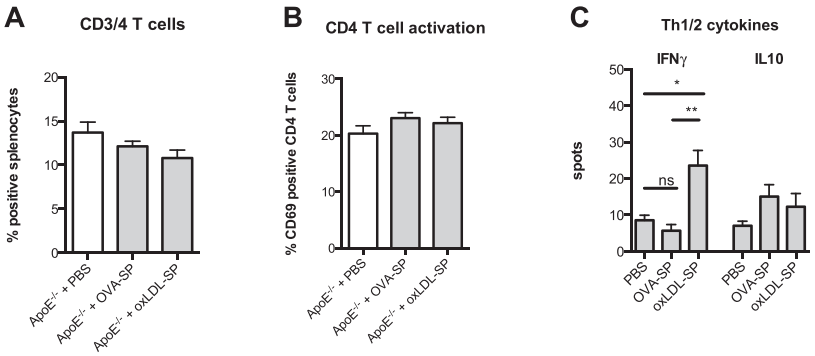

Fig. 1. ApoE$^{-/-}$ mice were injected either PBS, splenocytes coupled with OVA (OVA-SP) or oxLDL (oxLDL-SP). After 10 days, all mice received 50 μg oxLDL dissolved in PBS and complete Freund's adjuvant. Another 10 days later, all mice were sacrificed. A) CD3/4 positive T cells in the spleen. B) CD4 T cell activation in spleen. C) Cytokine ELISpots after OVA challenge in vitro; n = 4, *p ≤ 0.05, **p ≤ 0.01.

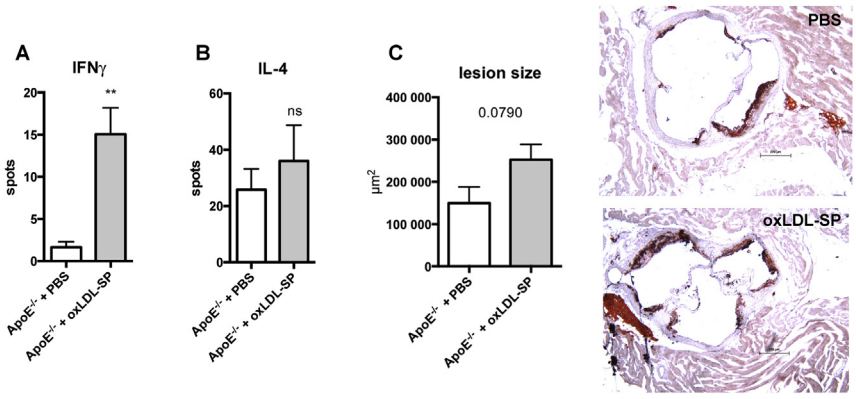

Fig. 2. ApoE$^{-/-}$ mice received either PBS or 50 × 10^6 oxLDL-coupled splenocytes (oxLDL-SP) every 5 weeks during 20 weeks. 5 weeks after the last injection, all mice were sacrificed. ELISpots for A) IFNγ and B) IL-4 after in vitro challenge with 25 µg/mL oxLDL. C) Atherosclerotic plaque in the aortic root; n = 7–8, $^{**}p \leq 0.01$.

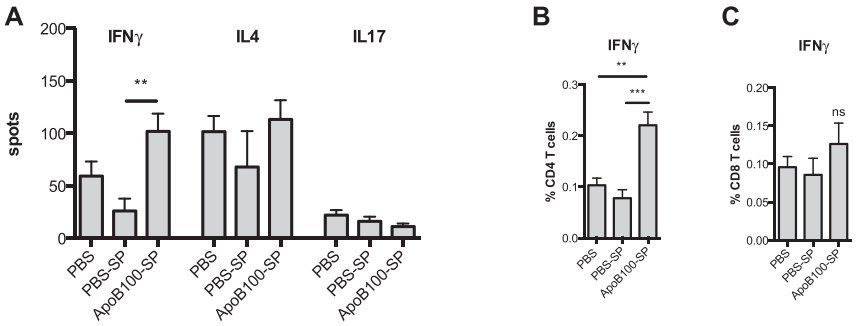

Fig. 3. Wild type mice (C57bl6J) were injected either PBS, ECDI-fixed splenocytes (PBS-SP), or splenocytes coupled with ApoB100 peptides 3 and 6 (ApoB100-SP). After 10 days, all mice received P3&6 (CFA/ApoB100; each 50 µg per mouse) dissolved in PBS and complete Freund's adjuvant. Another 10 days later, all mice were sacrificed. A) Cytokine ELISPots of splenocytes after in vitro challenge with P3&6 (each 25 µg/mL). Intracellular cytokine staining for IFNγ in B) CD4 positive and C) CD8 positive spleen cells after in vitro challenge with P3&6; n = 5, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

adapted a cell-transfusion based approach that has been successfully investigated in other mouse models for Th1-driven diseases like experimental encephalomyelitis [16] or type 1 diabetes [19], but also Th2 orchestrated allergic disease [20]. Moreover, this experimental treatment was recently investigated for multiple sclerosis in humans [21].

The alteration of immunoresponses through the injection of antigen-coupled splenocytes led to puzzling results in our study. In OT II mice whose immune system is highly preconditioned because the majority of TCR is specific against one single antigen, OVA-SP seemed capable of dampening proliferation and by trend reduced IFNγ. CD4 T cells expressed more CD69, which reflects general

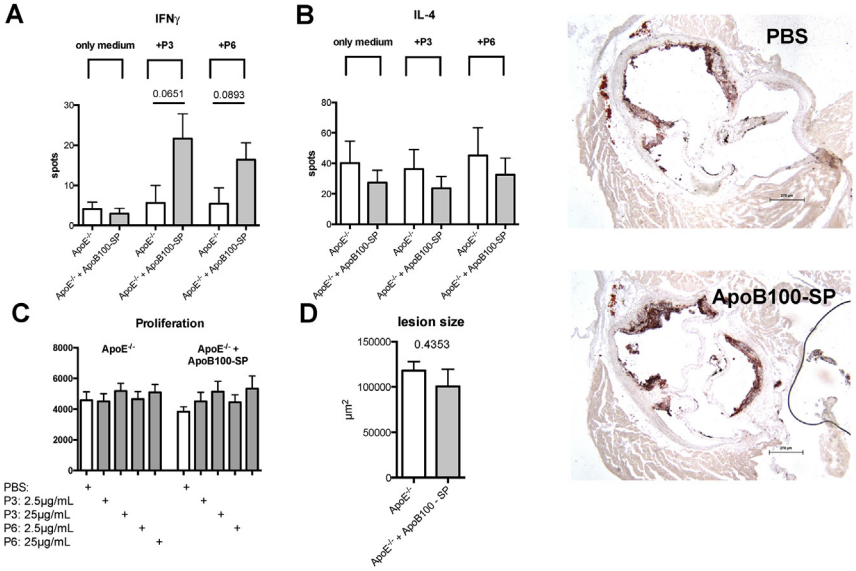

Fig. 4. ApoE$^{-/-}$ mice received either PBS or 50 × 10^6 P3&6-coupled splenocytes (ApoB100-SP) every 5 weeks during 20 weeks. 5 weeks after the last injection, all mice were sacrificed. ELISpots for A) IFNγ and B) IL-4 after in vitro challenge with P3 or P6 (each 25 µg/mL). C) Proliferation upon in vitro challenge with P3 or P6 (each 2,5 or 25 µg/mL). D) Atherosclerotic plaque in the aortic root; n = 7–8.

activation, but is also crucial for tolerance induction in Treg induction [22]. However, no Treg induction was observed in our experiments (data not shown). In vivo recall with OVA and complete Freund's adjuvant (CFA) after a single OVA-SP pretreatment overcame tolerance induction though, and skewed towards a Th1 response (as expected from CFA). This suggested already that a very strong inflammatory induction might easily out-balance immunosuppressive stimuli, especially if not a repetitive Ag-SP applications, but just a single one was performed.

To transfer the Ag-SP concept into ApoE−/− mice, we tested 2 groups of antigens that have been well explored in adjuvant-based vaccination against atherosclerosis. Both oxLDL [5,23–25] and ApoB100 petides P3&6 [26], did not inhibit or slow down atherosclerosis development. On the contrary, the chosen antigens and regimen even seemed to provoke more antigen-specific Th1 inflammation, though admittedly on a very low level.

Several possibilities for problems and pitfalls must be considered to explain those results. First, TCR diversity in atherosclerotic lesions is limited compared to the lymphnode TCR repertoire, which has been demonstrated by Paulsson and Hansson [4]. Only certain epitopes contribute to T cell activities in the plaques. These data were yielded from male ApoE−/− mice on a cholesterol−rich high fat diet without further interventions. In how far the level of hypercholesteremia further changes the composition of TCR in atherosclerotic lesions is not known. The same accounts for TCR variability and clonality in plaques of vaccinated mice, i.e. after a treatment with antigen and adjuvant: Whereas immunization with adjuvants might lead to the clonal amplification of one T cell type, it might as well result in the expansion of different T cell clones (which could be one explanation for unspecific adjuvant effects) with TCR towards multiple relevant antigens. A recent study by Oh and colleaques demonstrated the adjuvanicity of gut microbiota-derived flagellin on antigen processing and immunoresponses against influenza vaccine antigen that was administered to the skin [27], which underlines the systemic effects of local immunoadjuvants.

Antigen-specific tolerance induction should at best affect only one T cell clone, and therefore might not be efficient enough to modify atherosclerosis. Epitopes can be sub-divided into B cell and T cell epitopes whereas oxLDL is considered a B cell epitope which leads to the production of (protective) antibodies in adjuvant-based vaccination, T cell epitopes, such as ApoB100 peptides 3 and 6 change the T cell composition by e.g. induction of Treg or activation and proliferation of T effector cells [26,28]. Hence, the further identification of relevant TCR clones is necessary, and would facilitate the use of several antigens for Ag-SP simultaneously.

The experimental protocol was adapted from Niens and coworkers who treated their NOD mice every 5 weeks with $50*10^6$ Ag-SP to attenuate or prevent onset of type 1 diabetes [19]. In a different study addressing experimental autoimmune encephalomyelitis, Turley and Miller used the same amount of Ag-SP after a careful titration, but injected once at different time points, in which the overall time span for disease induction and observation was considerably shorter [16], which confirms that this amount of cells is needed and feasible to achieve a significant impact on immunoresponses, and seemed appropriate in our experiments. Less frequent administrations (i.e. >5 weeks) did not appear more promising in our study, but we cannot rule out that a higher frequency of injections e.g. at the start of treatment would have had a stronger impact, especially since the effect on proliferation appeared overcome in the ApoE−/− mice with repeated injections.

For our study, we chose to investigate ApoE−/− mice with standard chow at the age 6 months (whose treatment started at the age of 4–6 weeks). Most studies that investigated vaccination strategies against atherosclerosis, preferred a cholesterol-rich high fat diet (HFD). Immunoresponses between those regimen profoundly differ, because the T cell response seems less important when HFD is used [29] in ApoE−/−/RAG ± vs ApoE−/−/RAG−/− mice. Of course, overall deletion of RAG in ApoE−/− mice in that study affected all functional T/B lymphocytes including Tregs, whose induction seems to be highly interesting for the Ag/adjuvant effects in immunocompetent ApoE−/− mice with HFD. Further the induction of a Th2 response in ApoE−/− mice with HFD through Ag/adjuvant combination evidently overcomes this phenomenon. Last, we evaluated only one gender, i.e. male wildtype and ApoE−/− mice. A test of the experimental protocol in female ApoE−/− mice that - with and without HFD - show a stronger Th1 response and more atherosclerotic plaque burden, might be interesting for further studies.

With this study, we provide first insights into the effects of antigen-specific tolerance induction with antigen-coupled splenocytes in atherosclerosis. Despite the failure of our approach to effectively inhibit plaque development, we were able to modify the immunoresponses in an antigen-specific manner.

The concept of an adjuvant-free modification of immune responses in atherosclerosis is necessary to reduce general side effects, and trials to use in situ mimickry of cell−cell interactions to induce tolerance against specific antigens of interest deserves further investigation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.148.

Transparency document

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