Universitat Autònoma de Barcelona

Identification of differential biological mechanisms between pregnancy and postpartum in Rheumatoid Arthritis

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Doctoral Thesis

Identification of differential biological mechanisms between pregnancy and postpartum in Rheumatoid Arthritis

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Abbreviations

RA	Rheumatoid Arthritis
HLA	Human Leukocyte Antigen
GWAS	Genome wide association studies
ACR	American College of Rheumatology
EULAR	European League Against Rheumatism
ACPA	Anti-citrullinated antibodies
RF	Rheumatoid Factor
APC	Antigen presenting cells
ACPA	Anti-cyclic citrullinated peptide antibodies
FDA	Food and Drug Administration
PLLR	Pregnancy and Lactation Labelling Rule
NSAID	Non-steroidal anti-inflammatory drugs
COX	No selective cyclooxygenase
GC	Glucocorticosteroids
DMARDs	Disease modifying anti-rheumatic drugs
MTX	Methotrexate
LEF	Leflunomide
OTIS	Organization of Teratology Information Specialists
SSZ	Sulfasalazine
HCQ	Hydroxychloroquine
AZA	Azathioprine
CSA	Cyclosporine A
TNFα	Tumour necrosis factor α
FcRn	Neonatal Fc receptor
ETN	Etanercept

IFX	Infliximab
ADA	Adalimumab
CZP	Certolizumab Pegol
GOL	Golimumab
TCZ	Tocilizumab
SARI	Sarilumab
ABA	Abatacept
RTX	Rituximab
ANAK	Anakinra
TOFA	Tofacitinib
BARI	Bariticinib
EVT	Extravillous trophoblast
GFR	Glomerular filtration rate
GnRH	Gonadotrophin-releasing hormone
CRH	Corticotrophin-releasing hormone
ACTH	Adrenocorticotrophic hormone
dNK	Decidual Natural Killers
LDA	Low disease activity
ESR	Erythrocyte sedimentation rate
CRP	C-Reactive Protein
PBMCs	Peripheral blood mononuclear cells
INF	Interferon
sc-qPCR	Single-cell qPCR
scRNA-seq	Single-cell RNA sequencing
CIA	Chronic inflammatory arthritis
DAS28	Disease Activity Score 28

CDAI	Complex Disease Activity Index
SDAI	Simplify Disease Activity Index
NPJ	Number of 28 painful joints
NSJ	Number of 28 swollen joints
PhGV	Physician global evaluation
PtGV	Patient global evaluation
PPV	Patient pain evaluation
RT	Room temperature
HTO	Hash Tag Oligonucleotide
QC	Quality control
GEM	Gel beads in Emulsion
UMI	Unique Molecular Identifier
PCA	Principal component analysis
PCs	Principal components
LISI	Local Inverse Simpson’s Index
UMAP	Uniform Manifold Approximation and Projection
K-NN	k-Nearest Neighbour
DEG	Differentially expressed gene
HC	Healthy control
cm	Central memory
em	Effector memory
n	Naïve
cDCs	Conventional Dendritic cells

Index

Resum

L'embaràs a l'Artritis Reumatoide (AR) proporciona un model únic per l'estudi dels mecanismes biològics de la malaltia. Durant l'embaràs, generalment, es produeix una interrupció transitòria de l'activitat de l'AR que es reprèn després del part. En conseqüència, l'estudi dels patrons moleculars associats als canvis en l'activitat de la malaltia durant l'embaràs podria proporcionar una informació essencial sobre les principals vies biològiques de la malaltia. Aquí perfilem els transcriptomes de 8.604 cèl·lules mononuclears de sang perifèrica d'alta qualitat (CMSP) amb sc-RNAseq de dues dones amb AR a la setmana 30 de gestació i la setmana 8 del postpart. Es van estudiar les CMSP després de cultiu in vitro durant 48 hores amb i sense estimulació de cèl·lules T. El present estudi és el primer atles amb single cell de CMSP a AR durant l'embaràs i el postpart. L'anàlisi inicial agrupat de les cèl·lules va dividir-les en cèl·lules Natural Killer i T no estimulades, cèl·lules T estimulades, cèl·lules mieloides i cèl·lules B. Aquests clústers es van subdividir en 35 clústers més específics. Hem detectat un gradient d'activitat efectora al compartiment de cèl·lules T no estimulades. Al postpart, l'anàlisi de la composició cel·lular va revelar, en les cèl·lules no estimulades, un augment de les cèl·lules T naïf en repòs i de les cèl·lules T $CD8^+$ que produeixen $GZMA^+$ $GNLY^+$. En les cèl·lules estimulades, l'anàlisi va revelar un augment de cèl·lules de memòria central T activades amb alt contingut ribosòmic i les cèl·lules T naïf activades recentment. A les cèl·lules de l'embaràs, hem trobat una resistència immune a l'estimulació cel·lular en els $CD14^+$ monòcits i $CD16^+$ $CD56^{dim}$ NK. Finalment, l'expressió gènica diferencial en els diferents tipus cel·lulars va revelar gens potencialment relacionats amb la modulació de la malaltia durant l'embaràs. També gens que podrien estar implicats en l'embaràs i l'oncogènesi i, gens implicats en RA i preeclampsia.

Abstract

Pregnancy in Rheumatoid Arthritis (RA) provides a unique model to study the biological mechanisms of the disease. In RA pregnancy, there is generally a transitory interruption of disease activity that is resumed after delivery. Consequently, the study of the molecular patterns associated with changes in disease activity during pregnancy could provide essential information on the main biologic pathways in the disease. Here we profile the transcriptomes of 8,604 high quality peripheral blood mononuclear cells (PBMCs) with sc-RNAseq from two RA women at the 30^{th} week of gestation and at the 8^{th} week of postpartum. PBMCs were studied after *in vitro* culture for 48 hours with and without T cell stimulation. The present study is the first single-cell atlas of PBMCs of RA during pregnancy and postpartum. An initial cell clustering analysis divided the cells into NK and unstimulated T cells, stimulated T cells, myeloid cells and B cells. These clusters could be further subdivided into 35 highly specific clusters. We detected an effectorness gradient in the unstimulated T cell compartment. In postpartum, cell composition analysis revealed an increase in unstimulated resting naïve T cells and $GZMA^{+}$ $GNLY^{+}$ producing $CD8^{+}$ T cells. In stimulated cells, the analysis revealed an increase in activated T central memory cells with high ribosomal content and recently activated naïve T cells. In cells from pregnancy, we found an immune resistance to cell stimulation in $CD14^{+}$ monocytes and $CD16^{+}$ $CD56^{dim}$ NKs. Finally, differential gene expression between the different cells clusters revealed genes related possibly associated to modulation of disease during pregnancy. Also genes that could be involved in pregnancy and oncogenesis and genes involved in RA and preeclampsia.

1. Introduction

1.1. Rheumatoid Arthritis

1.1.1. Concept and epidemiology

Rheumatoid Arthritis (RA) is a systemic autoimmune disease, characterized by chronic inflammation of the synovial membrane of the diarthrodial joints (1). The main manifestation is joint involvement, but as a systemic disease can present multiple extra-articular manifestations (2).

RA is the most prevalent chronic inflammatory arthritis, affecting approximately 1% of the adult population with an estimated incidence between 20-25 cases per 100,000 inhabitants in North American and European countries (3).

RA is ~3 times more frequent in women than men and has a peak incidence between 40-50 years of age (1). Due to this, the course of this disease frequently coincides with the patient's reproductive age. The exact mechanism by which gender affects susceptibility for the development of RA is not known but female sex hormones may play a role (1). For example, in the SERA (Studies of the Etiology of Rheumatoid Arthritis) cohort, the use of contraceptives was associated with a decreased risk of seropositive RA regardless of age, educational level and smoking status (3,4). Moreover, a protective effect or decrease in inflammatory activity has been widely described during pregnancy. Also, increased onset risk of the disease during the postpartum period is described in the literature (4).

1.1.2. Aetiology

RA is a complex disease of unknown aetiology influenced by genetic and environmental risk factors (1). Genetic factors play a fundamental role in the risk of developing RA. It is estimated that these factors contribute approximately to 50-60% of the risk (5). Thanks to genome wide association studies (GWAS), multiple risk loci have been identified, although most of them have small effect size. Classically, the *HLA * DRB1* locus has been considered the most relevant genetic risk factor in RA. Variations in this gene are involved in a third of the genetic susceptibility of the disease. *HLA * DRB1* risk alleles *(* 0401, * 0404, * 0405, * 0408, * 0413, * 0101, * 0102, * 1001, * 1402*) encode an HLA (Human Leukocyte Antigen, HLA) class II molecule that has a highly conserved amino acid sequence

(QKRAA) located in the third hypervariable region of the β chain known as epitope sharing (ES). This genetic variation presents the most robust association with the RA susceptibility (6,7).

In recent years, an increased number of loci associated with the RA risk have been identified. For example, the loci *PTPN22* (type 22 non-receptor protein tyrosine phosphatase), *STAT4* (signal transducer and transcription activator 4), *TRAF1-C5* (factor associated with tumour necrosis factor), *PADI4* (peptidyl arginine deiminase type IV) and *CTLA4* (cytotoxic T lymphocyte antigen 4). These loci, however represent only 3-5% of the total genetic risk for developing RA (1,7). Also, a large group of genes with low effect sizes have also been associated with the genetic aetiology of RA. Many of this low-effect genes codify cytokines as well as T and B cell signalling processes (8). Also, epigenetic modifications like genomic DNA hypomethylation or the modification of histones could be associated with the expression changes observed in proinflammatory genes in RA (9).

Finally, there are environmental factors which contribute to the expression of the disease in genetically susceptible individuals. Non-genetic factors associated with the risk of RA include tobacco, alcohol consumption, diet, female sex, infectious agents and periodontal disease associated with *Porphyromonas gingivalis* (1,10).

1.1.3. Physiopathology

RA is characterized by a symmetric polyarthritis and, in a large number of patients, by the presence of extra-articular manifestations like subcutaneous nodules, interstitial pneumopathy, pericarditis, neuropathy and vacuities. It is accepted, however, that it is a complex disease where triggering environmental factors interact with genetically predisposed individuals. This interaction ultimately leads to a loss of the immune system tolerance and the uncontrolled activation of the inflammatory cascade (11).

The activation of innate immune response is probably the first pathogenic process in RA, followed by protein citrullination and autoantigen recognition by antigen presenting cells (APCs) involving the adaptative immune response. This former group of cells of the innate immune system will then migrate to the secondary

lymphoid organs where they will present and activate T cells, recognizing these autoantigens. These activated T cells will subsequently activate B cells and/or migrate to the synovial membrane (12). During this initial stages of RA and in the subsequent disease chronic stages, the proinflammatory cytokines will be important autocrine and paracrine mediators (13,14).

The main characteristic of RA is the inflammation of the synovial membrane (synovitis) of diarthrodial joints (15). In this inflammatory process, different immune cell types including B and T lymphocytes, macrophages and mastocytes, invade the synovial membrane. This pathological process is orchestrated by the expression of high levels of chemokines and proinflammatory cytokines. Also, autoreactive T cells are one of the key players in this process. This subgroup of T cells are stimulated by myeloid and dendritic cells (i.e. APCs) in the synovial membrane by the release of amounts of proinflammatory cytokines (e.g. IL-12, IL-18 and IL-2) and by the binding to type II HLA molecules as well as CD80 and CD86 co-stimulatory molecules. These latter cell-surface molecules facilitate the interaction between APCs and T lymphocytes and the presentation of antigens.

Innate immune cells in RA synovium produce a diverse set of inflammatory cytokines like TNFα, IL1, IL6, IL12, IL15, IL18 and IL23 (15). Also, the activation of synovial fibroblasts in RA leads to their hyperproliferation and subsequent production of extracellular matrix metalloproteinases and cytokines. These processes are accompanied by neovascularisation due to the presence of tissue hypoxia and the production of pro-angiogenic factors like the vascular endothelial growth factor (VEGF). The end-stage result of all these molecular and cellular changes is the structural damage of the joint that is characteristic of RA (15).

The identification of biological pathways associated with disease can help explain the molecular mechanisms associated with the development of complex diseases. In a recent study by Zhang *et al*, a pathway-based GWAS was performed in order to identify the pathways associated with RA (16). The results from this study identified nine pathways associated with RA risk: focal adhesion, extracellular matrix-receptor interaction, calcium signalling, dopaminergic synapse, long-term potentiating, retrograde endocannabinoid signalling, glutamatergic synapse, cholinergic synapse and morphine addiction. In previous publications four of these

pathways (focal adhesion, extracellular matrix-receptor interaction, calcium signalling and dopaminergic synapse) had been described as high-risk pathways for RA.

1.1.4. Clinical manifestations

RA is characterized by pain and symmetrical swelling of the peripheral joints secondary to chronic inflammation of the synovium. Most patients have morning joint stiffness lasting more than an hour. This symptom can appear before pain and may be related to the accumulation of oedema fluid within inflamed tissues (17). Pain is typically due to constant inflammation occurring at rest (18).

RA characteristically affected joints include the carpals, proximal metacarpophalangeal and interphalangeal joints of the hands and metatarsophalangeal joints of the feet. RA can also affect other joints such as elbows, shoulders, knees, ankles, and hip joints. The distal interphalangeal joints are very rarely affected (19). In addition to synovitis produced in the joint, non-articular structures such as bone, tendons and ligaments can be affected in RA. Chronic and persistent synovium inflammation leads to irreversible joint destruction and deformity. In the long term and in cases in which the activity of the disease has not been controlled, marked deformities such as swan-neck fingers (flexion of the distal interphalangeal joint and hyperextension of the proximal interphalangeal joint), boutonniere deformation (flexion of the proximal joint and hyperextension of the distal interphalangeal joint) and subluxation of the metacarpophalangeal joints with ulnar deviation can be observed.

Axial skeleton involvement is almost limited to the cervical spine, particularly in the upper part where it can produce an atlantoaxial subluxation. It is important to highlight this clinical manifestation due to its frequency (17-86% in different series) and the outcomes that it may cause. This feature of the disease can occur asymptomatically or cause clinical manifestations that include headache, neck pain, paraesthesia or myelopathy (19).

The disease may begin gradually, initially affecting one or a few joints and progressing to polyarthritis with a more typical pattern (18). RA begins as a joint

disease of one or more joints, although it can also begin with extra-articular or non-articular involvement, such as local bursitis, tenosynovitis, carpal tunnel syndrome, a systemic presentation with diffuse polyarthralgia or polymyalgia (20). Due to its systemic condition, RA can present as general symptoms such as fatigue, myalgia, low-grade fever, weight loss and depression (18).

Likewise, RA can be associated to extra-articular manifestations (Table 1) that occur in 18-41% of patients (21). These manifestations can precede the onset of polyarthritis by weeks or months, so a targeted systemic examination should be performed to detect possible organ involvement (18).

Table 1. Extra-articular manifestations in rheumatoid arthritis according to the Malmö criteria, table adapted from (22).

	Extra-articular manifestations	
	Mild	**Severe**
Skin	Nodules Raynaud Phenomena	Petechiae Purpura Gangrene ulcers
Lung	Obliterative bronchiolitis Organizational pneumonia	Pleuritis Interstitial lung disease
Heart	Valvular disease Myocarditis Arrhythmias	Pericarditis Coronary vacuities Aortitis
Central Nervous Systen		Mono / polyneuritis multiplex CNS vacuities
Eyes	Secondary Sjögren Syndrome Sicca Syndrome	Episcleritis Scleritis
Haematological involvement		Felty Syndrome
Kidney		Glomerulonephritis Interstitial nephritis Amyloid deposits
Bone		Osteoporosis

1.1.5. Autoantibodies

As previously mentioned, RA is an autoimmune disease and its immunopathology involves autoantibodies that are capable of recognizing various non-organ specific autoantigens such as different components of cartilage, enzymes, nuclear proteins and citrullinated peptides (18). Due to the aberrant production of autoantibodies by autoreactive B cells, we can detect these proteins many years before disease appears. Of these autoantibodies, rheumatoid factor and anti-citrullinated antibodies (ACPA) are the most frequently used in clinical practice (18).

Rheumatoid factor (RF) is an autoantibody that recognizes the constant region (Fc) of immunoglobulin (Ig) G (IgG) as an autoantigen. It is produced locally by B cells in lymphoid tissues and in inflamed synovial tissue. In RA patients, four isotypes (IgM, IgG, IgA and IgE) of high affinity and specificity against human IgG can be detected (17,18). The sensitivity and specificity of RF in RA varies in the different series. In this sense, a sensitivity of 60-90% and a specificity of 80-90% have been described (17,18).

Citrulline is a non-standard amino acid generated by the post-translational modification of arginine by peptidylarginine deiminase enzymes during a variety of biologic processes, including inflammation (23). This modification can be observed in numerous proteins such as filaggrin, vimentin, α and β fibrin, α enolase and collagen I and II peptides (17). These proteins, which are present in the inflamed joints of patients with RA, favour the development of an immune response with the consequent formation of ACPA. These autoantibodies show sensitivity similar to that of RF (70-80%) but higher specificity (90-98%) (17,18,23).

The presence of RF and ACPA antibodies in the serum of patients with RA have been shown to be associated with a more severe disease course (17). Thereby making these antibodies importance to assess, not only for diagnosis but also for prognosis and follow-up of these patients (17,18).

1.1.6. Diagnosis

The diagnosis of RA can be challenging due to a wide spectrum of presentation forms, progressive changes in the course of the disease and the scarcity of

specific tests that define the presence or absence of disease. The first established classification criteria were the 1987 American College of Rheumatology (ACR) criteria (24). These criteria are well accepted as providing the benchmark for disease definition, but have a significant limitation in that they were derived by trying to discriminate patients with established RA from those with a combination of other definite rheumatologic diagnoses. They are therefore not helpful in achieving the goal of identifying patients who present with early stages of the disease (25). In order to improve this limitation, the 2010 European League Against Rheumatism (EULAR)/ACR classification criteria were developed (Table 2) (25). These criteria include the presence of joint swelling, serological positivity of ACPA or RF, increased acute phase reactants and more than 6 weeks of symptoms duration. In these criteria, the presence of more than six points is suggestive of an RA diagnosis.

Table 2. The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis.

Domain	Category	Point score
A	**Joint involvement (0–5 points)[1]**	
	1 large joint	0
	2–10 large joints	1
	1–3 small joints (large joints not counted)	2
	4–10 small joints (large joints not counted)	3
	>10 joints including at least one small joint	5
B	**Serology (at least one test needed for classification; 0–3 points)[2]**	
	Negative RF and negative ACPA	0
	Low positive RF or low positive ACPA	2
	High positive RF or high positive ACPA	3
C	**Acute-phase reactants (at least one test needed for classification; 0–1 point)[3]**	
	Normal CRP and normal ESR	0
	Abnormal CRP or abnormal ESR	1
D	**Duration of symptoms[4]**	
	<6 weeks	0
	≥6 weeks	1

The points from each of domains A through D are added and the sum is considered to be the total score. A total score of ≥6 is needed to classify a patient as having definite RA.

[1] Joint involvement refers to any swollen or tender joint on examination, which may be confirmed by imaging evidence of synovitis. DIP joints, first CMC joints and first MTP joints are excluded from assessment. Large joints refer to shoulders, elbows, hips, knees and ankles. Small joints refer to MCP joints, PIP joints, second through fifth MTP joints, thumb IP joints and wrists. [2] Negative means less than or equal to the upper limit of normal (ULN); low positive means >ULN; high positive means >3× ULN. [3] Normal and abnormal are determined by local laboratory standards. [4] Duration of symptoms as per patient's self-report.

Table adapted from Ref. (25).

1.1.7. Treatment

In recent years there has been a radical change in RA treatment. Thanks to the availability of disease modifying drugs (synthetic, biological and targeted synthetic), a paradigm shift occurred in relation to the control of disease course. Consequently, it is possible to establish clinical remission or low disease activity as a therapeutic objective in these patients. A rapid and adequate induction of

remission is necessary to stop structural damage and prevent disability. Guideliness in RA treatment recommends the initiation of a disease-modifying drug (DMARD) at the time of clinical diagnosis is done (26). Currently, due to the fact that patients have long periods of remission or low activity, pregnancy is increasingly being considered in female patients.

Treatment of RA could require embryotoxic agents. Therefore, it is necessary to have a wide knowledge of the different drugs and use recommendations during pregnancy.

For more than 30 years, a 5-tier set of alphabet categories (ABCDX) have been used to designate the safety of a drug for use during pregnancy (Table 3). The objective of this categorization was to promote safety and avoid use of teratogenic agents by pregnant women. Despite this, the ABCDX letter categories have multiple problems, like misinterpretation, confusion and misuse (27). In 2015, the US Food and Drug Administration (FDA) decided to discontinue these pregnancy risk categories and replace it by the FDA Pregnancy and Lactation Labelling Rule (PLLR) (Figure 1). This new classification describes drug safety profiles during pregnancy with a narrative text. In this text a description of the risk information, clinical considerations and background data for the drug are included. The new rule includes three overarching categories: 1) pregnancy, which includes labour and birth; 2) lactation; and 3) females and males of reproductive potential (27).

Table 3. US Food and Drug Administration Letter Categories for Safety of Drug Use During Pregnancy.

Category	Definition	Clinical implications
A	Well-controlled studies in humans show no risk to foetus.	Few drugs have been the subject of double-blinded, randomized controlled trials that enrolled pregnant women as participants. There are very few drugs assigned to this category.
B	No well-controlled studies have been conducted in humans; animal studies show no risk to foetus.	This category includes drugs that have safety data from observational studies that may or may not be included on the drug label. Although somewhat helpful, these studies detect an association but cannot prove an etiologic relationship between a specific risk and the index drug. Similarly, animal studies are species-dependent and may not be easily generalizable to humans.
C	Either no well-controlled studies have been conducted in humans, or animal studies have demonstrated an adverse effect on foetus.	The majority of drugs (approximately 60%-70%) are assigned this category because no or little research about use in pregnancy has been conducted. Animal studies that have identified adverse foetal effects are included in this category. Thus, category C includes some information about animal studies that may or may not be concerning to humans and no data about human studies, which results in a category that has limited clinical utility and potential confusion for the health care professional.
D	Evidence of human risk to the foetus exists; however, benefits may outweigh risks in certain situations.	This category is assigned to drugs that have known risks to the foetus or new-born. However, most of the drugs in this category may be essential to conserve maternal health. An example is phenytoin (Dilantin) that is used to prevent seizures among women with epilepsy but is also known to cause foetal hydantoin syndrome. The problem is assigning risks versus benefits and often the incidence of risks associated with the drug is not well defined in the drug label nor are the potential benefits of treatment.
X	Controlled studies in animals or humans demonstrate foetal abnormalities; the risk in pregnant women clearly outweighs any possible benefit.	This category is reserved for known teratogens such as isotretinoin (Accutane) that is linked with adverse foetal outcomes and for which either alternative therapies can be found or discontinuation does not result in major maternal harm.

Table adapted from Ref. (27).

Figure 1. New Pregnancy and Lactation Rule Labelling system.

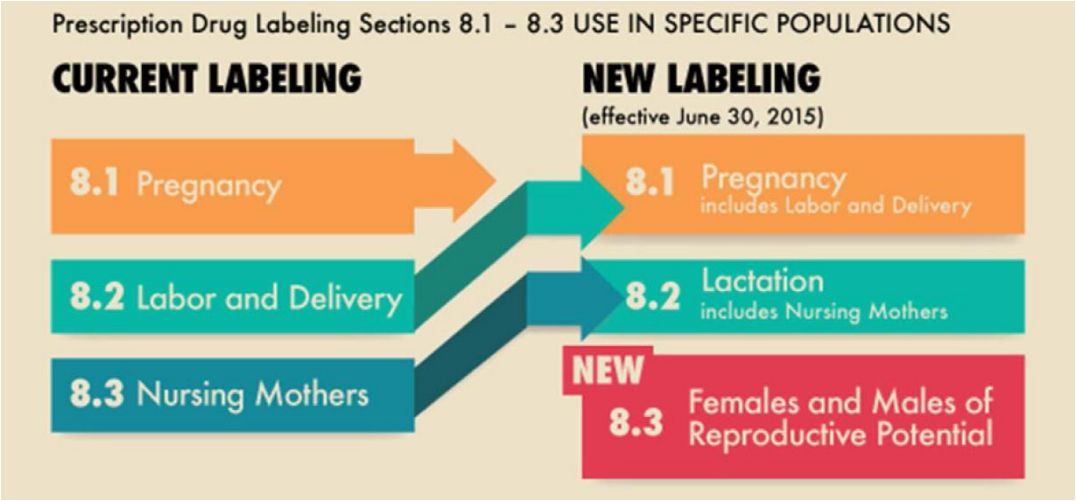


Image reproduced from Ref. (27).

Likewise, within rheumatologist, antirheumatic drug safety during pregnancy has provoked great interest. Thanks to this, various working groups have designed recommendations for the use of drugs during pregnancy.

The most widely accepted recommendations on which the vast majority of rheumatologists base our clinical practice are: *"The EULAR points to consider for use of antirheumatic drugs before pregnancy, and during pregnancy and lactation"* (28), *"BSR and BHPR guideline on prescribing drugs in pregnancy and breastfeeding-Part I-II: standard and biologic disease modifying anti-rheumatic drugs and corticosteroids"* (29,30) and *2020 American College of Rheumatology Guideline for the Management of Reproductive Health in Rheumatic and Musculoskeletal Diseases* (31).

In the next section, a description of each antirheumatic treatment and its recommendation for use in pregnancy will be described.

1.1.7.1. Non-steroidal anti-inflammatory drugs and Glucocorticosteroids

Non-steroidal anti-inflammatory drugs (NSAID)

NSAIDs are frequently used to control RA symptoms. Their chronic use is controversial due to their limited effectiveness and their inability to modify the course of the disease (32). There is no robust evidence to suggest that NSAIDs

can modify the course of RA, so they are considered purely symptomatic treatments. Among these, non-selective cyclooxygenase (COX) inhibitors are classified as not teratogenic and can be continued during the first and second trimester. All NSAIDs except low dose aspirin should be withdrawn at gestational week 32 because of the increased risk of early closure of the ductus arteriosus (30).

Glucocorticosteroids (GC)

The short-term use of GC reduces synovitis and in the long term reduces joint damage progression. Despite this, their use entails important adverse effects such as increased risk of infections and osteoporosis, showing an unfavourable risk/benefit balance. Its use is especially useful in periods of reactivation of the disease due to its rapid action (33).

Corticosteroids used for rheumatic disease treatment (prednisolone, prednisone and methylprednisolone) are metabolized in the placenta, so ≤10% of the active drug reaches the foetus. This drug class is classified as compatible with pregnancy (29).

1.1.7.2. Disease modifying anti-rheumatic drugs

The objective of the use of DMARDs (synthetic and biological) is to control inflammatory activity and induce clinical remission of the disease. Once this objective has been achieved, it is aimed at maintaining remission, reducing the frequency of relapses and allowing decreased use of symptomatic therapy, thus reducing possible adverse effects. The introduction of biological DMARDs therapies has radically changed the treatment strategy for patients with chronic inflammatory arthritis (CIA), and consequently the prescription of synthetic DMARDs. For this reason, many of these drugs are little used today. The most widely used drugs in routine clinical practice are methotrexate, leflunomide, salazopyrin, and antimalarials (34). This is different in the field of treatment of rheumatic diseases in pregnancy, since on many occasions, thanks to the safety profile and the accumulated experience in pregnancy, classic synthetic DMARDs are still used (29).

1.1.7.2.1. Conventional synthetic DMARDs

Methotrexate (MTX)

Methotrexate (MTX) has been considered the "gold standard" of chronic inflammatory arthritis treatment due to its powerful anti-inflammatory and immunosuppressive effects (35). MTX is an analogue of folic acid, it interferes with the action as a co-factor for a variety of essential enzymes related to the synthesis of purine and pyrimidine bases and consequently inhibits cell proliferation responsible for synovial inflammation. In addition, it inhibits leukocyte accumulation and endothelial injury in the inflamed area and induces apoptosis of activated lymphocytes (36).

MTX is a teratogenic drug that can provoke craniofacial, cardiac, pulmonary, gastrointestinal, genitourinary and musculoskeletal anomalies (37). These teratogenic effects have been described more frequently with high doses, but several studies have demonstrated that spontaneous abortion and birth defects can occur with low-dose exposure during the first trimester of pregnancy (37). This finding is consistent with disruption of organogenesis between weeks 6 and 8 of gestation, the most vulnerable period of teratogenicity (29,37). As a consequence, MTX is contraindicated during pregnancy and is recommended to be stopped 1-3 months prior to conception (28,29).

Leflunomide (LEF)

LEF is an isoxazole derivative with immunomodulatory and disease modifying proprieties. It is a pro-drug rapidly transformed to its active metabolite (A77 1726) after passing through the intestinal mucosa and the liver. The A77 1726 inhibits the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH), which has a critical role in the *de novo* synthesis of pyrimidine bases. This impacts the cellular cycle, arresting the cell growth in the G1 phase and consequently, blocking leukocyte proliferation and inhibiting neutrophil chemotaxis in the synovial tissue (19).

LEF has been found to be embryotoxic and teratogenic in rats and rabbits at therapeutic doses similar to those used in humans (38). Despite this and being

based on studies with relatively small sample size, the Organization of Teratology Information Specialists (OTIS) pregnancy registry did not find increased risk of major congenital malformation for this drug. However, current safety evidence in not sufficient to change the previous recommendation. Therefore, LEF is a contraindicated drug in pregnancy and cholestyramine washout should be performed (29).

Sulfasalazine (SSZ)

SSZ or salazopyrin is a drug first synthesized in the 1940s combining an antibiotic, sulphapyridine, with an anti-inflammatory, 5-aminosalicylic. Its mechanism of action is not fully understood. It has been shown to inhibit cyclooxygenase and consequently the production of prostaglandin E2 and leukotrienes (39).
Current evidence does not indicate an increase in the rate of congenital defects. Sulfasalazine can be continued at doses up to 2 g/day with concomitant folate supplementation throughout pregnancy (28).

Hydroxychloroquine (HCQ)

HCQ is an antimalarial drug quite frequently used for rheumatological diseases treatment. Anti-malarial drugs have several actions, but the exact mechanism by which they act in RA is unknown. A hypothesis is, due to the fact that they are weak bases, they interfere with pH-dependent immunological functions like the presentation of antigens (19).

HCQ is not associated with any increased risk of congenital defects, spontaneous abortion, foetal death, prematurity or decreased numbers of live births in pregnant patients with autoimmune diseases. HCQ is safe for use during pregnancy (40).

Azathioprine (AZA)

Azathioprine is a pro-drug of 6-mercaptopurine and was first synthesized in 1957 (41). AZA has a complex metabolism. After oral ingestion, it's absorbed from the whole intestinal tract. In the intestinal wall, liver and red blood cells, AZA is then converted to 6-mercaptopurine via a glutathione-mediated non-enzymatic process. This reaction provokes the removal of an imidazole group. Hypoxanthine-guanine phosphoribosyltransferase and other enzymes generate the formation of cytotoxic

nucleotides acting in the *de novo* purine synthesis inhibition, thereby decreasing leukocyte proliferation (41).

Early animal studies have reported foetal anomalies related to AZA exposure but this has not been shown in humans. A possible explanation is due human foetal liver lacks the enzyme inosinate pyrophosphorylase, which converts AZA into active metabolites and may potentiate foetal anomalies (42). AZA is compatible throughout pregnancy at a daily dose not exceeding 2 mg/kg/day. This dose recommendation is due to the fact that in two studies, a relationship between the dose of AZA with birth abnormalities and depressed haematopoiesis in pregnancies whose mothers were treated with >2 mg/kg/day, was detected (29).

Cyclosporine A (CSA)

Cyclosporine was initially isolated in 1971 from the fungus *Tolypocladium inflatum Gams* and its immunosuppressive effect was discovered in 1976 (43). Its mechanism of action works binding to cytoplasmic proteins and forming a calcineurin inhibitor complex that stops lymphocytes proliferation and lymphokines transcription of interleukin-2, tumour necrosis factor α (TNFα) and interferon γ (43).

Although, embryotoxicity has been reported in animals at high doses (25-100 mg/kg/day), this has not been confirmed in human pregnancies for the doses usually used in clinical practice (29). CSA is compatible throughout pregnancy at the lowest effective dose with suggested monitoring of maternal blood pressure, renal function, blood glucose and drug levels (29).

Tacrolimus

In 1982, researchers in Japan tested fermentation products of *Streptomyces* spp. to determine whether these products had immunosuppressant effects in a mixed lymphocyte reaction. FK506 (now called tacrolimus), one of these products, derived from *Streptomyces tsukubaensis* was shown to have potent suppressing response in a mixed lymphocyte culture, 100 times higher than that of cyclosporine (41).

In rats and rabbits, tacrolimus causes embryo-foetal toxicity at doses that demonstrated maternal toxicity (44). Despite this, it is not confirmed in human

pregnancies. Currently, tacrolimus can be continued during pregnancy at the lowest possible dose with suggested monitoring of maternal blood pressure, renal function, blood glucose and drug levels (28,29).

1.1.7.2.2. Biological DMARDs

TNFα inhibitors

The drugs included in this family act with a competitive inhibition of TNFα binding to its receptor or directly against TNFα. The drugs present several differences in molecular structure, route of administration and pharmacokinetics (45). In relation to the molecular structure, among the different anti-TNFα there are monoclonal antibodies and a fusion protein (45).

Etanercept (ETN)

ETN was the first biologic drug synthesized in 1998 for RA. It's a fusion protein composed of the 2 extracellular portion of the human TNF receptor 2 (TNF-R2) (p75 TNF receptor) and the Fc portion (hinge, CH2 and CH3 domains) of human IgG1 (46) (Figure 2). This fusion protein acts by competitively inhibiting the binding of TNFα to its receptor on the cell surface, thereby impeding the cellular response mediated by TNF.

Currently, there are few studies that have focused on the transplacental passage of ETN. The Birgitte G. Berthelsen *et al* study demonstrated ETN concentration ratio between maternal serum and umbilical cord serum was 14:1 at delivery (47). Later, in the study by Gaby A. M. Eliesen confirmed a low concentration of ETN in the umbilical cord blood supporting low transplacental transmission of this drug (48).

At present, with the available information, an increase in miscarriages or congenital malformations has not been detected in babies of mothers treated with this drug in relation to the control groups and/or background data (28,29).
Currently, treatment can be administered until 32 weeks' gestation. If the inflammatory activity of the maternal disease requires it, ETN treatment can be maintained throughout pregnancy (28).

Figure 2. Molecular structure of ETN.

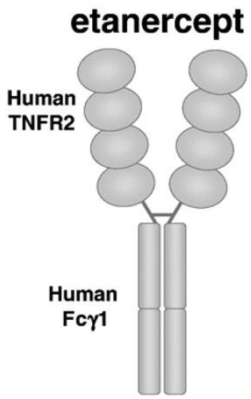


Image reproduced from Ref. (49)

Infliximab (IFX)

IFX is a chimeric IgG1 monoclonal antibody formed of a murine variable region and a constant human portion (Figure 3). IFX targets both soluble and transmembrane TNF-α, but does not bind lymphotoxinα (50).

In the study by Uma Mahadevan *et al* (51), serum concentrations of IFX at birth in the mother, infant and in cord blood were evaluated. Drug concentrations in cord and the infant at birth were compared with those of the mother. They detected higher concentrations in infants at birth and their cords than in their mothers. Specifically, IFX median level in the cord was 160% higher of that of the mother (51).

Despite transplacental passage, infliximab has not been shown to increase the rate of miscarriages or congenital malformations (28,29). With all this information, IFX is considered safe and can be continued until gestational week 20; if necessary, due to the inflammatory activity of maternal disease, it can be used throughout pregnancy (28).

Figure 3. Molecular structure of IFX

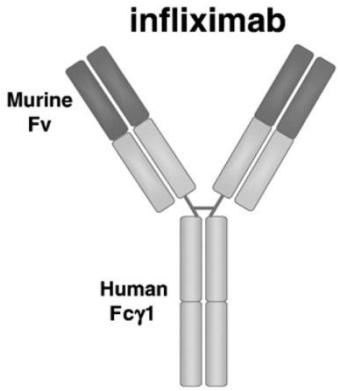


Image reproduced from Ref. (49).

Adalimumab (ADA)

ADA is a recombinant human IgG1 monoclonal antibody that binds human TNFα with high affinity (52) (Figure 4).

In the previously cited study by Uma Mahadevan *et al*, where anti-TNFα concentrations were studied in the mother, the child and the umbilical cord, ADA was also assessed. It was observed that the median level of ADA in the cord was 153% that of the mother and that ADA could be detected in the child up to 6 months after delivery (51).

Despite these data, as is the case with IFX, treatment with ADA during pregnancy has not shown an increase in the number of miscarriages or congenital malformations (28,29). It is currently considered that ADA can be used during the first and second trimesters and can be used throughout pregnancy, if indicated (28,29).

Figure 4. Molecular structure of ADA

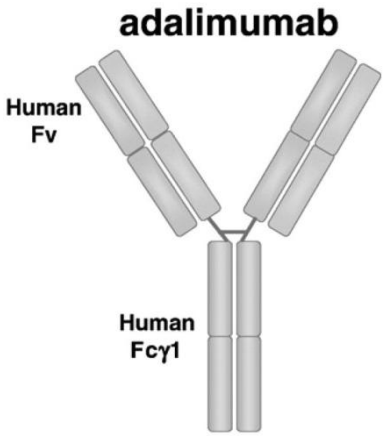


Image reproduced from Ref. (53).

Certolizumab Pegol (CZP)

CZP is TNFα inhibitor derived from a humanized antibody and with a different structure (Figure 5). It's a monovalent Fab antibody fragment covalently linked to polyethylene glycol (53).

Thanks to the drug structure, which does not have an Fc, it is considered that the immunoglobulin active transfer thought placenta FcRn is not carried out. The CRIB study (54) was designed as a pharmacokinetic study of pregnant women under CZP treatment to assess drug levels in umbilical cord blood and peripheral blood of new-borns. From a total of 14 infants, 13 infants had no quantifiable CZP levels at birth and 1 had a minimal CZP level of 0.042 μg/mL showing an infant/mother plasma ratio of 0.0009 (54).

Like others TNFα inhibitors, CZP does not show an increase in the number of miscarriages or congenital malformations (28,29). Currently, due to the structure of the drug and studies that are available, CZP can be maintained throughout pregnancy (28,29).

Figure 5. Molecular structure of CZP

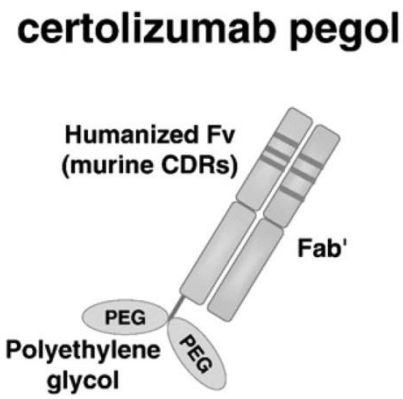


Image reproduced from Ref. (49).

Golimumab (GOL)

Golimumab is a fully human IgG1 kappa monoclonal antibody, which neutralizes TNFα actions by binding to both the soluble and transmembrane forms of TNFα, thus neutralizing its binding to receptors (55) (Figure 6).

Currently, there are no studies that have evaluated the passage through the placenta of this drug. Despite this, since it is a monoclonal immunoglobulin G1, it is considered to be actively transported across the placenta (56).

There are few cases of exposure during pregnancy, but unintended exposures during the first trimester does not indicate an increased rate of congenital malformations (29). Today, due to the limited data, alternative medications should be considered for treatment throughout pregnancy (28).

Figure 6. Molecular structure of GOL

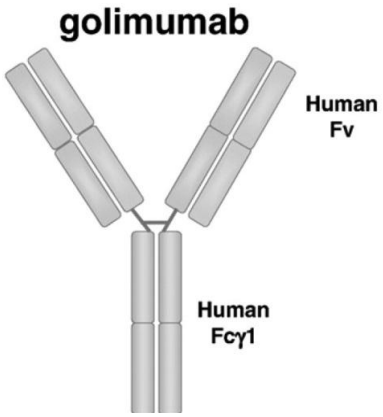


Image reproduced from Ref. (49).

IL6 inhibitors

Tocilizumab (TCZ)

TCZ is a monoclonal antibody that competitively inhibits the binding of IL6 to its receptor. Inhibiting the receptor complex prevents IL6 signal transduction to inflammatory mediators that recruit B and T cells (57) (Figure 7).

Currently, there is only one published case in which the drug concentration in umbilical cord blood was evaluated and compared with maternal levels (58). The researchers found that concentration in the umbilical cord was 7 times lower than in the maternal peripheral blood (58). To date, TCZ has not been associated with an increased risk of miscarriage or congenital malformations (28,29).

Current available information come from global safety databases, however the number of exposed cases is not sufficient to assess drug safety. TCZ should not be used in pregnancy unless there is no other alternative and that the activity of the maternal disease clearly requires it (59,60).

Figure 7. Molecular structure of TCZ

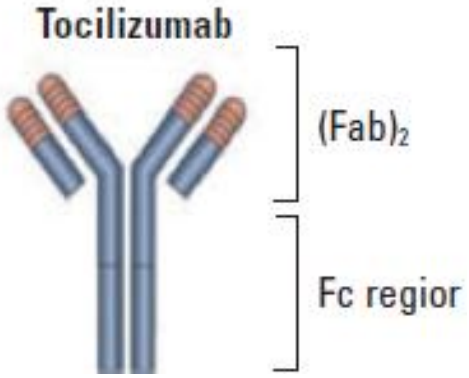


Image reproduced from Ref. (61).

Sarilumab (SARI)

SARI is human monoclonal antibody that blocks IL6 from binding to the alpha subunit of mIL-6R and sIL-6R, thereby inhibiting IL6-mediated cis- and trans-signalling (62).

There are currently no published cases of the use of sarilumab in pregnancy. The drug's summary of product characteristics are that SARI should not be used during pregnancy unless the clinical situation of the woman requires treatment with sarilumab (63).

CTLA4 inhibitor

Abatacept (ABA)

ABA is a fusion protein composed of the Fc region of the immunoglobulin G1 linked to the CTLA4, the extracellular domain of cytotoxic T lymphocyte-associated antigen. ABA modulates T-cell activation by interfering with co-stimulation of B/T lymphocytes (64) (Figure 8).

There are no published studies of transplacental passage of ABA in human gestation.

Although the number of exposed cases is not very high, the use of ABA presented an increase in the number of congenital malformations but without a pattern of congenital anomalies and in the context of multiple confounders like the use of other well-known teratogenic medications (65). ABA should not be used during pregnancy unless the clinical situation of the woman requires it (65).

Figure 8. Molecular structure of ABA

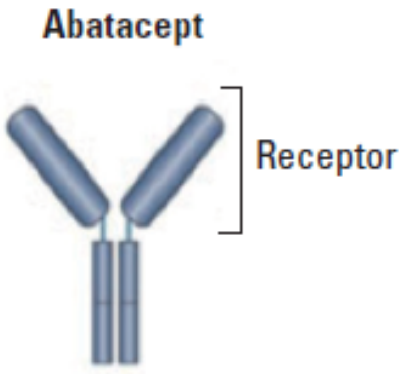


Image reproduced from Ref. (61).

CD20 inhibitor

Rituximab (RTX)

RTX is a chimeric monoclonal anti-CD20 antibody. CD20 is an antigen expressed in B lymphocytes and it's involved in the generation of B-cell responses against T-cell independent antigens. RTX induces B-cell depletion through complement-mediated cytotoxicity, antibody-dependent cellular cytotoxicity and direct signalling (66) (Figure 9).

In the literature, there are three published cases of RTX detection in new-borns of mothers under this treatment. In the first case, concentrations in the umbilical cord blood were three times higher than maternal levels. Likewise, RTX has been detected in the peripheral blood of two new-borns. The new-borns presented serum RTX levels of 30 and 6.7mg/L, which are about 150% and 30% of maternal levels. Exposure to RTX was at week 25 and 34 weeks' gestation, respectively (67,68).

Despite the limited information available, there is no evidence that the rate of abortion or birth defects increases due to RTX use during pregnancy (28,29). RTX should not be administered during pregnancy unless the expected benefit outweighs the potential risk. It is important to note that its use in the second and third trimesters can generate B-cell depletion and other cytopenias in the neonate (28,29).

Figure 9. Molecular structure of RTX

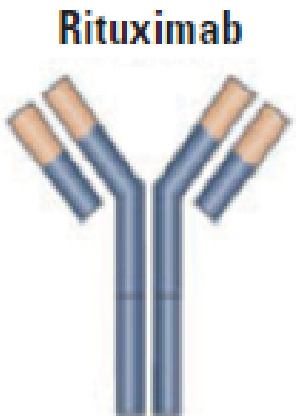


Image reproduced from Ref. (61).

IL1 inhibitor

Anakinra (ANAK)

ANAK is a human recombinant protein produced in *Escherichia coli* cells by recombinant DNA technology. It acts as antagonist of the human receptor for IL1 (r-metHuIL-1ra). It is important to note that ANAK is not a monoclonal antibody and that it's molecular weight of approximately 17.3 kilodaltons, much lower than that of classic monoclonal antibodies [74, 75] and currently, there are no published studies of transplacental cross of ANAK in human gestation.

Globally, current evidence does not indicate an increased rate of congenital malformations, but in the literature there are two cases of renal agenesis in foetuses of mothers undergoing ANAK treatment. In both cases, other risk factors related to renal agenesis coincided. In the first case, the foetus presented an ectopic neurohypophysis, resulting in growth hormone deficiency and the second case was a twin gestation of a diabetic mother in which one foetus died in utero and had bilateral renal agenesis. The surviving twin had no developmental abnormality (69). Important concerns remain about the incidence of renal tract abnormalities in the foetus described in the literature. It's mandatory to discuss this factor with potential parents at preconception counselling (69).

1.1.7.2.3. Targeted synthetic DMARDS

Janus Kinase (JAK) inhibitors

Tofacitinib (TOFA)

TOFA inhibits the signalling pathway through JAK1 and JAK3 with more selectivity over JAK2, modulating the signalling pathway and preventing the phosphorylation and activation of STATs and nuclear factor κB p65. Inhibition of JAKs may result in modulation of intracellular activities including gene expression and inflammation in articular and extra-articular locations (70).

Currently, there are no studies that have evaluated the placenta cross, however, TOFA is a small molecule that has the potential to cross it (71).

Animal studies have shown that TOFA was teratogenic in rats and rabbits showing membranous ventricular septal defects and skeletal/cranial malformations. It was also associated with an increase in post-implantation loss, a decrease in the number of viable foetuses and mean foetal body weight. These alterations appear at exposures 146 times and 13 times greater than the human dosage, respectively (71). Despite these animal data and based on limited human data, the Megan E. B. Clowse *et al* (71) study shows that unintentional exposure to TOFA during pregnancy does not seem to be associated with an increased risk to the foetus. Until now, the use of TOFA in pregnancy is contraindicated (72).

Baricitinib (BARI)

BARI acts through reversible inhibition of JAK1 and JAK2. As TOFA, BARI, through JAK inhibition, modulates intracellular activities including gene expression and inflammation (73).

BARI was teratogenic in rats and rabbits, showing alterations in bone development in utero at high doses (74). Currently, there are no studies that have evaluated whether BARI crosses the placenta but it must be noted that it is a small molecule that has the potential to cross it (71). BARI is currently contraindicated during pregnancy (74).

1.2. Pregnancy

1.2.1. Physiology of normal pregnancy

Pregnancy is the period that elapses from the implantation in the uterus of the fertilized ovum to the time of delivery. Human pregnancy lasts about 40 weeks from the first day of the last menstruation or 38 from fertilization (about 9 months). It is divided in 3 trimesters. The first trimester covers up to week 12, second trimester lasts from week 13 to week 26 and third trimester, starts at 27 weeks and finishes with partum at 40 weeks.

During pregnancy, pregnant women experience important anatomical and physiological changes. With regards to the haemopoietic system, plasma volume increases progressively throughout pregnancy. Plasma expansion is greater than

an increase in red blood cell mass, generating a reduction on haemoglobin concentration, haematocrit and red blood cell counts (75). Platelets are progressively reduced with the progression of pregnancy, but usually remains within normal limits (75). Pregnancy increases the requirements of iron 2-3 fold, the requirements of folate 10- 20 fold and the requirements of vitamin B12 2-fold (75). Pregnancy causes changes in the coagulation system generating a physiological hypercoagulative state (75).

The cardiovascular system undergoes multiples changes during normal pregnancy, including increases in cardiac output, arterial compliance, and extracellular fluid volume and decreases in blood pressure (BP) and total peripheral resistance (76). The anatomy of the heart also undergoes structural changes during pregnancy. The heart is pushed upwards and rotated forward with its left border laterally displaced. In addition, the ventricular wall muscle mass and the larger valvular annular diameters are increased (77). The rise in cardiac output plateaus after 20 weeks of gestation remaining elevated until term. The increase in cardiac output is associated with increases in stroke volume and heart rate. Mean circulatory filling pressure, a determinant of venous return, is also elevated during pregnancy. Furthermore, resistance to venous return is dramatically reduced during pregnancy (75–78).

Numerous changes occur in the respiratory system during pregnancy. Increasing abdominal pressure and the enlarging uterus produce an elevation of the diaphragm. Progesterone and relaxin cause ligaments connecting the ribs to the sternum to relax facilitating the chest circumference increase. Despite the increase in chest circumference, chest wall compliance is decreased (77). Lower chest wall compliance added with an elevated diaphragm results in a 5% decrease in total lung capacity. Vital capacity is unaltered, functional residual capacity reduces by 10–25% and oxygen consumption increases by 30%. All these changes result in a higher PaO_2 (13–14 kPA) and a lower $PaCO_2$ (3.7–4.2 kPA) in the maternal circulation (78). This scenario facilitates the transfer of oxygen from the maternal to foetal circulation. Inversely, CO_2 is transported from the foetus to the maternal circulation (77).

The kidneys are displaced in a cephalic direction by the enlarging uterus. They increase approximately 1 cm in size due to increased vasculature, interstitial volume and dead space. Due to the progesterone effect, and the compression of the ureters at the pelvic brim, the renal collecting system dilates from the first trimester (77). As a consequence of renal vasodilatation, renal plasma flow and glomerular filtration rate (GFR) both increase (75). Due to higher GFR, serum creatinine and urea concentrations decrease. Finally, as the increases in GFR and glomerular capillary permeability to albumin, the fractional excretion of protein increase up to 300 mg/day (79).

The endocrine system is modulated during pregnancy in order to cope with the increased requirements of the mother and the foetus (77). The thyroxin-binding globulin production by the liver is increased, as a consequence increased levels of thyroxin and tri-iodothyronine are detected (75). Pregnancy is also associated with a state of iodine-deficiency because of increased active transport across the foeto-placental unit and renal excretion (75). Gonadotrophin-releasing hormone (GnRH) and corticotrophin-releasing hormone (CRH) are expressed by the placenta, and their levels increase during pregnancy. GnRH is needed for placental growth and function. A rise in CRH may be important to the initiation of both term and preterm labour (77). Pregnancy is a state of hypercortisolism. The normal negative feedback loop of a high cortisol level suppressing adrenocorticotrophic hormone (ACTH) release is altered. The placenta secretes both CRH and ACTH hormones, leading to high levels of both free and bound cortisol during pregnancy (77). In order to prepare the woman's body for breastfeeding, an increase in prolactin secretion by the anterior pituitary is generated during pregnancy (77). Carbohydrate and fat metabolism also change. For example, fatty acids and glycerol are utilized for maternal energy, whereas glucose and amino acids are reserved for the foetus (77).

During pregnancy, due to the growing uterus, mechanical changes in the alimentary tract are present. The stomach is displaced upwards, increasing the intra-gastric pressure and the oesophageal sphincter tone decreases. These factors may predispose women to symptoms of reflux, as well as nausea and vomiting (75,77).

1.2.1.1. Immunological changes in pregnancy

Pregnancy is an immune challenge since a genetically different foetus must be maintained in the woman for 9 months (80). During pregnancy, there is an active process which promote tolerance toward the semi-allogeneic foetus, at the same time maintaining the immune competence towards infections and transformed cells (81–83).

Placentation is the strategy used by humans to protect the foetus and promote its growth. The maternal-foetal interface is composed of the maternal derived decidua and the foetal-derived placenta and is the site for gas, nutrient and waste interchange between the mother and the foetus (80). Approximately 40% of the decidua is made up of maternal leukocytes. The highest percentage of these immunological cells are decidual Natural Killers (dNK) comprising approximately 70%, followed by decidual macrophages (~20-25%) and T cells (3-10%). The proportion of these cells vary depending on the stage of gestation (80). An important part of this tolerance effect takes place locally in the uterus and, as mentioned, dNK are the most abundant subtype of the maternal leucocytes located at the maternal-foetal interface. These cells are highly granulated and distinguished as $CD56^{++}CD16^{-}$. Unlike their circulating counterparts in peripheral blood, dNK produce a large amount of growth factors, angiogenic factors and cytokines(80). These cells play an important role in the immune tolerance to the semi-allogeneic foetus and the remodelling of spiral uterine arteries (84).

In the maternofoetal interface, the primary APCs are the decidual macrophage. Phenotypically, these cells are $CD163^{+}$ $CD206^{+}$ $DC\text{-}SIGN^{+}$ and express IL-10, CCL2 and CCL18. They are thought to exist as regulatory/homeostatic, anti-inflammatory cells of an M2-like phenotype (80). The Th1/Th2/Th17 immune axis cells have an important role on pregnancy tolerance. In addition to effector cells, Th cells are regulated by regulatory T (Treg) cells. There is a predominance of Th2 cells on foetal-maternal interface inducing Th2 type immunity without a substantial change in Th1/Th2/Th17 immune axis in peripheral blood (85).

Currently, all the mechanisms that act at the maternofoetal interface to allow tolerance of the foetus are unknown. However, one known mechanism of

tolerance is HLA G molecules. Unlike classical HLA I molecules, where there are thousands of allelic variations in the encoding genes, in HLA G genetic region there are only 16 genetic variations and HLA G molecules are manly expressed extravillous trophoblast (EVT). HLA G binds to dNK inhibitory receptors KIR2DL4 and LILRB to protect the trophoblasts from NK-mediated cytolysis (80).

Within the immunological changes generated by pregnancy, it is important to note how the active acquisition of immunity by the foetus takes place. Placenta generates an active transport of IgG due the expression of the FcRn receptor. The Fc of the maternal Ig will bind to the receptor present in the placenta, generating an active transport to the foetus (Figure 10). This process begins approximately at 16 weeks' gestation and increases exponentially until the end of the pregnancy, where it is maximum (86).

Figure 10. Active transport of IgG in the materno-foetal barrier

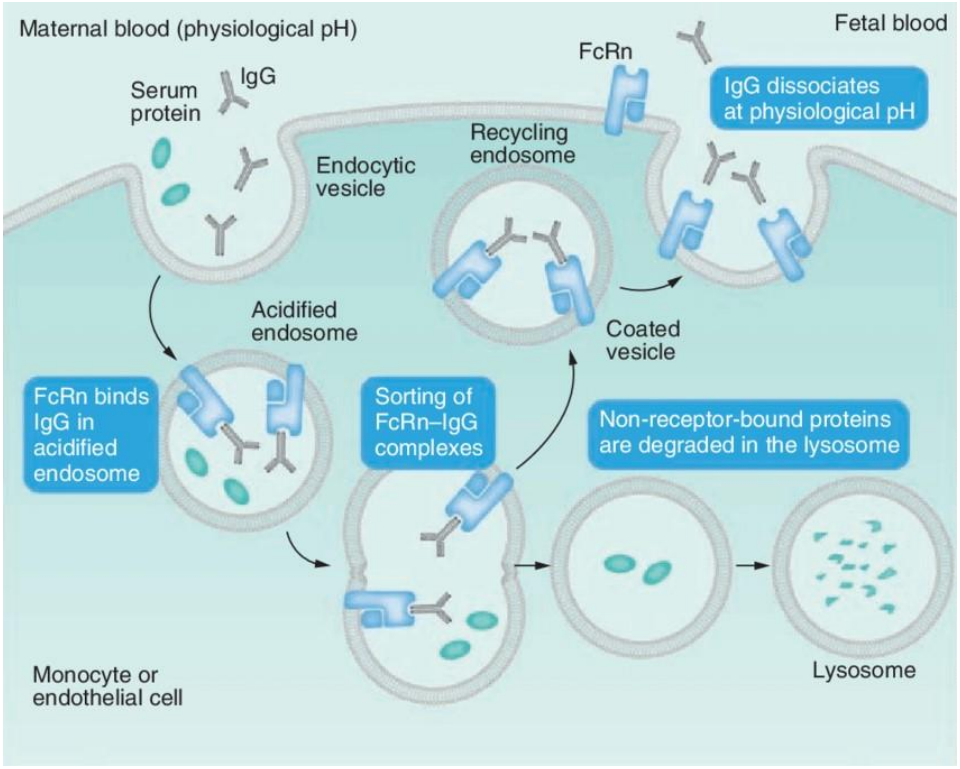


Image reproduced from (87)

1.3. Childbearing age and rheumatic diseases

1.3.1. Pregnancy and postpartum in rheumatic diseases

Chronic inflammatory rheumatologic diseases preferentially affect women of childbearing age (88). Pregnancy leads to changes in the maternal immune and neuroendocrine systems in order to create tolerance to the developing semi-allogeneic foetus while avoiding the generation of an immunodepressive state in the maternal immune system (89,90). These changes are coordinated with the progression of the pregnancy to ensure adequate foetal growth. During this period, the increase in steroidal hormones like glucocorticoids, oestrogens and progesterone induce functional changes in immunocompetent cells like B cells, T cells and monocytes.

The immunoregulation induced by pregnancy directly influences the activity of the chronic inflammatory or autoimmune diseases (91,92). Some of these diseases like RA show an improvement during pregnancy, while others like Spondyloarthropathies (SpA), show a reactivation during the second trimester and improvement towards the end of the pregnancy. Finally, there is a third group of autoimmune diseases like Systemic Lupus Erythematosus (SLE), that have a highly variable disease course (93,94).

The evolution of RA during pregnancy depends on the activity of the disease both before conception and during pregnancy, the extent of organ involvement and the presence of antiphospholipid autoantibodies (aPA) (95). Risks for prematurity and small for gestational age of the new-born are related with the disease activity in the mother and the treatment with middle-high doses of glucocorticoids (96–98).

The care of these patients in multidisciplinary units consisting of rheumatology, gynaecology, paediatric and nursery specialists is essential to maximize the probability of a successful pregnancy. The optimal control of the disease and, preferentially, its remission or low disease activity (LDA) before pregnancy is a fundamental requisite for successful outcomes (99). The pharmacologic treatment in rheumatic diseases is adapted to the type of affliction, the severity of the disease and individual response to treatment. In the pregnancy planning period

and during the pregnancy itself, the main objective of the pharmacological treatment is to control the disease activity in the mother and to ensure healthy development of the child (100). Hydroxychloroquine, salazopyrin and azathioprine, as well as low-dose glucocorticoids are compatible with pregnancy. Recently, it has been accepted that the treatment with TNFα antagonists can be continued during pregnancy (88,99,101).

1.3.2. Clinical evolution of Rheumatoid Arthritis during pregnancy

Since the studies of PS Hench 70 years ago (102), it has been repeatedly demonstrated that RA patients show a clear improvement of their disease status during pregnancy and a subsequent reactivation in the postpartum. In Hench's series from 1938, 37 pregnancies of 22 women were studied and a clinical improvement was observed for 20 of them (102). Later, a large number of studies have confirmed the high probability of disease improvement during pregnancy and a similar probability of reactivation during postpartum (102–106). In the last decade, the definitions of disease improvement and reactivation have significantly evolved and there are different standardized clinical scores that have been defined for this objective and which are widely accepted by the medical community (107).

The therapeutic option to use certain DMARDs during pregnancy positively influences the control of the disease and subsequently to measurement disease activity. Conversely, pregnancy increases the levels of acute phase reactants (i.e. ESR, CRP) and, therefore, it directly influences many disease activity scores. Additionally, some patients might obtain higher scoring in the Visual Analogue Scale due to the presence of unspecific symptoms (108). Thus, during pregnancy and postpartum, there are many factors that can influence the calculation of the disease activity scores and, therefore, provide suboptimal measures of the real activity of the disease.

In the study of pregnancy in RA, there are two prospective studies that clearly stand out. The study by de Man *et al* in 2009 (109) and the study by Barrett *et al in* 1999 (110) which analysed 140 and 84 pregnant RA patients, respectively. In both studies, the course of RA patients from before conception up to 6 months after delivery was evaluated. In these two studies they observed a highly significant

improvement in disease activity during pregnancy (62% and 48% of patients, respectively). Also, a complete remission of RA was observed in several patients (16% and 27% of patients, respectively). This improvement in disease activity was then maintained during the progression of the pregnancy. Of relevance, in these two studies, disease improvement was found to be more frequent in the second half of the pregnancy (111,112).

1.3.3. Clinical evolution of Rheumatoid Arthritis during postpartum

After delivery, the mother must to re-adapt to the non-pregnant state. This change, which might seem rather simple, is a very complex process at the biological level. In this period, some of the changes in the immune and hormonal systems that were initiated during pregnancy still persist. An example of this is the increase of prolactin levels, which are maintained in postpartum due to lactation (113) and could influence RA disease activity (114). In this period, not only the course of an established disease can change but also, can trigger the start of the disease in genetically susceptible individuals (93,115).

As previously mentioned, two studies have described the evolution of RA in pregnancy and postpartum (109,110). If we focus on the postpartum, in the Pregnancy-induced Amelioration of Rheumatoid Arthritis study (PARA) study published in 2009, a total of 39% of pregnant RA patients showed a moderate reactivation of the disease at postpartum (109). In addition, in the study by Barrett *et al* published in 1999, up to 66% of patients presented a worsening of the clinical symptoms during the first six months after delivery (110). The different percentages between the two studies may be due to the increase in available treatments. The study by Østensen *et al* (105) published in 1983, also evaluated the effect of pregnancy and pospartum on RA activity. The authors included 31 women who had given birth 49 infants after disease onset. The results showed a postpartum disease exacerbation in 62% of the cases, similar to the results described by Barret *et al* (110). Also the authors described that disease relapses were more often 4-10 weeks after delivery. The authors did not found relation with lactation or the time of the first menstrual period after partum (105).

1.3.4. Biological pathways activated during pregnancy and postpartum in Rheumatoid Arthritis

Several studies have attempted to characterize the biological changes occurring during pregnancy and postpartum that are associated with the modification of the clinical activity of the disease. To date, there are several hypotheses on the biological mechanisms that contribute to these changes. Some of these hypotheses are the maternofoetal HLA (90) incompatibility, the increase in IgG glycosylation (116), the presence of changes in IgG and IgA levels (117) or alterations of the balance between the different T cell subtypes and their associated cytokine profiles (118–120). With regard to the latter hypothesis, several studies have reported an increased prevalence of a T helper 2 (Th2) cytokine profile during pregnancy. Despite this evidence, however, it is important to emphasize that pregnancy not only promotes a Th2 type of response, but it rather exerts an immunomodulation on the different T-helper subtypes which will, in turn, depend on the stage of the pregnancy (92).

Previous studies have expanded the knowledge on the biological pathways associated with RA activation at postpartum. For example, in the study by Häupl *et al* published in 2008, the genome-wide expression profiles from peripheral blood mononuclear cells (PBMCs) were analysed during pregnancy and at postpartum in patients with RA and healthy controls (121). During pregnancy, authors described minor difference in gene expression between RA and controls. In the postpartum period, the authors observed significant changes in the gene expression profiles of PBMCs. In particular, the gene expression activity associated with monocytes was found to be reduced in healthy control individuals while it persisted in RA patients. From a total of 32 biological pathways analysed, a significant activation of adhesion, cell migration, defence against pathogens and cell activation pathways were observed in RA patients at postpartum (121). These pathways include Notch, phosphatidylinositol, mammalian Target of Rapamycin (mTOR), Wingless-integrated (Wnt) and Mitogen-Activated-Protein-Kinase (MAPK) signalling.

A posterior study analysed the relation between the gene expression patterns of PBMCs with the disease activity scores in RA (112). In this study, a correlation between the Rheumatoid Arthritis Disease Activity Index (RADAI) and C-Reactive

Protein (CRP) levels was observed with the transcriptional profile in monocytes of RA patients during and after pregnancy (112).

Later, Weix *et al* in 2012 performed a transcriptional study to identify the group of genes that are modulated during pregnancy and, therefore, could be likely candidates to modulate the activity in RA (122). PBMCs from pregnant healthy control were analysed. Authors described that adipokine genes and genes from the Peroxisome Proliferator-Activated Receptor (PPAR) signalling pathway were strongly associated with the modulation of disease activity in RA during pregnancy (122).

One year later, in 2013, this same research group, analysed the transcriptional changes of genes from the type I interferon (IFN) pathway during pregnancy in healthy pregnant women and pregnant RA patients (123). They found that IFN type I genes are significantly over-expressed in pregnant RA patients compared to controls, although they did not find an association of this gene expression profile with the improvement of disease during pregnancy.

A recent study published by Wright *et al* (2021) found dysregulated gene expression during the RA postpartum flare (124). Researchers used total RNA from frozen whole blood for RNA sequencing. Twelve women with RA and five healthy women were included. Investigators found that large proportion of gene expression changes between the third trimester and three months postpartum among RA women who had a postpartum flare. Interestingly, the results show normal postpartum changes in RA pregnant patients also seen among healthy women. However, while expression levels of most genes showing significant changes decreased postpartum in both groups of women, levels did not decrease to the same extent during an RA flare as they did in healthy women in numerous genes shuch as *DEFA1*, *DEFA3, LTF, MMP8, CAMP, CEACAM8 and CRISP3* (124). Another interesting finding is that, by three months postpartum, the gene expression profile had reverted to the pre-pregnancy state in healthy women. This transcriptional effect was not seen in the RA group where some genes *(CD177, PADI2, TLR9, NOTCH1, IL4R, IL12B, IL1R1)* had significantly different expression levels at three months postpartum compared to before pregnancy (124).

Pregnancy is a unique model for the analysis of the biological mechanisms that are relevant in the regulation of disease activity in RA. The reduction of disease activity during the third trimester of pregnancy could be highly useful study subject to identify the key immunopathological pathways in this disease. Importantly, this unique model of disease activity improvement occurs naturally, independent of the pharmacological treatment that the patient receives. Despite significant advances in the last decade in the knowledge of this area, there is still a lack of characterization and understanding of the pathophysiological pathways involved in disease activity regulation in RA pregnancy.

1.4. Single cell technology

Omics sciences are based on the integration of high throughput analysis technologies (e.g. microarrays, RMN, HTS, HPLC-MS) to the analysis of biological variability. High-throughput technologies allow the measurement of an extremely large number of biological variables per individual and, importantly, allow for large-scale quantifications in a fast and reliable way. As a consequence, large datasets of specific and sensitive biological variation from individuals can be obtained. Access to large and detailed information is helping to explain many complex biological processes which otherwise could not be understood (125). Importantly, the use of omics technologies is enabling the characterization of new biological pathways associated with diseases (126–128).

In the study of autoimmune diseases, like RA, the difficulty in accessing the target tissue, the synovial membrane, is an important limitation. However, the recirculation of immune system cells between the lymphatic system and the target tissue through blood is a property that can be exploited to analyse this group of diseases. For this reason, whole blood is considered a highly appealing source for the identification of surrogate biomarkers in autoimmune diseases like RA (129).

1.4.1. Single cell technology

Genomic medicine generated the way for identifying biomarkers and therapeutical targets for complex diseases, but is complicated by the involvement of thousands of variably expressed genes across multiple cell types (130,131). Single-cell

technology has revolutionized this field because it revealed a complete vision of transcription profile and the DNA content in one cell without requiring *a priori* knowledge of genes of interest (132). In 2013, *Nature Methods* selected this technique as technology of the year (132). In particular, single cell RNA-sequencing (sc-RNAseq) helped to identify complex and rare cell populations, uncover regulatory relationships between genes and track the trajectories of distinct cell lineages in development (133). scRNA-seq therefore allows a comprehensive analysis of the immune system in health and during pathology (130,131).

This technology, within its experimental analysis processing, presents a first part focused on capturing based on the isolation of individual cells were are lysed to allow the capture of as many RNA molecules as possible (134). Currently, there are three main capturing models including Microfluidic device; Plate-based or Drop-based protocols (135). Subsequently, there are different available techniques for secuencing, each one has differing advantages and disadvantages. For example, Pooled approaches (CEL-seq, MARS-seq, SCRB-seq, CEL-seq2) and massively-parallel approaches (Drop-seq, InDrop) are some of the single-cell technologies available to date. According to the scale of the experiment, the cost and sensitivity of each method and the biological question to be answered the technique will be selected (131). Plate-based protocols or commercial microfluidics solutions (Fluidigm C1) are capable of deep profiling of single cells but with increased cost. These technologies are better suited to study variability between single cells or to discover subtle transcriptomic differences in 'homogeneous' populations. Pooled approaches (CEL-seq, MARSseq, SCRB-seq, CEL-seq2) use automation and microfluidics to reduce costs and improve throughput. Finally, massively parallel approaches use massive parallelization to increase the number of cells that could be profiled in one run to tens of thousands (131).

Single-Cell RNA Sequencing with Drop-Seq

For the present study we used the scRNA-seq technique based on Drop-Seq technology (136). This technique uses encapsulation of single cells in individual droplets to allow library preparation to occur individually. Barcoded mRNA is co-confined with cells in a microfluidic device within the droplets. Within each droplet,

cells are lysed and RNA hybridisde. After that, a reverse transcription PCR its applied and sequencing in single reactions create data from thousands of single-cell transcriptomes (136).

1.4.2. Single cell technology in the study of Rheumatoid Arthritis

In RA, as in other pathologies, the use of the single cell is revolutionizing its knowledge and several studies that used this technology for increase the knowledge of the physiopathology of this disease have been published (137–139).

The study by Zhang *et al* (137) is one of the most recent and important in the knowledge of the pathophisiology of RA in which single cell technology has been used for its methodology. The study focused on defining the different inflammatory cell states in joint synovial tissues in rheumatoid arthritis. Authors included 36 patients with rheumatoid arthritis and 15 patients with osteoarthritis and obtained synovial tissues from ultrasound-guided biopsies or joint replacements. Investigators analysed 5,265 scRNA-seq profiles including 1,142 B cells, 1,844 fibroblasts, 750 monocytes and 1,529 T cells and used canonical variates to define 18 cell clusters that were independent of donor and technical plate effects. Combining mass cytometry and transcriptomics revealed cell states in synovial tissue: THY1(CD90)$^{+}$ HLA-DRA^{hi} sublining fibroblasts, IL1B^{+} pro-inflammatory monocytes, ITGAX^{+}TBX21^{+} autoimmune-associated B cells, PDCD1^{+} peripheral helper T cells and follicular helper T cells. They also defined distinct subsets of CD8^{+} T cells characterized by GZMK^{+}, GZMB^{+}, and GNLY^{+}. All these populations and cell states are potentially key mediators involved in RA pathogenesis.

The study performed by Wu *et al* (2021) (138) focused on cell composition, proportion, gene expression signature, and developmental trajectories of CD45^{+} cells in PBMCs and synovial membrane from ACPA^{+} and ACPA^{-} RA patients using sc-RNAseq. Authors included PBMCs from four healthy controls and PBMCs and synovial tissue mononuclear cells from ten ACPA^{+} RA patients and ten ACPA^{-} RA patients. Authors described a transcriptional landscape of immune cell status in ACPA^{-} and ACPA^{+} RA patients. They also found that HLA-DR15 as a risk factor for developing the active disease in ACPA^{+} RA patients. They described higher expression of CCL13, CCL18, and MMP3 in DC and macrophages. In addition to

poor B cell and T cell responses, there were immune features in synovial tissue of ACPA$^-$ RA patients.

Another interesting study is the one performed by Dana E. Orange *et al* (2020) focused on the activation pathways of RA in which single cell technology has been used as part of its methodology (139). In this study they carry out a novel clinical and technical protocol for repeated home collection of blood (data that can be used to identify changes in transcriptional profiles in blood weeks before the onset of symptoms) in patients with RA to allow for longitudinal RNA sequencing (139). Researchers detected consistent changes in blood transcriptional profiles 1-2 weeks before an RA flare. B-cell activation was followed by expansion of circulating CD45$^-$CD31$^-$PDPN$^+$ preinflammatory mesenchymal (PRIME) cells in the blood from patients with RA. PRIME cells decrease before a flare in peripheral blood. These findings support the hypothesis in which PRIME cells immigrate from blood to the synovium, where they contribute to the inflammatory process. These findings, apart from identifying this new cell type in RA and its involvement in reactivation pathways, reaffirm the concept of systemic disease due to the migration of this cell type (139).

1.4.3. Single cell technology in pregnancy research

The study of the immunological changes occurring during pregnancy is a vastly unexplored field. This is mainly due to the difficulty in accessing the samples where the greatest changes occur, like the fetoplacental interface. In this context, single-cell studies can help to deeply characterize the healthy and pathological changes that occur during and after pregnancy and ultimately determine the origins of the observed transitory tolerance.

There are very few studies on pregnancy using single cell approaches. The Vento-Tormo *et al* study (2018) used single-cell transcriptomics to comprehensively resolve the cell states that are involved in maternal–foetal communication in the decidua, during early pregnancy when the placenta is established (140). In this study the investigators performed a combined droplet-based encapsulation (using the 10x Genomics Chromium system) and plate-based Smart-seq214 single-cell transcriptome profiles from the maternal–foetal interface (11 deciduae and 5

placentas from 6–14 weeks' gestation) and six matched PBMCs (140). They studied T cell composition and clonal expansion using full-length transcriptomes using Smart-seq2 and reconstructed the T cell receptor sequences. The results showed expansion of CD8 T cells in the decidua (140) and also detected the three subtypes of dNK already known. Interestingly, dNK1 cells are characterized by active glycolytic metabolism, and show overexpression of KIR genes, LILRB1 and cytoplasmic granule proteins. These findings suggest that dNK1 cells could interact with trophoblast cells in a more intense form and be an important cell in the materofoetal tolerance.

The study by Pique-Regi *et al*, using scRNA–seq described a cell type profile and transcriptional activity in placentas of women with labour at term or preterm (141). Specifically, for this study they include placental villous tree, basal plate and the chorioamniotic membrane samples. Two new cell types were identified in this study: 1) lymphatic endothelial decidual cells in the chorioamniotic membranes, and 2) non-proliferative interstitial cytotrophoblasts in the placental villi (141). With gestation progression and overall delivery, maternal and foetal transcriptional signatures from placental scRNA-seq are modulated. The authors described that the expression signatures of NK-cells and activated T-cells were up-regulated in women with labour at term compared to gestational-age matched controls without labour suggesting possible involvement in labour onset (141).

Until now, the use of the scRNA-seq technology in the study of pregnancy has focused on the study of the placenta with the exception of the Vento Tormo *et al* study (140) where peripheral blood was included. To our knowledge no studies have been published that exclusively assess the molecular changes detected in peripheral blood during pregnancy. Accessing the placenta always carries risks and, when placentas are easily accessible, it's when pregnancy ends (abortion, childbirth or legal termination of pregnancy). Therefore, the information obtained from these samples, although very valuable, may be showing the final stages of both healthy and pathological gestation. Therefore, if we were able to describe the changes that occur in gestation from peripheral blood, we could prevent negative events or avoid performing more invasive techniques.

1.4.4. Single cell technology in Rheumatoid Arthritis pregnancy research

Today, after conducting an exhaustive search in the main bibliographic databases, we have not found publications regarding the application of this technology in the study of pregnancy in RA, using peripheral blood as study tissue. Possible reasons why studies with this methodology have not been published may be the novelty of the technology, the cost and likely the difficulty in obtaining longitudinal samples in pregnant women.

2. Hypothesis

Rheumatoid arthritis is the most prevalent chronic autoimmune arthritis and it is characterized by a fluctuating course, with phases of high activity and phases were disease activity is very low or even absent. It is clearly established that RA patients have a significant improvement during pregnancy and a subsequent worsening at postpartum. Pregnancy in RA induces changes in the adaptive immune system of the mother in order to establish tolerance to the semiallogeneic foetus while avoiding an immunodepression. After delivery, the adaptation to the immune system to the non-pregnancy status triggers the reactivation of the disease.

The hypothesis of the present study is that, at postpartum, the loss of the immune-regulation acquired during pregnancy, activates pathogenic pathways associated with disease relapses in Rheumatoid Arthritis patients.

3. Objectives

The objectives of this doctoral thesis project are:

3.1. Primary objective

The main objective of this study is the identification of differential biological mechanisms between pregnancy and postpartum in Rheumatoid Arthritis.

3.2. Secondary objectives

1. To describe the first single cell RNA seq atlas of blood cells in pregnant and postpartum in Rheumatoid Arthritis.
2. To identify and compare the abundance of different cell types in pregnancy and postpartum in Rheumatoid Arthritis *versus* Healthy Controls in stimulated and non-stimulated cells.
3. To describe, within each cell type, what genes are differentially expressed in pregnancy and postpartum in Rheumatoid Arthritis *versus* Healthy Controls in stimulated and non-stimulated cells.

4. Materials and methods

4.1. Study design

To achieve the objectives of the present study, a prospective cohort study has been performed.

4.2. Study subjects

4.2.1. Origins

The monographic outpatient's clinic on chronic inflammatory arthritis (CIA) from our centre, Vall d'Hebron University Hospital, was initiated by Prof S Marsal more than 25 years ago and was focused on the highly specialized follow-up of patients with these pathologies. From the beginning, a specific protocol was designed for patients with RA that includes epidemiological and clinical data in addition to serological and genetic biomarkers. The outpatients clinics has become a referential national centre for RA patients with the main objective to provide all patients control and the more advanced combinatorial therapies using conventional synthetic DMARDs.

In December 1999, the first two biological treatments were prescribed (i.e. anti-TNFα) in two patients with RA. From then onwards, the use of biologic therapies has spread very notably due to the marked efficacy and good safety profile of these treatments. The use of biological therapies blocking different mediators for the treatment of CIAs has modified the prognosis and also improved the quality of life of these patients. More recently, the introduction of small molecules (targeted DMARDs) into the therapeutic arsenal of RA, has increased the number of drugs with high efficacy and different mechanism of action.

Rheumatic diseases frequently affect young patients of reproductive age. Nowadays, and due to the introduction of new biological therapies, a large of our patients achive long periods of low activity or remission, which allows for pregnancy to be considered and attainable for those patients with gestational desire.

Currently, within Rheumatology, a subspecialty is being generated which is dedicated to the study of different topics related to sexuality and the use of

different contraceptive methods, fertility, pregnancy and pharmacological treatments during pregnancy, lactation and postpartum.

In our clinic, since its inception, there has been a special sensitivity for these patients, being pioneers in the use of anti-TNFα in pregnancy in RA patients. In 2013, we had the first pregnancy RA patient who, due to a high disease activity at the treatment initiation, required maintaince of her biological treatment with anti-TNFα. During pregnancy, the patient presented progressive improvement of joint inflammation, so it was possible to reduce her dose without withdrawing it. The patient had a healthy girl with no complications. Given the mother's desire to breastfeed and its benefits for the new-born, it was decided to maintain anti-TNFα therapy during this period as well. The patient was informed of the risks of both maintaining and withdrawing the anti-TNFα agent and we provided her a good understanding of the available science on the field. The patient singed the informed consent form to maintain the treatment and today she is a happy mother of a healthy girl. Currently she is in remission using low doses of etanercept.

In order to cover unmet needs, the specialized outpatient clinic for Pregnancy & Rheumatic Diseases was created in 2016 by Drs A Pluma and S Marsal, as a national reference centre to attend patients with rheumatic inflammatory and autoimmune disorders during preconception period, pregnancy, postpartum and breastfeeding. Our aim was to offer highly specialized clinical care in this filed for patients from our rheumatology service, from other hospital departments and from patients referred from other hospitals either advice or follow-up.

Since its inception, we started collaboration with the Gynaecology Department from our hospital under the direction of Prof. Elena Carreras. Specifically, we began to work together with Dr. M Casellas from the High-Risk Obstetric Unit. Initially, the patients were followed by both services but progressively, and thanks to the good harmony of the two departments and the favourable outcomes for the patients since this collaboration was initiated, we have managed to establish a multidisciplinary outpatient clinic.

Patients included in this research project were prospectively recruited from the Pregnancy & Rheumatic Diseases specialized outpatient clinic in the

Rheumatology Department of the Vall Hebron University Hospital. Pregnant healthy controls were hospital staff members and other healthy voluntary women.

4.2.2. Inclusion and exclusion criteria

The **inclusion criteria** of the RA patient cohort were:

- Pregnant women
- Between 18 and 45 years old
- Caucasian
- Fulfilment of the EULAR/ACR 2010 classification criteria for RA (25)
- Positive for RF and ACPA

Disease activity was evaluated using the Disease Activity Score (DAS28), Complex Disease Activity Index (CDAI) and Simplify Disease Activity Index (SDAI) scores (142). In the following table, the cut-off points are described (Table 4):

Table 4. Disease activity scores in Rheumatoid Arthritis

	DAS28	CDAI	SDAI
Remission	< 2.4	≤ 2.2	<3.3
Low activity	≤ 3.2	> 2.2 a ≤ 5	<11
Moderate activity	>3.2 to ≤ 5.1	> 5 a ≤ 21	>11 to < 26
High activity	> 5.1	> 21	≥26

The **inclusion criteria** for healthy controls were:

- Pregnant women
- Between 18 and 45 years old
- Caucasian
- No previous chronic autoimmune inflammatory disease

The **exclusion criteria** for RA patients and healthy controls were:

- Individuals under fertility treatment
- Active infections at recruitment

- Psychiatric condition that, according to the clinical researcher, could impair the ability to participate in this project

4.2.3. Definition of the study subjects groups

Clinical data and biological samples were obtained from n=3 individuals belonging to the RA group and n=3 individuals belonging to the healthy control group. A total number of 6 subjects were included in the present study.

4.2.4. Study protocol

The study protocol included a visit at 12-13 weeks' gestation, 25-26 weeks' gestations, 30-31 weeks' gestation, 8-9 weeks' postpartum (or two months postpartum) and six months postpartum for all participants. Blood sampling was done at 30-31 weeks' gestation and 8-9 weeks' postpartum.

In the next section, the protocol followed in the study is described:

First, it was assessed whether the patient met the inclusion criteria and none of the exclusion criteria and informed consent obtained. Afterwards, an exhaustive anamnesis was made to the participants to record the following clinical and epidemiological variables:

4.2.4.1. Epidemiological and non Rheumatoid Arthritis associated clinical variables

a) Weight (Kg) and height (cm) before pregnancy***.***

b) Maternal age at the moment of delivery.

c) Demographic data

- **Study level.** To determine the degree of studies, the following classification was used:
  - Primary School (until 12 years old)
  - Secondary School (until 16 years old)
  - College / Professional training

- University
- No studies

- **Profession.** To determine the profession, the International Standard Classification of Occupations (143) was used:
  - Manager
  - Professional
  - Technician and associate professional
  - Clerical support worker
  - Service and sales worker
  - Skilled agricultural, forestry and fishery worker
  - Craft and related trades worker
  - Plant and machine operator, and assembler
  - Elementary occupation
  - Armed forces occupation

d) Consumption of toxic substances

- **Smoking habit** (cigarettes, cigars, pipe) daily, occasionally and number of cigarettes per day.
- **Alcohol habit** (beer, wine, cocktails) daily, weekends and quantity.
- **Others**

e) Personal pathologies

If there was any previous pathology of interest, it was collected.

f) The obstetric history was recorded through TPAL

TPAL is one of the methods to provide a quick overview of a person's obstetric history. In TPAL, the T refers to term births (after 37 weeks' gestation), the P refers to premature births, the A refers to abortions, and the L refers to living children. Information regarding obstetric complications in previous pregnancies was also recorded.

g) Concomitant pharmacological/hormonal treatments

All types of hormonal treatment, drugs or vitamins supplement during pregnancy were noted.

4.2.4.2. Rheumatoid Arthritis clinical variables

a) Retrospective data on RA and previous therapies

The date of diagnosis of the disease and all the treatments performed from the beginning to the date of gestation were collected.

b) Physical general exploration

All patients underwent a general physical examination that included cardiopulmonary auscultation. Information regarding this exploration was collected.

c) Musculoskeletal exploration included the following explorations

- Number of 28 painful joints (NPJ)
- Number of 28 swollen joints (NSJ)
- Physician global evaluation (PhGV)
- Patient global evaluation (PtGV)
- Patient pain evaluation (PPV)

d) Calculation of disease activity scores

The following activity indices were calculated:

- Disease Activity Score C Reactive Protein (DAS28-CRP)
- Disease Activity Score Erythrocyte Sedimentation Rate (DAS28-ERS)
- Complex Disease Activity Index (CDAI)
- Simplify Disease Activity Index (SDAI)

e) Whole blood and urine sample collection

Samples were obtained in the laboratory department of the hospital by specialized personnel.

4.2.4.3. Pregnancy clinical variables

At each pregnancy trimester, the obstetrician performed an examination obtaining the following information:

a) First Trimester

- Clinical history and co-morbidities with special attention to infection and metabolic syndromes were reported.
- Diabetes screening results were reported if the test was performed.
- Weight (Kg) and arterial pressure (mmHg).

b) Second Trimester

- Clinical history and co-morbidities with special attention to infection and metabolic syndromes were reported.
- Diabetes screening results were reported if the test was performed.
- Weight (Kg) and arterial pressure (mmHg).
- Echography results from 18-22 weeks' gestation.
- Pathology record of the actual pregnancy regarding preeclampsia, eclampsia, HELLP syndrome, thrombosis, threat of abortion or other pathologies.

c) Third Trimester

- Clinical history and co-morbidities with special attention to infection and metabolic syndromes were reported.
- Diabetes screening results were reported if the test was performed.
- Weight (Kg) and arterial pressure (mmHg).
- Echography from 32-36 weeks' gestation.
- Pathology record of the actual pregnancy regarding preeclampsia, eclampsia, HELLP syndrome, thrombosis, threat of abortion or other pathologies.

4.2.4.4. Clinical variables of delivery

a) Weeks of gestation at the moment of parturition

b) Whether labour was induced

Induction of parturition if required and the motivation was recorded.

c) Delivery type

Information was collected in relation to the type of delivery (eutocic or dystocic). Information was collected in relation to whether instrumentation or caesarean section was required and the motivation.

4.2.4.5. Clinical variables of the new-born

The information regarding the new-born was obtained from the mother at the different visits.

a) At birth

- Weight (gr) and height (cm) of the baby.
- APGAR score at birth was reported. This score is a quick clinical test to evaluate the health of new-borns at 1 and 5 minutes after birth. A refers to Appearance (skin colour), P refers to Pulse (heart rate), G refers to Grimace (reflex irritability), A refers to Activity (muscle tone), and R refers to Respiration.
- Type of nutrition:
  - Maternal breastfeeding
  - Artificial breastfeeding
  - Mixed feeding

b) At eight weeks and six months of life

- Weight (gr) and height (cm) of the baby
- Type of nutrition:
  - Maternal breastfeeding
  - Artificial breastfeeding
  - Mix breastfeeding

4.3. Patient Data collection

The annotation of the clinical and epidemiological data obtained from each individual at each time point was registered in a study database. The

Rheumatology Research Group at Vall Hebron Research Institute (GRR-VHIR) has >15 years experience in coordinating data collection of different studies. The bioinformatic team coordinated by Dr Julià designed and implemented a specific study database. The database fields and data types were determined by the collaborating group of specialists (Drs Pluma, Förger, Østensen and Marsal), according to the objectives of the project.

4.4. Biological samples collection

Due to the enormous difficulty of obtaining longitudinal cohorts of patients and healthy pregnant women, it was decided to make an additional effort to take advantage of the potential derivatives from this study so that this project would constitute the beginning of a larger cohort of pregnant women for further studies. For this, a comprehensive protocol for obtaining samples was established, in which samples were, stored with maximum guarantees of conservation in the IMID-Biobank, allowing us to make use of samples in the future which were collected for this doctoral thesis.

The complete protocol for obtaining biological samples from RA patients and healthy pregnant women is described below.

Peripheral whole blood samples

Collection tubes: 2 x 10 ml BD Vacutainer® EDTA K2E tubes

Once whole blood samples were obtained by peripheral venepuncture (16 mL approximately), the tubes were sent to IMID-Biobank and processed approximately 24h after blood extraction. In the laboratory, blood samples were centrifuged for 10 min, at 1500×g and room temperature (RT). The resulting plasma layer was centrifuged again for 5 min, at 2500×g and RT to eliminate platelets. The clean plasma supernatant was then aliquoted and stored at -80ºC.

From the resulting cellular fraction, genomic DNA was extracted using the Chemagic™ MSM I robotic system (PerkinElmer) and Chemagic DNA Blood Kit special 7ml (PerkinElmer) extraction method. The isolated DNA was normalized at 100 ng/ul, aliquoted and stored at -80ºC.

Collection tubes: 2 x 60 ml BD Vacutainer® ACD solution B tubes

Once whole blood samples were obtained using peripheral venepuncture (12 mL approximately), the tubes were sent to IMID-Biobank and processed approximately, 24h after blood extraction. In the laboratory, blood samples were centrifuged for 30 min, at 500×g and RT. The resulting plasma layer was centrifuged again for 5 min, at 2500×g and RT to eliminate platelets. The clean plasma supernatant was aliquoted and stored at -80ºC.

From the remaining cellular fraction, PBMCs separation was performed using Ficoll density gradient centrifugation. The cellular fraction was diluted with an equal volume of pre-warmed RPMI 1640 culture medium (Lonza). The diluted blood was then carefully layered onto a Leucosep tube (Greiner Bio-One) prefilled with 15 ml of Ficoll-Plus (GE Healthcare Biosciences AB) and centrifuged for 15 min, at 800×g and RT (without acceleration and brake). After centrifugation, PBMC were collected with a sterile Pasteur pipette into a 50-ml tube, diluted up to 20 ml with pre-warmed RPMI 1640 culture medium and centrifuged for 10 min, at 400×g and RT. Following a second washing step with 10 ml of RPMI 1640 culture medium and a 5-min centrifugation, PBMC were resuspended in 6 aliquots of freezing media. Freezing media consisted of RPMI 1640 with 20% heat-inactivated foetal bovine serum (Sigma-Aldrich), 10% DMSO (Sigma-Aldrich), and penicillin-streptomycin 1:1000 (Lonza). One-millilitre aliquots were gradually frozen using a commercial freezing box (Mr. Frosty, Nalgene) at − 80 °C for 24 h and then stored in a vapor-phase liquid nitrogen tank at − 150 °C.

Collection tubes: 2 x 2.5 ml PAXgene® Blood RNA tubes.

Once the whole blood sample was obtained using peripheral venepuncture (5 mL approximately), the Paxgene tubes were sent to IMID-Biobank and processed approximately 24h after blood extraction. In the laboratory the samples were stored 2 hours at 4ºC, overnight at -20ºC and, on the next day, they were frozen to -80ºC for storage.

Urine samples

1x10 mL tube with 75 HCl µl

Once the urine sample was obtained (10mL approximately), the tube was sent to IMID-Biobank. Approximately 24h after obtaining, urine was centrifuged for 5 min, at 600xg and RT in order to eliminate any possible urinary sediment. Then, the clean urine supernatant was aliquoted and stored at -80ºC.

4.5. Single cell technology analysis

The sample processing has been carried out at the Centre Nacional d'Anàlisis Genòmica (CNAG) by the Single Cell Genomics Team leaded by Dr. Holger Heyn.

Cell culture and T cell activation

Cryopreserved (-80ºC) PBMC samples (matched GEST and POST time points) were thawed in culture media (Hibernate-A supplemented with 10% FCS; ThermoFisher), centrifuged for 5 min at 500 x g at room temperature, washed once with PBS 1x (2001201; Gibco) and resuspended in 1 ml of 1X PBS^{+}BSA 0.05% (130-091-376; Miltenyi). A TC20™ automated cell counter was used to assess cell number and viability. DAPI-negative cells were FACS sorted with a BD FACSAria™ Fusion Flow cytometer (BD Biosciences) in order to remove dead cells before seeding in pre-warmed culture media (RPMI, 1% Pyruvate, 10% FBS, Pen/Strep) at a density of 0.6-1 million cells/ml in a 24-well cell culture plate. Each sample was split in two experimental conditions, control (1ng/ml of human rIL-2, PHC0021; Thermo Fisher) and Tcell activation (1ng/ml of human rIL-2 + 25ul of Dynabeads Human T-Activator CD3/CD28, 11132D; Gibco). Cells were incubated for 48h at 37°C with 5% CO_2 and 5% humidity, collected in 1.5 ml tubes (Eppendorf) and separated from beads using a magnet (Invitrogen). Each of the four conditions (gestational (GEST) - control, GEST-T cells activation, postpartum (POST) - control, POST-T cells activation) were labelled using a specific oligo-tagged antibody following the Cell Hashing protocol (Cell hashing and Single Cell Proteogenomics Protocol Using TotalSeq™B Antibodies; BioLegend), in order to pool them before proceeding to scRNA-seq. Finally, cells were resuspended in an appropriate volume of 1X PBS+0.05% BSA to obtain a suitable cell concentration

for 10x Genomics scRNA-seq. An equal number of hashed cells from each of the conditions was mixed and filtered with a 40 µm strainer. Cell concentrations of the pool was verified by counting with a TC20™ Automated Cell Counter.

Single Cell 3' library construction

Cells were partitioned into Gel Bead in Emulsions with a Target Cell Recovery of 25,000 total cells. cDNA sequencing libraries were prepared using the Chromium Next GEM Single Cell 3' v3.1 kit (10X Genomics) following manufacturer's instructions (Chromium Next GEM Single Cell 3' kit with Feature Barcode Technology for Cell Multiplexing). Briefly, after GEM-RT clean up, cDNA was amplified using 11 cycles and cDNA QC and quantification were performed on an Agilent Bioanalyzer High Sensitivity chip (Agilent Technologies). 50 ng of cDNA were used for library preparation. Libraries were indexed by 13 cycles of PCR using the PN-220103 Chromiumi7 Sample Index Plate. Size distribution and concentration of Gene Expression (GEX) and Hash Tag Oligonucleotide library (HTO) libraries were verified on an Agilent Bioanalyzer High Sensitivity chip. Finally, sequencing was carried out on an Illumina NovaSeq 6000 sequencer to obtain approximately 20,000 reads per cell.

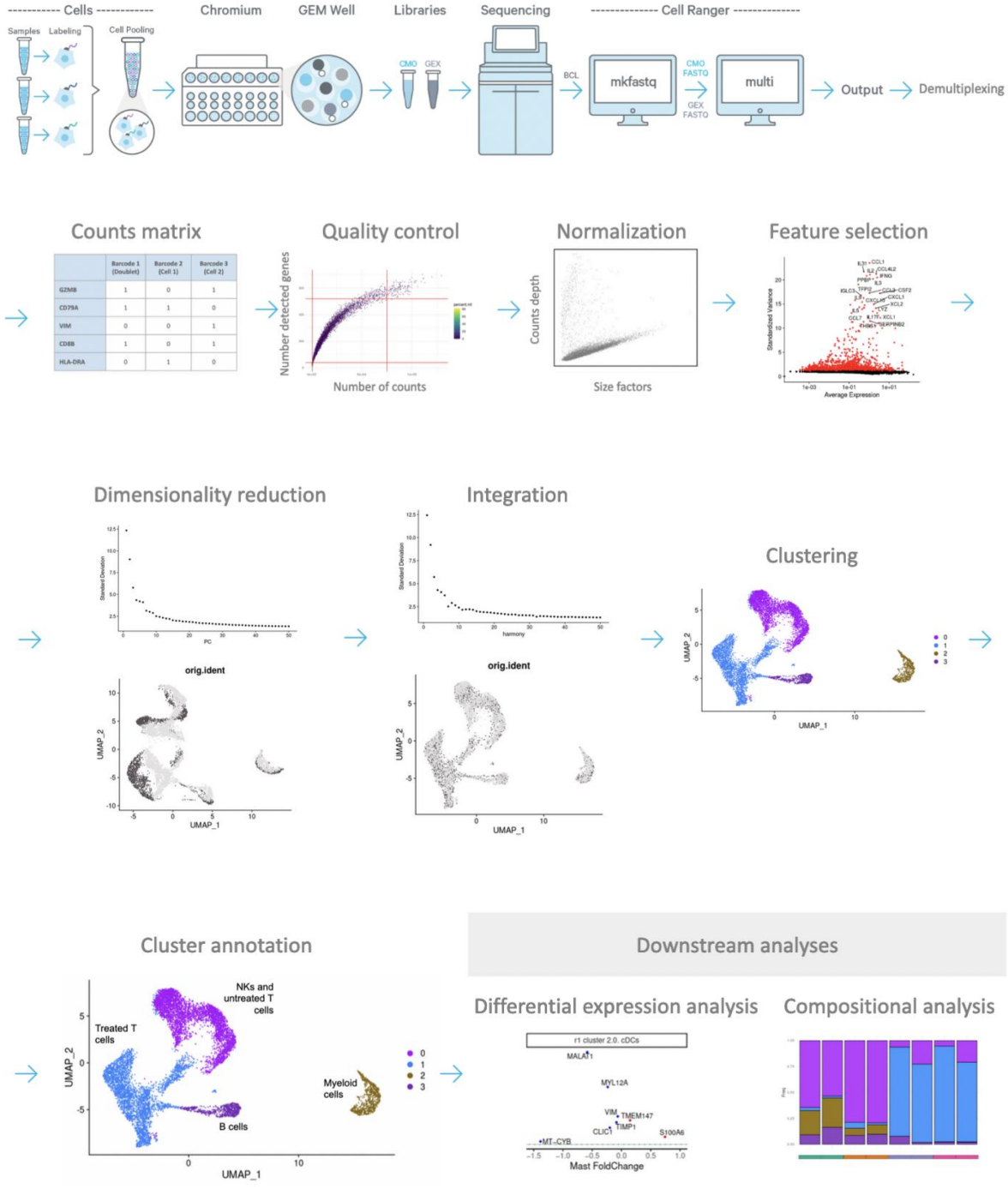
4.6. Advanced analytics

4.6.1. Single cell RNA-seq analysis methodology

Bioinformatic analyses were performed using the programming language *R*, version 4.04 (144). Most of the analyses were performed using *Seurat* version 4.0.1 (145), an *R* toolkit for single cell genomics designed for quality control (QC), analysis and exploration of scRNA-Seq datasets. Many of the newly developed methods for the different steps of the analysis of single cell data can be applied with this package. The analytical workflow is summarized in (Figure 11).

Figure 11. Single cell analysis workflow**.** Single-cell RNA is isolated and sequenced using the 10X Genomics protocol. Alignmentand gene counting is performed using Cell Ranger, which generates gene expression count matrices. Count matrices undergo quality control, normalization, feature selection,

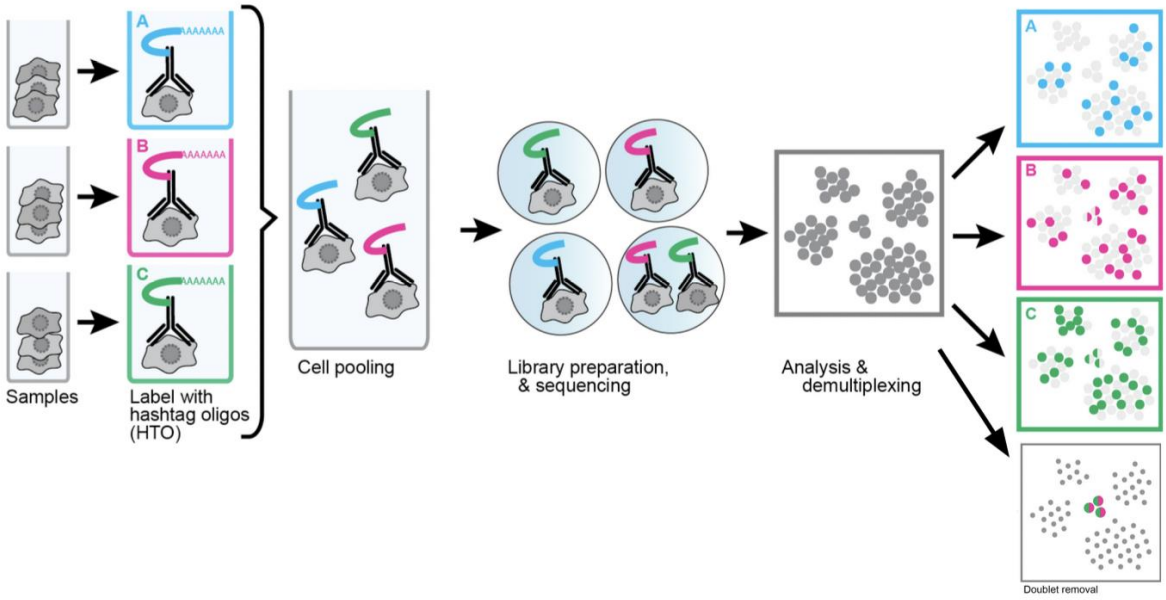
dimensionality reduction and integration to finally define batch-corrected cell clusters, which are annotated to cell populations. The defined cell populations are used for downstream analyses, including cell composition and differential expression analysis. Adapted from (146,147) and built using figures from our results that are explained in detail in the results section.



4.6.1.1. Alignment, demultiplexing and quality control

As explained in the single-cell technology analysis section, samples were labelled with oligo-tagged antibodies following the Cell Hashing protocol and pooled together in four different libraries before sequencing (Figure 12). This procedure allows processing and sequencing together different samples, which greatly reduces the strong batch effects that affect scRNAseq technologies.

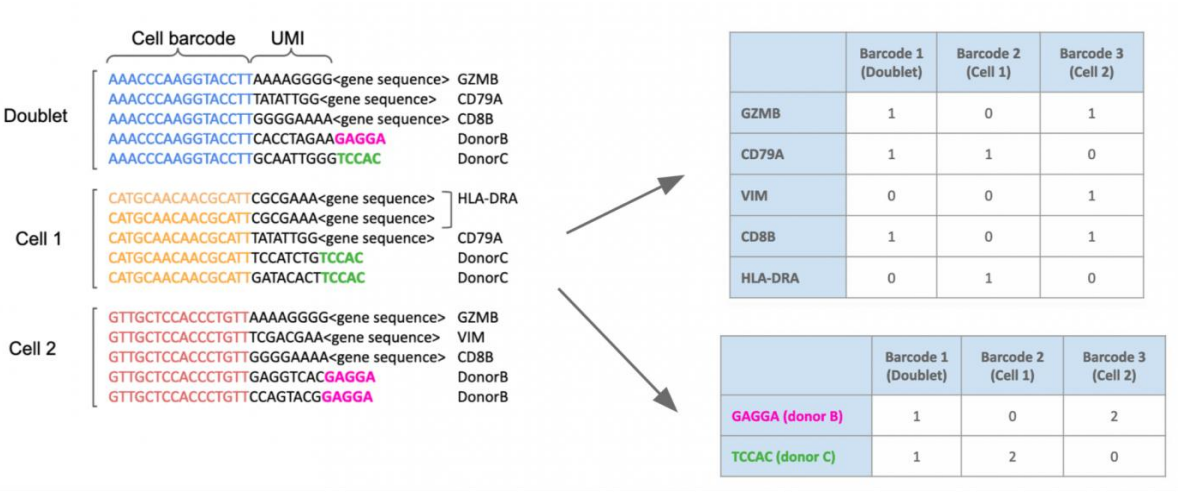
Figure 12. Schematic overview of sample multiplexing by cell hashing**.** Cells from different samples are incubated with DNA-barcoded antibodies recognizing ubiquitous cell surface proteins. Distinct barcodes (referred to as hashtag-oligos, HTO) on the antibodies allow the pooling of multiple samples into one scRNA-seq experiment. After sequencing, cells can be assigned to their sample of origin based on the detection of the HTO tags. Gel beads in EMulsion (GEM) that have captured more than one cell coming from two different samples can be easily detected by the presence of more than one HTO. Adapted from (148).



For each pool, a gene expression library and an antibody capture library were generated, and were sequenced to generate FASTQ files. Each read consisted on (i) Illumina sequencing adapters and primers, (ii) a 14 bp cell barcode identifying each GEM, (iii) a feature barcode or Unique Molecular Identifier (UMI) that is added to sequencing libraries before conducting any PCR amplification, to enable

accurate identification of PCR duplicates, (iv) an anchored 30 bp oligo-dT to prime polyadenylated mRNA transcripts and (v) the actual gene or HTO sequence (149) (Figure 13). FASTQ library files were aligned to the reference genome hg19 using the Cellranger (10x Genomics) count pipeline with feature barcode analysis, version 5.0.1. This generated a gene expression matrix, with genes in rows and cell barcodes in columns, as well as an antibody capture matrix, with the HTO feature barcodes in rows and cells in columns (Figure 13).

Figure 13. Schematic representation of single cell gene expression and antibody capture libraries and how these relate to the final count matrices. Note that cell barcodes allow identifying the cells, UMIs allow identifying PCR duplicates and HTOs allow identifying both the l donor and GEMs containing doublets.



	Barcode 1 (Doublet)	Barcode 2 (Cell 1)	Barcode 3 (Cell 2)
GZMB	1	0	1
CD79A	1	1	0
VIM	0	0	1
CD8B	1	0	1
HLA-DRA	0	1	0

	Barcode 1 (Doublet)	Barcode 2 (Cell 1)	Barcode 3 (Cell 2)
GAGGA (donor B)	1	0	2
TCCAC (donor C)	1	2	0

Library quality was controlled by evaluating the estimated number of recovered cells, the hashing efficacy (i.e. percentage of reads with a valid HTO barcode, a valid UMI and a valid cell barcode), the mean reads per cells and the fraction of reads mapping to exonic reads.

Libraries were demultiplexed by applying Seurat's *HTODemux* function to the antibody capture matrices, with previous centered Log Ratio transformation across cells (150). This method infers, for each library and based on the expression levels of the HTOs, the original condition of each barcode (i.e. gestational-control treatment, gestational-Tcell activation, postpartum-control treatment, postpartum-T cell activation). Additionally, it also allows for detection of negative and doublet GEMs. After this, a cell quality control filter was performed, by keeping only cells

identified as singlets and with a total number of counts between 750 and 75,000, a total number of detected genes between 500 and 6,500 and a percentage of mitochondrial gene counts lower than 15%. These filters are used to remove potentially empty GEMs, GEMs containing more than one cell or GEMs containing dying cells, cell with high mitochondrial gene expression, which are technical artifacts known to affect single cell technology.

4.6.1.2. Cell cluster identification

Clusters of cells with similar expression profiles were identified by applying the cluster identification pipeline in two subsequent rounds. The pipeline is composed of the following steps:

1. **Dimensionality reduction**. First, genes that are not detected in more than five cells are excluded from the dataset. Counts are then normalized for the total number of detected transcripts using log-transcript per 10,000 normalization (logTPK10): feature counts for each cell are divided by the total counts for that cell and multiplied by a scale factor of 10,000. The resulting data is then log-transformed. From the remaining genes, the top 2,500 most variable genes are selected using the Variance Stabilizing Transformation (VST) method (151). On this set of genes, principal component analysis (PCA) is performed. The obtained principal components (PCs) represent the major axes of variance of variation in the dataset. For downstream analysis, the top (<50) PCs, capturing most of the variance, are selected.
2. **Integration**. Library preparation is a major source of bias in scRNA-Seq analysis. To remove this bias, different methods have been developed. Here cells coming from different libraries are integrated by applying *harmony* (152) to the previously obtained PCs. *Harmony* projects cells into a shared embedding in which cells are grouped by cell type rather than library. The elbow rule (153) is used to select the final number of *Harmony*-corrected PCs that will be used to represent the cells. Library integration is assessed by computing the Local Inverse Simpson's Index (LISI) (152) and

controlling that its median value is close to two in the two sequencing libraries, and by visual inspection of the UMAPs.

3. **Visualization**. Uniform Manifold Approximation and Projection (UMAP) are two dimensional manifolds (154) useful to visually inspect the cell clustering procedure.

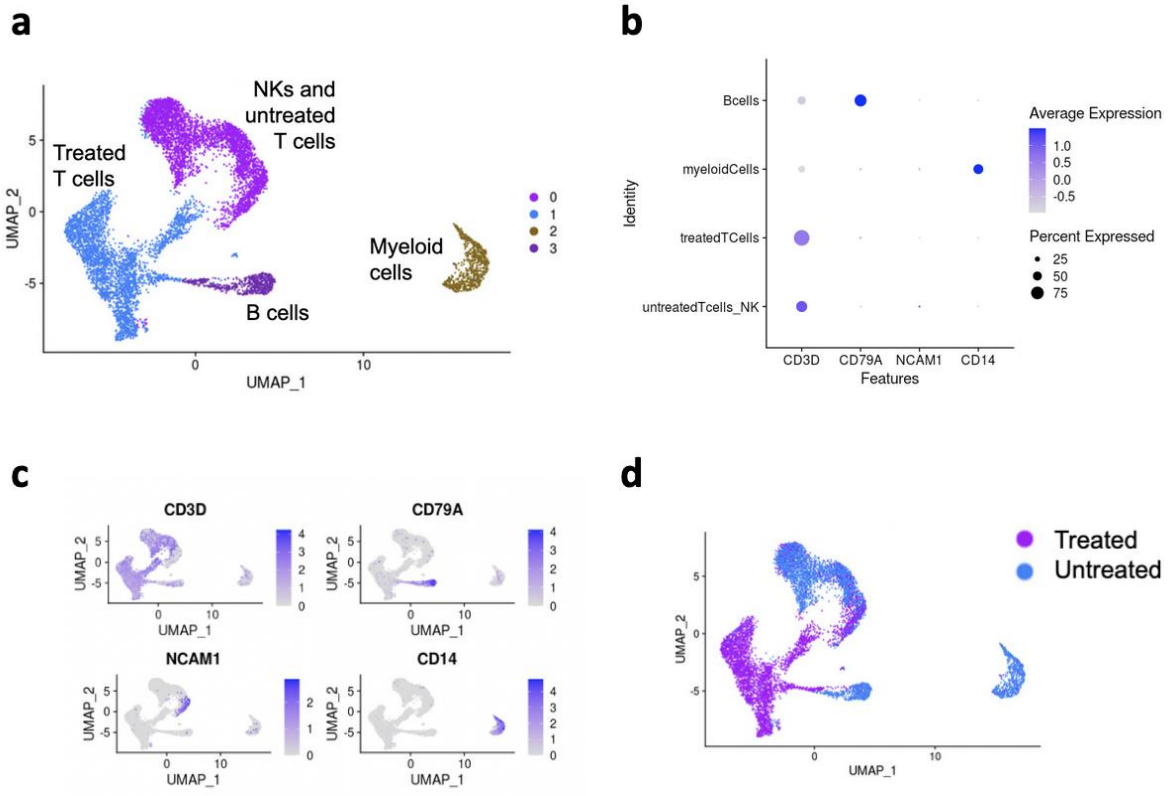
4. **Clustering**. Cells are finally clustered by building a k-Nearest Neighbour (k-NN) graph (155). The kNN graph is then transformed to a shared k-NN graph, by calculating the neighbourhood overlap between every pair of cells using the Jaccard index. The Louvain algorithm (156), a modularity optimization method, is used to define the cell clusters. For this objective, different resolutions can be used. The optimal resolution level is decided by carefully inspecting the clusters, and finding the resolution level where cell subtypes do not aggregate into the same cluster, rare cell types can be identified and, at the same time, cells belonging to the same population are not split across different clusters (157).

In the present study, the above clustering identification pipeline was performed in two rounds. The first round was used to identify the main immune lineages in PBMCs (i.e. T cells, B cells and myeloid cells). This clustering was found to also separate activated cells from non-activated cells. In the second round, the pipeline was applied independently to each of the identified lineages. This step allowed to focus on those features that are most variable within each specific population. Given the important impact of finding the optimal resolution on the final results, in the second round of clustering we decided to keep two different levels of resolution: resolution 1 (r1), which provided a more general view, and resolution 2 (r2) which provided a more granular separation of cell types and states. The differential composition analyses were performed at the two resolution levels and for the differential expression analysis between pregnant and postpartum samples we only used the first resolution level. This latter aspect was decided based on the fact that cell populations defined with the second level of resolution were very small and there would not have been enough power to detect differences in gene expression with such granularity.

4.6.1.3. Cell cluster annotation

One of the most crucial steps in single cell analysis is the interpretation and annotation of the identified clusters. This analytical step it helps to identify the underlying cell types, the states and the gradients that the clusters represent. Consequently, it is a necessary step to better understand the cell and tissue biology that is under study. To this aim, we used manual cell annotation. Up to date, manual annotation is the most reliable method for cell annotation (157).

Figure 14. Illustrative example of the cell cluster annotation process**.** In this example, (**a**) unlabeled clusters (0-3) are annotated as 0- NKs and untreated T cells, 1- treated T cells, 2- B cells and 3- myeloid cells based on the level of expression of classical lineage markers. This is visually assessed using (**b**) a dot plot and (**c**) UMAPs where the colour is proportional to the gene expression values. The UMAP helped to distinguish a subpopulation of NK cells ($CD3D^-$ $NCAM1^+$) within the cluster of untreated T cells. (**d**) Other cell characteristics can be studied using the UMAPs. In this case, the two T cell clusters are distinguished by the presence or absence of *in vitro* stimulation.



For manual cell annotation (Figure 14), we used several resources. First, we visualized the gene expression levels of literature-curated markers of circulating immune populations. For this objective, we used UMAPs and dot plots. Second, we identified and reviewed the genes that are more strongly over-expressed in each cluster compared to the rest of clusters (i.e. cluster marker genes, see below). We also performed functional enrichment analysis (see below) to help annotate the predominant biological function of the identified marker genes. Finally, we supported this approach with the visual and statistical inspection of other possible variables that may be driving the cluster identity (e.g. mitochondrial expression, number of expressed genes, library, treatment, pregnancy stage and cell cycle phase).

4.6.1.4. Differential Expression analysis

Differential expression refers to the identification of genes significantly over or under-expressed between two conditions. In this study, this analysis was performed with three objectives. The first objective was to identify cluster marker genes, that is, genes that are over-expressed in one cluster compared to all the other clusters. The second objective was to identify genes that are up-regulated by *in vitro* stimulation in pregnancy and in the post-partum, without taking into account the underlying cell populations. Finally, the third objective was to identify genes that are differentially expressed in pregnancy compared to the post-partum for each particular cell cluster.

In all three cases, differentially expressed genes (DEGs) were identified using MAST (158), a flexible statistical framework for the identification of transcriptional changes in scRNAseq data. A technical and analytical problem with scRNAseq data is that, due to the small amount of starting material, there is an increased likelihood of dropouts. This is a stochastic process by which we are not able to detect expression of many genes that are in fact expressed in the cells. MAST implements a generalized linear model that takes into account the stochastic dropout that affects scRNAseq data as well as its characteristic bimodal expression distribution. A hurdle model is proposed, where differences between

conditions both in levels of expression (i.e. fold change) and in percentage of expressing cells are taken into account.

In the three situations, we modelled gene expression by taking into account the cellular detection rate in each cell. To detect cell marker genes, the cell type is added to the model. To detect the effect of cell stimulation, the treatment each cell has received is considered, and also the donor. Finally, to detect genes differentially expressed during pregnancy in each cell type, an interaction model is defined, which considers the interactive effect of the pregnancy stage and the cell type in gene expression, adjusting for the donor.

4.6.1.5. Functional enrichment analysis

To help characterize the biological mechanisms underlying DEGs, an enrichment analysis was performed using the *GeneOverlap* R package. Functional enrichment analysis is a method to determine classes of genes that are over-represented in a large set of genes. For instance, functional enrichment analysis will identify that one cell cluster is enriched in antigen presentation if many of the marker genes of the cluster are annotated to be involved in this biological function. Briefly, we computed the odds ratio between the list of DEG and the genes defined to be involved in a particular biological process. We included in the analysis the curated biological processes from the gene ontology database (159,160), as implemented in the GOdb package version 3.12, the human blood atlas cell type marker genes (161) and the molecular pathway signature genes from PROGENy (162).

4.6.1.6. Analysis of differential cell type proportions

Changes in cell compositions between pregnancy and the postpartum were assessed with a mixed effects linear model (nlme *R* package version 3.1). In particular, differences were studied by modelling cell proportions in each sample based on the pregnancy stage including the donor as a random variable in the model.

The doctoral student has been involved in the data analysis process and has learned to perform clusters annotation based on markers and differentially

expressed genes. She also has learned to interpret the differential expression, functional enrichment and differential cell type proportions analysis.

4.7. Ethical aspects

The present project has been presented and approved by the Ethical Committee for Clinical Research of the Hospital Universitari Vall d'Hebron (HVH). It has been approved and assigned the Project code: PR(AG)52/2016. The study has been developed in accordance with the principles of the Declaration of Helsinki.

Informed consent and associated data obtainment

The doctoral student selected the patients according to the defined inclusion and exclusion criteria and requested their participation in the research. The doctoral student adequately informed the patients and healthy controls about the purpose and details of the project included in the information document prepared for this project. In case the subject agreed to participate in the research, she was invited to sign the Informed Consent document in triplicate, one for the patient, one for the clinical records and one for the IMID-Biobank's registry.

4.8. Work plan

The present doctoral thesis has been carried out in the following stages:

- Stage I. Study design. 2016
- Stage II. Database elaboration. 2016-2017
- Stage III: Patient's selection and follow-up. 2017-2020
- Stage IV: Longitudinal samples and clinical data collection. 2017-2020
- Stage V: Review of the different sources of information. 2016-2017
- Stage VI: Laboratory training in samples processing. 2017-2018
- Stage VII: Introduction of the data. 2017-2021
- Stage VIII: Molecular data generation. 2020
- Stage IX: Statistical analysis and data interpretation. 2020-2021
- Stage X: Writing and dissemination of results. 2021
- Stage XI: Defence of the doctoral thesis expected at the end of 2021

This project has been developed in accordance with the work plan and no relevant incidents have been identified in its timeline execution.

This doctoral thesis has represented a unique opportunity to apply single cell technology to describe the cells clusters presents in RA pregnant/postpartum patients and find possible shared biological pathways of both conditions, RA and pregnancy or postpartum. In this sense, the participation of the doctoral student has been essential for patient selection, sample and data collection, single cell data analysis and data interpretation. Throughout the entire pre-doctoral period, A Pluma has acquired the necessary knowledge to carry out a pathway disease study using a single cell technology strategy. Future studies as a principal investigator will only need some support from an expert in bioinformatics focused on single cell analysis.

5. Results

5.1. Cohort description

5.1.1. Epidemiological variables

From our outpatient's clinic, a total of 3 RA pregnant patients and 3 pregnant healthy controls (HC) were recruited. Table 5 shows the epidemiological characteristics of our cohort.

Table 5. Epidemiological variables at week 13 of gestation of RA patients and HC

Patient group	Age	Height (cm)	Weight (kg)	Education	Profes-sion	Tobac-co	Alcohol	Pathological record
RA1	33	159	47	University	1	No	No	CIN II cervix
RA2	41	173	63	College	2	No	No	Graves Basedow
RA3	37	163	76	University	3	No	No	Hypothyroi-dism
HC1	33	166	57	University	1	No	No	No
HC2	35	160	54	University	1	No	No	Hypoferriti-nemia
HC3	33	153	58	University	1	No	No	No

RA: rheumatoid arthritis, HC: healthy control, CIN: cervical intraepithelial neoplasia, 1: Health professionals, 2: Craft and related trades workers, 3: Elementary occupations

5.1.2. Rheumatoid Arthritis clinical variables

Table 6 describes the clinical variables related to RA in the patients group. It also shows the clinical activity indices DAS28-ESR, DAS28-CRP, SCAI and CDAI in pregnancy and the postpartum period.

Table 6. Rheumatoid Arthritis clinical variables

Patient	RA1					RA2					RA3				
State	Preg (wk)			PP (mth)		Preg (wk)			PP (mth)		Preg (wk)			PP (mth)	
Time	13	25	30	2	6	13	25	30	2	6	13	25	30	2	6
Symptom onset	2004					2015					2000				
Diagnosis	2004					2015					2001				
Erosion	Yes					No					Yes				
Previous treat	3, 4					6, 7					3, 4, 5, 2				
Actual treat	2	2	2	2,7	5, 7,8	9	9	9	7	7, 3	1	1	1	1	1, 7
PGE	No alteration					No alteration					No alteration				
NAD	0	0	5	0	0	1	0	0	5	4	1	0	1	2	2
NAT	2	0	10	0	0	0	0	0	6	2	1	0	0	2	2
PGV	15	10	43	3	10	4	3	3	20	25	40	29	36	60	60
PPV	15	10	40	8	6	4	3	3	20	25	40	11	36	70	60
PhGV	10	20	70	10	20	5	5	5	20	25	30	20	30	50	50
DAS28 ESR	2.6	2.9	5.3	1.7	1.5	2.7	3.1	3.3	4.8	4.3	4.2	3.5	4.1	4.7	4.9
DAS28 CRP	1.5	1.1	3.8	1.1	1.1	1.6	1.6	1.6	3.4	3.0	2.8	1.9	2.8	3.7	3.6
CDAI	5.0	3.0	25.3	1.8	3.0	1.0	1.0	1.0	15.0	12.0	9.0	3.0	8.0	17.0	15.0
SDAI	2.0	3.0	19.5	1.0	3.0	1.4	1.4	1.4	15.8	12.1	9.3	3.5	8.7	17.4	15.5

Preg (wk): Pregnancy weeks, PP (mth): Postpartum months, Symptoms onset: Start date of illness (year), Diagnosis: Illness diagnosis date (year), PGE: Pregnant / Postpartum Physical general exploration, NPJ: Number of painful joints, NSJ: Number of swollen, PGV: Patient global evaluation 0-100, PPV: Patient pain evaluation 0-100, PhGV: Physician global evaluation 0-100, 1: Certolizumab, 2: Etanercept, 3: Methotrexate, 4: Leflunomide, 5: Salazopyrin, 6: Hydroxychloroquine, 7: Prednisone (or equivalent to 5 mg), 8:Leflunomide, 9: No treatment

5.1.3. Pregnancy and postpartum clinical variables

5.1.3.1. Rheumatoid Arthritis patients

In relation to the clinical variables related to pregnancy and the postpartum (Table 7), no RA patient had received any fertility treatment, there were no pathologies in previous pregnancies and there were no pathologies related to pregnancy or postpartum. Patient RA1 suffered a mild dental infection that required antibiotic treatment one week before to the sample collection at two moths postpartum. The patient did not present with fever. RA patient 2 presented with an alteration in the oral glucose tolerance test but did not require any specific treatment.

Table 7. Pregnancy and postpartum clinical variable of RA patients

Patients group	RA1					RA2					RA3				
Pregnancy status	Preg (wk)			PP (mth)		Preg (wk)			PP (mth)		Preg (wk)			PP (mth)	
Time	13	25	30	2	6	13	25	30	2	6	13	25	30	2	6
TPAL	0010					1031					1001				
Pathology of previous offspring	NA					No					No				
Current treatments	1	1	1	No		1	1	1	No		1, 2	1,2	1,2	No	
Current Infection	No	No	No	Yes	No	No					No				
Pregnancy/ Postpartum Pathology	No					No					No				
Echography	Normal			NA		Normal			NA		Normal			NA	
Arterial Tension	Normal					Normal					Normal				

Preg (wk): Pregnancy weeks, PP (mth): Postpartum months, NA: not applicable, 1: pregnancy supplements, 2: Levothyroxine, 3: iron

5.1.3.2. Healthy controls

Regarding the clinical variables related to pregnancy and the postpartum (Table 8), no HC performed any fertility technique, there were no pathologies in previous pregnancies and oral glucose tolerance test were normal in the three participants. Also, no patient had altered blood pressure. There were two infections reports in the 30th week of pregnancy. HC1 presented an upper respiratory infection but no specific treatment was needed and HC3 had a skin infection on a finger that required topical and oral antibiotic one week before the 30 week blood collection. No patient presented with fever.

Gestational ultrasound follow-up was normal in HC patients 1 and 2. HC3 presented with an alteration in the corpus callosum of the foetus, detected by ultrasound at week 20 of gestation. Amniocentesis with no alteration was performed. In the following ultrasounds, the alteration resolved.

Table 8. Pregnancy and postpartum clinical variables of HC individuals

Patients group	HC1					HC2					HC3				
Pregnancy status	Preg (wk)			PP (mth)		Preg (wk)			PP (mth)		Preg (wk)			PP (mth)	
Week	13	25	30	2	6	13	25	30	2	6	13	25	30	2	6
TPAL	1001					1001					0010				
Pathology previous offspring	No					No					No				
Current treatments	1	1	1	No		1	1	1	No		1	1	1, 3	No	
Current Infection	No	No	Yes	No	No	No	No	No	No	No	No	Yes	No	No	No
Pregnancy/ Postpartum Pathology	No					No					No	No	Yes	No	No
Echography	Normal			NA		Normal			NA		Nor.	Alt.	Nor.	NA	
Arterial Tension	Normal					Normal					Normal				

Preg (wk): Pregnancy weeks, PP (mth): Postpartum months,1: pregnancy supplements, 2: Levothyroxine, 3: iron , Nor.: Normal, Alt.:Alterated

5.1.4. Clinical variables of delivery

Table 9 shows the information related to the delivery. No premature deliveries and no caesarean sections were required for any of the individuals.

Table 9. Clinical variables of the delivery

Patient group	Partum week	Induction	Reason for induction	Delivery type
RA1	40	No	NA	Instrumented
RA2	39	No	NA	Eutocic
RA3	40	Yes	PRM	Eutocic
HC1	37	No	NA	Eutocic
HC2	40	No	NA	Instrumented
HC3	40	No	2	Eutocic

PRM: premature rupture of membranes, NA: not applicable

5.1.5. Clinical variables of the new-born

Table 10 shows the information related to the clinical variables of the new-born. It's important to highlight that there were no cases of low birth weight or small for gestational age. There were no changes to nutritional and health status in any new-born between the two time points of the postpartum period.

Only the new-born from HC1 presented with a congenital cytomegalovirus infection. Despite this, his clinical evolution during the study did not show any alteration.

Table 10. Clinical variables of the new-borns of the study cohort

Patient Group	New-born evolution	Gender	Weight (mg)	Height (cm)	APGAR	Nutrition
RA1	Normal	Female	3320	49	9/10	Formula milk
RA2	Normal	Female	3020	48	9/10	Formula milk
RA3	Normal	Female	2860	51	9/10	Breastfeeding
HC1	Congenital CMV	Male	2890	47	9/10	Breastfeeding
HC2	Normal	Male	3570	52	9/10	Breastfeeding
HC3	Normal	Female	3220	49	5/9/10	Breastfeeding

CMV: cytomegalovirus

Due to the technical issues that compromised the samples explained in detail in 5.2.1. Quality Control section, only libraries from RA1 and RA2 could be included for the statistical analysis. In the next section a detailed description of both RA patients is provided.

Patient RA1 was a 33-year-old Caucasian woman. She had a weight at the time of conception of 47 kg and a height of 159 cm. The highest academic degree obtained was university. She developed her professional activity in the health sector. She did not smoke or drink alcohol. In relation to her previous pathologies, she presented a CIN II of the cervix that required conization.

In relation to her RA, the patient symptoms began in 2004, the same year as diagnosis. During the evolution of the disease, she presented with erosions. Regarding treatments carried out to control her pathology, the patient previously

followed treatment with MTX and LEF, which were withdrawn. At the time of pregnancy, she was receiving treatment with ETN. This treatment was optimized during the first two trimesters of pregnancy following a regimen of 50 mg every 15 days. Her inflammatory activity indices, calculated by DAS28-ERS, DAS28-PCR, CDAI and SDAI, were on LDA until the third trimester, when, due to an increase in inflammatory activity (DAS28-CRP of 3.76, DAS28-ERS 5.29, CDAI of 25.30, SDAI of 19.50), it was necessary to resume the usual regimen of ETN 50 mg weekly. Regarding postpartum inflammatory activity, although it is true that the patient showed low activity indices (DAS28-ERS, DAS28-PCR, CDAI and SDAI), she experienced a clinical worsening and the patient required an intensification of her treatment by adding prednisone after childbirth. Later, at 6 months postpartum, LEF was added due to an increase of painful inflammation.

In relation to the pregnancy-related variables, the patient presented a TPAL 0010. As a pregnancy-specific treatment, she only used a supplement of folic acid. She did not perform fertility techniques to achieve pregnancy and she had no infections during pregnancy. At two months postpartum, she developed a mild dental infection that required antibiotics at two months postpartum. She did not present pregnancy or postpartum pathologies and pregnancy ultrasound follow-up was normal. She did not show changes in blood pressure during the entire period, nor did she show any changes in the oral glucose overload test. She presented with an uncomplicated pregnancy overall.

Variables collected from the delivery of RA1, show a terminus delivery in the 40th week of gestation. Labour did not require induction but required instrumentation. The patient had a girl who weighed 3,320 mg and measured 49 cm at birth with an APGAR test of 9/10. The baby was breastfed with formula milk in the postpartum visits and her clinical evolution during the follow-up period did not reveal alterations.

Patient RA2 was a 41-year-old Caucasian woman. She showed a weight at the time of conception of 63 kg and a height of 173 cm. The highest academic degree obtained was college. She developed her professional activity in the metallurgical sector. She did not smoke or drink alcohol. In relation to her previous pathologies,

she presented with Graves' Basedow but didn't follow any specific treatment for this pathology during the study period.

In relation to her RA, the patient symptoms began in 2015, in the same year as diagnosis. During the evolution of the disease, she didn't present with erosions. Regarding treatments carried out to control her pathology, the patient previously followed treatment with HCQ and prednisone, which were withdrawn. At the time of pregnancy, she was without treatment. Her inflammatory activity indices, calculated by DAS28-ERS, DAS28-PCR, CDAI and SDAI, were on LDA until the third trimester, when, she presented a DAS28-ERS 3.31, DAS25-CRP of 1.57, CDAI of 1.0 and SDAI of 1.0. It was unnecessary to add any new treatment. Regarding postpartum inflammatory activity, the patient presented with an increase of the activity indices showing a DAS28-ERS 4.76, DAS28-PCR 3.39, CDAI 15.0 and SDAI15.8. In this occasion, the patient needed to add prednisone and MTX to her RA treatment at six-months postpartum.

In relation to the pregnancy-related variables, the patient presented a TPAL 0031. As a pregnancy-specific treatment she only used supplement of folic acid. She did not perform fertility techniques to achieve pregnancy and she had no infections during pregnancy or postpartum. She did not present with pregnancy or postpartum pathologies and ultrasound follow-up was normal on all assessments. She did not show changes in blood pressure during the entire period. She had an alteration in the oral glucose tolerance test but did not require specific treatment.

Variables collected from the delivery showed a terminus delivery in the 39th week of gestation. Labour did not require induction or instrumentation. The patient had a girl who weighed 3,020 mg and measured 48 cm at birth with an APGAR test of 9/10. The baby was breastfed with formula milk in the postpartum visits and her clinical evolution during the follow-up period did not reveal alterations.

5.2. Data analysis

Study overview

For the three RA patients, PBMCs from the 30th week of pregnancy and the eight/ninth week postpartum were isolated. PBMCs from the 30th week of pregnancy were also isolated for the three HC. To study the pregnancy-induced immune changes and the changes in the response to immune activation, for each sample, PBMCs were cultured in two different plates. One of the plates was activated with a T cell activation cocktail (recombinant IL2, anti-CD28 and anti-CD4; treated samples from hereafter) while the other plate received control treatment (recombinant IL2; untreated samples from hereafter). Samples were cultured for 48h and processed for single cell analysis using antibody hashing and 10x technology for a final theoretical number of 25,000 cells per GEM channel (see methods).

5.2.1. Quality control

Raw read expression data were processed into counts matrices using CellRanger and demultiplexed using the Seurat HTODemux function. Library quality control was performed by assessing the total number of recovered cells, the mean number of reads per cell and the proportion of negatives, singlets and doublets detected by demultiplexing (Table 11).

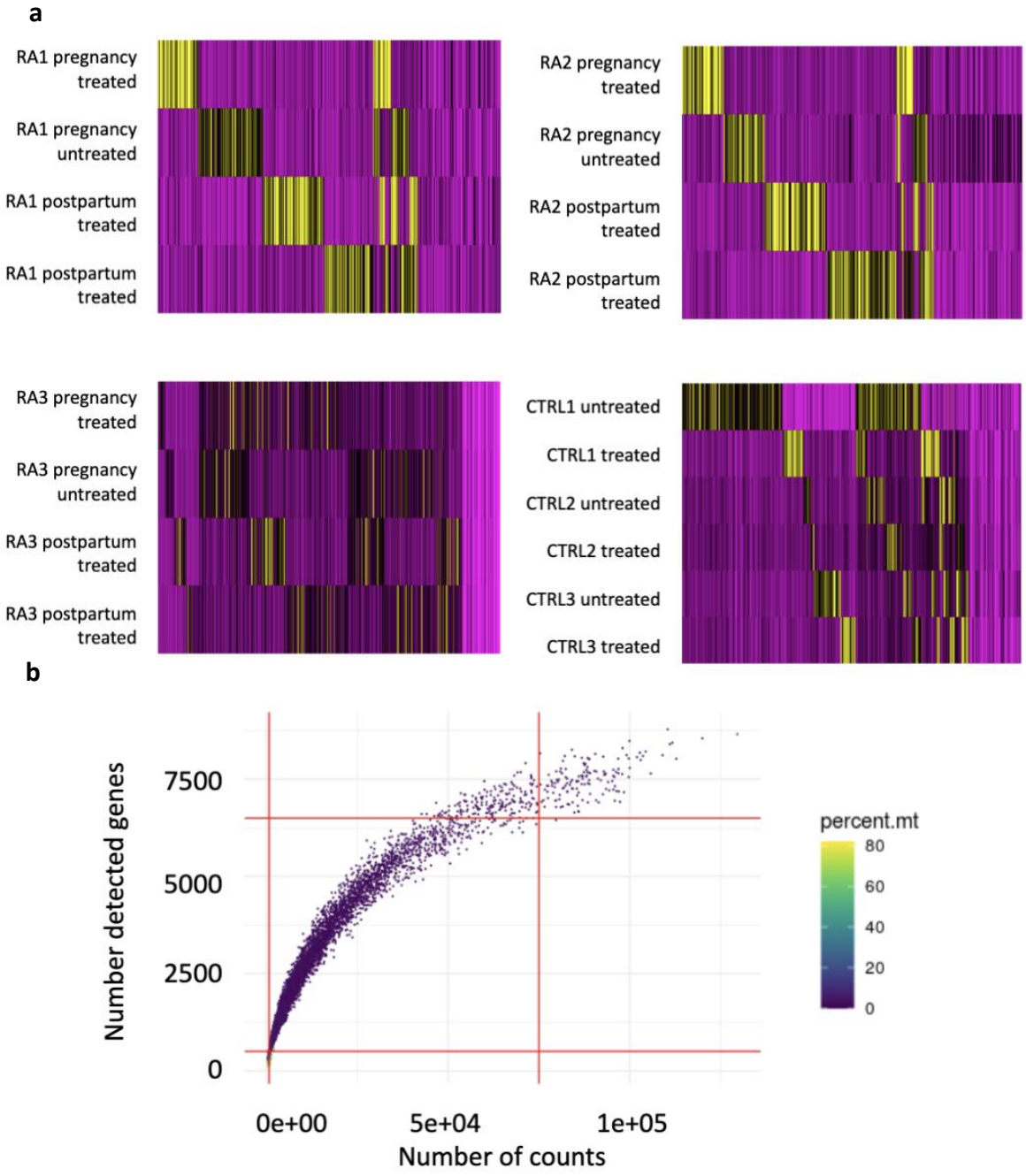
Table 11. Library and sample Quality Control. For each library, the estimated number of recovered cells (before quality control), as well as the number of singlet, doublet and negative barcodes are indicated. The mean number for reads per cell are also indicated (computed using only the singlet barcodes). Hashing efficacy is shown as the percentage of valid reads in the HTO library. For each sample, the final number of singlets and the mean number of reads per cell is indicated.

Library	Included Samples	Recovered cells	Singlets	Doublets	Negatives	Mean number of reads per cell	Hashing - Reads with HTO, UMI and cell barcode
RA1		5,789	3,626 (62.6%)	754 (13.0%)	1,409 (24.3%)	10,254	20%
	RA1 preg. untreated		1,095			2,691	
	RA1 preg. treated		688			17,673	
	RA1 post. untreated		838			2,414	
	RA1 post. treated		1,005			19,952	
RA2		9,108	5,699 (62.6%)	998 (11.0%)	2,411 (26.5%)	10,806	25.8%
	RA2 preg. untreated		1074			4,516	
	RA2 preg. treated		1171			15,053	
	RA2 post. untreated		1775			4,280	
	RA2 post. treated		1679			18,767	
RA3		5,630	692 (12.3%)	4,317 (76.7%)	1,409 (24.3%)	6,083	6.5%
	RA3 preg. untreated		134			4,239	
	RA3 preg. treated		150			3,254	
	RA3 post. untreated		212			2,789	
	RA3 post. treated		196			13,071	
CTRL		913	468 (51.3%)	304 (3330%)	141 (15.4%)	15,435	2.0%
	CTRL1 preg. untreated		272			19,169	
	CTRL1 preg. treated		56			12,2584	
	CTRL2 preg. untreated		19			5,369	
	CTRL2 preg. treated		10			3,514	
	CTRL3 preg. untreated		71			10,147	
	CTRL3 preg. treated		40			11,186	

preg.: pregnancy, post.: postpartum

As shown, the final number of recovered cells per library was lower than expected in the RA samples, ranging from 5,000 to 9,000 cells. These numbers may be related to cell loss provoked by the culturing protocol, but are sufficient to proceed with the single cell analysis. However, for the HC library, the number of recovered cells was extremely low, with only 913 sequenced cells for a total of 6 HC samples. This indicates that there was a severe problem either in cell handling, in the library preparation or in the sequencing quality. Due to the fact that the captured cells for the HC library are also affected by the technical issues that compromised the samples, and that the final number of cells is too low to be used for statistical analyses, all the cells from this library needed to be excluded. Therefore, no data from healthy samples was available for this study.

Figure 15. Cell quality control. (**a**)**.** Heatmaps showing, for each library, expression levels of the HTO identifiers (rows) in each cell (columns). Cells are assigned to a donor based on the expression levels of the HTO identifiers. Samples with high expression of more than one HTO identifier are labelled as doublets, while samples with low expression of all the HTO identifiers are labelled as negatives. (**b**)**.** Cell quality control. Number of counts, number of detected features and percentage of mitochondrial genes in each cell are depicted. Red lines indicate the exclusion criteria.



Regarding the demultiplexing process (Figure 15a), for the RA1 and the RA2 libraries, in 62% of the recovered cells, a single hashing identifier (HTO) was detected, indicating high hashing efficacy and that these barcodes actually correspond to a single cell (singlets). However, for the RA3 library, the hashing efficacy was extremely low, with only 6.5% of reads having an HTO barcode, a valid UMI and a cell barcode, which resulted in only 692 cells (12.3% of total cells, Table 11) identified as singlets. Therefore, the RA3 library needed also to be excluded from downstream analyses.

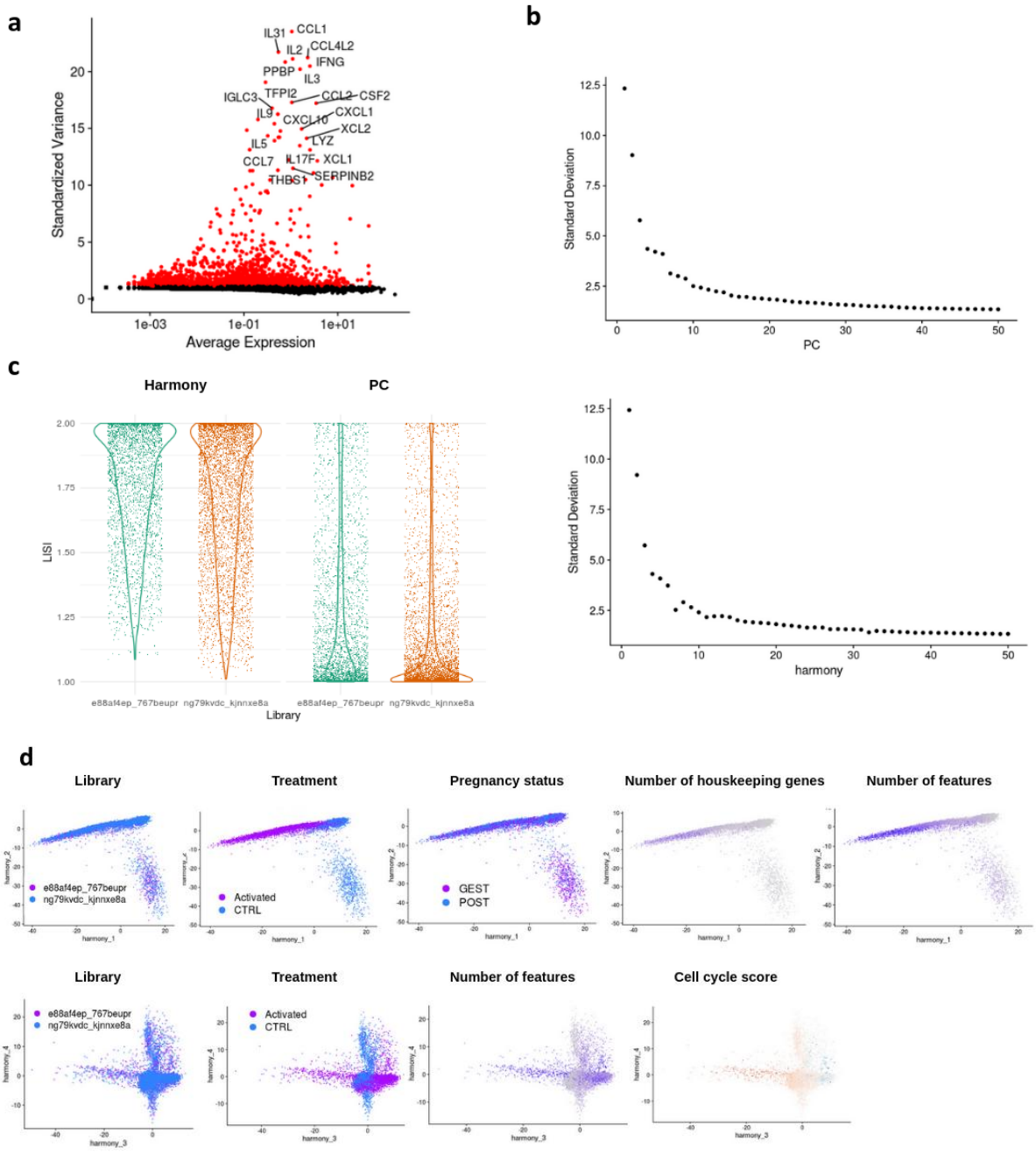
For the two remaining libraries, a final number of 3,626 and 5,699 cells were assigned to a single sample and selected for downstream analysis. To further assess that no doublets, negative barcodes or damaged cells were included in the analyses, we kept only cells with a total number of counts between 750 and 75,000, a total number of detected genes between 500 and 6,500 and a percentage of counts coming from mitochondrial genes lower than 15% (Figure15b). Finally, a total number of 8,604 high quality cells, coming from two RA donors and four different conditions (gestational and post-partum, treated and untreated) were kept for downstream analysis.

Due to these criteria, the RA 1, RA 2 samples were used for the present study. For each patient, to 2 time points (30 weeks' pregnancy and 8 weeks postpartum) were included (4 samples). These four samples were subdivided in stimulated and unstimulated and finally a total number of 8 samples.

5.2.2. Cell cluster identification

To describe the cellular and transcriptional landscape of RA patients during the course of pregnancy, the transcriptomes of 8,604 high quality cells were analysed together. First, we excluded non-expressed genes (genes detected in less than 5 cells) and normalized gene counts by taking the logarithm of the number of detected transcripts per 10,000 total counts (logTP10K normalization). To perform clustering, we kept the 2,500 most variable genes (VST method (151) and reduced the dimensionality by performing a PCA analysis (Figure 16).

Figure 16. Dimensionality reduction. (**a**)**.** Top variant genes selections. Genes are shown based on their Standard Variance and their Average Expression. Shown in red, are the 2,500 most variable genes selected for downstream analyses. (**b**)**.** PCA scree plot. The variance captured by the top 50 PCs and by the top 50 *Harmony* integrated PCs is shown. (**c**)**.** LISI integration score per library before and after *Harmony* integration. (**d**)**.** Exploration of driving variables in the dataset by visualizing the cells using the first 4 PCs and colouring the cells using variables of interest.



Exploratory analysis of the three first PCs (Figure 16d), explain a 27% of the total variance, revealed that the main source of variation in the dataset is the sample activation state. Treated cells show a higher number of detected features and a higher expression of housekeeping genes. PC2 represents an axis of variation that is exclusive for untreated cells. In this axis, we also see differences between pregnant and postpartum samples, with values lower than -20 being almost exclusive of gestational samples. Conversely, only treated cells vary across PC3. This component seems to be associated with different cell cycle phases.

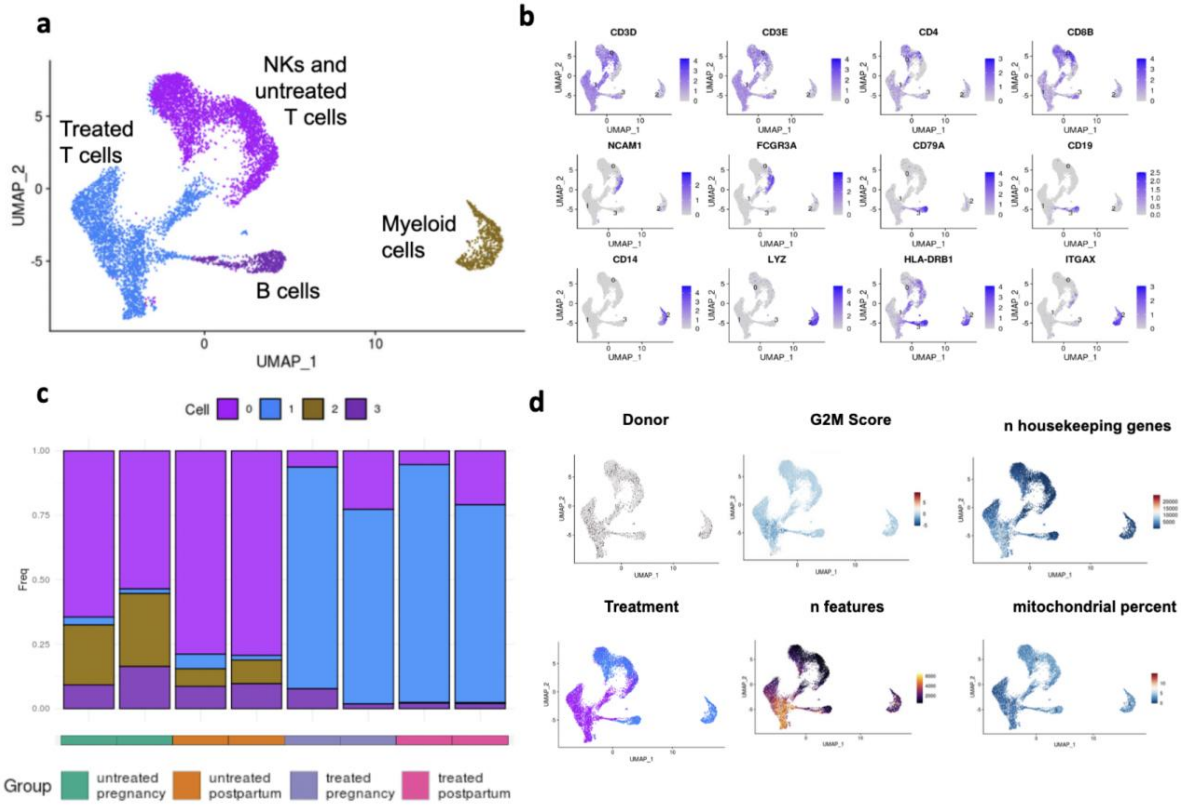
We also detected important differences for the two libraries in the PCs, representing processing batch effects. To integrate the two libraries, we corrected the PCs by sequencing libraries using the *harmony* method (152). This resulted in optimal sample integration, as reflected by a median LISI score of 1.80, compared to a median LISI score of 1.05 before integration (Figure 16c). Using the elbow rule (153), we selected the first 20 harmony integrated PCs to represent the dataset, which together explain 70% of the total variance. These 20 PCs were used to compute a two dimensional representation of the dataset with UMAP. UMAP is a dimensionality reduction algorithm that depicts the cell expression patterns using only two dimensions. We used them in downstream analyses to visualize our data and study the characteristics of the cells in our dataset. The 20 PCs were also used to find cell clusters by applying the Louvain clustering algorithm on a shared shared-kNN at different resolution levels (see methods).

Global cell lineages

By clustering the cells with a low resolution level (0.05), we identified four global clusters (Figure 17a). We annotated the cell types composing each cluster based on the expression of the canonical markers explained below and the absence of markers of other lineages (Figure 17b). Cluster 0 (n=3,803 cells) includes untreated T cells ($CD3D^{+}$) and both treated and untreated NKs (NKs, $NCAM1^{++}$, $FCGR3A^{+}$). Cluster 1 (n=3,437 cells) includes treated T cells ($CD3D^{+}$). Cluster 2 (n=748 cells) includes myeloid cells ($CD14^{+}$ monocytes and $HLA\text{-}DRB1^{+}$ IL3RAdim CD11c bright cDC, with some $CD3E^{+}$ $CD14^{+}$ T cell doublets). Cluster 3 (n=616 cells) includes treated and untreated B cells ($CD79A^{+}$ $CD19^{+}$), with some T cell doublets ($CD3D^{+}$ $CD79^{+}$). Visual inspection of these clusters (Figure 17d)

manifests good integration across libraries and increased gene expression in activated cells, with higher proportions of housekeeping genes and lower proportions of mitochondrial genes.

Figure 17. Global cell lineages**.** (**a**)**.** UMAP plot representing all the cells that passed quality control, coloured by global cell lineages. (**b**)**.** UMAP plot showing the expression level of well established lineages markers across cells. (**c**)**.** Proportion of each cluster across samples. (**d**)**.** UMAP plot showing different characteristics of the studied cells.



To better characterize the cell populations that compose our samples, we performed a second round of clustering in each of the four clusters independently. To this aim, we repeated the previous clustering pipeline: variable gene selection, PCA, PC integration with harmony, construction of the shared-kNN graph and clustering with the Louvain algorithm. With this approach, we were able to focus on the factors that distinguish cell populations within each of the global clusters.

Cell clusters can be defined at multiple resolution levels, depending on the level of granularity the researcher is interested in. In this case, motivated by the fact that

our dataset is relatively small, which leads to the identification of clusters with very few cells if looking for a high-resolution level, we have decided to keep clusters defined at two different resolutions for downstream analyses. We use a first mid-resolution level, more broad, to characterize the main populations that are present in our samples, the differences between them and how their transcriptomic profile changes when comparing pregnancy and postpartum. We used a second high-resolution level to identify specific cell populations, even if these were composed by low numbers of cells (<50), and to study how these changes, in terms of cell composition, in pregnancy compared to postpartum in RA patients.

For clarity, we have labelled cell populations by indicating the resolution level (r1 or r2), the global cluster they come from (0 to 3) and the sub-lineage cluster number.

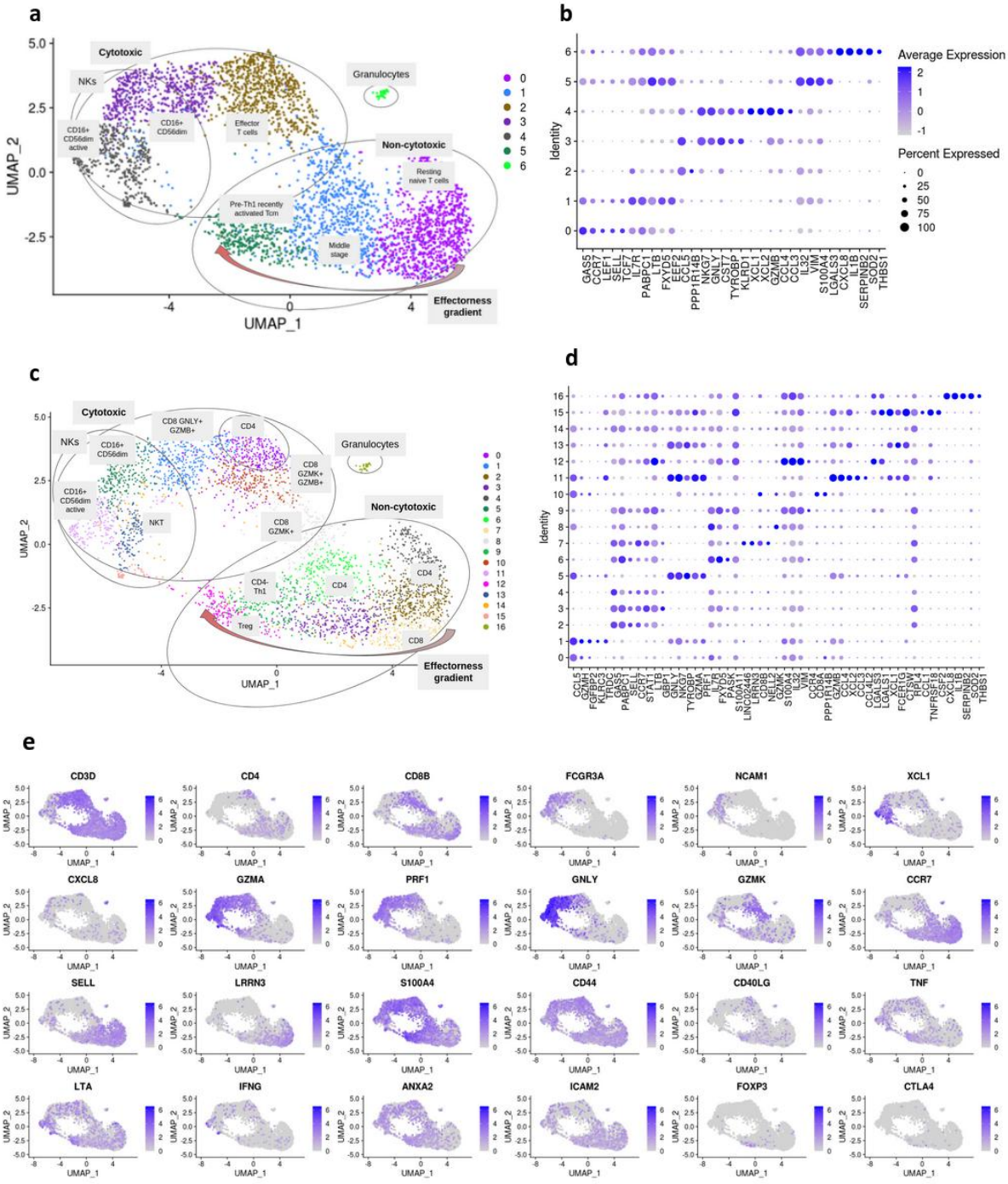
0 - Untreated T cells and treated and untreated NKs clusters

First resolution level

We re-applied the clustering pipeline to the *global cluster 0* (Figure 18). Cells were represented with the first 16 harmony integrated PCs, and clustered with the Louvain algorithm with a resolution of 0.5. This approach identified seven subclusters (Figure 18a). As seen in the UMAP, the cells divide in two main regions (Figure 18e and 19): first, a region with a cytotoxic profile composed by ***r1 cluster 0.4*** (NKs: $CD3^-$, $CD8^-$, $CD16a^+$), ***r1 cluster 0.3*** (NKs and a few effector $CD8^+$ T cells) and ***r1 cluster 0.2*** ($CD4^+$ and $CD8^+$ cytotoxic effector T cells) and characterized by the expression of cytotoxic molecules (*GZMA*, *GZMB*, *PRF1, GNLY, GZMK*) and effector cell marker genes (*S100A4*$^+$, *CD44*$^+$, *CCR*$^{7-}$ *LEF1*$^-$, *SELL*$^-$).

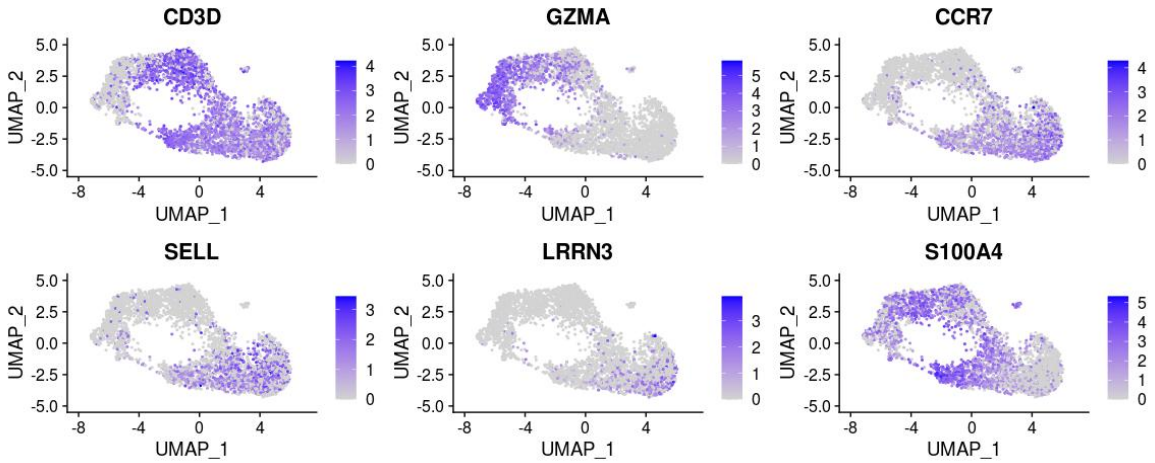
Figure 18. Untreated T cells and treated and untreated NK clusters**.** UMAP plots showing the identified clusters and its annotation at the (**a**) first resolution level and (**c**) second resolution level. Dotplot representing expression of top ten and five cluster marker genes for clusters identified at the (**b**) first resolution level and (**d**) second resolution level. Colour is proportional to the average expression level in each cluster and size is proportional to the percentage of expressing cells. (**e**)

UMAP plots showing the expression levels of selected genes that are relevant for cluster annotation.



A second region can be found (Figure 18a), which is composed mostly of non-cytotoxic T cells that express markers of naïve and memory T cells (Figure 19).

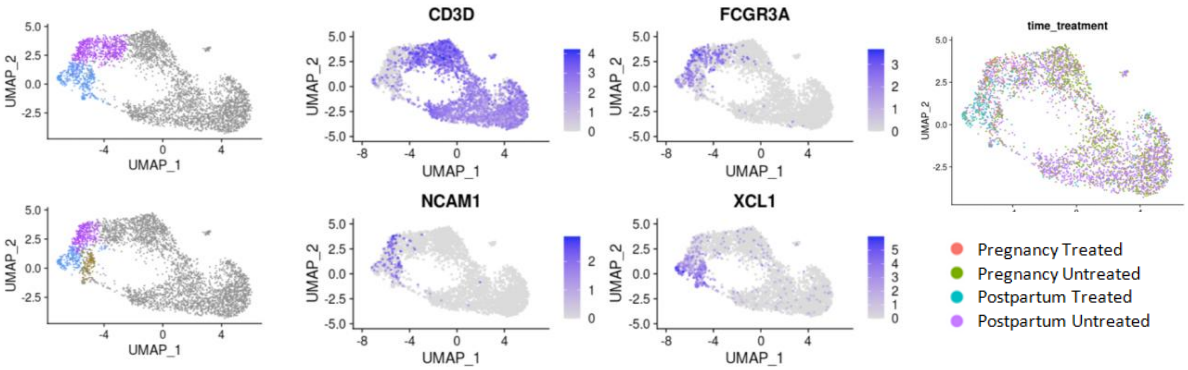
Figure 19. Cytotoxicity and effectorness gradients drive differences in unstimulated T cells. The UMAP of unstimulated T cells can be divided in a cytotoxic region ($GZMA^+$), with T cells ($CD3D^+$) and NKs ($CD3D^-$), and in a non-cytotoxic region, with resting T cells that follow an effectorness gradient.



Particularly, an effectorness gradient was detected in this region, starting with naïve T cells expressing very high levels of *SELL* and *CCR7*, and low levels of *S100A4* and *CD44* (*r1 cluster 0.0*) and ending with *r1 cluster 0.5.* This second cluster has lower levels of the central memory (CM) and naïve T cell markers *SELL* and *CCR7*, and higher levels of the effector memory (EM) and CM T cell markers *CD44* and *S100A4*, together with effector molecules such as granzymes and cytokines and several activation markers. *R1 cluster 0.1,* in the middle, can be considered a middle point in this activation continuum. Of note, cytotoxicity has already been reported to be the main factor distinguishing circulating T cells at the transcriptomic level (163) and, when focusing on resting $CD4^+$ T cells, the effectorness gradient that we report has also been identified as the principal source of variation (164). Finally, we detected *r1 cluster 6.0* (Figure 18b), which expresses high levels of *IL1B, SERPINB2, CXCL8, SOD2* and *THBS1*, genes that are exclusively expressed in granulocytes (165–169), and in which we also detect $CD3^+$ cells. This cluster corresponds to artifact doublets of T cells and granulocytes, and will be excluded from downstream analyses.

Identification of marker genes across these subclusters allows further characterization of their transcriptional profile (Figure 18b). Regarding the two NKs clusters (*r1 cluster 0.4.* and *r1 cluster 0.3*), both of them correspond to CD16a^{+} CD56^{dim} NKs. In ***r1 cluster 0.4*** we mostly find treated NKs (Figure 20), while ***r1 cluster 0.3*** is composed of both treated and untreated cells. This is accompanied by higher expression levels of chemokines and granzymes in *r1 cluster 0.4*, including *XCL1, XCL2, GZMB, CCL3* and *CCL4*, showing that this cluster is made of NK cells that are somehow responsive to the activation treatment. This response may be either directly mediated by IL2 or indirectly induced by activated T cells.

Figure 20. NK clusters characterization**.** All NK cells (CD3D^{-}) are CD16A(*FCGR3A*)$^{+}$ CD56(*NCAM1*)$^{dim+}$ NKs. *r1 cluster 0.4* has only NK cells that have been activated by treatment and show increased production of chemokines and granzymes, including *XCL1*.



Regarding the non-cytotoxic region of the UMAP, ***r1 cluster 0.5*** has some CCR7 expression but also expresses effector molecules (granzymes and perforins, T_{reg} markers, IL32 and TNF among others, Figure 18e). This suggests that this cluster corresponds to central memory T cells that have been recently activated. This is supported by the identification of cluster marker genes (Figure 18b), which shows that *r1 cluster 0.5* has a strong transcriptional signature (with 220 Differentially Expressed Genes, DEG) that involves the over-expression of many cell adhesion and cytoskeleton remodelling genes *(VIM, ANXA2, S100A4, LGALS3, ANXA5*). These molecules are necessary for T cell interaction with APCs (170). Other important molecules for the process of T cell activation (*TAGLN2* (171), *ICAM2*

(172)) are also overexpressed, as well as genes involved in cytokine signalling, including interleukin *IL32* -which increases after T cell activation-, IFN stimulated genes, TNF receptors, *LTA* and *TNF*, *IL2RA* and *IL27RA*. The expression of genes related to IL12, TNF, IFN and IL2 signalling suggests that these cells are being activated towards a Th1 effector profile. Additionally, these cells show high biosynthetic activity.

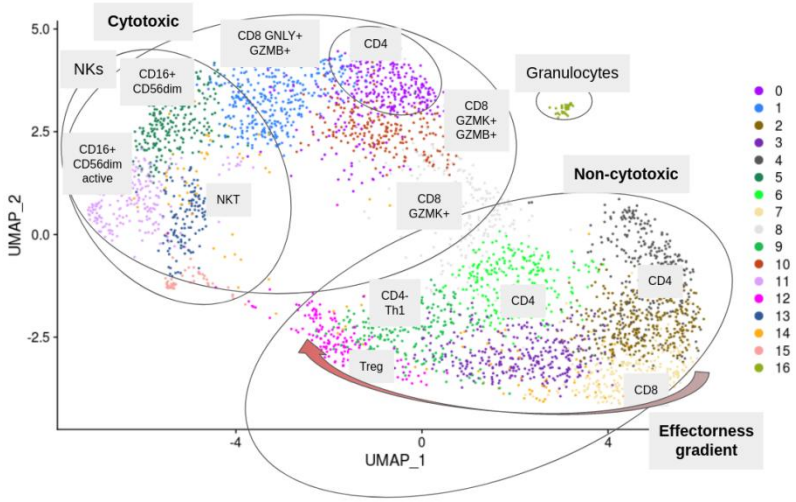
Conversely, ***r1 cluster 0.0***, was found to only upregulate 8 genes (Figure 18b). Most of these genes are markers of resting T cells (T_{cm} or naïve T cells): *GAS5* (a growth arrest protein used by T cells)*, LEF1* (a transcription factor that regulates T cell differentiation)*, SELL* (a L-selectin precursor) and *CCR7* (molecules necessary for the migration of T cells to the lymph nodes)*, TCF7, RPS17, LDHB, and SNHG6.* Consequently, this cluster was considered a resting state T cell cluster.
As commented previously, ***r1 cluster 0.1*** (Figure 18b) represents T cells in a middle activation stage, in between *cluster 0.0*. and *0.5.*, with the expression of some genes that characterize *r1 cluster 0.0* (*GAS5* and *LHDB*) but also some genes characteristic of *r1 cluster 0.5* (*LTB*, produced in recently activated T cells (173)) and genes involved in translational activity (*RPL4, RPS20, PABPC1* and *EEF2*).

These three non-cytotoxic clusters, r1 cluster *0.2, r1 cluster 0.0* and *r1 cluster 0.5* seem to be composed of both a $CD4^+$ and $CD8^+$ T cells (Figure 18e).

Second resolution level

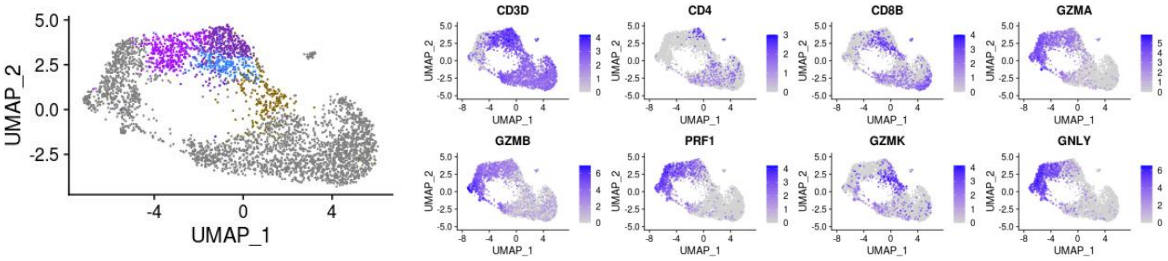
To gain more insights on the cells composing the previous clusters, we increased the resolution of the clustering analysis. Using a 2.25 resolution parameter, we identified 16 cell clusters. As expected, they generally correspond to more granular versions of the previous ones.

Figure 21. Untreated cell clusters identified with the second resolution level. Amplification of Figure 18c.



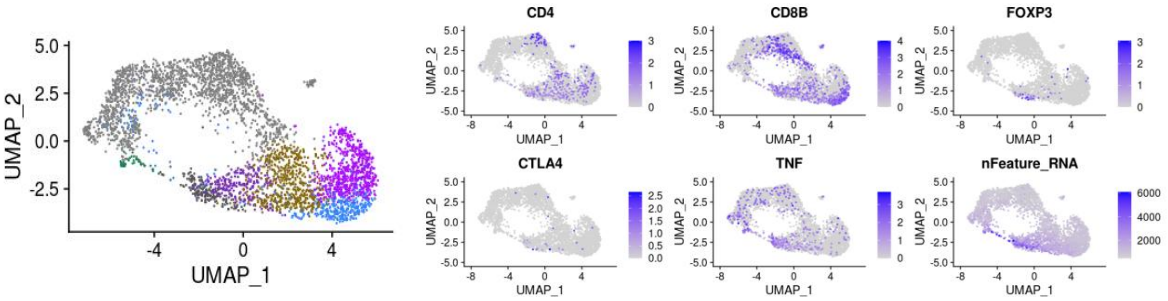
The second level of resolution divided the cytotoxic region of the UMAP into three NK clusters, three $CD8^+$ T cells clusters and one $CD4^+$ T cells cluster (Figure 21). Regarding the NK clusters, the previous *r1 cluster 0.4,* containing treated $CD16a^+$ $CD56^{dim}$ NK cells, was subdivided in two clusters, *r2 cluster 0.11* and 0.13. ***r2 cluster 0.11*** is consistent with the previous classification and shows the same signs of activation (i.e. chemokine production). ***r2 cluster 0.13*** is a new small cluster that expresses markers consistent with a described NKT subpopulation ($CD3^+$ $CD16a^-$ $CD56^+$ (174), Figure 20) and over-expresses KIR2DL4, an HLA-G receptor (175). Previous *r1 cluster 0.3* ($CD16a^+$ $CD56^{dim}$ NK cells and some $CD8^+$ T cells) is subdivided in a pure $CD16a^+$ $CD56^{dim}$ NK cells cluster (***r2 cluster 0.5***) and a cytotoxic $CD8^+$ T cells cluster (*r2 cluster 0.1*). r*1 cluster 0.2.* of cytotoxic T cells can be divided into an *r2 cluster 0.10* of $CD8^+$ cytotoxic T cells and an *r2 cluster 0.0.* of $CD4^+$ cytotoxic T cells ($CD4^+$ cells with production of granzymes [GZMA and GZMB] and perforins [PRF1], as well as *CCL5*, an important chemokine for effector T cells homing). Finally, an additional *r2 cluster 0.8.* of $CD8^+$ cytotoxic T cells was revealed.

Figure 22. $CD8^+$ Cytotoxic T cell clusters are distinguished by GZMA, GZMK and GNLY expression level. A $CD4^+$ Cytotoxic T cells cluster is also distinguished.



Regarding the three $CD8^+$ cytotoxic T cell clusters, three clearly different cytotoxic profiles are distinguished based on the expression of effector molecules (Figure 22): ***r2 cluster 0.1*** produces *GZMA, GZMB, PRF1* and *GNLY*, but is negative for *GZMK*; ***r2 cluster 0.10*** also produces some *GZMA, GZMB* and *PRF1* but expresses *GZMK* instead of *GNLY*; finally, ***r2 cluster 0.8*** is only positive for *GZMK*, and does not produce any *GZMA, GZMB, PRF1* and *GNLY*. These clusters coincide with the $CD8^+$ clusters that have previously been identified in the RA and osteoarthritis synovial tissue (137).

Figure 23. Non-cytotoxic clusters subdivision into $CD4^+$, $CD8^+$, pre regulatory T cells and pre Th1 clusters, and detection of a cluster of stressed $CD8^+$ T cells.



Increased resolution also revealed several subtypes in the non-cytotoxic region of the UMAP (Figure 23). r*1 cluster 0.0*, the resting naïve T cells cluster, was divided in two $CD4^+$ resting naïve T cells clusters (***r2 cluster 0.4*** and ***r2 cluster 0.2***) and two $CD8^+$ resting naïve T cells cluster (***r2 cluster 0.7*** and ***r2 cluster 0.14***). *r1 cluster 0.1*, corresponding to a middle point in the effectorness gradient, was divided in two $CD4^+$ subclusters (***r2 cluster 0.6*** and ***r2 cluster 0.3***). Finally, the cluster of activated pre-Th1 cm cells (*r1 subcluster 0.5*) was divided in a pre-Th1

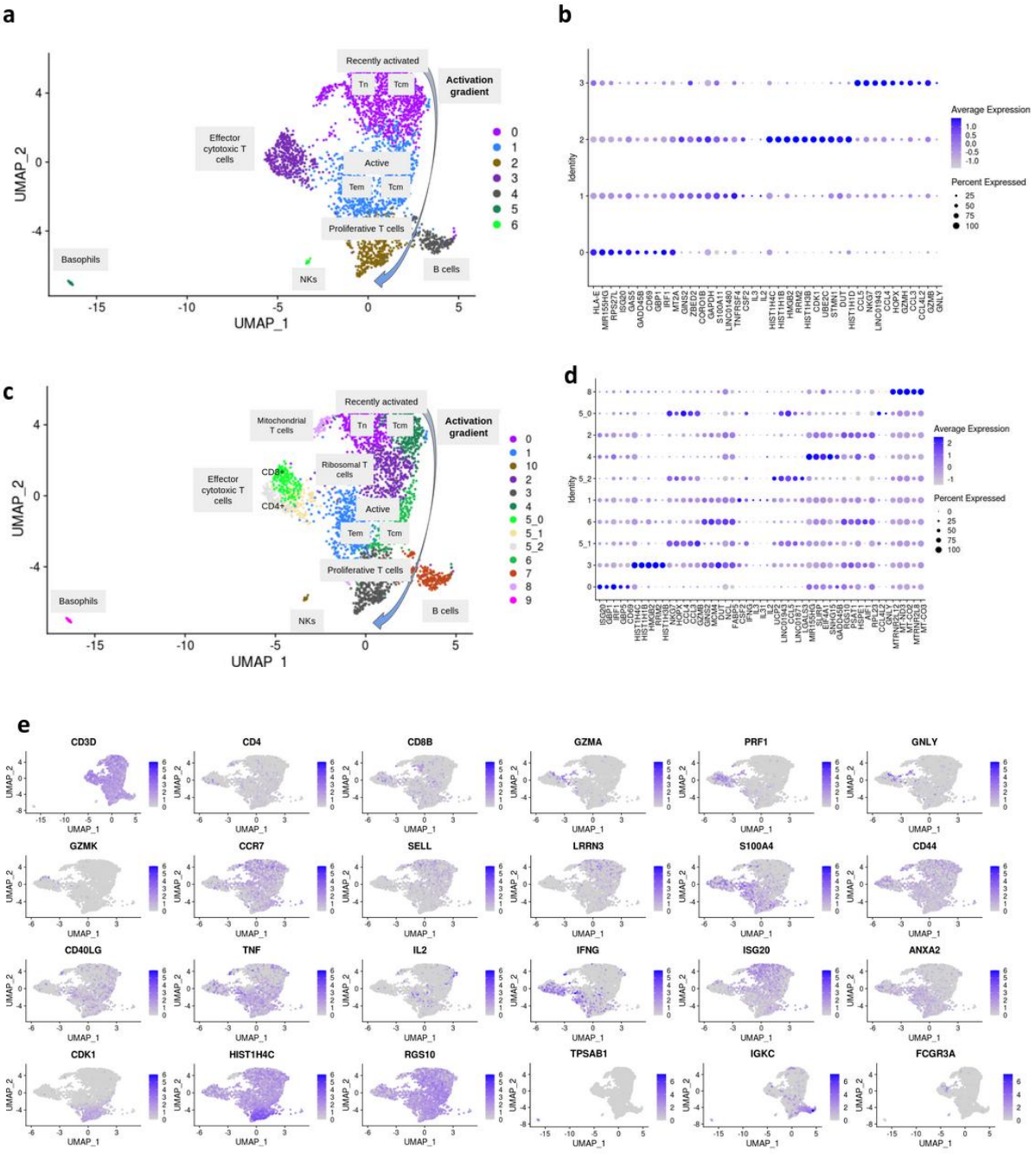
activated Tcm cluster (***r2 subcluster 0.9***) and a pre-Treg activated Tcm cluster (***r2 subcluster 0.12***, FOXP3^{+} CTLA4^{+}). Finally, a small cluster (*r2 subcluster 0.15,* n=43 cells) of proliferative T cells, with high expression of housekeeping genes, high number of detected features and high expression of DNA repair genes, heat shock proteins, ribosomal proteins, and low levels of NK and T cell markers, were detected and excluded. Clusters with the same cell type label, where no differences in cellular activity were found, were aggregated for downstream analyses.

1 - Treated T cells clusters

First resolution level

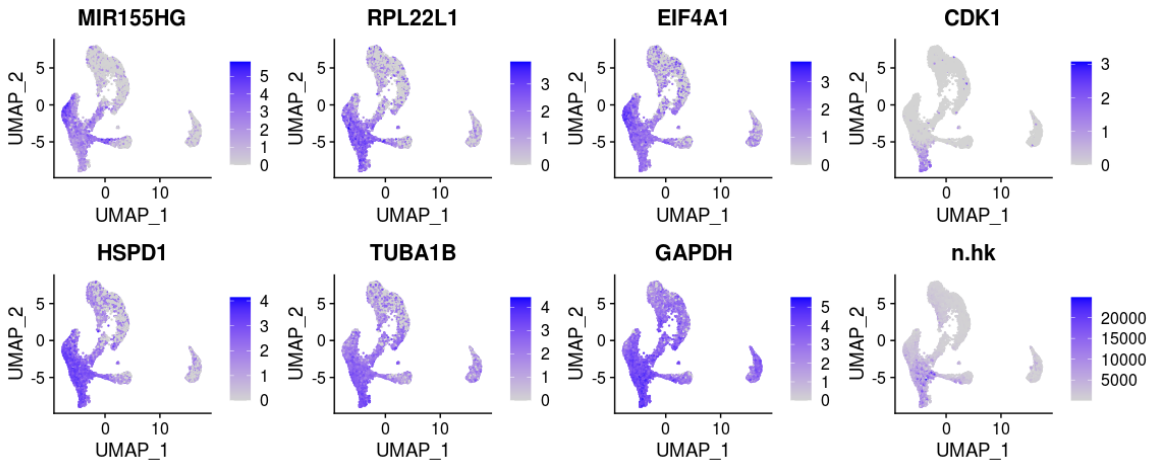
Global cluster 1, with treated T cells, was subclustered by computing its harmony corrected PCs and using the first 22 components to cluster the cells with a low resolution level of 0.3, which revealed seven different clusters (Figure 24).

Figure 24. Treated T cells. UMAP plots showing the identified clusters and its annotation at the (**a**) first resolution level and (**c**) second resolution level. Dotplot representing expression of top ten and five cluster marker genes for clusters identified at the (**b**) first resolution level and (**d**) second resolution level. Colour is proportional to the average expression level in each cluster and size is proportional to the percentage of expressing cells. (**e**) UMAP plots showing the expression levels of selected genes that are relevant for cluster annotation.



Two outlier clusters, *r1 cluster 1.5* (n=24 cells) and *r1 cluster 1.6* (n=22 cells) were detected and excluded from downstream analyses (Figure 24a). *r1 cluster 1.5* overexpresses genes that are exclusively expressed in basophils (*TPSAB1*, *TPSB2* and *GATA2*, among others, Figure 24e). *r1 cluster 1.6* is enriched in NK markers, such as *GNLY, GZMA* and *XCL2*, and does not express CD3 (Figure 24e). *r1 cluster 1.4* (n=215 cells) contains B cells with T cells doublets, with expression of *CD3E, CD3D, CD79A* and *MS4A1*, and was also excluded from downstream analyses (Figure 24e).

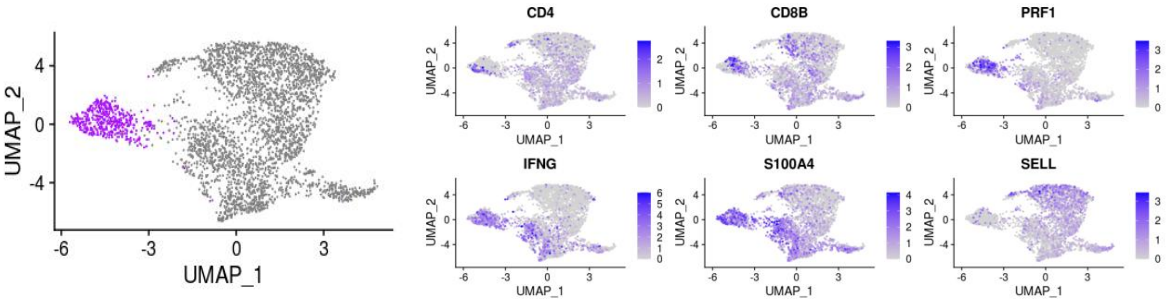
Figure 25. High expression of genes involved in cell cycle and cell housekeeping functions in treated T cells.



For the remaining clusters, we detect a similar pattern as for untreated cells but with much higher levels of genes that are required for cell proliferation and development, such as heat shock protein and genes involved cell metabolism, DNA replication and cell cycle progression (Figure 25). Interestingly, the top cluster markers in this case do not generally have immune roles but the aforementioned functions (Figure 24b). This shows that *in vitro* immunological cell activation deeply modifies the cell transcriptional signature, and that this signature is not dominated by the activated immune processes, but by the housekeeping processes that are required to maintain the activation. However, we can still detect the main expected cell populations.

First, a clearly differentiated cluster with both $CD4^+$ and $CD8^+$ cells with effector cytotoxic functions is distinguished (***r1 cluster 1.3***, n=425 cells, Figure 24a). This cluster is characterized by the secretion of granzymes, perforins, cytokines and chemokines (Figure 24e). Regarding its maturation state, the cluster shows high levels of effectorness markers *S100A4* and *CD44*, very low levels of *CCR7* and no expression of *SELL* nor *CD40LG* (which is up-regulated in T_{cm} (176)), suggesting an effector profile.

Figure 26. Treated cytotoxic T cells. These cells are characterized by the expression of granzymes, perforins and cytokines, by high expression of effector T cell markers and low expression of resting T cell markers. A CD4 region and a CD8 region can be distinguished.



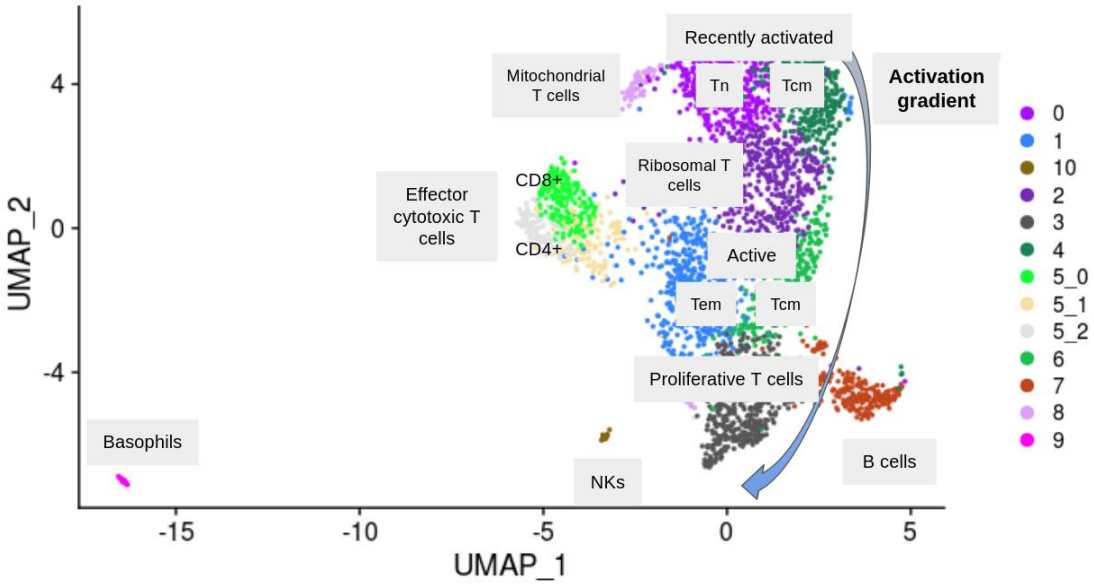
Clusters 0 to 2 show a gradual increase in activation (Figure 24a), with a gradient of recently activated T_{cm} and T_n in *r1 cluster 1.0.* (n=1,204 cells), activated T_{cm} and T_{em} cells in *r1 cluster 1.1* (n=1,101 cells) and proliferative T cells in *r1 cluster 1.2* (n=446). ***r1 cluster 1.0*** has the highest expression of the T_{cm} and Tnaïve markers *SELL, CCR7* and *LEF1* and shows, in contrast with the rest of clusters, no cytokine production (Figure 24e). There is a gradient from left to right of decreasing LRRN3 levels (which is increased in T_n) and increasing CD44 and CD40LG levels (which are increased in T_{cm}), suggesting a T_n to T_{cm} gradient from left to right (Figure 28). Clear signs of recent activation are manifested in this cluster (Figure 24b), with over-expression of genes reported to be rapidly induced after T cell activation and with early immunoregulatory roles (*HLA-E* (177), *CD69* and *GADD45B* (178)), high levels of *MIR155HG*, which we find increased in clusters of treated cells, and genes involved in interferon signalling (*ISG20, IRF1, GBP1, GBP4, GBP5* and *STAT1*). ***r1 cluster 1.1*** groups activated T_{cm} and T_{em} cells and is characterized by the over-expression of genes with housekeeping functions (*GINS2, CORO1B, GADPH...*) but also genes with important immune roles such as *TNFRSF4* (a co-stimulatory molecule expressed 24-72h after T cell activation (179)), *CSF2*, *IL3*, *IL2, LTA, TNF, IL13, IL24* and *CCL20* (Figure 24b). *CD40LG* is also over-expressed and *S100A4* expression is detected in some regions, with low *SELL* and *CCR7* expression, which supports a T_{cm} and T_{em} differentiation stage (Figure 28). ***r1 cluster 1.2*** contains proliferative T cells (Figure 29) and has a transcriptional profile dominated by the over-expression of

genes involved in cell cycle and DNA replication (e.g. *HIST1H4C, HIST1H1B, HMGB2, RRM2, HIST1H3B, RRM2, HIST1H3B* and *CDK1*, Figure 24b).

Second resolution level

To gain granularity on the defined populations, we studied clusters defined with a higher level of resolution and subclustered a particular cluster containing a $CD4^+$ and a $CD8^+$ region (Figure 27).

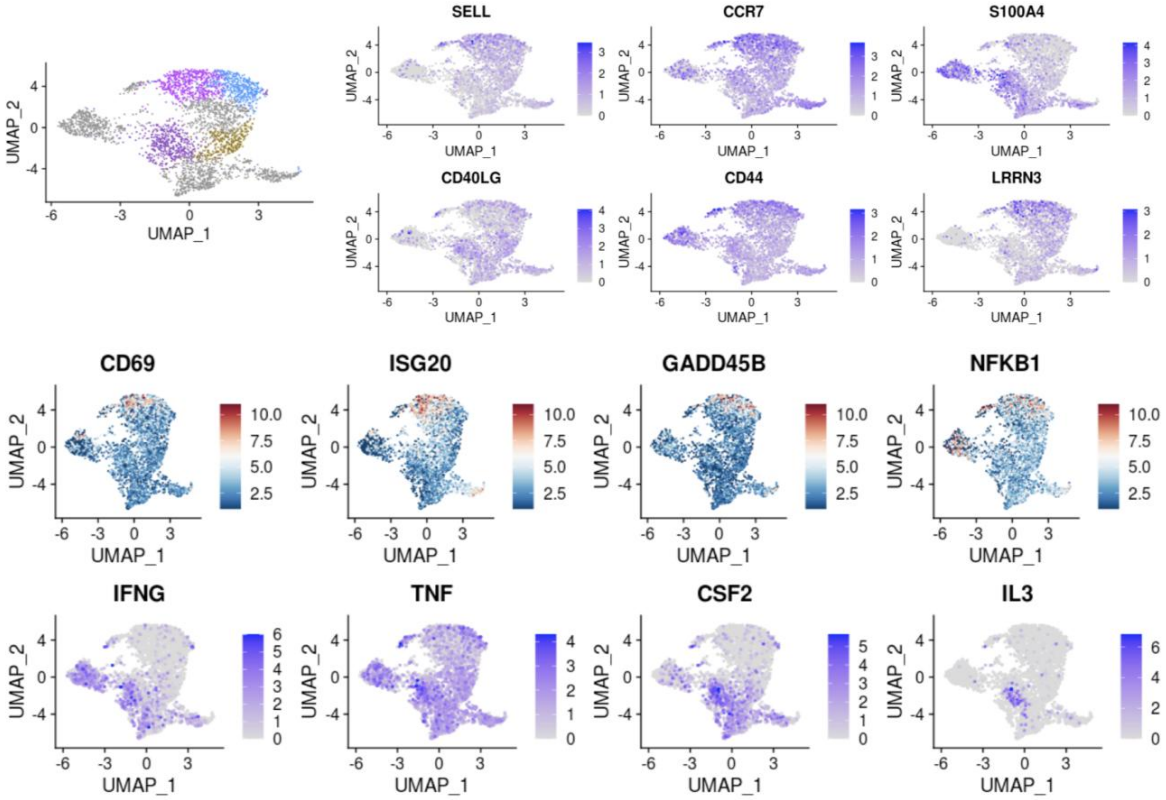
Figure 27. Treated cell clusters identified with the second resolution level. Amplification of Figure 24c.



This approach revealed a population of high mitochondrial T cells (***r2 cluster 1.8***, Figure 29) that was previously part of *r1 cluster 1.0* and *r1 cluster 1.2.* We subclustered the effector cytotoxic T cells cluster (*r1 cluster 1.3*) into a $CD8^+$ cluster, a $CD4^+$ cluster and a small cluster with both $CD4^+$ and $CD8^+$ cells (n=130) (Figure 26). The $CD8^+$ T cells cluster (***r2 cluster 1.5.0***, n=180) was $GZMA^+$ $GZMB^+$ $GNLY^+$ $GZMK^+$ (Figure 24e). A cluster with this granzyme expression pattern was not found among the untreated effector $CD8^+$ clusters. Additionally, $CD8^+$ T cells showed high levels of cytotoxic activity markers, chemokines (*CCL4L2, CCL3, CCL4* and *CCL5*), cytokines (*IFNG* and *IL32*) and *HLA-DRB1* (an indicator of T cell activation found to be associated to several autoimmune diseases (180)), among many others differentially expressed genes (Figure 24d).

The cytotoxic CD4$^+$ T cells cluster (***r2 cluster 1.5.2***, n=114) had a very similar expression profile as the cytotoxic CD8$^+$ cluster, but with relatively lower levels of granzymes (Figure 24e).

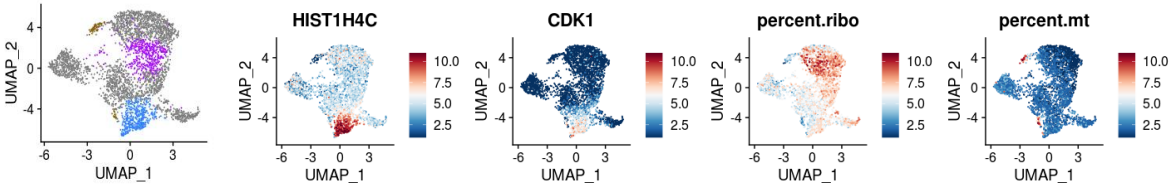
Figure 28. Naïve, central memory and effector memory T cells at different activation stages.



The *r1 cluster 1.0* of recently activated T_{cm} and naïve T cells was splitted into two clusters, one with naïve T cells and the other with T_{cm} cells (Figure 27). The recently activated T cells cluster (***r2 cluster 1.0***, n=524) shows a clear IFN response signature (*ISG20, CBP1, GBP2, IFT20, IRF1, IRF9* and *STAT1,* (Figure 24d) but with no IFN production, which suggests that they are pre-Th1 naïve T cells that have just been activated. These cells have no cytokine production, have high levels of *CD69, SELL, CCR7, LRRN7* and *LEF1* (Figure 24e, 28), and are enriched in processes related to translation, JAK-STAT and IFN signalling, antigen presentation and naïve T cell differentiation. The recently activated Tcm cluster (***r2 cluster 1.4***, n=427) also showed *SELL* and *CCR7* expression, but with higher levels of *CD44* and *CD40LG* and lower *LRRN3*, supporting that these T cells are in a central memory differentiation phase (Figure 28). This cluster shows slightly

higher levels of cytokine production than the naïve T cells cluster and has higher levels of GADD45B, HLA-E and MIR155HG (Figure 24d, 28), suggesting that they are more advanced in the activation process than the previous cluster. The cluster marker genes also include NFkB and genes involved in NFkB signalling as well as genes with housekeeping functions (translation, rRNA metabolism, mitochondrial activity and protein catabolism), which are necessary to support T cell activation (Figure 24c, 28).

Figure 29. Identification of T cell clusters with high ribosomal and high mitochondrial expression, and a cluster of proliferative T cells.



The *r1 cluster 1.1* of activated T_{cm} and T_{em} cells was subclustered into three different clusters. First, a cluster of activated, ribosomal, T_{cm} cells (***r2 cluster 1.2***, n=463, Figure 24f), which is also $CCR7^+$ $SELL^+$ $CD44^+$ $CD40LG^+$ (Figure 24e). This cluster has slightly higher levels of cytokine expression, compared to the previous ones, but is mainly characterized by the expression of ribosomal genes (Figure 29). Besides ribosomal genes, this cluster has very high levels of expression of *RGS10, PSAT1, AIF1, CD7* and *CD27* (Figure 24d). Second, a cluster of activated T_{cm} cells (***r2 cluster 1.6***, n=332) was identified (Figure 28). This cluster is dominated by a proliferative transcriptional profile, with genes involved with functions necessary to support T cell proliferation, such as genes involved in DNA replication, protein folding, RNA processes, translation and lymphocyte proliferation (Figure 24d). It also over-expresses some genes involved in IL12 and IL4 signalling, showing that Th1 and Th2 activation profiles cannot be distinguished. Finally, a cluster of T_{em} cells (***r2 cluster 1.1***, n=488) was identified. This cluster has the activation profile that we also detected in untreated cells, with expression of genes involved in cytoskeleton remodelling or necessary for cell migration and T cells homing (*ANXA2, S100A11, LGALS3, ITGB7, CD226* (181) and *CCR4*). The cells in this cluster produce very high levels of cytokines,

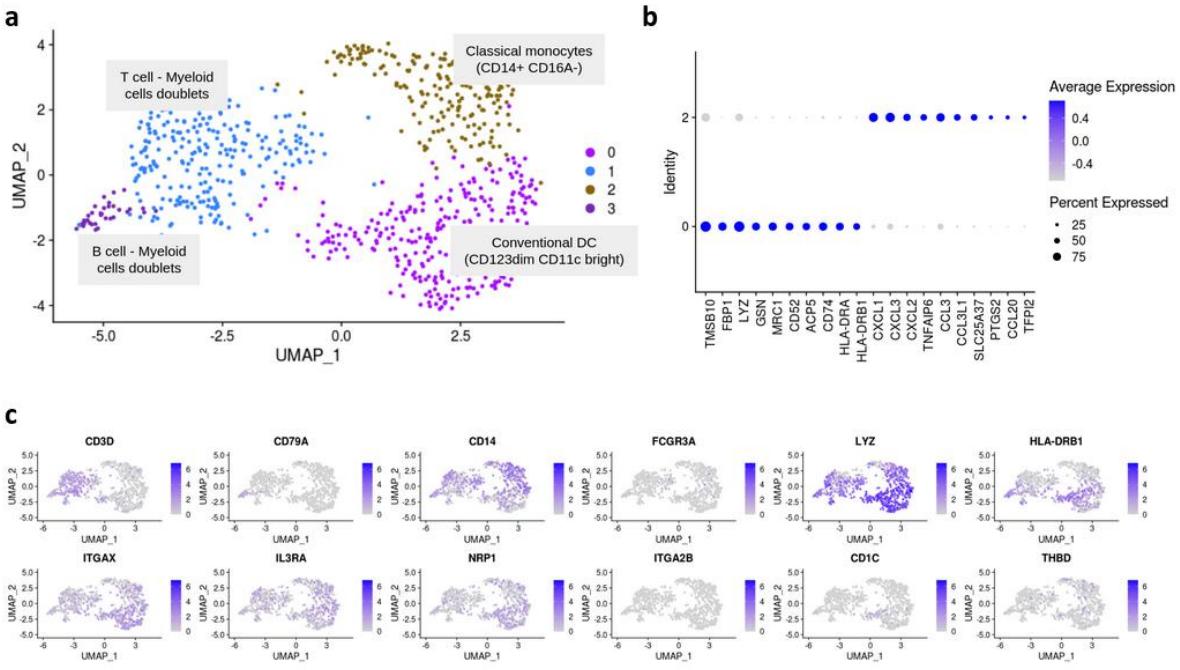
chemokines and immune regulators such as *CSF2, IL3, IFNG, IL31, IL2, IL13, TNF, CCL3* and *CCL20*, do not express *SELL, CCR7* or *LRRN3* and express S1004 (Figure 24e, 28). This shows a transcriptional profile of activated, migratory, effector or effector memory T cells.

The *r1 cluster 1.2* of proliferative T cells (Figure 29), and the one that is apparently more affected by activation, was renamed to ***r2 cluster 1.3*** but remained unchanged, besides the already explained detection of a small cluster of high mitochondrial T cells.

2 - Myeloid cells

Sub-clustering the *global cluster 2* with 15 harmony corrected PCs and a resolution of 0.55 revealed four different clusters (Figure 30).

Figure 30. Myeloid cells**.** (**a**). UMAP plot showing the identified clusters and its annotation. (**b**). Dotplot representing the expression levels of top ten cluster marker genes. Colour is proportional to the average expression level in each cluster and size is proportional to the percentage of expressing cells. (**c**). UMAP plots showing the expression levels of selected genes that are relevant for cluster anotation.

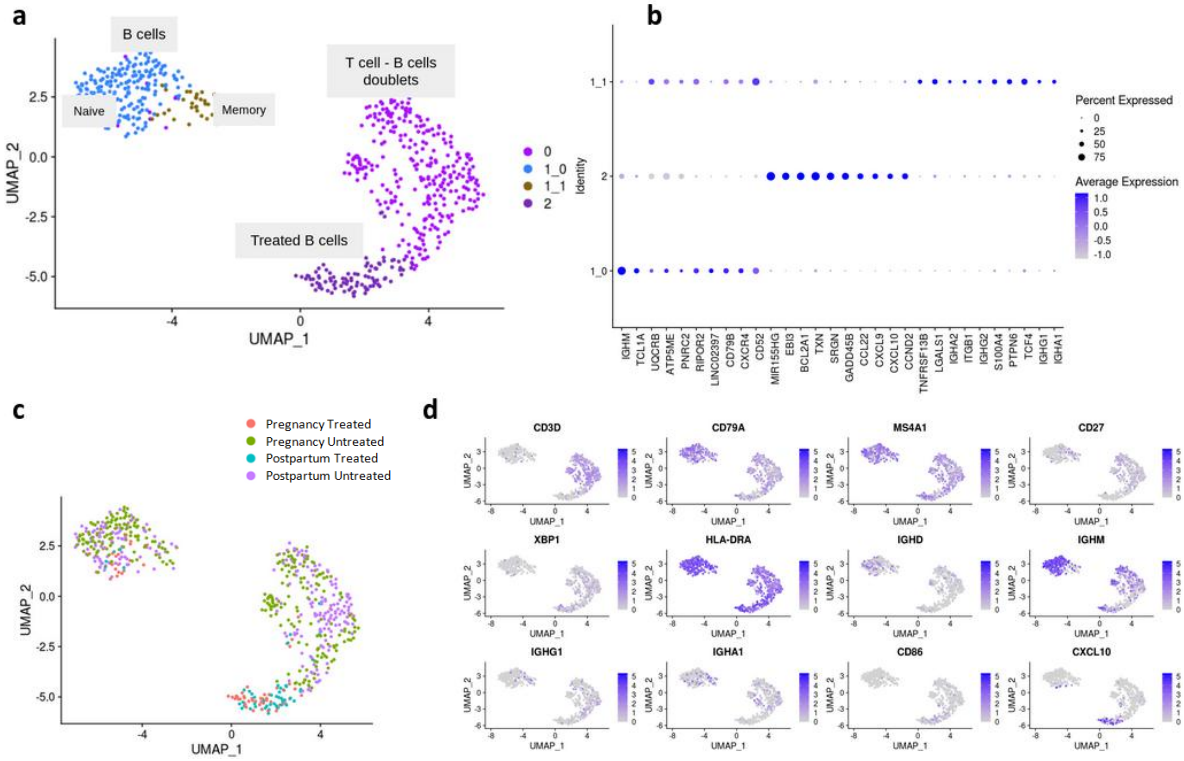


r1 cluster 2.1 (n=254 cells) corresponds to myeloid cells with T cells doublets ($CD3^+$ $CD14^+$ cells, Figure 30c). *r1 cluster 2.3* (n=33 cells) corresponds to myeloid cells with B cells doublets ($CD79A^+$ $CD14^+$ cells, Figure 30c). These clusters were excluded from downstream analyses. ***r1 cluster 2.2*** (n=183 cells) represents $CD14^+$ $CD16a^-$ classical monocytes and expresses molecules such as *CXCL1, CXCL2, TNFAIP6, THBS1* and *CCL3L1* (Figure 30b). Finally, ***r1 cluster 2.0*** (n=278 cells), is made of $CD123^{dim}$ (*IL3RA*) $CD11c^{bright}$ (*ITGAX*) myeloid/conventional dendritic cells (cDC, Figure 30c), with high expression of MHC class II molecules (HLA-DR, Figure 30b). A small sub-population of $CD1C^+$ cDC2 is observed (Figure 30c). Given the small size of these clusters and the lack of heterogeneity in the explored myeloid cell markers, we did not perform a second round of clustering for these cells.

3 - B cells

Sub-clustering the *global cluster 3* with the 15 first harmony corrected PCs and a resolution of 0.25 defined two B cell clusters and a cluster of $CD3^+$ $CD79^+$ cells (*r1 cluster 3.0,* n=298 cells) that we defined as T cells with B cells doublets and excluded from downstream analyses (Figure 31a).

Figure 31. B cells**.** (**a**) UMAP plot showing the identified clusters and its annotation. (**b**) Dotplot representing the expression levels of the top ten cluster marker genes. Colour is proportional to the average expression level in each cluster and size is proportional to the percentage of expressing cells. (**c**) UMAP plot showing the sample activation and pregnancy status for each cell. (**d**) UMAP plots showing the expression levels of selected genes that are relevant for cluster annotation.

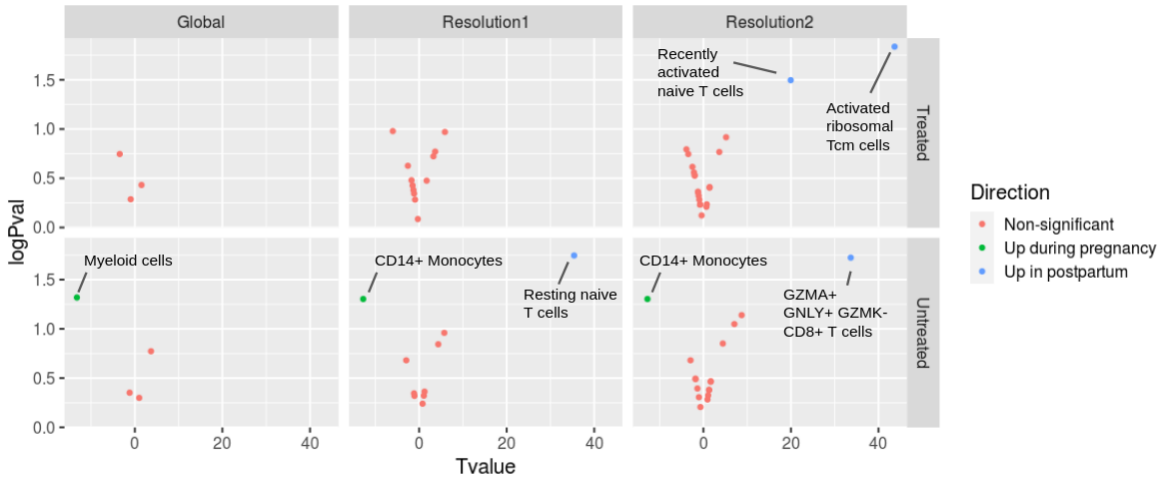


r1 cluster 3.2. (n=91) corresponds to treated B cells (Figure 31c) and, besides having moderate expression of classical B cell markers (*CD79A, MS4A1, HLA-DRA, IGHD, IGHM…,* Figure 31d), over-expresses genes such as *MIR155HG, EBI3, CXCL10, CCL22* and the B cell activation marker *CD86* (Figure 31b). r***1 cluster 3.1***. (n=227) is made of both treated and untreated cells. Sub-clustering of this cluster revealed a majority $CD27^-$ naïve B cells cluster (n=197, *r1 cluster 3.1.0,* Figure 31d), where expression of IgM dominates; and a cluster of $CD27^+$ memory B cells (n=30, *r1 cluster 3.1.1,* Figure 31d), with high expression of the genes codifying for the heavy chains of IgA and IgG, among others (Figure 31b).

5.2.3. Differences in cell proportions

To study the effect of pregnancy on the circulating immune populations in RA women, we studied differences in the proportions of the identified populations for treated and un-treated samples independently. To do this, we fitted linear mixed-effects models that measured differences in cell proportions based on the pregnancy stage (pregnancy or postpartum), including the sample donor as a random effect in the model. It is important to note that this analysis is strongly limited by our sample size, with only two available samples per contrast. The results of this analysis are shown in Figure 32 and described in detail below.

Figure 32. Differences in cell proportions**.** Results from the differential cell composition analysis, for the three resolution levels and stimulated and unstimulated samples are shown. The y-axis represents the minus logarithm of the p-value from the linear model, and the x-axis represents the T-value. Dots are coloured based on significance and direction of change.

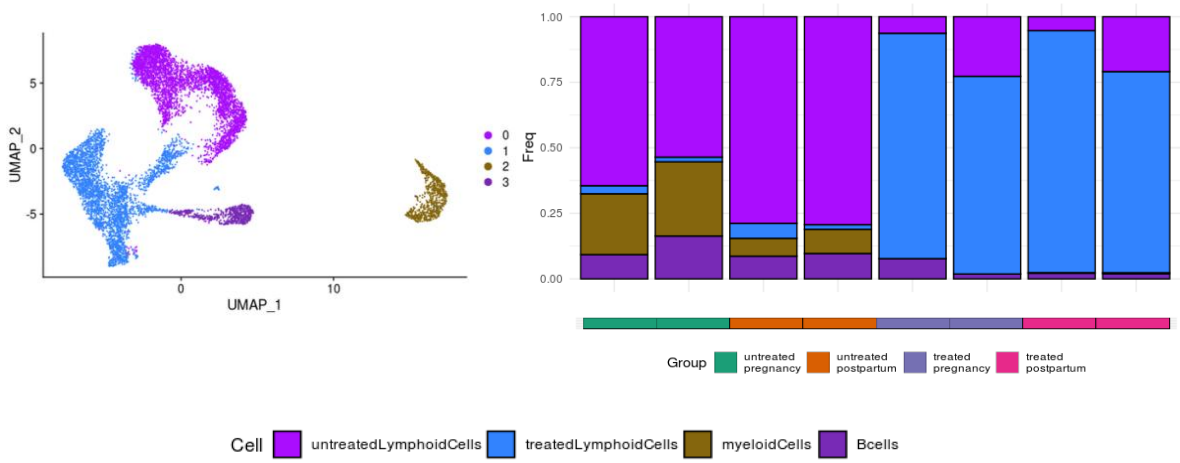


Global lineages

At the global level (Figure 33, Annex table 1) we detected a remarkable increase in myeloid cells during pregnancy (mean=25.75%, SD=3.59), compared to the postpartum (mean=7.98%, SD=1.69) in untreated samples (p-value=0.048). This finding should be interpreted with caution because it is affected by technical confounders. Myeloid cells are very sensitive to activation due to technical processing steps, which induces cell adhesion to the culturing plate and prevents

further cell sequencing. This explains why no myeloid cells are detected in treated samples. The higher proportions of myeloid cells in pregnant samples, more than suggesting that in post-partum women only a 7.98% of total PBMCs are myeloid cells, supports the presence of an immunological break during pregnancy, which gives myeloid cells certain resistance to activation, making them less adherent and easier to recover. The low percentage of recovered myeloid cells in postpartum samples suggests the absence of this immunological break during postpartum, which may lead to higher cell activation by processing and to a lower percentage of recovered myeloid cells.

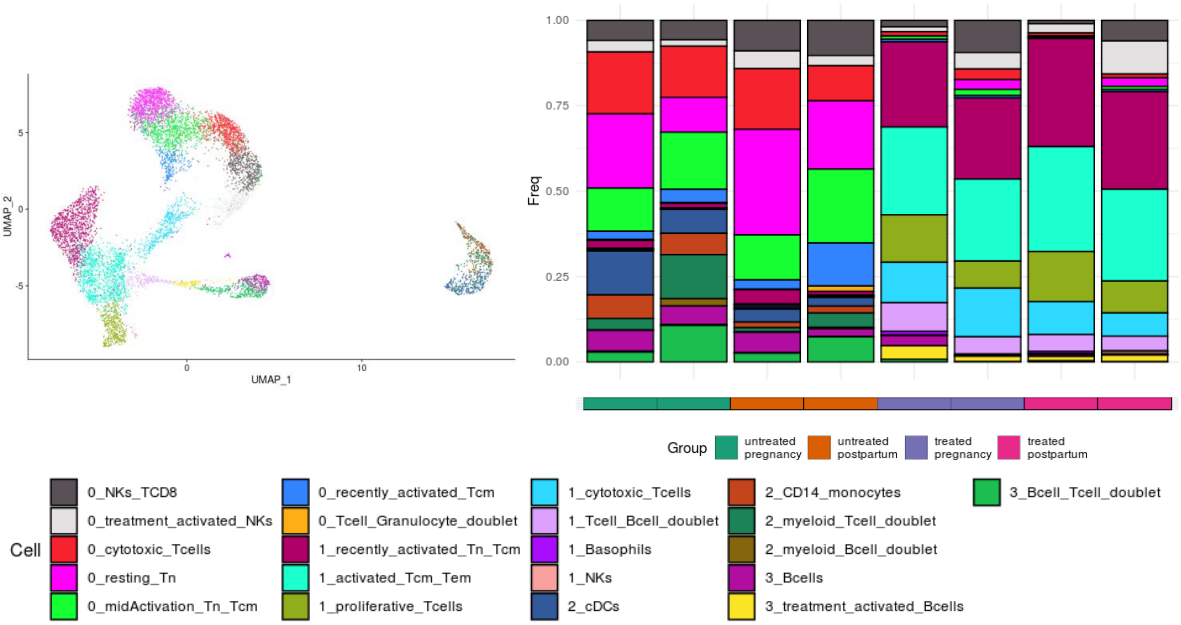
Figure 33. Barplots showing the cell composition of each sample based on the global lineages cell annotation. UMAP is added as reference to identify cell populations. The thin line indicates the sample group (pregnancy, postpartum, stimulated and unstimulated).



Resolution level 1

Using the cell populations defined by subclustering global lineages at low-resolution level, the previous findings were confirmed and refined (Figure 34, Annex table 2). Particularly, we find that the myeloid population that is increased during pregnancy in untreated samples corresponds to $CD14^{+}$ monocytes (p-value=0.049; pregnancy mean=6.56%, SD=0.44; postpartum mean=1.82%, SD=0.28), but not to cDCs (p-value = 0.208). Additionally, we detect an important increase in resting naïve T cells in untreated samples (*r1 cluster 0.0*) in the postpartum (p-value=0.018; pregnancy mean=15.97%, SD=8.13; postpartum mean=25.46%, SD=7.75).

Figure 34. Barplots showing the cell composition of each sample based on the first resolution level annotations. UMAP is added as reference to identify cell populations. The thin line indicates the sample group (pregnancy, postpartum, stimulated and unstimulated).

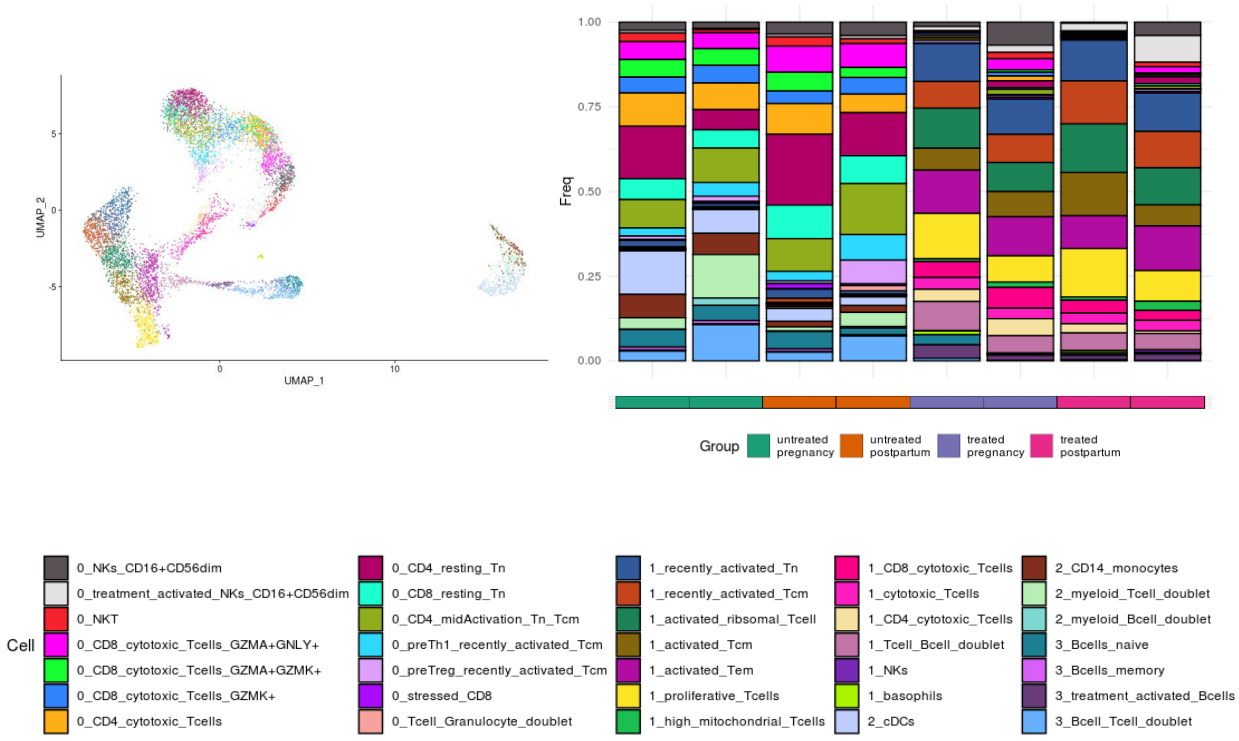


Resolution level 2

Using the highest level of resolution, which reveals the most granular cell populations identified, besides the reported increase in $CD14^{+}$ monocytes during pregnancy, we find three new associations (Figure 35, Annex table 3). In treated samples, both activated ribosomal T_{cm} cells (*r2 cluster 1.2*; p-value=0.015; pregnancy mean=10.20%, SD=2.31; postpartum mean=12.63%, SD=2.39) and recently activated naïve T cells (*r2 cluster 1.0*; p-value=0.032; pregnancy mean=10.81%, SD=0.54; postpartum mean=11.70%, SD=0.48) were found to be augmented in the postpartum. In untreated samples, $GZMB^{+}$ $GNLY^{+}$ $GZMK^{-}$ $CD8^{+}$ T cells (*r2 cluster 0.1.*; p-value=0.019; pregnancy mean=5.00%, SD=0.39; postpartum mean=7.39%, SD=0.49) were also found to be augmented in the postpartum. Regarding $CD16A^{+}$ $CD56^{dim}$ NKs and T_{reg} populations, differences in cell proportions are observed in one of the patients (RA2). For treated $CD16A^{+}$ $CD56^{dim}$ NKs, we observe that most of the gestational cells correspond to the cluster that is not affected by treatment (*r2 cluster 0.5*, with 6.80% of cells, compared to 2.14% of cells in the affected by treatment cluster r2 0.11). Instead,

postpartum cells mostly belong to the affected by treatment *cluster r2 0.11* (7.89% of cells, compared to a 3.85% of cells in the not affected by treatment *cluster r2 0.5*). Untreated recently activated pre $T_{reg\text{-}cm}$ cells *(r2 cluster 0.12)* presented a percentage that increased from 0.81% of cells during pregnancy to 6.99% of cells in the postpartum period. A higher sample size would be required to assess the statistical significance of these changes.

Figure 35. Barplots showing the cell composition of each sample based on the second resolution level cell annotation. UMAP is added as reference to identify cell populations. The thin line indicates the sample group (pregnancy, postpartum, stimulated and unstimulated).

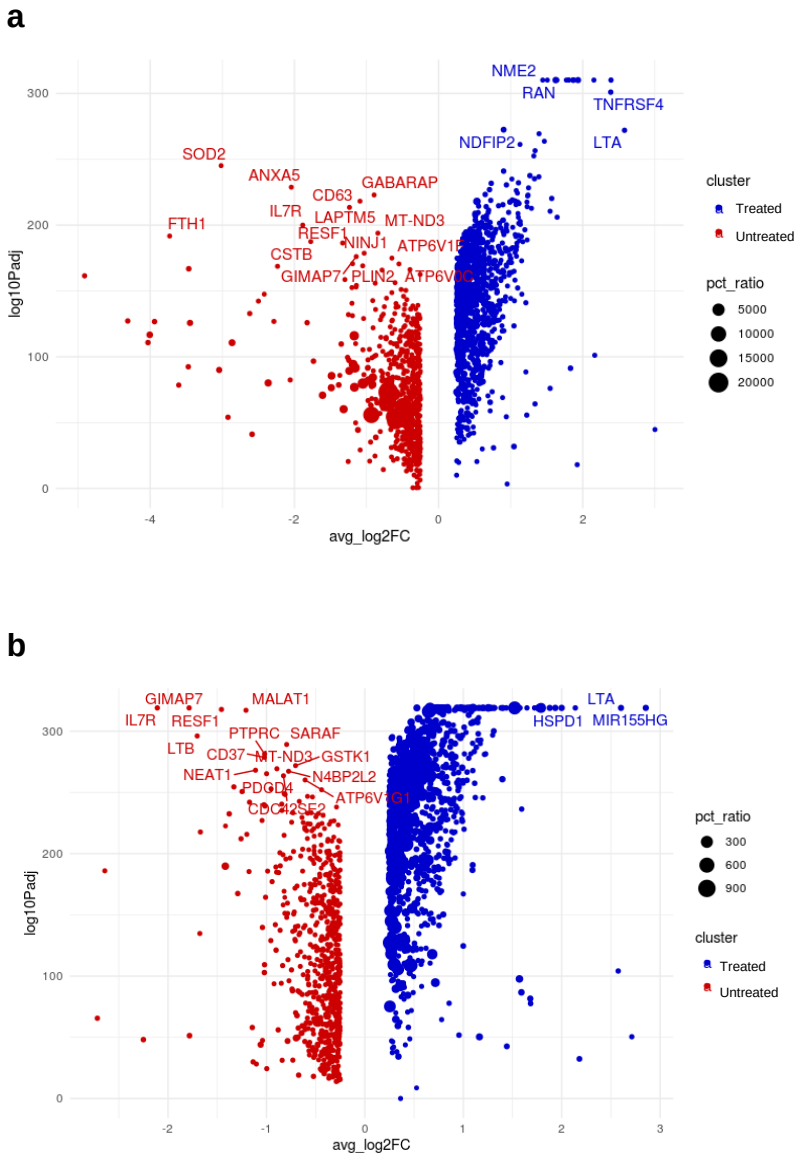


5.2.4. Differences in gene expression

5.2.4.1. Globally differential expression of genes due to cell treatment

To be able to directly compare the changes induced by cell treatment in pregnant and postpartum samples, we first detected differentially expressed genes across treatment conditions, in the pregnant and postpartum stage independently. Due to the fact that treated and non-treated cells generally belong to different clusters; it was not possible to perform this analysis at the cluster (cell-type) level. Instead, we looked for global genes in PBMCs gene expression, without taking into account the underlying variations in cell populations. In pregnancy samples we detected n=1259 genes up-regulated in treated cells and n=618 genes up-regulated in non-treated cells (Figure 36a). In postpartum samples we detected n=1302 DEG up-regulated in treated cells and n=699 DEG up-regulated in non-treated cells (Figure 36b). Of the total number of differentially expressed genes, n=1,523 genes were differentially expressed in both conditions, with n=478 postpartum-exclusive genes and n=354 pregnancy-exclusive genes. This result indicates that cell treatment induces a very strong transcriptional response, with the up-regulation of more than 1,000 genes independent pregnancy stage.

Figure 36. Differentially expressed genes in response to cell treatment in (**a**) pregnant samples and (**b**) postpartum samples. The y-axis represents the minus logarithm of the FDR-adjusted p-value from the MAST model, and the x axis represents the average log fold change (log2FC). Dots are coloured based on significance and direction of the change and proportional in size to the ratio of percentage of expressing cells between conditions.

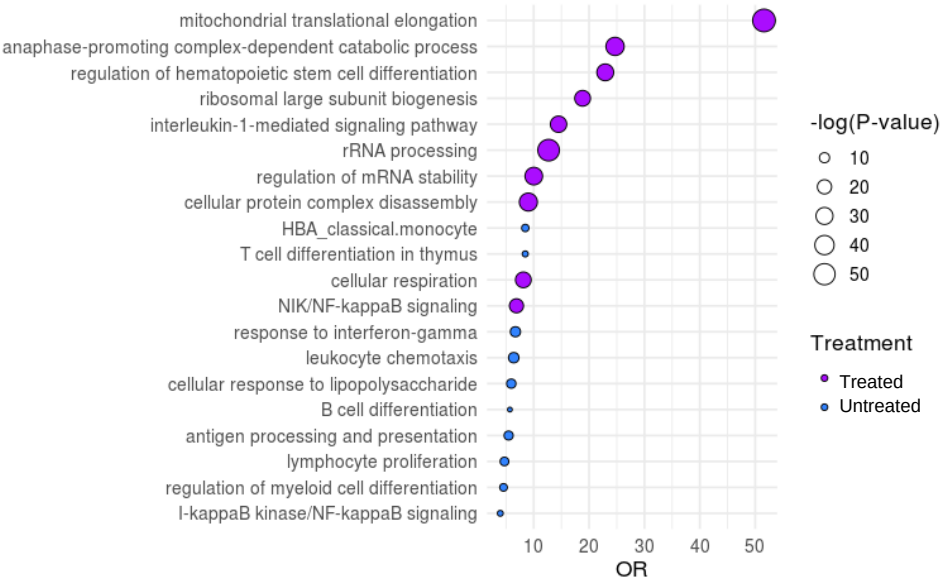


We performed a pathway enrichment analysis to the biological function of the genes activated by cell stimulation. This identified n=218 processes induced by treatment and n=157 processes inhibited by treatment in pregnancy samples (Figure 37a), and n=123 induced by treatment and n=90 inhibited by treatment processes in postpartum samples (Figure 37b). This analysis revealed that, in both

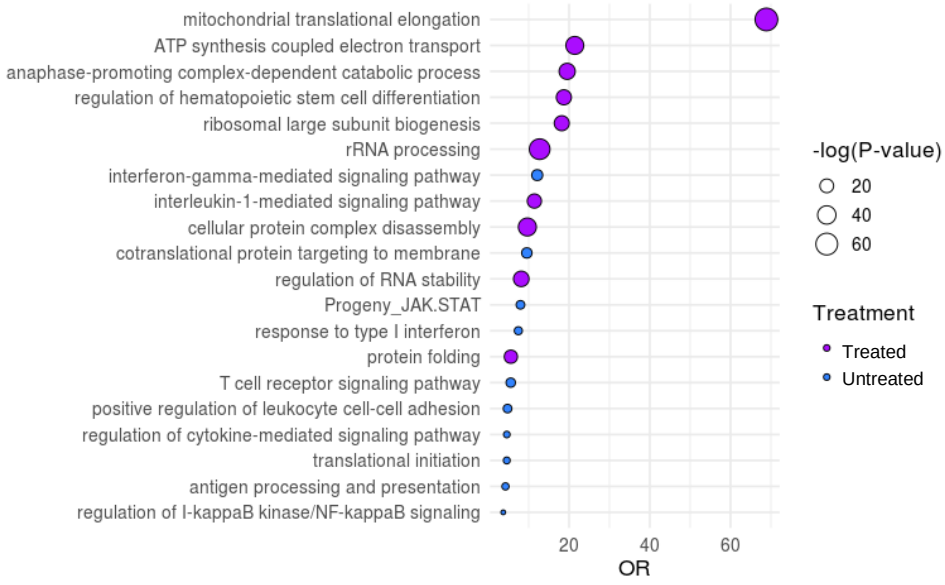
cases, the CD3/CD28 stimulation induces a transcriptomic signature that consists on a massive up-regulation of processes that are related to the metabolic and proliferative processes (i.e. mitochondrial activity, RNA and protein metabolism, DNA replication and translation, among others). These processes do not seem to reflect immune activation *per se*, but the cellular processes that are necessary to support cell activation. Conversely, in un-treated samples the immunological signal was not hidden by activation processes, and we were able to detect an up-regulation in processes related to leukocyte migration and differentiation, antigen presentation and cytokine signalling and production, among others.

Figure 37. Top 10 biological processes induced and repressed by treatment during (**a**) pregnancy and (**b**) postpartum. (**c**) Top 15 pregnancy-specific and postpartum-specific treatment induced biological processes. x-axis represents the odds ratio (OR) of the overlap between differential genes and genes in the biological process. Dot size is proportional to the minus log of the associated P-value.

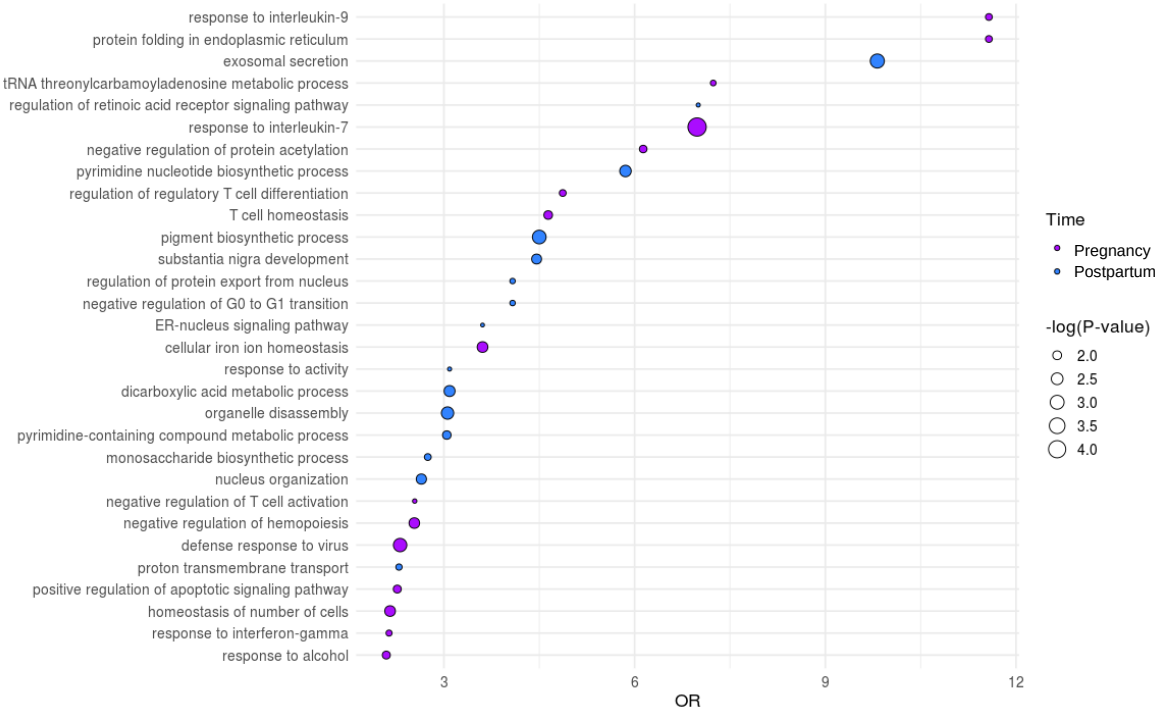
a



b



c



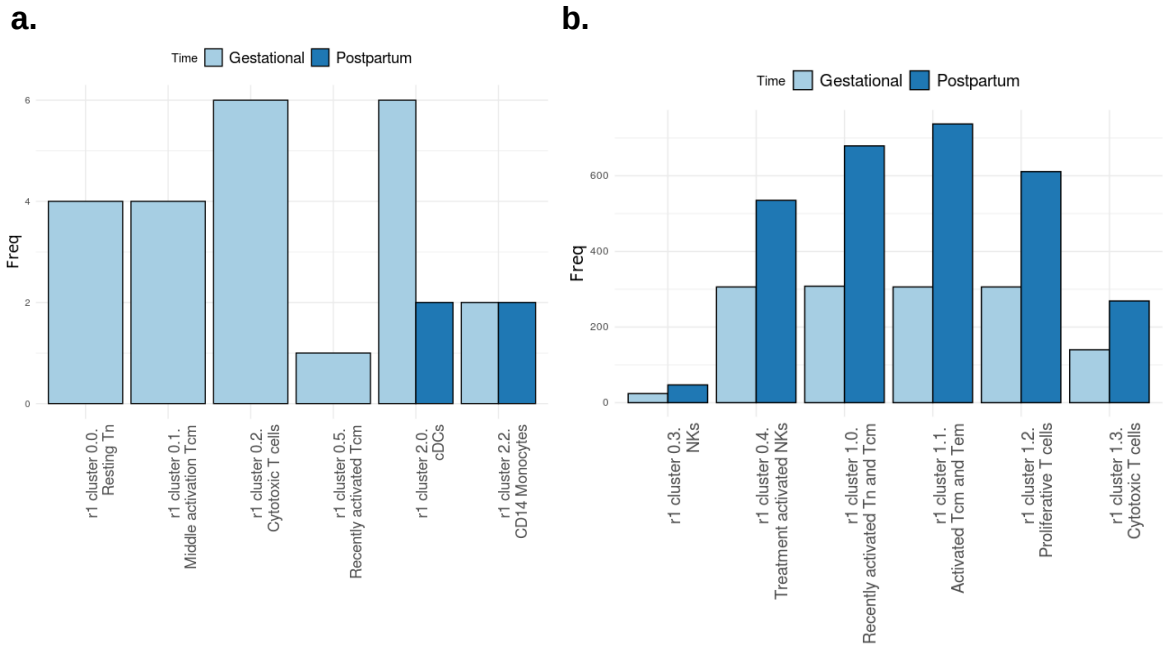
Finally, to detect pregnancy-specific or postpartum-specific effects of cell treatment, we identified the processes that are up-regulated by cell treatment exclusively during pregnancy or in the postpartum (i.e. in one condition but not in the other). We found n=39 pregnancy-specific treatment-induced processes and n=31 postpartum specific treatment up-regulated processes (Figure 37c). Interestingly, pregnancy-specific processes were mostly related to immune processes, while postpartum-specific processes involved again processes necessary for the support of immune cell activation. This suggests that treatment-induced activation in pregnant samples is less pronounced.

5.2.4.2. Differentially expressed genes during pregnancy in particular clusters

To find differences in cell activity during pregnancy and in the postpartum, we used a regression model to study the interactive effects of pregnancy/postpartum and cell cluster on gene expression, for treated and un-treated samples independently. For this analysis, we used the first level of cluster resolution. Figure 38 shows the number of genes found to be up-regulated during pregnancy or in

the postpartum in each cell cluster, for treated and un-treated samples (FDR adjusted p-value < 0.05).

Figure 38. Number of genes significantly up-regulated during pregnancy or in the postpartum period in each of the clusters identified in (**a**) untreated and (**b**) treated samples.



In the unstimulated cells, we detect a total of 30 DEG. In all but the $CD14^{+}$ monocytes cluster (*r1 cluster 2.2*) we found that most of the genes were up-regulated during pregnancy compared to the post-partum.

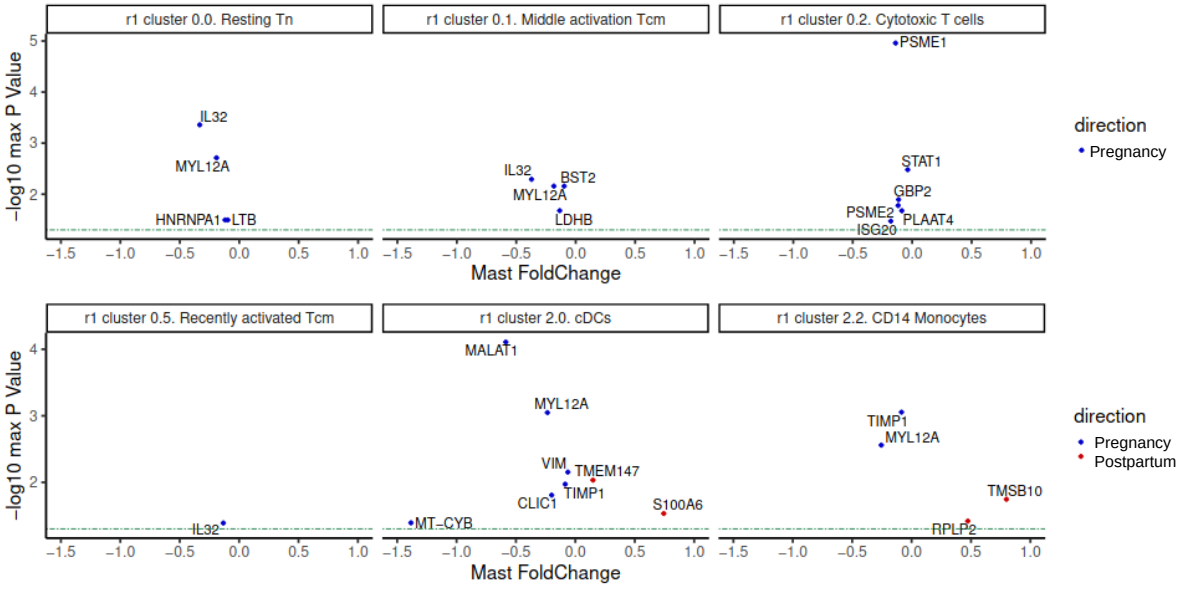
In stimulated cells, we detected a much higher number of DEGs, with up to 5,075 DEG. In contrast to what we observe in untreated cells, in all clusters we see that most of the genes are up-regulated in the postpartum compared to pregnancy. This, again, indicates that cell *in vitro* stimulation has a greater effect in post-partum samples.

5.2.4.2.1. Differential gene expression by the cell cluster in unstimulated cells

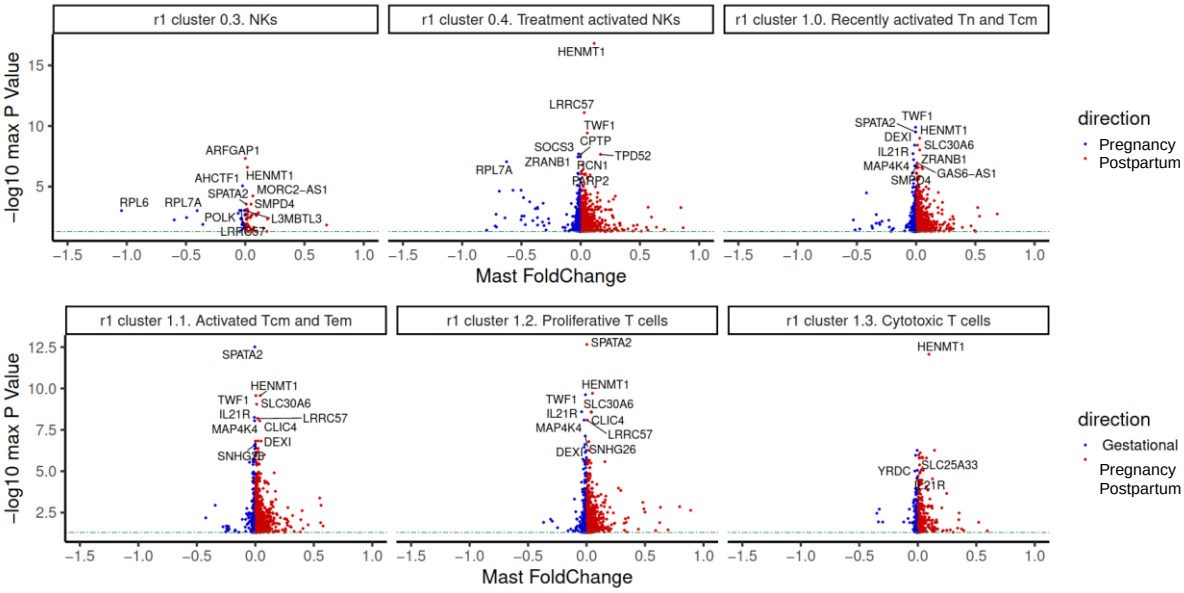
The following section describes the DEG found to be up-regulated during pregnancy and in the postpartum in each of the clusters identified in untreated samples (Figure 39a).

Figure 39. Volcano plots representing the DEG in each cell cluster in (**a**) untreated and (**b**) treated samples. The x-axis represents the FoldChange from the MAST model and the y-axis represents the minus logarithm of the FDR adjusted p-value. Dots are coloured based on the direction of the change. Only significant results (FDR < 0.05) are shown.

a



b



In conventional DCs (*cDCs, r1 cluster 2.0*) in pregnancy samples we found an upregulation of genes related to cell homeostasis such as *MALAT1* involved in regulation gene expression (182), *MYL12A* involved in DNA damage repair (183) or *MT-CYB* that is part of the respirasome complex (184). We also found genes related with physical cell support like *VIM*, implicated in cytoskeleton formation (185) and *TIMP1*, involved in remodelling and homeostasis of the extracellular matrix (186). Finally we detected an up-regulation of *CLIC1*, a gene that, participates in the regulation of cellular metabolism, but also has been described to be involved in pathological processes like inflammation in immune diseases (187).

If we focus on the same cluster in postpartum samples, genes upregulated are *TMEM147* related to NF-KB pathways (188), and *S100A6*, a gene that regulates cell cycle progression and cell differentiation (189).

In $CD14^{+}$ monocytes (*r1 cluster 2.2*) in pregnancy samples, housekeeping related genes like *TIMP1* (190) and *MYL12A* (183) (functions described previously) are found to be over-expressed. In postpartum samples, we detected *TMSB10* (191) (function described previously) also *RPLP2* (192), a gene that encodes a ribosomal protein that has been involved in immune-mediated diseases.

For the cell clusters below, we only detected significant up-regulated genes in the pregnancy samples.

In resting naïve T cells (*r1 cluster 0.0*) we detected an increase of housekeeping genes such us *MYL12A* (183) or *HNRPA1*(193) involved in regulation of alternative splicing. Also an up regulations of genes clearly involved in immunological process like *IL32* (194) or *LTB* a membrane protein of the TNF family (195).

In the middle activation stage (between 0.0 and 0.5) in the cells (*r1 cluster 0.1)* we detected an up regulation of homeostasis maintenance genes like *MYL12A* (183), *BST2* involved in growth and development of B-cells (196) and *LDHB* that presents cellular energy production functions (124). Also, an up regulation of the *IL32* gene with immunological function was detected (197).

In the effector cytotoxic T cells (*r1 cluster 0.2*) we detected an up regulation of genes related to immunological interferons pathways like *STAT1* (198), *GBP2* (199) and *ISG20* (200). We detected genes such as *PSME1/2* (201) that show housekeeping function since it acts in protein degradation but also seems to have immunological functions in the presentation of antigens. Finally, we detect *PLAAT4* (202) which is related to gene cell death.

Finally, in pre-Th1 recently activated T_{cm} cells (*r1 cluster 0.5*) we only detect the up regulation of the gene encoding for IL32 cytokine (194).

5.2.4.2.2. Differential gene expression by the cell cluster in stimulated cells

The following section describes particular DEG found to be up-regulated during pregnancy and in the postpartum in the clusters identified in stimulated samples (Figure 39b). As shown previously, *in vitro* stimulated cells show a very high number of DEG. The most significant genes and present in a high number of cell clusters are *HENMT1, SPATA2 and TWF1.* These three genes are involved in proliferation and metabolic responses indicating changes to support effector responses (203–205). Also, genes with a important immune function are detected (*IL21R* and *SOCS3*) and described here (206,207).

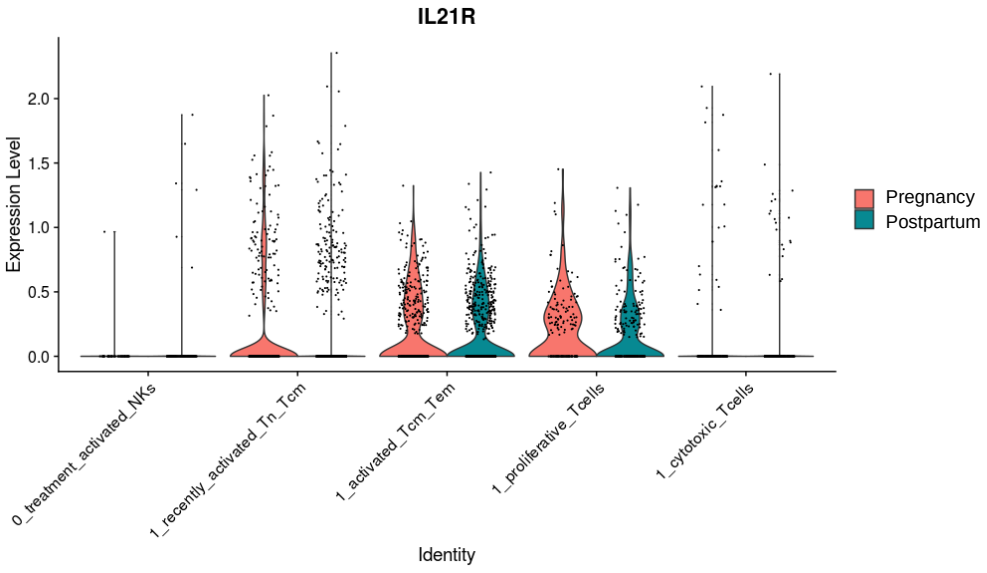
HEN Methyltransferase 1 (HENMT1) is a protein coding gene involved in RNA binding and RNA methyltransferase activity (203) and is upregulated in all treated cells clusters only in postpartum. Also its overexpressed in treated cells from NK (NKs (and some effector $CD8^+$ T cells) (*r1 cluster 0.3)* and treated NKs (*r1 cluster 0.4*)) which is part of the global untreated cluster but remember that there is a cell part of that cluster that mixes treated and untreated cells.

Spermatogenesis Associated 2 (SPATA2) is involved in the regulation of necroptotic process (204) and in our study was up-regulated in treated cells in postpartum samples in proliferative T cells (*r1 cluster 1.2*) and effector cytotoxic T cells (*r1 cluster 1.3*). In pregnancy samples is detected in recently activated T_n and T_{cm} cells (*r1 cluster 1.0*.) and activated T_{cm} and T_{em} cells (*r1 cluster 1.1*). This gene is also overexpressed in treated cell from the *r1 cluster 0.3,* a cluster with NKs cells and some effector $CD8^+$ T cells.

Twinfilin Actin Binding Protein 1 (TWF1) encodes for a protein that may be an actin monomer-binding protein (205) and was found to be upregulated in treated pregnancy samples in recently activated T_n and T_{cm} (*r1 cluster 1.0*) cells and proliferative T cells (*r1 cluster 1.2*). In postpartum stimulated samples, this gene was found to be up-regulated in Activated T_{cm} and T_{em} cells (*r1 cluster 1.1*) and effector cytotoxic T cells (*r1 cluster 1.3*). We found an up-regulation of this gene in cells from the un-stimulated cluster from postpartum samples in NKs (and some effector $CD8^+$ T cells) (*r1 cluster 0.3).*

Interleukin 21 Receptor (IL21R) encodes the receptor for IL21 (206). It was found to be upregulated in treated pregnancy simple in recently activated T_n and T_{cm} cells (*r1 cluster 1.0*), activated T_{cm} and T_{em} cells (*r1 cluster 1.1),* proliferative T cells (*r1 cluster 1.2*) and effector cytotoxic T cells (*r1 cluster 1.3*). In postpartum treated cells we detected an up-regulation in untreated cells in treated NKs (*r1 cluster 0.4*) (Figure 40).

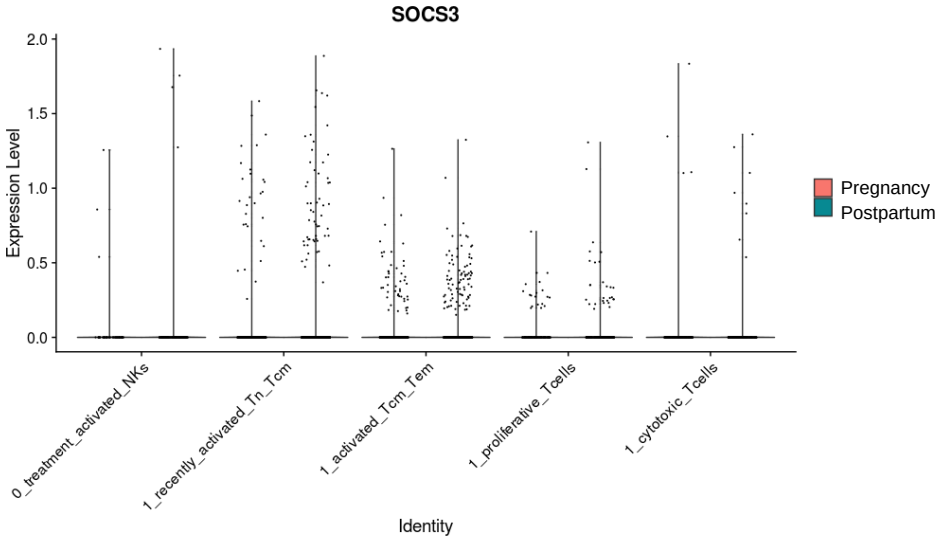
Figure 40. Violin plots representing *IL21R* gene in each cell cluster in pregnancy and postpartum samples.



Suppressor Of Cytokine Signalling 3 (SOCS3) encodes a suppressor of cytokine signalling (207). It is overexpressed in postpartum samples in treated cells in recently activated T_n and T_{cm} cells (*r1 cluster 1.0*), activated T_{cm} and T_{em} cells (*r1 cluster 1.1*), proliferative T cells (*r1 cluster 1.2*) and effector cytotoxic T cells (*r1*

cluster 1.3). In pregnancy samples it is over-expressed in untreated treated NKs (*r1 cluster 0.4*) (Figure 41).

Figure 41. Violin plots representing *SOCS3* gene in each cell cluster in pregnancy and postpartum samples.



6. Discussion

6.1. Cohort description

The studies that involve pregnant women are extremely challenging to perform due to the difficulty in obtaining biological samples during pregnancy. Certainly, one of the most difficult points to achieve is the inclusion of patients and even more difficult is to recruit healthy pregnant controls. On top of that, one strong additional inconvenience is to obtain longitudinal samples across the pregnancy and postpartum periods. There is a highly sensitive matter behind this, and the pregnancy status makes all participative efforts in any given volunteer more secondary. In addition, there is a time-sensitive aspect to any study involving pregnancy.

The present project has included a cohort of 6 individuals: 3 affected by RA and 3 HC. Although we were aware of the difficulties around this type of project, we established a very restrictive protocol to obtain a homogeneous cohort and subsequent samples at different time points. We prioritized the quality and adhesion to the protocol. The revolutionary technology we used in this study allowed us to obtain robust results even with low sample sizes, as has been demonstrated in multiple references published so far. At the time of recruitment, the number of candidates was very low, and the stringency of the inclusion criteria made selection more difficult. Another difficulty with patient inclusion is that even patients who are willing to participate prefer not to undergo additional blood extractions than those scheduled in pregnancy care. An important challenge is the collection of blood samples at each time point, as samples must be obtained in a very short window of time due to the marked and rapid changes in the development of a pregnancy. For this reason, each scheduled visit with blood collection could only be held in a two-week margin in order to assure comparable data between study participants.

As previously described, to ensure the most homogeneous cohort, the inclusion criteria were very strict. For this reason, only patients who presented RF and ACPA auto-antibodies and fulfilled diagnostic criteria for RA following the ACR 2010 Criteria were included. Furthermore, from the whole group of pregnant patients that Dr Pluma is prospectively collecting data in our unit (not described in

the present document); only those in which we could obtain the samples according to the protocol were finally included in this present study.

As mentioned in the results, due to technical problems during the data generation processing in the central facility, only the single-cell data from two RA patients could be analysed making the comparison with healthy controls, both in pregnancy and postpartum, not possible. Due to this, we focused our study in to identify the differential mechanisms between pregnancy and postpartum in Rheumatoid Arthritis patients and to described for the first time the single cell RNA seq atlas of blood cells in pregnant and postpartum women with Rheumatoid Arthritis.

Despite this, as shown in the results section, both patients had very similar characteristics: time to diagnosis, smoking and alcohol intake, absence of previous pathologies that required specific treatment during pregnancy, no fertility techniques, no pathologies related to pregnancy or postpartum, normal ultrasound follow-up, normal blood pressure, partum at terminus, lactation with formula milk and normal development of the babies. In addition, both required increased RA treatment due to an exacerbation of inflammatory activity. The similarities between the two patients help to reduce the biases of the study and contribute to the robustness of the results obtained in the molecular analysis. The differences in treatment during pregnancy, do not clearly affect our results as we have not performed direct comparison between patients but longitudinal compararisons at two time points. Further studies are needed to precisely define the effects of DMARDs in the biological pathways during pregnancy in RA patients.

6.2. Cell cluster identification

Despite having a low number of patients to analyse, thanks to the power of single cell technology the results are robust and meaningful. We included a final number of 2 RA patients, 2 time points for each patient (4 samples) and finally we analysed a total number of 8 samples (stimulated and unstimulated cells). Single-cell RNA-seq is a technique that is capable of analysing the cell types with a degree of detail not possible until now with bulk techniques (208). Several reference studies in the literature a have similar number of patients as ours. For example, in the study by Orange *et al* (139), where PRIME novel fibroblasts cells

were identified for the first time using single-cell technology as a part of the methods used, they included 4 patients. The authors performed a study design where blood sampling was done longitudinally over the time during flares in RA patients. The authors detected changes in blood transcriptional profiles 1 to 2 weeks before an RA flare. In those patients who presented a flare, B-cell activation followed by expansion of circulating $CD45^{-}CD31^{-}PDPN^{+}$ preinflammatory mesenchymal cells in the blood were observed. The authors named these cells PRIME and were found to share characteristics with inflammatory synovial fibroblasts. In addition, they suggested that these cells could be activated before a flare and migrate from blood to the synovium. Another example is the pre-print manuscript by Qingliang Zheng *et al* (2021) (209). Despite not yet going through the peer review process, is a very relevant study for pregnancy. In this study, they analysed the decidua macrophages during normal and recurrent spontaneous abortion by using scRNA-seq. A total of four patients were included in this study (2 normal and 2 with recurrent spontaneous abortion). The authors were able to describe a higher percentage of M1 cells (70.6%) in the normal deciduae in contrast with a higher percentage of M2 cells (68.3%) in the recurrent spontaneous abortion deciduae. Finally, in the area of pregnancy, a relevant study was performed by Vento-Tormo (140). In this study they focused on the foetal-placental interface at the beginning of gestation. To do this, unicellular transcriptome profiles using single cell of the maternal-foetal interface (11 deciduae and 5 placentas at 6-14 weeks' gestation) and six paired PBMCs were obtained. They identified three different types of dNK. Although there cell types are already known, they found novel functions for some of them. In particular, dNK1 was found to have receptors for trophoblast HLA-C, HLA-E and HLA-G molecules, and could be metabolically primed through increased expression of glycolytic enzymes. This has helped to increase the knowledge of the molecular mechanisms that act at the materno-foetal interface, trying to clarify how this microenvironment of immunological tolerance is generated.

In our analysis of circulating cells, we have found 12 cell clusters for untreated T cells and treated and untreated NKs; 10 cell clusters for treated T cells; 2 cell clusters for myeloid cells and 3 cells clusters for B cells. To our knowledge, the different cell clusters present in peripheral blood samples from patients with RA in

pregnancy and postpartum had not been described previously. The few previously carried out single cell studies recruited healthy pregnant women (140), at the beginning of pregnancy and used peripheral blood and placenta. Due to the differences in methodology or the type of population included in both studies (marked cellular changes that occur during pregnancy, the influence of the disease) it is difficult to compare our results with those of this study. Despite this, if we compare the results with the Vento-Tormo study (since it used peripheral blood), they described the presence of T cells, Nks, NK $CD16^{+}$, monocytes and DC in peripheral blood that we also found, but in our case with a deeper resolution.

Within all these new cell categorization we have been able to carry out, we have found a small cluster *(r2 cluster 0.13.)* which expresses markers consistent with a cluster described in the literature NKT subpopulation ($CD3^{+}$ $CD16a^{-}$ CD56) (174) and over-expressed KIR2DL4, an HLA-G receptor (175). The interaction of KIR2DL4 with the HLA-G receptor in the placenta acts as an inhibition mechanism, facilitating the tolerance to the foetus (80). NKT cells are different from NK cells since they express T cell receptors (T cell receptor/CD3 complex) that recognize glycolipid antigens bound with the antigen-presenting molecule CD1D. An important role of this cell is the capacity to produce immunomodulatory cytokines in front of antigenic stimulation, giving them an important immunoregulatory role (210). Interestingly, this cell has also been described in the placento-foetal barrier modulating innate and adaptive immune responses, but its exact function is unknown (211). An association with preeclampsia has been described (212). As far as we know, the marker KIR2DL4 has been described in NKs, but its biology is not as well understood as with NKTs (213).

6.2.1. Effectorness gradient

When defining the different untreated lymphoid cells clusters, we clearly detect cells separating according to their ability of cytotoxicity. In one extreme, we detect non-cytotoxic cells and, at the other extreme, cells with clear cytotoxic activity like NK and $CD8^{+}$ cells (showing markers such as GZMK and GZMA). In the non-cytotoxic region we can detect an effectorness gradient. In one extreme, we detect

more naïve T cells (i.e. showing high expression of receptors such as CCR7 and SELL). In the other, we found cells such as T Cell $CD4^+$ Th1 that are already specializing in a more effector subtype. In the intermediate zone, cell progressing from one phenotype to other can be found. This result is very interesting as it coincides with the findings previously described in the literature (163,164).

The Hashimoto *et al* study (163) focused on immune cytotoxicity. The authors performed single-cell transcriptome analysis of 61,202 PBMCs from 7 supercentenarians and 5 younger controls. Results from the cluster analysis showed that cytotoxicity was the primary factor separating them. In one extreme, cell with cytotoxic effector molecules like GZMH, GZMB, GZMA, and PRF1 were detected. In the other extreme, cells presented with CCR7 and SELL, indicating more naïve T cells. We have detected the same pattern in our data.

The Cano-Gomez *et al* study (164) focused on the effects of cytokines on human naïve and memory $CD4^+$ T cells using scRNA-seq and included 4 patients. They demonstrated that $CD4^+$ T cells form a continuum, describing a progression from T naïve to T central memory, T effector memory and effector memory T cells reexpressing CD45RA cells, with natural T_{regs} located separately. This progressive modification involved an up-regulation of chemokine and cytokine genes. The authors conclude that these results could indicate that T cells at the end of this trajectory are ready to start a rapid effector response upon activation (164).

As described in the two cited studies (163,164), we have found the same results as those described and our findings support the increasingly more established idea of plasticity of cells (214). Until recently, the concept that the differentiation of $CD4^+$ T cells into various effector subsets was an irreversible event (215). This concept has been progressively modified and, currently, there are studies that suggest effector T cells retain some degree of functional plasticity and these cells can change their effector phenotype. The mechanisms by which cells can change their phenotype are not clearly understood but it’s hypothesized that the cytokine milieu environment plays a very important role(215).

6.3. Proportions across samples

We have detected important changes in cell composition when comparing pregnancy and postpartum. The two analysed patients showed a similar cell composition. One of the most interesting results we found in untreated samples was the increased proportion of myeloid cells in pregnancy compared with postpartum. More in detail *(r1 cluster 2.2)* $CD14^+$ monocytes. Regarding T cells, with the more general resolution (Resolution 1) we detected an increased proportion of *(r1 cluster 0.0)* resting naïve T cells in postpartum and with the more granular resolution (Resolution 2) an increase of *(r2 cluster 0.1)* $GZMB^+$ $GNLY^+$ $GZMK^-$ $CD8^+$ T cells. In treated samples we found an increase of (*r2 cluster 1.0*) recently activated naïve T cell and (*r2 cluster 1.2*) activated ribosomal T_{cm}. We want to emphasize that the RA2 patient has a proportion of NK (*r2 cluster 0.11*) and T_{reg} *(r2 cluster 0.12)* in pregnancy untreated samples that increases markedly in the postpartum period. For this reason and the importance of these two cell types in pregnancy, although the results have not been significant, we include it in the discussion.

6.3.1. Myeloid cells

Myeloid cells include granulocytes, monocytes, macrophages and dendritic cells. Like lymphoid cells, these cells are generated from hematopoietic stem cells located in the bone marrow (216,217). Myeloid cells, and more specifically, dendritic cells work as an antigen presenting cell and also mediate immune tolerance by acting on T_{regs}.

The study of Häupl *et al* (2008) (121), was a prospective cohort study focused on the mechanism involved in RA postpartum reactivation. Six pregnant patients with RA and 8 healthy pregnant controls were included and transcriptomes of PBMCs were generated. Blood sampling was done at gestational weeks 32–34 and 24 weeks postpartum. Focusing on $CD14^+$ monocytes, the authors described a decrease on monocytes in postpartum compared with pregnancy in healthy controls. In RA, the authors described a persistent elevation of this cell type in postpartum vs. pregnancy and expressed more genes related to activation. In our

study we have detected an increased proportion on monocytes in pregnancy samples compared with postpartum. In our case, we were not able to recover the monocytes in the postpartum. This could be because postpartum cells are more active and stick to the petri dish. For this reason we consider that our results are in line with what is described by the authors.

The study of Luppi *et al* (218) (2002), performed a prospective study in healthy pregnant women to study the changes in the proportion of different subsets of circulating leukocytes (218). They included 17 healthy pregnant women and blood sampling was done once per trimester. The first blood sample was obtained from 8 participants between 1-12 weeks' gestation, the second sample was obtained from 17 participants between 13-28 weeks' gestation, and a third sample was obtained from 13 participants between 29-36 weeks' gestation. Flow cytometry was applied to determine the main cell populations. Focusing on myeloid cells and more in detail $CD14^{+}$ monocytes, the authors did not find any significant changes in the proportion at any time-points during gestation. Unlike these findings, in our study, we detected an increase in these cells in pregnancy compared to postpartum. One possible explanation for the difference in the results is the different design of the studies as postpartum was not included.

The study by NM Shah *et al* (2017), described the possible role of myeloid cells during pregnancy (219). They performed enzyme-linked immunosorbent spot assays in blood from 15 healthy volunteers and 10 pregnant women using a longitudinal design obtaining samples from the second and third trimester of pregnancy. Also, they performed a flow cytometry assay using PBMCs from 20 healthy pregnant (10 second trimester (12–28 weeks' gestation) and 10 third trimester (> 28 weeks of gestation) and nine healthy non-pregnant female control. Interestingly, profiling of dendritic cells showed a decrease in myeloid dendritic cells but not plasmacytoid dendritic cells in peripheral blood during pregnancy (219).

The study by Ulrike Friebe-Hoffmann *et al* (2017) (220), focused on peripheral immunological cells in pregnant women and their change in type 1 diabetes. A flow colour cytometry analysis was applied. They included 106 patients divided into the following groups: 24 non-pregnant controls, 24 healthy pregnant women, 24 non-

pregnant women with type 1 diabetes, 15 pregnant women with type 1 diabetes and 18 pregnant women with gestational diabetes mellitus. Blood sampling was done from 27-31 weeks' gestation. First, they described a significantly lower rate of dendritic cells in pregnant healthy controls than in non-pregnant healthy controls. They describe that the numbers of dendritic cells were significantly lower in pregnant type1 diabetes than in non-pregnant type 1 diabetes. Regarding the apoptosis rates of the myeloid and lymphoid subgroups of the dendritic cells, they reported significantly higher levels in pregnant women with type 1 diabetes in comparison with non pregnant type 1 diabetic women.

The second paper described a decrease of myeloid dendritic cells but not plasmacytoid dendritic cell in peripheral blood during pregnancy (219). In third, dendritic cells in healthy pregnant women were lower than healthy non-pregnant controls (220). In both cases, the data seems to indicate a reduction in some of the dendritic cell subtypes during gestation. In our case, we did not find statistically significant differences between the proportion of dendritic cells (cDCs) in pregnancy or postpartum. A possible hypothesis for the difference in these results is that we have carried out the study in patients with RA and do not know if the disease itself can modify these cell proportions.

In another relevant study (221), the peripheral blood samples from 25 women with preeclampsia and 30 women with normal pregnancies were studied. Blood sampling was done from 27-40 weeks' gestation. Flow cytometry was performed. Results revealed that, the ratio of myeloid and lymphoid dendritic cell in preeclampsia was significantly higher than normal pregnancy.

The last one described an increase in the ratio of myeloid and lymphoid dendritic cell in preeclampsia (221). Interestingly, they found in preeclampsia, a disease exclusively of pregnant women, it seems that the ratio of these cells could increase. In our study, we did not observe this increase and our patients did not present with preeclampsia.

With the exception of the first paper described, where the differences between cellular proportions during the progression of gestation were studied, the rest of the articles made a comparison between pregnancy vs. non-pregnancy, or healthy

vs. disease during pregnancy. The main limitation when comparing our results with previous studies is they do not include the postpartum comparison, which is an advantage in our study, and we could not include our healthy controls samples. In addition, the deep analysis we have performed using single-cell technology differs from the techniques used in the reported papers.

6.3.1.1. Immune brake

In the results obtained in the myeloid cells proportions, we found that myeloid cells could only be recovered in pregnancy samples. The most likely explanation for this is that, in the postpartum samples, the monocytes are activated in a more powerful way. This makes them adhere to the petri dish and likely could not be recovered for scRNAseq. In pregnancy, these cells were recoverable. Our hypothesis is that during pregnancy monocytes are subject to regulatory pathways that impose a sort of “immune brake” that does not allow these cells to respond to the cellular stress that we have artificially generated by culturing *in vitro*. This finding could coincide with the natural evolution of RA during pregnancy that shows an improvement in inflammatory activity. A possible hypothesis would be that this “immune brake” also exerts its action on the control of RA. This hypothesis requires validation in independent patients’ samples.

The Häupl *et al* (121) study previously reported performed a pathway analysis that showed similar pathway activity in patients with RA and healthy controls in pregnancy. In contrast, postpartum RA patients showed that monocyte-associated pathways were strongly increased. In addition, this increase exceeded the level expected by the increase in monocyte cell numbers. These results indicate, not only the persistence of elevated monocytes in postpartum, but an increase on gene activation pathways. This can indicate a resistance of the cells in gestation to the monocyte-associated pathway activation and in line with what found.

6.3.2. T cells

Lymphoid cells are a major cell involved in the adaptative immune response. The two main types of lymphocytes are B lymphocytes and T lymphocytes. B

lymphocytes are responsible for the antibody production. T lymphocyte have a role in executing and controlling immune responses (222,223).

Some studies have focused on the study of modulation of these cells during pregnancy and postpartum. All have shown data in relation to the variation of these cells during pregnancy both in healthy women and those affected by pathologies of immunological origin (224,225).

The article by Häupl *et al* (2008) (121) study previously described, found an increase of $CD4^+$ T cells and $CD8^+$ T cells in postpartum of healthy controls and RA patients with no differences between the groups. Similar to these findings, we have observed an increase of T cells in postpartum. In fact, we have found an increase of $CD8^+$ T cells *(r2 cluster 0.1)* $GZMB^+$ $GNLY^+$ $GZMK^-$ $CD8^+$ T cells could coincide with results described by the authors.

The article by Mikio Watanabe *et al* (1997)(224), focused on the study of changes in T cells, B cells, and NK lymphocytes in pregnancy and postpartum. They included a relatively large sized cohort: 5 healthy non-pregnant women, 106 healthy pregnant women divided in 38 in the first trimester (7-13 weeks'), 30 in the second trimester (19-25 weeks') and 38 in the third trimester (30-38 weeks') and 148 healthy postpartum women divided into 30 at 1 month postpartum, 35 at 4 months postpartum, 33 at 7 months postpartum, 27 at 10 months postpartum and 23 at 13 months postpartum. Lymphocyte subsets were analysed by flow cytometry. This study showed an increase in peripheral lymphocytes in the postpartum period due to an increase in both T and B cells. More specifically, it describes that the increase in T cells in the postpartum period was caused by an increase in TCRαβ-negative T cells. These results suggested and increased activity of maternal immunity. This could be related to the increase in these cells in the postpartum period and the reactivation of RA during that time (224). Even though our patients presented with an increased inflammatory activity in the postpartum we did not find an increase of this cell type.

Another relevant paper from Annette Langer-Gould *et al* (2010), focused on studying interferon-producing T cells and the relation to pregnancy and postpartum relapses in Multiple Sclerosis (MS) (225). Interestingly, MS, a chronic

T cell mediated inflammatory disease, shares the pattern of pregnancy-associated remission and postpartum flares with RA (225). The authors included 26 women with MS and 24 healthy pregnant controls at less than 35 weeks of pregnancy. A blood sample was obtained in each trimester and at 2, 4, 6, 9, and 12 months postpartum. PBMCs were analysed by flow cytometry. The results showed that during pregnancy, the absolute proportion of the IFNγ producing $CD4^+$ $CD45RA^-$ lymphocytes was higher in women with MS than healthy controls. Interestingly, the pattern that these cells follow was different. In women with MS, IFNγ producing $CD4^+$ $CD45RA^-$ cells significantly declined during pregnancy. Nevertheless, in the postpartum, those women who presented a reactivation, the progressive reduction of these cells persisted. Moreover, in healthy pregnancy, these cells did not change. Given the similarities between the two pathologies, RA and MS in their pathophysiology as well as in the response to pregnancy, we expected to find a similar pattern but, we have not detected this cell cluster.

If we focus more on the maturation of T cell subtypes during pregnancy, we find a review by Kieffer *et al* (226). A good balance of memory T cells could be necessary for the success of the pregnancy and an immune memory in reproduction could be necessary and not harmful. The maternal immune system is exposed to paternal-foetal antigens (exposure to semen even before and after pregnancy, paternal-foetal antigens are exposed at the foetal-maternal interface in pregnancy and microchimerism during the course of pregnancy and postpartum). Memory cells allow the immune system to efficiently protect the body from pathogens by generating a more appropriate immune response to a known antigen (in this case, paternal antigens). Furthermore, there is evidence (226) that memory cells may present a different response to paternal antigens in relation to their response to other antigens. This different response could lead the tolerance rather than rejection of paternal antigens.

If we focus on the postpartum, we find an observational study by the same author Kieffer *et al* (2017) (227). In this study, peripheral blood from 14 nulligravid, 12 primigravid and 15 parous women that were on average 18 months postpartum were analysed using flow cytometry. Results showed significantly higher proportions of $CD4^+$ effector memory, $CD4^+$ central memory and activated memory

T cells in parous women compared to nulligravid women. In contrast to $CD4^+$ cells, the activation status of $CD8^+$ memory cells did not differ between the groups. In this case, we have detected these cell clusters (*r2 cluster 0.9* and *r1 cluster 0.1*) but they did not show significant differences in their portions when comparing pregnancy and postpartum. One possible explanation is that these cells may be modulated differently in an RA pregnancy.

Again, it is difficult to compare these results to ours given the different design of the study and the applied technique. We have not found studies with a similar design and focused on cell clusters, where we have found a significant increase in the postpartum period in untreated resting naïve T cells and $GNLY^+$ $GZMK^-$ $CD8^+$ T. In treated samples we have found an increase in activated T_{cm} cells with high ribosomal content and recently activated naïve T cells. The increase in these cell clusters, especially CD8 from our results, may indicate an increase in postpartum cytotoxic activity that would coincide with the increase in inflammatory activity in this period.

6.3.3. Natural Killer cells

Globally, in our results, we have not detected the different proportions presence of NK. Despite this, we see that one of our patients shows a markedly increase in NK in the postpartum period. For this reason and due to the important role of this cell type in pregnancy, postpartum and rheumatic diseases; it is described here.

NK cells are an innate immune system cell that plays a crucial role in defence against viruses, parasites, bacteria, and tumour cells responses. Furthermore, NK cells, participate on the adaptive immune system connecting innate and the adaptive immunity through their receptor FcγRIIIA (CD16) (228).

One of the first groups focused in the study of NK cells during pregnancy and postpartum was Y Hidaka *et al* (1991) (229). The authors included 111 healthy pregnant women: 34 in the first trimester (7-13 weeks'), 41 in the second trimester (14-27 weeks'), 36 in the third trimester (28-40 weeks'), 126 healthy postpartum women: 36 at 1 month postpartum, 40 at 4 months', 30 at 7 months', 20 at 10 months', and 58 healthy non-pregnant women. Lymphocyte subpopulations from

peripheral blood were analysed by cytometry. Interestingly, they described that the relative NK activity increased significantly in the first trimester and also at 1 month postpartum. Furthermore, a decrease in the activity of these cells was detected during the third trimester.

As previously described, Mikio Watanabe *et al*, studied NK lymphocytes from peripheral blood in pregnancy and postpartum (224). Their work described that $CD16^{+}$ $CD57^{-}$ NK cells characterized by an intense cytotoxic activity, were expanded during the first trimester and reduced in the third trimester reaching to the normal levels or non-pregnant level, after delivery. They also showed that NK $CD16^{+}$, $CD57^{+}$ (which presented a weak cytotoxic activity) did not presented significant change during pregnancy but expanded 1-4 months after delivery.

Studying the functionality of these cells more in-deep during postpartum, M Groer *et al* (230) focused on this period. They included data from 103 women at 2 months postpartum, 89 at 3 months postpartum, 89 at 4 months postpartum, 84 at 5 months postpartum, and 79 at 6 months postpartum. Flow cytometry cytotoxicity assays on PBMCs and NK cells found a decrease in NK cytotoxicity in postpartum women compared to controls through the first 6 months postpartum. At 9 months postpartum, levels became normal. As a possible explanation for these results, the authors propose the hypothesis that this could be the response to interaction with foetal microchimeric cells persisting in maternal blood and tissues in the postpartum (230).

In a similar line as the first two studies described (224,229), our study detected a disbalance in NK populations in one patient during the postpartum, more specifically $CD16A^{+}$ $CD56^{dim}$ NKs. We have detected two populations of $CD16A^{+}$ $CD56^{dim}$ NKs in treated samples. One of these (*r2 cluster 0.11*) has only treated samples and is made of cells that are more responsive to treatment, with increased expression of chemokines and granzymes (*XCL1, XCL2, GZMB, CCL3* and *CCL4)*. The other NK cell cluster (*r2 cluster 0.5*) showed a conventional $CD16A^{+}$ $CD56^{dim}$ NKs phenotype and had cells from both treated and untreated samples, indicating that the treated cells in this cluster are not affected by treatment. In one patient, we observed that treated gestational $CD16A^{+}$ $CD56^{dim}$ NK mostly belong to the cluster not affected by treatment (*r2 cluster*

0.5, with a percentage change from 6.80% of cells during pregnancy vs. 3.95% of cells in the post-partum). Conversely, cells in the post-partum mostly correspond to the treatment responsive CD16A^{+} CD56^{dim} NK population (*r2 cluster 0.11,* with a percentage change from 2.14% in pregnancy to 7.89% postpartum). Our findings show, more than an increase in the NK populations in the post-partum, that, in this patient, NKs are more responsive to T cell activation in the post-partum than during pregnancy. This, again, suggests the presence of an immune-break during pregnancy, that is also affecting CD16A^{+} CD56^{dim} NKs. Despite this, we again highlight the difference in the design of the studies in order to compare our results. We have not carried out a cytotoxicity assay, so it is difficult to compare the results with the third study (230).

6.3.4. Regulatory T cells

T_{reg} cells suppress activation, proliferation, and cytokine production by CD4^{+} and CD8^{+} T cells B lymphocytes and dendritic cells. T_{reg} cells can produce soluble messengers with immunosuppressive function like IL10, IL2 exhaustion and direct cell-cell inhibition (231).

T_{regs} are a cellular type with a key role in regulating the immune system during pregnancy. T_{regs} prevent maternal T-cell activation against foetal cells. However, its variation during the progress of pregnancy or postpartum is not well understood. The study by Lima *et al* (232), focused on this gap of knowledge. They carried out a prospective study including 43 healthy pregnant women and 35 nonpregnant women during the third trimester, on the day of delivery, and postpartum. They performed a flow cytometry to analyze T_{reg} subsets in the peripheral blood of pregnant women from late pregnancy through the postpartum period. The authors described how all T_{reg} subsets significantly increased postpartum compared with the third trimester and the day of delivery (232).

In our case, in unstimulated cells, one of the patients in pregnancy had 0.81% *(r2 cluster 0.12)* pre-T_{reg} recently activated T_{cm} cells that increased to 6.99% in the postpartum period, a result that would be in line with what was detected by the previous study. However, we have only detected this pattern in one of the patients and we must interpret it with caution.

6.4. Differential gene expression

6.4.1. Differential gene expression due to cell treatment

Our study presents a novel experimental design including *in vitro* stimulation. PBMCs are located in peripheral blood and therefore not in the target tissue for RA, the synovium, or the placenta for gestation. For this reason, we decided to stimulate our cells and thus be able to study whether, due to *in vitro* stimulation, they showed a differential or pathological phenotype (as if they were in the target tissues).

Results showed that in pregnancy and postpartum samples, stimulation induced a massive up-regulation of processes that are related, not to immune activation, but to the metabolic and proliferative processes that are necessary to maintain this activation. Conversely, in untreated samples, the immunological signal is not hidden by stimulation processes, and we were able to detect an up-regulation in immunological processes. In addition, to detect pregnancy-specific or postpartum-specific effects of cell treatment, we selected processes that were up-regulated by cell treatment only in pregnancy or postpartum samples. We found 39 pregnancy-specific treatment-induced processes and 31 postpartum-specific treatment up-regulated processes. Pregnancy-specific processes were more related to immune processes, while postpartum-specific processes were related to metabolic functions to support cell activation. This suggests that treatment-induced activation in pregnant samples is less pronounced.

Therefore, during pregnancy, cells are more resistant to cell activation generating a potential new mechanism of tolerance. We have observed this same response to cell activation in the cell proportion sections, specifically on myeloid cells. These findings are in line with previously described hypotheses. RA improves during pregnancy, then immune resistance in cells during pregnancy act like an "immune brake" that exert its action on RA disease activity control.

6.4.2. Differential gene expression in unstimulated cells

For the discussion of DEGs, we have focused on untreated cells since this is where we have detected the most immune processes. We have selected the most significant ones which are detailed below. We will also describe two genes detected in the treated cells for their immunological implications. In this section, we first describe DEGs found and the knowledge we have of their role in RA and pregnancy. Second, we contextualize their possible role in our study in the Related Genes section (6.4.4).

Signal Transducer and Activator Of Transcription 1 (STAT1)

We have found an up-regulation of this gene during pregnancy, specifically in effector cytotoxic T cells (*r1 cluster 0.2*). The function of this gene on $CD8^+$ effector cells is not clear, but recently *STAT1* has been shown to play a cell-intrinsic role in maintaining naïve $CD8^+$ T cell quiescence (233).

The protein encoded by *STAT1* belongs to the STAT protein family. STAT family members can be activated depending on the cytokines and growth factors. Once activated, they can act in the nucleus as a transcription activators and are involved in the IFN-α and INFγ pathways presenting an important role in immune responses (198).

The Weix *et al* study (122) research on detecting candidate genes regulated during human pregnancy which have the potential to modulate RA disease activity. The study included 3 different patient cohorts. Cohort 1 included a total of 32 participants: 8 at gestational weeks 11–14, 8 at gestational weeks 20–23, 8 at gestational weeks 30–33 and 8 at 6 weeks postpartum. Cohort 2 included 8 healthy pregnant women followed during the third trimester and cohort 3 included 8 RA patients followed during the third trimester and postpartum. They performed a gene chip analysis using Affymetrix GeneChips and secuently a real-time quantitative polymerase chain reaction using PBMCs. They detected and up regulation of STAT1 during third trimester in comparison to the postpartum period in healthy controls and even higher in RA patients (122).

Furthermore, if we focus on the modulation of *STAT1* by progesterone, a crucial hormone in pregnancy, we find a recent study by Hellebeg *et al* (234). The researchers from this study included 13 healthy non-pregnant women. Blood sampling was done during the menstrual cycle (seven women were in the luteal and six in the follicular phase). Next-generation sequencing on PBMCs was used to investigate the direct effects of progesterone on $CD4^+$ T cell activation. They found that *STAT1* and *STAT3* were significantly down-regulated by progesterone. As described, progesterone generates transcriptional changes in genes associated with immune-mediated diseases such as RA that present a modulation of their activity during pregnancy. Furthermore, the researchers described these changes at the protein level, including RA-related cytokines, such as IL12B (234).

Interleukin 32 (IL32)

We have detected an up-regulation of this gene in pregnancy samples from resting naïve T cells (*r1 cluster 0.0*), middle activation stage (between 0.0 and 0.5) (*r1 cluster 0.1*) and pre-Th1 recently activated T_{cm} (*r1 cluster 0.5*).

IL32 encodes a member of the cytokine family. The expression of the protein causes immune functions on monocyte, macrophages, dendritic cells and T cells and can induce the TNFα production (194). Also it promotes the dendritic cells maturation and activation increasing the IL12 and IL6 production (235). Interestingly, T_{regs} are the main cell producing this cytokine presenting an important role in the immune responses (197).

This cytokine has been shown to play a crucial role in RA animal models with pro-inflammatory effects and as potential therapeutic target (235). The study of Chao Lin *et al* focused on the identification of differential gene expression comparing RA and osteoarthritis. In this work, researchers compared the gene expression profiles in synovial tissue between RA and osteoarthritis from the National Centre of Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database and performed a Gene Ontology analysis for study the DEGs. The authors detected that the DEGs between RA and osteoarthritis that could play a role in the pathogenesis or therapy of RA are the following *CXCL13, CD247, CCL5, GZMB,*

IGKC, IL7R, ADAMDEC1, BTC, AIM2, SHANK2, CCL18, LAMP3, CR1 and *IL32*. As we can see, *IL32* is one of them.

The Mazlum *et al* study included 199 Iranian pregnant women suffering from preeclampsia and 228 healthy women. Polymerase chain reaction–restriction fragment length polymorphism for two selected single-nucleotide polymorphisms (SNP) in the *IL32* gene (*rs9927163, rs4786370*) was done. Results from this study showed that for the *rs4786370* C/T SNP, there was a significant difference in genotype and allele frequencies between patients with preeclampsia and the healthy group. They concluded that variety of allele as well as an increase in serum IL32 could be a risk biomarker for preeclampsia (236). Also preeclampsia could be augmented in autoimmune diseases pregnancies like RA (237).

Vimentin (VIM)

In our results *VIM* is upregulated in conventional DCs (cDCs, $CD123^{dim}$ $CD11c^{bright}$) (*r1 cluster 2.0*), in pregnancy samples. Its described in the literature that citrullinated VIM could facilitate the phenotypic maturation of dendritic cells and increase inflammatory cytokine production (238).

This gene encodes a type III intermediate filament protein that is involved in the cytoskeleton formation. In particular, *VIM* is responsible for maintaining cell shape and integrity of the cytoplasm (185).

Cintrullinated *VIM* its described to play a role in the stimulation of cell proliferation and pro-inflammatory cytokine secretion in the cultured fibroblast-like synoviocytes of RA patients (239). Recently, anti-mutated citrullinated vimentin antibodies (anti-MCV) have been studied as one of the possible markers for RA and, Liming Tan *et al* provided evidence of its utility as a diagnostic biomarker in RA (240).

In a study aiming to discover endometrial stromal cell proteome differences between repeated implantation failure, recurrent pregnancy loss and normal fertile women showed that, in the normal fertile group, *VIM* was down-regulated during pregnancy (241).

S100 Calcium Binding Protein A6 (S100A6)

We found an up regulation of this gene in conventional DCs (cDCs, $CD123^{dim}$ $CD11c^{bright}$) (*r1 cluster 2.0*) in postpartum samples. S100 proteins are described as a possible tumour activator in dendritic cells (242,243).

The protein encoded by this gene belongs to the S100 family of proteins. These proteins are involved in the regulation of cell cycle progression and cell differentiation (189).

Svendsen *et al* (244) performed an explorative epigenome-wide association study in monozygotic 28 twins discordant for RA. Genomic DNA was isolated from whole blood and DNA methylation was measured using the Human Methylation 450 Bead Chips. Investigators found that the S100A6 promoter region was hypomethylated in RA patients. The authors hypothesized that active gene could be a predictor for cartilage and bone destruction (244).

Jurewicz *et al* (245) studied the umbilical cords from 10 new-borns delivered by healthy and 10 newborns delivered by preeclamptic mothers were included in order to study the role of *S100A6* in preeclampsia Wharton jelly. Investigators used immunoblotting and immunohistochemistry for characterize *S100A6* in Wharton's jelly. The authors concluded that *S100A6* levels were higher in patients with preeclampsia than in healthy controls suggesting an involvement in this pathology.

Heterogeneous Nuclear Ribonucleoprotein A1 (HNRNPA1)

We found *HNRNPA1* upregulated in resting naïve T cells (*r1 cluster 0.0*) in our pregnancy samples.

This gene encodes a member of a family of ubiquitously expressed heterogeneous nuclear ribonucleoproteins. The protein encoded presents an important role in the regulation of alternative splicing (193).

The Vordenbäumen *et al* study focused on novel diagnostic autoantibodies for RA. For this, the authors included four different groups of patients, including 72 patients with established RA; 116 patients with early RA, 51 CCP-negative patients with early RA, 184 patients with early seronegative RA and 184 matched

healthy controls). They applied a multiplex bead-based for autoantibody detection method. Increased reactivity to HNRNPA1 antigens was found in all cohorts. The authors conclude that this gene could be novel candidate marker for RA (246).

Its described that polarisation of SF2/ASF and hnRNPA1 proteins presents in the myometrium during pregnancy modulates the expression of alternate spliced myometrial proteins (247). In addition, hnRNP A1/A1^{B} its described to be involved in the myometrium contraction during parturition (248).

Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT 1)

We detected an upregulation of *MALAT1* in pregnancy samples in conventional DCs (cDCs, CD123^{dim} CD11c^{bright}) (*r1 cluster 2.0*). *MALAT1* is described as tolerance regulator in immunity in dendritic cells (249).

MALAT1 is a long non-coding RNA related to metastasis in lung cancer. This gene, when located in the nucleus, interacts with proteins rich in serine and arginine, acting in the regulation of splicing. Also, due to the interaction with the 3 'end sequences of various genes, it is involved in the regulation of gene expression (182).

The Chatterjee *et al* study focused on the expression pattern of several long non-coding RNAs, including *MALAT1*, in RA patients. They included plasma samples from 82 RA patients matched with 15 controls; and 24 RA plasma knee fluid of 24 RA matched with 10 osteoarthritis controls. Total RNA was isolated from plasma PBMC and synovial fluid with standard TRIzol method. The authors described an increase of *MALAT1* in plasma PBMCs from RA patients and hypothesize that could be involved in increased angiogenesis and cell proliferation and decreased synovial cells apoptosis (250).

In addition, *MALAT1* is highly expressed in placenta accrete tissues (251). In this line the study of Qin Li *et al* included 40 placentas from preeclamptic patients matched with 40 placentas from healthy controls. qRT-PCR was used to determine RNA levels and western blot and immunohistochemistry analysis were used for protein expression. Results revealed lowed expression of this gene in

preeclampsia placentas and inhibition of trophoblast functionality. This impairs the remodelling of spiral artery in the uterus (251).

Transmembrane Protein 147 (TMEM147)

We detected this gene upregulated in postpartum in conventional DCs (cDCs, $CD123^{dim}$ $CD11c^{bright}$) (*r1 cluster 2.0*). The role of this gene in dendritic cells it is not well described.

TMEM147 is a 7 transmembrane protein whose functions could be related to the NF-KB pathways but it remains unclear (188).

Ota *et al*, using samples from RA patients and animal, models focused on this gene function in chondrocytes. They analysed 5 RA patients and 6 osteoarthritis patients from knee arthroplasty and, as a control group, 6 polydactyl patients from reconstructive surgery. A quantitative polymerase chain reaction was applied. The authors found that this gene was up-regulated in samples from RA patients and anti-TMEM147 antibody treatment reduced the cytokine-mediated activation of NF-Kb *in vitro* suggesting a drug target (188).

The role of this gene in pregnancy or postpartum is not clearly defined. Despite this, its involvement in the early embryonic nodal signalling in vertebrates is documented in the literature (252).

TIMP Metallopeptidase Inhibitor 1 (TIMP1)

We found an up-regulation of *TIMP1* in pregnancy in conventional DCs (cDCs, $CD123^{dim}$ $CD11c^{bright}$) (*r1 cluster 2.0*) and $CD14^{+}$ monocytes (*r1 cluster 2.2*). The specific role of this gene in these specific cell types is not well known.

TIMP1 belongs to the matrix remodelling factors. It is involved in toll-like receptor signalling, mechanotransduction and the extracellular matrix biology (190).

Matrix metalloproteinases are endopeptidases involved in remodelling and homeostasis of extracellular matrix. Its activity is regulated by specific tissue inhibitors of matrix metalloproteinases (TIMPs). For example,*TIMP1*, a tissue inhibitor of matrix metalloproteinase could be involved in cartilage damage in RA via SIRT1 (253). This was described by Guo *et al* where they included samples of

synovial tissue from patients with RA and controls with knee joint trauma. RNA isolation and real-time PCR were applied (253).

Regarding pregnancy, it seems that the equilibrium between metalloproteinases and TIMPs could play a role in remodelling uterine arteries. This balance seems to be responsible for maintaining vasodilatation at the end of gestation and its dysregulation could be involved in pregnancy hypertensive disorders (254). In Montagnana *et al* study, they included 14 preeclamptic women, 37 normotensive pregnant women (10 in first trimester, 10 in second trimester and 17 in third trimester) and 21 non-pregnant women. A multiplexed sandwich enzyme-linked immunosorbent assay was used. Results showed serum levels of *TIMP1* were significantly higher in preeclamptic women in comparison to non-pregnant and normotensive pregnant women (254).

Lactate Dehydrogenase B (LDHB)

We detected an up-regulation of this gene in naïve middle activation stage (between 0.0. and 0.5.) cells (*r1 cluster 0.1*) in pregnancy samples. The role of this gene in these cells is not clearly described.

The LDHB encodes the B subunit of lactate dehydrogenase enzyme. This enzyme acts in the NADH post-glycolysis process (255) facilitating the energy production under the hypoxic cellular state (256).

Its role in RA is not completely elucidated. The Wright *et al* (2021) (256) study performed their analysis including blood and synovial fluid samples from patients with active RA applying RNA-seq technology. They concluded that this gene was increased in neutrophils from RA synovial fluid (256).

The role of this gene during pregnancy has not yet been clearly described. Kay *et al*, studied this gene in the trophoblast and the authors described how this gene appears to be down-regulated during hypoxia (257).

Chloride Intracellular Channel 1 (CLIC 1)

We detected an upregulation of *CLIC1* in conventional DCs (cDCs, CD123^{dim} CD11c ^{bright}) (*r1 cluster 2.0*) in pregnancy samples. *CLIC* function on dendritic cells could indicate that this gene regulates DC phagosomal pH of the antigen before presentation to antigen-specific T cells (258).

CLIC1 belongs to the *CLICs* family. These proteins participate in different pathological processes like inflammation and different functions involving regulation of cellular metabolism. CLIC1 is overexpressed in macrophages and is also present in connective tissue fibroblasts and myofibroblasts, cells found in different inflammatory lesions (187).

In the Bordean *et al* study, 22 skin biopsies from RA patients and 8 skin biopsies from Psoriatic Arthritis (PsA) patients with clinical signs of vasculitis were analysed for immunohistochemistry. The authors described *CLIC1* expression in rheumatoid nodules, and stromal and vascular compartments in skin biopsies from patients with RA and PsA (187).

Also, it's interesting to note the presence of this gene in high levels in the human foetal lung, kidney, and liver (187). Focused on pregnancy area, the role of this gen has been related to hydatidiform moles (259). This pathology is a gestational trophoblastic disease due to an abnormal fertilization characterized by trophoblastic proliferation (260). It is considered a benign pathology but potentially can become malignant and invasive (261). For example in the Zhong-Hua Shi *et al* study, they applied a proteomic analysis in 3 samples of complete benign moles and 3 malignant-transformed moles and found that *CLIC1* expression was upregulated in malignant-transformed hydatiform moles in comparation to compete benign moles (259).

Mitochondrially Encoded Cytochrome B (MT-CYB)

We found an up regulation of this gene in conventional DCs (cDCs, CD123^{dim} CD11c ^{bright}) (*r1 cluster 2.0*) during pregnancy. Is not well described the possible role of this gene in dendritic cells.

MT-CYB is a member of the oxidative phosphorylation system and play a crucial role on respirasome super complex (184).

We have not found literature regarding the role of this gene in RA or pregnancy.

Ribosomal Protein Lateral Stalk Subunit P2 (RPLP2)

We detected an upregulation of this gene in $CD14^{+}$ monocytes (*r1 cluster 2.2*) in postpartum samples. The exactly role of this gene in monocytes it is not fully understood.

This gene codifies a ribosomal protein. It is believed that these proteins stabilize specific rRNA structures and promote correct folding in order to maintain the cell metabolic functions. It could also act as a transcription factors and play a role in the oncogenesis. The PRLP2 protein belongs to a group of acidic ribosomal proteins named P proteins. P proteins are highly expressed in normal lymph nodes, skin, spleen, and foetal brain tissue, expressed at lower levels in normal lung, bladder, and placenta. In addition it's been described that this proteins act in immune-mediated diseases like lupus where autoantibodies against the P proteins are detected (192).

The role on RA or pregnancy is not clearly described.

Myosin Light Chain 12A (MYL12A)

We found an up regulation of this gene in conventional DCs (cDCs, $CD123^{dim}$ $CD11c^{bright}$) *(r1 cluster 2.0*), $CD14^{+}$ monocytes (*r1 cluster 2.2*), resting naïve T cells (*r1 cluster 0.0*) and middle activation stage (between 0.0. and 0.5.) cells (*r1 cluster 0.1*) in pregnancy samples. The role of this gene in these cells clusters is not fully described.

This gene encodes a non-sarcomeric myosin regulatory light chain. This protein regulates smooth muscle and non-muscle cell contraction. This protein seems be involved in DNA damage repair by sequestering the transcriptional regulator apoptosis-antagonizing transcription factor (183).

The Yokoyama *et al* study focused on Myl9 and Myl12a/ Myl12b (Myl9/12) in the pathogenesis of inflammation in inflammatory bowel disease. The authors included

10 samples of the colon from Ulcerative Colitis (UC) patients and blood samples from 47 UC patients (20 patients with active disease and 27 patients in remission) and 11 healthy controls. In addition they perform a colitis model in mice and applied ELISA. Results showed highly Myl9/12 expression in inflamed gut of ulcerative patients and mice. The administration of anti-Myl9/12 antibody to mice with induced colitis ameliorated the inflammation (262).

The role of this gene in the human pregnancy or RA it's not clearly described.

Lymphotoxin Beta (LTB)

We detected an up regulation in resting naïve T cells (*r1 cluster 0.0*) from pregnancy samples. Currently, the function of this gene in this cell type is not clearly known.

LTB encodes a type II membrane protein of the TNF family (195). This protein is involved in lymphoid follicle development, pro-inflammatory cytokines production, and fibroblasts and synoviocytes proliferation (263).

The study of O'Rourke *et al* included 21 patients diagnosed with RA and 20 healthy controls and obtained peripheral blood and synovial samples. The authors applied a multiplex RT-PCR for the analysis. Results showed, in synovial samples from patients with RA, a marked inflammatory activity, this gene was significantly elevated compared to samples from patients who did not present marked inflammatory activity. Thus, this gene could be implicated on RA pathogenesis (263).

In relation to its role in pregnancy, it has not been clearly studied and at the moment there is no solid idea of its role. Despite this, the Windsperger *et al* study shows how decidua parietalis tissues contain higher levels of the high endothelial venules-maintaining LTB (264).

Bone Marrow Stromal Cell Antigen 2 (BST2)

We found an upregulation during pregnancy in middle activation stage (between 0.0. and 0.5.) cell (*r1 cluster 0.1*). This gene present a low cell type specificity (191) and its function in this cells cluster is not well known.

BST2 is involved in the growth and development of B-cells (196).

The role of this gene has not been thoroughly studied in RA. Despite this, there is a paper published in the nineties that describes 7% of patients with RA presented elevated levels of BTS2 compared to healthy controls. In addition, patients with elevated levels had the highest inflammatory load (265).

Its function in pregnancy is not well known.

Proteasome Activator Subunit 1 and 2 (PSME1/2)

We found an upregulation in effector cytotoxic T cells (*r1 cluster 0.2*) in pregnancy samples. These genes presents a low cell specificity (266,267).

PSME1/2 interplays with 19S particle generating large complexes and acts in ATP-dependent degradation of proteins. It's postulated that acts in MHC class I antigen presentation but the exact mechanism isn't known (201). The protein encoded by this gene is involved in the proteasome. More in detail, proteasome is a protease complex focused on protein degradation. Proteasomes are induced in parenchymal cells in intracellular pathogen infection context and are necessary for effective CD8 T-cell-mediated immune responses against infected cells (268).

Currently its role in RA or pregnancy is not known.

Guanylate Binding Protein 2 (GBP2)

We detected an increase in this gene in effector cytotoxic T cells (*r1 cluster 0.2*) during pregnancy. The role of this gene in this cell cluster is not fully described.

The encoded protein is a GTPase that hydrolyses GTP. The gene belongs to interferon-induced proteins that can bind to guanine nucleotides (199).

In a recent article published by Gaurav *et al*, they focus on the analysis of immune cells from a murine model of lung disease in the context of RA using scRNA-seq. The authors describe interferon-related and autoimmune genes were disproportionately expressed among collagen-induced arthritis model and collagen-induced arthritis with organic dust extract induced airway inflammation. These genes were detailed below: *OASL1, OAS2, LFIT3, GBP2, LFI44 and ZBP1*. Specifically, GBP2 was detected in monocytes (269).

Regarding their role in pregnancy, we have not found any relevant evidence in the literature.

Thymosin Beta 10 (TMSB10)

We found this gene up-regulated this gene in $CD14^+$ monocytes (*r1 cluster 2.2*) in postpartum samples. This gene presents a low cell type specificity (270) and the exactly role on monocytes is not well known.

TMBS 10 is a Protein Coding gene. This gene function in not exactly known but in Gene Ontology annotations it is related to actin binding and actin monomer binding (191). *TMSB10* has been demonstrated to be overexpressed in most types of cancer, as an oncogene, and participate in regulating cell proliferation and motility (271).

Its role in RA or pregnancy is not well defined.

Phospholipase A And Acyltransferase 4 (PLAAT4)

We detected an increase in this gene in effector cytotoxic T cells (*r1 cluster 0.2*). The role of this gene in this cell cluster is not fully described.

PLAAT4 is a member of the HREV107 tumour suppressor gene family. The expression of PLAAT4 has been shown to induce cell death (202).

Currently, the role of this gene in pregnancy or RA has not been clearly studied.

Interferon Stimulated Exonuclease Gene 20 (ISG20)

We found an increase in this gene in effector cytotoxic T cells (*r1 cluster 0.2*) of the pregnancy samples. This gene presents a low cell type specificity (272).

ISG20 belongs to the interferon pathways genes. This gene seems to be involved in response to RNA viral infections (200).

Yoshida *et al* investigation focus on evaluate gene expression in synovial lining cells of RA patient. They included 11 RA and five osteoarthritis patients and performed a microarray analysis and immunohistochemistry. Results showed an up regulation of this gene in RA synovial tissues.

The role of this gene in human pregnancy has been little studied and, at most, its role in ZIKA infection during pregnancy has been investigated (273).

6.4.3. Differential gene expression in stimulated cells

In the *in vitro* stimulated cells we have detected more than 5,000 differently expressed genes. When reviewing the most significant ones, we observe that they are present in practically all cell clusters and show metabolic or proliferative functions. Altogether this indicates a cell response to our activation. Despite this, we want to highlight two differentially expressed genes due to their important immunological involvement in the case of IL21R (206) and that have already been previously described in a postpartum RA reactivation study, such as SOCS3 (207).

Interleukin 21 Receptor (IL21R)

We found an up regulation of *IL21R* in stimulated pregnancy samples in recently activated T_n and T_{cm} cells (*r1 cluster 1.0*); activated T_{cm} and T_{em} cells (*r1 cluster 1.1*), proliferative T cells (*r1 cluster 1.2*) and effector cytotoxic T cells (*r1 cluster 1.3*). In postpartum treated cells we detect an upregulation in treated NKs (*r1 cluster 0.4*). This gene presents a low cell type specificity and is found in many cell types (274).

The protein encoded by this gene is a cytokine receptor for IL21 that belongs to the type I cytokine receptors. IL21 cytokine acts in the proliferation and differentiation of T cells, B cells, and NK cells. It is involved in activation of downstream signalling molecules like JAK1, JAK3, STAT1, and STAT3 (206). The literature referenced here is related to IL21 itself. Regarding IL21 receptor, we have not found a clear bibliography regarding its role in pregnancy or RA.

IL-21 and IL-23 activate STAT3, which is critical for Th17 cell differentiation, a cell well known for its involvement in RA pathophysiology (275,276). In line with this, the study of A Paradowska-Gorycka *et al* focused on determining the correlation between transcription factors expression and Th17/T_{reg} ratio and cytokine expression in RA. The authors included 45 RA patients, 27 osteoarthritis patients and 46 healthy controls. The study methodology chosen was flow cytometry for Th17 and T_{reg} frequency, qPCR for gene expression and ELISA for cytokine levels.

They conclude that T_{reg}/Th17 ratio in RA patients was lower than in HC blood, showing an alteration on Th response. Focusing in the role of IL21, the authors described an increased serum levels range of IL17 and IL21 RA patients in comparation with osteoarthritis patients (275).

In pregnancy, the C Monteiro *et at* study focused on the immunological study of follicular helper T (TFH) in pregnancy. Blood samples from 35 pregnant women, in the third trimester, and 35 non-pregnant women were included. A Flow cytometry analysis and ELISA were applied. Results showed $IL21^+$ TFH cells were increased in pregnant women and levels of IgG anti-HBsAg were directly associated with the proportion of $IL21^+$ TFH cells. The authors proposed that circulating TFH cells are elevated in pregnancy and thus can favour humoral immune response since this T cell subset aids antibody production. Interestingly, TFH cells are able to produce IL21(277) and are described to be involved in the pathogenesis of IMIDs such as SLE (277).

Suppressor of Cytokine Signalling 3 (SOCS3)

We detected an upregulation of this gene in postpartum samples in stimulated cells in recently activated T_n and T_{cm} cells (*r1 cluster 1.0*), activated T_{cm} and T_{em} cells (*r1 cluster 1.1*), proliferative T cells (*r1 cluster 1.2*), effector cytotoxic T cells (*r1 cluster 1.3*). In pregnancy samples is overexpressed treated NKs (*r1 cluster 0.4*). The RNA of this gene seem to be enhanced on neutrophils (278) and a clearly description of this functions in this cells clusters is not well described.

This gene encodes a STAT-induced STAT inhibitor (SSI) member that acts as a suppressor of cytokine signalling. SSI family members are negative regulators of cytokine signalling induced by cytokines. For example, IL6, IL10 and IFNγ induce its expression.

This gene has previously been studied in RA during pregnancy and postpartum by Weix *et al*. In their study, they detected SOCS3 expression in a mixed pattern among RA patients with a tendency toward a higher postpartum increase. The authors explained these results because this protein has presents a short half-life and is rapidly up-regulated in response to cytokines (122).

In pregnancy, its role has been studied by Zhou *et al* (90). In this investigation, the authors focused on describing changes of IL6, JAK/STAT3 and SOSC3 in human placenta during preterm and term labour. Fifteen preterm labours women placentas and 15 term labour placentas were included. ELISA and Western blood were applied. The authors described in the preterm labour group an overexpression of placental *SOCS3* and its expression was significantly up-regulated after IL6 stimulation in trophoblasts cells (279). The exactly role of this gene is not fully understood in pregnancy but it's involved as a negative feedback molecule in the IL-mediated start of labour (preterm and term) (279).

6.4.4. Related genes

On various occasions, when investigating the different genes that we have detected as over-expressed, we observe that their association, either with RA or pregnancy, is not confirmed and require subsequent validation studies. Despite this, the results help to expand the current knowledge of the modulation of rheumatological diseases, and specifically of RA, in pregnancy.

If we take a more global view of these genes, we observe that there are certain patterns that emerge which are detailed below.

Rheumatoid arthritis and pregnancy

Candidate genes regulated during human pregnancy which have the potential to modulate RA disease activity have been studied (122). Interestingly, within the genes detected by researchers, we also detected two of them *STAT1* and *SOCS3*. Specifically, we have detected an increase in *STA1* in the pregnant PBMCs. In relation to *SOCS3*, we have detected an increase in gene expression in pregnancy and postpartum cell clusters. These data support previously published results.

As we have detailed in the previous section, we have detected several genes that potentially have a role in RA and pregnancy-postpartum or pregnancy-related disorders. The genes are *VIM, HNRNPA1, TMEM147, LDHB, CLIC1, LTB* and *IL21R*. We want to emphasize that in many cases this association is initial and requires additional study. Despite this, we have not found evidence in the literature of pregnancy and postpartum RA studies and further study would be interesting.

Immune tolerance breakdown in pregnancy in RA

The principal objective of conducting this study was to know which biological mechanisms are activated in postpartum as they could potentially help to identify new targets of the pathogenic pathways of RA.

We showed that, cells from pregnancy samples were less over-activated with *in vitro* treatment. This suggests the presence of a type of immune control over the hyperactivation. *SOCS3,* a known negative regulator of cytokine signalling could be a candidate gene involved in this, we detected gene expression differences in pregnancy vs. postpartum from this gene in treated NKs (*r1 cluster 0.4*). Therefore, a possible hypothesis would be, to exert this control over hyperactivation, an increase in negative regulators would be needed. We also found an up-regulation of this gene in postpartum samples in recently activated T_n and T_{cm} cells (*r1 cluster 1.0*), activated T_{cm} and T_{em} cells (*r1 cluster 1.1*), proliferative T cells (*r1 cluster 1.2*) and effector cytotoxic T cells (*r1 cluster 1.3*). So, it's not clear whether this gene is acting as a negative regulator in pregnancy. A possible explanation for not having found more genes with negative regulatory function could be that the inhibitory signals come from the placenta, and we do not detect them in peripheral blood.

Pregnancy and tumorigenesis genes

We have detected five differentially expressed genes in our samples (either pregnancy compared to postpartum or *vice versa*) related to the regulation of tumorigenesis. These genes are *S100A6, MALAT1, RPLP2, TMBS10* and *PLAAT4.*

This is a very interesting result given the parallelism between these two situations (120). Both pregnancy and cancers have shared processes focused on evading host immune responses. Both scenarios present similar mechanisms in proliferation, invasion and immune privilege. This is very striking since they are opposite clinical situations. In gestation, the objective is to create in a controlled process a new life and, in tumorigenesis, the lack of control, would lead to massive cell growth (120,280).

Both tumours and trophoblast cells have a high proliferative capacity. For example, in both situations, high telomerase activity is present, not seen in normal somatic cells (80,280). In relation to invasion, both situations require the establishment of blood and nutrient supply. For example, a shared mechanism is epithelial-mesenchymal transition, which results in loss of cell-to-cell contact inhibition. In parallel, changes in integrin expression and loss of E cadherin appear, leading to enhanced cellular motility. To survive, both situations require nourishment through vascular supply. They achieve this by performing vasculogenic mimicry in cells other than endothelial cells form vascular structures (280). Once the vessel has been created, it must proliferate, giving rise to neoangiogenesis. For example, angiopoietins and VEGF family members are extremely important in spiral artery remodelling during placentation and the growth of many tumour types (280). Throughout this process, immunomodulations have been generated in the fetoplacental barrier that makes it unique. HLA G is involved in the immunomodulatory effects on trophoblast and cancer cells. HLAG interacts with NK cells via inhibitory receptors (CD94 / NKG2A, ILT2, KIR2DL4). This inhibitory interaction acts in NK and cytotoxic T cell, regulates cytokine production in blood mononuclear cells and impairs maturation of DCs. So, the presence of this molecule prevents the cytotoxic cells from being activated, allowing the progression of new cells (120,280). Finally, once the pregnancy or the tumour has been tolerated, it must be maintained over time and therefore there must necessarily be an effect at the systemic level. In the case of pregnancy, we detect foetal DNA in maternal blood. For cancer, cell-derived microparticles of tumours can be detected. These particles have the capacity of down-regulate T cell activity (120,280).

Understanding more deeply the similarities that exist in both situations, the detection of genes involved in the regulation of tumorigenesis in our study seems relevant and indirectly could support this parallelism. However, we must bear in mind that our study was carried out in patients with a disease (RA) and in peripheral blood, not in cells of the maternal-foetal barrier. We do not know how these factors influence the results but further investigations in this group of genes could increase the knowledge of this parallelism.

Rheumatoid Arthritis and preeclampsia genes

We have found several over-expressed genes with a possible role in RA and preeclampsia which have previously been described. These genes are *IL32*, *S100A6*, *MALAT1* and *TIMP1*.

Preeclampsia is a pregnancy-specific syndrome is one of the main causes of maternal and foetal mortality and is a major risk factor for preterm birth. Preeclampsia is characterized by hypertension, proteinuria and an exaggerated maternal systemic inflammatory response. Both innate and adaptative immunity play an important role in this pathology. Interestingly, a marked increase of circulating monocytes, neutrophils and NK cells are seen in preeclampsia (212).

In relation to the possible association of RA and preeclampsia, the initial studies have shown contradictory data regarding the risk of preeclampsia in RA pregnant women. For example, studies from Bernabe *et al* and Reed *et al* reported no risk and studies from Chakravarty *et al*, Lin *et al*, Norgaard *et al* and Wallenius *et al* reported increased risk (237). The Aljary *et al* study, focused on the analysis of adverse maternal and neonatal outcomes in RA patients, performing a Population cohort study using the Healthcare Cost and Utilization Project National Inpatient Sample (HCUP-NIS). The authors included 8,417,607 women, of whom 6,068 had RA. Results showed an increased risk of preeclampsia in RA pregnant patients. The authors hypothesized that indicate there could be a common autoimmune factor in both pathologies (237).

In our study, we have detected four genes over-expressed that previously have been related to preeclampsia. A hypothesis would be that our patients had an increased risk of preeclampsia, they also present an over-expression of genes related to this pathology. Although it is true that the two patients included did not present these hypertensive complications of pregnancy, having an over-expression of a gene may not be enough to develop the pathology, requiring a specific microenvironment.

Rheumatoid Arthritis related genes

The following DEGs detailed here *BST2, GBP2* and *ISG20*, seemed to only been studied within the pathogenesis of RA. Currently we do not know what role they can play in pregnancy or postpartum.

Other related genes

We have also detected 3 genes, *MTCYB, MYL12A* and *PSME1/2*, of which, at present, their involvement in RA, pregnancy or postpartum is not clear.

All these results are of great value, and lay the foundations for further studies about the potential implicate of these genes in modulating the activity of RA during pregnancy and postpartum.

7. Conclusions

1. We have described the first single cell RNA seq atlas of blood cells in pregnant (gestational week 30) and postpartum (week 8) women with Rheumatoid Arthritis under physiological conditions and in response to *in vitro* T cell stimulation. To the best of our knowledge, this approach has not been performed before.

2. In the annotated atlas that we have generated, we identify NK and unstimulated T cells, stimulated T cells, myeloid cells and B cells, as the main source of transcriptomic differences between the studied cells. This highlights the important transcriptional effect *in vitro* T cell stimulation has, which may guide the design of future studies.

3. In unstimulated T cells, we confirm previous findings from studies in healthy non-pregnant women, which show the presence of a gradient in the effectorness of T cells. In stimulated T cells, we detect a gradient in T cell activation. This agrees with the hypothesis of possible cellular plasticity depending on the microenvironment where the different cells are found.

4. We have detected, in the postpartum, an increase in unstimulated resting naïve T cells and $GZMA^{+}$ $GNLY^{+}$ producing $CD8^{+}$ T cells and in stimulated activated T central memory cells with high ribosomal content and recently activated naïve T cells. To the best of our knowledge, these differences have not been reported before.

5. We have observed in $CD14^{+}$ monocytes and, for one patient, in $CD16^{+}$ $CD56^{dim}$ NKs a resistance to exogenous stimulation in pregnancy samples, which is not observed in cells from the postpartum period. We have also observed this resistance when directly comparing the transcriptomic effects of T cell stimulation in pregnancy and postpartum samples. This finding introduces the hypothesis of the presence of some type of immunological repression in these cells during pregnancy.

6. The study of the differentially expression identified genes with a possible implication in Rheumatoid Arthritis in pregnancy or postpartum.

a) Two of these genes *STAT1* and *SOCS3*, have already been described as candidates for modulating the activity of Rheumatoid Arthritis in pregnancy. We have also found a new set of genes that could also be associated to both conditions, such as *VIM, HNRNPA1, TMEM147, LDHB, CLIC1, LTB* and *IL21R*.

b) We have detected a shared set of genes that potentially have an involvement in both pregnancy and oncogenesis, such as *S100A6*, *MALAT1, RPLP2, TMBS10* and *PLAAT4*.

c) Finally, we have described a group of genes that can potentially be involved in both, Rheumatoid Arthritis and preeclampsia, such as *IL32, S100A6, MALAT1* and *TIMP1*.

8. Future steps

The main limitation of our study was the impossibility to generate data from samples donated by healthy pregnant women. Despite this, thanks to the power of the technology used in the present work, scRNA-seq, we decided to analysed all available data using advanced analytical tools. We focus our efforts to determine the differential biological mechanism between pregnancy and postpartum in RA patients as well as to describe the first single cell RNA seq atlas of blood cells in pregnant and postpartum women with Rheumatoid Arthritis. To the best of our knowledge, this approach has not been performed before not only in pregnant RA patients but also in RA patients.

This doctoral thesis project is the first study utilizing a promising area of research in our unit led by Dr Pluma in collaboration with basic and data analysis scientists. All the results obtained in this project will help us to direct future research projects to better understand the relationship between pregnancy and disease, at the clinical and molecular level. It is our aim to generate a large body of clinical and molecular data, in several rheumatic diseases and healthy controls during pregnancy. Dr Pluma continues the recruitment of both healthy controls and patients with RA and will shortly initiate new cohorts of patients with other inflammatory or autoimmune rheumatic conditions. In collaboration with the IMID-Biobank, a growing set of high-quality biological samples will be the basis of future studies in this field.

An important point to take into consideration in our study is the technology we have used in the present project. Single cell analysis is a recently developed technology that, thanks to the analysis at cellular level, it is undoubtedly revolutionizing the world of immunology. It is a powerful technique in which we still do not understand the full scope that it can offer. Thanks to it, new cell types, new cell states, new cell processes are being described and, globally, we could even say that immunology will be one of the areas with the greatest changes in its fundamental knowledge in the coming years. As far as we know, we have applied this technology in an area where it had not been previously explored. scRNA-seq will help us to better understand the cell implications and biological pathways involved in the reactivation of RA in the postpartum period. Given the pioneering nature of our study, there are practically no data on which we can perform direct

comparations. It is one of our future objectives to validate the results presented here in an independent data set and using targeted and less expensive technologies.

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10. Annexes

Annex Table 1. Cell lineage description

	RA1 preg treated	RA1preg untreated	RA1 post treated	RA1 post untreated	RA2 preg treated	RA2 preg untreated	RA2 post treated	RA2 post untreated
Untreated T cells NK, n (%)	40 (6.31)	647 (64.44)	49 (5.31)	581 (78.83)	244 (22.72)	566 (53.55)	302 (20.91)	1374 (79.33)
Treated T Cells, n (%)	545 (85.96)	31 (3.09)	851 (92.3)	42 (5.7)	810 (75.42)	19 (1.8)	1108 (76.73)	31 (1.79)
Myeloid cells, n (%)	0 (0)	233 (23.21)	2 (0.22)	50 (6.78)	0 (0)	299 (28.29)	5 (0.35)	159 (9.18)
B cells, n (%)	49 (7.73)	93 (9.26)	20 (2.17)	64 (8.68)	20 (1.86)	173 (16.37)	29 (2.01)	168 (9.7)

preg: pregnancy, post: postpartum, n:number of cells, (%): percentage

Annex Table 2. Cell sublineage description (resolution 1)

	RA1 preg treated	RA1preg untreated	RA1 post treated	RA1 post untreated	RA2 preg treated	RA2 preg untreated	RA2 post treated	RA2 post untreated
0-NKs TCD8, n (%)	12 (1.89)	59 (5.88)	9 (0.98)	66 (8.96)	101 (9.4)	60 (5.68)	87 (6.02)	178 (10.28)
0-treated activated NKs, n (%)	9 (1.42)	34 (3.39)	25 (2.71)	38 (5.16)	52 (4.84)	20 (1.89)	140 (9.7)	52 (3)
0-cytotoxic Tcells, n (%)	8 (1.26)	182 (18.13)	8 (0.87)	131 (17.77)	33 (3.07)	158 (14.95)	16 (1.11)	178 (10.28)
0-resting $T_{n,}$ n (%)	0 (0)	218 (21.71)	2 (0.22)	228 (30.94)	31 (2.89)	108 (10.22)	36 (2.49)	346 (19.98)
0-midActivation T_n $T_{cm,}$ n (%)	6 (0.95)	127 (12.65)	3 (0.33)	97 (13.16)	19 (1.77)	177 (16.75)	13 (0.9)	375 (21.65)
0-recently activated $T_{cm,}$ n (%)	5 (0.79)	24 (2.39)	2 (0.22)	20 (2.71)	8 (0.74)	41 (3.88)	10 (0.69)	218 (12.59)
0-Tcell Granulocyte doublet, n (%)	0 (0)	3 (0.3)	0 (0)	1 (0.14)	0 (0)	2 (0.19)	0 (0)	27 (1.56)
1-recently activated T_n $T_{cm,}$ n (%)	158 (24.92)	23 (2.29)	292 (31.67)	31 (4.21)	255 (23.74)	14 (1.32)	412 (28.53)	19 (1.1)
1-activated T_{cm} $T_{em,}$ n (%)	163 (25.71)	2 (0.2)	283 (30.69)	2 (0.27)	258 (24.02)	2 (0.19)	388 (26.87)	3 (0.17)
1-proliferative Tcells, n (%)	88 (13.88)	0 (0)	135 (14.64)	0 (0)	85 (7.91)	0 (0)	135 (9.35)	3 (0.17)
1-cytotoxic Tcells, n (%)	75 (11.83)	3 (0.3)	89 (9.65)	4 (0.54)	153 (14.25)	0 (0)	98 (6.79)	3 (0.17)
1-Tcell Bcell doublet, n (%)	53 (8.36)	0 (0)	45 (4.88)	0 (0)	54 (5.03)	1 (0.09)	62 (4.29)	0 (0)
1-Basophils, n (%)	7 (1.1)	3 (0.3)	6 (0.65)	4 (0.54)	2 (0.19)	0 (0)	2 (0.14)	0 (0)
1-NKs, n (%)	1 (0.16)	0 (0)	1 (0.11)	1 (0.14)	3 (0.28)	2 (0.19)	11 (0.76)	3 (0.17)
2-cDCs, n (%)	0 (0)	129 (12.85)	0 (0)	28 (3.8)	0 (0)	74 (7)	3 (0.21)	44 (2.54)
2-CD14-monocytes, n (%)	0 (0)	69 (6.87)	0 (0)	12 (1.63)	0 (0)	66 (6.24)	1 (0.07)	35 (2.02)
2-myeloid Tcell doublet, n (%)	0 (0)	34 (3.39)	2 (0.22)	9 (1.22)	0 (0)	136 (12.87)	1 (0.07)	72 (4.16)
2-myeloid Bcell doublet, n (%)	0 (0)	1 (0.1)	0 (0)	1 (0.14)	0 (0)	23 (2.18)	0 (0)	8 (0.46)
3-Bcells, n (%)	19 (3)	61 (6.08)	5 (0.54)	44 (5.97)	2 (0.19)	57 (5.39)	0 (0)	39 (2.25)
3-treatment activated Bcells, n (%)	25 (3.94)	3 (0.3)	12 (1.3)	1 (0.14)	17 (1.58)	3 (0.28)	28 (1.94)	2 (0.12)
3-Bcell Tcell doublet, n (%)	5 (0.79)	29 (2.89)	3 (0.33)	19 (2.58)	1 (0.09)	113 (10.69)	1 (0.07)	127 (7.33)

preg: pregnancy, post: postpartum, n:number of cells, (%): percentage

Annex Table 3. Cell sublineage description (resolution 2)

	RA1 preg treated	RA1preg untreated	RA1 post treated	RA1 post untreated	RA2 preg treated	RA2 preg untreated	RA2 post treated	RA2 post untreated
0-NKs $CD16^{+}CD56^{dim}$, n (%)	8 (1.26)	24 (2.39)	3 (0.33)	26 (3.53)	73 (6.8)	19 (1.8)	57 (3.95)	69 (3.98)
0-treatment activated NKs $CD16^{+}CD56^{dim}$, n (%)	8 (1.26)	8 (0.8)	21 (2.28)	6 (0.81)	23 (2.14)	4 (0.38)	114 (7.89)	16 (0.92)
0-NKT, n (%)	1 (0.16)	26 (2.59)	1 (0.11)	20 (2.71)	20 (1.86)	10 (0.95)	19 (1.32)	25 (1.44)
0-CD8 cytotoxic Tcells $GZMA^{+}GNLY^{+}$ n (%)	3 (0.47)	53 (5.28)	3 (0.33)	57 (7.73)	35 (3.26)	50 (4.73)	27 (1.87)	122 (7.04)
0-CD8 cytotoxic Tcells $GZMA^{+}GZMK^{+}$, n (%)	1 (0.16)	52 (5.18)	3 (0.33)	41 (5.56)	8 (0.74)	52 (4.92)	3 (0.21)	51 (2.94)
0-CD8 cytotoxic Tcells $GZMK^{+}$, n (%)	4 (0.63)	47 (4.68)	2 (0.22)	27 (3.66)	12 (1.12)	55 (5.2)	6 (0.42)	85 (4.91)
0-CD4 cytotoxic Tcells, n (%)	4 (0.63)	98 (9.76)	3 (0.33)	67 (9.09)	15 (1.4)	83 (7.85)	7 (0.48)	95 (5.48)
0-CD4 resting Tn, n (%)	0 (0)	156 (15.54)	2 (0.22)	154 (20.9)	22 (2.05)	63 (5.96)	29 (2.01)	220 (12.7)
0-CD8 resting $T_{n,}$ n (%)	1 (0.16)	62 (6.18)	2 (0.22)	73 (9.91)	5 (0.47)	57 (5.39)	10 (0.69)	142 (8.2)
0-CD4 midActivation $T_n T_{cm,}$ n (%)	4 (0.63)	84 (8.37)	2 (0.22)	71 (9.63)	17 (1.58)	107 (10.12)	12 (0.83)	261 (15.07)
0-preTh1 recently activated $T_{cm,}$ n (%)	0 (0)	24 (2.39)	0 (0)	21 (2.85)	2 (0.19)	43 (4.07)	1 (0.07)	131 (7.56)
0-preTreg recently activated $T_{cm,}$ n (%)	5 (0.79)	10 (1)	3 (0.33)	6 (0.81)	6 (0.56)	16 (1.51)	10 (0.69)	121 (6.99)
0-stressed CD8, n (%)	1 (0.16)	0 (0)	4 (0.43)	11 (1.49)	6 (0.56)	5 (0.47)	7 (0.48)	9 (0.52)
0-Tcell granulocyte doublet, n (%)	0 (0)	3 (0.3)	0 (0)	1 (0.14)	0 (0)	2 (0.19)	0 (0)	27 (1.56)
1-recently activated $T_{n,}$ n (%)	71 (11.2)	19 (1.89)	111 (12.04)	20 (2.71)	112 (10.43)	10 (0.95)	164 (11.36)	17 (0.98)
1-recently activated $T_{cm,}$ n (%)	50 (7.89)	2 (0.2)	117 (12.69)	9 (1.22)	89 (8.29)	3 (0.28)	155 (10.73)	2 (0.12)
1-activated ribsomal Tcell, n (%)	75 (11.83)	3 (0.3)	132 (14.32)	1 (0.14)	92 (8.57)	1 (0.09)	158 (10.94)	1 (0.06)
1-activated $T_{cm,}$ n (%)	41 (6.47)	0 (0)	118 (12.8)	1 (0.14)	80 (7.45)	1 (0.09)	90 (6.23)	1 (0.06)
1-activated $T_{em,}$ n (%)	81 (12.78)	1 (0.1)	89 (9.65)	1 (0.14)	124 (11.55)	0 (0)	190 (13.16)	2 (0.12)
1-proliferative Tcells, n (%)	85 (13.41)	0 (0)	132 (14.32)	0 (0)	83 (7.73)	0 (0)	131 (9.07)	3 (0.17)
1-high mitocondrial Tcells, n (%)	5 (0.79)	0 (0)	9 (0.98)	1 (0.14)	17 (1.58)	1 (0.09)	39 (2.7)	0 (0)
1-CD8 cytotoxic Tcells, n (%)	30 (4.73)	1 (0.1)	35 (3.8)	4 (0.54)	66 (6.15)	0 (0)	42 (2.91)	2 (0.12)
1-cytotoxic Tcells, n (%)	22 (3.47)	1 (0.1)	29 (3.15)	0 (0)	33 (3.07)	0 (0)	45 (3.12)	0 (0)

Annex Table 3 *(continuation)*. Cell sublineage description (resolution 2)

	RA1 preg treated	RA1preg untreated	RA1 post treated	RA1 post untreated	RA2 preg treated	RA2 preg untreated	RA2 post treated	RA2 post untreated
1-CD4 cytotoxic Tcells, n (%)	23 (3.63)	1 (0.1)	25 (2.71)	0 (0)	54 (5.03)	0 (0)	11 (0.76)	0 (0)
1-Tcell Bcell doublet, n (%)	54 (8.52)	0 (0)	47 (5.1)	0 (0)	55 (5.12)	1 (0.09)	70 (4.85)	0 (0)
1-NKs, n (%)	1 (0.16)	0 (0)	1 (0.11)	1 (0.14)	3 (0.28)	2 (0.19)	11 (0.76)	3 (0.17)
1-basophils, n (%)	7 (1.1)	3 (0.3)	6 (0.65)	4 (0.54)	2 (0.19)	0 (0)	2 (0.14)	0 (0)
2-cDCs, n (%)	0 (0)	129 (12.85)	0 (0)	28 (3.8)	0 (0)	74 (7)	3 (0.21)	44 (2.54)
2-CD14 monocytes, n (%)	0 (0)	69 (6.87)	0 (0)	12 (1.63)	0 (0)	66 (6.24)	1 (0.07)	35 (2.02)
2-myeloid Tcell doublet, n (%)	0 (0)	34 (3.39)	2 (0.22)	9 (1.22)	0 (0)	136 (12.87)	1 (0.07)	72 (4.16)
2-myeloid Bcell doublet, n (%)	0 (0)	1 (0.1)	0 (0)	1 (0.14)	0 (0)	23 (2.18)	0 (0)	8 (0.46)
3-Bcells naive, n (%)	19 (3)	52 (5.18)	5 (0.54)	38 (5.16)	1 (0.09)	47 (4.45)	0 (0)	35 (2.02)
3-Bcells memory, n (%)	0 (0)	9 (0.9)	0 (0)	6 (0.81)	1 (0.09)	10 (0.95)	0 (0)	4 (0.23)
3-treatment activated Bcells, n (%)	25 (3.94)	3 (0.3)	12 (1.3)	1 (0.14)	17 (1.58)	3 (0.28)	28 (1.94)	2 (0.12)
3-Bcell Tcell doublet, n (%)	5 (0.79)	29 (2.89)	3 (0.33)	19 (2.58)	1 (0.09)	113 (10.69)	1 (0.07)	127 (7.33)

preg: pregnancy, post: postpartum, n:number of cells, (%): percentage