Final report
Leibniz Collaborative Excellence
Project title: TargArt

Project number: K259/2019
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Executive Summary

Oligoarticular (oligo) juvenile idiopathic arthritis (JIA) is the most common rheumatic disease in children. In contrast to the chronic course of rheumatoid arthritis (RA) in adults, oligo JIA can undergo medication-free remission in a significant fraction of patients for reasons, which remain unclear. This project aimed to characterize the immune cell compartment infiltrating the inflamed synovia of JIA patients and to identify cells and molecular pathways associated with chronic inflammation and remission. To this aim, we have uncovered the heterogeneity of immune cells derived from the synovial fluid (SF) and peripheral blood (PB) of children at the onset of oligo JIA, using single cell RNA sequencing and flow cytometry. This work enabled the identification of unique populations of activated effector T cells and Natural Killer (NK) cells, as well as of potential mediators regulating immune cell recruitment and activation. Importantly, the presence of a specific subset of T cells in blood of patients was associated with JIA remission. In perspective, the cell/targets identified could have prognostic value in oligoarticular JIA, and could represent interesting candidates to modulate chronic inflammatory diseases.

1. Achievement of objectives and milestones

The project comprised of four main WPs:

WP1: Identification of cellular and molecular signatures correlating with disease remission or persistence and of immune-check points for tuning inflammation
Partners: Kallinich, Mashreghi, Romagnani
Uncovering the heterogeneity of T cells and ILCs of the SF and the paired peripheral blood (PB) of patients at the onset of oligo JIA, combining Flow Cytometry (FACS) and single cell RNA sequencing (scRNAseq), and then correlating the signatures obtained with clinical data.
Implementation:
Patients recruitment: 100% completed
scRNAseq analysis and FACS analysis of T and NK/ILC cells: 100% completed
Correlation with clinical data: 100% completed
This data identified a population of recirculating effector T cells as well as SF specific populations of NK cells activated in situ.

WP2: Role of innate cross-reactivity in the pathogenesis of JIA
Testing whether, in the presence of an inflammatory and hypoxic milieu such as the synovia of JIA patients, cross-reactivity of the innate receptors NKG2A and NKG2C towards self or danger peptides may contribute to elicit auto-reactive responses and exacerbate inflammation.

Partners: Romagnani
Implementation:
Ex vivo analysis of NKG2C$^+$ and NKG2A$^+$ NK cell activation: 100% completed
As NKG2C$^+$ NK cells from SF did not show any selective sign of activation, and ligand up-regulation could not be prominently observed, in vitro stimulations were not performed.

WP3: In vivo validation by targeting of identified candidates
Partners: Romagnani, Mashreghi, Menger
Validation of selected targets identified in our pre-work and in WP1 for their ability to modulate joint inflammation *in vivo*. To this aim, we used existing genetic or biological tools and tried to develop new reagents, which can down-tune or eliminate pathogenic T cells and ILCs by targeting of immune checkpoints.

Implementation:
Arthritis induction in NKG2A$^{-/-}$ mice: 100% completed.
Antigen-induced arthritis was performed in NKG2A$^{-/-}$ vs WT mice. The experiments did not show a role for NKG2A in modulating arthritis.
PD-1 targeting could not be accomplished due to the failure of specific aptamer development

WP4. Validation of identified signatures in patients
Partners: Kallinich, Mashreghi, Romagnani
Validating the value of cellular/molecular signatures identified in WP1 as early prognostic markers in an independent cohort of patients,
Implementation: 100% completed in JIA patients.

2. Activities and obstacles

The COVID-19 pandemic has led to several disruptions of the scientific activities of the TargArt project in 2020 and 2021. In particular, it has dramatically affected patients sample collection, laboratory experiments, recruitment for scientific positions and general activities, therefore a cost-neutral 1-year extension of the project was requested and granted. At the same time, MF Mashreghi's team dedicated its unique scientific and technological expertise to the study of the dysregulated, exuberant immune response to the SARS-CoV-2 virus.
The granted extension enabled the PIs to execute the planned working packages, although some obstacles were encountered. For WP3, the plan was to develop aptamers against surface proteins to modulate the function of pro-inflammatory tissue-resident T cells in JIA. The first candidate was the molecule PD-1 on T cells, which defined a subset of memory T cells enriched in the SF of patients. Usually associated with T cell exhaustion, in the context of JIA, these cells are stimulated and actively produce cytokines in the tissue (*Maschmeyer et al., EJI 2021*). Therefore, an aptamer against PD-1 was to be designed to act as an agonist and turn off T cells via PD-1-mediated signal transduction. However, developing a PD-1-specific aptamer proved more challenging than expected. Despite multiple attempts, the Fraunhofer Institute for Cell Therapy (Menger) could not generate such an aptamer, and the objectives of WP3b could not be achieved. This might be due to the nature and polarity of PD-1 protein, which made it impossible to get a PD-1 binding aptamer. Nevertheless, efforts will continue to develop agonistic antibodies instead of aptamers for this purpose.

3. Results and successes

Publications
Since the beginning of the project, results from the project have been included in several publications (see attached table).
The review article Maschmeyer et al (*Nat Rev Rheumatol 2021*) provides an overview on our concept of the generation and maintenance of inflammatory rheumatic diseases such as JIA. It summarizes our knowledge on the so far identified cells, which adapt to the inflammation and are the cause of the inflammation turning into a chronic disease. In Maschmeyer et al (*Eur J Immunol. 2021*), the team of T. Kallinich and MF Mashreghi dissected the T cells in inflamed joints of patients with JIA. Single-cell transcriptome analysis identified different populations of T cells, including a population that drive inflammation, and T cells with immunoregulatory functions. This reference is related to WP1. Moreover, the work from the Mashreghi and

Kallinich groups led to successful collaborations with two groups from the Netherlands and Italy, respectively. In the first collaboration, it was shown that regulatory T cells at the site of inflammation differentiate into effector Tregs, which have a predominantly suppressive and cytotoxic phenotype with a distinct TCR repertoire (*Lutter et al., Clin Transl Immunology 2023*). The transcription factor BHLHE40, which supports this phenotype, is also expressed in conventional effector T helper cells that are present in the inflamed tissue. Further investigation is needed to understand how eTregs modulate inflammation. In the second collaboration, colleagues in Italy were able to verify our data (*Maschmeyer et al., EJI 2021*) in a subsequent study (*Vanni et al., EJI 2023*).

The team of C. Romagnani established an arthritis model and tested NKG2D as a modulator of arthritis *in vivo* (*Babic et al, J Exp Med. 2020*), related to WP3. Moreover, the team studied signals driving activation and differentiation of ILCs as well as clonal expansion and epigenetic remodeling of NK cells (*Stehle et al, Nat Immunol. 2021; Hernández et al, Immunity 2021; Rückert et al, Nat Immunol. 2022*) related to WPs 1-2.
In the emerging corona pandemic, MF Mashreghi's team used their expertise in single cell analysis to uncover the key role of TGFβ in severe disease progression: TGFβ normally terminates the immune reaction at the end of a successful virus control. In severe corona-infections, SARS-CoV-2 already induces the expression of this cytokine at the beginning of the infection. Thus, NK cells, which are crucial for the initial fight against virus-infected cells, are not activated, and the virus can replicate unchecked (*Witkowski et al. Nature 2021*).

Unpublished results
Analysis of single-cell data from peripheral blood and inflamed tissue of children with JIA identified strong enrichment of in situ activated and proliferating NK cells in the SF of JIA patients. Rather than expression of inflammatory cytokines or cytotoxic molecules, a central feature of SF NK cells was the enrichment in chemokine expression, suggesting a role for NK in regulating immune cell infiltrate, especially of dendritic cells (WP1). We are currently preparing a manuscript to publish these results. Conversely, a role for NKG2C- or NKG2A-mediated activation in JIA could not be detected (WP2). Accordingly, in vivo experiments showed that NKG2A deficiency did not affect arthritis severity (WP3). Analysis of single-cell T cell data from PB and inflamed tissue of children (WP1) with JIA also led to the identification of a unique population of SF HLA-DR+PD1+CXCR5-CD39- CD4 memory T helper cells which recirculate in PB, as described above (*Maschmeyer et al., EJI 2021*). Importantly, those data obtained in WP1 were validated in an independent cohort (WP4). Previously identified HLA-DR+PD1+CXCR5-CD39- CD4 memory T helper cells were found in the PB of patients in therapy-free remission. This data suggest that the presence of this inflamed tissue-resident T cell population in the PB is associated with potential resolution of JIA. Frequent monitoring of this population in the PB of children with JIA could predict disease activity and guide therapy adjustments. We are currently preparing a manuscript to publish these results.

4. Equal opportunities, career development and internationalisation

The project has strived to promote equal opportunities to scientists and other staff members. When advertising open positions, preference was given to women with equal qualifications. PIs and staff recruited had a good balance of gender and internationality, representing six different nationalities.
The hired scientists have benefited from the outstanding scientific environment at the DRFZ and Charité University. In particular, PhD candidates funded or associated to the project have been embedded in structured graduate programs, such as the Leibniz Graduate School for Chronic Inflammation (LeGCI), the ZIBI Graduate program for Immunology, Infectious Diseases and Inflammation and the International Max Planck Research School for Infectious Diseases and Immunology (IMPRS-IDI), benefiting from an excellent, diverse and international

environment. PostDocs funded or associated to the project have been embedded in the Leibniz Postdoc College on Chronic Inflammation, offering specific training opportunities, including mentoring in academic and non-academic career orientation, support in writing grant applications and papers, with the aim to foster early independence, and a successful academic career.
Measures in line with the DFG and Leibniz gender equality standards and goals of diversity were put in place at the DRFZ in conjunction with the gender equality commission, to specifically support the career development of young female scientists. These included mentoring programs, symposia and networks for “Women in science”, complementing the activities at the Charité University, such as the Rahel Hirsch Scholarship and Lydia-Rabinowitsch Fellowship.
As project members suffered from the corona-pandemic restrictions accompanied by home schooling of kids, mobile working helped to keep in touch with colleagues. Beyond the regular daycare opportunities at the Charité, flexible childcare options have been available within the Campus funding.

5. Structures and collaboration

C. Romagnani was the project leader and ensured regular meetings between the PIs and the collaboration partner. Progress reports were also regularly conducted among staff members, who shared samples, technologies, and knowledge on scientific advances. The groups of C. Romagnani and M-F Mashreghi had a long-standing collaboration in understanding the molecular mechanisms of T cells and ILC differentiation and activation, which has been further reinforced during the funding period, with a total of more than 10 joint publications. A close cooperation with the group of T. Kallinich in characterizing T cells and ILC/NK cells in the SF and PB of oligo JIA patients was established and strengthened during the funding period, resulting in more than five joint publications. The collaboration with the Menger group should have enabled the development of new therapeutic tools, including PD-1 aptamers, which could not be successfully generated.

6. Quality assurance

In the last years we have made major efforts to publish open access (see Annex Table ad 3.1). Costs for open access publications have been partially supported by the grant.
The applicant institute DRFZ and the Charité have guidelines for good scientific practice, which is handed out to all new employees. As described above, doctoral students are enrolled in graduate programs, which also has guidelines in place and offer events on this topic.
Working with laboratory animals is strictly regulated at the DRFZ. In order to comply with legal requirements for animal welfare, the scientists are advised on their projects, but also on breeding and husbandry, by the animal welfare officers who are independent of instructions and who ensure and control the welfare of the animals and compliance with all guidelines and regulations. The Animal Welfare Officers are supported in their work by the Animal Welfare Committee, which meets regularly. In addition, monthly project presentations take place to discuss and improve the knowledge gained and the actual burdens of animal experimentation. All scientists performing animal experiments have to pass a basic expertise course with examination before they can do animal experiments and they have to undergo regular training on the topics of animal welfare and the 3R principle (Replace, Reduce, Refine).
The scientists at the DRFZ are very aware of their responsibility towards animals and always limit animal suffering to an indispensable level. The researchers at the DRFZ conduct as many experiments as possible without the use of animals ("Reduce & Replace"), for example by using cell cultures. When conducting animal experiments, a statistically calculated minimum number of animals is used, but no more animals than necessary ("Reduce"). In addition, a well thought-out and controlled breeding management is applied to keep the number of surplus animals as low as possible. In addition to species-appropriate housing with nesting material, hiding places and occupational material, the animals are trained in tunnel handling from the

time they are weaned in order to make handling as stress-free as possible. Before the experiments begin, the animals are familiarized with their environment and the experimenter by training with the animals in preparation. In the context of animal experiments, the DRFZ takes public concerns such as transparency and communication very seriously. The Leibniz-initiative *Tierversuche verstehen* has awarded the DRFZ a prize for "Exemplary Communication of Animal Experimental Research". This award honors research institutions and scientific organizations that show exemplary commitment to transparent and open communication and dialogue about animal experiments and animal experimental research.
Even during the Covid19 pandemic, all animal welfare concerns were met. A well thought-out animal house management ensured both, the health protection of the employees and the continued operation of the animal husbandry, so that the continuation of research could be guaranteed.

7. Additional resources

The DRFZ and the Charité Universitätsmedizin have supported the project co-financing the PIs as well as technical assistent and student positions, which have contributed to the project by establishing technologies, executing part of the working program as well as associated projects. Moreover, the institutions have supported the consortium with core funding for basic consumables as well as for core facility staff and equipment usage. The project has been a catalyzer for other funding acquisition, including an ERC Advanced Grant to C.Romagnani, as described in Annex Table 3.4.

8. Outlook

This work has identified a potential role for NK cells and of a subset of $CD4^+$ memory T cells in regulating JIA and has shown the association between the presence of the latter in PB of JIA patients with possible disease remission. Future work will be dedicated to further explore the validity of these findings as prognostic disease marker. Moreover, this research opens new avenues to target NK cell effector molecules and chemokines in order to modulate JIA severity.