Part A – Applicant

A.1 Applicant

Name student (initials, first name, last name, student number):	H. (Heleen) van der Veen 5843278
Affiliation (university/institute + department):	Utrecht University / Veterinary Sciences, Dept. of Infectious diseases and Immunology
Name first examiner:	Prof. dr. F. Broere
Affiliation (university/institute + department):	Utrecht University / Veterinary Sciences, Dept. of Infectious diseases and Immunology
Name second examiner:	Dr. N. Benne
Affiliation (university/institute + department):	Utrecht University / Veterinary Sciences, Dept. of Infectious diseases and Immunology

Part B – Scientific proposal

B.1 BASIC DETAILS

B.1.1 Title
Unravelling the mechanisms underlying the induction and immunological function of tolerogenic dendritic cells by immunomodulators.

B.1.2 Abstract
Autoimmune diseases such as rheumatoid arthritis are severe and common diseases that together affect ~3-5% of the population, and for which a new treatment strategy is needed. Current treatments are associated with side effects caused by broad immunosuppression, and are ineffective for some patients. Tolerogenic dendritic cells have the potential to induce antigen-specific tolerance and thereby restore the immune balance without side effects caused by broad immunosuppression. Tolerogenic dendritic cell-therapy is currently investigated in early-phase clinical trials. Tolerogenic dendritic cells induced using immunosuppressants have shown promising results in these clinical trials and other experiments. These include, dexamethasone, Vitamin D3 (1,25(OH)2D3), and all-trans retinoic acid. However, the exact mechanisms through which these tolerogenic dendritic cells are induced, and through which they exert their immunologic functions are largely unknown. Therefore, with this proposal, we aim to study the molecular mechanisms underlying antigen-specific tolerogenic dendritic cell induction by dexamethasone, Vitamin D3, and all-trans retinoic acid. We will do this in both murine and human cells. In addition, we aim to study the effects of these induced antigen-specific tolerogenic dendritic cells on other immune cells and rheumatoid arthritis development in an in vivo model. The end goal of this research is to contribute to improving tolerogenic dendritic cell therapies and to contribute to finding the optimal tolerogenic dendritic cell strategy for specific autoimmune diseases, such as rheumatoid arthritis.

B.1.3 Layman's summary
Auto-immuunziekten zijn veel voorkomende ziekten die vaak veel impact hebben op het leven van patiënten. Bij deze ziekten is het immuunsysteem, welke normaal ziektekiemen aanvalt (immuun activatie) en eigen cellen herkent als eigen (immuun tolerantie), uit balans. Deze disbalans zorgt ervoor dat het lichaam eigen cellen aanvalt. Een voorbeeld hiervan is reumatoïde artritis, waarbij de disbalans van het immuunsysteem leidt tot schade aan gewrichten. Deze patiënten moeten vaak levenslang immuun onderdrukkende medicijnen gebruiken. Deze medicijnen hebben veel bijwerkingen en zijn niet voor iedereen effectief. Daarom is er een nieuwe behandelstrategie nodig voor deze ziekten.

Bij reumatoïde artritis zijn veel verschillende immuuncellen betrokken. Belangrijke cellen in het immuunsysteem zijn dendritische cellen. Deze dendritische cellen initiëren de immuunreactie tegen de eigen cellen in reumatoïde artritis. Interessant aan deze dendritische cellen is dat ze ook een rol kunnen spelen immuun tolerantie. Daarom wordt onderzocht of

deze dendritische cellen het immuunsysteem van patiënten kunnen 'resetten' waardoor het lichaam weer tolerant wordt voor zijn eigen cellen, terwijl de immuun activatie tegen ziektekiemen blijft bestaan.

Er zijn verschillende mogelijkheden om dendritische cellen tolerant te maken. Een manier hiervoor is het gebruik van zogeheten immunomodulatoren. Dit zijn stoffen met een effect op het immuunsysteem, zoals dexamethason, vitamine D3, en een variant van Vitamine A. Deze stoffen kunnen buiten het lichaam worden toegevoegd aan dendritische cellen. De tolerant gemaakte dendritische cellen kunnen daarna in het lichaam worden gebracht, waar ze verdere immuun tolerantie kunnen veroorzaken door interactie met andere immuuncellen. Deze behandelstrategie wordt al onderzocht in kleine groepen patiënten in klinische studies.

In klinische studies hebben tolerant gemaakte dendritische cellen al laten zien dat ze symptomen van auto-immuunziekten zoals reumatoïde artritis kunnen verminderen. Maar sommige dingen moeten nog verder onderzocht worden om tot een optimale behandelstrategie te komen. Zo zijn de exacte mechanismen waarmee immunomodulatoren dendritische cellen tolerant maken nog niet helemaal bekend. Ook is nog niet volledig onderzocht met welke immuuncellen de tolerante dendritische cellen een interactie aangaan nadat ze zijn teruggebracht in het lichaam.

Met dit onderzoeksvoorstel willen we bovenstaande punten graag onderzoeken. Dit vinden wij belangrijk omdat het in kaart brengen van de onderliggende mechanismen bijvoorbeeld kennis kan geven over welke immunomodulator het best gebruikt kan worden voor een bepaalde ziekte. Ook het onderzoeken van welke immuuncellen betrokken zijn bij de inductie van verdere immuun tolerantie door tolerante dendritische cellen kan hieraan bijdragen.

De manier waarop wij dit willen onderzoeken is door eerst mogelijke onderliggende mechanismen te bestuderen in muizencellen. Daarna willen wij de mechanismen die we hebben gevonden in muizencellen graag verder onderzoeken in menselijke cellen. Omdat er veel verschillende immuuncellen betrokken kunnen zijn bij de verdere immuun tolerantie door de dendritische cellen, is het nodig om dit te onderzoeken in dierexperimenten. Voor deze onderzoeken willen wij daarom tolerante dendritische cellen toedienen aan muizen met reumatoïde artritis. Met deze muizen kunnen we onderzoeken welk effect de tolerante dendritische cellen hebben op de symptomen van reumatoïde artritis en welke andere immuuncellen hierbij betrokken zijn.

B.1.4 Keywords

Tolerogenic dendritic cells, mechanistic studies, tolerance induction, immunomodulators, rheumatoid arthritis

B.2 SCIENTIFIC PROPOSAL

B.2.1 Research topic

B.2.1.a Background

B.2.1.a.1. Autoimmune diseases

Autoimmune diseases, such as rheumatoid arthritis (RA), are major public health concerns that together affect ~3-5% of the population[1,2]. In autoimmune diseases, the immune system is unbalanced[3], leading to a loss of self-tolerance where the body attacks its own cells. Most patients require life-long treatment with immunosuppressants. This can be accompanied by severe side effects caused by broad immune suppression[4,5].

In RA, a breach of self-tolerance leads to autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA)[6]. Key players in the pathogenesis of RA are dendritic cells (DCs) and T cells[7]. Amongst others, DCs are involved in the pathogenesis of RA by the presentation of self-peptides to T cells. These T cells then get activated and differentiate into autoreactive T cells[7]. This further activates immune cascades and thereby leads to extracellular matrix degeneration and cartilage damage[8]. In case of insufficient treatment, this can lead to irreversible joint damage and disability[9].

Treatments for RA often include disease-modifying antirheumatic drugs, such as methotrexate, or glucocorticoids, such as dexamethasone (DEX). However, these drugs are associated with a wide range of side effects[4], and are not effective for all patients[10]. New treatments that restore the immune balance instead of suppressing the immune system are therefore needed[11]. A promising new approach to induce immune tolerance in an antigen-specific manner is the use of tolerogenic DCs (tolDCs)[12].

B.2.1.a.2. DCs in RA

DCs play important roles in RA pathogenesis. They present antigens, prime lymphocytes, and secrete proinflammatory cytokines, contributing to induction and maintenance of RA[13]. DCs are key players in adaptive and innate immune responses, and are pivotal regulators of immunity as well as tolerance[14,15]. The function of DCs is dependent on their maturation stage[14], and subtype[16]. Immature DCs (imDC) reside in the blood and peripheral tissues. There, they capture antigens, after which they migrate to the lymphoid organs and present the antigens to naïve T cells[14,17].

In immunity, antigen processing leads to maturation of the DCs, recognised by the presentation of antigen-derived epitopes on major histocompatibility complex (MHC) molecules, the expression of costimulatory molecules, such as CD80, CD86, and CD83 on their surface, and production and secretion of cytokines[14]. These signals stimulate T cells to differentiate into effector T cells (Teff) and advance the inflammatory immune cascade. In the presence of anti-inflammatory signals during antigen uptake, imDCs can become tolDCs, which is characterised by a semi-mature phenotype[14]. These tolDCs dampen the immune response by dampening Teff activation and increasing regulatory T cells (Tregs). This way, tolDCs can initiate immune tolerance in an antigen-specific manner[12,14].

B.2.1.a.3. T cells in RA

Several T cell subsets have been reported to be involved in RA, some of which are discussed below.

In RA, the balance between Th1 and Th2 is shifted towards Th1 cells, with activated Th1 cells and insufficient Th2 cells[18]. Th1 and Th2 cells are subsets of CD4+ T cells, which are known to control immune responses in autoimmune diseases[18]. Th1 cells secrete proinflammatory cytokines and activate macrophages, contributing to cellular immunity[18]. Th2 cells secrete anti-inflammatory cytokines, suppress macrophage activation, and contribute to humoral immunity[18].

Also T follicular helper (Tfh) cells and Th17 cells are thought to be involved in RA pathogenesis[19,20]. Tfh cells are CD4+ T cells which can activate B cells to produce high-affinity antibodies[21,22]. Th17 cells can stimulate macrophages and synovial fibroblasts, and can promote osteoclastogenesis and cartilage degeneration[20]. In RA patients, higher numbers of Tfh cells in synovial tissue, and Th17 cells in peripheral blood and synovial fluid, are present compared to healthy individuals[20,21].

It is thought that in RA, also an imbalance between Th17 cells and Tregs contributes to the pathogenesis[23]. Tregs control the immune system via suppressive cytokines such as IL-10, TGF-β, and IL-35. In addition, they can induce apoptosis in several cell types, including Teff cells, natural killer (NK) cells, and DCs[23]. Although contrasting results on the amount of Tregs in RA patients compared to healthy individuals exist, Treg dysfunction is associated with RA[23,24]. Improving Treg function is an interesting strategy for the treatment of RA[24].

B.2.1.a.4. Other immune cells in RA

Although DCs and T cells are key in RA pathogenesis[7], other immune cells are also involved. A selection of these is outlined below.

NK cells are innate lymphoid cells which are involved in both innate and adaptive immune responses[25]. Two major subgroups exist; CD16+CD56^{dim} which is known for cytotoxic functions, and CD16-CD56^{bright} which produces cytokines similar

to CD4+ T helper cells[25]. There are also regulatory NK cells, which produce IL-10[25]. NK cell dysregulation could contribute to various autoimmune diseases, including RA[25–27]. NK cells are involved in the pathogenesis of RA and may control osteoclasts in their role in bone absorption[27,28]. The NK cell percentage in peripheral blood is increased in RA patients compared to healthy individuals, but their function is decreased[28]. In addition, NK cell populations, numbers, and activity in inflammatory synovial sites differ between RA patients and healthy individuals[27].

B cells are antibody-secreting cells which can also function as antigen-presenting cells (APCs)[29]. In RA, B cells can activate Teff cells by antigen presentation, and are the main secretors of autoantibodies such as RF and ACPA[30]. In addition, B cells can secrete inflammatory cytokines including TNF-α, IFN-γ, and IL-6, which are involved in bone destruction[30]. In contrast, regulatory B cells (Bregs) are of interest because they can produce anti-inflammatory cytokines including IL-10, TGF-β, and IL-35, by which they can inhibit the activation of T cells[30,31]. Bregs are associated with RA disease activity and could function as biomarkers of treatment responses[30–33].

Macrophages contribute to the pathogenesis of RA, amongst others, by secreting pro-inflammatory cytokines, chemokines, and degradative enzymes, like matrix metalloproteinases, that drive joint inflammation and contribute to joint destruction[34]. In addition, they are thought to be involved in synovial angiogenesis[34].

Neutrophils are important effector cells in the innate immune system[35]. Dysregulated neutrophil activation is thought to contribute to RA pathogenesis by activating the immune response and inducing damage to local tissues[36].

Mast cells are innate immune cells, which have been observed in higher amounts in RA synovium than healthy synovium[37]. They are thought to play a role in RA via angiogenesis of synovium, recruitment and activation of other immune cells, and osteoclastogenesis by histamine secretion[37].

B.2.1.a.5. TolDCs as strategy for treatment

As noted above, a variety of immune cells are involved in RA pathogenesis. DCs are interesting targets for the treatment of autoimmune diseases, because DCs are key regulators of the immune system[14,15], and can thereby exert effects on a wide variety of immune cells in a direct or indirect manner. Because tolDCs can induce antigen-specific tolerance, they have the potential to restore the immune balance in RA patients, without the side effects associated with general immunosuppression[12].

TolDCs have already been investigated in early-phase clinical trials, including tolDCs for RA. For example, DEX-induced tolDCs (DEX-tolDC), and tolDCs induced by a combination of DEX and Vitamin D3 (VitD3), have shown promising results in phase I clinical trials by a reduction of RA symptoms[12].

TolDCs can have different phenotypes[16]. In general, tolDCs have altered chemokine receptor expression, a low expression of costimulatory molecules CD40, CD80, CD86, and CD83, and a low excretion of pro-inflammatory cytokines compared to mature DCs (mDCs)[12,15,17,38,39]. In contrast, expression of inhibitory and modulatory receptors, such as PD-1, and secretion of anti-inflammatory cytokines, such as IL-10 are upregulated in tolDCs compared to mDCs[12,15,17,38,39]. In addition, the expression of annexin A1, glucocorticoid-induced leucine zipper (GILZ), IDO, C1q, and stabilin-1 are reported to be altered in tolDCs compared to mDCs[12]. The exact mechanisms through which tolDCs are induced are largely unknown. It is thought that, next to tolDC inducer-specific mechanisms, ERK, JNK, cathepsins, and TGF-β are involved[40,41].

Several ways have been developed to generate tolDCs. For the generation of human tolDCs in vitro, the use of monocyte-derived DCs (moDCs) is the industry standard, which also seems to be the best clinical option[12]. For the generation of murine tolDCs, bone marrow progenitor cells are often used, which are differentiated to bone marrow-derived DCs (BMDCs). These human and murine cells are similarly differentiated towards imDCs, using GM-CSF and IL-4[12]. Immunomodulatory agents such as DEX, VitD3, and all-trans retinoic acid (ATRA) can be used to induce tolDCs[12,42–45]. Similar to tolDCs in our body, these induced tolDCs have a semi-mature phenotype[44,46], and can induce tolerance in various ways, including induction of Tregs and anergy and apoptosis of autoreactive T cells[43,47,48].

B.2.1.a.6. TolDC induction by DEX

DEX is a long-acting glucocorticoid, with anti-inflammatory and immunosuppressive properties. Glucocorticoids exert effects on many cells and tissues throughout the body, and are known to bring side effects[4]. DEX mainly binds to the glucocorticoid receptor, which is found throughout the body, and is involved in the anti-inflammatory effects of glucocorticoids[4]. Glucocorticoid receptors are heterogeneous and complex. The mechanisms through which glucocorticoid receptors function can be broadly categorised as transactivation and transrepression[4].

When DEX is present during antigen-uptake of DCs, this results in a semi-mature phenotype[49]. These DEX- tolDCs can induce immune tolerance[49,50]. DEX-tolDCs have low expression of MHCII, CD1a, CD40, CD83, CD86, and CD54, high expression of CD14, CD16, CD32, CD11/CD18, CD54, CD163, mannose receptor, MERTK, CCL18, ILT2, ILT3, and GILZ, low secretion of IL-12, and high secretion of IL-10, compared to human mDCs[43,51–53].

Interesting signalling pathways glucocorticoids act on, which could be involved in tolDC induction, are NF-κB and Akt[54,55]. Glucocorticoids inhibit NF-κB activity through induction of IκB synthesis[55]. The NF-κB pathway is required for DC development, maturation, and function[56,57]. Inhibition of NF-κB can block DC maturation and the capacity of DCs to secrete proinflammatory cytokines and activate T cells[57,58]. Related to this, glucocorticoids can stimulate protein kinase Akt[54]. Akt can regulate NF-κB function through the PI3K/Akt pathway[56], and is important for DC survival[59]. In addition, the PI3K/Akt pathway is an upstream activator of mTORC1[60], which is interesting because this relates to the function of rapamycin, which has also been demonstrated to be a tolDC inducer[43,61].

B.2.1.a.7. TolDC induction by VitD3

The active form of VitD3, 1,25(OH)2D3, is a steroid hormone that is important in calcium and bone metabolism. In addition, it has effects on the growth and differentiation of cells, as well as immunoregulatory properties[62]. VitD3 binds to the vitamin D receptor (VDR), which acts as a nuclear transcription factor, and binds to vitamin D responsive elements on the DNA[62]. This leads to the transcription of vitamin D responsive genes[62]. The vitamin D receptor is expressed on various cells, including DCs[62]. VitD3 has been shown to prevent the maturation of imDCs during stimulation with lipopolysaccharides[62].

The phenotype of VitD3-induced tolDCs (VitD3-tolDCs) is semi-mature[49], and is characterised by high expression of mannose receptor and low expression of CD83, CD40, CD80, CD86, and MHCII molecules compared to mDCs[62,63]. In addition, VitD3 leads to the inhibition of IL-12p75, enhances IL-10 secretion in maturing DCs, and promotes apoptosis of mDCs[62,63]. Furthermore, VitD3-tolDCs lead to T cell hyporesponsiveness[63]. Notably, VitD3 exerts different effects on different DC subtypes. It was shown to upregulate CCL22, downregulate CCL17, and inhibit IL-12p75 when added to blood myeloid DCs. However, these effects were not seen when VitD3 was added to plasmacytoid DCs, which could be caused by differences in NF-κB phosphorylation[63]. This, together with the effect DEX has on NF-κB[55], could suggest an important role for NF-κB in tolDC induction. Next to using VitD3 on its own, it has also been used to generate tolDCs in combination with IFN-γ[64], and in combination with DEX[65]. The latter combination results in stable tolDCs with intermediate levels of CD80 and CD86, low levels of IL-12 and TNF-α, and very high levels of IL-10[51,65]. These tolDCs in part exert their function through TGF-β1[65].

Several studies have investigated mechanisms that could underly tolDC induction by VitD3. Nurminen et al.[66] indicated several primary target genes of VitD3 with a high fold change in VitD3-tolDCs: *CD14, ORM1, CAMP, FBP1*, and *CYP26B1*. A study on the transcriptomic profile of VitD3-tolDCs showed overexpression of the genes *MUCl1, CYP24A1*, and *MAP7* compared to immature and mature DCs[43,67]. In addition, also a study on VitD3-tolDCs from healthy and multiple sclerosis patients pointed to *MUCL1, CYP24A1*, and *MAP7* as biomarkers for these tolDCs[67]. The VitD3 pathways of inducing tolDCs have also been suggested to be dependent on the PI3K/AKT pathway[68]. Interestingly, a study by Ferreira et al.[68] suggests that the dependency of VitD3-tolDC induction on PI3K/AKT is unique, and that other compounds such as DEX do not rely on this pathway. In addition, Català-Moll et al.[69] showed that the JAK/STAT pathway plays an important role in tolDC induction using VitD3. They showed VDR, STAT3, and TET2 interact with each other and that inhibition of JAK2 reverts tolDC induction by VitD3[69].

B.2.1.a.8. TolDC induction by ATRA

ATRA is an ortholog of retinol with a high ability to diffuse through water-soluble phases and hydrophobic membranes[70]. It is a ligand for nuclear retinoic acid receptors. These receptors are transcription factors that regulate the expression of target genes. This way, ATRA can regulate differentiation, proliferation, and apoptosis of cells[70].

Retinoic acid plays an important role in tolDC function in the intestine[71]. Administration of ATRA can induce a semi-mature phenotype in DCs[44,72]. These ATRA-induced tolDCs (ATRA-tolDCs) are functional in inducing a tolerogenic profile in T cells in vitro and in vivo[44,72]. Target genes of ATRA[73] with a strong and direct link to immune functioning include *CD38[74], Drd2[75], EGR1[76], IL2RA (CD25)[77], RARA[78], RARB[78], RARG[78], and TGM2[79]*. ATRA-tolDCs express high levels of IL-10, IL-27, and aldehyde dehydrogenase 1A2, and low levels of IL-12 and IL-35 compared to mDCs[72]. In addition, arginase -1 and Inducible NO synthase are shown to be upregulated in ATRA-tolDCs compared to imDCs[80]. Antigen-specific ATRA-tolDCs can inhibit allergic responses in mice through induction of Foxp3-regulatory T cells[80]. Furthermore, ATRA administered together with TGF-β, was shown to be able to prevent type 1 diabetes in mice[81].

B.2.1.a.7. Optimising tolDC therapy

The characteristics of induced tolDCs are heterogenous, depending on which protocol is used to induce them[43]. Because DCs influence many processes and immune cells which are involved in autoimmune diseases such as RA, the therapeutic results of differently induced tolDCs might vary depending on the immunological pathways triggered by the tolDCs. Several mechanisms by which tolDCs are induced have already been investigated. However, a full-picture of the exact mechanisms has not yet been defined, whereas this could help to identify which tolDC would be best for a specific disease, based on the disease pathogenesis. In addition, most research focuses on murine DCs, whilst several important differences between murine and

human DCs exist, making the translation from one species to the other difficult[16]. Therefore, it is vital to fully understand the mechanisms underlying tolDC induction and function for the application of tolDCs in new therapies[17]. Only then, we can optimally choose the best tolDC for specific autoimmune diseases such as RA.

B.2.1.b Overall aim

In summary, autoimmune diseases, such as RA are severe diseases, and current treatments are often accompanied by severe side effects and lack efficacy for a significant proportion of patients[4,5,10]. TolDCs provide a promising new strategy for the treatment of these diseases because they can induce antigen-specific tolerance, and thereby restore the balance of the immune system[12,14,15]. TolDCs have shown promising results in early-phase clinical trials[12]. However, there are gaps in knowledge about the mechanisms by which immunomodulators induce tolDCs, differences between these mechanisms in human and murine cells, and how tolDCs affect other immune cells in the body. More knowledge on these topics could speed the progress of the application of tolDC therapies, and could help to find the optimal tolDC for specific autoimmune diseases. Therefore, the overall aim of this proposal is to study the mechanisms underlying the induction of tolDCs by DEX, VitD3, and ATRA, and to study the effects of these tolDCs on other immune cells, with a focus on finding the optimal DC for RA. We hypothesise that the tolDC from witch the molecular mechanisms, and the affected immune cells, are most closely related to RA pathogenesis, is the most effective in RA symptom reduction.

B.2.1.c Objectives

To answer our overall aim, this proposal focuses on three objectives:

1. Study molecular mechanisms underlying antigen-specific tolDC induction by DEX, VitD3, and ATRA in murine cells in vitro.
2. Investigate if molecular mechanisms underlying tolDC induction by DEX, VitD3, and ATRA found in murine cells, are the same in human cells.
3. Study the effects of antigen-specific tolDCs induced by DEX, VitD3, and ATRA on other immune cells and RA development, in an in vivo RA model.

B.2.2 Approach

B.2.2.a Description of proposed research

This proposal consists of three research lines:

1. Molecular mechanisms underlying antigen-specific tolDC induction by DEX, VitD3, and ATRA in murine cells.
2. Molecular mechanisms underlying tolDC induction by DEX, VitD3, and ATRA found in murine cells, compared to human cells.
3. Effects of antigen-specific tolDCs induced by DEX, VitD3, and ATRA on other immune cells and RA development in an RA model.

The results of these research lines together will indicate which tolDC is the most optimal for RA based on molecular mechanisms of induction, and involvement of other immune cells, in relation to RA pathogenesis, together with symptom reduction. The findings of the research lines will be shared via publications in peer-reviewed open-access journals and via communication with ReumaNL.

B.2.2.a.1. Research line 1: molecular mechanisms underlying antigen-specific tolDC induction by DEX, VitD3, and ATRA in murine cells.

The use of tolDCs, induced by immunomodulators like DEX, VitD3, and ATRA, is a promising strategy for the treatment of autoimmune diseases. However, because these immunomodulators act through different mechanisms, these different immunomodulators can result in differences in phenotype and function of tolDCs. Literature, as discussed in section B.2.1. indicates a variety of interesting pathways and genes that could be involved in tolDC induction by DEX, VitD3, and ATRA. However, currently, the exact mechanisms through which the different immunomodulators induce tolDCs are largely unknown. Therefore, this research line will focus on investigating the molecular mechanisms underlying antigen-specific tolDC induction by DEX, VitD3 (1,25(OH)2D3), and ATRA in murine cells.

For this, first, possible molecular mechanisms will be indicated via literature and RNA sequencing (RNA-seq) experiments. These will then be further investigated, by blocking or inhibiting the targets of interest, and analysis with flow cytometry, phospho-flow cytometry, and RT-qPCR. TolDC induction will be confirmed by common markers and cytokine production. In addition, tolDC induction will be assessed with a T cell co-culture, where we will look at antigen-specific induction and proliferation of Tregs, and inhibition of proliferation of Teff cells. TolDCs will be loaded with human-proteoglycan-derived peptide (hPG) for antigen-specificity.

B.2.2.a.1.1. Methods and techniques

For this research line, first, cells need to be retrieved and selected, and tolDCs need to be induced using DEX, VitD3, and ATRA. Second, induction of tolDCs will be confirmed by flow cytometry and T cell co-cultures. Third, targets of interest will be identified using RNA-seq. Fourth, targets of interest are further investigated by blockage or inhibition, and analysed with flow cytometry, phospho-flow cytometry, and RT-qPCR, to assess the effects of the targets of interest on receptors and other involved molecules including cytokines, and on up- and downregulation of genes of interest.

B.2.2.a.1.1.1. Obtaining cells and induction of tolDCs

For the experiments in this research line, primary DCs and T cells from Balb/cAnNCrl mice will be used. Cells will be obtained using previously established protocols[44,45]. BMDCs will be generated from bone marrow collected from femurs and tibias of Balb/cAnNCrl mice[44]. Murine T cells will be extracted from spleens of hPG TCR transgenic Balb/cAnNCrl mice, in order to be able to induce antigen-specific responses[44]. Naïve CD4+ T cells can be negatively selected using Dynabeads and anti-CD8, anti-CD11b, anti-MHCII, anti-CD25, and anti-B220[44]. TolDCs will be induced using DEX, VitD3, and ATRA, and loaded with dye-conjugated hPG, by adding it to the culture medium, following established protocols[44,45].

B.2.2.a.1.1.2. Confirmation of tolDC induction

To evaluate if tolDC induction is successful, flow cytometry on BMDCs will be performed for well-known markers, CD11c, MHCII, CD40, and CD86, as previously described[44]. To assess the functionality of tolDCs, T cell co-culture experiments will be performed using an established protocol[44,45]. Naïve CD4+ T cells are co-cultured with hPG-loaded tolDCs for 3 days and subsequently analysed using flow cytometry[44,45]. For co-culture experiments, CD4+ T cells can be labelled with carboxyfluorescein succinimidyl ester (CFSE) for tracking, and stained for CD4, CD25, CD49b, Lag-3, and FoxP3 to look at Treg induction and proliferation, and inhibition of proliferation of Teff cells[44]. In addition, cytokine expression of tolDCs and T cells can be investigated. hPG loading of tolDCs can be confirmed using the dye-conjugate via flow cytometry[44].

B.2.2.a.1.1.3. Identification of targets of interest

In literature, several targets/pathways that could underly tolDC induction by DEX, VitD3, and ATRA, have been described. To further identify targets of interest, RNA-seq experiments will be performed, following existing protocols[82]. RNA-seq is chosen because it allows for a broad analysis of the transcriptome. In the RNA-seq experiments, imDCs, mDCs, DEX-tolDCs, VitD3-tolDCs, and ATRA-tolDCs will be compared.

B.2.2.a.1.1.4. Further investigation of targets of interest

Targets of interest identified by literature and RNA-seq will be further investigated. This will be done by using commercially available small-molecule inhibitors, siRNA, or monoclonal antibodies, added before and during tolDC induction.

Flow cytometry will be used to measure intracellular and cell surface markers and cytokine production. In addition, phospho-flow cytometry will be used. The benefit of this technique is that it allows for the measurement of multiple phosphorylation events[83], which is important when investigating for example the phosphorylation-dependent NF-κB pathway. Because both flow cytometry and phospho-flow cytometry allow for the measurement of relatively large panels simultaneously, we can use these techniques to link signalling cascades to cellular markers. The panels used will depend on the identified targets of interest.

RT-qPCR will be performed to assess gene transcription. The methods used will be based on previous experiments[84]. Primer pairs will be optimised and constructed for the identified targets of interest.

B.2.2.a.1.2. Applicability and accessibility

The transgenic mouse model allows for antigen-specific measurement of tolDC induction and is, therefore, most applicable since in therapy settings, tolDCs will also work in an antigen-specific manner. The transgenic mouse model and instruments needed for the experiments are available at the department, allowing for easy accessibility. The immunomodulators DEX, VitD3, and ATRA are chosen because of their promising results in previous experiments in the Broere lab[44,45], and in clinical trials[12].

RNA-seq is chosen to identify targets to further investigate. Compared to other techniques such as microarray analysis, RNA-seq has a broader dynamic range and is superior in detecting low abundance transcripts[85]. In addition, RNA-seq is better in differentiating isoforms and detecting genetic variance[85]. Therefore, we deem RNA-seq to be the most suitable technique for a first broad search on possibly involved mechanisms in tolDC induction. Because RNA-seq is expensive and time-consuming, this technique will only be used to identify targets of interest, which will be further examined in follow-up experiments using flow cytometry and RT-qPCR. The combination of these techniques allows for the analysis of transcription, translation, and phosphorylation of genes and proteins of interest.

To our goal, flow cytometry and phospho-flow cytometry have advantages over other techniques such as western blotting and ELISA because multiple analytes can be measured simultaneously. For (phospho-) flow cytometry, conditions and compensations need to be optimised, so preparation time is needed before further experiments can start. The benefit of RT-PCR is that it is very sensitive and can detect therefore relatively small differences in gene expression[86].

B.2.2.a.1.3. Data collection and statistical analysis

For all experiments, appropriate positive and negative controls will be used, such as untreated and stimulated (matured) controls, with and without the addition of an immunomodulator. Both flow cytometry and RT-qPCR protocols are available at the department. In addition, elaborate guidelines exist on how to optimally design, validate, and perform experiments using flow cytometry[87,88] and RT-qPCR[89,90], which will be followed.

All experiments will be performed in triplicate. Power analysis will be done before experiments are performed in order to determine the optimal sample size. A P value of <0.05 will be seen as significant. Flow cytometry can be analysed using ANOVA and Tukey's multiple analysis tests. RNA-seq and RT-qPCR will be analysed according to the descriptions of Conesa et al.[91] and Yuan et al.[92]. Experiments are documented in an electronic lab-journal.

B.2.2.a.2. Research line 2: molecular mechanisms underlying tolDC induction by DEX, VitD3, and ATRA found in murine cells, compared to human cells.

Some important differences exist between the human and murine immune system[93]. Therefore, a response in mice may not occur in the same way in humans[93]. Human and mouse DCs share similarities, but also differences exist, for example in cytokine secretion and some cellular markers[94]. A main difference limiting direct translatability between human and murine cells are differences in DC subtype[94], using moDCs and BMDCs respectively, as experimental standards[12]. To increase the translatability of the results of this proposal, we will investigate if the mechanisms underlying tolDC induction found in research line 1 in murine cells are similar to those in human cells.

B.2.2.a.2.1. Methods and techniques

The most promising mechanisms underlying tolDC induction by DEX, VitD3, and ATRA, found in research line 1 will be investigated in human cells.

For these experiments, imDCs will be generated from buffy coats using established protocols[95]. To reach a significant amount of DCs, they will be generated ex vivo from monocytes[95]. Naïve T cells can be extracted from a buffy coat using kits like the Dynabeads™ Untouched™ Human T Cells Kit (Invitrogen), selecting for CD4+CD25- cells.

Induction of tolDCs and experiments will be similar to those described in B.2.2.a.1.

B.2.2.a.2.2. Applicability and accessibility

The main limitation of this research line is the availability of samples. Buffy coat can be attained from Sanquin, with whom there is a collaboration. Because only the most promising mechanisms in murine cells are studied in this research line, the use of human samples is minimized. See B.2.2.a.1.2. for more information on applicability and accessibility. An assumption in this research line is that the mechanisms underlying tolDC induction are to some extent similar. However, it is known that of for example, LL-37, encoded by *CAMP*, which is upregulated in VitD3-tolDCs[66], the function might be specific for primates and could therefore not show results in murine cells[96]. This will be taken into account when designing the experiments and when choosing the most promising targets to move on from murine to human DC experiments.

B.2.2.a.2.3. Data collection and statistical analysis

As described in B.2.2.a.1.3.

B.2.2.a.3. Research line 3: effects of antigen-specific tolDCs induced by DEX, VitD3, and ATRA on other immune cells and RA development in an RA model.

In vivo tolDC function is normally mainly measured by its effectiveness to inhibit Teff cell activation, induce Tregs, and reduce disease symptoms. However, within RA pathogenesis many processes are involved. TolDCs can influence other immune cells besides T cells, and also T cells exert their role in RA pathogenesis through interaction with other cells, as described in B.2.1.a. Therefore, we want to investigate which RA-related immune cells are influenced by DEX-tolDC, VitD3-tolDC, and ATRA-tolDC therapy.

Immune cells we will investigate in this research line are DCs, T cells, NK cells, B cells, macrophages, neutrophils, and mast cells. These cells will be evaluated for quantity, subtype, and functionality. Because these cells all interact with each other, we will perform these experiments in vivo. Appropriate controls will be included in all experiments.

B.2.2.a.3.1. Methods and techniques

The model and experiments used in this research line will be similar to previous experiments[44,45]. For this research line, wildtype Balb/cAnNCrl mice and hPG T-cell receptor transgenic Thy1.1+ Balb/cAnNCrl mice will be used[44,45]. There are two types of animal experiments in this research line; a PGIA model to investigate the effects of tolDCs on RA symptoms and biodistribution, and an adoptive transfer model to look at the antigen-specific effects of hPG-loaded tolDCs on other immune cells[44,45]. TolDCs are obtained, induced, and loaded as described in B.2.2.a.1.

B.2.2.a.3.1.1. tolDCs in PGIA model – RA symptoms and biodistribution

To investigate the effects of tolDCs on RA symptoms, PGIA is induced using established methods[45]. hPG is injected into female Balb/cAnNCrl mice at days 0 and 21[45]. TolDCs are administered at day 17[45]. Mice will be sacrificed when the disease score plateaus, as done in previous experiments[45]. Time until the onset of PGIA and maximum arthritis score will be assessed[45]. Spleens and draining lymph nodes will be investigated for the presence of T cell and APC subsets using flow cytometry, and RA symptom scores will be assessed using a visual scoring system[45,97].

Biodistribution studies are performed in order to investigate where the tolDCs localise and exert their effects. TolDCs will be fluorescently labelled. This can for example be done using DiR (1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide)[98]. Data collection can be done using an in vivo imaging system, such as the IVIS system (PerkinElmer) at 4h, 24h, 5days, 10days, and 20 days after administration[98]. After imaging, mice will be sacrificed and major organs can be excised followed by ex vivo imaging to further investigate tolDC biodistribution, following existing protocols[98]. These biodistribution studies will be performed to determine where tolDCs migrate to and thereby indicates which organs should be investigated in the following experiments.

B.2.2.a.3.1.2. tolDCs in adoptive transfer studies – antigen-specific effects of tolDCs

Here, we will investigate if tolDCs induce antigen-specific immune tolerance in vivo, and which other immune cells are affected by the tolDCs and might therefore play a role in tolerance induction by tolDCs. For the adoptive transfer studies, naïve CD4+ T cells are isolated from spleens of hPG TCR transgenic Thy1.1+ Balb/cAnNCrl mice, by means of magnet-based negative selection for CD4+CD25- T cells[44,45]. CD4+CD25- T cells are labelled with CFSE, after which they are injected intravenously into wildtype Balb/cAnNCrl mice[44,45]. 1 day later, hPG-loaded tolDCs are injected into the wildtype mice[44,45]. 3 days later[44,45] spleens, draining lymph nodes, and other major organs as determined in the biodistribution study are harvested and cells are extracted and analysed using flow cytometry.

Using flow cytometry, immune cells of interest can be identified. DCs, T cells, NK cells, B cells, macrophages, neutrophils, and mast cells, will be analysed for (relative) quantity, subtype, phenotype, and functionality. Previously used protocols will be used to assess markers and cytokine production[44,45,99–103].

B.2.2.a.3.2. Applicability and accessibility

A variety of immune cells are included in this study because they are known to be involved in RA and could potentially be involved in tolerance induction by tolDC therapy. Therefore, we believe that the immune cells investigated in this research line can provide important information on the effects of tolDC in vivo.

The PGIA mouse model is used regularly in the Broere lab, and the transgenic mice are bred in-house, making them readily available. The PGIA mouse model shares several features with human RA, like deposition of immune complexes, involvement of DCs, T cells, and B cells, swelling of joints, and the presence of RF[30,104]. In the PGIA model, only female mice are used, which will increase comparability between our study and other studies because the use of only female mice is standard in RA research[104].

A limitation of the PGIA model used is that it is a preventative model. In RA patients, administration of tolDCs would likely be in later stages of RA. Therefore, administration to the mice in later stages, for example at a predetermined arthritis severity score, would be more translatable to the human situation. However, because of preparation times and limited storage possibilities of tolDCs, this is not feasible in practice.

B.2.2.a.3.3. Data collection and statistical analysis plans

Before the start of the experiments, a power calculation will be performed in order to choose the correct sample sizes for the experiments. Logbooks will be kept. Data between the experimental groups can be compared using ANOVA, Tukey's, and Bonferroni's multiple comparisons tests[44,45]. Mice will be randomised and proper positive and negative control groups, such as saline-injected mice and mice injected with mDCs, are present. Logbooks of all experiments and events will be kept.

B.2.2.b Work plan

The research will be performed in 4 years. The first 6 months will be used to get acquainted with techniques and optimise methodology. Throughout the 4 years, optimisation of methodology is ongoing. Once tolDCs are reliably induced, RNA-seq experiments of research line 1 will start. Once finished around the end of year 1, experiments on pathways and genes of interest in research line 1 will continue until around half of year 3. Next to these experiments, experiments of research line 2 will start to investigate the most promising mechanisms in human cells. Research line 3 will start with experiments with the PGIA model (~ 1 year), after which the adoptive transfer studies will start (~1 ½ year). An overview of the proposed work plan can be found in Figure 1.

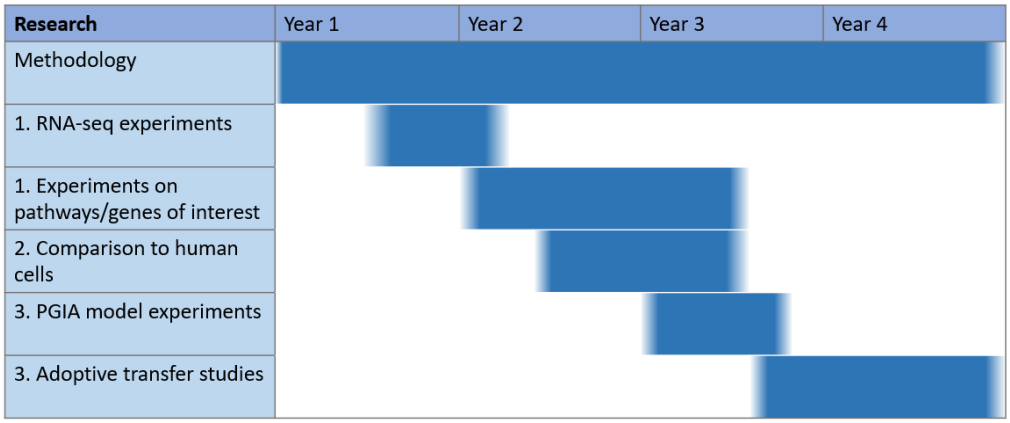

Figure 1. Work plan of project elements throughout the 4 years, planned periods in blue. Methodological development and optimisation are done throughout the project. Research line 1 starts with RNA-seq experiments, followed by experiments on pathways/genes of interest, to which a comparison to human cells (research line 2) runs parallel. Research line 3 includes PGIA model experiments, followed by adoptive transfer studies.

B.2.3 Feasibility / Risk assessment

Although the proposed experiments are largely designed to connect to research which is already performed within the Broere lab, all research lines come with their own risks. The most prominent risks are noted below. The proposed techniques are well established, and current research at the Broere lab indicates our goals are feasible.

B.2.3.1. Feasibility and time frame

The apparatus, mice, and facilities are available at the department. In addition, there is experience with the proposed techniques in the department.

The proposed research fits into the time frame with time to divert to alternatives, as shown in the work plan in B.2.2.b. Some experiments, such as flow cytometry, will take a lot of preparation time. However, once optimised, the same settings can be used for all experiments which saves a lot of time. Time for methodology optimisation is included in the proposed work plan.

B.2.3.2. Risks and alternatives

As with all experiments, risks are present within the research proposed. Below, the major risks we envision for the proposed research are discussed.

B.3.2.2.1. In vitro experiments

The cells, mice, and techniques proposed are well established, minimising methodological risks. Nonetheless, primary cells are sensitive, so some experiments might fail. In case the primary cell experiments do not advance as expected after the first time period, cell lines can be used for research line 1. Immortalised cell lines are available for murine and human DCs, such as D1 and THP cells[105]. However, primary cells more closely resemble the in vitro situation than the cell lines[105]. Therefore, we would propose to only use the cell lines for the first experiments until primary cell experiments advance enough.

Another risk related to the cells we propose to use is limited availability, especially for research line 2. To minimise the number of cells needed, optimised protocols from research line 1 will be used, and only the most interesting pathways will be investigated in the human cells.

B.3.2.2.2. In vivo experiments

The PGIA mouse model is a model for RA with remissions and exacerbations[106]. It is chosen because of its suitability and because there is a lot of experience with this model within the Broere group. A risk associated with the model we propose is that it is used less than the collagen-induced arthritis (CIA) model[107]. If needed, an alternative for the PGIA model could be to switch to the CIA model. This model is currently not used in the Broere group but should be achievable[108].

In case serious off-target effects or other adverse events are seen, the cause of these has to be determined. Hopefully, the off-target effects seen can be investigated and determined by the broader immune system that we will investigate, which can also contribute to the advancement of tolDCs in the clinic.

B.3.2.3. Embedding

The Broere lab focuses on treatments for chronic inflammatory diseases like RA, including tolDCs induced with DEX, VitD3, and ATRA. Therefore, the protocols of making tolDCs, analysing tolDC markers, and co-cultures with T cells to evaluate efficacy are well established in the lab. The group regularly uses the hPG TCR transgenic Thy1.1+ mouse model for experiments with a similar set-up as the proposed experiments.

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

B.2.4.a. Scientific impact

B.2.4.a.1. Impact on research field

Current therapies for autoimmune diseases like RA are suboptimal in efficacy and bring side effects caused by broad immune suppression. Therefore, strategies like tolDCs are developed to induce antigen-specific tolerance. In the short term, the proposed research can deepen the understanding of the ongoing developments in tolDC therapy. In the long term, the proposed research can contribute finding the best tolDC inducer for specific autoimmune diseases.

B.2.4.a.2. Impact on other scientific areas

The knowledge gained by these experiments can also be used in other scientific areas. An interesting area is that of biomimetic particles. The mechanisms that underlie tolerance induction could be used as a basis to design nanoparticles that induce antigen-specific tolerance. These tolDC-like biomimetic nanoparticles could be used for several cargos, including CRISPR-Cas[109–111]. CRISPR-Cas is used ex vivo, but for broader application of this promising protein, in vivo delivery is needed[109–111]. However, immune-recognition poses challenges for in vivo CRISPR-Cas therapy[112]. Inducing CRISPR-Cas-specific antigen tolerance could provide a solution to these limiting factors. Experiments in this field could for example be performed in collaboration with the group of Prof. dr. Mastrobattista, who focuses on in vivo delivery of CRISPR-Cas, and also has an ongoing research line on tolerance induction to CRISPR-Cas.

B.2.4.b. Societal impact

The new technology of tolDC therapy brings hope to patients with autoimmune diseases who often have to take immunosuppressants for the rest of their lives and experience severe side effects. The proposed research focuses on RA. This disease is common and treatments are sometimes inefficient and often bring side effects[4,10]. TolDC therapy bears the potential to restore normal immune function[12], and thereby lessen the need for immunosuppressants by these patients.

The proposed research deepens understanding of tolDCs and can thereby increase the speed of the journey of tolDC therapy towards the clinic for RA, as well as for other autoimmune diseases. In the future, also the contribution the proposed research can have to in vivo delivery of CRISPR Cas, can have large implications for society, for example in the treatment of severe genetic diseases.

To contribute to societal impact, the results of the proposed research will be shared with ReumaNL, in order to reach the public that will benefit most directly from it, RA patients.

B.2.5 Ethical considerations

In the execution of the proposal, the Netherlands Code of Conduct for Research Integrity (2018)[113] will be followed. Two large ethical issues of the proposal are the use of mice for animal experiments and the use of human materials.

B.2.5.1. Animal experiments

For the animal experiments, strict conditions, regulated by law and enforced by Utrecht University[114] are followed, as discussed below.

1) There is no other way to achieve our research goal. Because the immune system and RA are complex, cell lines are not sufficient to study the function of induced tolDCs. In addition, sources for human primary cells are limited. Therefore, primary cells from animals and animal experiments are needed.

2) We ensure the replacement, reduction, and refinement of animals. There are several developments which can replace the need for animal experiments, such as the use of organoids. Organoid systems have also been researched for immunological research[115]. However, we do not believe that organoids can add significantly to the research we propose because tolDCs can exert actions in too many ways to investigate in vitro. Good statistics, power calculations, documentation, and information gained from literature and previous experiments are applied to ensure the amount of animals needed is kept to a minimum whilst still generating statistically significant results. Further reducing the suffering of animals needed is the performance of co-culture experiments of DCs and T cells, to gain extra functional information before starting in vivo experiments, because the animals needed for cell retrieval do not undergo harmful experimental procedures

(partial replacement). Refinement is applied by housing the animals under standard conditions at the Gemeenschappelijk Dierenlaboratorium of Utrecht University or the Animal Research Center Poonawalla Science Park, including cage enrichment and at libitum food and water. A humane endpoint is applied during experiments to ensure the animals do not suffer beyond humane.

3) The research is important for society. Current treatments for RA often cause side effects, including systemic suppression, leaving patients more susceptible to pathogens. A promising new strategy is using antigen-loaded tolDCs to induce antigen-specific immune responses against RA-relevant antigens. TolDCs are being used in clinical trials at the moment[12], but underlying mechanisms of tolDC induction remain largely unknown. The aim of this project is therefore to investigate the mechanisms underlying the induction of tolDCs by DEX, VitD3, and ATRA, and to study the effects of these tolDCs on other immune cells. This research has the end goal to contribute to improving tolDC therapies and to contribute to finding the optimal tolDC strategy for RA.

4) The Central Animal Experiments Committee will be contacted to assess and licence the experiments. The Central Animal Experiments Committee will assess if the suffering caused to the mice is outweighed by the importance of the experiments. They are also the party that will licence our experiments.

5) The Utrecht Animal Welfare Body will check the work protocol and guide the experiments if needed. The experimental logbook at the animal facility will be updated in order to record all handling and experiments. In case of irregularities, the researchers will be contacted, and will be guided by the Utrecht Animal Welfare Body if needed.

B.2.5.2. Human materials

The buffy coats used in the second research line are subjected to WMO approval. This approval and informed consent is obtained by Sanquin before sample collection. Therefore, this approval does not have to be obtained again by the researchers before starting the experiments.

B.2.6 Literature/references

1. Wang, L., Wang, F. S. & Gershwin, M. E. Human autoimmune diseases: a comprehensive update. *J. Intern. Med.* **278**, 369–395 (2015).
2. Willame, C. *et al.* Incidence Rates of Autoimmune Diseases in European Healthcare Databases: A Contribution of the ADVANCE Project. *Drug Saf.* **44**, 383–395 (2021).
3. Chaplin, D. D. Overview of the immune response. *J. Allergy Clin. Immunol.* **125**, (2010).
4. Hardy, R. S., Raza, K. & Cooper, M. S. Therapeutic glucocorticoids: mechanisms of actions in rheumatic diseases. *Nat. Rev. Rheumatol.* **16**, 133–144 (2020).
5. Wang, W., Zhou, H. & Liu, L. Side effects of methotrexate therapy for rheumatoid arthritis: A systematic review. *Eur. J. Med. Chem.* **158**, 502–516 (2018).
6. Rönnelid, J., Turesson, C. & Kastbom, A. Autoantibodies in Rheumatoid Arthritis – Laboratory and Clinical Perspectives. *Front. Immunol.* **12**, 1619 (2021).
7. Wehr, P., Purvis, H., Law, S. C. & Thomas, R. Dendritic cells, T cells and their interaction in rheumatoid arthritis. *Clin. Exp. Immunol.* **196**, 12 (2019).
8. Mueller, A. L. *et al.* Recent Advances in Understanding the Pathogenesis of Rheumatoid Arthritis: New Treatment Strategies. *Cells 2021, Vol. 10, Page 3017* **10**, 3017 (2021).
9. Smolen, J. S. *et al.* Rheumatoid arthritis. *Nat. Rev. Dis. Prim.* **4**, (2018).
10. Guo, Q. *et al.* Rheumatoid arthritis: pathological mechanisms and modern pharmacologic therapies. *Bone Res.* **6**, (2018).
11. Miller, S. D., Turley, D. M. & Podojil, J. R. Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. *Nat. Rev. Immunol.* **7**, 665–677 (2007).
12. Ness, S., Lin, S. & Gordon, J. R. Regulatory Dendritic Cells, T Cell Tolerance, and Dendritic Cell Therapy for Immunologic Disease. *Front. Immunol.* **12**, 195 (2021).
13. Lutzky, V., Hannawi, S. & Thomas, R. Cells of the synovium in rheumatoid arthritis. Dendritic cells. *Arthritis Res. Ther.* **9**, 1–12 (2007).
14. Banchereau, J. *et al.* Immunobiology of dendritic cells. *Annu. Rev. Immunol.* **18**, 767–811 (2000).
15. Audiger, C., Rahman, M. J., Yun, T. J., Tarbell, K. V. & Lesage, S. The Importance of Dendritic Cells in Maintaining Immune Tolerance. *J. Immunol.* **198**, 2223–2231 (2017).
16. Castenmiller, C., Keumatio-Doungtsop, B. C., van Ree, R., de Jong, E. C. & van Kooyk, Y. Tolerogenic Immunotherapy: Targeting DC Surface Receptors to Induce Antigen-Specific Tolerance. *Front. Immunol.* **12**, 422 (2021).
17. Suuring, M. & Moreau, A. Regulatory Macrophages and Tolerogenic Dendritic Cells in Myeloid Regulatory Cell-Based Therapies. *Int. J. Mol. Sci. 2021, Vol. 22, Page 7970* **22**, 7970 (2021).
18. Skapenko, A., Leipe, J., Lipsky, P. E. & Schulze-Koops, H. The role of the T cell in autoimmune inflammation. *Arthritis Res. Ther. 2005 72* **7**, 1–11 (2005).
19. Lucas, C., Perdriger, A. & Amé, P. Definition of B cell helper T cells in rheumatoid arthritis and their behavior during treatment. *Semin. Arthritis Rheum.* **50**, 867–872 (2020).
20. Leipe, J. *et al.* Role of Th17 cells in human autoimmune arthritis. *Arthritis Rheum.* **62**, 2876–2885 (2010).
21. Lu, J., Wu, J., Xia, X., Peng, H. & Wang, S. Follicular helper T cells: potential therapeutic targets in rheumatoid arthritis. *Cell. Mol. Life Sci.* **78**, 5095–5106 (2021).
22. Kondo, N., Kuroda, T. & Kobayashi, D. Cytokine Networks in the Pathogenesis of Rheumatoid Arthritis. *Int. J. Mol. Sci. 2021, Vol. 22, Page 10922* **22**, 10922 (2021).
23. Li, X. *et al.* Dysfunctions, Molecular Mechanisms, and Therapeutic Strategies of Regulatory T Cells in Rheumatoid Arthritis. *Front. Pharmacol.* **12**, 1973 (2021).
24. Jiang, Q., Yang, G., Liu, Q., Wang, S. & Cui, D. Function and Role of Regulatory T Cells in Rheumatoid Arthritis. *Front. Immunol.* **12**, 1021 (2021).
25. Kucuksezer, U. C. *et al.* The Role of Natural Killer Cells in Autoimmune Diseases. *Front. Immunol.* **12**, (2021).
26. Gianchecchi, E., Delfino, D. V. & Fierabracci, A. Natural Killer Cells: Potential Biomarkers and Therapeutic Target in Autoimmune Diseases? *Front. Immunol.* **12**, (2021).
27. Yang, Y., Day, J., Souza-Fonseca Guimaraes, F., Wicks, I. P. & Louis, C. Natural killer cells in inflammatory autoimmune diseases. *Clin. Transl. Immunol.* **10**, (2021).
28. Fathollahi, A. *et al.* The role of NK cells in rheumatoid arthritis. *Inflamm. Res.* **70**, 1063–1073 (2021).
29. Silverman, G. J. & Carson, D. A. Roles of B cells in rheumatoid arthritis. *Arthritis Res. Ther.* **5**, 1–6 (2003).
30. Wu, F. *et al.* B Cells in Rheumatoid Arthritis ： Pathogenic Mechanisms and Treatment Prospects. *Front. Immunol.* **12**, 3987 (2021).
31. Zhu, Q., Rui, K., Wang, S. & Tian, J. Advances of Regulatory B Cells in Autoimmune Diseases. *Front. Immunol.* **12**, 1131 (2021).
32. Ma, L., Liu, B., Jiang, Z. & Jiang, Y. Reduced numbers of regulatory B cells are negatively correlated with

disease activity in patients with new-onset rheumatoid arthritis. *Clin. Rheumatol.* **33**, 187–195 (2014).
33. Kalampokis, I., Yoshizaki, A. & Tedder, T. F. IL-10-producing regulatory B cells (B10 cells) in autoimmune disease. *Arthritis Res. Ther.* **15 Suppl 1**, (2013).
34. Edilova, M. I., Akram, A. & Abdul-Sater, A. A. Innate immunity drives pathogenesis of rheumatoid arthritis. *Biomed. J.* **44**, 172–182 (2021).
35. Rosales, C. Neutrophil: A cell with many roles in inflammation or several cell types? *Front. Physiol.* **9**, 113 (2018).
36. Fresneda Alarcon, M., McLaren, Z. & Wright, H. L. Neutrophils in the Pathogenesis of Rheumatoid Arthritis and Systemic Lupus Erythematosus: Same Foe Different M.O. *Front. Immunol.* **12**, 570 (2021).
37. Min, H. K., Kim, K. W., Lee, S. H. & Kim, H. R. Roles of mast cells in rheumatoid arthritis. *Korean J. Intern. Med.* **35**, 12 (2020).
38. Merad, M., Sathe, P., Helft, J., Miller, J. & Mortha, A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu. Rev. Immunol.* **31**, 563–604 (2013).
39. Fucikova, J., Palova-Jelinkova, L., Bartunkova, J. & Spisek, R. Induction of tolerance and immunity by dendritic cells: Mechanisms and clinical applications. *Front. Immunol.* **10**, 2393 (2019).
40. Arce, F. *et al.* Selective ERK Activation Differentiates Mouse and Human Tolerogenic Dendritic Cells, Expands Antigen-Specific Regulatory T Cells, and Suppresses Experimental Inflammatory Arthritis. *Arthritis Rheum.* **63**, 84 (2011).
41. Nakahara, T. *et al.* Role of c-Jun N-terminal kinase on lipopolysaccharide induced maturation of human monocyte-derived dendritic cells. *Int. Immunol.* **16**, 1701–1709 (2004).
42. Cauwels, A. & Tavernier, J. Tolerizing Strategies for the Treatment of Autoimmune Diseases: From ex vivo to in vivo Strategies. *Front. Immunol.* **11**, 674 (2020).
43. Navarro-Barriuso, J. *et al.* Comparative transcriptomic profile of tolerogenic dendritic cells differentiated with vitamin D3, dexamethasone and rapamycin. *Sci. Rep.* **8**, 14985 (2018).
44. Braake, D. Ter, Benne, N., Lau, C. Y. J., Mastrobattista, E. & Broere, F. Retinoic acid-containing liposomes for the induction of antigen-specific regulatory T cells as a treatment for autoimmune diseases. *Pharmaceutics* **13**, 1949 (2021).
45. Jansen, M. A. A. *et al.* Matured tolerogenic dendritic cells effectively inhibit autoantigen specific CD4+ T cells in a murine arthritis model. *Front. Immunol.* **10**, 2068 (2019).
46. Harry, R. A., Anderson, A. E., Isaacs, J. D. & Hilkens, C. M. U. Extended report: Generation and characterisation of therapeutic tolerogenic dendritic cells for rheumatoid arthritis. *Ann. Rheum. Dis.* **69**, 2042 (2010).
47. Obregon, C., Kumar, R., Pascual, M. A., Vassalli, G. & Golshayan, D. Update on dendritic cell-induced immunological and clinical tolerance. *Frontiers in Immunology* **8**, 1514 (2017).
48. ten Brinke, A. *et al.* Ways forward for tolerance-inducing cellular therapies- An afactt perspective. *Front. Immunol.* **10**, 181 (2019).
49. Naranjo-Gómez, M. *et al.* Comparative study of clinical grade human tolerogenic dendritic cells. *J. Transl. Med.* **9**, 89 (2011).
50. Xia, C.-Q., Peng, R., Beato, F. & Clare-Salzler, M. J. Dexamethasone Induces IL-10-Producing Monocyte-Derived Dendritic Cells with Durable Immaturity. *Scand. J. Immunol.* **62**, 45–54 (2005).
51. Gordon, J. R., Ma, Y., Churchman, L., Gordon, S. A. & Dawicki, W. Regulatory dendritic cells for immunotherapy in immunologic diseases. *Front. Immunol.* **5**, 7 (2014).
52. Piemonti, L. *et al.* Glucocorticoids Affect Human Dendritic Cell Differentiation and Maturation. *J. Immunol.* **162**, (1999).
53. Cabezo´n, R. *et al.* MERTK as negative regulator of human T cell activation. *J. Leukoc. Biol.* **97**, 751–760 (2015).
54. Hafezi-Moghadam, A. *et al.* Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nat. Med.* **8**, 473–479 (2002).
55. Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A. & Karin, M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* **270**, 286–290 (1995).
56. Bhattacharyya, S. *et al.* Immunoregulation of dendritic cells by IL-10 is mediated through suppression of the PI3K/Akt pathway and of IκB kinase activity. *Blood* **104**, 1100–1109 (2004).
57. Hayden, M. S. & Ghosh, S. NF-κB in immunobiology. *Cell Res. 2011 212* **21**, 223–244 (2011).
58. Tas, S. W. *et al.* Selective inhibition of NF-kappaB in dendritic cells by the NEMO-binding domain peptide blocks maturation and prevents T cell proliferation and polarization. *Eur. J. Immunol.* **35**, 1164–1174 (2005).
59. Park, D., Lapteva, N., Seethammagari, M., Slawin, K. M. & Spencer, D. M. An essential role for Akt1 in dendritic cell function and tumor immunotherapy. *Nat. Biotechnol. 2006 2412* **24**, 1581–1590 (2006).
60. Manning, B. D. & Cantley, L. C. AKT/PKB Signaling: Navigating Downstream. *Cell* **129**, 1261–1274 (2007).
61. Macedo, C., Turquist, H., Metes, D. & Thomson, A. W. Immunoregulatory properties of rapamycin-conditioned monocyte-derived dendritic cells and their role in transplantation. *Transplant. Res.* **1**, 16 (2012).

62. Penna, G. *et al.* 1,25-Dihydroxyvitamin D3 Selectively Modulates Tolerogenic Properties in Myeloid but Not Plasmacytoid Dendritic Cells. *J. Immunol.* **178**, 145–153 (2007).
63. Penna, G. & Adorini, L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J. Immunol.* **164**, 2405–2411 (2000).
64. Švajger, U. & Rožman, P. J. Synergistic Effects of Interferon-γ and Vitamin D3 Signaling in Induction of ILT-3highPDL-1high Tolerogenic Dendritic Cells. *Front. Immunol.* **10**, 2627 (2019).
65. Anderson, A. E. *et al.* Tolerogenic dendritic cells generated with dexamethasone and vitamin D3 regulate rheumatoid arthritis CD4+ T cells partly via transforming growth factor-β1. *Clin. Exp. Immunol.* **187**, 113–123 (2017).
66. Nurminen, V., Seuter, S. & Carlberg, C. Primary Vitamin D target genes of human monocytes. *Front. Physiol.* **10**, 194 (2019).
67. Navarro-Barriuso, J. *et al.* MAP7 and MUCL1 are biomarkers of Vitamin D3-induced tolerogenic dendritic cells in multiple sclerosis patients. *Front. Immunol.* **10**, 1251 (2019).
68. Ferreira, G. B. *et al.* Vitamin D3 induces tolerance in human dendritic cells by activation of intracellular metabolic pathways. *Cell Rep.* **10**, 711–725 (2015).
69. Català-Moll, F. *et al.* Vitamin D receptor, STAT3, and TET2 cooperate to establish tolerogenesis. *Cell Rep.* **38**, 110244 (2022).
70. Blomhoff, R. & Blomhoff, H. K. Overview of retinoid metabolism and function. *J. Neurobiol.* **66**, 606–630 (2006).
71. Weiner, H. L., da Cunha, A. P., Quintana, F. & Wu, H. Oral tolerance. *Immunol. Rev.* **241**, 241–259 (2011).
72. Dawicki, W., Li, C., Town, J., Zhang, X. & Gordon, J. R. Therapeutic reversal of food allergen sensitivity by mature retinoic acid-differentiated dendritic cell induction of LAG3 + CD49b - Foxp3 - regulatory T cells. *J. Allergy Clin. Immunol.* **139**, 1608-1620.e3 (2017).
73. Balmer, J. E. & Blomhoff, R. Gene expression regulation by retinoic acid. *J. Lipid Res.* **43**, 1773–1808 (2002).
74. Piedra-Quintero, Z. L., Wilson, Z., Nava, P. & Guerau-de-Arellano, M. CD38: An Immunomodulatory Molecule in Inflammation and Autoimmunity. *Front. Immunol.* **11**, 3111 (2020).
75. Feng, Y. & Lu, Y. Immunomodulatory Effects of Dopamine in Inflammatory Diseases. *Front. Immunol.* **12**, 987 (2021).
76. ten Hoeve, A. L., Hakimi, M. A. & Barragan, A. Sustained Egr-1 Response via p38 MAP Kinase Signaling Modulates Early Immune Responses of Dendritic Cells Parasitized by Toxoplasma gondii. *Front. Cell. Infect. Microbiol.* **9**, 349 (2019).
77. Driesen, J., Popov, A. & Schultze, J. L. CD25 as an immune regulatory molecule expressed on myeloid dendritic cells. *Immunobiology* **213**, 849–858 (2008).
78. Hall, J. A., Grainger, J. R., Spencer, S. P. & Belkaid, Y. The Role of Retinoic Acid in Tolerance and Immunity. *Immunity* **35**, 13–22 (2011).
79. Kim, J. H. *et al.* Transglutaminase 2 on the surface of dendritic cells is proposed to be involved in dendritic cell–T cell interaction. *Cell. Immunol.* **289**, 55–62 (2014).
80. Bhatt, S. *et al.* All-trans retinoic acid induces arginase-1 and inducible nitric oxide synthase-producing dendritic cells with T cell inhibitory function. *J. Immunol.* **192**, 5098–5108 (2014).
81. Koprivica, I. *et al.* Orally delivered all-trans-retinoic acid- and transforming growth factor-β-loaded microparticles ameliorate type 1 diabetes in mice. *Eur. J. Pharmacol.* **864**, (2019).
82. Trapnell, C. *et al.* Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc. 2012 73* **7**, 562–578 (2012).
83. Krutzik, P. O., Trejo, A., Schulz, K. R. & Nolan, G. P. Phospho flow cytometry methods for the analysis of kinase signaling in cell lines and primary human blood samples. *Methods Mol. Biol.* **699**, 179–202 (2011).
84. Derdelinckx, J. *et al.* Clinical and immunological control of experimental autoimmune encephalomyelitis by tolerogenic dendritic cells loaded with MOG-encoding mRNA. *J. Neuroinflammation* **16**, 1–20 (2019).
85. Zhao, S., Fung-Leung, W. P., Bittner, A., Ngo, K. & Liu, X. Comparison of RNA-Seq and Microarray in Transcriptome Profiling of Activated T Cells. *PLoS One* **9**, e78644 (2014).
86. Wong, M. L. & Medrano, J. F. Real-time PCR for mRNA quantitation. *Biotechniques* **39**, 75–85 (2005).
87. Van Der Strate, B. *et al.* Best practices in performing flow cytometry in a regulated environment: Feedback from experience within the European Bioanalysis Forum. *Bioanalysis* **9**, 1253–1264 (2017).
88. Selliah, N. *et al.* Flow Cytometry Method Validation Protocols. *Curr. Protoc. Cytom.* **87**, (2019).
89. Broeders, S. *et al.* Guidelines for validation of qualitative real-time PCR methods. *Trends Food Sci. Technol.* **37**, 115–126 (2014).
90. Ma, H., Bell, K. N. & Loker, R. N. qPCR and qRT-PCR analysis: Regulatory points to consider when conducting biodistribution and vector shedding studies. *Mol. Ther. Methods Clin. Dev.* **20**, 152 (2021).
91. Conesa, A. *et al.* A survey of best practices for RNA-seq data analysis. *Genome Biol. 2016 171* **17**, 1–19 (2016).
92. Yuan, J. S., Reed, A., Chen, F. & Stewart, C. N. Statistical analysis of real-time PCR data. *BMC Bioinformatics* **7**,

1–12 (2006).
93. Mestas, J. & Hughes, C. C. W. Of Mice and Not Men: Differences between Mouse and Human Immunology. *J. Immunol.* **172**, 2731–2738 (2004).
94. Shortman, K. & Liu, Y. J. Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol. 2002 23* **2**, 151–161 (2002).
95. Nair, S., Archer, G. E. & Tedder, T. F. Isolation and generation of human dendritic cells. *Curr. Protoc. Immunol.* **Chapter 7**, (2012).
96. Gombart, A. F., Borregaard, N. & Koeffler, H. P. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB J.* **19**, 1067–1077 (2005).
97. Chondrex. Mouse Arthritis Scoring System. Available at: https://www.chondrex.com/documents/Scoring-System.pdf. (Accessed: 13th February 2022)
98. Tian, Y. *et al.* DiR-labeled tolerogenic dendritic cells for targeted imaging in collagen- induced arthritis rats. *Int. Immunopharmacol.* **91**, 107273 (2021).
99. Lo, C. K. C. *et al.* Natural killer cell degeneration exacerbates experimental arthritis in mice via enhanced interleukin-17 production. *Arthritis Rheum.* **58**, 2700–2711 (2008).
100. Luo, L. *et al.* The number of regulatory B cells is increased in mice with collagen-induced arthritis. *Open Life Sci.* **14**, 12–18 (2019).
101. Eyles, J. L. *et al.* A key role for G-CSF–induced neutrophil production and trafficking during inflammatory arthritis. *Blood* **112**, 5193–5201 (2008).
102. Mast Cell Markers: R&D Systems. Available at: https://www.rndsystems.com/resources/cell-markers/immune-cells/granulocytes/mast-cell-markers. (Accessed: 13th February 2022)
103. Ye, L. *et al.* Interleukin-10 attenuation of collagen-induced arthritis is associated with suppression of interleukin-17 and retinoid-related orphan receptor γt production in macrophages and repression of classically activated macrophages. *Arthritis Res. Ther.* **16**, 1–14 (2014).
104. Schinnerling, K., Rosas, C., Soto, L., Thomas, R. & Aguillón, J. C. Humanized Mouse Models of Rheumatoid Arthritis for Studies on Immunopathogenesis and Preclinical Testing of Cell-Based Therapies. *Front. Immunol.* **10**, 203 (2019).
105. van Helden, S. F. G., van Leeuwen, F. N. & Figdor, C. G. Human and murine model cell lines for dendritic cell biology evaluated. *Immunol. Lett.* **117**, 191–197 (2008).
106. Rajaiah, R. & Moudgil, K. D. Animal Models. *Rheum. Arthritis* 218–224 (2009). doi:10.1016/B978-032305475-1.50031-8
107. Lewis, B. J. B. & Branch, D. R. Mouse Models of Rheumatoid Arthritis for Studies on Immunopathogenesis and Preclinical Testing of Fc Receptor-Targeting Biologics. *Pharmacology* **105**, 618–629 (2020).
108. Brand, D. D., Latham, K. A. & Rosloniec, E. F. Collagen-induced arthritis. *Nat. Protoc.* **2**, 1269–1275 (2007).
109. Duan, L. *et al.* Nanoparticle Delivery of CRISPR/Cas9 for Genome Editing. *Front. Genet.* **12**, 788 (2021).
110. Wei, T., Cheng, Q., Min, Y. L., Olson, E. N. & Siegwart, D. J. Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing. *Nat. Commun. 2020 111* **11**, 1–12 (2020).
111. Walther, J. *et al.* Impact of Formulation Conditions on Lipid Nanoparticle Characteristics and Functional Delivery of CRISPR RNP for Gene Knock-Out and Correction. *Pharmaceutics* **14**, 213 (2022).
112. Crudele, J. M. & Chamberlain, J. S. Cas9 immunity creates challenges for CRISPR gene editing therapies. *Nat. Commun.* **9**, 9–11 (2018).
113. Netherlands Code of Conduct for Research Integrity. (2018). doi:10.17026/dans-2cj-nvwu
114. Responsible animal testing - Research - Utrecht University. Available at: https://www.uu.nl/en/research/research-at-utrecht-university/quality-and-integrity/responsible-animal-testing. (Accessed: 13th February 2022)
115. Bar-Ephraim, Y. E., Kretzschmar, K. & Clevers, H. Organoids in immunological research. *Nat. Rev. Immunol. 2019 205* **20**, 279–293 (2019).