Part A – Applicant

A.1 Applicant

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Part B – Scientific proposal

B.1 BASIC DETAILS

B.1.1 Title

Extracellular vesicles: the application to tolerogenic vaccination for Rheumatoid Arthritis

B.1.2 Abstract

Rheumatoid Arthritis (RA) is a chronic autoimmune disease which results in joint inflammation. Joint inflammations are characterized by a pro-inflammatory environment in which immune cells attack the synovium, cartilage and bone. Current treatments are lifelong immunosuppressive drugs focused on treating the symptoms and not the disease, therefore new treatments are required. An upcoming treatment for autoimmune diseases is tolerogenic vaccination, in which antigen-specific control over humoral and cellular responses against self-proteins is induced. However, to date there is no approved tolerogenic vaccination on the market. For tolerogenic vaccination, the use of extracellular vesicles (EV) could be promising as it has several benefits compared to current tolerogenic vaccination strategies. EVs function as intercellular messengers which contain biological information and can cross barriers, therefore EVs could be a promising vaccine carrier. Research in which tolerogenic EVs from primary cells are tested as tolerogenic treatment showed promising results, however yield, culture conditions and manufacturability are a major concern. In this research proposal we aim to create an EV candidate vaccine for tolerogenic vaccination to RA. To this end, we aim to design an immortalized EV-producing platform in which EVs can be loaded with self-antigens, tolerogenic mRNA/miRNAs and dendritic cell (DC) targeting antibodies. The self-antigens determine antigen specificity of the tolerogenic response, the tolerogenic mRNA/miRNAs will induce tolerogenicity and DC targeting of the vaccine will enhance effectiveness. If successful, the EV candidate vaccine could eventually be applied in the clinic to treat RA. Furthermore, the EV platform could be adapted for treating other diseases.

B.1.3 Layman's summary

Rheumatoid Arthritis (RA) is a severe autoimmune disease, affecting 1% of the world population. Autoimmune diseases are disorders in which the immune system attacks self proteins, causing severe inflammation and symptoms. In the case of RA, the immune system attacks proteins in the joints which can lead to joint pain and eventually inability to walk.

The immune reaction towards proteins is mainly organized by dendritic cells (DC), which are immune cells specialized in either the activation or suppression of the immune system. DCs present foreign proteins from e.g., viruses to T cells. T cells protect the body against infection by executing the immune response to these foreign proteins. However, in the case of autoimmune diseases, DCs accidently present self-proteins to T cells, which subsequently execute an immune response to self-proteins.

Current treatments for RA are focused on treating the symptoms instead of the cause of the disease and require lifelong medication. Therefore, new treatments are required. A new upcoming treatment is tolerogenic vaccines. Tolerogenic

vaccines teach the immune system which proteins are 'self', and therefore should not be attacked by T cells. Generally, the vaccine contains self-proteins and specific tolerogenic signals. The vaccine is targeted to DCs where the tolerogenic signals tell the DC that this self-protein should not be attacked. The DC then teaches the T cells that they are not allowed to attack these self-proteins.

To make these tolerogenic vaccines, several different techniques have been tested in a broad range of research efforts with some promising results. There is however no approved tolerogenic vaccine on the market yet. Therefore, it is important to keep exploring our possibilities. In this research proposal we are going to explore a relatively infrequently used technique to design a tolerogenic vaccine for RA. This technique has several advantages compared to the currently used techniques. It makes use of extracellular vesicles (EV), which are small, encapsulated particles secreted by cells and contain biological information. EVs function as messengers between cells. Therefore, EVs might be used as nature's own means of transport to transport a vaccine to DCs.

We aim to design a candidate vaccine for RA by using the EV technique. We aim to load the EVs with an RA-specific protein, tolerogenic signals and DC-targeting proteins. The RA-specific proteins and the tolerogenic signals should tell the DC that the RA-specific protein should not be attacked. The DC-targeting proteins are meant to increase the effectiveness of the vaccine by recruiting the vaccine towards DCs. The DCs should then tell the T cells that they are not allowed to attack the RA-specific protein in the joints. In the end, if our designed EV vaccine appears to be effective, it may subsequently be used to treat RA in the clinic.

B.1.4 Keywords

Rheumatoid Arthritis
Tolerogenicity
Vaccination
Dendritic Cells
Extracellular vesicles

B.2 SCIENTIFIC PROPOSAL

B.2.1 Research topic

RA is a chronic autoimmune disease which affects approximately 1% of the world population. RA is characterized by an autoimmune reaction towards self-antigens causing chronic inflammation in the joints, which leads to cartilage and bone damage. Symptoms range from morning stiffness, pain, inability to walk and eventually life threatening diseases when left untreated[1]. RA is currently treated with symptom-targeting therapies, there are no curing treatments on the market[1].

Current treatments for RA consist of nonsteroidal anti-inflammatory drugs, corticosteroids, disease modifying anti-rheumatic drugs and newer anti-inflammatory treatments. These treatments aim to relieve pain and decrease inflammation and disease progression[1]. However, these treatments often have side effects and are targeted against symptoms, but not the cause of disease. These treatments are non-specific and require lifelong therapy. Moreover, they can be toxic or cause secondary problems like cancer or infections[2]. Therefore, new treatments, which target the cause of RA, are required. To this end, tolerogenic therapies have emerged as a promising new strategy. Tolerogenic therapies aim to induce antigen-specific tolerance to control both humoral and cellular responses against self proteins[3]. This immune reprogramming towards self tolerance should be permanent and lead to remission of immunopathologies.

Tolerogenic therapies are often targeted towards DCs due to the DCs ability to differentiate the immune system to a anti-inflammatory phenotype[2]. These tolerogenic therapies present self-antigens to DCs and differentiate the DCs to a tolerogenic phenotype. The induced tolerogenic DCs (tDC) subsequently present the self-antigen to naïve T cells and induce T cell anergy or regulatory T cells[4]. The regulatory T cells are able to suppress autoimmune T effector cells by secreting anti-inflammatory cytokines[5]. In this way, the tDCs and Tregs combined can dampen inflammatory responses. Therefore, DCs are an attractive target for new experimental tolerogenic therapies[4,6]. This strategy is deployed by tolerogenic autologous cell transfer therapies and tolerogenic vaccines[3].

Autologous cell transfer is a tolerogenic inducing treatment in which patient derived immune cells are differentiated towards tDCs in vitro, and subsequently are given back to the patient. The tDCs subsequently induce a tolerogenic response towards the self-antigens[2,3]. However, autologous cell transfer is expensive, time consuming and invasive[3]. A more suitable treatment could be tolerogenic vaccines. Current tolerogenic vaccination designs can be split up in peptide-based vaccines, nanoparticle based vaccines, DNA- and mRNA-based vaccines[7]. Tolerogenic vaccines aim to induce antigen-specific tolerance via several mechanisms, mainly by targeting DCs[7].

These vaccination strategies however come with some difficulties. Peptide-based vaccines are hampered by their single protein approach, as it is difficult to introduce both DC-targeting and tolerogenic properties in a single protein. Nanoparticle vaccines are ideal for combining these properties, however nanoparticles have a higher degree of toxicity compared to other vaccines due to the usage of chemicals during production[8]. DNA vaccines contain DNA which is a danger signal capable of activating the immune system, which could hamper tolerogenic induction[9]. DNA vaccines have for example occasionally sensitized the response to myelin antigens in a EAE mouse model[7]. Lastly, mRNA vaccines are a relatively new strategy in the tolerogenic vaccination field, therefore not much is known about these vaccines. However, the stability and efficacy are controversial[9].

Although several of the tolerogenic vaccines have showed promising results in in vivo studies and in clinical phase I/II studies, there is no approved tolerogenic vaccine on the market yet[7]. Multiple of the promising tolerogenic vaccines are halted in phase I/II clinical trials due to adverse events or a lack of efficiency[9]. Therefore, it is crucial to keep exploring the possibilities of tolerogenic vaccinations. A promising possibility could be the EV-based vaccines.

EVs are cell-derived messengers which are able to transport biological cargo like proteins, RNAs and bioactive lipids over biological barriers[10]. Therefore, EVs can be used as natures own means of transport and may be transformed to promising vaccine carriers. Furthermore, the EV-composition is adaptable with EV-engineering and therefore EVs are deployable in multiple contexts. Thus, EVs have emerged as a promising new vaccine platform with therapeutic potential[11]. We argue that EVs might overcome the mentioned difficulties associated with peptide based, nanoparticle, DNA and mRNA vaccines.

EVs can be loaded with both antigens and adjuvants[8,12] contrary to peptide-based vaccines. Furthermore, compared to soluble antigens, EV-mediated antigen presentation to DCs is more efficient[13]. EVs are also less toxic to healthy cells compared to nanoparticles[8]. EV isolation is environmentally friendly whereas nanoparticle isolation requires several chemicals and solvents.

EVs have enhanced stability compared to peptide-based and nanoparticle vaccine as they are easier to store and can withstand several storage conditions[8]. Furthermore, numerous studies have indicated that MSC-EVs are well tolerated and safe in rodents[14] and in humans[15,16]. EV vaccines also have shown promising results in research focussed on cancer treatments and infectious diseases[8,12]. Thus, EVs sit in between the ideal combination of biologics, drug delivery and cell therapies[10].

Tolerogenic EVs from primary cell sources are being investigated as potential tolerogenic therapy for autoimmune diseases, which has come with promising results[17]. MSC-derived EVs and autologous DC-derived EVs have been shown to contain intrinsic tolerogenic capacities[10]. However, the yield, culture conditions and manufacturability of primary cell derived EVs are a major concern[9]. Primary cells are also difficult to edit and therefore difficult to load with self-antigens and DC-targeting capacities. We believe these difficulties can be addressed by engineering an EV-producing immortalized cell line with tolerogenic characteristics and specific self-antigens. To our knowledge, there are no immortalized tolerogenic EV-producing platforms which have been inserted with self-antigens. Furthermore, the effectivity of tolerogenic vaccines can be enhanced with DC targeting[18]. To our knowledge tolerogenic EV targeting towards DCs has been done in only 1 study via overexpressing lactadherin[19]. Therefore, we believe advancements have to be made in tolerogenic EV targeting towards DCs. By addressing these tolerogenic EV related issues with EV engineering, significant steps can be taken towards a potentially effective tolerogenic EV vaccination.

In this research proposal we aim to create an EV-based tolerogenic candidate vaccine for RA which we will test in RA-model mice. To this end, we aim to design an immortalized EV-producing platform in which EVs are loaded with RA-specific self-antigens, tolerogenic mRNA/miRNAs and DC-targeting antibodies. We will make use of EV-targeting proteins to load the EVs with RA-specific self antigens, tolerogenic mRNA/miRNAs and DC-targeting antibodies. In the end, we aim to induce an RA-antigen-specific tolerogenic response in mice with this EV vaccine.

This project will be addressed in the following three work packages (Figure 1).

1. Design of an immortalized EV-producing platform in which EVs can be loaded with self-antigens, mRNAs/miRNAs and DC-targeting antibodies
2. Optimize capacity of EVs to induce tDCs and subsequently regulatory T cells
3. Investigate the preventive and therapeutic capacity of the EV vaccine in vivo

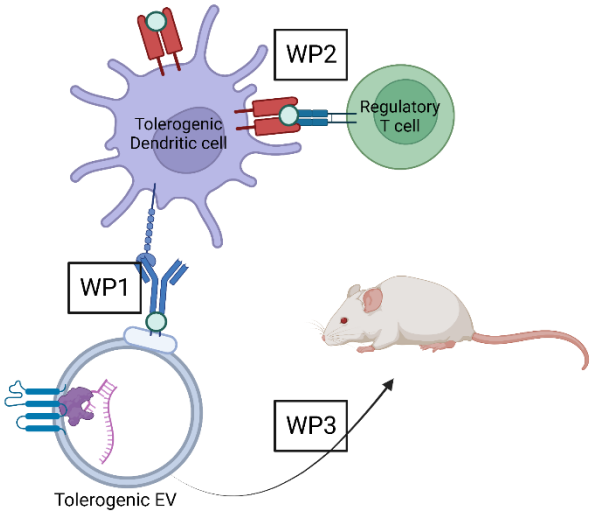

Figure 1: *Our research proposal will be split up in 3 WPs. WP1 addresses the EV platform, WP2 addresses the tolerogenic induction in both DCs and T cells, WP3 addresses the in vivo effectiveness of the EV vaccine. Created with Biorender.*

The combination of state of the art EV-engineering techniques in an immortalized cell line to create an effective tolerogenic EV candidate vaccine is what makes this research proposal unique.

B.2.2 Approach

WP 1: Design of an immortalized EV-producing platform in which EVs can be loaded with self-antigens, mRNAs/miRNAs and DC-targeting antibodies

Self-antigen and mRNA/miRNA recruitment and DC targeting of EVs is important for the EV functionality. The self-antigens determine the antigen specificity of the EV vaccine and the mRNA/miRNA determine the tolerogenic properties of the EV vaccine. EV targeting to DCs with antibodies has the purpose to increase tolerogenic vaccination effectiveness as DCs are the central organizing cells of the adaptive immune response due to their capacity to polarise effector T cells and in this way elicit the tolerogenic immune response. Furthermore, active recruitment of self-antigens, mRNA/miRNAs and DC-targeting antibodies increases the EV-localized cargo concentration, and thereby likely increases EV vaccine effectiveness. In this WP, we therefore aim to create an EV platform in which self-antigens, mRNA/miRNAs and DC-targeting antibodies can be recruited.

In this research project we will sort out the yield and culturing problem of primary cells by using the immortalized Expi293F cell line which has high EV yield, easy culturing conditions and high transfection/transduction efficiency. Furthermore, Expi293F EVs are immune inert and well-tolerated in mice[20]. Throughout the entire research project, cells will be cultured in EV-free medium, and EVs will be harvested by collecting supernatant. EVs will be isolated by ultracentrifugation, which results in concentrated EVs, however often together with a lot of debris. Therefore, we will further purify the EVs with iodixanol-based density gradient ultracentrifugation[21]. The purified EVs can subsequently be analysed with western blotting and RT-qPCR. Purified EVs will also be used for cellular essays which study the effect of EVs. For single particle analysis, EVs will be stained with BODIPY TR ceramide or PKH67 in advance of density gradient ultracentrifugation[22-24]. We will optimize the EV staining and choose the most effective lipid dye for Expi293F EVs. After EV staining and density gradient ultracentrifugation, EVs will be analysed in a highly modified flow cytometer for single particle detection, of which the modifications and protocols are described previously by the Wauben lab[24,25].

Specifically, we will investigate 1) how we can target self-antigens towards EVs, 2) how we can target mRNA/miRNAs to EVs, 3) how we can target the EVs towards DCs, 4) which fusion constructs are most effective for antigen presentation, and lastly, 5) we will optimize the endosomal delivery of the self-antigens and the cytosolic delivery of mRNA/miRNA.

1.1 Self-antigen loading in EVs. To produce functional EVs, vaccine cargo needs to be actively recruited to EVs. We will recruit RA-specific self-antigens and DC-targeting antibodies in EVs to increase their EV concentration and thereby increase EV functionality. To recruit the mentioned proteins in EVs, we will link the proteins to EV-targeting proteins which have a physiological EV localisation. The tetraspanin CD9 is an EV-targeting protein often used for cargo recruitment[26]. We will recruit proteins of interest to the EVs interior by linking them with a peptide linker to CD9. To recruit proteins to the EVs exterior, we will use the C1C2 domain of lactadherin. $C1C2_{lactadherin}$ localizes on the outside of EVs by binding phosphatidylserines which are enriched on EVs[27]. Antigen fusion constructs with $C1C2_{lactadherin}$ improved antigen presentation and in vivo immunogenicity[28]. By linking our EV cargo to CD9 and $C1C2_{lactadherin}$ in fusion constructs, we aim to elevate the vaccine cargo concentration in EVs.

We will use the RA-associated immunogenic proteoglycan (PG) aggrecan, specifically the immunodominant epitope PG_{70-84}, as antigen in the EV-vaccine[29]. We will link the N-terminus of $C1C2_{lactadherin}$ to mTurqoise and PG_{70-84}, via linkers. Furthermore, we will link the N-terminus of CD9 to the green-fluorescent eGFP/the red-fluorescent mScarlett protein and PG_{70-84}. The CD9-fluorescent tag will be dependent on the lipid dye used for EV staining: BODIPY TR ceramide will be combined with eGFP while PKH67 will be combined with mScarlett. These $C1C2_{lactadherin}$/CD9-fluorescent protein-PG_{70-84} fusion constructs (and other additional fusion constructs in this project) will be stably expressed in Expi293F cells with transduction. Fusion constructs will be expressed individually or together. We will characterize the fusion construct recruitment to EVs by western blot and by measuring the fluorescence signal in EVs with single particle detection. In this way we can establish the degree of EVs containing one or both constructs and thus targeting efficiency. These experiments will result in EVs in which proteins are efficiently recruited.

1.2 *Recruitment of mRNA/miRNA to EVs.* To create EVs with tolerogenic properties, we will recruit tolerogenic mRNAs or miRNAs in EVs. For mRNA/miRNAs recruitment towards EVs we will make use of the mRNA recruiting method developed by Zhang et al[30]. The authors designed a DNA aptamer consisting of 2 parts, the first part binds to the mRNA of interest around the AUG codon to inhibit translation, while the second part is a double strand that can be recognized by zinc finger domains.

Furthermore, Zhang and co-authors linked a zinc finger domain to the EV marker CD9. Coexpression of the CD9 fusion construct, IL-10 mRNA and the DNA aptamer in a cell resulted in IL-10 mRNA recruitment to EVs[30]. This method has been proven effective in vivo for IL-10 mRNA delivery in inflammatory bowel disease[30]. We will collaborate with the group of Zhang to get the CD9-ZF, IL-10 mRNA and the suitable DNA aptamer. We will apply this technique in our experimental setting by linking eGFP/mScarlett with a linker to the N-terminus of the zinc domain of their CD9-zinc fusion construct. Furthermore, we will also create this fusion construct with the addition of PG_{70-84} (Figure 2). The fusion constructs, IL-10 mRNA and DNA aptamer will be expressed in Expi293F cells. Fusion construct recruitment to EVs will be assessed with single particle detection and western blotting. IL-10 mRNA recruitment to EVs by the CD9-zinc constructs will be quantified by measuring mRNA expression in EVs with RT-qPCR. miRNA recruitment in EVs will also be measured with RT-qPCR during this proposal. Furthermore, EVs will be cocultured with mouse bone-marrow derived DCs (mBMDC). IL-10 expression levels in mBMDCs will be measured by western blot to address mRNA delivery. These experiments will result in EVs to which mRNA/miRNAs can effectivity be recruited.

1.3 *EV targeting to DCs with DC-targeting antibodies.* To increase EV effectiveness, EVs need to be taken up by DCs in vivo[18]. Therefore, we will target EVs to DCs with DC-targeting antibodies. Multiple studies have successfully targeted DCs with the anti-CD205 antibody which targets CD205 localized on DCs[18]. The CD205 antibody targeting has been applied in tolerogenic context and has been proven effective in mouse models for EAE, IBD and RA[9]. CD205 targeting has so far only been investigated with antigen-CD205antibody fusion peptides and not in EV context. We aim to load mouse and human CD205 antibodies on EVs and investigate whether tolerogenic EVs can be targeted to DC. CD205 is expressed on mouse and human DCs and has been proven to be an excellent target in mouse[31] and humans[18,32]. The eukaryotic expression vector encoding mouse IgG1 CD205 antibody (mCD205ab) is present in the Broere lab. We will obtain the eukaryotic expression vector encoding human CD205 antibody (hCD205ab), from the Ludwig institute in New York[33]. The mCD205ab and hCD205ab will be fused to $C1C2_{lactadherin}$. Fusion constructs of homing peptides or tissue-specific antibodies with $C1C2_{lactadherin}$ were shown to be effective for EV recruitment[34,35].

The C terminus of the mCD205ab/hCD205ab heavy chain will be linked to the N terminus of $C1C2_{lactadherin}$ with the blue-fluorescent peptide mTurqoise in between by using peptide linkers. We will also make a fusion construct which includes PG_{70-84} (Figure 2). These constructs will be expressed together with their respective Ig-k light chain in Expi293F cells. Recruitment of fusion constructs to EVs will be assessed with single particle detection and western blotting. EV recruitment towards DCs will be tested in both mBMDCs and human monocyte derived DCs (hMDC), both express CD205[36,37]. mBMDCs and hMDCs will be cocultured in different ratios with suspension cells lacking CD205. EV-mCD205ab and EV-hCD205ab uptake in mBMDCs and hMDCs respectively will be tested by measuring the mTurqoise signal of the fusion constructs in mBMDC/hMDC with flow cytometry. Targeting efficiency and specificity will also be measured ex-vivo by coculturing mouse splenocytes and human PBMCs with EVs containing respectively mCD205 or hCD205. Splenocytes/PBMCs will be stained for different cellular markers (identifying myeloid cells, B-cells and T cells) and mTurqoise expression will be analysed using flow cytometry. In this way the ex vivo EV uptake in myeloid cells can be measured. The mentioned experiments will include an extra control where the CD205 receptor is blocked to correct for the constitutive uptake of myeloid cells. These experiments will induce CD205 antibody mediated EV uptake in DCs.

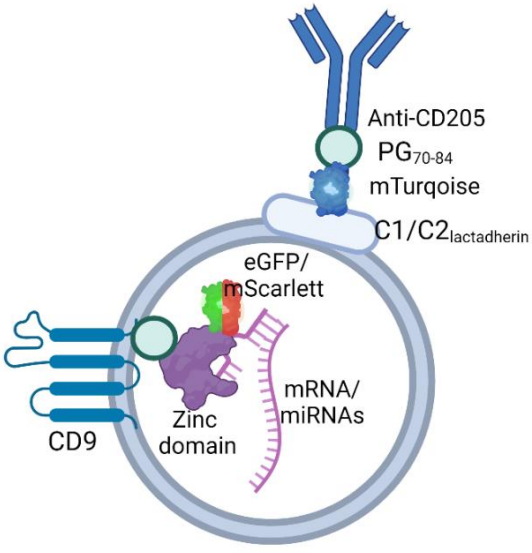

Figure 2: EV loaded with the fusion constructs: the C1C2-mTurqoise-PG-mCD205ab construct and the CD9-PG-zinc-eGFP/mScarlett construct. Together these constructs load the EV-vaccine with the required properties: PG-antigens, mRNA/miRNAs and DC-targeting antibodies. Created with Biorender.

1.4 *Antigen presentation capacity of fusion constructs.* For EV vaccine effectivity, the EVs have to induce PG_{70-84} antigen presentation in DCs. To find most effective EV-composition for this, EVs containing the created antigen-fusion constructs from wp1.1-wp1.3 will be tested for their antigen presentation induction in DCs. The EVs will be coincubated with mBMDCs and mBMDCs will subsequently be cocultured with proliferation dye-labelled PG-specific T cells. T cell proliferation will be mapped using flow cytometry. High T cell proliferation suggests effective antigen presentation by the DCs. The Broere lab has the PG-specific T cells available to conduct these experiments.

In case the C1C2-mTurqoise-PG_{70-84}-antibody and the CD9-PG_{70-84}-zinc domain-eGFP/mScarlett fusion constructs (figure 2) do not induce sufficient antigen presentation in DCs, we will add the most efficient antigen presenting construct from the C1C2-antigen and CD9-antigen fusion constructs in EVs. With the results of wp1.1-1.4, we aim to create EVs which are recruited to DCs, can induce antigen presentation in DCs and in which mRNA/miRNAs can be recruited.

1.5 *Assessing endosomal delivery of self-antigens and cytosolic delivery of mRNA/miRNAs.* EV cargo needs to be delivered to the right subcellular locations to be effective. The PG_{70-84} antigen requires MHCII loading, which happens in the endosomes. The mRNA/miRNAs require a cytosolic localisation as the translational machinery and miRNA targets are localised in the cytosol. EV uptake often results in EV localisation in endosomes and eventually in lysosomes. EV cargo can subsequently be released in the cytosol upon EV fusion to the endosomal membrane[38]. We designed the EVs in such way that after endosome fusion, PG_{70-84} is still endosome-localized while the mRNA/miRNAs are cytosolic-localized. EV-cargo which requires cytosolic delivery is often not optimally effective due to inefficient EV fusion with the endosomal membrane to release cargo before lysosomal degradation[39]. Therefore, we want to ensure that our EV cargo is efficiently taken up by cells.

EVs from wp1.4 will be coincubated with mBMDCs and endosomal delivery of EVs will be studied with fluorescent microscopy and staining for endosomal and lysosomal markers. The fluorescent fusion constructs present in the EVs will reveal the subcellular localization of EVs. Cytosolic delivery of soluble EV cargo will be assessed using the anti-GFP fluobody[40]. Anti-GFP fluobody will be expressed in mBMDCs, via electroporation mediated transfection[41]. When the EVs fuse with the endosomal membrane of mBMDCs, the anti-GFP fluobody will bind at the endosomal membrane fusion sites to the exposed GFP from the CD9 fusion construct. The anti-GFP fluobody will subsequently emit fluorescent red light[40]. With this set up we will be able to live cell image cytosolic uptake of EV cargo during mBMDC coculture with EVs. The department has state of the art fluorescent microscopes (Olympus IXplore spin microscope) capable of looking to subcellular localisation at vesicle level. We aim to get insights in EV cargo delivery to both endosomes and cytosol.

When cytosolic uptake is inefficient, we will improve this with the GALA fusion peptide[42]. The GALA fusion activity is triggered by a decrease in endosomal PH. A decreased PH leads to a conformation change which leads to interaction with the endosomal membrane and subsequently fusion[43]. Nakase et al showed that EV fusion with endosomes could be enhanced by preincubation of EVs with cationic lipids and the GALA peptide[39]. In case the cytosolic uptake of our EV-cargo is inefficient, we will determine an EV-GALA ratio resulting in both endosomal and cytosolic localisation of our EV cargo. EV uptake will be studied using the beforementioned methods. These insights will be used to optimize the EV uptake which should lead to an enhanced EV functionality.

Collectively the results of WP1 will contribute to a functional immortalized EV-producing platform in which we aim to efficiently recruit self-antigens, mRNAs/miRNAs and DC-targeting antibodies. Furthermore, the EVs should be capable of inducing PG_{70-84} presentation in DCs and delivering cargo at the right subcellular locations.

WP 2: Optimize capacity of EVs to induce tDCs and subsequently regulatory T cells

To become a functional tolerogenic vaccine, our designed EVs need to contain tolerogenic cargo. We will make use of mRNA/miRNAs as these are frequently used for safer and more effective therapies[44]. Thus, we need mRNA/miRNAs which are capable of inducing tolerogenicity in DCs and T cells. Therefore, we will 1) test multiple mRNA/miRNAs for their capacity to induce tolerogenicity in DCs and T cells and we will 2) select the most effective tolerogenic mRNA/miRNAs and recruit these in EVs. In this way we aim to design EVs which are able to efficiently induce tDCs and subsequently Tregs.

Several mRNA/miRNAs are known to induce tDCs or are involved in tDC function. Tolerogenicity in DCs could for example be induced with miRNA-23b which inhibits the NF-kB pathway[45]. miRNAs are also involved in cytokine expression: miR-150-5P upregulates IL-10 expression and miR-142-3p is associated with decrease of the inflammatory IL-6[46], it remains to be

elucidated whether these miRNAs are capable of inducing tDCs by themselves. miR-142 could also induce a tolerogenic response in mice[47] while miR-17, miR-133b, miR-203 are solely expressed in tDCs, which makes them all potential tolerogenic miRNAs. Another example is the EV mediated delivery of IL-10 mRNA which proved to be effective in atherosclerosis treatment in mice[48].

We will also test new innovative mRNA/miRNAs. The ERK-RALDH and Wnt-β-catenin pathways are important for tDC induction[49]. ERK-RALDH and Wnt-β-catenin pathway activation leads to transcription of RA producing enzymes and IL10. Wnt-β-catenin pathway activation also leads to transcription of TGF-β and IDO1/2[49,50] and DC trafficking via upregulating chemokine receptors[50,51]. RA, IL-10, TGF-β, and IDO are important anti-inflammatory factors and described to induce tolerogenicity. The mentioned factors can induce SOCS1/3 transcription which inhibits proinflammatory cytokine production[49,52]. Furthermore, TAM receptor, inhibitory Fc receptor and ILT3/4 receptor activation is described to induce tDCs[49]. We will stimulate these pathways via increasing transcription of pathway-activating proteins with mRNA and decreasing transcription of pathway-inhibiting proteins with miRNAs. Furthermore, we will directly increase the transcription of RA, IL-10, TGF-β, and IDO with mRNAs.

2.1 *Testing multiple mRNAs and miRNAs for tDC and regulatory T cell inducing capacity*. To create effective tolerogenic EVs, we want to select mRNA/miRNAs which induce a strong tolerogenic phenotype in DCs and T cells. Therefore, we will evaluate the tolerogenic effect of multiple mRNAs and miRNAs. We will screen the effect of the aforementioned mRNAs and miRNAs individually by transfecting mBMDCs with the mRNA/miRNAs. The mBMDCs will be incubated with purified PG_{70-84} to induce antigen-specific DCs. After transfection and PG_{70-84} incubation, DC expression of CD11, CD83, CD86, CD40 and MHCII will be analysed by staining with mAbs and expression of TNF-a, IL-6, IL-10 and TGF-β will be analysed by intracellular staining with mAbs. Expression levels will be quantified with flow cytometry. Tolerogenic induction should correlate with low levels of DC surface marker expression, TNF-a, IL-6 and increase in IL-10 and TGF-β[53]. Results will be compared to PG_{70-84} control without mRNA/miRNA transfection. Expression-levels of these markers will be used to measure the mRNA/miRNA mediated tolerogenic induction of DCs. Due to the immature phenotype of mBMDCs it could be that no decrease in cell surface marker expression is seen upon tolerogenic treatment. In this case, the mBMDCs transfected with mRNA/miRNA will be incubated with LPS to test whether the tolerogenic mRNA/miRNAs have the tolerogenic capacity to prevent pro-inflammatory differentiation by LPS.

The PG_{70-84}-incubated, mRNA/miRNA-transfected DCs will also be cocultured with naïve mouse PG-specific T cells (purified by negative selection[54]) to assess whether the DCs are capable of inducing regulatory T cells. Regulatory T cell induction will be quantified by measuring expression of FOXP3, ICOS, PD-1, CD73 and IL10. These markers enable to detect and differentiate regulatory T cells: Treg and Tr1[55,56]. Furthermore, CD5 will be measured to assess anergy induction. Pro and anti-inflammatory cytokines, IL17, IFN-y and IL-10 will also be measured. Expression levels of mentioned markers will be analysed by (intracellular) antibody staining and subsequent flow cytometry. Collectively, these results will show the most effective mRNAs and miRNAs for inducing tDCs and subsequently regulatory T cells. These results can also be useful for other researchers performing research on tolerogenic vaccination.

2.2. *Recruiting effective mRNA/miRNAs in EVs and testing EV effectiveness in vitro.* To create EVs with the capacity to induce tDCs and regulatory T cells, the tolerogenic mRNA/miRNAs need to be recruited to EVs. The most effective 2-3 tolerogenic mRNA/miRNAs from wp2.1 will be selected. Recruitment of the selected mRNA/miRNAs will be done by the method of Zhang et al. described in wp1.2. For the EV recruitment, mRNA/miRNA specific DNA aptamers will be designed. We will test whether recruitment of selected tolerogenic mRNA/miRNAs can be increased by designing 1 RNA combining the 2-3 tolerogenic mRNA/miRNAs.

The designed DNA aptamers and mRNA/miRNAs will be transfected in the Expi293F cells designed in WP1. Recruitment of mRNA/miRNAs to EVs will be tested with RT-qPCR of EVs. EVs will be cocultured with mBMDCs and tolerogenic profile of DCs will be measured as described in wp2.1. Furthermore, increase of EV-mRNA expression and decrease of EV-miRNA target expression will be measured by western blotting of the mBMDCs. mBMDCs will also be cocultured with purified naïve mouse PG-specific T cells. T cell phenotype will be measured as described in wp2.1. Thus, the created tolerogenic vesicles will be tested for their ability to induce tDCs and regulatory T cells. Collectively, these results will ultimately lead to an effective tolerogenic EV vaccine in vitro, which can be tested in vivo.

WP 3: Investigate the preventive and therapeutic capacity of the EV vaccine in vivo

The designed EV candidate vaccine needs to be effective in RA mouse models. The previous results give an indication of the vaccines functionality in vitro, however, cannot indicate whether the EV vaccine is functional in vivo. Therefore, we will assess in vivo whether the created EV vaccine is capable of 1) preventing or 2) treating RA in an RA mouse model. Thus, in this WP we aim to obtain insights in the vaccines capacity to ameliorate RA in an RA mouse model.

3.1 Testing preventive capacity of EV vaccine in vivo. To assess whether the EV vaccine is effective in vivo, the vaccine needs to be tested on mouse models for RA. We will make use of a proteoglycan induced arthritis (PGIA) mice model. PGIA will be induced with a standard immunization protocol. This consists of intra-peritoneal injection of 250 µL PG protein with 2mg DDA at day 0 and 21. PG will be purified from human articular cartilage and glycosaminoglycan side chains will be depleted[31,57]. For this WP, we will use congenic CD90.1 BALB/c mice and conventional CD90.2 TCR-5/4E8 BALB/c mice, which are both available in the Broere lab. The CD90.2 TCR-5/4E8 BALB/c mice have T cells containing a T cell receptor specific for $PG_{70\text{-}84}$[31]. For experiments, EVs will likely be injected intravenously as this is the general administration method for applying EVs currently. Furthermore, intravenous injection results in uptake of exosomes in the splenic immune cells[58].

Firstly, we will address the tolerogenic response induced by the EVs in healthy mice. We will transfer CD90.2 PG-TCR T cells with proliferation dye in CD90.1 mice. We will then treat mice with EVs or PBS control. Splenoctyes and LN cells will subsequently be analysed. Proliferation of CD90.2 PG-TCR T cells will be quantified with flow cytometry and FOXP3 and IL-10 expression will be quantified by intracellular staining of cells with antibodies and subsequent flow cytometry. T cell proliferation and phenotype will indicate whether the EV vaccine induces a tolerogenic response in vivo.

The preventive capacity of the EV vaccine will be assessed by EV administration before PGIA induction in CD90.1 mice. The vaccine effect on RA development/severity will be assessed with a arthritis score based on swelling and redness of paws[57]. After the experiment, FOXP3, CD5, IL17, IFN-γ and IL-10 expression will be measured in splenocytes and lymph nodes of mice by (intracellular) staining and subsequent flow cytometry. Furthermore, we will measure PG-specific IgG2a antibodies with Elisa. These results will give insights in the preventive capacity of the EVs and whether a tolerogenic immune response is induced. Furthermore, this experiment will be repeated with transfer of CD90.2 PG-TCR T cells in CD90.1 mice prior to vaccination. These data will be used to determine the vaccine dose needed to ameliorate PGIA when additional PG-specific T cells are added. These data are needed for the next experiment.

To measure the T cell specific effect of the vaccine, CD90.1 mice will get adoptive transfer of proliferation dye labelled CD90.2 PG-TCR T cells. After adoptive transfer, mice will receive the EV vaccine, an EV control without CD205 targeting (to check effect of DC-targeting) and a PBS control. PGIA will then be induced to evaluate protection of EV vaccine. The EV vaccine effect on CD90.2 PG-TCR T cells will be assessed by measuring proliferation. We will also measure FOXP3, ICOS, PD-1, CD73 and IL10 expression in CD90.2 PG-TCR T cells to measure regulatory T cell induction. In this way we will measure the vaccine effect on PG-specific T cells in preventive context.

Collectively these results will reveal the preventive capacity of the EV vaccine in a PGIA mouse model. Furthermore, these results will elucidate the direct effect of the EV vaccine on PG-specific T cells and thus whether the tolerogenic response is antigen specific.

3.2 *Testing therapeutic capacity of vaccine in vivo.* The vaccine might also have a therapeutic capacity, meaning it would be able to treat RA disease. To assess this, we will induce PGIA in CD90.1 mice and test the EV-vaccine at different timepoints after PGIA induction. The vaccine effect on arthritis development will be assessed with the arthritis score. Furthermore, we will analyse splenocytes and LN cells for FOXP3, CD5, IL17, IFN-γ and IL-10 expression with (intra)cellular staining and subsequent flow cytometry to measure pro- and anti-inflammatory mediators, regulatory T cell induction and T cell anergy induction.

Collectively these results will give insights in the EV vaccine's capability to prevent or treat RA.

Work plan

			Year1		Year2		Year3		Year4	
	Milestone/Deliverable	**Description**	6	12	18	24	30	36	42	48
WP1	Milestone/Deliverable:1.1	EVs to which CD9 and C1C2 fusion constructs are targeted.		*						
	Milestone/Deliverable:1.2	EVs to which IL-10 mRNA is recruited in effective concentrations.		*						
	Milestone:1.3	mCD205ab/hCD205ab expression on EVs.		*						
	Milestone/Deliverable:1.4	EV which are targeted to DCs by DC-targeting antibodies.			*					
	Milestone/Deliverable:1.5	EVs, loaded with PG_{70-84}, IL-10 mRNA and anti-mCD205, are able to induce antigen presentation in DCs.				*				
	Milestone:1.6	EV uptake is visualized .			*					
	Milestone/Deliverable:1.7	EVs which are efficiently taken up by DCs				*				
WP2	Milestone:2.1	Overview of mRNA/miRNAs which induce tDCs and regulatory T cells.				*				
	Milestone/Deliverable:2.2	Functional tolerogenic EVs which are able to induce tDCs and regulatory T cells in vitro.						*		
WP3	Milestone:3.1	Preventive capacity of EV vaccine is quantified.								*
	Milestone:3.2	Therapeutic capacity of EV vaccine is quantified.								*
	Deliverable:3.3	Insights in the potential of EVs for treating RA.								*

The time schedule of deliverables and milestones for this research proposal.

B.2.3 Feasibility / Risk assessment

For this proposal, we will combine the tools and expertise present in the Broere and the Nolte lab in cell editing, EV editing and DC targeting. This will contribute to the quality of this proposed research as it will reduce the time needed for procedural optimization and will help overcome obstacles. The Broere lab also has extensive experience in measuring the tolerogenic effect on DCs and T cells individually and the tolerogenic effect in the immune system of mice systemically. The Nolte lab has extensive experience in experiments combining EVs and immune cells which will contribute to the EV-related part of this research proposal.

We will purify EVs and assess them with western blotting, RT-qPCR, single particle detection, or use them in cellular essays. For single particle detection experiments, there is a possibility that the EVs are not stained well by one of the lipid dyes (BODIPY TR ceramide or PKH67), therefore we will firstly assess which lipid dye stains the EVs more efficiently and subsequently optimize the staining. Dependent on the lipid dye we will tag the CD9 construct with eGFP or mScarlett as described. Furthermore, western blotting requires samples with high EV concentrations, therefore it might be that the density gradient purifying protocol has a insufficient EV-yield for these experiments. In this case we will use ultracentrifugation only for western blot samples.

WP1:

This work package combines several, proven effective, techniques to create an EV vaccine platform. The first technique, targeting vaccine cargo to EVs with EV-targeting proteins has been successfully used by several companies to produce a SARS-VOV-2 EV vaccine[12]. Furthermore, a significant number of papers have used and described the EV-targeting proteins we plan to use as effective. For example, CD9 was shown to successfully recruit cargo to Expi293F EVs[26]. The constructs for $C1C2_{lactadherin}$ are already present in the lab and were targeted to EVs effectively. Therefore, the risk of failure to recruit EV-targeting proteins towards EVs is low. In case this still happens, we plan to use TSPAN14 as EV-targeting protein, as it is described to target to EVs efficiently[59]. Next to TSPAN14 there are multiple other proteins which target to EVs efficiently[59].

The second technique which recruits mRNA/miRNAs to EVs, should be of low risk as we will use the same, shown effective, setup as Zhang et al. The same CD9-ZF constructs, DNA aptamer and IL-10 mRNA will be used. In case mRNA/miRNA recruitment fails, EVs can still be passively loaded with mRNA/miRNAs via transfection[48].

The third technique, DC-targeting antibody mediated recruitment towards DCs, has been employed by multiple groups and used in multiple tested vaccines[7]. The Broere lab has experience in antibody-mediated targeting of antigens towards CD205 and has an effective mCD205ab[31]. Therefore, the risk of ineffective targeting of the mCD205ab is low. We will express the mCD205ab on EVs via the so called surface display technique, which constitutes antibody linking to $C1C2_{lectadherin}$. This technique has been executed before by Wang and coauthors[60], who fused a anti-HER2 antibody to $C1C2_{lactadherin}$. This was however a single chain antibody and therefore not completely representative for our experimental setup, as we also co-express the light chain of the antibody. Therefore, the mCD205ab expression on EVs has a risk, but is also innovative. In case we fail to express the mCD205ab on EVs we plan collaborate with the group of Antes to use the membrane cloacking technique they designed[35]. Furthermore, EV recruitment towards DCs will probably not be specific, as the CD205 receptors are also present on other immune cells like monocytes, macrophages and B cells[18]. This should not be problematic as these immune cells are also antigen presenting cells, meaning our EVs still can be effective.

The fourth technique, testing antigen presentation, will likely not result in difficulties. The experience and tools present in the Broere lab for testing PG_{70-84} antigen presenting capacity of DCs to T cells will be of great use. Furthermore, we optimized the EV composition in such way that PG_{70-84} is efficiently targeted to EVs, and therefore is likely to be delivered in DCs.

The fifth technique, increasing cytosolic uptake of EV cargo with the GALA peptide, mitigates the risk of ineffective EVs. There is a possibility the EVs cannot escape the endosome after internalization. This would probably result in ineffective EVs as the tolerogenic mRNA/miRNAs are not delivered in the cytosol. The GALA peptide enhances membrane fusion with the endosomal membrane, thereby increasing the chance of cytosolic delivery of the EV-loaded mRNA/miRNAs[39,43].

WP2:

WP2 aims to create tolerogenic EVs by loading them with tolerogenic mRNA/miRNAs. The tolerogenic mRNA/miRNAs evaluated in wp2.1 will be a combination of mRNA/miRNAs known to be involved in tolerogenicity and new innovative mRNAs/miRNAs which are rationally likely to be involved in tolerogenicity. As there are tolerogenic mRNA/miRNAs included which are known to induce tolerogenicity, failure of inducing tolerogenic DCs and Tregs therefore seems not likely. For example IL-10 mRNA has been shown to have tolerogenic capacities in vivo[48].

The second part, recruiting the tolerogenic molecules in EVs, also has a low risk. The described techniques we are planning to use have been shown effective[30]. In case of failure to recruit mRNA/miRNAs in EVs, they will be passively loaded with transfection.
By combining mRNA/miRNAs, which have been associated with tolerogenicity before, with proven effective techniques, we minimize the risks associated with this WP. The experience and tools of the Broere lab in investigating DC antigen presentation towards naïve T cells and determining tolerogenicity of DCs and T cells will be of great use in this work package.

WP3
WP3 will investigate the EV vaccine effectiveness in vivo. The Broere lab has extensive experience in PGIA model animal testing, which will be of great value for this work package. Furthermore, the mice and tools are available in the lab. The risk for insufficient quantities of the EV vaccine is also low as our EV platform is purposely designed in Expi293F cells which have a high yield and can easily be cultured to high numbers. Furthermore, we have optimized the EV composition in vitro in such way the EVs effectively target DCs, induce tDCs, lead to effective PG_{70-84} antigen presentation and induce regulatory T cells.

B.2.4 Scientific (a) and societal (b) impact

Currently there are no approved curing treatments for RA on the market, however tolerogenic vaccinations are a promising future treatment. In this research proposal we aim to design an EV candidate tolerogenic vaccine for RA. If effective, the EV vaccine could eventually contribute to RA treatment. Furthermore, the EV-engineering concepts combined in this proposal could contribute to the scientific knowledge and may initiate multiple research projects on EV-based tolerogenic vaccination. This EV vaccine is designed in Expi293F cells to get high yield, easy culturing conditions and good manufacturability. These characteristics increase the chance that biotech companies are willing to take on this concept and expand it. This might increase the probability that the EV-vaccine will eventually be approved and developed by biotech companies. Furthermore, researchers can adapt this EV vaccine to other auto-immune diseases by changing the self-antigen loaded in the EVs. Therefore, this vaccine might also contribute to treatments of other autoimmune diseases. Researchers could also apply this vaccine to prevent/treat infectious diseases or cancer by changing the loaded antigens and mRNA/miRNAs. The vaccine might also be adapted to answer fundamental immunology questions in which DCs have to be induced with certain characteristics in vivo. Therefore, this vaccine is not limited to RA, but can also be adapted to other diseases or research questions by altering the antigens and mRNA/miRNAs. On the short term, this vaccine may be beneficial for fundamental research questions and for new therapy efforts. On the long term, this vaccine might lead to new insights and effective EV based therapies for multiple diseases.

As mentioned, 1% of the society suffers from some form of RA, while currently there is no curing treatment for RA. If effective, our candidate vaccine might contribute to the development of an effective tolerogenic EV vaccine for RA treatment. This would potentially be beneficial for a large population. This would not only be beneficial on a personal level by relieving disease, but also on a societal level as curing RA patients increases their contribution to society. This is also beneficial for the economy because curing RA patients enables them to work more hours and also work more efficiently as the pain is not hampering them anymore. Another economical benefit is that tolerogenic vaccination is more affordable and would relieve the work package for healthcare workers. The affordability is also beneficial for insurance companies as their costs are reduced. The personal, societal and economic benefits could also extend to other patient groups if this EV vaccine concept becomes successful for other diseases.

B.2.5 Ethical considerations

The main ethical consideration for this project is animal testing with mice. We think animal testing for our project is justified as there is no other way to test and analyse the in vivo characteristics of our vaccine. We will perform the animal testing according to the Dutch law for animal testing. We have considered the 3 Rs: Replacement, Reduction and Refinement. Regarding replacement, we will test everything possible in advance in vitro which will indicate whether our candidate vaccine is effective. Thus, animals are not sacrificed without significant results. Furthermore, we will use as few animals as possible while still having sufficient animals for statistically significant results. We also have maximally reduced the experiments in which animals are needed. Combined, this ensures we use the lowest number of animals possible for this project. Lastly, we will minimize the harm to the animals and ensure the highest possible welfare for them. It is however not possible to prevent the animals from having PGIA-related pain, as this is the only valid testing model available for RA. We believe the few animals sacrificed in this project is justified as the animal related results will give a lot of information about the working mechanisms and effectiveness of the vaccine and therefore might have a significant scientific and societal impact for a large population.

The experience of the Broere lab in animal testing will ensure the animals are treated optimally and the dignity of the animals is respected and taken care of.

Some experiments make use of blood samples derived from humans to obtain human PBMCs. We will ensure the experiments and blood collection are in line with the WMA Helsinki declaration rules. We will use the leftovers from blood donations, which contain a lot of white blood cells. In this way there are no blood donations required specifically for our research project. Furthermore, we will also use a limited number and low volume of blood samples to prevent any wasting. Furthermore, blood samples will be taken in the hospital by experts with the least invasive method to ensure the donor has no to very little harm.

We will ensure the research project adheres to the ethical standards of conducting research by preventing scientific misconduct and honestly reporting the methods, results and data. Furthermore, research results will be published in peer-reviewed and open-access journals. The results will also be published on the open-access service BioRxiv and scientific exchange platforms like ResearchGate to ensure the open access of this research project for society.

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