**Aberrant autophagy and skewed inflammatory and tolerogenic functions in STAT1 gain-of-function dendritic cells**

***Running title:*** DCs in STAT GOF

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**Declaration of interests**

The authors have declared that no conflict of interest exists.

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# **Abstract**

Signal transducer and activator of transcription 1 (STAT1) gain-of-function (GOF) mutations underlie an inborn error of immunity called chronic mucocutaneous candidiasis. Beyond the fungal susceptibility, attributed to Th17 failure, over half of the reported patients suffer from autoimmune manifestations, the mechanism of which has not been explained yet. Dendritic cells (DCs) have been implicated in the pathogenesis of various autoimmune disorders, however, to date they have not been studied in STAT1 GOF CMC. We hypothesized that the STAT1 mutations would affect DCs' properties and alter their inflammatory and tolerogenic functions. To test the hypothesis, we generated monocyte-derived DCs (moDCs) and tolerogenic DCs (tDCs) from freshly isolated STAT1 GOF patients' monocytes cultivated in the presence of IL-4 and GM-CSF (moDCs), and tolerogenic factors vitamin D2 and dexamethasone (tDCs). Functional and signaling studies, co-culture experiments and RNA sequencing demonstrated that STAT1 GOF DCs were profoundly altered in their phenotype and functions, characterized by defective autophagy, proinflammatory skew and loss of tolerogenic functions. The results suggested that DCs play an important role in the immune dysregulation in STAT1 GOF CMC and may contribute to the disease-associated autoimmune manifestations via alteration in various cellular mechanism, including autophagic processes.

***Key words:*** autophagy; candidiasis; CMC; dendritic cells; ruxolitinib; STAT1; tolerogenic

# **Introduction**

Germline autosomal dominant gain-of-function (GOF) mutations in signal transducer and activator of transcription 1 (*STAT1*) gene cause an inborn error of immunity called chronic mucocutaneous candidiasis (CMC). This highly heterogeneous disease, characterized by CMC, increased viral, bacterial and mycobacterial susceptibility, vascular pathology and cancer predisposition, is also associated with markedly increased risk of autoimmune phenomena, such as cytopenias, thyropathy, diabetes mellitus, hepatitis and others. In fact, autoimmunity appears to affect as many as 60% of the over 450 reported patients [1]. While the hallmark susceptibility to *Candida* microorganism has been mechanistically linked to impaired Th17 development and functions due to STAT1/STAT3 disbalance [2], the pathophysiological background of autoimmunity in STAT1 GOF patients has not yet been elucidated. Several theories, derived mainly from similarities with other inborn errors of immunity with diverse autoimmune dysregulation, such as *autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy* (AIRE deficiency) [3], *immunodysregulation-polyendocrinopathy-enteropathy X-linked syndrome* (IPEX syndrome) [4] or type I interferonopathies (eg. Aicardi-Goutières syndrome, systemic lupus erythematodes) [5, 6] were probed, however no universally applicable mechanism was identified. The few published studies concentrated on the detection of autoantibodies or intrinsic defects of B cells and their signaling [7, 8], the role of regulatory cells (Treg) [9] and the pro-autoimmune capacity of enhanced type I and II signaling [2, 10].  Despite the well appreciated role of innate immune cells in autoimmune diseases [11–13] only two studies, describing the NK cell deficiencies, addressed the constituents of innate immunity in STAT1 GOF [14, 15], none concerned the dendritic cells (DCs) and none addressed the possible involvement of autophagy.

DCs are professional antigen-presenting cells that bridge the acquired and innate immunity. They are also key mediators in immune tolerance and various strategies of DCs manipulations were proposed as therapeutic approaches in human autoimmunity and malignancy [16–18]. Previous reports showed that DCs’ differentiation and maturation requires STAT1 signaling [19] and that a tightly regulated mechanisms of autophagy are also required for the development of their tolerogenic properties [20]. Thus, we examined the phenotype and functions of DCs of STAT1 GOF patients, implementing an *in vitro* model of human monocyte-derived DCs (moDCs) and vitamin D2/dexamethasone-generated tolerogenic DCs (tDCs) to elucidate DCs’ involvement in human STAT1 GOF CMC.

# **Results**

***The STAT1 GOF cohort characteristics***

Eight patients, three male and five female, median age 45 years (range 8–52 years), from six non-consanguineous Czech families of Caucasian ethnicity with genetically confirmed *STAT1* GOF mutation (Figure 1A and B), were included in this study. The patients harbored previously described heterozygous mutations [21] affecting the N-terminal (p.E29A, p.Y68C), coiled-coil (p.A267V, p.T288N) and DNA-binding (p.N357D, p.M390T) protein domains (Figure 1C), which resulted in functional hypersignaling downstream from IFNα- and/or IFNγ-recruited STAT1 pathway in T lymphocytes. All patients suffered from CMC of various severity, as well as from increased infectious susceptibility to bacterial or viral pathogens. Three patients had clinically manifest autoimmunity and six patients had detectable autoantibodies against various, predominantly organ-nonspecific antigens. One patient had aortic aneurysm; no patient was diagnosed with malignancy. All patients received antifungal prophylaxis, three patients were treated with selective inhibitor of Janus kinases (JAK) 1/2 ruxolitinib (P1 for CMC and multiple autoimmune features; P2 for refractory CMC and severe keratitis; P8 for refractory CMC and severe lung disease).

All but one patient had markedly decreased peripheral Th17 lymphocyte count, while the rest of the T cell pool was unaffected. Three patients had low circulating numbers of mature B cells and all patients displayed some degree of dysgammaglobulinemia. No patient had monocytopenia at the time of sample acquisition. The summary of genotypes, clinical phenotypes and an overview of routine immunological investigations is available in Supplementary Table 1 and 2.

***STAT1 and STAT3 phosphorylation is augmented in STAT1 GOF moDCs***

To study the effects of *STAT1* mutations on DCs unaffected by *in vivo* extrinsic factors, such as therapy, infections or allergens, a model of moDCs and tDCs was established (Figure 1D).

As anticipated, both the ligand-independent and IFNα- and IFNγ-induced STAT1 phosphorylation were increased in STAT1 GOF moDCs compared to healthy donors (HDs) (Figure 1E). Additionally, STAT3 phosphorylation was also found to be increased compared to HDs. moDCs from patients treated with JAK inhibitor had comparable STAT1 phosphorylation profiles as untreated patients, likely due to the waning effect of the inhibitor after *ex-vivo* cultivation. STAT1 GOF cells were unable to dephosphorylate STAT1 to unstimulated levels even after 120 min from stimulation, while HD cells achieved basal levels within 60 minutes (Figure 1F). The expressions of *STAT1* and *STAT3* genes in moDCs were similar in STAT1 GOF and HD groups (Figure 1G), while in tDCs, higher expression of *STAT1* gene was detected in patient cells.

***5703 genes distinguish between HDs’ moDCs and tDCs***

When transcriptomic profiles of HDs’ moDCs versus HDs’ tDCs were compared, a total of 5703 differentially expressed genes (DEGs) were revealed. According to the gene ontology enrichment analysis, these DEGs are involved in various biological processes, such as immune activities, cytokine mediated signaling pathways, metabolism, migration, and others. A similar number of DEGs (n=5759) was discovered when comparing STAT1 moDCs and STAT1 tDCs (Figure 2A, B).

Correspondingly, the HD moDCs and tDCs retained their respective phenotypical and functional differences. Compared to HD moDCs, HD tDCs expressed increased levels of inhibitory molecules, such as PD-L1, ILT-3, Tim-3 and reduced levels of maturation molecules, for instance CD83 and CD40 (Figure 2C). HDs’ tDCs also released higher levels of IL-10 and lower levels of TNFα (Figure 2D) and induced more regulatory T lymphocytes (Tregs) (Figure 2E). Assessing a key characteristic of tDCs, i.e., the ability to maintain a stable phenotype under inflammatory conditions, we stimulated HDs’ tDCs with fungal glucan zymosan, lipopolysaccharide (LPS) and *C. albicans* and evaluated their phenotype. Compared to HDs’ moDCs, HDs’ tDCs upregulated the expression of their maturation molecules (CD80, CD83, CD86, CD40) poorly, implying their stability (Figure 2F).

***JAK1/2 inhibition increased tolerogenic functions of HD moDCs***

The addition of selective JAK1/2 inhibitor ruxolitinib to adherent HDs’ monocytes at the beginning of the cultivation arrested the moDCs’ differentiation, implying the JAK signaling indispensability in moDCs development. The addition of ruxolitinib to differentiated immature moDCs (rDCs) for 24 hours resulted in an increase of HLA-DR and PD-L1 expression and decrease of Tim-3 expression, which is suggestive of upregulation of tolerogenic mechanisms. Additionally, no significant changes were observed in the surface expressions of co-stimulatory CD80, CD83 and CD40 molecules (Supplementary Figure 1A).

***Multiple signaling pathways are affected in STAT1 GOF moDCs and tDCs***

To elucidate the impact of altered STAT1 signaling we first performed a transcriptomic profile analysis of STAT1 GOF moDCs. In total, 388 DEGs were found (Figure 3A). Out of these, 157 DEGs were upregulated and 231 downregulated. The pathway enrichment analysis (KEGG pathway enricher) revealed that the most enriched categories in STAT1 GOF moDCs included the cytokine-cytokine receptor interactions and chemokine signaling. While the involvement of IL-17 pathway was not surprising, several other DEGs were involved in less expected pathways, such as in MAPK, PI3K-Akt, TLR, HIF-1 and NFκB signaling, suggesting either a direct involvement of STAT1 molecule in these pathways or their recruitment secondary to the STAT1 dysregulation (Figure 3C). Moreover, the pathway enrichment analysis uncovered DEGs involved in antimicrobial inflammatory responses, such as in legionellosis, salmonellosis, staphylococcal or influenza A infections, and cancer (Figure 3C).  When an interaction network was constructed, visualizing the ten pathways with the largest number of DEGs as hubs, autophagy, metabolic processes and cytokine signaling were shown to be the most affected processes (Figure 3E).

Analogically, we compared the transcriptomic profile of STAT1 GOF tDCs to HDs’ tDCs. Altogether, 846 DEGs were found (Figure 3B), 360 upregulated and 486 downregulated and an apparent clustering of group-specific variables was noted (Figure 3B). The most enriched categories in STAT1 GOF tDCs’ transcriptome resembled that of moDCs, i.e., cytokine-cytokine receptor interactions, chemokine signaling and various signaling cascades, such as IL-17, TNF, TLR, HIF-1, NOD or NFκB signaling (Figure 3D). Similar to moDCs, genes involved in antimicrobial inflammatory responses, such as in legionellosis, salmonellosis or pertussis were affected (Figure 3D). Again, the interaction network visualization revealed that autophagy, metabolic processes and cytokine signaling were the most affected cellular processes (Figure 3E).

***Autophagy is decreased in STAT1 GOF moDCs and tDCs***

The KEGG pathway relationship network analysis detected 135 and 56 differentially expressed autophagy related genes in tDCs and moDCs, respectively (Figure 4A), majority of which were downregulated (Figure 4A). Therefore, we assessed the overall level of autophagy in STAT1 GOF moDCs by flow cytometry and found it to be significantly decreased compared to HDs (Figure 4B). When moDCs were treated with bafilomycin, an autophagy inhibitor, autophagy decreased in HDs’ moDCs but not in STAT1 GOF moDCs (Figure 4C). Correspondingly, western blotting demonstrated that the expression of LC3B, a structural protein of autophagosomal membranes, was decreased in moDCs compared to HDs’ DCs (Figure 4D).

Conversely, when STAT1 GOF moDCs were treated with rapamycin, an autophagy inducer, a substantial decrease of surface maturation markers was observed (Figure 4E), implying a role of autophagy in downregulation of inflammatory attributes.

***STAT1 GOF tDCs fail to induce Tregs***

A mild, yet significant decrease in circulating Tregs was noted in this patient cohort (p=0,0346). To define the effects of altered DCs on Tregs induction, we first co-cultured the STAT1 GOF DCs with autologous T lymphocytes and evaluated the Tregs count (Figure 5A). Only HDs’ tDCs were able to induce Tregs, while STAT1 GOF tDCs failed to do so.

To ascertain that the observed reduction of Tregs was not due to the autologous T lymphocytes characteristics, we co-cultured the STAT1 GOF tDCs with HDs’ T lymphocytes (Supplementary Figure 1B). In this allogeneic system, similar results were achieved (Supplementary Figure 1C), i.e., STAT1 GOF tDCs failed to induce Tregs even from healthy T lymhocytes.

Next, we evaluated IL-10 levels in tDCs: T cells co-cultures (Figure 5C) and noted reduced levels of IL-10 in the STAT1 GOF samples in both the autologous and allogeneic co-cultures. This may be due to the diminished production of IL-10 by either/both the STAT1 GOF tDCs and Tregs.

***STAT1 GOF moDCs capacity to induce IFNγ-producing T cells is increased***

In a similar experimental set-up, we co-cultured the STAT1 moDCs with autologous T lymphocytes and evaluated the production of IFNγ by the T cells (Figure 5B). The moDCs had been unstimulated or stimulated with *C. albicans* for 24 hours prior to the co-culture experiments. STAT1 GOF moDCs induced higher percentage of IFNγ+ CD4+ T cells (Th1), both in the immature moDCs and the *C. albicans-*stimulated moDCs settings (Figure 5B), however statistically significant difference was only achieved in the unstimulated co-culture. A similar trend in CD8+ T cells was noted; however, it was not statistically significant.

Again, to verify that the higher percentage of IFNγ+ T cells was due to STAT1 moDCs characteristics and not the T lymphocytes' intrinsic properties, we co-cultured STAT1 moDCs with HDs’ T lymphocytes (Supplementary Figure 1D). This experiment yielded similar results, i.e., the STAT1 GOF moDCs induced comparable numbers of IFNγ+ T cells, regardless of the T cell origin (Supplementary Figure 1E).

***STAT1 GOF DCs are involved in failure of Th17 induction***

Low peripheral blood Th17 count was observed in all our STAT1 GOF patients (Supplementary Table 1 and Figure 5D). We hypothesized that *STAT1* GOF mutation would affect moDCs' ability to induce Th17. When STAT1 GOF moDCs were co-cultured in an allogeneic system with HDs’ T cells, reduction of Th17 counts was observed compared to HDs’ moDCs: T cells co-cultures (Figure 5E). Moreover, RNA sequencing revealed decreased expression of Th17-related genes in both STAT1 GOF moDCs and tDCs (Figure 5F). Given the reported restriction of Th17 differentiation under increased TGFβ stimulation, we measured TGFβ1 and TGFβ2 production by the STAT1 GOF moDCs and tDCs and found it to be correspondingly elevated (Figure 5G).

***STAT1 GOF moDCs and tDCs are characterized by ligand-independent inflammatory bias and enhanced phagocytic activity***

To further characterize the altered cytokine and chemokine signaling inferred by the transcriptomic analysis, we assessed the cytokine production profiles of STAT1 GOF moDCs and tDCs (Figure 6A-D). The unstimulated STAT1 GOF moDCs released significantly higher levels of pro-inflammatory cytokines, such as IL-1β, IL-6, TNFα, IL-8 and CXCL10, compared to HDs (Figure 6A, C). The ligand-independent production of anti-inflammatory IL-10 was similar in STAT1 GOF and HD group. However, upon stimulation with LPS, zymosan or *C. albicans*, the STAT1 GOF moDCs produced significantly lower amounts of IL-10 than HDs (Figure 6E).

Similarly, STAT1 GOF tDCs released substantially elevated levels of pro-inflammatory cytokines, such as IL-1β, IL-6, TNFα and IL-8 (Figure 6B, D). Unexpectedly, STAT1 GOF tDCs also released higher amounts of IL-10 in a ligand-independent experiment, however upon stimulation with zymosan and LPS, the IL-10 production was markedly reduced compared to HDs. After *C. albicans* exposure, tDCs produced increased levels of IL-10 (Figure 6F).

Furthermore, we analyzed the STAT1 GOF DCs’ capacity to phagocyte. Enhanced ingestion of fluorescently labeled zymosan and *E. coli* was noted by both moDCs and tDCs, implying the involvement of STAT1 molecule in phagocytosis in DCs, as well as STAT1 GOF DCs’ priming towards the phagocytic activities (Figure 6G, H).

***STAT1 GOF moDCs and tDCs exhibit mature phenotypes***

Lastly, a multidimensional analysis of DCs’ phenotype revealed that both the STAT1 GOF moDCs and tDCs overexpressed maturation markers CD80, CD83, CD40, HLA-DR compared to HDs (Figure 7A-F). The moDCs expressed higher levels of some inhibitory molecules, such as PD-L1 and Tim-3 (Figure 7A, C and E), while the tDCs’ expression of these molecules was similar to HDs (Figure 7B, D and F).  Inhibitory ILT-3 was only increased in tDCs, but not in moDCs.

When moDCs and tDCs were stimulated with *C. albicans*, zymosan and LPS overnight, an overall diminished expression of maturation as well as inhibitory molecules was seen (Figure 7G, H). This may be due to the pre-existing ligand-independent inflammatory bias of the cells.

# **Discussion**

Despite the mounting evidence of the relevance of STAT signaling in DCs development, maturation and functions [19, 22, 23], DCs have not yet been explored in the context of STAT1 GOF CMC. In this work, the *in vitro* model moDCs and tDCs allowed observations of cells unaffected (to a certain degree) by extrinsic factors, such as therapy, infections, or allergens.

The transcriptomic profiling suggested that STAT1 GOF DCs have aberrant autophagy, are metabolically altered and that their cellular processes are affected by multidirectionally abnormal interactions within the cytokine signaling network. We showed that STAT1 GOF moDCs are primed towards heightened inflammatory states, which is evidenced by increased production of inflammatory cytokines, expression of maturation markers, increased phagocytic activities and, consequently, the increased capacity to polarize T cells into the inflammatory IFNγ-producing Th1. Tolerogenic DCs, whose dysregulation has been implicated in number of self-reactive immune pathologies [24], also displayed inflammatory bias and, importantly, their immunosuppressive properties were impaired in STAT1 GOF. This was suggested by the disbalanced production of inflammatory and anti-inflammatory cytokines, increased ligand-independent expression of maturation markers, increased phagocytosis and, most illustratively, by the diminished tDCs' ability to drive T cells to differentiate into the immunosuppressive Tregs. Consistently, decreased Tregs counts were observed in our patient cohort. This is interesting, as peripheral blood Tregs’ counts and functions were previously reported normal in the STAT1 GOF CMC patients [9].

The ascertainment of decreased autophagy in DCs unraveled a new possible pathophysiological mechanism behind autoimmunity in STAT1 GOF patients. Autophagy has recently been associated with various autoimmune diseases, such as systemic lupus erythematosus, multiple sclerosis or rheumatoid arthritis [25].  It is an archaic intracellular degradation process involved in antigen processing and presentation, pro-inflammatory signaling, and cell growth and differentiation. While some studies described increased autophagy in various classic autoimmune disorders [26–30], others demonstrated its decrease [31–34]. Undoubtedly, the complexity of autoimmune pathophysiology, as well as immune regulation during induction and progression of autoimmunity, renders a simplistic all-in-one model unlikely and context-dependent (and tissue-specific) outcomes of autophagy deregulation far more probable.

In the context of this study, autophagy has, indeed, been described as a mechanism with positive effects on DCs' tolerogenic ontogeny [20]. Several mechanisms may underlie the impaired autophagy in DCs. Firstly, STAT1 itself has been described as a transcriptional suppressor of autophagy genes and autophagic activity [35]. Furthermore, the increased STAT3 phosphorylation, detected here in STAT1 GOF DCs, but not described in the differentiating healthy moDCs [36], may negatively regulate autophagy in various ways; for instance by sequestering EIF2AK2 (eukaryotic translation initiation factor 2-α kinase 2), by interacting with other autophagy-related signaling molecules such as FOXO1 and FOXO3 or by suppressing the oxidative stress in mitochondria [37]. Thus, the STAT1-driven perturbation of autophagy may account for the DCs’ tolerogenic failure, such as the inability to induce Tregs or produce IL-10 observed in our experiments.

The transcriptomic analysis revealed that MAPK and PI3K pathways were also affected by STAT1 GOF mutation. These signaling cascades are activated by myriad of stimuli and exhibit a context-dependent stimulatory and inhibitory effects in various cellular actions, including autophagy [38–42]. The control of PI3K and MAPK signaling at the sequestration and maturation of autophagy checkpoints may represent an important mechanism for autophagy-related diseases, including autoimmunity [41]. However, the origin of the MAPK and PI3K pathways dysregulation in our cohort may be caused by either the STAT1 mutation itself or be secondary to the highly proinflammatory environment created by the cells themselves.

Concerning the hallmark clinical feature of STAT1 GOF CMC, i.e., the increased *Candida spp.* susceptibility, it is vastly attributed to disturbed Th17 immunity, likely due to STAT3 signaling diminution in T cells under the increased STAT1 activity [2]. Here, we show that STAT1 GOF DCs have impaired ability to induce Th17 and contemplate two new possible mechanisms behind the failed fungal clearance. Firstly, previous research demonstrated that TGFβ signaling in DCs may restrict Th17 differentiation [43] and we have noted increased TGFβ production by STAT1 GOF DCs.  Secondly, mice lacking autophagy-related protein-7, essential for autophagosome formation, have diminished autophagic sequestration of A20 in tissue-resident macrophages. This results in aberrant NFκB activation and disturbs the induction of early-stage anti-*Candida* immune responses [44]. Thus, the impaired autophagy may directly affect the innate immune antifungal activities.

Moreover, STAT1 GOF CMC patients often display increased viral susceptibility, which has not been explained. Interestingly, autophagy might be integrated into type I interferon-driven innate antiviral immune responses via the shared principal signaling components within JAK/STAT, PI3K, mTOR, and MAPK pathways [45].

Lastly, the disturbed autophagy-phagocytosis interaction may contribute to the increased phagocytic activity observed in the STAT1 GOF DCs in our experiments [46].

This study is limited by the size and heterogeneity of the patient cohort which lacks the statistical strength to allow for clinical and cellular phenotype correlations. Studies on larger cohorts may address this issue. Furthermore, the mechanisms involved in skewed autophagy were not studied in comprehensive details, nevertheless the pilot data obtained here pave the path for future investigations of STAT1 role in the autophagic activities in DCs. Lastly, the dysregulation of MAPK and PI3K signaling pathways in the context of JAK/STAT dysfunction also warrants dedicated research efforts to elucidate a possible crosstalk between these pathways in regulation of autophagy in DCs.

In summary, we demonstrate severe impairment of DCs in STAT1 GOF CMC, particularly their impaired autophagic processes, proinflammatory bias, aberrant tolerogenic functions, and suppressed Th17 induction capacity. Our findings imply the involvement of DCs in both the autoimmunity and the failure of antifungal defense, providing a background for future research. Finally, our observations translate to the possible utility of DC-based tolerance-inducing immunotherapies in STAT1 GOF patients, such as *ex vivo* generated autologous tDCs or *in vivo* immunomodulatory targeting of the DCs' surface receptors and provide rationale for the potential usefulness of therapeutic autophagy-inducing mTOR inhibition in severe STAT1 GOF-associated autoimmunity.

# **Patients and methods**

***Patients***

The biologic material was obtained from patients followed at the Department of Immunology, 2nd Faculty of Medicine, Charles University and University Hospital in Motol, who were diagnosed with CMC and carried STAT1 mutations (detailed patients’ data are available in Supplementary Table 1) and from age and sex-matched healthy donors (HDs). The study was carried out in accordance with the recommendations of the Ethical Committee of the second Faculty of Medicine, Charles University in Prague, and University Hospital in Motol, Czech Republic. The study protocol was approved by the institutional Ethical Committee. All subjects or their legal guardians gave written informed consents with the research and publication in accordance with the Declaration of Helsinki.

***Cell isolation and culture***

Peripheral blood was collected from patients and HDs into EDTA-coated tubes. First, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (GE Healthcare Biosciences, Uppsala, Sweden). The obtained cultures of PBMCs were resuspended in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin and 1% Glutamax (ThermoFisher Scientific, Waltham, USA).

***Generation of monocyte-derived dendritic cells***

Monocyte-derived DCs (moDCs) were generated from adherent monocytes cultured in IL-4 (20ng/mL) and GM-CSF (500IU/mL) (CellGenix, Freiburg, Germany) presence for 6 days. The cytokines were replenished on day 3. On day 6, the cells were harvested, seeded in 96-well plates at 1x106/mL concentration and stimulated.

To induce tolerogenic dendritic cells (tDCs), DCs were treated with dexamethasone (1µM, Medochemie) and vitamin D2 (1,5ng/ml, Zemplar). tDCs were then harvested, stimulated as moDCs and then phenotype and cytokine production were determined.

***STAT phosphoflow***

moDCs were stimulated with 1µg/mLl IFNγ or IFNα (Abcam, Cambridge, UK) for 5, 15, 30, 60 and 120 minutes or left untreated at 37°C. Intracellular signaling was prevented by using 4% paraformaldehyde without methanol for 10 minutes at room temperature. Erythrocytes were lysed using 0,1% Triton-X for 20 minutes (Sigma Aldrich, St. Luis, USA) at 37°C, leukocytes were permeabilized with ice-cold 80% methanol for 30 minutes and stained with anti-phosphoSTAT1-BV421 (Tyr701) (clone 4a) and anti-phosphoSTAT3-PE (Tyr705) (clone 4/5-STAT3) (both from BD Bioscience, San Jose, USA), anti CD11-APC (clone BU15) (Exbio, Vestec, Czech Republic). The samples were acquired on BD Fortessa (BD Biosciences), and data analysis was performed using FlowJo (TreeStar).

***moDCs and tDCs phenotype***

moDCs and tDCs were harvested, seeded in 96-well plates at 1x106/mL concentration and stimulated with 100ng/mL LPS, 100ng/mL zymosan or heat-killed *C. albicans* in ratio 1:10 for 24 hours. After 24 hours, the cells were stained with anti CD11c-APC (clone BU15), CD14-PEDy590 (clone MEM15), CD86-PerCP (clone BU63) (Exbio), CD80-FITC (clone MAB104) (Beckman Coulter, Brea, USA), CD83-BV421 (clone HB15e), HLA-DR-A700 (clone L243), CD40-BV650 (clone 5C3), ILT3-PC7 (clone ZM4.1), PD-L1-BV510 (clone 29E.2A3), Tim3-PE (clone F38-2E2) (BioLegend). The samples were collected using BD Fortessa (BD Biosciences) and BD FACSDiva software (BD Biosciences) was used for signal acquisition.

***Cytokine production***

moDCs and tDCs were harvested, seeded in 96-well plates at 1x106/mL concentration and stimulated with 100ng/mL LPS, 100ng/mL zymosan or heat-killed *C. albicans* in ratio 1:10 for 24 hours. Cytokine levels in cell-free supernatants of moDCs and tDCs were determined by multiplex Luminex cytokine bead-based immunoassay (Millipore).

***Phagocytosis***

The capacity to phagocyte zymosan (Green Zymosan) and *E. coli* (Red *E. coli*) was determined using commercially available kits from Abcam.

***moDCs: T cells cultures***

The capacity of moDCs and tDCs to activate T lymphocytes or induce Tregs (CD3+CD4+CD25+) was evaluated in DCs: T cells co-cultures experiments. The assays were carried out for 7 days at a 1:5 DCs: T cells ratio. IL-2 (20 U/mL) was added on days 2 and 5. On day 7, Treg induction and IFNγ and IL-17 production were analyzed according to already published protocols (Parackova *et al.*, 2016; Bloomfield *et al.*, 2018). IL-10 levels in the co-cultures were determined by ELISA (R&D Systems).

***RNA isolation and RNA-seq***

Total RNA was isolated using the RNeasy Mini kit following the manufacturer's instructions (Qiagen, Hilden, Germany). RNA quality and quantification was determined by TapeStation 4200 (Agilent, St. Clara, USA) following manufacturer’s instructions. Strand specific library construction was completed by enriching mRNA from total RNA by using oligo (dT)-attached magnetic beads, validated on the Agilent Technologies 2100 bioanalyzer and amplified with phi29 to make DNA nanoball (DNB) which had more than 300 copies of one molecular. The DNBs were loaded into the patterned nanoarray and single end 50 (pair end 100/150) bases reads were generated in the way of combinatorial Probe-Anchor Synthesis (cPAS). Differential expression was determined by employing a Mann-Whitney U test, followed by estimation of false discovery rate. Only genes with a p-value ≤ 0.05 and a FDR ≤ 5% were considered differentially expressed.

***Real time PCR***

moDCs’ total RNA was isolated using RNeasy Mini Kit following manufacturer’s instructions (Qiagen) and complementary DNA (cDNA) was synthesized using M-MLV Reverse Transcriptase (ThermoFisher Scientific). RT-PCR was performed in duplicates using the cDNA and Platinum Taq polymerase (ThermoFisher Scientific), 200 nM dNTP (Promega, Southampton, UK), 50mM MgCl2 (ThermoFisher Scientific) and TaqMan primer/probe sets (ThermoFisher Scientific). Samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR reaction and normalized to GAPDH (forward primers GAAGGTGAAGGTCGGAGTC; reverse primers GAAGATGGTGATGGGATTTC; FAM/TAMRA CAAGCTTCCCGTTCTCAGCC) (TIB MOLBIOL, Berlin, Germany) to obtain the relative expression value. Real time assays were run on FX96 Cycler (Bio-Rad). Primes used: STAT1 (Hs01013996\_m1), STAT2 (Hs01013115\_g1) and STAT3 (Hs00374280\_m1) (all from ThermoFisher Scientific).

***Western blot***

moDCs were washed and lysed in RIPA lysis buffer and PMSF (CellSignaling, Danvers, USA), placed on ice, sonicated, and then centrifuged at 14000g to remove cell debris. For western blot analysis, samples were resuspended in Laemmli buffer (Sigma Aldrich) at 1:1 ratio and boiled for 5 min. Proteins were separated by SDS-PAGE, transferred to the PVDF membrane. After blocking with 5% BSA in TBST (TBS and 0,1% Tween, both from Bio-Rad, Hercules, USA), membranes were incubated with the primary antibodies anti LC3B (#2775) (CellSignalling) overnight, followed by incubation with peroxidase-conjugated anti rabbit or anti mouse secondary antibodies for 2 hours. Membranes were developed using SuperSignal West Femto (ThermoFisher Scientific). Densitometry was performed with ImageJ software (National Institutes of Health, USA). Band area values were used for semi-quantification. Graphs are expressed as ratio of stimulated/unstimulated cells of band area value calculated from band area of phosphorylated forms/band area of unphosphorylated forms.

***Autophagy***

The autophagy was assessed when moDCs were harvested, and after 48 hours, by flow cytometry using a commercially available cell-based kit (Autophagy Assay Kit, AB139484, Abcam) which selectively labels autophagy vacuoles. Cells were treated with 50nM bafilomycin or 500nM rapamycin overnight and then autophagy was analyzed.

***Statistical analysis***

The results obtained from at least three independent experiments are given as the medians ± SDs. Not all patients were involved in all experiments due to the limited amount of blood available per sample. Statistical analysis was performed using non-parametric one-way analysis of variance (ANOVA) with multiple comparisons Dunn’s post-test where applicable. A two-tailed paired Wilcoxon or unpaired Mann-Whitney *t*-test was also applied for data analysis using GraphPad Prism 8. Values of p<0.05 (\*), p<0.01 (\*\*) p<0.001 (\*\*\*) and p<0.0001 (\*\*\*\*) were considered statistically significant.

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# **Disclosure statement**

All authors declare no conflict of interest.

# **Data availability**

Data are available upon request in the corresponding author.

# **Author contribution**

ZP designed the study, performed experiments, analyzed data, and wrote the manuscript.

IZ performed experiments and reviewed the manuscript

PV performed autophagy experiments and reviewed the manuscript.

AS reviewed the manuscript.

MB treated the patients, provided biological material, and co-wrote the manuscript.

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# **Figure legends**

**Figure 1: STAT1 mutations in the study cohort**

1. STAT1 protein structure, position of each mutation is shown; ND - N-terminal domain, CCD - coiled-coil domain, DBD - DNA-binding domain, LD - linker domain, SH2 - Src homology 2 domains, TAD - tyrosine activation domain and a transcriptional activation domain
2. Pedigrees and segregationsof *STAT1* mutations in individual kindreds
3. 3D structure of STAT1 dimer, position of each mutation is highlighted; Red – E29A, Lime – Y68C, Cyan – A268V, Blue – T288N, Magenta – N357D, Grey - M390T
4. The experiment setup
5. Phosphorylation of STAT1 (pSTAT1; Tyr701) and STAT3 (pSTAT3; Tyr705) in moDCs upon IFNγ and IFNα stimulation in STAT1 GOF patients (n=7) and HD (n=9) detected by flow cytometry
6. Kinetics of STAT1 and STAT3 phosphorylation upon IFNα stimulation in moDCs detected in STAT1 GOF patients (n=3) and HD (n=2) by flow cytometry
7. *STAT1, STAT2* and *STAT3* genes relative expressions detected in STAT1 GOF (n=7) and HD (n=7) moDCs by real-time PCR

Values are standardized and expressed as median values. Statistical analyses were performed using paired t-tests. Values of p<0.05 (\*), p<0.01 (\*\*), p<0.001 (\*\*\*), and p<0.0001 (\*\*\*\*) were considered statistically significant.

**Figure 2: Model of healthy donors’ (HDs) monocyte-derived dendritic cells (moDCs) and tolerogenic dendritic cells (tDCs)**

1. Volcano map and heatmap of differentially expressed genes (DEGs) in HDs’ moDCs vs. tDCs (n=7)
2. Bubble chart showing the enrichment of DEGs in the biological processes in which DEGs are involved; The y-axis annotates the process; the x-axis label represents the Rich ratio. The size of the bubble represents the amount of DEGs involved, the color reflects the Q value of each pathway.
3. Phenotypes of HDs’ moDCs (n=13) and tDCs (n=10) detected by flow cytometry
4. IL-10 and TNFα production by HDs’ moDCs (n=13) and tDCs (n=10) detected by Luminex
5. Regulatory T lymphocytes (Tregs) induction by HDs’ moDCs and tDCs (n=7) in autologous co-culture system.
6. HDs’ moDCs (n=13) and tDCs (n=10) phenotype stability upon Zymosan, LPS and *C. albicans* stimulation

LPS – lipopolysaccharide. TCO - T cell only. Values are standardized and expressed as median values. Statistical analyses were performed using paired t-tests. Values of p<0.05 (\*), p<0.01 (\*\*), p<0.001 (\*\*\*), and p<0.0001 (\*\*\*\*) were considered statistically significant.

**Figure 3: Gene expression profiling in STAT1 GOF monocyte-derived dendritic cells (moDCs) and tolerogenic dendritic cells (tDCs)**

1. Scatter plot and heatmap of differentially expressed genes (DEGs) in healthy donors’ (HDs) (n=7) vs. STAT1 GOF moDCs (n=7)
2. Volcano maps and heatmap of DEGs in HDs’ vs. STAT1 GOF tDCs
3. Enrichment bubble chart of signaling pathways where moDCs DEGs are involved. X-axis is the enrichment ratio [the ratio of the number of genes annotated to an entry in the selected gene set to the total number of genes annotated to the entry in the species, calculated as Rich Ratio = Term Candidate Gene Num/Term Gene Num] It is GO Term. The size of the bubble represents the number of DEGs annotated to a GO Term. The color represents the enriched significance. The redder the color, the smaller the significance value.
4. Bubble chart showing the enrichment of DEGs in the biological processes in which DEGs are involved in STAT1 GOF tDCs; The y-axis annotates the process; the x-axis label represents the Rich ratio. The size of the bubble represents the amount of DEGs involved, the color reflects the Q value of each pathway.
5. The KEGG pathway interaction network of moDCs’ DEGs. The affected pathways are ranked by the number of genes in each pathway; only the top 10 pathways with the largest number of DEGs are displayed.
6. The KEGG pathway interaction network of tDCs’ DEGs. The affected pathways are ranked by the number of genes in each pathway; only the top 10 pathways with the largest number of DEGs are displayed.

**Figure 4: Autophagy**

1. Heatmap of differentially expressed genes (DEGs) involved in autophagy in STAT1 GOF monocyte-derived dendritic cells (moDCs)
2. Representative dot plot of autophagy detection in moDCs and quantification of autophagosomal content in STAT1 GOF (n=4) and HD moDCs (n=3) detected by flow cytometry
3. Impact of autophagy inhibitor bafilomycin on autophagy in STAT1 GOF moDCs (n=4) and HD (n=2)
4. Expression of LC3B protein in STAT1 GOF (n=2) and HD moDCs (n=3) detected by Western Blot
5. Phenotype of STAT1 GOF moDCs (n=2) treated with autophagy inducer rapamycin

HD - healthy donors. Values are standardized and expressed as median values. Statistical analyses were performed using paired t-tests. Values of p<0.05 (\*), p<0.01 (\*\*), p<0.001 (\*\*\*), and p<0.0001 (\*\*\*\*) were considered statistically significant.

**Figure 5: T cell induction by STAT1 GOF monocyte-derived dendritic cells (moDCs) and tolerogenic dendritic cells (tDCs)**

1. Regulatory T cells (Tregs; CD3+CD4+CD25+FoxP3+) induced by STAT1 GOF (n=5) and HD tDCs (n=8) in autologous co-culture.
2. IFNγ+ CD4+ and CD8+ T cells induced by STAT1 GOF (n=5) and HD moDCs (n=9) in autologous co-cultures, with or without *C. albicans*
3. IL-10 concentration in tDCs: T cells co-cultures in STAT1 GOF (n=5) and HD (n=8) detected by ELISA
4. IL-17+CD4+ T lymphocytes in STAT1 GOF (n=6) and HD (n=6) periphery
5. IL-17+CD4+ T lymphocytes induced by STAT1 GOF (n=6) and HD (n=8) moDCs in allogenous co-cultures
6. Heatmap of Th17-related differentially expressed genes (DEGs) in STAT1 GOF moDCs
7. TGFβ1 and TGBβ2 production by STAT1 GOF (n=8) and HD (n=10) moDCs and tDCs (n=7; n=10) detected by LUMINEX

HD - healthy donors; TCO - T cell only. Values are standardized and expressed as median values. Statistical analyses were performed using paired t-tests. Values of p<0.05 (\*), p<0.01 (\*\*), p<0.001 (\*\*\*), and p<0.0001 (\*\*\*\*) were considered statistically significant.

**Figure 6: Cytokine production by STAT1 GOF monocyte-derived dendritic cells (moDCs) and tolerogenic dendritic cells (tDCs)**

1. Radar graph of STAT1 GOF (n=9) and HD (n=13) moDCs’ basal cytokine production
2. Radar graph of STAT1 GOF (n=7) and HD (n=11) tDCs basal cytokine production
3. Cytokine production by STAT1 GOF (n=9) and HD (n=13) moDCs detected by LUMINEX
4. Cytokine production by STAT1 GOF(n=7) and HD (n=11) tDCs detected by LUMINEX
5. IL-10 production by STAT1 GOF (n=9) and HD (n=13) moDCs upon LPS, zymosan and *C. albicans* stimulation detected by LUMINEX
6. IL-10 production by STAT1 GOF (n=7) and HD (n=11) tDCs upon LPS, zymosan and *C. albicans* stimulation detected by LUMINEX
7. STAT1 GOF (n=5) and HD (n=6) moDCs’ phagocytosis of fluorescently labeled zymosan or *E. coli*
8. STAT1 GOF (n=6) and HD (n=6) tDCs’ phagocytosis of fluorescently labeled zymosan or *E. coli*

HD - healthy donors; LPS – lipopolysaccharide. Values are standardized and expressed as median values. Statistical analyses were performed using paired t-tests. Values of p<0.05 (\*), p<0.01 (\*\*), p<0.001 (\*\*\*), and p<0.0001 (\*\*\*\*) were considered statistically significant.

**Figure 7: Phenotype of STAT1 GOF monocyte-derived dendritic cells (moDCs) and tolerogenic dendritic cells (tDCs)**

1. Radar graph of STAT1 GOF (n=9) and HD (n=13) moDCs phenotype markers. Black circles represent patients treated with ruxolitinib.
2. Radar graph of STAT1 GOF (n=7) and HD (n=10) tDCs phenotype markers
3. t-distributed stochastic neighbor embedding (tSNE) visualization of STAT1 GOF moDCs’ basal phenotype
4. tSNE visualization of STAT1 GOF tDCs’ phenotype
5. Expression of surface maturation and inhibition markers on STAT1 GOF (n=9) and HD (n=13) moDCs detected by flow cytometry
6. Expression of surface maturation and inhibition markers on STAT1 GOF (n=7) and HD (n=10) tDCs detected by flow cytometry
7. STAT1 GOF (n=9) and HD (n=13) moDCs’ response to *C. albicans*, zymosan and LPS stimulation
8. STAT1 GOF (n=7) and HD (n=10) tDCs’ response to *C. albicans*, zymosan and LPS stimulation

HD – healthy donors. Values are standardized and expressed as median values. Statistical analyses were performed using paired t-tests. Values of p<0.05 (\*), p<0.01 (\*\*), p<0.001 (\*\*\*), and p<0.0001 (\*\*\*\*) were considered statistically significant.

**Supplementary Material**

**Supplementary Figure 1**

1. Ruxolitinib-treated (1uM overnight) STAT1 GOF moDCs’ phenotype
2. Tregs induced in allogenous experiment setup (STAT1 GOF moDCs: HDs’ T cells)
3. IFNγ+CD4+ and CD8+ T cells induced in allogenous experiment setup (STAT1 GOF tDCs: HDs’ T cells)

HD – healthy donors; rDC – ruxolitinib-treated moDCs. Values are standardized and expressed as median values. Statistical analyses were performed using paired t-tests. Values of p<0.05 (\*), p<0.01 (\*\*), p<0.001 (\*\*\*), and p<0.0001 (\*\*\*\*) were considered statistically significant.

**Supplementary Table 1:** Patients’ genetic and clinical characteristics

**Supplementary Table 2:** Patients’ basic immune parameters