Vaccination strategies in atherosclerosis

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Summary
The treatment of atherosclerosis is currently based on lipid lowering in combination with anti-inflammatory therapies that slow the progression of atherosclerosis. Still, we are not able to fully inhibit the formation or progression of atherosclerotic lesions. A very effective strategy in other disease pathologies is vaccination, in which the body is challenged with the culprit protein or micro-organism in order to create a highly specific humoral immune-response. Immunisation can typically be divided into active or passive immunisation. Active immunisation occurs naturally when the body is exposed to certain microbes or antigens, but also artificially in the case of vaccination. Exposure to a microbe or antigen will result in the production of (antigen specific) antibodies. Passive immunisation is defined as the transfer of humoral immunity (as a result of antibody transfer). Another mechanism to ensure immune-protection is tolerance induction. Immune tolerance occurs naturally to prevent immune responses to ‘self-antigens’, but can also be induced to non-self antigens. Acquired tolerance to foreign antigens is accompanied by suppression of cellular and/or humoral immune response to the introduced antigen. In its most effective way, vaccination can result in a lifelong protection against the targeted pathology, and therefore the development of an atherosclerosis-specific vaccination is of high importance in the future prevention of atherosclerosis. One of the difficulties in developing effective vaccination strategies for atherosclerosis is the selection of a specific antigen to target. So far vaccination strategies have been based on targeting of lipid-antigens, inflammation-derived antigens, and recently cell-based vaccination strategies have been employed; but also the cardiovascular ‘side-effects’ of infection-based vaccines are worthy of our attention. This review describes the current status-quo on classical antibody associated vaccination strategies but also includes promising immunomodulation approaches that may lead to a clinical application.

Keywords
Atherosclerosis, vaccination, immunology

Effect of influenza and pneumococcal vaccines on atherosclerosis
Over the past decades several studies have suggested that respiratory infections can trigger acute cardiovascular syndromes (1–3). It has been established that the incidence of myocardial infarction peaks at winter time (4) and particularly in years associated with epidemic influenza A infections (5). Indeed, infection with influenza A virus has been shown to increase the progression of atherosclerosis in humans, and the virus can infect and reside in arteries of atherosclerotic mice (6). A first influenza vaccine pilot study was started in 2001 by the FLUVACS study group, in which myocardial infarction patients were treated with a single injection of influenza vaccine. Cardiovascular death occurred four times less frequently in vaccinated patients (7), and this protective effect of the vaccination was maintained over a two-year follow-up period (8, 9). Similar results were obtained in the Probe study, where acute coronary syndrome (ACS) patients received inactivated influenza vaccine, which resulted in a 10% reduced occurrence of combined major cardiovascular events during follow-up (10). Moreover, it has also been shown that influenza vaccination is related with reduced risk of venous thromboembolism, a frequent cause of morbidity and mortality potentially associated to acute cardiovascular syndromes (11).

Immunisation of mice with heat-killed R36a, Streptococcus pneumoniae induced high titres of oxidised low-density lipoprotein (ox-LDL)-specific IgM, which subsequently inhibited atherosclerotic lesion formation as a result of decreased ox-LDL uptake by macrophages (12). This study nicely depicts the molecular mimicry between phosphorylcholine epitopes on S. pneumoniae and ox-LDL epitopes. Translation of these data into the human situation has not led to a clear conclusion on the cardiovascular effects of pneumococci vaccination, as some have shown a correlation between vaccination and cardiovascular outcome (13), while others do not (14, 15). The discrepancies in these studies may be explained by the use of different adjuvants, the applied vaccination regime or the fact that phosphorylcholine epitopes are not always included in human vaccine strategies as they do not always elicit strong immune responses in humans.

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The beneficial effect of influenza vaccination on cardiovascular outcome may make it worthwhile to educate cardiovascular patients more on the benefits of yearly flu-vaccinations.

**Lipid-based vaccination strategies**

**Cholesteryl ester transfer protein**

As lipids are one of the major players in atherosclerosis, several vaccination strategies have targeted different proteins central in lipid metabolism. Over the last decade interest has turned to modulation of cholesteryl ester transfer protein (CETP) activity, thereby raising HDL levels. As pharmacological inhibitors of CETP have not been very successful so far (16), due to detrimental non-specific side effects, the development of a specific CETP vaccine is worthwhile exploring. CETP vaccination was conducted in rabbits, which were repeatedly injected with a peptide-based vaccine, containing a B cell-derived CETP epitope in combination with a T helper cell epitope (to overcome B cell non-responsiveness towards self antigens) as a chaperone molecule. In the first study by Rittershaus et al. rabbits were vaccinated by repeated intramuscular (i.m.) injections of the TT/CETP vaccine (synthetic chimeric peptide containing an N-terminal cysteine, a T-cell epitope of tetanus toxin, and a B-cell epitope of human CETP) in Complete Freund’s Adjuvant (CFA). The authors used an irrelevant hCG vaccine as negative control. This approach resulted in inhibited atherosclerotic lesion formation, associated with increased CETP antibodies, decreased CETP activity and modified lipoprotein profiles (17). A similar approach was conducted five years later, where rabbits were now vaccinated by repeated subcutaneous (s.c.) injections using rHSP65-CETPC (chimeric peptide containing a helper T-cell epitope of HSP65 and a B-Cell Epitope of human CETP) in Alum adjuvant. rHSP65– and ovalbumin-vaccinated animals served as controls. This vaccination strategy increased CETP antibodies, but also HSP65 antibodies. Additionally, a more athero-protective lipoprotein profile and attenuated atherosclerotic lesion formation was observed in rHSP65-CETPC only (18). It remains interesting to see if the HSP65 chaperone protein is fully functional in the vaccinated animals, as it may have been impaired by the produced antibodies.

Intramuscular DNA vaccination targeting CETP has been shown to be similarly effective as peptide-based vaccination (19). In an effort to find a less invasive vaccination strategy the authors employed a nasal immunisation protocol in a separate set of experiments. Nanoparticles were prepared from a DNA plasmid pCETP (containing the C-terminal region of human CETP) inserted into the Hepatitis B virus core (HBc) gene, containing eight CpG motifs as immunostimulatory sequence) and chitosan. Mucosal immunisation induced CETP antibodies, modified the lipoprotein profile and attenuated atherosclerosis in a similar extent as i.m. immunisation (20). Though the authors had already established that the observed effects were specific for the pCR-X8-HBc-CETP plasmid in the i.m. immunisation set-up (19), a pCR-X8-HBc immunised group should have been included in the nasal immunisation protocol as well.

Recent progress was made by employing human intestinal trefoil factor (TFF3) as a molecular vehicle for the CETP B cell epitope, which allows oral administration of the chimeric peptide (21). Repeated oral administration of the chimeric peptide containing a CETP B cell epitope flanked by two tetanus toxin helper T cell epitopes connected to TFF3, which was subsequently fused to gluthathione S-transferase as a vehicle) again ameliorated atherosclerotic lesion development via induction of CETP-specific IgG/IgA antibodies. An overview of the different approaches is provided in Table 1. Though these observations point to CETP-specific effects of the used vaccine construct, non-specific side effects of the vaccine construct cannot be excluded, as there was a lack of controls in the study.

**Vaccination against oxidised LDL**

Modification of LDL, as a result of oxidation, transforms the lipoprotein into a highly immunogenic form (22, 23). Antibodies directed against ox-LDL are prevalent in humans and are able to bind to ox-LDL in atherosclerotic lesions (24, 25). To establish whether antibodies against modified LDL can modulate atherosclerosis, rabbits were immunised with malondialdehyde-modified LDL (MDA-LDL) or ox-LDL (an overview of the different approaches is provided in Table 2). LDL receptor(LDLr)-deficient rabbits were immunised s.c. with MDA-LDL or the irrelevant antigen keyhole limpet hemocyanin (KLH) in the presence of CFA. Immunisation resulted in increased MDA-LDL auto-antibody IgA and IgG titres in the MDA-LDL immunised animals, while no effect on IgM

**Table 1: CETP-based vaccination strategies.**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Source</th>
<th>Helper epitopes</th>
<th>Adjuvant/ Vehicle</th>
<th>Immunisation</th>
<th>Species</th>
<th>Antibodies</th>
<th>Atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT/CETP (17)</td>
<td>peptide</td>
<td>Tetanus toxin</td>
<td>CFA</td>
<td>i.m.</td>
<td>Rabbit</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>rHSP65-CETP (18)</td>
<td>peptide</td>
<td>HSP65</td>
<td>Alum</td>
<td>s.c.</td>
<td>Rabbit</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>pCR-X8-HBc-CETP (20)</td>
<td>DNA</td>
<td>CPG motifs</td>
<td>Chitosan</td>
<td>mucosal</td>
<td>Rabbit</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>GST-TFF3 (21)</td>
<td>peptide</td>
<td>Tetanus toxin</td>
<td>GST</td>
<td>oral</td>
<td>Rabbit</td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
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# increased CETP and HSP antibodies. CFA: Complete Freund’s Adjuvant; GST: Glutathione S-transferase. i.m.: intra-muscular; s.c.: subcutaneous.
titres was observed. More importantly, MDA-LDL immunisation inhibited atherosclerotic lesion formation in both the thoracic and abdominal aorta (26). Immunisation s.c. with native LDL or ox-LDL in the presence of AdjuPrime resulted in a two-fold increase of ox-LDL antibody titres compared to non-immunised hypercholesterolaemic rabbits. Immunisation with native LDL significantly inhibited atherosclerotic lesion formation in the proximal, mid and distal part of the aorta, while ox-LDL immunisation had a less pronounced effect (27). To further explore the efficacy of LDL vaccination, the effect of continued MDA-LDL immunisation in a murine model of atherosclerosis was determined. Animals were repeatedly immunised with native LDL, MDA-LDL (in Complete/Incomplete Freund’s Adjuvant; CFA/IFA) or PBS for seven weeks. Prolonged immunisation with both native and MDA-LDL slightly, but not significantly, reduced circulating cholesterol levels. Atherosclerotic lesion formation in the aortic sinus was similarly attenuated by both vaccination strategies; however, this reduction was associated with enhanced antibody titres in the MDA-LDL immunised mice only (28). This observation led the authors to conclude that the anti-atherogenic effect of immunisation is not primarily dependent on antibody titres but may also be a result of enhanced cellular immunity. Involvement of cellular and humoral immune responses in this was further explored by immunising apolipoprotein E (ApoE)-deficient mice with MDA-LDL or atherosclerotic plaque homogenates (in CFA). Both immunisations resulted in decreased lesion formation in the aortic root when compared to phosphate-buffered saline (PBS)-treated animals. The titre of T cell-dependent IgG antibody against MDA-LDL was considerably increased in both groups, while no effects on IgM titres were observed. Additionally, IgG antibody titres correlated with decreased lesion formation and lowered cholesterol levels (29). Despite the vast amount of experiments, showing protective effects of ox-LDL vaccination on atherosclerosis, the underlying mechanisms remain elusive.

**ApoB100 p210 peptide vaccination**

As these initial data clearly demonstrated that immunisation with ox-LDL may provide a novel therapeutic entrance, experiments were set out to further characterise the antigenic structures of oxidised LDL. This research focused on ApoB100, the primary apolipoprotein of LDL. Screening of a peptide library resulted in binding of (mainly IgM) auto-antibodies to more than 100 different ApoB100 peptides, allowing further characterisation of the peptide library for the identification of possible epitopes with therapeutic implications (30). Immunisation with ApoB100 peptide sequences p143 and p210 combined (with Alum) inhibited atherosclerotic lesion in the descending aorta of cholesterol-fed ApoE-deficient mice. This effect was not observed in the subvalvular plaques, but these plaques did show a more stable phenotype as they contained significantly more collagen (31). Immunisation with ApoB100 p210 peptide (with Alum) in human ApoB100 transgenic LDLr−/− mice on chow diet did not affect lesion formation in the descending aorta (32) and was not associated with increased antibody production against the ApoB100 p210 peptide. The authors suggested that a mechanism involving the induction of regulatory T cells and the used adjuvant Alum could be the alternative route via which these vaccines function. Indeed, it was later demonstrated that immunisation with the ApoB100 p210 peptide (in the presence of cBSA and Alum) induces CD25 expressing regulatory T cells, accompanied by interleukin(IL)-10 production, and depletion of Treg cells, completely abolishing the athero-protective effect of the ApoB100 p210 immunisation (33). However, it must be noted that the authors used PBS as a control treatment, and it cannot be excluded that the induction of regulatory T cells was specifically due to the ApoB100 p210 immunisation or an effect of Alum adjuvant.

**2D03-IgG antibodies**

Recombinant human antibodies against MDA-ApoB100, 2D03-IgG, were very effective in promoting atherosclerotic lesion regression in ApoB100-expressing LDLr−/− mice, which has been suggested to be a result of reduced macrophage infiltration and enhanced cholesterol efflux (34). The latest development in this field focuses on the therapeutic potential of the 2D03 antibody. A panel of clinically symptomatic and silent human carotid artery plaques was screened for the presence of 2D03 epitopes. The 2D03 epitope is expressed in atherosclerotic lesions, but is more prominently present in clinically symptomatic specimens. The presence of 2D03 epitope was associated with the presence of cholesterol esters and not with circulating ox-LDL and ApoB100 auto-antibodies (35). These latter findings support the possibility of clinically applied 2D03 antibody therapy and most certainly warrants further development.
Inflammation-based vaccination strategies

Apart from the lipid-associated aspect of atherosclerosis, atherosclerosis is also recognised as a chronic inflammatory disease, and several vaccination strategies are based on that feature.

Heat shock proteins

Autoimmunity to heat shock proteins (HSP) is one element in atherosclerosis-induced immune responses. HSP are a class of functionally related, highly conserved, proteins that function as sentinels in the so-called cellular stress response (36). HSP function as intra-cellular chaperones for other proteins, e.g. HSP90 is indirectly involved in vascular relaxation as it binds endothelial nitric oxide synthase (37), while HSP70 is involved in antigen binding and presentation (38). HSP are regulated in endothelial cells and macrophages upon shear stress, exposure to ox-LDL and hypoxia, all atherosclerosis-related stress responses (39–41). Initial findings show that active s.c. immunisation with heat-killed Mycobacterium tuberculosis and HSP65 (in the presence of IFA) resulted in larger fatty streaks in the aortic sinus of LDLr-deficient mice (42). Repeated mucosal administration of Mycobacterium HSP60/65, both orally and nasally, inhibited atherosclerotic lesion formation in LDLr-deficient mice when compared to OVA-treated controls. The observed attenuation of atherosclerosis was associated with decreased macrophage and T cell numbers, and enhanced IL-10 intimal expression, but this was observed in the in-transasally treated animals only (43). Oral tolerance induction to Mycobacterium HSP60 derivates attenuates atherosclerotic lesion formation by 80% in a model of flow-induced carotid artery atherosclerosis. The protective effect of oral HSP60 administration is due to enhanced regulatory T cell expansion and function, rather than the induction of HSP-specific antibodies (44).

Cytokines/growth factors

The adaptive immune response in atherosclerosis is mainly recognised as T helper 1 (Th1)-related. IL-12 is the main cytokine to drive polarisation of naïve CD4 T cells into Th1 cells, and therefore was identified as a potential target for therapeutic intervention. Pan-DR epitopes (PADRE) are highly immunogenic peptides binding human HLA and mouse MHC molecules, thereby providing efficient and powerful T cell activation (45). The p35 subunit of IL-12 was coupled to PADRE and subsequently injected [in the presence of monophosphoryl lipid A and an immunostimulatory saponin (of Quillaja saponaria Molina) adjuvant] five times into the gastrocnemius muscle of atherosclerosis prone LDLr-deficient mice. Control animals received sham-PADRE complexes in the presence of adjuvant. Immunisation resulted in high levels of IL-12 auto-antibodies and decreased serum interferon γ levels (46). This vaccination strategy resulted in a significant inhibition of carotid artery lesion formation (after perivascular collar placement), while the lesions also showed marked increase of different plaque stability markers (46).

Other studies used DNA vaccination techniques to target atherosclerosis. In these techniques the target of interest is cloned into the eukaryotic expression plasmid pcDNA3.1 and subsequently incorporated into Salmonella typhimurium by means of electroporation. IL-15 is a pro-inflammatory cytokine that promotes T cell proliferation in non antigen-specific way, has chemo-attractive properties and is a regulator of cytokine production (47–49). High cholesterol feeding stimulates IL-15 production in the spleen of LDLr deficient mice, where macrophages appear the main source of IL-15. Vaccination against IL-15, by oral delivery of 10^8 colony forming units of S. typhimurium transformed with pcDNA3.1-IL-15 (or empty pcDNA3.1 as a control) inhibited atherosclerotic lesion formation after perivascular collar placement in the carotid arteries (50).

Endothelial cells

DNA vaccination, similar to the method described above, targeted to vascular endothelial growth factor receptor-2 (VEGFR-2) induced specific lysis of VEGFR-2 expressing cells, anti-angiogenic effects and inhibited both the initiation and progression of carotid artery atherosclerotic lesions. However, the vaccination also had detrimental side effect as it inhibited arteriogenesis after ischaemia and promoted restenosis (51). A different independent study showed similar effects on lesion formation in the aortic root, accompanied by attenuated neovessel formation (52). DNA vaccination against other endothelial cell markers, CD99 and TIE2, inhibited atherosclerotic lesion formation both in the carotid arteries and the aortic root, which was mainly due to cell-specific lysis (53, 54). TIE2 vaccination induced noticeable plaque stabilisation and, in agreement with the VEGFR2 study, reduced capillary formation.

Metabolic factors

Metabolic syndrome is defined as a combination of medical disorders, including high blood pressure, high glucose levels, high cholesterol levels and abdominal fat accumulation, that increase the risk of developing atherosclerosis or diabetes (55). Nowadays, approximately 50% of all cardiovascular patients meet the criteria for metabolic syndrome. In view of this, interest has grown in the occurrence of metabolic abnormalities and their relation to atherosclerosis. In addition to unravelling the interactions between the different players in metabolic syndrome, effort is being put into targeting both metabolic disorders and atherosclerosis.

Ghrelin is a peptide hormone involved in feeding behaviour, energy metabolism, and glucose/insulin metabolism (56–58). It has been suggested that Ghrelin plays a regulatory role in the car-
A very small clinical study utilised oral immunisation with pooled adipose antigens in obese subjects. The pooled antigens were obtained from pig adipose tissue and were administered daily per os for three months, and the effect on plasma cholesterol and bodyweight were assessed. Whether this prolonged immunisation protocol resulted in antibody formation to the antigens is not demonstrated in the paper. The authors do claim an effect on plasma high-density lipoprotein (HDL) levels, which increased by 25%, while total cholesterol levels remained similar when compared to baseline levels. The effect on body mass index (BMI) was insignificant, though waist diameter slightly decreased.

Though the above studies are very preliminary with several aspects to be improved, an initial step has been made towards the discovery of obesity- and atherosclerosis-specific antigens. The chosen targets were selected from a ‘metabolic’ perspective, but can also be employed the other way around. Indeed it has been shown that auto-antibodies against ApoB100 peptides are present in patients with type 2 diabetes (without clinically evident coronary heart disease) (64). In fact, patients with diabetic retinopathy had higher levels of IgG against MDA modified p45 and p210 ApoB100 peptides, but also of native p45 and p210 ApoB100 peptides. Auto-antibodies against native ApoB peptides were associated to coronary calcifications, in which increased antibodies to native p210 ApoB100 peptide related to the severity of coronary calcification.

These findings suggest that the human 2D03 antibodies may serve as a therapeutic intervention for vascular complications in type 2 diabetes patients.

**Dendritic cell-based vaccination strategies**

A new development in the vaccination field is the use of dendritic cells (DC), due to their capacity to modulate T cell responses (65, 66). DCs pulsed with the antigen of interest can be re-introduced in vivo and elicit a highly specific humoral immune response. Ox-LDL pulsed dendritic cells were used as an immunotherapy for atherosclerosis. Vaccination i.v. with ox-LDL pulsed DCs almost completely attenuated atherosclerotic lesion formation in a model for flow induced atherosclerosis in the carotid arteries. Additionally, DC therapy redirected the plaques to a more stable phenotype. Lesion formation in the aortic root was not affected. The repeated exposure to ox-LDL pulsed DCs nicely induced an antibody response to ox-LDL (67). A similar approach was conducted using ApoB100 peptide pulsed DCs in human ApoB100 transgenic mice. Different from the approach by Habets et al., differentiated DCs were treated with the immunosuppressive cytokine IL-10 to induce tolerogenic DCs. Additionally, the mice received cytokine treatment, at the time of DC infusion, to retain the tolerogenic properties of the DCs. Tolerogenic DCs have a decreased production of pro-inflammatory cytokines, promote the generation of regulatory T cells and induce suppression of antigen-specific T cells (68–71). The authors elegantly show by in vitro experiments that treatment with IL-10 induces a tolerogenic phenotype in ApoB100 pulsed DCs, resulting in decreased Th1 and Th2 responses most likely due to enhanced regulatory T cell expansion. Furthermore, they show that only the combined infusion of ApoB100 pulsed DCs and IL-10 resulted in attenuation of atherosclerotic lesion development in the aorta which was associated with decreased cellular immunity to ApoB100 (72).

**Figure 1:** Schematic overview of different vaccination strategies. Vaccination strategies are targeted at lipid-antigens and inflammation-derived antigens. Recently dendritic cell-based vaccination strategies have also been employed. The figure represents the three main location sites for targeting: the circulation (lipid antigens), the atherosclerotic lesion (lipid and inflammatory antigens) and lymphoid organs (inflammatory antigens).
Another DC-based strategy was used to deplete a specific subset of immune cells, the regulatory T cell. DCs were cultured from bone marrow and were electroporated with a Foxp3-encoding plasmid. Presentation of plasmid-derived peptides resulted in partial lysis of Foxp3-expressing regulatory T cells in the circulation, spleen and lymph nodes. As expected, depletion of regulatory T cells resulted in increased atherosclerotic lesion formation (73). It would be very interesting to see if this strategy could also be used for the depletion of more detrimental T cells, like the Th1 or Th17 cell, thereby strengthening the technique for use in therapeutic intervention.

Word of caution: Effects of used adjuvants?

Although most of the described vaccination therapies seem very promising, a word of caution should be applied regarding the use of adjuvant and its effect on the outcome. For instance, several adjuvants have athero-protective properties. Treatment of hyperlipidaemic mice with complete Freund’s Adjuvant/Incomplete Freund’s Adjuvant, or Alum without any antigen significantly reduced atherosclerotic lesion formation (74). The athero-protective effect of Freund’s Adjuvant was attributable to increased Th2 responses, MDA-LDL antibody titres and athero-protective lipoprotein profiles (74). The athero-protective effects of Alum were confirmed in a different study, where it was additionally shown that this protective effect was associated with increased regulatory T cell numbers as a result of tolerance induction to ox-LDL (75). These observations clearly show that the use of proper controls in vaccination studies is crucial to prevent any overestimation of the vaccine functionality and specificity.

Concluding remarks

Though the different vaccination strategies and targets (depicted in ▶ Fig. 1) look very promising, it remains very important to establish a highly atherosclerosis-specific antigen. Targeting of inflammatory molecules may be very useful in the clinic as has been shown for other chronic inflammatory diseases like rheumatoid arthritis. Rheumatoid arthritis is relatively confined to a certain area (the inflamed joint), but atherosclerosis is spread out through the body. This more systemic nature of the disease may be problematic when targeting components of the immune system, since we do not want to imbalance the immune system too much and leave our patients immune compromised after they have been cured for atherosclerosis.

Furthermore, additional studies on the effect of the different vaccine strategies on progression of atherosclerosis are needed in order to implement the therapy in patients. Currently, most studies have focused on atherosclerotic lesion development, while lesion progression or even regression are, at this time, more clinically relevant as patients are usually only seen by the cardiologist when they have clinically manifest atherosclerosis.

Clearly, vaccination approaches can have beneficial effects on the progression of atherosclerosis. Further research should provide more insight on the protective mechanisms of vaccination and the possible therapeutic implementation of an atherosclerosis vaccine.

Conflict of interest
None declared.

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