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Design of TOLERANT: phase I/II safety assessment of intranodal administration of HSP70/mB29a self-peptide antigen-loaded autologous tolerogenic dendritic cells in patients with rheumatoid arthritis

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ABSTRACT

Introduction In rheumatoid arthritis (RA), immunosuppressive therapies may achieve symptomatic relief, but do not induce long-term, drug-free remission. Meanwhile, the lifelong use of immunosuppressive drugs confers increased risk for malignancy and infections. As such, there is an unmet need for novel treatments that selectively target the pathogenic immune response in RA by inducing tolerance to autoantigens. Autologous cell therapy using antigen-loaded tolerogenic dendritic cells (tolDCs) aims to reinstate autoantigen-specific immunological tolerance in RA and could potentially meet this need.

Methods and analysis We report here the design of the phase I/II, investigator-initiated, open-label, dose-escalation trial TOLERANT. In this study, we will evaluate the intranodal administration of tolDCs in patients with RA that are in remission under immunosuppressive therapy. The tolDCs in this trial are loaded with the heat shock protein 70-derived peptide mB29a, which is an effective surrogate autoantigen in animal models of arthritis. Within this study, three dose-escalation cohorts (two intranodal injections of 5×10^6, 10×10^6 and 15×10^6 tolDCs), each consisting of three patients, are evaluated to identify the highest safe dose (recommended dose), and an extension cohort of nine patients will be treated with the recommended dose. The (co-)primary endpoints of this study are safety and feasibility, which we assess by the number of AEs and the successful production of tolDCs. The secondary endpoints include the immunological effects of the treatment, which we assess with a variety of high-dimensional and antigen-specific immunological assays. Clinical effects are exploratory outcomes.

Ethics and dissemination Ethical approval for this study has been obtained from the Netherlands Central Committee on Research Involving Human Subjects. The outcomes of the trial will be disseminated through publications in open-access, peer-reviewed scientific journals, scientific conferences and to patient associations.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Echo-guided administration of tolerogenic dendritic cells (tolDCs) into the inguinal lymph node ensures that the cells reach the intended site of action.
⇒ The use of cryopreserved tolDCs stored as single-dose aliquots facilitates trial logistics and planning and reduces production costs compared to fresh tolDCs for repeated administrations.
⇒ The choice of a broadly major histocompatibility complex class II -presented surrogate autoantigen as the target eliminates the need for human leucocyte antigen type as an inclusion criterion.
⇒ The selection of patients who are in remission under maintained immunosuppressive therapy allows the detection of potential treatment-induced rheumatoid arthritis flares but limits the potential to observe clinical effects.

Trial registration numbers NCT05251870; 2019-003620-20 (EudraCT); NL71296.000.20 (CCMO register).

INTRODUCTION

Rheumatoid arthritis (RA) is a debilitating autoimmune disease characterised by synovial inflammation of the joints. In recent decades, the prospects of patients with RA have improved significantly due to the availability of more effective drugs. However, these do not fundamentally correct the immune abnormalities that underlie RA. As a result, most patients depend on the long-term use of immunosuppressant drugs, including expensive biological and targeted synthetic disease-modifying antirheumatic drugs (DMARDs). RA results from a breakdown in immunoregulation, leading to the loss of tolerance

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towards antigens that are expressed in the joints. For example, cartilage-specific T cells are present both in healthy individuals and patients with RA. Yet, in healthy individuals these cartilage-specific T cells include regulatory T cells (Tregs), whereas in patients with RA they are predominantly proinflammatory effector T cells.[1] Therapeutic tolerance induction is a novel treatment strategy that aims to reinstate immunoregulatory responses in patients with RA and may open a path towards sustained drug-free remission.

Successful therapeutic tolerance in RA requires the induction and expansion of Tregs that are specific for disease-relevant antigens. Tregs are a specialised subset of T cells that can suppress local immune responses when activated through their antigen receptor and thus play important roles in establishing and maintaining self-tolerance.[2,3] Various subtypes of Tregs are recognised, including FoxP3+ thymus-derived natural Tregs, FoxP3+ peripherally induced Tregs and peripherally induced Treg that are FoxP3− such as Type 1 regulatory T cells (Tr1 cells). Where FoxP3+ Treg are thought important for initial tolerance induction, Tr1 cells are required to maintain long-term tolerance.[4]

Therapeutic tolerance in autoimmunity ideally targets the disease-causing antigen. However, the antigen-specificity of pathogenic autoreactive T cells in individual patients with RA is generally unknown and varies between individual patients with different human leucocyte antigen (HLA) haplotypes. This hampers the targeting of tolerogenic therapies towards relevant populations of autoreactive T cells. Several citrullinated peptides have been identified as disease-relevant T cell epitopes in patients that are hallmarked by shared-epitope HLA alleles and circulating anti-citrullinated protein antibodies (ACPA),[5,6] comprising approximately two-thirds of patients with RA. Yet, individual patients within this subgroup respond to different citrullinated autoantigens, and citrullinated peptide-specific T cell responses are absent in the remaining one-third of patients with RA that are ACPA negative. A more universally applicable strategy would be directed at T cells that recognise a surrogate autoantigen; a conserved, non-pathogenic self-antigen which is expressed in the inflamed joint and is naturally involved in immune regulation.[7]

HSP70 peptide mB29a as surrogate autoantigen in RA

The inducible heat-shock protein 70 (HSP70) is upregulated during conditions of cellular stress, such as chronic inflammation. Consequently, this protein is highly expressed in the inflamed joints of patients with RA but not, for example, in those of patients who have osteoarthritis.[8] HSP-specific T cells can be detected both in patients suffering from autoimmune diseases and in healthy individuals.[9,10]

Remarkably, increased frequencies of HSP-specific T cells are associated with remitting disease in juvenile idiopathic arthritis,[11,12] and HSP70-specific autoantibodies are inversely correlated with systemic tumor necrosis factor alpha in RA[13] and are exclusively seen in the relapsing-remitting form of multiple sclerosis (MS) and not in progressive MS.[14] These observations suggest that HSP-specific responses in autoimmune diseases may be beneficial.[15]

Using the murine proteoglycan-induced arthritis (PGIA) model, we previously showed that HSP-specific Tregs can control auto-inflammation against cartilage associated proteoglycan antigens.[16,17] Adoptive transfer of Tregs recognising the B29 epitope, a highly conserved 15-aminoacid peptide sequence of mycobacterial HSP70, inhibited PGIA both prophylactically[16,17] and therapeutically.[16] This effect was specific for B29 as ovalbumin-specific Tregs did not affect the disease. Depletion of the transferred B29-specific Tregs abrogated the therapeutic effect. As such, the HSP70-derived B29 peptide is a potential surrogate autoantigen for immune regulation; B29-specific Tregs can control inflammation targeted towards different autoantigens.

B29-specific T cells are highly cross-reactive to the mammalian homologues mB29a and mB29b.[18] *In vitro* studies using a human lymphoblast cell line showed that mammalian B29 peptides are naturally presented epitopes in the context of major histocompatibility complex class II.[19] Using peptide binding assays, we previously demonstrated that B29 variants are moderate to high-affinity binders to most major HLA class II supertypes, notably including the RA-associated HLA-DR1 and HLA-DR4.[18] Consequently, B29-specific T cells are detected in low numbers in peripheral blood mononuclear cells (PBMCs) from both healthy individuals and patients with RA.[9] We hypothesise that increasing the number and activity of B29-specific Tregs through tolerogenic immunotherapy may aid in restoring immunological tolerance towards pathogenic autoantigens in patients with RA.

Tolerogenic dendritic cells for B29-targeted tolerance induction

Antigen-specific Tregs can be induced and/or expanded *in vivo* using autologous tolerance-inducing or tolerogenic dendritic cells (tolDCs). We and others have previously demonstrated that adoptive transfer of antigen-loaded tolDCs,[20,21] including HSP70-treated DC,[22] results in increased numbers of antigen-specific Tregs and reduced disease scores in experimental models of arthritis in mice.

Harry *et al*[23] previously showed that monocytes of patients with RA may be differentiated into tolDCs using interleukin-4 (IL-4), granulocyte-macrophage colony-stimulating factor (GM-CSF), dexamethasone and 1α,25-dihydroxyvitamin D3 (Dex/VitD3 tolDCs). Thus generated tolDCs have a characteristic phenotype as CD11c+HLADR+ cells with low expression levels of the DC maturation marker CD83 and co-stimulatory molecules CD80 and CD86, low CCR7 expression and high expression of toll-like receptor 2 and IL-10,[23,24] which is maintained under proinflammatory conditions.[23] Co-culture of PBMC from patients who had arthritis with autologous Dex/VitD3 tolDCs loaded with HSP peptides,

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including B29, resulted in an induction of Lag3+CD49b+, IL-10 producing Tr1-like regulatory T cells and a reduction of Th1 and Th17 cells.[9]

In addition to Dex/VitD3 tolDC, several protocols for good manufacturing practice (GMP)-compliant manufacturing of tolDCs have been developed.[25] Such tolDCs, loaded with disease-relevant autoantigens, have been studied for safety using different administration routes in various autoimmune diseases, ie, RA,[26 27] MS,[28] and type 1 diabetes,[29] with several other phase I/II clinical trials currently ongoing (table 1). The results thus far are encouraging. In each of these trials, tolDCs were well tolerated, and autoimmunity did not appear exacerbated by the treatment. Moreover, several of the studies reported some indications of beneficial immunological and/or clinical effects.[27–30] TolDCs that are not antigen loaded are also investigated in clinical trials, especially in the context of organ transplantation, and are elaborated on in recent reviews.[31 32]

Which administration route is most effective remains unclear and may depend on the characteristics of the tolDCs as well as the indication. To date, no results have been published for intranodal tolDC therapy, although a comparative study for intranodal and intradermal administration of tolDCs in MS is currently ongoing in two linked phase I trials.[33] In oncology trials, however, only a minute proportion of proinflammatory DC reach a lymph node when intradermally administered, with the remaining cells retained and likely dying at the injection site.[34] This was despite the high expression of the lymph node homing receptor CCR7 by proinflammatory DC. These observations are highly relevant for Dex/VitD3 tolDC, which lack CCR7 and are therefore even less prone to migrate to the lymph node. We therefore hypothesise that intranodal injection may be the best-suited route of administration for Dex/VitD3 tolDCs.

In the TOLERANT study, we are the first to investigate the safety, tolerability and feasibility of intranodal therapy with autologous Dex/VitD3, mB29a-loaded, tolDCs ($tolDC_{B29}$) in patients with RA.

METHODS AND ANALYSIS

Study design

The TOLERANT trial is an investigator-initiated, phase I/II, open-label, dose-escalation multi-centre clinical trial with 20 weeks of follow-up. A total of 18 patients with RA that are in stable remission or low-disease activity under DMARD treatment will be enrolled into the study. The study encompasses three dosage discovery cohorts of at least three patients each, as well as an extension cohort of up to nine patients at the identified recommended dose.

Patient screening, inclusion, treatment and follow-up are conducted at the University Medical Center Utrecht (UMCU), the Netherlands. Leukapheresis and manufacturing of the $tolDC_{B29}$ cellular product are performed by the GMP-licensed manufacturer Radboud university medical centre (Radboudumc) in Nijmegen, the Netherlands. Patient recruitment has started in August 2021 and is anticipated to end in 2024.

Primary objectives

The primary objectives of TOLERANT are to determine safety, tolerability and feasibility of $tolDC_{B29}$ therapy in RA. To determine the safety and tolerability of intranodal $tolDC_{B29}$ administration, we record the occurrence and severity of adverse events (AEs), with specific emphasis on the occurrence of RA disease flares.

To assess the feasibility of GMP-grade manufacturing of $tolDC_{B29}$ from patient with RA apheresis starting material, we determine if the phenotype, potency, yield, viability, sterility and endotoxin content meet the release criteria as stated in the investigative medicinal product dossier (IMPD).

Secondary objectives

Our secondary objective is to investigate the immunological effects of $tolDC_{B29}$ administration. We will assess the impact of $tolDC_{B29}$ administration by extensive leucocyte and cytokine profiling. To measure antigen-specific effects in response to $tolDC_{B29}$ therapy, we will monitor the HSP70/B29 specific reactivity using quantitative and qualitative T cell assays.

Exploratory objectives

TOLERANT is not designed, nor powered, to assess (preliminary) clinical efficacy of $tolDC_{B29}$ therapy, in part due to the inclusion of patients with RA with quiescent disease. Still, we will monitor the effects on patient well-being and clinical parameters including autoantibody levels, disease activity scores and patient-reported outcomes to explore any indications for clinically beneficial effects.

Patient and public involvement

Patients with RA were consulted regarding acceptability of the frequency and nature of study procedures and were actively involved in writing the patient information brochures.

Study population

Eligibility and enrolment

The TOLERANT study population comprises of adult patients with RA with low disease activity or remission under stable medication for at least 3 months. We selected this patient population as we anticipate that the absence of active inflammation facilitates the induction of antigen-specific tolerance by tolDCs. Furthermore, this patient population permits the assessment of inadvertent RA flares as a potential treatment-related AE.

There are no further limitations on age, disease duration or medication, except the use of janus kinase (JAK) inhibitors and the use of high dose (>7.5 mg/day) or intra-articular glucocorticoids. Patients using JAK inhibitors are excluded from participation, as these drugs interfere with the signal transduction processes downstream of cytokine receptors. Cytokine receptor signalling is required for

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Table 1 List of clinical trials using antigen-loaded tolerogenic dendritic cells in autoimmune diseases

Indication	Number and route of administrations	Dose	TolDC type	Antigen	Number of patients	Phase and status	Outcome	Reference
Multiple sclerosis	Three intravenous administrations with 2-week interval	Dose escalating Low dose (50×10^6) Intermediate dose (150×10^6) High dose (300×10^6)	Dexamethasone tolDCs matured with IL-1β, IL-6, TNF-α and PGE2 for 24 hours	Peptide pool comprising seven myelin peptides for MS + $AQP4_{63-76}$ for neuromyelitis optica	Eight relapsing-remitting patients with MS, 4 neuromyelitis optica patients	Phase Ib, Completed	TolDC treatment was considered safe and well tolerated. Immunomonitoring showed increased production of IL-10 on antigen restimulation of PBMC and a reduction in memory CD8 and NK cells at week 12.	NCT02283671 TolDec-EM-NMO Zubizarreta *et al*[28]
	Three intravenous administrations with 2-week interval, Combination with low-to-moderate efficacy immunomodulatory drugs	Not reported	Dexamethasone tolDCs matured with IL-1β, IL-6, TNF-α and PGE2 for 24 hours	Peptide pool comprising seven myelin peptides	45 relapsing-remitting patients with MS	Phase II, Ongoing, recruiting	–	NCT04530318 TolDec-COMBINEM
	Six intradermal administrations; four injections every 2 weeks, after 8 weeks two injections every 4 weeks	Dose escalating Low dose (5×10^6) Intermediate dose (10×10^6) High dose (15×10^6)	Vitamin D3 tolDCs matured with IL-1β, TNF-α and PGE2 for 24 hours	Peptide pool comprising seven myelin peptides	Nine active patients with MS	Phase I, Ongoing, not recruiting	–	NCT02618902 MS-tolDCs Willekens *et al*[33]
		Dose escalating Low dose (5×10^6) Intermediate dose (10×10^6) High dose (15×10^6) + an additional cohort treated with IFN-β + highest well-tolerated dose of tolDCs	Vitamin D3 tolDCs matured with IL-1β, TNF-α and PGE2 for 24 hours	Peptide pool comprising seven myelin peptides	12 active patients with MS	Phase I, Ongoing, recruiting	–	NCT02903537 TOLERVIT-MS Willekens *et al*[33]
Type I diabetes	Two intradermal administrations with 4-week interval	Dose escalating Low dose (5×10^6) Intermediate dose (10×10^6) High dose (15×10^6)	Dexamethasone+Vitamin D3 tolDCs, matured with IL-1β, IL-6, TNF-α and PGE2 for 24 hours	Proinsulin peptide (C19-A3)	9 HLA-DRB1 04:01$^+$ patients with type I diabetes	Phase I, Completed	TolDC treatment was considered safe and well tolerated. Immunomonitoring showed a decline in pre-existing autoimmune responses to the Proinsulin peptide upto 3 years after therapy and a temporary decline in T cell responses to other islet autoantigens.	CCMO registry (Netherlands) NL48984.000.14 PlpepTolDC, D-sense trial Nikolic *et al*[29 30]
	Two intradermal administrations with 4-week interval	Not reported	Not reported	Proinsulin peptide (C19-A3)	seven patients with type I diabetes	Phase I, Ongoing, recruiting	–	NCT04590872 PlpepTolDC

Continued

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Table 1 Continued

Indication	Number and route of administrations	Dose	TolDC type	Antigen	Number of patients	Phase and status	Outcome	Reference
Rheumatoid arthritis	Single intradermal administration	Low dose (0.5–1×10^6) High dose (2–4.5×10^6)	NF-κB-inhibitor Bay 11–7082 tolDCs, no maturation	Peptide pool comprising four citrullinated peptides	18 ACPA$^+$ HLA-DRB1 SE$^+$ patients with RA	Phase I, Completed	TolDC treatment was considered safe and well tolerated. Immunomonitoring showed a reduction in effector T cells and an increased ratio of regulatory to effector T cells at 1 month after treatment.	Rheumavax Benham *et al*[27]
	Five subcutaneous administrations; three injections every 2 weeks, after 4 weeks two injections every 2 weeks	Low dose (5×10^6) High dose (15×10^6)	Not reported	Recombinant PAD4, RA33, citrullinated filaggrin and vimentin	12 patients with RA	Phase I, Completed	TolDC treatment was considered safe and well tolerated. Immunomonitoring showed a decrease in antigen-specific autoantibodies. An indication of dose-dependent clinical efficacy was reported only in patients with autoantibodies.	CRiS (South-Korea) KCT0000035 CreaVax-RA Young Bin Joo *et al*. ACR 2014 abstract[41]
	Single intra-articular administration	Dose escalating Low dose (1×10^6) Intermediate dose (3×10^6) High dose (10×10^6)	Dexamethasone+Vitamin D3 tolDCs, matured with 1 µg/mL MPLA for 20 hours	Autologous synovial fluid	Nine patients with RA with active disease	Phase I, Completed	TolDC treatment was considered safe, feasible and acceptable to patients. Immunomonitoring did not reveal consistent immunomodulatory effects in peripheral blood.	NTC01352858 AuToDeCRA Bell *et al*[26]

ACPA, anti-citrullinated protein antibodies; MS, multiple sclerosis; RA, rheumatoid arthritis; tolDCs, tolerogenic dendritic cells.

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Table 2 TOLERANT inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Adult aged 18 years or older	Intramuscular or intra-articular glucocorticoid injection during 12 weeks prior to inclusion
Diagnosis of rheumatoid arthritis according to ACR or EULAR classification criteria	Use of JAK inhibitors or more than 7.5 mg/day of glucocorticoids
Stable dose of any combination of DMARDs and glucocorticoids, with exception of those drugs that are part of the exclusion criteria	Ongoing active or chronic infection, excepting fungal nail infection
Disease in remission or in low disease activity, measured by DAS28 <3.2 for at least 12 weeks	Recent infection requiring hospitalisation or class IV antibiotics within 6 weeks of baseline
Able and willing to give informed consent and to comply with the study protocol	Recent immunisation with live vaccine within 6 weeks of baseline
	History of malignancy, excepting treated basal cell carcinoma of the skin
	Major surgery within 8 weeks of baseline or planned within 12 weeks from baseline
	Pregnancy, individuals planning to become pregnant within the study period or individuals that are breastfeeding
	Individuals of childbearing potential unwilling to use adequate contraception for the duration of the study
	Hb <6 mmol/L; neutrophils $<2.00\times10^9$/L; thrombocytes $<150\times10^9$/L; ALAT or ALP >2× upper limit of normal; renal insufficiency (clearance <60 mL/min) at screening visit
	Poor venous access or medical condition precluding leukapheresis
	Any other serious or unstable co-morbidity deemed unsuitable for inclusion by the principal investigator
	Recent use of any other investigational medicinal products within 30 days prior to study entry

DAS28, disease activity score of 28 joints; DMARDs, disease-modifying antirheumatic drugs; JAK inhibitors, Janus Kinase inhibitors.

the manufacturing of tolDC$_{B29}$ and plays a central role in the induction of antigen-specific Tr1 cells, the proposed mechanism of action of Dex/VitD3 tolDCs.[9] We therefore strongly suspect that JAK inhibitors may interfere with the tolDC$_{B29}$ differentiation process and their *in vivo* potency. Indeed, a recent study by Vogel *et al* supports the notion that JAK signalling may be required for peripheral tolerance induction *in vivo*.[35] High dose glucocorticoids affect Treg and Tr1 cells in a context-dependent fashion,[36] and therefore may impact the *in vivo* effects of tolDC$_{B29}$ treatment. Patients are included after written informed consent and when the inclusion and exclusion criteria are met (table 2).

Dose-escalation procedure and data safety monitoring

Both the dosage discovery phase and the extension phase of this study are overseen by an independent data safety monitoring board (DSMB) consisting of international experts in the fields of tolDC therapy and biostatistics. This DSMB will assess participant safety at regular intervals during the conduct of the trial and advise on dose-escalation decisions as well as on the recommended dose for the extension phase.

The dosage discovery phase follows a 3+3 dose-escalation design comprising of three dosage cohorts and three dosages. Each patient will receive two intranodal injections of 5×10^6, 10×10^6 or 15×10^6 viable tolDC$_{B29}$ with a 4-week interval. These cell numbers are similar to the dosages that are intranodally administered in oncology trials[37,38] and in an ongoing tolDC trial in MS.[33] Allocation of participants in dosage cohorts is based on the order of inclusion into the trial. The decision to start inclusion in a new dose cohort, continue within the same cohort, or stop further dose escalation will be made after all subjects in the cohort have completed at least 8 weeks of follow-up after the first tolDC$_{B29}$ administration and when the safety and tolerability results thus far obtained are satisfactory to the DSMB (figure 1A). Enrolment and/or dose escalation will be halted if one or more of the following occurs at that dose or previous dosages:

- Two or more subjects experience an AE of at least moderate severity that is possibly related to the intervention.
- One or more subjects experience a severe AE that is possibly related to the intervention.

In such event, the DSMB will evaluate the safety results and advise on the continuation of the trial. On conclusion of the dose-escalation phase, the DSMB will advise on the recommended dose for the extension study, based on

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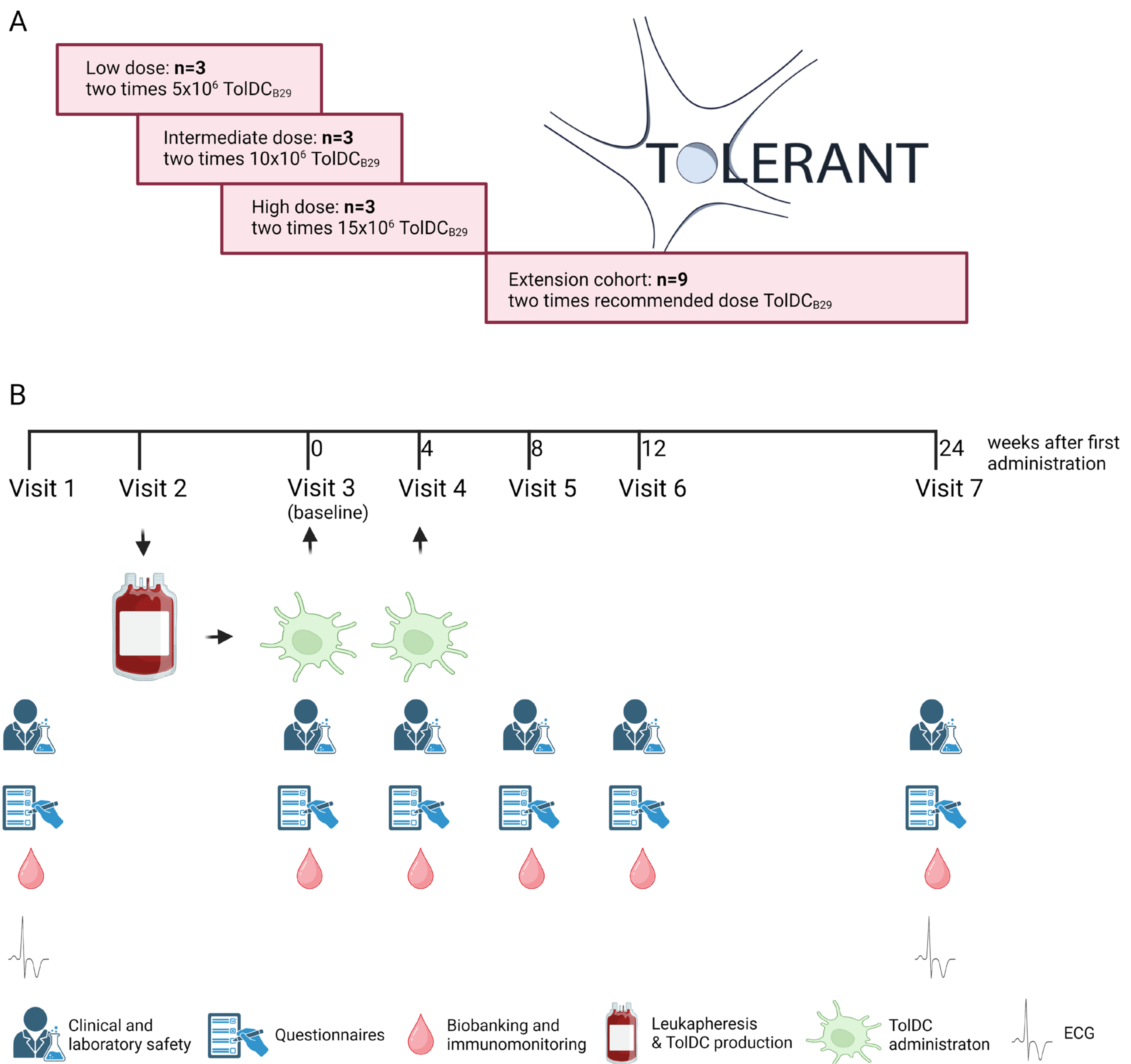

Figure 1 Overview of the design, dosing and procedures in the TOLERANT trial. (A) Outline of the cohorts and respective dosing. The ability to dose escalate will be evaluated when the last patient within a dose cohort has completed the 8-week follow-up timepoint. The recommended dose is determined after all patients within the dose-escalation study have completed 24-week follow-up. (B) The treatment will start at Visit 3 (baseline), at least 5 weeks after leukaferesis (Visit 2). Patients will receive two tolDC$_{B29}$ administrations with a 4-week interval and are followed up to 24 weeks after start of treatment. Clinical and laboratory safety assessments, questionnaires and blood for immunomonitoring are collected at Visit 1 and Visits 3–7. An ECG is taken at Visit 1 and Visit 7. The image was generated using Biorender. tolDC, tolerogenic dendritic cell.

combined safety and immunological effectivity data thus far obtained.

Study medication

Autologous tolDCs are generated from CD14$^+$ monocytes using Dex/VitD3 according to the NewCastle protocol[23] and loaded with the mB29a peptide. We manufacture tolDC$_{B29}$ in adherence to the principles of GMP as laid down in Directive 2003/94/EC. The designated GMP facility for this trial is the Radboudumc, Nijmegen, the Netherlands.

Manufacturing and preparation of tolDC$_{B29}$

Starting from leukapheresis product, CD14$^+$ monocytes are selected using the CliniMACS Prodigy system (Miltenyi Biotec). The enriched CD14+fraction is cultured for 7 days in GMP DC medium (CellGenix) supplemented with GM-CSF and IL-4. On day 3 of culture, fresh medium

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containing GM-CSF, IL-4 and dexamethasone is added. DCs are further differentiated into matured tolDCs and antigen loaded by addition of dexamethasone, vitamin D3, monophosphoryl lipid A and the mB29a peptide (VLRVINEPTAAALAY) on day 6 of culture. The resulting tolDC$_{B29}$ product is harvested on day 7 of culture and cryopreserved at −150°C. Each vial contains two times the number of tolDC$_{B29}$ required for a single administration, to mitigate the inevitable loss of cells during reconstitution.

Quality control testing for release consists of: sterility testing, endotoxin testing, cell count, viability, phenotypic characterisation by flow cytometry and T cell responsiveness in an allogeneic mixed leucocyte reaction. Additionally, tolDC$_{B29}$ are more extensively phenotyped and tested for their capacity to dampen autologous mature moDC-mediated T cell activation in a competitive allogeneic mixed leucocyte reaction. These additional characterisations are not part of the tolDC$_{B29}$ criteria for batch release.

Vials of cryopreserved and batch-released tolDC$_{B29}$ are shipped to the cell therapy facility of the UMCU clinical pharmacy (Utrecht, The Netherlands) by courier at −190°C, where the vials are stored in liquid nitrogen until reconstitution for administration. For reconstitution prior to administration, a single vial of tolDC$_{B29}$ is thawed. The cells are washed three times to remove cryoprotectant DMSO, and an aliquot is counted in trypan blue for quantification and viability assessment. The remaining cells are reconstituted in a syringe at the required cell density of viable cells for the intended dose in 150–200 µL of 0.9% NaCl, 25% Albuman (Sanquin, the Netherlands). An air bubble of ~150 µL is included in the syringe to fill the dead volume in the needle on injection and thus ensure complete administration of the product.

Product characteristics

Clinical grade tolDC$_{B29}$ are sterile HLADR$^+$ CD11c$^+$ cells (>90%) with tolDC morphology and a low level of CD83 expression (<25%), low T cell stimulatory capacity in a mixed lymphocyte reaction (potency index <10.0), and contain <7 EU/mL endotoxin. The potency index is defined as the fold increase in T cell activation after adding tolDC$_{B29}$ to a culture of allogeneic peripheral blood lymphocytes.

The required viability at time of cryopreservation and after reconstitution are >80% and >60%, respectively. The specified cell numbers depend on the intended dose for each individual patient within the dose-escalation regime; a 25% deviation from the intended dose is permitted, as is reallocation of patients to a lower dose cohort if the intended dose is not met.

Out of specification (OOS) products may be administered in this study in accordance with EudraLex Volume 4 GMP, part IV. We estimate the rate of OOS product manufacturing at 10%, based on the frequency of OOS products in the AuToDeCRA trial, which used the same tolDC generation protocol.[26]

Trial intervention: tolDC$_{B29}$ administration

Each participant enrolled into TOLERANT will receive two autologous tolDC$_{B29}$ doses of each 150–200 µL with a 4-week interval. Both tolDC$_{B29}$ doses will be injected ipsilaterally into an inguinal lymph node by a trained radiologist under echographic guidance. Echographic images are captured to demonstrate successful administration. After administration, participants remain at the day unit for 2 hours of observation, during which the patient's vital signs will be monitored at regular intervals. The administration site is inspected for bleeding, bruising and other abnormalities prior to patient discharge. Patients are followed-up at 4, 8 and 20 weeks after administration of the second tolDC$_{B29}$ dose.

Primary outcome measures

Safety

The safety of tolDC$_{B29}$ is defined by the occurrence and severity of potentially treatment-related AEs. AEs will be recorded using clinical and non-clinical outcome measures. To this end, we will perform physical examinations at each patient visit. RA disease activity will be monitored using the disease activity score of 28 joints (DAS28), which is the gold standard for grading RA disease activity and is calculated using the tender- and swollen joint counts of 28 joints, blood C reactive protein (CRP) level or erythrocyte sedimentation rate (ESR) and patient self-assessment (visual analogue scale, VAS). The non-clinical outcome measures include cardiac, haematological, hepatic and renal safety, as measured by ECG, blood cell frequencies, chemistry (ALAT, ALP, Creatinine) and urinalysis (table 3).

Feasibility

To evaluate the feasibility of tolDC$_{B29}$ treatment for patients with RA, we will evaluate the success of tolDC$_{B29}$ production within this trial. This includes the production of sufficient numbers of tolDC$_{B29}$ for two administrations of each intended dose, and compliance of the final product to the release criteria as specified in the IMPD. We will evaluate tolDC$_{B29}$ manufacturing for each patient based on:

- The number of tolDC$_{B29}$ manufactured in relation to the intended dose and to the number of monocytes at the start of culture.
- The phenotypic characteristics and purity of tolDC$_{B29}$ in relation to the release criteria defined in the IMPD.
- The potency of tolDC$_{B29}$ according to the release criteria defined in the IMPD.
- The occurrence and nature of OOS products.

In our analysis, we include both the viability and the live cell dose of the final reconstituted product.

Secondary outcome measures

The secondary aim of the study is to evaluate tolDC$_{B29}$-related changes in the immune cell and autoantibody profiles of patients with RA. To this end, we will collect peripheral blood at multiple timepoints before and after

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Table 3 Calendar detailing procedures and assessments for an individual patient throughout the study

	Visit 1 inclusion/screening	Visit 2 leukapheresis	Visit 3 treatment I+baseline	Visit 4 treatment II+monitoring	Visit 5 monitoring	Visit 6 monitoring	Visit 7 monitoring
	Week −7 to −10	Week −3 to −6	Week 0	Week 4	Week 8	Week 12	Week 24
Study information	X						
Informed consent	X						
Inclusion/exclusion criteria	X		X				
Medical history	X						
Physical examination	X		X	X	X	X	X
Vital signs	X		X	X	X	X	X
Routine blood exam*	X		X	X	X	X	X
Blood magnesium	X						
Blood infectious screening†	X						
Blood autoantibodies‡	X		X	X	X	X	X
Urinalysis	X		X	X	X		
ECG	X						X
Joint count	X		X	X	X	X	X
Pregnancy test	X						
Questionnaires	X		X	X	X	X	X
HLA-DR/DQ/DP typing	X						
Leukapheresis		X					
$TolDC_{B29}$ administration			X	X			
Recording (S)AEs			X	X	X	X	X
Immunomonitoring (blood)	X		X	X	X	X	X

*Creatinine, ALAT, CRP, ESR, Hb, leucocytes, thrombocytes.
†human immunodeficiency virus, hepatitis B, hepatitis C, hepatitis E, Treponema pallidum haemagglutination assay, human T-lymphotropic virus type 1+2.
‡RF, ACPA, ANA.
ACPA, anti-citrullinated protein antibodies; ALAT, alanine aminotransferase; ANA, antinuclear antibodies; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; RF, rheumatoid factor; tolDCs, tolerogenic dendritic cells.

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treatment (figure 1B) and perform extensive immunomonitoring. At each time point, we will collect 60 mL sodium heparin blood, from which we will isolate and cryopreserve plasma and PBMC, and 1 mL EDTA blood which we will cryopreserve as whole blood for epigenetic analysis.

Immunomonitoring

Our immunomonitoring strategy includes B29-specific assays as well as high-dimensional immune profiling. We will perform immunomonitoring assays on longitudinal samples of individual patients simultaneously in a per-cohort fashion. In doing so, we will limit effects of inter-assay variation on our outcomes.

To measure effects of tolDC$_{B29}$ on B29-specific T cell responses, we will measure antigen-specific T cell reactivity at all time points before, during, and after treatment. We anticipate that this will allow us to map the presence of circulating B29-specific T cells in response to tolDC$_{B29}$ over time. Additionally, we will address responses to a pool of unrelated pathogen-derived peptide antigens (CEFTA), to ex vivo assess off-target effects of tolDC$_{B29}$ and potential risk for opportunistic infections. Furthermore, for selected ACPA positive participants, we will assess the responsiveness towards a pool of 10 citrullinated peptides to explore effects on RA-relevant T cell responses.

To gain additional insights in the immunological effects of tolDC$_{B29}$ treatment, we will perform high-dimensional analysis techniques including spectral flow cytometry and single-cell RNA sequencing. In these analyses, we study the proportions and activation status of a variety of immune cells, especially focussing on CD4 T cells. Also, autoantibody levels and memory B cell analysis will be performed. Whole blood epigenetic analysis will be performed by Epimune GmbH, allowing quantification of absolute numbers of immune cell lineages, including FoxP3+ Tregs.

Exploratory outcome measures

TOLERANT is not designed to study effects of tolDC$_{B29}$ on clinical outcomes. Our selected patient population is characterised by low disease activity/remission, which makes it unlikely to achieve observable clinical improvements. Nonetheless, we will explore the effects of tolDC$_{B29}$ on RA-relevant clinical parameters. To this end, we will analyse longitudinal samples from all timepoints for changes in autoantibody levels (RF, ANA, ACPA), proinflammatory and anti-inflammatory mediators and acute phase measures such as CRP and ESR. Furthermore, we will assess effects on disease activity based on changes in the DAS28 score and on patient-reported outcomes.

Patient-reported outcomes

Patients will be asked to fill out the following questionnaires during screening, baseline and follow-up visits to document changes in their functional ability and quality of life:

- Dutch consensus Health Assessment Questionnaire[39]: functional ability questionnaire specifically developed to support RA research.
- EuroQol-5D-5L[40] generic quality of life instrument.

Patients will be asked to provide a rating of the pain and physical discomfort due to arthritis at every visit (VAS ranging from 0 to 100).

Data management and monitoring

To ensure data integrity and good clinical practise (GCP)-compliance, data will be collected in an electronic case report form (eCRF, Castor) in a pseudonymised fashion. This includes AE reports, patient questionnaires and limited tolDC$_{B29}$ manufacturing data. Individual data points are entered into the eCRF by authorised personnel from source documents, that is, electronic patient records, DAS28-assessment forms and manufacturing and QC logs. Patient-reported outcomes are submitted directly into the eCRF. Source data and GCP compliance will be regularly verified by an external monitor affiliated to a clinical research organisation. Data will be handled according to FAIR data standards to enable future reuse.

ETHICS AND DISSEMINATION

Patient protection

This study (protocol V.3.1, 06-07-2022) was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO, NL71296.000.20) and will be conducted in agreement with the Declaration of Helsinki and the Dutch Medical Research Involving Human Subjects act ('Wet medisch-wetenschappelijk onderzoek met mensen (WMO)'). We will adhere to the ICH Harmonised Tripartite Guideline for Good Clinical Practise. We ensure that patient anonymity is maintained, according to the Dutch Personal Data Protection Act and the EU General Data Protection Regulation.

Informed consent

We will inform participants using a patient information letter on the aim of the study, the involved procedures, and potential AEs or hazard to which they may be exposed. We will also inform them that several authorised individuals other than their treating physician may access their medical records for trial purposes, and that biomaterial or data may be exchanged with parties outside of the EU where different privacy laws apply. Patients are included no less than 2 weeks after first being informed and will have extensive opportunity to request further information prior to inclusion.

Dissemination

We will publish the results of this clinical trial in a peer-reviewed open-access journal. Furthermore, we will present our findings to our peers at national and international scientific meetings and to patients, other stakeholders and the general public through press releases and official social media accounts in collaboration with

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the Dutch Arthritis Society. Data requests for reuse may be submitted to the researchers after completion of the trial and publication of the results.

DISCUSSION

Several phase I/IIa clinical trials to date have reported on the safety of autologous tolerogenic DCs for administration to patients with immune mediated diseases. These previous trials primarily used tolDCs that were loaded with cocktails of disease specific autoantigens, which ensure the direct targeting of tolerance induction towards autoreactive T cells. However, the antigen-specificity of pathogenic autoreactive T cells for patients is generally unknown and varies between individual patients, in part due to HLA-restriction. As such, tolDC therapy may be limited to selected patients with known antigen-specific T cell responses.

In the current dose-escalation trial, we therefore investigate tolDCs that are loaded with the surrogate autoantigen mB29a, which is a HSP70-derived peptide that can be presented by all major HLA variants. We propose that the B29 antigen is more universally applicable and would therefore enable tolDC therapy to a wide variety of patients with RA and potentially other disease indications.

While the B29 antigen offers a promising approach to deal with limitations regarding antigen specificity, application of tolDC therapy in future clinical practise faces numerous other challenges and unknowns. For example, the effects of systemic immunosuppressive drugs and proinflammatory mediators on the ability to generate optimally functioning tolDCs are poorly understood. Also the possible interactions of these drugs and inflammatory mediators with the *in vivo* functional potency of these cells are unknown. In the current study, we have therefore elected to include patients that are in stable remission under DMARD therapy and excluded patients that use JAK inhibitors and high-dose corticosteroids. We look forward to interaction studies that may provide a better insight into the patient populations that are most eligible for tolDC therapy.

In conclusion, our protocol aims to restore tolerance in patients with RA using tolDCs loaded with the surrogate autoantigen mB29a. Our trial will provide insights into the feasibility of clinical grade tolDC production from medicated patients with RA and the optimal dosing of tolDCs for intranodal administration. Through extensive immunomonitoring, we will detail the immunological effects of $tolDC_{B29}$. Combined, the results will guide the design of future phase II studies to investigate the efficacy of this widely applicable tolerogenic treatment modality in RA.

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Contributors AJS, FB, WvE and JMvL conceived and designed the study. AJS, BK, E-JB, GS and JMvL participated in the logistical planning of the study. PW provided statistical support. GS, TD-dB and IJMdV established the to IDC manufacturing process at Radboudumc and wrote the IMPD. AJS, E-JB, LL, AdG and FB critically reviewed the IMPD. AJS wrote the initial draft of the manuscript. All co-authors reviewed draft versions of this paper and approved the final manuscript. JL is the guarantor for this study.

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Competing interests Trajectum Pharma B.V. has licensed IP relating to the B29 antigen from Utrecht University. AJS, FB, WvE and PL have a financial interest in Trajectum Pharma B.V. The other authors declare that they have no conflict of interest.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Methods section for further details.

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