PROTOCOL

Title: Phenotyping immune cells in peripheral blood before and after 4 months of treatment with Methotrexate, TNFα-, or IL17 inhibitors in patients with psoriatic arthritis

Authors: Marie Skougaard[1,2], Lars Erik Kristensen[1]

[1] The Parker Institute, Bispebjerg and Frederiksberg Hospital, Frederiksberg, Denmark
[2] Copenhagen Center for Translational Research, Bispebjerg and Frederiksberg Hospital, Copenhagen, Denmark

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INTRODUCTION

Psoriatic Arthritis (PsA) is a chronic immune-mediated inflammatory disease with heterogenous symptoms that affect a variety of structures in a distinct individual way. Symptoms experienced by the individual patient might include clinical manifestations involving skin, joints, entheses, nails, and/or other connective tissues. Nonetheless, patients diagnosed with PsA suffer from the alleged same disease they experience a diverse range of symptoms and manifestations which respond very differently to available treatments.

Current treatment strategies include first line treatment with disease modifying anti-rheumatic drugs (DMARDs) with little and/or unsatisfactory effect in PsA (1-3) . Biological treatment of PsA include Tumor Necrosis Factor α (TNFα) inhibitors and Interleukin 17 (IL-17) inhibitors (4). Nevertheless, 20-30% of PsA patients fail to respond to available biological agents (5, 6) which might be explained by different response to drugs due to different patient- and/or disease specific characteristics as implied in one study of survival rates of TNFα inhibitor in patients with two different PsA phenotypes (7). This is demonstrating the importance in comparing the immune pathological findings with patient- and disease specific characteristics and treatment response.

T-cell differentiation with development of the T-cell into specific T-cell phenotypes is believed to play an important role in the immune pathological pathway of PsA (8, 9). While T helper cell type (th) 1 and th17 cells are believed to play a part in development of adaptive autoimmune response and tissue damage in PsA, recent knowledge further implement an innate autoinflammatory response (10). Nevertheless, the association between different T-cell phenotypes in PsA remain unclear and current evidence has also been associating several additional cell types of both innate and adaptive immunity with the developing inflammatory response leading to PsA (8).

Studies on the effect of treatment on human T cell phenotypes is limited. In PsO, TNFα inhibition has shown decreased levels of circulating Th17 cell and reduced Th1 activity (11, 12), while one study showed increased numbers of circulating Th17 cells in patients with inflammatory arthritis (including PsA) during TNFα inhibitor treatment (13). Studies regarding effect of treatment on immune cell phenotypes, including both methotrexate (MTX), IL17 inihibitors (IL17i) and TNFα inhibitors (TNFi), are needed in order to further understand immune pathological mechanisms of PsA. Furthermore, it is important to investigate immune cell phenotypes and associations with different clinical PsA phenotype that might provide knowledge on how to choose between treatments to ensure optimal effect on various symptoms.

OBJECTIVES

The objectives of the study include:

1) an examination of baseline immune cellular characteristics in PsA patients initiating new treatment
2) an examination of levels of immune cells e.g. Th1, Th17, regulatory T-cells (Tregs), dendritic cells (DCs), monocytes, and natural killer (NK) cells before and after treatment in PsA patients initiating TNFi, IL17i or MTX in association with effect of treatment

STUDY DESIGN

Before conducting the study an initial literature search was performed on July 10, 2019 in pubmed: (psoriatic arthritis)AND(t-cell)AND(phenotype OR receptor)AND(flow cytometry).

In accordance with Danish law, the project has been approved by the Regional Committee on Health Research Ethics in the Capital Region of Denmark and meets GDPR requirements approved by the Capital Region of Denmark.

Study population

70 patients are recruited from Danish Departments of Rheumatology in Region Zealand and the Capital Region of Denmark; 20, 30, 20 initiating treatment with IL17i, TNFi or MTX, respectively. PsA patients are included if following eligibility criteria are fulfilled

Inclusion criteria:

- age ≥18 years
- diagnosed with PsA fulfilling the CASPAR classification criteria
- initiation treatment with either IL17 inhibitor, TNFα inhibitor, or Methotrexate

Exclusion criteria:

- other rheumatic inflammatory diseases than PsA
- prior exposure to IL23- or IL17- inhibitors
- previous treatment with cell-depleting therapies
- active systemic infection during the last two weeks (exception: common cold).

Clinical examination

Patients are included in the Parker Institute consecutive PsA patient cohort (PIPA) (14) where all visits consist of a thorough clinical interview and examination including the listed:

TABLE 1		
	Baseline	4 month
Clinical interview		
Age	X	
Gender	X	
Disease duration	X	
Spinal involvement (yes/no)	X	
Medical history	X	
Clinical examination		
PsA phenotype	X	X
Swollen joint count (66 joints)	X	X
Tender joint count (68 joints)	X	X
SPARCC enthesitis score (number)	X	X
Dactylitis (number)	X	X
Psoriatic nail lesions (number)	X	X
PASI score	X	X
Patient reported outcome		
VAS (global, pain, fatigue),	X	X
HAQ	X	X
PsAID	X	X
PDQ score	X	X
Paraclinical examination		
Ultrasound	X	X
Blood sampling standard biochemistry	X	X
Blood sampling biobank	X	X

PsA: Psoriatic arthritis, BSA: Psoriatic body surface area, PASI: Psoriasis Area Severity Index, VAS: Visual Analogue Scale, HAQ: Health Assessment Questionnaire, PsAID: Psoriatic Arthritis Impact of Disease, PDQ: Pain Detect Questionnaire

Definition of responders and non-responders of treatment

Overall, evaluation of patients' response to treatment (responder versus non-responder) will be conducted by rheumatic evaluation of Disease Activity in Psoriatic Arthritis (DAPSA) for assessment of DAPSA50 reflecting 50% improvement in DAPSA from baseline to follow up (15). Some patients might experience response to treatment by improvement in joint disease, but no change in skin psoriasis or vice versa, why secondary outcome measures is included (table 2).

TABLE 2		
PRIMARY OUTCOME MEASURE	Outcome measure	Definition of responder vs. non-responders
Disease activity	DAPSA	DAPSA50
SECONDARY OUTCOME MEASURES	Outcome measure	
Joint involvement	66/68 swollen/tender joint count	
Skin psoriasis	PASI	

DAPSA: Disease Activity in Psoriatic Arthritis, PASI: Psoriasis Area Severity Index, VAS: Visual Analogue Scale, PDQ: Pain De tect Questionnaire.

Isolation and cryopreservation of buffy coat

Peripheral blood is collected in EDTA vacutainer tubes and lysed in lysis buffer to retrieve blood buffy coat. Cells are cryopreserved in fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO). Cryotubes are transferred to a CoolCell container to ensure controlled freezing of -1°C /min and placed in a -80°C freezer. After a minimun of 24 hours cells are transferred into a liquid nitrogen tank.

Preparation of cells

Cells are transferred from the nitrogen tank to a 37 °C water bath until small bits of ice remain in the cryovial. Culture medium (RPMI + 10% FBS) is added and 2 wash cycles are conducted. Pellet is dissolved in staining buffer and immediate staining of cells. A volume corresponding to $1x10^6$ cells is added to each Eppendorf tube and centrifuged. Cells are resuspended in BD staining and relevant antibodies are added. Prior to the study, antibody titration has been conducted. Cells and antibodies are incubated for 20 min (4 °C), followed by 2 wash cycles. Cells are resuspended in BD staining buffer and ready for flow cytometry

Cells are characterized by cells surface markers:

- CD3+ CD4+ CXCR3+ CCR6- T helper cell type 1 (Th1)
- CD3+ CD4+ CXCR3+ CCR6- T helper cell type 17 (Th17)
- CD4+ CD25+ $CD127^{low}$ CD45RO+ Memory T regulatory cells (mTreg)
- CD4+ CD25+ $CD127^{low}$ CD45RO- Naïve T regulatory cells (nTregs)
- CD3- CD19- CD20- CD14- HLA-DR+ Dendritic cells (DC)
- CD3- CD19- CD20- CD14- CD16+ Natural killer cells (NK cells)
- CD3- CD19- CD14+ Monocytes

Tregs, Th1 and Th17 cells are further characterized by activation markers HLA-DR and CD38. Surface staining is conducted in line with table 3 adapted to local conditions from the antibody panel proposed by the Human Immunophenotyping Consortium (17):

Table 3			
Fluorochrome	Cell surface marker		
	Th1 / Th17	Tregs	Monocytes, DCs, NK cells
PE	CXCR3	CD25	-
PerCP-Cy5.5	CD4	CD4	-
PE-Cy7	CCR6	-	-
APC	CD38	-	CD16
Alexa Flour 647	-	CD127	-
APC-H7	CD8	CD45RO	CD3/CD19/CD20
V450	CD3	CD3	CD14
V500	HLA-DR	HLA-DR	HLA-DR

Cell phenotyping are conducted in line with the Human Immunology Project. PE: phycoerythrin, PerCP-CY5.5: Peridinin-chlorophyl-protein cyanine 5.5, PE-CY7: phycoerythrin cyanine 7, APC: allophycocyanin, APC-H7: allophycocyanin cyanine H7, V450: violet 450, V500: violet 500. CXCR: CXC-chemokine receptor, CD: cluster of differentiation, CCR: CC-chemokine receptor, Th: T helper cells, Treg: regulatory T cells, DC: dendritic cells, NK: natural killer cells

Flow cytometry

Flow cytometry is conducted on the Beckman Coulter Gallios Flow Cytometer (Three laser, 10 Color Configuration System).

EXPLORATORY OUTCOME MEASURES

Primary outcome: components assessing possible of immune cellular phenotypes at baseline in association retrieved by principal component analysis

Secondary outcomes:

- the frequency of immune cells (Th1, Th17, Tregs, DCs, NK cells and monocytes) before and after treatment stratified by effect of treatment defined by DAPSA response e.g. patients with ≥ DAPSA 50% improvement between visits are considered responders to treatment and patients with < DAPSA 50% improvement are considered non-responders of treatment.
- Analyses will further be conducted stratifying patients as responders or non-responders by variables PASI and swollen joint count

DATA ANALYSIS AND STATISTICAL ANALYSIS PLAN

Flow cytometry data is analysed with Kaluza software for Gallios. Statistical analysis will be carried out using the statistical software R with additional relevant packages. Patients characteristics at baseline will be presented as medians with corresponding interquartile range for continuous variables and number with corresponding percentages. Kruskal-Wallis test will be applied for comparison of treatment groups e.g. TNFi, IL17i and MTX treated patients, Wilcoxon's signed rank for the paired comparison of outcome measures before and after treatment, and Mann-Whitney U-test for the comparison of responders and non-responders of

treatment at baseline. Boxplots will be illustrating immune cell frequencies before and after treatment with additional mean changes and 95% confidence intervals comparing baseline and follow up frequencies. Further, statistically significance levels will be evaluated using Wilcoxon's signed rank test. To examine possible immune cellular phenotypes at baseline, principal component analysis (PCA) will be implemented for dimensionality reduction exploring the variance and association between all evaluated cell types. Number of components included for interpretation will be decided by the Eigenvalues (proportion of variation explained by each component) at the point where remaining eigenvalues will be of comparable sizes on the scree plot (18). The contribution of each variable to the component will be considered significant/important if being above the expected average contribution defined as the contribution of which variables were uniform e.g. 1/length of variables (number of cells examined) ~ 1/9 ~ 11.1%. Spearman's rho correlation coefficient will be applied for the evaluation of correlation between components (immune cellular phenotypes), individual clinical manifestation of PsA and treatment effect outcome measures.

Table 1: Patients characteristics grouped by type of treatment
Table 2: PCA including contribution and loadings
Table 3: Mean changes from baseline to follow up (clinical outcome)
Table 4: Correlations between components and individual clinical manifestations
Figure 1: Boxplot with immune cell frequencies before and after treatment stratified by type of treatment

Gating strategy and additional explanatory plots will be included as supplementary files.

PUBLICATION STRATEGY

Results of the study will be disseminated through (a) publication(s) in international peer-reviewed journals. All outcomes will be published, i.e. either positive, negative, or inconclusive results. Public outreach will be performed by layman articles at the Parker Institutes' website www.parkerinst.dk

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