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Exposure to *Candida albicans* Polarizes a T-Cell Driven Arthritis Model towards Th17 Responses, Resulting in a More Destructive Arthritis

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**Abstract**

**Background:** Fungal components have been shown very effective in generating Th17 responses. We investigated whether exposure to a minute amount of *C. albicans* in the arthritic joint altered the local cytokine environment, leading to enhanced Th17 expansion and resulting in a more destructive arthritis.

**Methodology:** Chronic SCW arthritis was induced by repeated injection with *Streptococcus pyogenes* (SCW) cell wall fragments into the knee joint of C57BL/6 mice, alone or in combination with the yeast of *C. albicans* or *Zymosan A*. During the chronic phase of the arthritis, the cytokine levels, mRNA expression and histopathological analysis of the joints were performed. To investigate the phenotype of the IL-17 producing T-cells, synovial cells were isolated and analyzed by flowcytometry.

**Principal Findings:** Intra-articular injection of either *Zymosan A* or *C. albicans* on top of the SCW injection both resulted in enhanced joint swelling and inflammation compared to the normal SCW group. However, only the addition of *C. albicans* during SCW arthritis resulted in severe chondrocyte death and enhanced destruction of cartilage and bone. Additionally, exposure to *C. albicans* led to increased IL-17 in the arthritic joint, which was accompanied by an increased synovial mRNA expression of T-bet and *RORγt*. Moreover, the *C. albicans*-injected mice had significantly more Th17 cells in the synovium, of which a large population also produced IFN-γ.

**Conclusion:** This study clearly shows that minute amounts of fungal components, like *C. albicans*, are very potent in interfering with the local cytokine environment in an arthritic joint, thereby polarizing arthritis towards a more destructive phenotype.

**Introduction**

Rheumatoid arthritis (RA) is a systemic joint disease with an unknown etiology, characterized by a chronic inflammatory infiltration of the synovial membrane, and associated with destruction of cartilage and bone. Both genetic and environmental factors contribute to the development of disease. Various infectious agents, such as bacteria and viruses have long been associated with the pathogenesis of RA [1,2]. The involvement of these microorganisms has not only been proposed in the initiation of arthritis, but also in the progression and exacerbations of the disease.

Microorganisms are recognized by pattern recognition receptors (PRR), such as Toll-like receptors (TLRs) and C-type lectins, which are essential components of the innate immune system and form the bridge with adaptive immunity. If the inflammatory response is not adequate and/or the infection is not cleared properly, persistence can eventually result in chronic or even autoimmune inflammation [2]. Particularly TLR signaling has been shown to play an intrinsic role in the inflammatory processes of arthritis [3–5]. The concept that commensal pathogens affect the pathogenesis of RA is strengthened by data from spontaneous murine arthritis models and the notion that spontaneous arthritis does not develop in animals kept under germ free conditions [3,6]. This implies the importance of (memory) responses for the ‘break of tolerance’ and induction of auto-immunization/immunity. *Candida albicans* is the most common opportunistic fungal pathogen in humans. Infection with *C. albicans* induces IL-17 producing T helper (Th17) cells *in vitro* and *in vivo* in naive mice [7–
were examined. Antibodies, secretion of T-cell cytokines and presence of T-cells in cartilage, and bone were determined. Furthermore, the levels of joint swelling and histopathological changes in synovium, cartilage, and bone were determined. The purpose of the present study was to demonstrate the ability of C. albicans to skew the T-cell balance in the chronic murine SCW model. This model initiates as a local TNF-dependent macrophage-driven inflammation, at which repeated antigen exposure results in a chronic T-cell dependent arthritic process [17]. A small quantity of C. albicans or Zymosan A (<10% of mass) was added to the cell wall fragments of Streptococcus pyogenes (SCW) that were repeatedly injected into the knee joint. During the chronic phase of the arthritis, the development of macroscopic joint swelling and histopathological changes in synovium, cartilage, and bone were determined. Furthermore, the levels of antibodies, secretion of T-cell cytokines and presence of T-cells were examined.

Materials and Methods

Animals

Male C57Bl/6 mice were purchased from Janvier, France. The mice were housed in filter-top cages; water and food were provided ad libitum. The mice used were between 10–12 weeks of age. All animal procedures were approved by the animal ethics committee of the Radboud University Nijmegen.

Study protocol

Streptococcus pyogenes T12 organisms were cultured and prepared as described previously [17]. For the fungal components, the blastomycidia of Candida albicans (ATCC MYA-3573 (UC 820)) were used [8]. Zymosan A (Saccharomyces Cerevisiae) was purchased from Sigma-Aldrich and prepared as described earlier [18]. Chronic unilateral arthritis was induced by repeated intra-articular (i.a.) injection of 25 µg SCW on days 0, 7, 14, and 21, whether or not with 1*10⁷ particles of C. albicans (1*10⁷–1 µg) or 2 µg Zymosan, in 7 µl phosphate buffered saline (PBS) into the right knee joint of naive mice. As a control, additional groups were injected with the fungal particles alone. On day 22, twenty-four hours after the last injection, a subgroup of mice was sacrificed for the collection of synovial washouts. Accordingly, patellae with surrounding soft tissue were isolated from inflamed knee joints and cultured 1 hour at RT in RPMI-1640 medium containing 0.1% BSA (200 µl/patella). In addition, the draining lymph nodes (popliteal and inguinal) were collected and cells were isolated. Then, 1*10⁶ cells were stimulated for 72 hours with 2 µg/ml plate bound anti-CD3 (R&D systems) and 2 µg/ml plate bound anti-CD28 (BD Biosciences). Thereafter, supernatants were collected, centrifuged and stored for cytokine determination. On day 28, during the chronic joint inflammation, the sera from the remaining mice were collected, the mice were sacrificed, and knee joints were prepared for histology.

Measurement of joint swelling

Joint swelling was assessed by measuring the accumulation of ⁹⁹ᵐTc in the inflamed joint due to increased blood flow and edema. Therefore, 0.74 MBq of ⁹⁹ᵐTc in 200 µl of saline was injected subcutaneously. After several minutes of distribution throughout the body, external gamma radiation in the knee joints was measured. Swelling was expressed as the ratio of gamma counts in the right (inflamed) knee joint to gamma counts in the left (control) knee joint. Values higher than 1.1 counts per minute were considered to represent joint swelling.

Histopathology

For standard histological assessment, the isolated joints were fixed for 4 days in 10% formalin, decalcified in 5% formic acid, and the specimens were processed for paraffin embedding. Tissue sections were stained with hematoxylin and cosin. The severity of inflammation in the joints was scored on a scale of 0–3 (0 = no cells, 1 = mild cellularity, 2 = moderate cellularity, and 3 = maximal cellularity). Bone destruction was graded on a scale of 0–3, ranging from no damage to the complete loss of bone structure. Proteoglycan (PG) depletion was determined using Safranin O staining. Loss of proteoglycans was scored on a scale of 0–3, ranging from normal, fully stained cartilage to destained cartilage, fully depleted of PG. The scoring of the sections was performed in a blinded manner.

Cytokine detection

Cytokine levels were measured using Luminex multi-analyte technology in combination with Bio-Plex cytokine kits (Bio-Rad; IL-17, IL-4, IFN-γ, IL-10) and performed according to manufacturer’s instructions.

Quantitative PCR

RNA was isolated from synovial knee biopsies using TRI-reagent (Sigma) and treated with DNase to remove genomic DNA. The RNA was subsequently reverse transcribed with oligo(dT) primers in a reverse transcriptase procedure. Quantitative real-time PCR was performed with cDNA specific primers (Biologio) and SYBR Green PCR Master Mix (Applied Biosystems). The ACT method was used to normalize transcripts to GAPDH and to calculate relative mRNA expression (2⁻⁰ΔCt), for which the expression in the SCW injected group was arbitrarily set to 1. All primer pairs were developed using Primer Express 2.0 (Applied Biosystems) and validated according to protocol.

Isolation and stimulation of synovial cells

After sacrificing the mice, the ankle joint synovium was dissected for single cell isolation. In short, synovial biopsies were incubated with enzymatic digestion buffers (Liberase Blendzyme, Roche) for 30 minutes at 37°C. Next, a 70 µm nylon cell strainer (BD Falcon) was used to process the digested tissue. The cell preparation was collected in RPMI with 10% FCS. To isolate the single mononuclear cells, Lympholyte-M (Cederlane) was used according to manufacturer’s protocol. All cells were cultured in RPMI-1640 (Gibco; Invitrogen) supplemented with 10% FCS. Subsequently, the cells were prepared for intracellular flowcytometry.

Flow cytometry

Synovial cells were stimulated for 5 hours with PMA (50 ng/ml; Sigma) and ionomycin (1 µg/ml; Sigma) in the presence of Golgiplug (BD Biosciences) according to manufacturers protocol. After staining the cells extracellularly with anti-CD3 APC (BD Biosciences), the cells were fixed and permeabilized with Cytofix/ Cytoyperm solution (BD Biosciences). Subsequently, they were intracellularly stained with anti-IFN-γ PE (BD Biosciences) and anti-IL-17a APC (BD Biosciences).
anti-IL-17 FITC (BD Biosciences). Samples were measured on a FACS Calibur and data were analyzed using FlowJo software.

Determination of anti-SCW antibody levels

Levels of anti-SCW antibodies in the serum of mice with chronic SCW-induced arthritis (day 28) were analyzed according to standard methods [17]. Briefly, 10 ng of SCW fragments were coated overnight onto 96-well plates. Thereafter, plates were washed, and nonspecific binding was blocked with 1% BSA in PBS-Tween 80 (0.05%). Anti-SCW antibodies were examined in serial 2× dilutions, starting with an initial dilution of 20×. After incubation for 1 hour, plates were washed and isotype-specific goat anti-mouse Ig–HRP (1:1,000) was added for 1 hour at room temperature.

Statistical analysis

Results are expressed as the mean ± SEM. Differences between experimental groups were tested using Mann-Whitney U-test or
one-way analysis of variance with Dunnett’s multiple comparison test, as appropriate. \( P \)-values less than 0.05 were considered significant.

Results

Exposure to fungal particles aggravates chronic SCW arthritis

Repeated intra-articular injection of SCW bacterial fragments induce arthritic flares that shift from a predominantly macrophage-driven acute inflammation to a T-cell driven chronic inflammation [17]. To investigate the ability of \( C. \) albicans to effectively alter the local synovial inflammatory process, heat-killed conidia of \( C. \) albicans were co-injected with 25 \( \mu \)g SCW fragments in the knee joint on day 0, 7, 14 and 21. In preliminary experiments, exposure to differential doses of \( C. \) albicans to murine peritoneal macrophages resulted in the production of various innate cytokines like TNF-\( \alpha \), IL-1\( \beta \) and IL-6 (figure S1). Subsequently, during a pilot experiment, we selected a dose of \( 1 \times 10^4 \) conidia of \( C. \) albicans, which we co-injected with the SCW fragments into the knee joint. This dose did not show a significant alteration in arthritis severity/score (data not shown). We therefore increased the dose of \( C. \) Albicans to \( 1 \times 10^5 \) on top of the SCW dose of 25 \( \mu \)g. For comparison, we injected 2 \( \mu \)g Zymosan A, a glucan derived from the yeast cell wall of \( S. \) cerevisiae, known for its adjuvant properties [19–21]. As control, mice were injected i.a. with SCW, \( C. \) albicans or Zymosan A alone.

The control mice injected with \( C. \) albicans or Zymosan A alone did not develop joint swelling after the injections (figure 1A). Furthermore, the co-injection of \( C. \) Albicans or Zymosan A on top of the SCW fragments did not result in an increased joint swelling compared to the SCW injected group during the acute phase of the model. On the contrary, one day after the final injection, on day 22, the \( C. \) Albicans co-injected group showed a significant increase in the technetium measurements, whereas the Zymosan A co-injected group only showed a trend for increase. Accordingly, we analyzed the joint swelling and histology during the chronic phase of the model, on day 28. During this phase, the joint swelling in the mice injected with SCW remained significantly increased (figure 1B). Interestingly, exposure to minute amounts of \( C. \) albicans or Zymosan A on top of the SCW fragments significantly aggravated the joint swelling. This increase in joint swelling in both the \( C. \) albicans and Zymosan A injected mice was supported by a significant increase in the influx of inflammatory cells after histopathological analysis (figure 1 panels C and D), indicating that both fungal particles aggravated the inflammatory process during the chronic phase of the model.

Increased joint destruction in SCW arthritis after co-exposure to \( C. \) albicans

To compare the ability of \( C. \) albicans and Zymosan A to affect the cartilage and bone destruction, we performed a detailed histological analysis. Compared to the SCW-injected mice, the increased joint inflammation at day 28 after co-injection of SCW with fungal particles was accompanied by marked proteoglycan depletion (figure 2A). Remarkably, although the influx of inflammatory cells was comparable after addition of either \( C. \) albicans or Zymosan A on top of SCW fragments, we observed a significantly higher increase in cartilage erosion, chondrocyte death and bone erosion in the \( C. \) albicans co-injected group compared to the SCW injected group (figure 2A), suggesting that Candida exposure modulates towards a more destructive immune response.

In addition, we analyzed the mRNA expression of several inflammatory genes involved in bone and cartilage destruction...
using synovial tissue collected during the chronic phase of the arthritis. While the osteoclast marker Cathepsin K (Cat.K) was not significantly enhanced, RANKL was upregulated in the synovium in both the C. albicans and Zymosan A induced groups, in line with the increase in bone erosion. Moreover, the matrix metalloproteinase’s MMP3 and MMP13 were significantly increased in the synovium of C. albicans inoculated mice compared to the single SCW group, corresponding to the increased cartilage erosion (figure 2B).

To support that the increase in cartilage destruction was induced by a matrix metalloproteinase-mediated process, we examined the expression of the MMP-specific aggregan neo-epitope VDIPEN by immunohistochemistry. Repeated SCW injections were sufficient to induce VDIPEN expression at the cartilage sites (figure 2C). The expression of VDIPEN after four weeks of SCW arthritis was comparable to the Zymosan A co-injected mice. However, in line with the increase in MMP mRNA expression, the amount of VDIPEN expression increased dramatically when C. albicans had been co-injected with SCW into the joints. These findings show that a small amount of C. albicans is sufficient to exacerbate the chronic murine SCW arthritis model by increasing the cartilage and bone destruction.

**Exposure to C. albicans during chronic SCW arthritis skews the T cell cytokine profile towards Th17 responses**

To study modification of the adaptive immune responses, we initially studied the B-cell compartment and determined the levels of anti-SCW antibodies of total IgG, IgG1 and IgG3 in the sera of the mice. We previously demonstrated that in the chronic SCW model, antigen-specific antibodies are induced after the fourth i.a. injection of SCW fragments [22]. As can be appreciated from figure 3A, we observed no differences in the serum levels of SCW specific antibodies between the groups, indicating that the B-cell antibody production was not altered after addition of C. albicans or Zymosan A.

Since T helper cells are crucial for the chronic phase of the SCW arthritis model, and Candida is known for the induction of Th17 cells, we explored alterations in T-cell responses. Pan T-cell stimulation with anti-CD3 and anti-CD28 of draining lymph node cells on day 22 revealed no difference in the levels of IL-4 and IL-10 production. Interestingly, a significant increase in IL-17 production and a tendency for increased IFN-γ production were found, suggesting an enhanced helper T cell response of mainly the Th17 lineage by the addition of C. albicans (figure 3B).

To conclude whether this peripheral increase in IL-17 and IFN-γ represented a shift in the local T-cell balance in the arthritic joint, we measured the same T-cell derived cytokines in the synovial washouts of the mice. IFN-γ and IL-10 levels were not altered, and IL-4 was undetectable (figure 3C). In contrast, the IL-17 levels were significantly increased in the C. albicans co-injected group, while the cytokine levels in the Zymosan A co-injected group were not different from the SCW group. All together, these data suggest that C. albicans induces a shift in the T cell compartment during SCW arthritis, favoring Th17.

**C. albicans mainly induces IL-17-producing T cells in the joint**

To establish a shift in the T-cell compartment, we subsequently determined the levels of T-cell transcriptional lineage factors, which specify the different T helper cell lineages. FOXP3 (Treg) and GATA3 (Th2) mRNA expression levels were not significantly different between the SCW-arthritis groups (figure 4A). In line with the enhanced IL-17 production from the lymph nodes, we observed a significant increase in the mRNA expression of the Th17 transcription factor RORγT after C. albicans injection. Remarkably, although the level of IFN-γ was not significantly increased in the synovial washouts, we did observe an increase in Th1 transcriptional lineage factor T-bet. Co-exposure to Zymosan A did not influence the other transcription factors. To further confirm the Th17 profile, we analyzed the expression of IL-17A, IL-17F, IL-21, IL-22 and IFNγ (figure 4B). Here we again observed a significant increase in the expression of IL-17A, IL-21 and IFNγ. Although a trend for an increase was found for the other Th17 profile cytokines, they were not significantly increased in the C. albicans co-injected group compared to the SCW and SCW+Zymosan A groups.

Next, we determined whether the inoculation of C. albicans in the chronic SCW model affected the phenotype of the infiltrated T-cells. On day 22, at the peak of the inflammation, we dissected the inflamed synovium and isolated the residing cells. Intracellular cytokine staining revealed an increased percentage of single IL-17 producing T-cells (figure 4B, C). Further analysis revealed that a subpopulation of these IL-17 producing T cells, also produced IFN-γ, partly explaining the increase in the Th1 transcription factor T-bet. This indicates that in the presence of C. albicans the T-cell balance during SCW-arthritis is shifted towards a pronounced Th17/Th1 profile.

**Discussion**

Our main finding is that a low-grade exposure to C. albicans can skew the T cell balance in the SCW arthritis model, by inducing Th17 cells and pushing it towards a more destructive arthritis model. This indicates that the presence of a minute amount of C. albicans antigen, after e.g. a mucosal infection, might be sufficient to skew an inflammatory cytokine profile.

Microbial infections have long been associated with the pathogenesis of RA. Therefore, several fungi and fungal-derived ligands have been used to provoke or accelerate arthritic processes. In the last decade, several PRR’s that recognize fungal PAMPs have been identified, in particular Toll-like receptors (TLR-2 and TLR-4) and C-type lectin family members (Dectin-1, Dectin-2 and mannose receptor) [6,15,16,18,20,23]. C. albicans is especially known for the induction of Th17 cells via Dectin-1, TLR-2, TLR4, Mannose receptor, and complement [3,24] Earlier, it has been shown that gut colonization by C. albicans or exposure to C. albicans derived β-glucans can initiate and aggravate collagen-induced arthritis, probably via an increased and/or altered cytokine profile [16,25]. Although this shows that C. albicans can initiate and aggravate arthritis models, it was not known whether and how Th17 responses were mechanismically involved.

To explore the potency of a minute amount of C. albicans on skewing T-cell responses during arthritis, we investigated the chronic SCW model. In this model, chronic T cell dependent arthritis is induced by repetitive intra-articular injection of *Streptococcus pyogenes* cell wall (SCW) fragments [17]. *S. pyogenes* is a common Gram-positive bacteria that can induce severe rheumatic fever and/or reactive arthritis, depending on the host response [26]. Previously, we have shown that intra-articular exposure to SCW mainly depends on macrophage activation via TLR-2/MyD88 pathway [27]. Repeated exposure results in a chronic arthritis involving SCW-specific T- and B-cell memory responses, without the need for adjuvants like Freund’s complete adjuvant. Furthermore, although the model involves a chronic phase that is characterized by an IL-1 and IL-17 dependent cartilage destruction, the model is less destructive compared to collagen-induced arthritis (CIA) and hardly involves bone erosions. We hypothesized
that co-exposure to *C. albicans*, would alter the macrophage-derived cytokine production during the flares, aggravating the chronic disease.

Monocytes and macrophages stimulated with *C. albicans* induce pro-inflammatory cytokine production, like TNF-α, IL-1 and IL-6 [24]. To prevent an aggravation of the SCW-induced arthritis by the addition of Candida, we choose the dose of *C. Albicans* at such a subtle level that it did not cause a significant increase in the joint swelling on top of the *S. pyogenes* cell wall (SCW) induced local inflammation. During this acute phase of the SCW arthritis model, joint swelling is TNF-α dependent [28]. We therefore conclude that a possible Candida-induced increase in TNF-α could not be observed by our TNF-dependent Tc-measurement. Even three weeks of repeated co-injections with *C. albicans* or Zymosan did not significantly accelerate the model, showing that the co-exposure to *C. albicans* did not increase the innate immune response like a classical adjuvant [29], but may have prolonged or altered the immune response.
Candida Skews T-Cell Balance during Arthritis

A

Relative mRNA expression

T-bet | GATA-3 | RORγt | FOXP3

SCW | SCW + Candida | SCW + Zymosan

B

Relative mRNA expression

IFNγ | IL-17A | IL-17F | IL-21 | IL-22 | IL-10

SCW | SCW + C. Albicans | SCW + Zymosan

C

SCW | SCW + Candida

IL-17 vs IFNγ

D

Percentage (%)

IL-17+IFNγ+ | IL-17+IFNγ+ | IL-17+IFNγ+

SCW | SCW + Candida | SCW | SCW + Candida | SCW | SCW + Candida
This was further strengthened by the fact that we observed no changes in classes of SCW-specific immunoglobulin production. These SCW specific antibodies can be detected in sera during the late chronic stage, and are also TNF-α dependent [17]. Interestingly, B-cells were reported to be involved in the Th17 response after C. albicans exposure [30]. And although B-cells play a minor role in the repeated SCW arthritis model [17], co-exposure to C. albicans might have altered the antigen presenting function of B-cells. The presence of autoantibodies, like rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA) in the sera of patients are early predictors of disease and correlate with arthritis severity [31].

At day 22, one day after the last injection, when the model shifts from a predominately macrophage driven model to a T cell dependent model, we observed a significantly increased joint swelling in the Candida co-injected group, with a trend for increase in the Zymosan co-injected mice. Furthermore, during the late chronic phase of the model (at day 28) we observed a prolonged increase in joint swelling after co-exposure to both C. albicans or Zymosan A. Interestingly, although the increase in joint swelling and inflammatory cell influx in the C. albicans co-injected mice was comparable to Zymosan A co-injected mice, the chondrocyte death, bone erosion and cartilage erosion were significantly elevated by C. albicans. Additional analysis revealed that during the chronic phase of the model, the C. albicans exposure contributed to the increased MMP-mediated cartilage destruction and increased bone erosion, suggesting that additional processes were involved in the C. albicans exposed joints. This increase in joint destruction was accompanied by local synovial IL-17 expression, which is thought to may enhance the role of TNF-α in the progression of the destructive process [32–34]. Synergy between IL-17 and TNF-α may underlie the increased pathology in the C. albicans co-injected group. Together, we concluded that the low levels of C. albicans and Zymosan A did not accelerate the model during the first three weeks of repeated antigen injections, but addition of C. albicans did alter the T cell balance and arthritis outcome at the end of the experiment.

Focusing on the T-cell cytokine production, we observed an increased Th17/Th1 cytokine profile. In line with the increase in the transcriptional factors T-bet and RORγT, this increase was related to an increase in IL-17 single and double positive T-cells. Furthermore, over 90% of the total IL-17 producing cells were CD3+ (data not shown). This supports that T lymphocytes are the main source of IL-17 in this model, rather than innate immune cells capable of producing IL-17 [35]. Th helper cells are defined T lymphocytes expressing CD4 glycoprotein on their cell surface. We chose CD3 staining over CD4 staining due to the reported sensitivity of CD4 to cleavage during enzymatic digestion of the studied tissue [36]. Only recently, we have altered the protocol of enzymatic digestion slightly, to prevent CD4 cleavage. In a yet unpublished observation (submitted manuscript) we observed that in the SCW model more than 80% of the IL-17+ producing cells express CD4.

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Supporting Information

Figure S1 C. albicans and Zymosan A induce a different cytokine profile in vitro. 1×10⁵ peritoneal macrophages from naive C57Bl/6 mice were stimulated with different concentrations Zymosan or C. Albicans for 16 hours (n = 5). Levels of IL-1β, IL-6, IL-10 and TNF-α were determined by Luminex in the supernatants. Results are mean ± SEM.

Acknowledgments

We would like to thank Dr. Annemie van Spriel for her valuable comments and suggestions. We thank Birgitte Walgreen, Monique Helsen and Luidine van den Bersselaar for excellent technical assistance.
Author Contributions

Conceived and designed the experiments: RJM MIK FLV MGN JD AMHB LABJ WBB. Performed the experiments: RJM MIK LABJ.

References


Analyzed the data: RJM MIK. Contributed reagents/materials/analysis tools: RJM MIK LABJ. Wrote the paper: RJM MIK MGN AMHB LABJ WBB.