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Vogt-Koyanagi-Harada (VKH) disease is an important refractory uveitis mediated by pathological T cells (TCs). Tofacitinib (TOFA) is a Janus kinases (JAKs) targeted therapy for several autoimmune diseases. However, the specific pathogenesis and targeted therapeutics for VKH remain largely unknown. Based on single-cell RNA sequencing and mass cytometry, we present the first multimodal high-dimensional analysis to determine a comprehensive human immune atlas of VKH patients undergoing TOFA therapy in the context of subset composition, gene signatures, enriched pathways, and intercellular interactions. VKH patients are characterized by TCs polarization from naive to effector and memory subsets, altogether with accrued monocytes, upregulated cytokines and JAK-STAT signaling pathways. In vitro, TOFA reversed Th17/ regulatory T-cell (Treg) imbalance and inhibited IL-2-induced STAT1/3 phosphorylation. TOFA alleviated VKH symptoms by restoring pathological TCs polarization and functional marker expression and downregulating cytokine signaling and lymphocyte function. Remarkably, inflammation-related responses and intercellular interactions decreased after TOFA treatment, particularly in monocytes. Notably, we identified two inflammation- and JAK-associated monocyte subpopulations that were strongly implicated in VKH pathogenesis and mechanisms involved in TOFA treatment. Here, we provide a novel JAK-targeted therapy for VKH and elaborate on the possible therapeutic mechanisms of TOFA, expanding our knowledge of VKH pathological patterns.

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Insights gained from single-cell analysis of immune cells in tofacitinib treatment of Vogt-Koyanagi-Harada disease

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Abstract

Vogt-Koyanagi-Harada (VKH) disease is an important refractory uveitis mediated by pathological T cells (TCs). Tofacitinib (TOFA) is a Janus kinases (JAKs) targeted therapy for several autoimmune diseases. However, the specific pathogenesis and targeted therapeutics for VKH remain largely unknown. Based on single-cell RNA sequencing and mass cytometry, we present the first multimodal high-dimensional analysis to determine a comprehensive human immune atlas of VKH patients undergoing TOFA therapy in the context of subset composition, gene signatures, enriched pathways, and intercellular interactions. VKH patients are characterized by TCs polarization from naive to effector and memory subsets, altogether with accrued monocytes, upregulated cytokines and JAK-STAT signaling pathways. In vitro, TOFA reversed Th17/regulatory T-cell (Treg) imbalance and inhibited IL-2-induced STAT1/3 phosphorylation. TOFA alleviated VKH symptoms by restoring pathological TCs polarization and functional marker expression and downregulating cytokine signaling and lymphocyte function. Remarkably, inflammation-related responses and intercellular interactions decreased after TOFA treatment, particularly in monocytes. Notably, we identified two inflammation- and JAK-associated monocyte subpopulations that were strongly implicated in VKH pathogenesis and mechanisms involved in TOFA treatment. Here, we provide a novel JAK-targeted therapy for VKH and elaborate on the possible therapeutic mechanisms of TOFA, expanding our knowledge of VKH pathological patterns.

Keywords: Vogt-Koyanagi-Harada disease, Tofacitinib, Single-cell RNA sequencing, Mass cytometry, Monocytes
Introduction

Uveitis is the leading cause of vision loss, accounting for an estimated 25% of all blindness cases in the western and the developing world (1, 2). VKH is among the most important types of uveitis, characterized by rapid onset, recurrent inflammation, and multiple systems involvement (3). Besides binocular involvement and hearing loss, VKH affects the brain and spinal cord, involved in alopecia, and causes headache (4-6). Currently, VKH patients are primarily treated with systemic and topical glucocorticoids with or without immunosuppressive agents. Although high-dose systemic corticosteroids remain the gold standard therapy, refractory cases may require alternative agents (7). Furthermore, conventional treatment with early high-dose systemic corticosteroids is insufficient to prevent chronicity and vision-threatening complications, including cataracts, glaucoma, and even blindness, which have been reported in the chronic recurrent phase (8). In addition, VKH patients with osteoporosis, severe diabetes, hypertension, and glucocorticoid intolerance have limited access to effective therapy. Therefore, exploring effective and safe medications for VKH patients is necessary.

Although previous studies have shown that immune cells participate in the autoimmune destruction of melanocyte-enriched organs in VKH, the major immune cells and inflammatory pathways involved in VKH remain poorly understood. During immune dysfunction, antigens in eye tissues can be identified, resulting in immunoreaction induced by pathological TCs, such as Th17 and Th1, and related cytokines (9). For example, interferon-gamma (IFN-γ)-secreting Th1 and IL-17-secreting Th17 accumulate in the blood, aqueous fluid, and other tissues of VKH patients (4, 10). Additionally, lymphocytic infiltration, B cells (BCs) chemoattractant, and autoantibodies have been detected in aqueous humor (11, 12). In addition, inflammatory cytokines, such as IL-2, IL-6, IL-23, and IFN-γ, can induce TCs proliferation and the differentiation of BCs and TCs into effector and pathogenic types. These results indicate a key role of inflammatory cytokines and lymphocyte activation in VKH development. Among the cytokine-induced downstream pathways, JAKs are widely involved because they are shared by members of the receptors. Upon binding to
cytokine receptors, JAKs are activated and transduce signals through phosphorylation of downstream molecules, such as STAT and other transcription factors, to trigger immune responses. IL-2 is vital in maintaining and activating lymphocytes. Genetic evidence has connected the association of JAK1/3 and IL-2 with lymphocyte proliferation and immune homeostasis (13). A knock-in murine model study indicated that the Tyk2 P (a JAK family member) allele could effectively shield animals from the disease by altering Th1 and Th17 signaling (14). STAT3 is vital for Th17 TCS polarization (15), and the deletion of STAT3-related genes reduces pathogenic Th17 TCS proliferation and cytokine production (16). However, the role of JAK-STAT signaling in VKH pathogenesis is still poorly understood, indicating that the global and explicit demonstration of immune interactions in VKH remains unfulfilled.

As a JAK targeting inhibitor, TOFA (Tofacitinib, Pfizer, Inc., New York City, NY) is a U.S. Food and Drug Administration (FDA)-approved oral medication for rheumatoid and psoriatic arthritis and ulcerative colitis (17, 18). Evidence indicates that anti-JAK targeting can downregulate the JAK-STAT signaling pathway in TCS and BCs and reduce the inflammatory response (19). TOFA has been implicated in inhibiting TCS activation by downregulating cytokines produced by Th17 TCS during chronic intestinal inflammation (20). Another study further supported the role of TOFA in suppressing culprit-induced TCS proliferation in vitro and the JAK-STAT pathway in mediating adverse drug reactions (21). TOFA suppresses pathogenic immune responses in vessel vasculitis and minimizes CD4+CD103+ tissue-resident memory TCS with minimal production of IFN-γ, IL-17, and IL-21 (22). However, the therapeutic effects of TOFA on VKH and the molecular and cellular mechanisms of JAK blockade in the human immune system remain unclear. Previous approaches are largely restricted to well-established antibody panels or sorted cell samples based on prior knowledge and, therefore, preclude the comprehensive characterization of TOFA-induced immune modulation in inflammatory diseases. Unbiased high-throughput single-cell RNA sequencing (scRNA-seq) provides unique opportunities to gain novel insights into molecular mechanisms, allowing researchers to explore the overall remodeling effects of targeted therapy in
drug-induced hypersensitivity syndrome (21). Thus, it was desirable to establish a comprehensive atlas of immune modulation to facilitate our understanding of the therapeutic mechanism of TOFA treatment and provide an alternative approach to personalized medicine.

Therefore, we affiliated mass cytometry with time of flight (CyTOF) and scRNA-seq to generate a proteomic and transcriptomic landscape of peripheral blood mononuclear cells (PBMCs) from VKH patients. Furthermore, we compared blood immune cell properties before and after TOFA treatment to explore the therapeutic mechanisms. Our study is the first to treat VKH patients with TOFA to provide a comprehensive profile of its effects on the immune system.

Results

TOFA treatment effect on VKH symptoms and pathological TCs subset ratio

Ten patients with VKH treated by TOFA who had not previously received systemic therapy and were glucocorticoid intolerant were included in this study. We found that a dose (5 mg, bid) of TOFA combined with a peribulbar injection of triamcinolone acetonide was safe and not associated with severe adverse events. According to the optical coherence tomography (OCT) examination, the inflammation gradually resolved with enhanced best-corrected visual acuity (BCVA) after 3 months of treatment (Fig. 1A–B, Supplementary Tables 1-2). TOFA treatment sufficiently controlled VKH symptoms in a similar manner when compared with 18 subjects treated with conventional therapy (systemic glucocorticoids and peribulbar injection of triamcinolone acetonide) (Fig. 1A).

The differentiation of pathological TCs subsets, Th1 and Th17, are mediated by inflammatory cytokines that signal through JAK-STAT pathways. We found that TOFA treatment reduced the percentages of IFN-γ+ Th1 and IL-17A+ Th17 (Fig. 1C–D). Additionally, TOFA treatment increased FOXP3 expression in CD4+ TCs (Fig. 1E), indicating that TOFA treatment reversed Th17/Treg imbalance. We also investigated the ability of TOFA to inhibit cytokine signaling in TCs. As shown in Supplementary Fig. 1A–B, IL-2 induced STAT1 and STAT3 phosphorylation,
and TOFA inhibited both events. TOFA cytotoxicity was assessed using a CCK8 assay, and we found no substantial cytotoxicity of the drug when concentrations less than 10 μM were used (Supplementary Fig. 1C). Our results showed favorable anti-inflammatory activity of TOFA in vitro.

**Excogitation for single cell immunophenotyping on peripheral blood from healthy individuals and VKH patients**

To draft the circulating immune modulation and explore the mechanisms of TOFA in VKH, we conducted single-cell test using blood samples collected from healthy individuals (HC, n = 5) and patients before (VKH, n = 5) and after TOFA treatment (TOFA, n = 5), and wielded CyTOF and scRNA-seq analysis (Supplementary Table 3-4). CyTOF nodes were annotated into three main immune types [natural killer and TCs (NK&TCs), BCs, and myeloid cells (MYEs)] (Supplementary Fig. 1D-E). These populations were reorganized into 23 subsets (Supplementary Fig. 2F). With scRNA-seq, unsupervised analysis authenticated megakaryocytes, CD34+ cells, and three major immune populations (NK&TCs, BCs, and MYEs) according to the expression of canonical markers (Supplementary Fig. 2A). Consistent with CyTOF data, we reclassified these into 25 transcriptionally conventional subsets (Supplementary Fig. 2B-D). The clustering analysis was based on published studies (23, 24). To unveil the transcriptional events implicated in VKH and TOFA treatment, differentially expressed genes (DEGs) between VKH and HC and between TOFA and VKH were identified as “VKH-DEGs” and “TOFA-DEGs,” respectively.

**Reconstituting the circulating cellular ecosystem of VKH**

Based on the subpopulation results, we classified the five immune cell populations and projected a t-SNE diagram using CyTOF. By comparing the ratio between the HC and VKH groups, we noticed an increase in the percentage of monocytes (MCs) (Supplementary Fig. 3A–B), mainly due to the increase of CD14+ classical MCs (CMCs) (Supplementary Fig. 3C). Subsequently, we found that VKH altered the composition and functional protein expression of TCs. Using CyTOF,
we validated the downregulation in naïve TCs (Na) and the increase in effector memory TCs (Tem) among both CD4+ and CD8+ TCs (Fig. 2A–B). The naïve markers (CD45RA and LEF1) were decreased, and the memory and effector marker CD45RO was increased, with diminished CCR4, a Treg marker (Supplementary Fig. 3D). Moreover, VKH increased several effector markers, including the proliferating marker Ki67, exhausted marker CD279, cytotoxic marker CD57, and T-bet (Fig. 2C–D, Supplementary Fig. 3D). Altogether, the results validated the complex cell dynamics in circulation altered by VKH, using CyTOF analysis.

Next, we explored transcriptional patterns of VKH. According to the number of VKH-DEGs, MCs were the subset most affected by VKH (Supplementary Fig. 3E). We investigated the biological impact of the upregulated and downregulated VKH-DEGs via gene ontology (GO) and pathway analysis. The upregulated DEGs in CD4+ TCs were enriched in the IL-6 and JAK-STAT pathways (Fig. 2E). VKH also upregulated multiple pathways involved in leukocyte activation and cytokine signaling, especially in MCs. In addition, the commonly downregulated genes were enriched in oxidative phosphorylation and ATP metabolism (Fig. 2F). We then generated Venn diagrams to identify the interactions of VKH-DEGs in the seven subsets (Fig. 2G) and demonstrated that the commonly upregulated DEGs were DDIT4, NFKBIA, and CXCR4. DEGs related to inflammatory responses, including CXCL8, CCL3, IER2, and TNF, were only upregulated in MCs. Additionally, IL1B and ISG15 were elevated in MCs and conventional DCs (CDCs). DEGs were only elevated in CD4+ TCs, including genes involved in JAK-STAT signaling (PIM1, IL6ST), and TCs activation (GZMA, CD69, and PRF1). Similarly, the upregulated VKH-DEGs specific to BCs were related to JAK-STAT signaling (PIM2 and IL10RA) and BCs activation (IGHG1, IGH1, and TNFRSF13C). In addition, the downregulation of the cellular defense response in CD8+ TCs was driven by KLRC2, EOMES, KLRG1, and SH2D1A (Fig. 2G).

According to the upregulation of JAK-STAT pathways in the VKH group, we explored the levels of genes associated with JAK-STAT signaling in different cells (Fig. 3A). We found that most genes were highly expressed in MCs. The genes that were highly expressed in CD4+ TCs included IL6ST, JAK3, IL7R, and PIM1, and several others related to transcription factors (STAT1,
SOCS3, and STAT5A) (Fig. 3A). The biological scores were calculated to appraise the extent of
the manifestation enhanced by VKH. The JAK-STAT signaling score was similar among subsets
and was upregulated in VKH subjects compared with that in HC subjects (Fig. 3B). Furthermore,
we found that VKH upregulated the inflammatory response score, with MCs showing the highest
inflammatory response scores (Fig. 3C). Finally, we averaged the scores and found that the
mean scores in the TOFA group were significantly higher than those in the control group (Fig.
3B–C). Altogether, we constructed a blood immune cell map of VKH patients using CyTOF and
scRNA-seq. The decrease in the naïve subset and increase in memory and effector subsets, cell
activation upregulation, and the JAK-STAT pathway demonstrates that VKH induces cell-specific
inflammatory states.

Remodeling of the compartment and function of blood NKs and TCs by TOFA
Enhanced lymphocyte function is important for autoimmune diseases. As shown in the violin
plots, the JAK-STAT signaling score was lowest in naïve subsets and higher in highly
differentiated memory and effector subsets (Fig. 4A). By exploring the expression heterogeneity
of JAK-related genes, we found that some genes (PIM1 and IL6ST) were highly expressed in
 naïve subsets, whereas most genes were highly expressed in memory and effector subsets (Fig.
4B). The polarized and activated states of CD4+ and CD8+ TCs in VKH patients indicated the
importance of JAK-STAT signaling in the disease course. Notably, CyTOF validated that the
increase of CD45RO, as well as the decrease of CD45RA, LEF1, and CCR4, were rescued by
TOFA treatment (Supplementary Fig. 4A–B). In addition, TOFA inhibited the expression of
markers related to TCs polarization, such as CD279, CD57, and T-bet. Accordingly, TOFA
treatment led to a decrease in cytotoxic TCs and an increase in the naïve CD4+ and CD8+ TCs
(Supplementary Fig. 4C–D). Moreover, KI67 was downregulated after treatment (Fig. 4C–D).
Collectively, TOFA treatment reversed VKH-induced cell polarization and activation of CD4+ and CD8+ TCs.

Next, we explored the transcriptional patterns of TOFA in NKs and TCs subsets. According to the downregulated TOFA-DEGs number, the subset most altered by TOFA was the CD56<sup>mid</sup> CD57<sup>-</sup> NK (NK2), followed by proliferating TCs (Fig. 4E). Unsupervised analysis revealed that TOFA therapy weakened CD27 expression and enhanced the expression of genes associated with inflammatory activation (*NFKBIA, FOS*), JAK-STAT signaling (*IFNG, IL2RB, TIMP1*, and *CCND3*), and DNA damage (*DDIT3, GADD45B*) in the proliferating subsets (Fig. 4F). In addition, CD8+ TCs and NKs were most affected by TOFA treatment (Fig. 4E). Using CyTOF, we validated that the levels of cytotoxic factors were decreased by TOFA, such as CCL5 and GZMK in NKs and CCL5 and GZMB in CD8+ TCs (Supplementary Fig. 4E–F). We further investigated the transcriptional characteristics modified by TOFA by performing functional analysis. We found that TOFA treatment downregulated various cytotoxicity-related pathways, including cytokine signaling, cell activation and differentiation, especially in NK2, CD8Tem, and CD8CTL (Supplementary Fig. 4G).

The inhibitory effects of TOFA on pathological CD4+ subsets (Th1 and Th17) in vitro are shown above (Fig. 1C-D). Therefore, we explored the transcriptional patterns of TOFA on CD4+ TCs. Functional analyses revealed that the VKH-induced over-presentation of JAK-STAT signaling in CD4+ TCs was reduced by TOFA treatment (Fig. 4G). In the CD4CTL subset, TOFA downregulated leukocyte migration, TCR and MAPK signaling. In addition, cell activation and IL-6 signaling were downregulated, especially in the CD4Tem subset, whereas IL-17 signaling was downregulated, especially in the CD4Na subset (Fig. 4G). Using Venn plots to assess the subset-specific signatures of TOFA, we found that all CD4+ subsets showed decreased expression of genes related to cytokine and JAK-STAT signaling (*STAT1*) (Fig. 4H). Downregulation of the IL-17 signaling pathway in CD4Na was attributed to *HSP90AB1, NFKBIZ, TNFAIP3*, and *TRADD*, and that of the IL-6 signaling pathway in CD4Tem was driven by *JAK3* and *PRDM1* (Fig. 4G–H).

Notably, several inflammation-related genes (*TNFSF10, ISG15*, and *CASP1*) were inhibited by
TOFA in CD4 Treg, indicating that TOFA had a therapeutic effect in reversing inflammatory damage in Treg. Next, we performed an integrative comparative analysis of the VKH- and TOFA-DEGs. We found that genes related to JAK-STAT signaling (JAK1, STAT1, and IL10RA) and TCs activation (CCR7, IRF1, and IL2RG) were increased in VKH and decreased after TOFA treatment (Fig. 4I).

Collectively, TOFA remodeled the compartment and function of blood NKs and TCs, along with decreased markers and processes related to cell polarization, cytotoxic functions, and autoimmune signaling pathways.

**TOFA rescuing of VKH-induced interferon signaling and activation of BCs**

Among the BCs, we found that the JAK-STAT signaling score was lowest in naïve subsets and highest in highly differentiated subsets (Fig. 5A) in accordance with the expression heterogeneity of JAK-related genes (Fig. 5B). We found that the memory subset was increased in the VKH group and downregulated in the TOFA counterpart, and the naïve subset showed the opposite transition, indicating the TOFA reversal effect (Fig. 5C–D, Supplementary Fig. 5A–B). Similarly, using CyTOF, we validated that TOFA treatment reduced the expression of Ki67 and the activation markers (CD27 and CD38) and downregulated the autoimmune-related BCs markers; T-bet and CD11C (Fig. 5E).

Next, we assessed the TOFA-modulated transcriptional signatures of BCs subsets. TOFA treatment decreased the expression of genes related to inflammation, oxidative phosphorylation and BCs activation (Supplementary Fig. 5C). In addition, the downregulated TOFA-DEGs in naïve BCs were enriched in the regulation of lymphocyte activation, antigen processing, and presentation, and the IFN-γ signaling pathway (Fig. 5F). Using Venn plots to appraise the subtype-specific characteristics, we found that all BCs subsets showed decreased S100A9 expression. Besides, we ascertained subtype-specific profiles of TOFA, incorporating IFN-related genes (IFITM1, IFI30, IRF1, and IRF9) in naïve and memory BCs, BCs differentiation-related genes (CD38) in plasma BCs, HLA-DRA and CD74 in autoimmune-related BCs, and
inflammation-related genes (S100A8, STAT1, and HLA-A) in three BCs subsets (Fig. 5G). In addition, downregulation of biological processes indicated that TOFA treatment had extensive modulatory functions in naïve BCs (Fig. 5F–G).

Protein–protein interactions (PPI) are a complex web of functional associations between biomolecules (25). Potential protein complexes can be identified computationally from PPI networks by applying molecular community detection methods (MCODEs), which flag groups of entities interacting in certain patterns (26). Therefore, we performed PPI analysis on DEGs of BCs subsets to explore subset-specific gene networks. The top two signaling pathways corresponding to each MCODE were shown. In the memory BCs, two types of MCODEs were identified, with the most enriched being interferon signaling, antigen processing, and presentation processes (Fig. 5H). Notably, IRF9 had the strongest correlation with other genes, which is vital in BCs activation and inflammatory response mediated by IFN-β and downstream JAK-STAT pathway (27, 28) (Fig. 5H). For naïve BCs, the most enriched MCODEs were in interferon and cytokine signaling (Supplementary Fig. 5D). Additionally, we targeted genes related to three specific pathways among groups. TOFA treatment rescued the VKH-induced upregulation of genes related to the JAK-STAT pathway (IRF9, JAK1, STAT3, and PIM1), antigen processing and presentation, and cytokine signaling (Fig. 5I). Taken together, these findings highlight the rescuing effects of TOFA treatment on BCs polarization and activation, and the signaling pathways related to inflammatory and autoimmune disorders.

**Effect of TOFA treatment on inflammation- and JAK-associated CMCs subset**

Analysis of patients’ data indicated that VKH patients had an increase of MCs and CMCs, suggesting the possible role of MCs in VKH disease. Notably, TOFA treatment decreased the abnormal immune cell changes (Fig. 6A–B). Next, we compared the expression of functional markers in MYEs among three groups. We demonstrated that the upregulated markers in VKH
were decreased after TOFA treatment, including CD14, CD16, and markers related to antigen presentation (HLA-DR and CD11C) (Fig. 6C).

Thereafter, we elucidated transcriptional changes in MYEs. The CMCs subset showed the highest score in inflammatory response and had the widest span in the JAK-STAT signaling score, as indicated in the violin plots (Fig. 6D). By exploring the expression heterogeneity across subpopulations, we found that IFNAR1, IFNAR2, and JAK1 were highly expressed in the plasmacytoid DCs, whereas some genes (JAK3, STAT3, IL6R, IL6ST, PIM1, and CSF3R) were highly expressed in CMCs (Supplementary Fig. 6A). Consistent with patients’ data, MCs (especially CMCs) were the subset most influenced by TOFA (Fig. 6E). Accordingly, we found that TOFA treatment downregulated cytokine signaling, myeloid leukocyte activation, and JAK-STAT signaling, all of which contribute to inflammatory disorders. The modulatory effects were especially prominent in CMCs (Fig. 6F). Next, we separated the TOFA-DEGs on each MYEs subset (Fig. 6G). The expression of inflammation-related genes decreased in all MCs subsets, including CCL3, CCL4, IL1B, TNF, and IER2. In addition, subtype-specific patterns were also identified, including JAK-related genes (STAT1 and PIM1) in CDCs and all MC subsets, PIM3 in CMCs, ISG15 and IFI6 in CMCs and CDCs (Fig. 6G).

It is known that CMCs belong to the first line of immune defense cells and are important antigen-presenting cells recruited to lesion sites during infection or sterile injury and contribute to autoimmune disease development (7, 29-31). However, disease-specific monocyte populations with unique functions and signatures remain largely unknown. Therefore, we re-clustered the CMCs into five subclusters (SC1–5) (Fig. 7A). Both SC2 and SC4 had high expression of several inflammatory genes (IER2 and NFKBIA) and were characterized by the highest inflammatory response score (Fig. 7A–B). In addition, both SC4 and SC5 had higher JAK-STAT signaling scores than other SCs, with PIM3, PIM1, and STAT1 and cytokine receptors (IL7R and IL2RG), respectively (Fig. 7A–B). Notably, the two VKH-expanded subclusters, SC2 and SC4, which were related to inflammatory response and JAK-STAT signaling, were diminished by TOFA treatment (Fig. 7C, Supplementary Fig. 6B). These results suggest that inflammation- and JAK-related
CMCs subgroups are highly associated with VKH disease and TOFA treatment. Seven MCODEs were identified from the downregulated TOFA-DEGs of CMCs, all of which were related to inflammatory response, myeloid leukocyte activation, and JAK-STAT signaling (Supplementary Fig. 6C). Integrative comparative analysis also indicated that the expression of related genes was increased in the VKH group and rescued by TOFA treatment (Supplementary Fig. 6D).

**Demonstration of TOFA rescuing effect via predisposing genes and intercellular communication profiles of VKH**

Genetic factors have been shown to be implicated in the development of VKH (32). The identification of susceptibility genes for VKH can be used as a reliable basis to recognize similarities as well as differences among VKH and other types of uveitis. As such, we first addressed the cellular distribution of VKH susceptibility genes across the PBMCs subclusters by referring to a study employing single nucleotide polymorphisms associated with VKH (33). These are some genes that encode proteins involved in multifarious pathways connected to VKH pathogenesis, including the antigen presentation processes (*HLA-DQA1, HLA-DRB1, HLA-DRA*, and *HLA-DRB5*), inflammatory activation (*CXCL12, FCRL3, MMP9*, and *TRAF5*), cytokine signaling pathways (*TNF, IL23R, and JAK1*), as well as the regulation of immune response (*EGR2* and *PTPN22*). In this study, we first analyzed the expression of the genes in blood immune cells (Fig. 8A) and found that *EGR2, TNF*, and genes related to antigen presentation were mainly expressed in MYE cells, while *JAK1, TNFAIP3*, and *PTPN22* were primarily expressed in lymphocytes (Fig. 8A). We then illustrated how the transcriptional levels of these genes were influenced by VKH or TOFA treatment. We found that *TNF* was increased in MYE cells of VKH group and decreased after TOFA therapy (Fig. 8B). Interestingly, *JAK1* showed the similar variation trends in lymphocytes, as well as *HLA-DQA1* and *TNFAIP3* in almost all types of immune cells. The detailed information combined the published risk loci of VKH susceptibility genes (33) with our results were summarized in Supplementary Table 5. In addition, TNFAIP3 has been involved in the negative feedback regulation of the inflammatory NF-κB pathway, and
its expression has decreased in ocular Behcet's disease, which is another type of uveitis (34).

Employing real-time quantitative PCR, we found that TNFAIP3 gene expression level was increased in VKH relative to other types of uveitis. Similar trends were noted for other gene families involved in the NF-κB signaling pathway, NOD1 and NOD2 (Supplementary Fig. 7A); this was in agreement with other studies (35). These data, therefore, provided an opportunity to shed more light on the potential functional mechanisms for genetic risk loci in VKH disease particularly the cell-specific increase of VKH susceptibility genes, which can be partly suppressed by TOFA treatment.

Furthermore, recent advances in biotechnology provided opportunities to define function- or character-specific cell–cell communications (36). Although immune rescue by TOFA treatment was emphasized in the above results, the specific TOFA-modulated intercellular interactions in human circulating immune system have not been investigated. To identify the intercellular interactions modified by TOFA, we first investigated the intercellular communication between VKH vs. HC and TOFA vs. VKH using the iTALK tool. We focused on the upregulated ligand-receptor pairs in VKH vs. HC and the downregulated ligand-receptor (L-R) pairs in TOFA vs. VKH. We found that VKH increased several interactions between MCs and other cells, which were mainly involved in the inflammatory activation and chemotaxis of MCs to other cells (Fig. 8C). MCs-secreted cytokines and chemokines encoded by IL1B, TNF, CCL3, and CCL4, all of which contribute to autoimmunity and inflammation, may stimulate other cell populations expressing their homologous receptors. TOFA treatment rescued the interactions involving these pairs. Notably, the therapeutic effects of TOFA were also reflected in the reduced communication between inflammatory genes (TNF and IL6) and the corresponding receptors between MCs and other cells (Supplementary Fig. 7B). Additionally, we focused on the VKH-specific cell–cell L-R pairs, which were only detected in the VKH group (Fig. 8D). Functional enrichment analysis
indicated that these pairs were enriched in cell activation, leukocyte migration, and TNF and JAK-STAT pathways, which were driven by TNF- and CCL-related ligand-receptor pairs (Fig. 8E).

Afterward, we explored intercellular signaling using CellChat. This tool detected the TNF signaling pathway in the VKH group but not in the HC or TOFA counterparts, indicating the specific correlation between TNF signaling and VKH (Fig. 9A). MCs were the primary TNF source, which acted in a paracrine manner towards other cells, with the TNF-TNFRSF1B L-R pair being the major driver (Fig. 9A, Supplementary Fig. 7C). The upregulation of TNF in MCs and TNFRSF1B in other cells was rescued by TOFA treatment (Fig. 9B), which was confirmed by the protein level of TNF-α in the serum (Fig. 9C). Moreover, we found that VKH upregulated the CCL signaling pathway mainly among MCs, TCs, and NKs, which were decreased in the TOFA group (Fig. 9D). CCL signaling is involved in cell migration and activation, with the CCL5-CCR1 L-R pair being a major signaling driver (Supplementary Fig. 7D). The expression of related ligands and receptors in immune cells increased in the VKH group and decreased in the TOFA group (Fig. 9E). We also found that several cytokine pathways related to JAK-STAT signaling were altered in specific cells. The IL6 signaling pathway was driven by the intercellular IL6-(IL6R+IL6ST) pairs among cells (Supplementary Fig. 7E–G). Similar results were observed for the IFNG signaling pathway network between MCs and NKs (Supplementary Fig. 7H). IFNG-(IFNGR1+IFNGR2) was the dominant contributor, and the upregulation of receptors in NKs and MCs in VKH was rescued by TOFA treatment (Supplementary Fig. 7I–J). These findings reveal that VKH-induced cell-cell pathways are implicated in inflammatory and autoimmune disorders and highlight the reversal effects of TOFA treatment.

In this study, we explored the cell-specific pathogenesis of VKH. The upregulation of JAK pathway, cell activation and polarization suggested the therapeutic effect of TOFA targeting JAK pathway in VKH. Therefore, we further mined our data to predict other potential therapeutic agents for VKH. Specifically, the upregulated genes in VKH were annotated by Metascape tool. Then, we screened the 32 uveitis-associated genes based on DisGeNET annotation. Finally, we screened the 16 uveitis-associated genes and their targeted drugs by referring to the DrugBank.
These genes were involved in immune cell chemotaxis (CCL3, CCL4, CCL5, CXCL8), T cell activation and differentiation (CXCR4, S100A8), and cytokine pathways (TNF, IFNGR1, JAK1, STAT3). We next explore the cell-specific patterns and found that most of these candidate genes were upregulated in MCs of VKH group, like TNF and STAT3 (Fig. 9F). The upregulation of these genes suggested the potential therapeutic effect of these novel targeted drugs for VKH treatment. For example, the agents targeting TNF pathway (like adalimumab) have been very effective in the treatment of autoimmune diseases and have been the most selling biologics on market (37, 38). The increased expression of TNF in MCs from VKH patients indicated the adalimumab may be the effective therapeutic drug for VKH disease. In addition, ENMD-1198 can directly inhibited STAT3, a key factor in TH17 differentiation and VKH development (39), suggesting the potential applications in future studies. As such, our data may provide a basis for the subsequent studies of these targeted drugs for VKH treatment.

Discussion

Here, we first comprehensively demonstrated the implication of various immune parameters in VKH at proteomic and transcriptomic levels using CyTOF and scRNA-seq. We systematically evaluated the effects of TOFA treatment on VKH according to cell type composition, subtype-specific gene expression, enriched pathways, and intercellular communication. The primary findings of the current study were as follows: 1) The immune dysregulations in VKH were characterized by TCs polarization from naïve to effector, memory, and cytotoxic cells, along with increased inflammation- and JAK-associated MCs, upregulated cell activation, cytokine and JAK-STAT signaling. 2) In vitro, TOFA reversed the Th17/Treg imbalance and inhibited IL-2-induced STAT1/3 phosphorylation. 3) Comparable improvement in clinical symptoms was noted between patients treated with TOFA and those receiving conventional therapy, suggesting that a systemic “glucocorticoid free” strategy is feasible for treating VKH. 4) TOFA treatment reversed the VKH-induced downregulation of naïve subsets and the increase in effector memory subsets among CD4+ and CD8+ TCs, as well as various pathways related to cytokine signaling, JAK-STAT.
pathway, and lymphocyte function. 5) The upregulation of antigen presentation and processing, inflammation activation, and over-presentation of two inflammation- and JAK-related CMCs subpopulations of the VKH group were rescued after TOFA treatment. 6) TOFA rescued VKH-induced upregulation of specific predisposing genes and cell–cell interactions implicated in the inflammatory response, cell migration, and cell activation.

VKH is a severe bilateral granulomatous intraocular autoimmune inflammatory disorder mediated by TCs, characterized by rapid onset, and recurrent inflammation (5). Previous studies on VKH pathogenesis have mainly concentrated on the imbalance between pathological and regulatory TCs (40-42). However, some patients with little response to traditional therapy lack access to effective therapies. Therefore, a more specific understanding of VKH pathogenesis is necessary to facilitate the development of optimized therapies for patients with the disease. This study elucidated the VKH immune landscape with a high resolution and precision using multimodal studies. Our data supported the documented increase of cell polarization in TCs and BCs (43, 44), characterized by increased proliferation, exhaustion, and cytotoxicity. Common VKH-upregulated pathways were involved in leukocyte activation, cytokine signaling, and the JAK-STAT pathway. Particularly, VKH commonly increased DDIT4, NFKBIA, and CXCR4 expression levels. CXCR4 has been shown to contribute to the pathogenesis of Lewy body dementia (45) and multiple sclerosis (46) by promoting TCs recruitment and Th17/Treg imbalance. Combined with the results of VKH-induced cell-specific genes, such as the upregulation of IGHG1, IGHA1, and TNFRSF13C in BCs, VKH induced a proinflammatory and autoreactive status in immune system circulation. The higher expression of genes related to the JAK-STAT pathway and the polarized and activated states of CD4+ and CD8+ TCs indicated the importance of JAK-STAT signaling in VKH disease. In addition, VKH downregulated ATP metabolism and the Treg marker, CCR4, which is specifically expressed by effector Treg cells and suppress aberrant immune responses against self-antigens (47). Furthermore, VKH was characterized by an increased proportion of MCs, especially CD14+ CMCs. The upregulated genes in CMCs were enriched in
cell migration (CCL3, CCL4, and CXCL8) and inflammation (TNF, IL1B, and ISG15). As an important proinflammatory factor, the increased expression of TNF in MCs from patients with VKH could be a significant therapeutic target for the disease (38). Moreover, ISG15 has been identified as a disease-specific gene that is preferentially enriched in the proinflammatory subset of VKH patients (48). These results suggest the vital role of MCs in the development of VKH disease. We mapped the human circulating immune system of patients with VKH using multimodal tools, which were characterized by increased peripheral lymphocyte polarization, cell activation, JAK-STAT signaling, and inflammatory activation.

Early and aggressive systemic corticosteroids are still the mainstream therapy for VKH, notwithstanding immunosuppressive and biological agents, which are not as potent as instant interventions, have been becoming the norm in adjuvant therapy for corticosteroids (8). The ocular and systemic side effects of corticosteroids preclude prolonged use. Knowledge of inflammatory mediators in autoimmune disorders has facilitated the development of novel therapeutics that selectively target individual molecules. As a JAK1/3 inhibitor, TOFA was approved by the FDA for rheumatoid and psoriatic arthritis and ulcerative colitis (17, 18). The effectiveness identified in other autoimmune diseases and the suppression of TCs proliferation are practical foundations for using TOFA as a novel intervention in VKH. We present a comprehensive and integrated circulating immune cell landscape of VKH patients characterized by upregulated cytokine and JAK-STAT pathways. Our study is the first to confirm the validity of TOFA treatment in VKH patients, showing no discrepancy with conventional therapy. The OCT examination revealed that ocular inflammation was gradually resolved with the enhancement of BCVA after TOFA treatment. More importantly, we confirmed a novel and successful “zero systemic corticosteroids” approach, independent of systemic corticosteroids, controlling VKH with minimal side effects, which is a breakthrough in the context of VKH treatment. In addition, the study conducted by Zaka-ur-Rab et al. revealed that the peribulbar injection of triamcinolone acetonide at a dose of 40 mg would result in a detectable triamcinolone acetonide in blood, with
an obvious decrease at 48 h after injection (49). In our study, TOFA was administered orally every day, in contrast to peribulbar injection of 20 mg triamcinolone acetonide once a month, indicating TOFA was the dominant treatment in our study. On the other hand, the blood triamcinolone acetonide level was much lower than the blood level of glucocorticoid that reached significant anti-inflammatory effects after conventional oral prednisolone administration (0.5 mg/kg), as well as the daily physiological dose in glucocorticoid replacement therapy. Moreover, the detectable triamcinolone acetonide level was also much lower than the effective concentration for the inhibition of IL-6 bioactivity (50), B cell response (51), and inflammatory cytokine production of human retinal endothelial cells (52). From the collective evidence, we conclude that the therapeutic effect in the TOFA group is very likely due to the immune regulatory effect of TOFA rather than triamcinolone acetonide.

Currently, targeting JAK-associated pathways using JAK inhibitors such as TOFA has been increasingly applied clinically in various diseases (53). In animal models, TOFA increased Treg numbers and reduced Th17 counterparts (22, 54). Antiviral drugs combined with JAK inhibitors have yielded positive results for rapidly improving COVID-19 symptoms (55). Here, we first revealed the therapeutic effect of TOFA in VKH and comprehensively demonstrated its therapeutic mechanisms. The study demonstrated the heterogeneity of JAK-related genes which were highly expressed in highly differentiated subsets. TOFA treatment inhibited the cytokine and JAK-STAT pathways in vitro and in vivo, and rescued VKH-induced cell polarization and upregulated lymphocyte migration and activation. In addition, the inflammatory environment can result in Treg apoptosis and dampened anti-inflammatory response (56). Notably, several apoptosis- and inflammation-related genes were inhibited by TOFA in CD4+ Treg, indicating its therapeutic effect in reversing inflammatory damage caused by Treg. TOFA also reduced interferon signaling and antigen presentation in BCs, which are important for BCs and TCs activation. In addition, PPI analysis of downregulated memory BCs DEGs showed that IRF9 had the strongest correlation with other genes. Type I IFN-induced BCs activation primarily induces
interferon-stimulated genes via the JAK-STAT pathway involving complexes comprising IRF9

Collectively, these results revealed cell-specific patterns in response to TOFA treatment and
highlighted the therapeutic mechanisms of TOFA in TCs and BCs activation.

Human blood MYEs, including MCs and DCs, facilitate antigen presentation and inflammatory
processes. The initiation and development of inflammatory responses, as in COVID-19 (57) and
vascular inflammation (22), are closely linked to the key role of MCs. In autoimmune diseases,
MCs have been regarded as the primary producers of inflammatory cytokines and mediate
persistent inflammation, which involves the cytokine and JAK-STAT pathways (58-61). MCs from
JAK2V617F-mutated myelofibrosis patients show altered expression of chemokines and cytokine
receptors (62). JAK inhibitors, including TOFA, had therapeutic effects in COVID-19 by
suppressing cytokine storm progress related to MYEs (55, 57). However, the involvement of JAK-
STAT pathway in MCs activation in VKH pathogenesis is unknown, and the impact of JAKs
targeting on a broader spectrum of MCs states remains to be determined. We noticed that MCs,
especially CMCs, which were most strongly affected by VKH and TOFA, exhibited an apparent
inclination towards inflammation. In addition, VKH enhanced monocyte–lymphocyte interactions,
characterized by increased cell activation and chemotaxis of MCs to other cells. This immune
dysregulation was rescued by TOFA treatment. With the advantage of the single-cell method, we
re-clustered CMCs and identified two subgroups that are strongly associated with VKH disease
and response to TOFA treatment. The two subclusters which were increased in VKH patients and
decreased after TOFA treatment, one had high inflammatory gene expression (IL1B and IER2),
and the other had high JAK-related gene expression (PIM1 and STAT1). The previous rationale
for using TOFA to treat inflammatory diseases relied on the central role of TCs in disease
pathogenesis and the effective inhibition of JAK inhibitors on TCs activation (21). Thus, we
demonstrated the therapeutic effect of TOFA in VKH patients and broadened our knowledge of
the therapeutic mechanisms of TOFA treatment on circulating MCs.
Nevertheless, there were some limitations to our study. All patients included in this study have not received systemic glucocorticoid therapy before, while the patients in TOFA group were intolerant to systemic glucocorticoids. A small sample size was another one. Therefore, the findings might not be universally applicable to all types of patients with VKH.

In summary, we determined the therapeutic effect of TOFA on VKH disease and explored its action on the immune system using single-cell approaches. To delineate the pivotal cellular and molecular differences before and after TOFA treatment using CyTOF and scRNA-seq, such as the JAK-STAT pathway in effector CD4+ TCs, BCs, and inflammatory MCs, we explored the potential contributions of TOFA in reducing inflammation, cellular polarization, and autoreactive signatures. The multimodal adhition of single-cell technique conducted in this study furnishes a comprehensive profile of the immune pathogenesis of VKH and novel insights into the feasibility and therapeutic mechanism of TOFA treatment for VKH patients.

Materials and Methods

Human subjects and ethics statement

To explore the therapeutic effect of tofacitinib (TOFA) as initial treatments in VKH patients, we performed a retrospective study of 28 VKH patients (Supplementary Table 1). All patients were diagnosed based on the disease manifestations, and the results of standard coherent optical tomography, and fluorescein angiography, according to the Revised Diagnostic Criteria (RDC) of VKH disease (63). Only patients first diagnosed with VKH who had not received systematic treatment were reviewed. Medical records of VKH patients treated with conventional therapy (CT, refers to systemic glucocorticoids plus peribulbar injection of triamcinolone acetonide) or systemic tofacitinib therapy plus peribulbar injection of triamcinolone acetonide at 3 months of follow-up were analyzed. In CT group, 18 VKH subjects were enrolled and glucocorticoids were administrated from an initial oral 1.2 mg/kg and a gradually decreased dose combined with a peribulbar injection of triamcinolone acetonide (20 mg) monthly. In TOFA group, 10 subjects
diagnosed with VKH had not previously received systemic conventional therapy because they
could not tolerate glucocorticoids. Tofacitinib was orally administered at 5 mg, twice a day,
combined with a peribulbar injection of triamcinolone acetonide (20 mg) monthly. No remarkable
complications were observed in either group. Eyesight was tested, and an OCT examination was
performed every month to assess disease severity. In addition, five healthy participants met the
following criteria were included for the study: physical and psychological health; no clinically
significant abnormalities in blood chemistry; no medication, smoking, obesity.

Study protocol of single-cell analysis

Blood samples of VKH patients were obtained at the day before and one month after starting
tofacitinib treatment. Five samples each from the HC group, VKH group (before TOFA treatment),
and TOFA group (after TOFA treatment) were included for single-cell analysis. PBMCs were
isolated by standard density gradient centrifugation. Trypan Blue was used to identify the viability
and quantity of PBMCs; cell viability of all samples exceeded 90% with > 1 × 10^7 viable cells. A
proportion of PBMCs was allocated for scRNA-seq analysis and another was used for mass
cytometry. To elucidate the impacts of VKH and TOFA on blood immune cells, we measured
single-cell protein expression using a 39-marker CyTOF panel and RNA level using scRNA-seq
(n = 15; Supplementary Table 2-4). By combining CyTOF and scRNA-seq, we created a
comparative atlas detailing the impact of VKH and TOFA on cell type distribution, gene
expression changes, and cell-cell interaction analyses.

scRNA-seq
data alignment, processing, and sample aggregation

Single-cell suspensions were transformed into barcoded scRNA-seq libraries using the Chromium
Single Cell 5′ Library (10X Genomics, Genomics chromium platform Illumina NovaSeq 6000),
Gel Bead and Multiplex Kit, and Chip Kit (10X Genomics). According to the manufacturer’s
instructions, single-cell RNA libraries were prepared using the Chromium Single Cell 5′ v2
Reagent Kit (120237; 10X Genomics). FastQC software was used to check the quality of the library. Sequencing data was initially processed using CellRanger software (version 4.0; 10X Genomics). The count pipeline in the CellRanger Software Suite was used to demultiplex and barcode the sequences. Based on the calculation of the single-cell expression matrix by CellRanger, filtration, normalization, linear dimensionality reduction, selecting the dimension of dimensionality reduction for subsequent analysis, clustering, nonlinear dimensionality reduction visualization and differential gene expression analysis were performed using the Seurat package (version 3.0). We removed the cell population that expressed HBB, HBA1, and several light and heavy chain transcripts, which are considered erythrocyte contamination, before filtration using the Seurat package. Next, cells with less than 200 detected genes and a mitochondrial gene ratio greater than 15% were excluded. After quality control, a total of 15 libraries were sequenced, and 160,176 cells (HC: 59,350 cells; VKH: 54,720 cells; TOFA: 46,106 cells) were analyzed in subsequent studies.

**Dimensionality reduction and clustering analysis**

The "NormalizeData" function was used to logarithmically normalize the counts per cell (1 + counts per 10,000). The "FindVariableGenes" function in Seurat extracted the top 10 most variable genes with the default parameters. Dimensionality was implemented with the "RunPCA" function. Significant clusters were identified by the "FindNeighbors" and "FindClusters" functions at an appropriate resolution. The 2-dimensional t-SNE algorithm based on "RuntSNE" function was used to visualize cells. Marker genes of each significant cluster were identified by "FindAllMarkers" function.

**Differential expression analysis**

For each cell type between different groups, the Wilcoxon rank-sum test implemented in the "FindMarkers" function of the Seurat package (version 3.0) was used for differential expression
analysis. After identifying DEGs after pairwise comparison, VKH-related DEGs dataset and VKH-related DEGs were filtered and established (adjusted P-value < 0.05, |Log2FC| > 0.25).

**Analysis of gene functional enrichment**

The Metascape webtool (www.metascape.org) (64) was used to perform GO biological process and pathway analysis, and protein-protein interactions, which allowed us to visualize the functional patterns of DEGs and conduct statistical analysis. We visualized the top 10 of 30 VKH or TOFA-related terms using the ggplot2 R package. In addition, we performed the drug discovery bioinformatics analysis based on the Metascape webtool. After gene annotation of DisGeNET and DrugBank databases, 16 uveitis-associated genes and their targeted drugs were screened to predict other potential therapeutic for the VKH treatment (Supplementary Table 6).

**Scoring of biological processes**

By calculating the mean normalized expression of corresponding genes, individual cells were scored for their expression of gene signatures which represented certain biological functions. Functional signature with the full gene list is provided in Supplementary Table 7. For instance, the inflammatory response score was measured by calculating the mean expression of genes in the GO term “inflammatory response” (GO: 0006954). Genes related to JAK-STAT pathway were obtained from the KEGG pathways dataset “Jak Stat Signaling Pathway” (PW_0000209).

**Determination of cell-cell interactions**

With the help of CellChat (https://github.com/sqjin/CellChat) R packages and iTALK (https://github.com/Coolgenome/iTALK), cell-cell communication between different cells was predicted based on the scRNA-seq data. iTALK tool was also applied to analyze and visualize the differences in cellular communication between different groups. If the ligands and receptors were not detected, communication is considered to be absent. Thus, VKH-specific cell-cell L-R pairs, which were only detected in VKH group, were selected. TBtools (www.tbtools.com) was used to
normalize the data and draw a heatmap. In addition, we used CellChat R package to analyze and visualize signaling pathway networks among groups with default parameters.

**Mass cytometry**

**Antibodies and reagents**

Monoclonal anti-human antibodies for mass cytometry were either acquired preconjugated with heavy metal isotopes (Fluidigm, South San Francisco, CA) or conjugated via the MaxPar X8 Chelating Polymer Kit (Fluidigm). The 39 antibodies were used to recognize immune cells and detect the markers expression (Supplementary Table 4). The following steps were adapted from the published article (65).

**Live cell barcoding and surface staining**

To decrease inter-sample staining variability, sample processing time, and antibody consumption, a live cell barcoding methodology was applied. 0.5 μmol/L viability dyes (cisplatin-195pt; #201064; Fluidigm) was applied to stain the barcoded and combined samples, vortexed for 2 min at room temperature (RT), and then terminated the reaction with Maxpar Cell Staining Buffer on a rotating shaker (400 rcf) at RT. Then, on a rotary shaker (500 rpm), 1.6% paraformaldehyde in PBS was used to wash and fix the cells for 10 min at RT. To slow the fixation reaction, pre-cooled Maxpar Cell Staining Buffer was applied to resuspend the cells, and then washed twice with PBS/bovine serum albumin and once with double-distilled water. Finally, resuspend the cells in 400 μL surface antibody mixture and incubate at 37 °C for 30 min on a rotating shaker (500 rpm) for surface staining. Then the samples were stored overnight at 4 °C in freshly diluted 2% formaldehyde in PBS which contained 0.125 nmol/L iridium 191/193 intercalator (Fluidigm, 201192).

**Intracellular factor staining**
The cells were washed twice with permeabilization buffer [0.5% saponin, 2% bovine serum albumin, and 0.01% sodium azide (all Sigma-Aldrich) in PBS]. Then resuspended the cells in 50 μL of intracellular antibody mixture in permeabilization buffer for 1 h on a rotary shaker at 4 °C. Then washed the samples; removed the supernatant, and resuspended the cells in 1X iridium intercalator solution (Fluidigm) overnight. At last, the sample was washed twice with PBS/bovine serum albumin and once with double-distilled water before acquisition.

Mass cytometry acquiring, processing and quality control

At an event rate of < 400 /s, CyTOF data were acquired from a SuperSampler fluidics CyTOF2 system (Victorian Airships, Alamo, CA), and then normalized with the help of Helios normalizer software (version 6.7.1016; Fluidigm). The CyTOF2 mass cytometer (Fluidigm) was quality controlled and tuned daily. Cytobank software (version 7.0; https://mtsinai.cytobank.org) was applied to deconvolute barcoded samples and filter cross-sample doublets. Cytobank was applied to sequentially remove calibration beads, dead cells, debris, and barcodes of CD45+ PBMCs based on event length and live cell (195Pt) and DNA (191Ir and 193Ir) channels. Then, the FCS files were exported for downstream analysis. All cytometry data were converted to an inverse hyperbolic sine (arcsinh) function (mass cytometry: cofactor of 5) using R.

Mass cytometry visualization and clustering

FlowCore was used to read and process the FCS files for further analysis. For samples with over 12,000 cells, we selected 12,000 cells randomly to ensure that samples were equally representative. The CATALYST R package was applied to integrate data for analysis. To identify specific populations, all FlowSOM-based clustering and subclustering were performed on the dataset. Mass cytometry datasets from all individuals of each cell type were created for analysis. The detailed cell counts are provided in Supplementary Table 8.

Flow Cytometry of PBMCs in vitro
After isolation, PBMCs \((3 \times 10^5)\) were cultured in the presence of anti-CD3/CD28 in a 1:1 ratio and treated with TOFA (0.08–2 µM) for 2 days. To investigate the intercellular cytokines in TCs, hemocytes were stimulated by a leukocyte activator, incorporating phorbol myristate acetate ionomycin (500 ng/mL; Sigma-Aldrich) and (PMA, 50 ng/mL; Sigma-Aldrich, St. Louis, MO), and then inhibited by brefeldin A (1 µg/mL; Sigma-Aldrich) in a 5% CO\(_2\) environment at 37 °C for 4 hours. Thereafter, processed cells were stained with anti-human CD3 Brilliant Violet 421 (BioLegend, San Diego, CA), anti-human CD8 Pe-cy7 (BioLegend), and anti-human IFN-γ APC (BD Biosciences, Franklin Lakes, NJ) for Th1 cell analysis, anti-human IL-17A PE (BioLegend) for Th17 cell analysis, and anti-human FOXP3 PE (BioLegend) for Treg analysis. The labeled cells were measured using a BD LSR Fortessa flow cytometer (BD Biosciences). Ultimately, the harvested data were analyzed with FlowJo software (version 10.0; TreeStar, Ashland, OR).

**Phosphospecific Flow Cytometry (Phospho-Flow Analysis)**

PBMCs \((3 \times 10^5)\) were pretreated with TOFA (0.08–2 µM) and cultured in the presence of anti-CD3/CD28 in a 1:1 ratio for 2 days. Then the cells were stimulated using IL-2 (200 U/mL) for 30 minutes. The cells were fixed for 20 minutes at RT with 4% paraformaldehyde. Washed cells were permeabilized in ice-cold 75% methanol for 30 minutes, and simultaneously incubated with the following antibodies: anti-human CD3 Brilliant Violet 421, CD8 Pe-cy7, p-STAT1 (tyr701) PE, and p-STAT3 (tyr705) Alexa Fluor® 488 (BioLegend). Flow cytometry analysis was performed using a BD LSR Fortessa flow cytometer (BD Biosciences). Ultimately, the harvested data were analyzed with FlowJo software (version 10.0 TreeStar). STAT phosphorylation was appraised in CD3+ and CD8 + TCs.

**Cell viability assay**

PBMCs \((5 \times 10^3/{\text{well}})\) were seeded into a 96-well plate, treated with various concentrations of TOFA (0.08–10 µM), and incubated at 37 °C in a humidified incubator under 5% CO\(_2\) for 24 h.
Cell viability was determined using a Cell Counting Kit-8 (CCK-8), according to the manufacturer’s protocol.

**RNA isolation and real-time quantitative PCR**

Total RNA from PBMCs was extracted by Trizol reagent (Invitrogen), and then quantified with a NanoDrop spectrophotometer (NanoDrop Technologies). Next, cDNAs were synthesized using the PrimeScript™ RT Master Mix (Perfect Real Time, TaKaRa Bio Inc.). Real-time quantitative PCR was performed using SYBR Premix Ex Taq™ II (TaKaRa Bio Inc.) in accordance with the manufacturer’s instructions. Primer sequences were as follows: human *GAPDH* (Forward: 5′-TCGGAGTCAACGGATTTGGT-3′, Reverse: 5′-TTCCCGTTCTCAGCCTTGAC-3′); human *TNFAIP3* (Forward: 5′-CACGCTCAAGGAAACAGACA-3′, Reverse: 5′-CATGGGTGTGTCTGTGGAAG-3′); human *NOD1* (Forward: 5′-CAGAGTCTCACCCCCACATT-3′, Reverse: 5′-CGGCCGAGAAGTAGTCATTC-3′); human *NOD2* (Forward: 5′-GCGCGATAACAATATCTCAGA-3′, Reverse: 5′-CAGAGTTCTTCTAGCATGACG-3′). The relative mRNA levels of *TNFAIP3*, *NOD1*, and *NOD2* were determined using the 2−ΔΔCt method and the level of *GAPDH* mRNA was used as a loading control.

**ELISA analysis**

After PBMC isolation, sera were collected from the samples and stored at -80 °C for further analysis. Serum TNF-α levels were determined using an ELISA kit (#88-7346-88; Invitrogen, Carlsbad, CA).

**Statistics and reproducibility**

The cell percentages between the VKH and HC groups and TOFA and VKH groups were compared using two-tailed unpaired Student’s *t*-test and two-tailed paired *t*-tests, respectively, and data analysis and presentation were performed using GraphPad Prism (version 8.0.2; GraphPad Software Inc., La Jolla, CA). To compare the markers or genes at protein levels among
groups, a two-sided Wilcoxon test, which was accomplished in the function "compare_means" of the ggpubr R package using default parameters, was used to calculate the P-value. When computing the GO biological process and pathway, P-values were obtained through a hypergeometric test with default parameters in the Metascape webtool. Details on the size of biological replicates and assays are provided in each figure legend. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

**Study approval**

The study was registered in the clinical trial (ID: ChiCTR2000030237) and approved by the Ethics Committee of Zhongshan Ophthalmic Center, China (ID: 2020KYPJ124). Written informed consent was obtained from all participants and all procedures were performed according to the International Ethical Guidelines for Research Involving Human subjects, as stated in the Declaration of Helsinki.

**Data availability**

The scRNA-seq data is deposited in the Genome Sequence Archive in BIG Data Center, Beijing Institute of Genomics (BIG, https://bigd.big.ac.cn/gsa-human/), Chinese Academy of Sciences, under the Project Accession No. PRJCA009601 and GSA Accession No. HRA002400. Experimental protocols and the data analysis pipeline used in scRNA-seq follow those described on the 10X Genomics and Seurat official websites.

**Author Contributions:** W.S. and Y.Z. designed the study; X.L., Q.J. and J.L. led the bioinformatic analyses; X.L. and S.Y. performed the experiments; X.L., Q.J., J.L. and S.Y. took care of subjects and provided the clinical information; Z.H., R.D., T.T., Z.L., and R.J. provided intellectual input and comments into this study; X.L., Q.J. and J.L. wrote the paper. All authors have read and approved the final manuscript.

**Competing Interest Statement:** The authors declare no conflict of interest.
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References


**Figures and Figure legend**

**Figure 1**

A. The best-corrected visual acuity from baseline to 1, 2 and 3 months after using systemic tofacitinib (10 individuals, 20 eyes) and glucocorticoid (18 individuals, 36 eyes) respectively.

B. The representative OCT examination of pre- or post-treatment.

C. The representative OCT examination of pre- or post-treatment.

D. The flow cytometry histograms (left) and box plots (right) showing the percentage of IFN-γ (C), IL-17A (D), FOXP3 (E) in CD3+CD8− TCs (n = 6/group). Significance in C-E was calculated using two-tailed unpaired Student's t-test; **P < 0.01.
Figure 2. Reconstitution of the circulating cellular ecosystem by VKH.

A. The flow cytometry histograms showing the expression of CD45RA and CD45RO on CD4+ Tcs, and the percentage of CD4Na and CD4Tem in CD4+ Tcs between HC and VKH groups (n = 5/group).

B. The flow cytometry histograms showing the expression of CD45RA and CD45RO on CD8+ Tcs, and the percentage of CD8Na and CD8Tem in CD8+ Tcs between HC and VKH groups (n = 5/group).

Violin plot showing the expression of KI67, CD279, CD57 in CD4+ (C), CD8+ (D) Tcs between HC and VKH groups in CyTOF.

Representative gene ontology (GO) biological process and pathways enriched in upregulated (E) and downregulated (F) VKH-DEGs among immune subsets.
G. Venn diagram showing the interactions of upregulated (left) and downregulated (right) VKH-DEGs among immune subsets.

Significance in A, B was calculated using two-tailed unpaired Student's t-test; *P < 0.05, **P < 0.01, ****P < 0.0001.
Figure 3

A. The heatmap showing the levels of genes associated with JAK-STAT signaling among immune subsets.

B. Violin plot showing the JAK-STAT signaling scores among different cells and samples. The scores were averaged between HC and VKH groups (n = 5/group).

C. Violin plot showing the inflammatory response scores among different cells and samples. The scores were averaged between HC and VKH groups (n = 5/group).

Significance in B, C was calculated using two-tailed unpaired Student’s t-test; **P < 0.01, ***P < 0.001.

Figure 3. VKH induced the activation of JAK-STAT signaling and inflammatory response.
Figure 4. TOFA treatment downregulated cell activation of NKs and TCs.

A. Violin plot showing the JAK-STAT signaling score among NK&T subsets.
B. The heatmap showing the levels of genes associated with JAK-STAT signaling among NK&T subsets.
Violin plot showing the expression of Ki67, CD279, CD57 in CD4+ (C), CD8+ (D) TCs between HC and VKH groups in CyTOF.

E. The up- and down-regulated TOFA-DEGs among NK&T subsets.

F. Volcano plot showing TOFA-DEGs of proliferating TCs.

G. Representative GO biological process and pathways enriched in downregulated TOFA-DEGs among CD4+ TCs subsets.

H. Venn diagram shows the interactions of downregulated TOFA-DEGs among CD4+ TCs subsets.

I. Violin plot showing the expression of JAK1, STAT1, IL10RA, CCR7, IRF1 and IL2RG in CD4+ TCs among HC, VKH and TOFA groups in scRNA-seq.
Figure 5. TOFA treatment downregulated cell activation of BCs.

A. Violin plot showing the JAK-STAT signaling score among BCs subsets.
B. The heatmap showing the levels of genes associated with JAK-STAT signaling among BCs subsets.
C. t-SNE plot of BCs subsets among three groups.
D. The percentage of NBC in BCs or PBMCs between HC and VKH groups (n = 5/group).
E. Violin plot showing the expression of Ki67, CD27, CD38, CA11C and T-bet in BCs among three groups in CyTOF.

F. Representative GO biological process and pathways enriched in downregulated TOFA-DEGs among BCs subsets.

G. Venn diagram shows the interactions of downregulated TOFA-DEGs among BCs subsets.

H. The gene network showing protein-protein interaction (PPI) analysis of downregulated TOFA-DEGs in MBC subset.

I. The heatmap showing the levels of genes associated with specific pathways in BCs among HC, VKH and TOFA groups.

Significance in D was calculated using two-tailed paired t-test; *P < 0.05, ****P < 0.0001.
Figure 6. TOFA treatment downregulated cell activation of MYEs.

A. t-SNE plot of immune cells in VKH and TOFA groups.
B. The percentage of MCs and CMCs in PBMCs between HC and VKH groups (n = 5/group).
C. Violin plot showing the expression of CD14, CD16, HLA-DR and CD11C in MYEs among HC, VKH and TOFA groups in CyTOF.
D. Violin plot showing the inflammatory response score and JAK-STAT signaling score among MYEs subsets.
E. The up- and down-regulated TOFA-DEGs in MYEs subsets.
F. Representative GO biological process and pathways enriched in downregulated DEGs among MYEs subsets.
G. UpSet Plot showing the integrated comparative analysis of downregulated TOFA-DEGs in MYEs subsets.

Significance in B was calculated using two-tailed paired t-test; *P < 0.05, **P < 0.01, ****P < 0.0001.
Figure 7. TOFA treatment downregulated cell activation of CMCs.

A. The heatmap showing the scaled expression of discriminative gene sets of the CMCs-SCs (left), and violin plot showing the expression of specific genes among CMCs-SCs (right).

B. Violin plot showing the inflammatory response score and JAK-STAT signaling score among CMCs-SCs.

C. t-SNE plot of CMCs-SCs among three groups.
Figure 8. TOFA treatment rescued the susceptibility genes and cell-cell interaction influenced by VKH.
A. The heat map representing scaled expression levels of VKH susceptibility genes (n=21) across the 23 immune subclusters.

B. The heat map representing the variation trends of VKH susceptibility genes across the 23 immune subclusters. The expression differences of related genes influenced by VKH and TOFA treatment are indicated by color. Asterisks indicate significant differences in gene expression between VKH and HC, or between TOFA and VKH. The violin plot shows the relative level of JAK1 in CD4Na subset among the three groups.

C. Circle plot showing the upregulated L-R pairs in VKH/HC comparison.

D. Venn diagram showing the analysis of VKH-specific cell-cell L-R pairs.

E. Representative GO biological process and pathways enriched in VKH-specific cell-cell L-R pairs.
Figure 9. TOFA treatment reduced the inflammatory cytokine signaling influenced by VKH.

A. Circle plot showing TNF signaling pathway network only in VKH group.

B. The heatmap showing the levels of TNF and TNFRSF1B in immune cells among groups.

C. Protein level of TNF-α in serum of HC, VKH and TOFA groups detected by ELISA.

D. Circle plot showing CCL signaling pathway network in HC, VKH and TOFA groups.

E. The heatmap showing the levels of ligands and receptors related to CCL signaling in immune cells among groups.

F. UpSet Plot showing the cellular distribution of 16 candidate genes among immune subsets.

Significance in C was calculated using two-tailed unpaired Student's t-test; ***P < 0.001, ****P < 0.0001.