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Next generation therapeutic modality to cure autoimmune diseases

Edited by
Ce Wang, Guobao Chen, Qi Wan, Feng Dong, Timothy Radstake, Asif Amin Dar and Sergio Piñ Piñeiro

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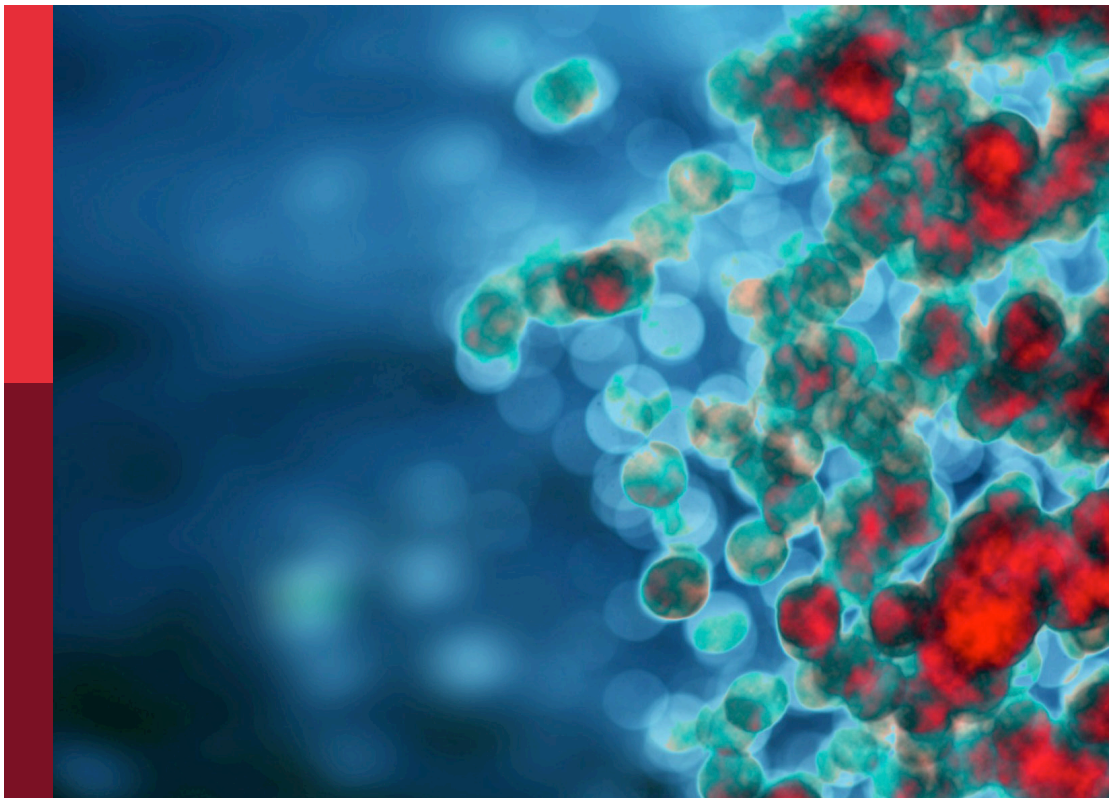

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Next generation therapeutic modality to cure autoimmune diseases

Topic editors

Ce Wang — AbbVie, United States
Guobao Chen — AbbVie's Cambridge Research Center, United States
Qi Wan — AbbVie's Cambridge Research Center, United States
Feng Dong — AbbVie, United States
Timothy Radstake — Radboud University, Netherlands
Asif Amin Dar — Children's Hospital of Philadelphia, United States
Sergio Piñ Piñeiro — Fundación Rioja Salud, Spain

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EDITED AND REVIEWED BY
Raphaela Goldbach-Mansky,
National Institute of Allergy and Infectious Diseases (NIH), United States

*CORRESPONDENCE
Guobao Chen
guobao.chen@abbvie.com

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Editorial: Next generation therapeutic modality to cure autoimmune diseases

Guobao Chen*, Ce Wang, Qi Wan, Feng Dong and Timothy Radstake

Cambridge Research Center, AbbVie Inc, Cambridge, MA, United States

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Editorial on the Research Topic
Next generation therapeutic modality to cure autoimmune diseases

Autoimmune diseases pose a major medical challenge as the immune system erroneously targets the body's own tissues, resulting in persistent inflammation and significant health consequences. The traditional treatment regime generally suppresses the immune system broadly, offering only symptomatic relief and increasing susceptibility to infections. However, recent strides in understanding the immune system coupled with breakthroughs in technology have sparked a new wave of treatments aimed at specific pathogenic pathways to restore balance and potentially offer a long-lasting cure without compromising overall immune function. Recent advancements in technologies such as stem cell therapies, chimeric antigen receptor (CAR) T cell therapies, targeted protein degradation approaches, and nucleotide-based therapies, hold the promise of drug-free remission and potential cures by focusing precisely on pathogenic cells without overall immune suppression.

To encourage more researchers to work towards novel therapies that could potentially bring cures to autoimmune disease patients, a Research Topic on *Next Generation Therapeutic Modality to Cure Autoimmune Diseases* was proposed and hosted by a group of AbbVie scientists working on novel modalities and joined by a few co-editors from other organizations in 2024/2025. This Research Topic has attracted many submissions on different novel drug modalities and resulted in 11 successful publications covering topics from novel small molecule drugs to different types of cell therapies, that are revolutionizing the therapeutic options for patients and challenging the status quo of standard care.

Although small molecule drugs are considered as traditional drug modalities, new class of small molecule modulators are still emerging as novel immune system regimens to reach immune balance in a milder way. There are 6 publications focusing on novel small molecule modulator approaches, covering from novel small molecule discovery platform to novel classes, combinations and novel analytic methods to connect drugs with new indications. The novel small molecule discovery platform designed by Dada et al., the Sulfur-Fluoride Exchange (SuFEx) click chemistry-based high-throughput medicinal chemistry (HTMC)

platform used a "Direct-to-Biology" approach to generate a focused library of tamoxifen analogs and screened them in a cell-based pseudo-Ebola virus infection assay, that greatly shortened the timeline for drug discovery. Li et al. have examined the therapeutic potential of coumarins, a novel class of aromatic natural products capable of modulating immune cells and regulating inflammatory cytokines. This research highlights coumarins' promise in treating autoimmune diseases like type 1 diabetes, ulcerative colitis, systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis by affecting key signaling pathways. This review provides the insight into the connection between coumarins and autoimmune diseases and enable the discovery of effective and safe drugs for autoimmune diseases. Similarly, Jonić et al. reported their discovery of a new class of fluorescent aryl hydrocarbon receptor (AHR) ligands, AGT-5, that promoted a general immunosuppressive environment in their mouse model in the pancreas and small intestine lamina propria at the early phase of disease, and thereby inhibit the severity of Type 1 diabetes (T1D) in mice. On the other hand, Zhang et al., reported the potential expansion of abrocitinib, an oral small-molecule Janus kinase 1 (JAK1) inhibitor, to be used for Lichen amyloidosis (LA) associated with severe atopic dermatitis (AD). A novel combination of low-dose cyclophosphamide with Chinese herbal medicine Shuli Fenxiao formula was reported by Du et al., for the treatment of intermediate-to-high risk primary membranous nephropathy (PMN), which provided an efficacious and safe option for intermediate-to-high risk PMN patients, particularly elderly patients with contraindications to corticosteroid use or those with refractory disease. Lastly, Fu et al. applied drug-target Mendelian Randomization (MR) to study IL-6 receptor inhibitor effects and utilized novel analytic methods to explore the therapeutic potential across multiple diseases.

While cell therapies were not initially a primary focus for treating autoimmune diseases, recent advancements have led to increased interest and research in this area with 5 publications recruited in this Research Topic. Traditionally, autoimmune diseases have been managed with immunosuppressive medications and other conventional treatments. However, cell therapies, which involve using living cells to modulate the immune system or regenerate damaged tissues, are gaining more tractions as potential options for treating autoimmune conditions. Research is ongoing to explore their effectiveness and safety in this context. Bode et al. reported a new therapeutic option: using genetically engineered beta cells to shape autoimmunity, giving valuable insights for future therapeutic advancements to treat and cure Type 1 diabetes (T1D). Arve-Butler and Moorman provided a comprehensive examination of tolerogenic adjuvants currently utilized in tolerogenic vaccines, which induce antigen-specific tolerance by promoting tolerogenic antigen presenting cells, regulatory T cells, and regulatory B cells, and/or by suppressing/depleting antigen-specific pathogenic T and B cells. In addition, Wong et al., Liang et al. and Bulliard et al. further illustrated the potential of novel cell therapies to cure autoimmune diseases.

Hematopoietic stem cell transplant (HSCT) therapies are emerging as a rescue approach for patients in whom many other immunomodulatory therapies are not successful. Wong et al. reported a detailed clinical and electrophysiological response to HSCT in a patient with autoimmune retinopathy (AIR). After undergoing HSCT, symptoms of photopsia rapidly discontinued, evidenced by improvement of retinal function and visual field recovery via objective electroretinography and optical coherence tomography. Additionally, a 22-month follow-up demonstrated a sustained clinical response. This case report highlights the novelty that HSCT may work as a therapeutic method for selected patients of refractory AIR that failed to respond to traditional immunotherapy.

Although cell therapies are emerging to address the unmet needs that are not covered by traditional therapies, such as small molecule immune suppressants and biologics, there are critical differences between different types of cell therapies. Liang et al. summarized the recent advances in cell therapies and delineated the differences between stem cell transplantation and CAR T therapy. The authors listed the advantages of targeted cell therapy over stem cell therapy on specific elimination of pathogenic cells directly in disease tissue to achieve complete immune reset, exemplified by the success of CD19 CAR T therapies in the clinic on achieving complete immune reset in lupus and a few other B-cell driven autoimmune diseases. The chimeric autoantibody receptor (CAAR) T cell approaches were also discussed, which could potentially deplete only specific autoantibody producing cells, including B cells and plasma cells. The authors evaluated the status of CAR T therapies against different target cells and different disease types and listed the potential and limitations of these therapies for immune mediated inflammatory diseases.

With the latest advancements in the rapidly evolving cell therapies, Bulliard et al. further summarized and discussed current innovative advancements in regulatory T cell (Treg)-based cell therapies in offering targeted and durable disease remission for autoimmune and immune mediated diseases. In addition to highlighting the groundbreaking advances in CD19 CAR T cell therapy for B cell-mediated autoimmune diseases, the authors explore the therapeutic potential of Type 1 and Foxp3+ CAR Treg cells. These strategies aim to restore immune homeostasis locally and softly, minimizing adverse effects associated with generalized immunosuppression. This review emphasizes the transformative potential of cutting-edge therapeutic strategies to redefine treatment paradigms, address critical unmet needs, and advance toward curing autoimmune diseases.

In summary, emerging novel drug modalities and innovative therapies are leading the revolution of the treatment regimens for autoimmune diseases. These innovative therapies hold the promise of drug-free remission and potential cures by focusing precisely on pathogenic cells or specific mechanisms without overall immune suppression. With the advancements in novel technologies, the

standard of care will be advancing more and more towards the cures for all autoimmune diseases.

Author contributions

GC: Writing – original draft. CW: Writing – review & editing. QW: Writing – review & editing. FD: Writing – review & editing. TR: Writing – review & editing.

Conflict of interest

GC, CW, QW, FD, and TR were employed by the company AbbVie Inc.

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EDITED BY
Guobao Chen,
AbbVie's Cambridge Research Center,
United States

REVIEWED BY
François-Xavier Mauvais,
Université Paris Cité, France
Gustaf Christoffersson,
Uppsala University, Sweden

*CORRESPONDENCE
Kevin Bode
k-bode@web.de
Peng Yi
peng.yi@joslin.harvard.edu

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Beta cells deficient for *Renalase* counteract autoimmunity by shaping natural killer cell activity

Kevin Bode[1,2,3]*, Siying Wei[2,3], Isabella Gruber[2], Jian Li[2,3], Stephan Kissler[1,3,4] and Peng Yi[2,3,4]*

[1]Section for Immunobiology, Joslin Diabetes Center, Boston, MA, United States, [2]Section for Islet Cell and Regenerative Biology, Joslin Diabetes Center, Boston, MA, United States, [3]Department of Medicine, Harvard Medical School, Boston, MA, United States, [4]Diabetes Program, Harvard Stem Cell Institute, Cambridge, MA, United States

Type 1 diabetes (T1D) arises from autoimmune-mediated destruction of insulin-producing pancreatic beta cells. Recent advancements in the technology of generating pancreatic beta cells from human pluripotent stem cells (SC-beta cells) have facilitated the exploration of cell replacement therapies for treating T1D. However, the persistent threat of autoimmunity poses a significant challenge to the survival of transplanted SC-beta cells. Genetic engineering is a promising approach to enhance immune resistance of beta cells as we previously showed by inactivating the *Renalase* (*Rnls*) gene. Here, we demonstrate that *Rnls* loss of function in beta cells shapes autoimmunity by mediating a regulatory natural killer (NK) cell phenotype important for the induction of tolerogenic antigen-presenting cells. *Rnls*-deficient beta cells mediate cell–cell contact-independent induction of hallmark anti-inflammatory cytokine Tgfβ1 in NK cells. In addition, surface expression of regulatory NK immune checkpoints CD47 and Ceacam1 is markedly elevated on beta cells deficient for *Rnls*. Altered glucose metabolism in *Rnls* mutant beta cells is involved in the upregulation of CD47 surface expression. These findings are crucial to better understand how genetically engineered beta cells shape autoimmunity, giving valuable insights for future therapeutic advancements to treat and cure T1D.

KEYWORDS

autoimmunity, type 1 diabetes, transplantation, beta cell, NK cell, CD47, Ceacam1, Tgfβ1

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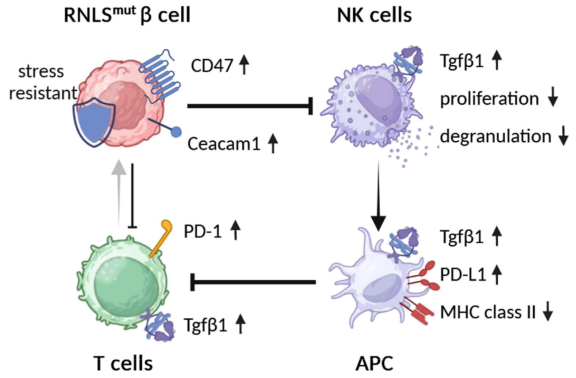

GRAPHICAL ABSTRACT

Introduction

In type 1 diabetes (T1D), the autoimmune process leads to the selective destruction of insulin-producing beta cells within pancreatic islets. Upon extensive elimination of the majority of beta cell mass by autoimmunity, a curative strategy for T1D requires the replenishment of functional beta cell mass to completely restore the patient's capacity for insulin production (1). The latest progress in the manufacturing of beta cells derived from stem cells (SCs) has rendered beta cell replacement therapy a viable promise (2). Despite these advancements, a significant challenge in translating this strategy to clinical application lies in our current inability to protect beta cells against recurrent autoimmune attacks without resorting to broad immunosuppressive treatments. Exacerbating this obstacle is the existing limitation in generating patient-specific SC-derived beta cells, underscoring the need to address both autoimmunity and alloimmune responses in any cellular therapy for T1D in the foreseeable future (3). To address these challenges, various research groups have started to genetically engineer SC-derived beta cells for enhanced resilience against immune-mediated destruction. Notably, most efforts focus on the deletion of genes encoding major histocompatibility complex (MHC) molecules mandatory for antigen presentation and activation of T cells, coupled with the incorporation of immune inhibitory ligands such as CD47 to prevent stimulation of innate immune cells (4–10).

In our pursuit of discovering novel immune-regulatory targets to protect beta cells from autoimmune destruction, we previously performed an unbiased genome-wide *in vivo* CRISPR screen, and found that *Renalase* (*Rnls*) deletion is able to protect beta cells from stress-induced cell death and autoimmunity (9). Beta cells lacking *Rnls* not only exhibit enhanced resilience to stress but also undergo comprehensive metabolic alterations favoring glucose metabolism (9, 10). Moreover, we have shown previously that *Rnls*-deficient beta cells orchestrate localized shifts in the overall immune cell composition within the graft, primarily characterized by the enhanced infiltration of CD4$^+$ T cells, reduced numbers of natural killer (NK) cells, and the accumulation of tolerogenic antigen-presenting cells (APCs) defined by diminished MHC class II expression coupled with elevated levels of programmed cell death 1 ligand 1 (PD-L1). We also demonstrated that the survival advantage of beta cells lacking *Rnls* is attributed to the induced expression of PD-L1 on APCs. Beta cells deficient for *Rnls* promote a significant transcriptional change in CD45$^+$ immune cells within the graft towards hallmark anti-inflammatory genes such as transforming growth factor beta 1 (Tgfβ1) (10). However, the immune cell population responsible for initiating the immune-regulatory cross-talk to reduce autoimmunity against *Rnls*-deficient beta cell grafts remained elusive.

As Tgfβ1 is a known inducer of PD-L1 upregulation on APCs in certain tumors as well as in pancreatic islet transplanation (11), and NK cells harbor a significant source of Tgfβ1 production (12), we have now investigated if graft-infiltrating NK cells play an important role for mediating the immuno-protective regulation of *Rnls* mutant beta cells. NK cells are not solely responsible for killing transformed or stressed target cells, but are also crucial to modulate the activation and phenotype of immune cells such as APCs (13). Here, we show that depletion of NKp46$^+$ innate lympoid cells in an experimental model of beta cell transplantation abrogated the induction of tolerogenic APCs consequently preventing the survival advantage of beta cell grafts deficient for *Rnls*. Using *in vitro* co-culture systems, we demonstrated that beta cells lacking *Rnls* are potent modulators of NK cell activation shown for both mouse-derived NIT-1 beta cells and human SC-derived beta-like cells. Elevated cell surface expression of key NK cell inhibitory molecules on beta cells deficient for *Rnls* such as Cluster of differentiation 47 (CD47) and Carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam1) are likely to play a major role in suppressing NK cell activation by cell–cell interaction. However, upregulation of Tgfβ1 in NK cells mediated by *Rnls* deletion in beta cells is independent of cell–cell contact demonstrating multifaced propsects of NK regulation.

Results

Protective immune-regulation by Rnls^{mut} beta cells requires NKp46^{+}innate lymphoid cells

We previously investigated the consequences of *Rnls* deletion on immune cell infiltration and activation by transplantation of the syngeneic mouse beta cell line NIT-1 into Non-obese diabetic (NOD) mice, a model of T1D. Single-cell RNA sequencing (scRNAseq) of graft-infiltrating CD45^{+} cells revealed that *Rnls* mutant (Rnls^{mut}) beta cells broadly influence immune cell activation and metabolism towards anti-inflammatory oncostatin M and Tgfβ1 expression, accompanied by enriched expression of genes important for glycolysis. In addition to elevated numbers of CD4^{+} T cells and tolerogenic PD-L1^{+} APCs, beta cell grafts deficient for *Rnls* showed markedly reduced frequency of NK cells. Because we previously demonstrated that PD-L1 blockade abrogates the advantage of Rnls^{mut} beta cells to survive autoimmunity, and NK cells are known to modulate APC maturation and activation, we now investigated the role of NK cells in protection of Rnls^{mut} beta cells in more detail (10). The gene expression profile of both graft-infiltrating NK cells and closely related type 1 innate lymphoid cells (ILC1) demonstrated markedly increased expression of genes involved in pathways of cellular activation and inflammation (interferon responses, allograft rejection, etc.) when derived from WT beta cell grafts compared to Rnls^{mut} (Figure 1A and Supplementary Figures 1A–C). Whereas ILC1 showed downregulated gene expression of inflammatory cytokines interferon gamma (Ifng) and tumor necrosis factor alpha (Tnf), NK cells upregulated gene expression of immune-regulatory Tgfβ1 when infiltrated in Rnls^{mut} beta cell grafts (Supplementary Figures 1A, B). Interestingly, the frequency of NK cells in Rnls^{mut} beta cell grafts was reduced not only in late stages (10), but also at earlier time points preceding the detection of graft weight difference between control and Rnls^{mut} (Supplementary Figures 1D, E; see Supplementary Figure 2 for gating strategy). Of note, the ILC1 frequency was not changed between the grafts and only represented a vast minority of infiltrating immune cells (≤1%) compared to NK cells (up to 14% according to scRNAseq analysis) (10), indicating that ILC1 likely does not play a major role in mediating the protective immune-regulation caused by Rnls^{mut} beta cells. These observations strengthened our hypothesis that NK cells might be crucial for regulating autoimmunity within Rnls^{mut} grafts. To investigate the importance of NK cells in shaping autoimmunity *in vivo*, we depleted NKp46^{+} innate lymphoid cells from autoreactive splenocytes before adoptive transfer into NIT-1 beta cell graft-bearing mice (Figure 1B, Supplementary Figures 1F–H). The isogenic WT and Rnls^{mut} beta cells were transplanted subcutaneously (s.c.) on opposite flanks of the same recipient mice following intravenous (i.v.) injection of diabetogenic splenocytes similar to described before (9, 10). Strikingly, NKp46-depleted autoreactive splenocytes lost the ability to protect Rnls^{mut} cells from autoimmunity in both immunodeficient recipients, NOD-*Prkdc*^{scid} (NOD.scid) mice and NOD.scid gamma (NSG) mice completely deficient for functional T, B, and NK cells (Figure 1C). Whereas the accumulation of CD4^{+} T cells was independent of innate lymphoid cells, the reduced frequency of mature MHCII^{high+} APCs and downregulation of MHCII expression level on APCs in Rnls^{mut} grafts completely depended on the presence of graft-infiltrating NKp46^{+} immune cells (Figures 1D, E, Supplementary Figure 1I). In the absence of innate lymphoid cells within Rnls^{mut} beta cell grafts, MHCII^{high+} APCs lost the ability to upregulate the immune checkpoint PD-L1, thus forfeiting the survival advantage over WT beta cells as shown before (Figures 1G, H) (10). In summary, these observations clearly demonstrated that graft-infiltrating NKp46^{+} innate lymphoid cells are crucial for the induction of a tolerogenic APC phenotype important to reduce autoimmune destruction of Rnls^{mut} beta cells.

Rnls^{mut} *NIT-1 beta cells shape NK cell activation towards a Tgfβ1^{+} regulatory phenotype*

After showing that NKp46^{+} innate lymphoid cells are important to mediate protective immune-regulation leading to prolonged graft survival of Rnls^{mut} beta cells, we subsequently investigated if Rnls^{mut} beta cells directly regulate the activation of innate lymphoid cells. Hence, we purified splenic NKp46^{+} cells (further declared as NK cells as ILC1 only represents approximately 5%–10% of all NKp46^{+} cells in the spleen) (14) by negative selection to collect "untouched" cells for functional *in vitro* co-culture experiments. Activation of NK cells was achieved by supplementation of interleukin-2 (IL2), well-known to enhance the proliferation and effector function of NK cells (15). Co-culture with Rnls^{mut} NIT-1 beta cells significantly reduced NK cell activation indicated by impaired expression of activation marker CD44 and CD69 compared to WT, but did not completely abolished NK cell activation (Figures 2A, B). However, the surface expression of degranulation marker CD107a (also known as LAMP1) on NK cells, a molecule that predicts cytotoxic activity (16), was strikingly abolished when co-cultured with Rnls^{mut} NIT-1 beta cells (Figures 2C, D). The ability of NK cells to proliferate following repeated IL2 stimulations was also completely prevented when co-cultured with Rnls^{mut} NIT-1 beta cells. This effect was solely dependent on cell–cell contact as separation of NK cells and Rnls^{mut} beta cells in trans-well abrogated the anti-proliferative function of Rnls^{mut} beta cells (Figures 2E, F). The cell–cell contact dependency on NK cell regulation by Rnls^{mut} NIT-1 beta cells also became evident for regulation of activation marker CD69 (Figure 2G). As described above, differential gene expression analysis of graft-infiltrated NK cells showed enriched Tgfβ1 when derived from Rnls^{mut} NIT-1 beta cell grafts (Supplementary Figure 1A). To investigate if Tgfβ1 is also upregulated on protein level, we stained NK cells for latency-associated peptide (LAP) representing membrane-bound Tgfβ1. Indeed, NK cells co-cultured with Rnls^{mut} NIT-1 beta cells demonstrated elevated Tgfβ1 cell surface expression compared to WT NIT-1 beta cells. In contrast to the modulation in NK

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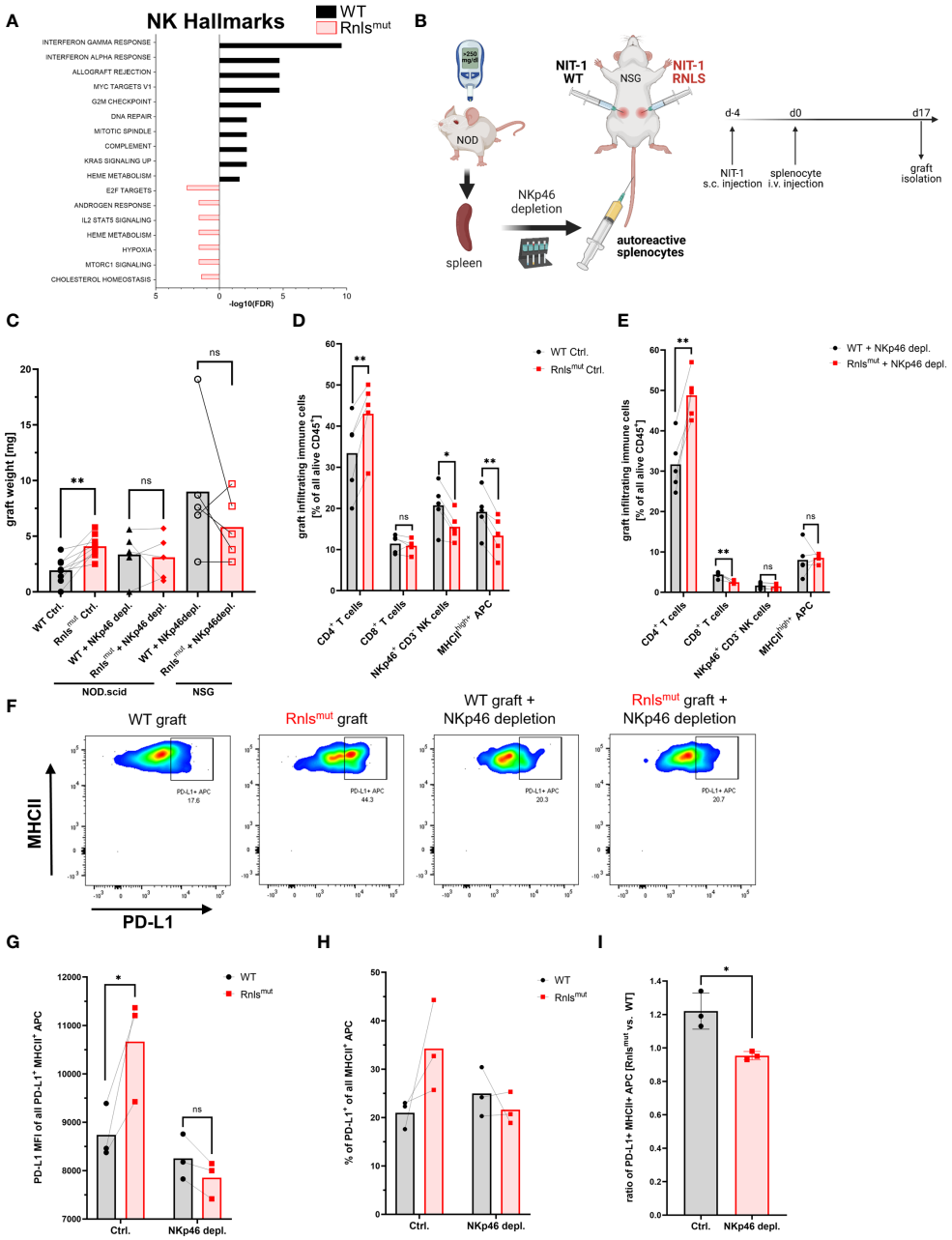

FIGURE 1
NKp46^{+} innate lymphoid cells are crucial for protective immune-regulation by *Renalase* mutant (Rnls^{mut}) NIT-1 beta cells *in vivo*. **(A)** Hallmark gene expression analysis of natural killer (NK) cells derived from indicated NIT-1 beta cell grafts showing the 10 most significantly changed pathways using 213 (Rnls^{mut}) or 506 (WT) most significantly upregulated genes ($p \leq 0.05$) as input. Raw data used in this panel were obtained from experiments performed previously. Datasets from our scRNAseq experiments are available from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE226361 as described before (10). **(B)** Schematic representation of the experimental design comparing paired WT and Rnls^{mut} NIT-1 beta cell grafts. Cells were injected s.c. into opposite flanks of immunodeficient mice, followed by i.v. injection of autoreactive splenocytes with or without preceding depletion of NKp46^{+} innate lymphoid cells. Grafts were harvested and scaled, and immune cells were characterized by flow cytometry. **(C)** Weight of paired grafts from mice with or without depletion of NKp46^{+} cells from autoreactive splenocytes is shown. Results represent the mean of nine (Ctrl. condition) or five (NKp46^{+} depletion) paired biological replicates from two combined independent experiments using NOD.scid or NSG recipient mice as indicated. **(D, E)** Quantification of immune cell subpopulations derived from five paired ctrl. NIT-1 beta cell grafts **(D)** or NKp46^{+} depleted grafts **(E)** as determined by flow cytometry. Results represent the mean of five paired biological replicates. **(F–I)** Representative flow cytometry data **(F)** or quantification **(G–I)** showing indicated expression level of PD-L1 on indicated immune cells derived from paired WT and *Rnls*-deficient grafts as determined by flow cytometry. The ratio of PD-L1^{+} MHCII^{+} cells comparing Rnls^{mut} *vs.* WT NIT-1 cell grafts shown in **(I)** are derived from data shown in **(H)**. Results represent the mean of three paired biological replicates. *$p < 0.05$, **$p < 0.01$, ns $p > 0.05$ (paired two-tailed *t*-test). Data obtained from scRNAseq **(A)** and from NKp46^{+} depletion experiments using NSG **(C-E)** or NOD.scid **(C, F–I)** recipient mice are derived from independent experiments. Data of four out of nine mice shown for the Ctrl. condition in **(C)** are partially derived from one previously shown experiment performed in parallel with NKp46 depletion using NOD.scid mice [10]. Data of five out of nine mice are derived from one newly performed experiment in parallel with NKp46 depletion using NSG mice.

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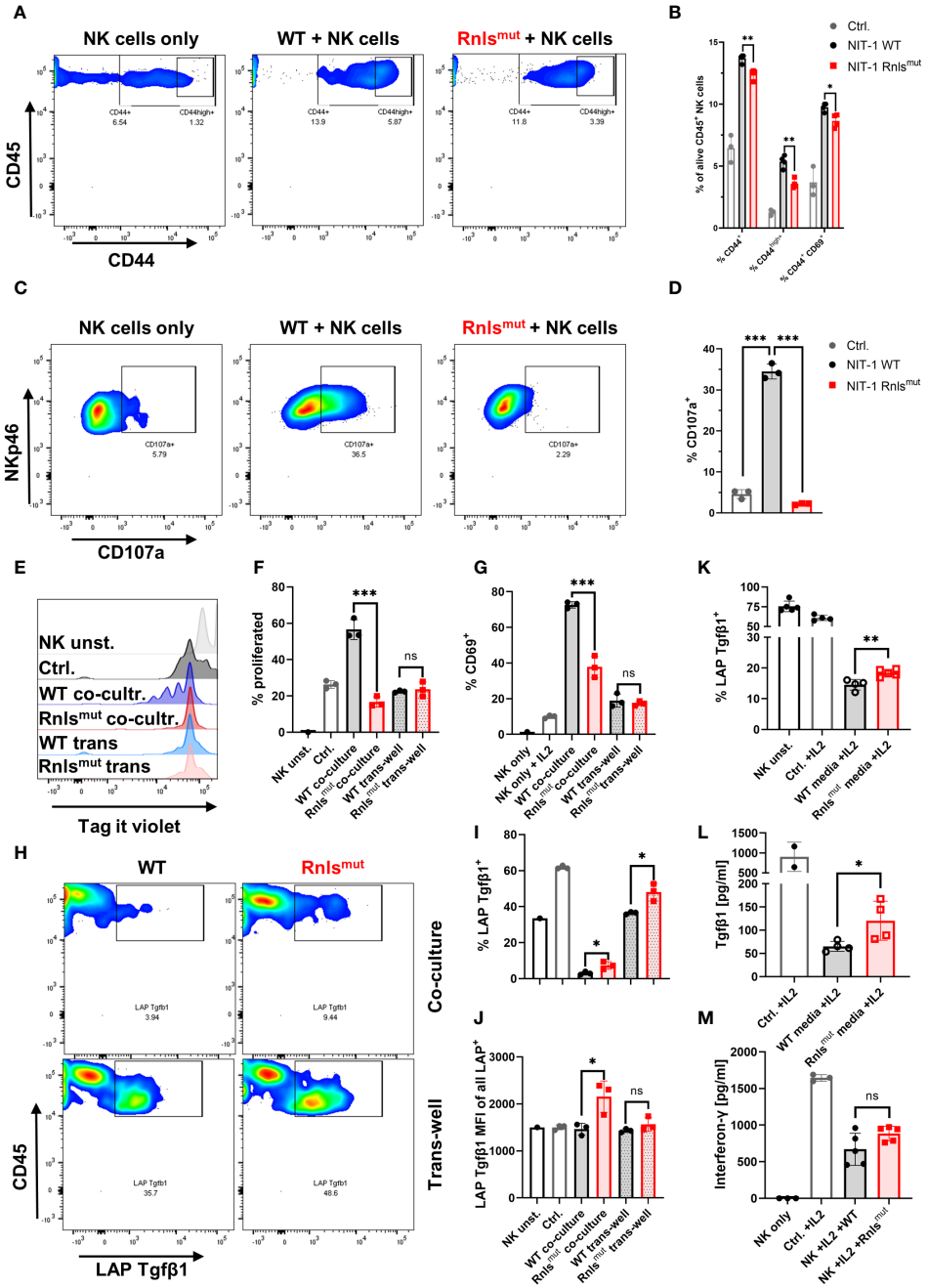

FIGURE 2
Rnls^{mut} NIT-1 beta cells broadly affect natural killer (NK) cell function *in vitro*. Interleukin 2 (IL2) stimulated primary mouse NK cells were co-cultured with indicated NIT-1 beta cells for 72 h. **(A–D)** Data derived from single-dose IL2 (20 ng/mL)-treated co-culture experiments showing representative flow cytometry plots **(A, C)** or related quantifications **(B, D)** of indicated activation marker **(A, B)** or cytotoxic activity marker CD107a **(C, D)**. **(E-I)** NK cells were repetitively stimulated with IL2 (100 ng/mL) every 24 h together with indicated NIT-1 cells in a co-culture or trans-well setting. **(E, F)** Representative flow cytometry data **(E)** or quantifications **(F)** showing NK cell proliferation of indicated conditions. **(G)** Quantification of surface expression of activation marker CD69 on NK cells. **(H–J)** Representative flow cytometry data **(H)** or quantifications of indicated conditions **(I, J)** showing expression level of membrane-bound latency-associated peptide (LAP)/Tgfβ1 expression on NK cells. **(K, L)** Conditioned media experiments showing LAP/Tgfβ1 surface expression on NK cells **(K)** or secreted amounts of Tgfβ1 derived from NK cells **(L)**. **(M)** Quantification of interferon-γ (Ifng) secretion by NK cells in co-culture with indicated NIT-1 cells. Results represent the mean ± SD from one out of two **(A, B, E–J, L)** or three **(C, D, K, M)** independent experiments (n = 3–5). ***p < 0.001, **p < 0.01, *p < 0.05, ns p > 0.05, (unpaired, two-tailed t-test).

proliferation, Rnls^{mut} NIT-1 beta cells drive Tgfβ1 expression independent of cell–cell contact (Figures 2H, I, L). The fact that Rnls^{mut} NIT-1 beta cells significantly upregulated Tgfβ1 expression and secretion in NK cells by soluble factors was demonstrated by two different methods, in a trans-well assay (Figures 2H–L) and by supplementation of conditioned media derived from Rnls^{mut} NIT-1 beta cells in comparison to supernatant of WT cells (Figures 2K, L). However, the increased intensity of Tgfβ1 expression on Tgfβ1^{+} cells mediated by Rnls^{mut} NIT-1 beta cells was dependent on cell–cell contact (Figure 2J). Next to Tgfβ1, the pro-inflammatory

cytokine Ifng is also known to facilitate the upregulation of PD-L1 (11). However, the secretion of Ifng was not significantly affected when NK cells were co-cultured with Rnls^{mut} NIT-1 cells compared to WT (Figure 2M). In summary, Rnls^{mut} NIT-1 beta cells directly shape NK cell activity in multiple modes, dependent and independent of cell–cell contact. The presence of NIT-1 beta cells deficient for *Rnls* causes NK cells to fail to proliferate as well as to lose cytotoxic activity in response to IL2, but at the same time elevate the expression level of anti-inflammatory Tgfβ1.

NIT-1 *beta cells deficient for Rnls upregulate NK immune checkpoint molecules CD47 and Ceacam1*

We demonstrated that Rnls^{mut} NIT-1 beta cells are potent modulators of NK cell activation (Figures 1, 2). To explore how *Rnls*-deficient beta cells might influence the activity of NK cells, we re-analyzed bulk RNA sequencing comparing differential gene expression data of WT and Rnls^{mut} NIT-1 cells (10). Rnls^{mut} NIT-1 beta cells showed an enrichment of genes involved in immune-regulatory interactions between a lymphoid and a non-lymphoid cell (Supplementary Figure 1J), indicating that upregulated expression of inhibitory cell surface molecules may regulate cell contact-dependent NK cell stimulation. Next, we selected all candidates from the top 1,500 most significant upregulated genes in Rnls^{mut} NIT-1 cells that are known to be involved in modulation of immune cell activity according to the GSEA-MSigDB data base (https://www.gsea-msigdb.org). In addition to the elevated expression of CD44 and Itga4 important for cell–cell adhesion processes (17, 18), Rnls^{mut} NIT-1 beta cells also upregulate key immune checkpoint surface molecules such as Ceacam1 (also known as CD66a), CD47 (also known as integrin associated protein), and CD200 (also known as OX-2), which are all eminent potent inhibitors of NK cell activation (Figure 3A) [19-22]. To validate if elevated mRNA expression correspond to higher protein expression, we performed cell surface staining for these three key inhibitory NK cell ligands analyzed by flow cytometry. Rnls^{mut} NIT-1 beta cells showed significant upregulated surface expression of CD47 for both percentage of cells and mean fluorescence intensity (MFI, Figures 3B–D). The surface expression of Ceacam1 was also significantly upregulated in terms of Ceacam1^{high+}-expressing cells, whereas Ceacam1 MFI showed a tendency of elevated expression on Rnls^{mut} NIT-1 beta cells (Figures 3E–G). Although the percentage of cells positive for cell surface CD200 was not dramatically changed between WT and Rnls^{mut}, the expression level of CD200 (MFI) on Rnls^{mut} NIT-1 beta cells was significantly upregulated (Figures 3H–J). These observations indicate that Rnls^{mut} NIT-1 beta cells could shape NK cell activation at least partially by the upregulation of multiple inhibitory NK cell ligands on their cell surface.

In a first attempt to investigate why Rnls^{mut} cells upregulate the expression of NK inhibitory molecules, we wondered if the changes in Rnls^{mut} beta cell metabolism towards increased glucose metabolism (10) could influence the cell surface expression of CD47. As CD47 expression has been described to be similarly regulated like the immune checkpoint PD-L1 (19), and PD-L1 is positively regulated by glycolysis (20, 21), we treated NIT-1 cells with 2-deoxy-d-glucose (2DG) to inhibit glucose metabolism. Strikingly, 2DG treatment for 48 h dramatically downregulated the surface expression of CD47 on both alive WT and Rnls^{mut} NIT-1 cells (Supplementary Figures 3A–D). Whereas treatment with low concentration of 2DG (1 mM) decreased the percentage of CD47-expressing Rnls^{mut} cells to about the level of untreated WT cells, small amounts of 2DG had no effect on the percentage of CD47 expression on WT cells (Supplementary Figures 3A, C). This observation indicates that enhanced glucose metabolism in Rnls^{mut} beta cells might be responsible for the upregulation of CD47 surface expression.

Human RNLS^{mut} SC-derived beta-like cells recapitulate key features of NK cell regulation

Rnls^{mut} NIT-1 beta cells strongly modulate NK cell activity towards a regulatory phenotype. To make sure that our observations are not restricted to mouse-derived NIT-1 beta cells, we also analyzed human iPSC-derived beta-like cells (SCBCs) lacking the *RNLS* gene (9) for NK regulatory characteristics (Figure 4A). Intracellular staining for beta cell marker demonstrated that SCs differentiate into beta-like cells regardless of genotype, as previously described (Supplementary Figures 4A, B) (9). Rnls^{mut} NIT-1 beta cells showed elevated surface expression of NK inhibitory molecules CD47, Ceacam1, and CD200 (Figure 3). In line with Rnls^{mut} NIT-1 beta cells, RNLS^{mut} SCBCs also demonstrated significantly upregulated expression of NK inhibitory ligands CD47 and CD66a/c/e, whereas, in contrast to NIT-1 beta cells, CD200 surface expression was not elevated on RNLS^{mut} SCBCs (Figures 4B, C, Supplementary Figures 4C–H). We co-cultured WT and RNLS^{mut} with allogenic peripheral blood mononuclear cells (PBMCs) stimulated with IL2 for 3 days (Figure 4A). Although surface expression of the NK cell activation marker was only modestly changed in this highly stimulatory allogenic setting (Figure 4D and data not shown), RNLS^{mut} SCBCs significantly upregulated the immune-regulatory cytokine TGFβ1 on human CD3^{-}CD56^{+} NK cells (Figures 4E, F). These findings clearly show that human beta cells lacking *RNLS* acquire key characteristics to induce a regulatory NK cell phenotype. Because we previously demonstrated that Tgfβ1 expression is elevated on other NOD-derived immune cells such as CD3^{+} CD4^{+} T cells when infiltrating in Rnls^{mut} beta cell grafts (10), we stained human CD3^{+} T cells for membrane-bound TGFβ1 as well. Indeed, PBMC-derived CD3^{+} T cells showed elevated surface expression of LAP/TGFβ1 when co-cultured with RNLS^{mut} SCBCs compared to WT SCBCs (Figure 4G). Finally, we tested if RNLS^{mut} SCBCs had higher resistance to immune-mediated destruction by IL2-stimulated allogenic PBMCs. The total number of alive CD45 negative SCBCs as well as the ratio of alive/dead SCBCs were significantly higher for RNLS^{mut} SCBC compared to WT indicating that the protective immune-regulatory effects of *RNLS*-deficient beta cells also translate, at least to some extent, to the human system (Figures 4H, I). Our results highlight that knocking out a single gene in beta cells can drive multifaceted modulation on the immunogenicity of beta cells to prevent autoimmunity.

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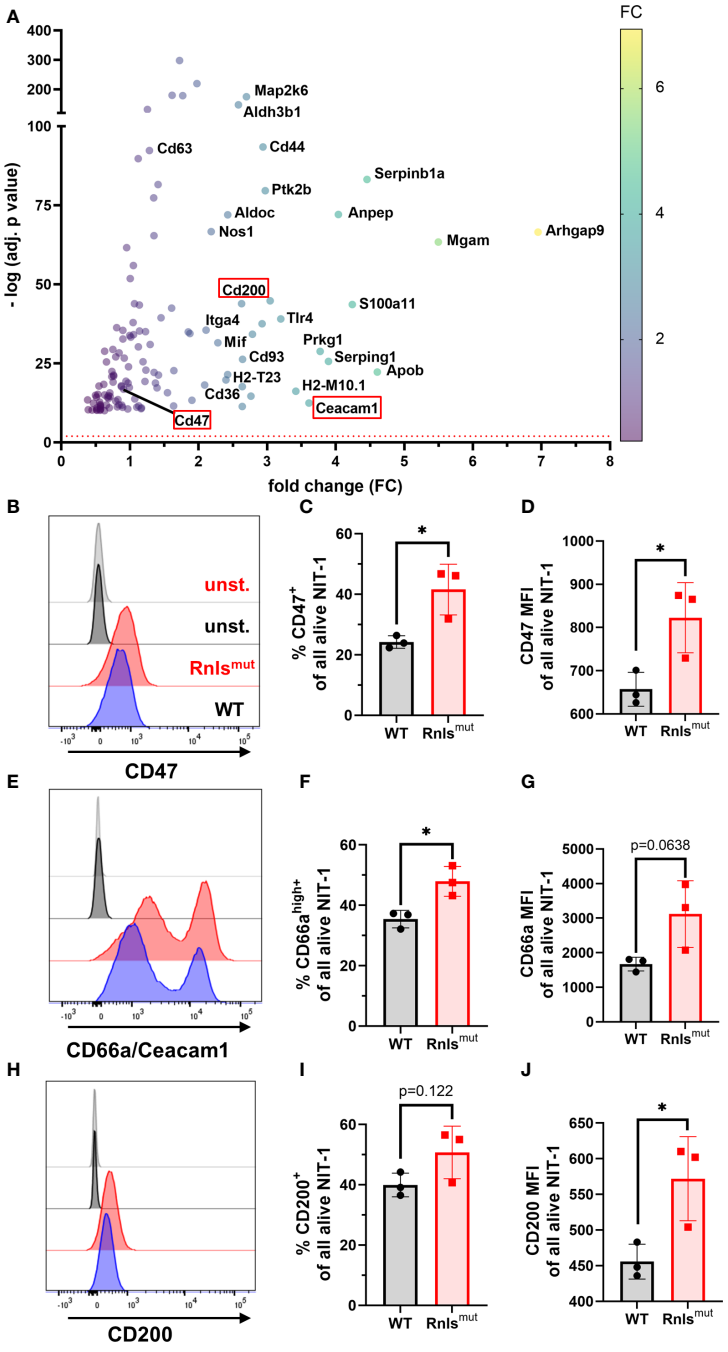

FIGURE 3
Rnls^{mut} NIT-1 beta cells show upregulated expression of key NK inhibitory ligands on their cell surface **(A)** RNA sequencing data showing the top 1,500 upregulated immune cell function-related genes (defined in GSEA-MSigDB) in Rnls^{mut} NIT-1 beta cells in comparison to WT control. Cell surface molecules known to reduce NK cell activity are highlighted in a red box. The red dotted line indicates the threshold for significantly changed genes [–log10 (*p* adj.) ≥ 2]. The fold change of gene expression is represented by colored dots from low (purple) to high (yellow). Raw data used in this panel were obtained from a bulk RNA sequencing experiment performed previously (10). **(B–J)** Representative histogram plots or quantifications of indicated inhibitory NK cell ligands expressed on the cell surface of Rnls^{mut} and WT NIT-1 beta cells characterized by flow cytometry. Results represent the mean ± SD from one out of three independent experiments ($n = 3$). *$p < 0.05$ (unpaired, two-tailed *t*-test).

Discussion

In this work, we provide compelling evidence supporting the critical involvement of NK cells in orchestrating the immune-regulatory milieu surrounding Rnls^{mut} beta cells. In our previous study, we already observed a marked reduction in NK cell frequency within Rnls^{mut} beta cell grafts compared to WT counterparts, suggesting a potential link between NK cells and the protective effects conferred by Rnls^{mut} beta cells (10). Here, further characterization of graft-infiltrating NK cells revealed distinct gene expression profiles, with NK cells derived from Rnls^{mut} beta cell grafts exhibiting upregulated expression of immune-regulatory

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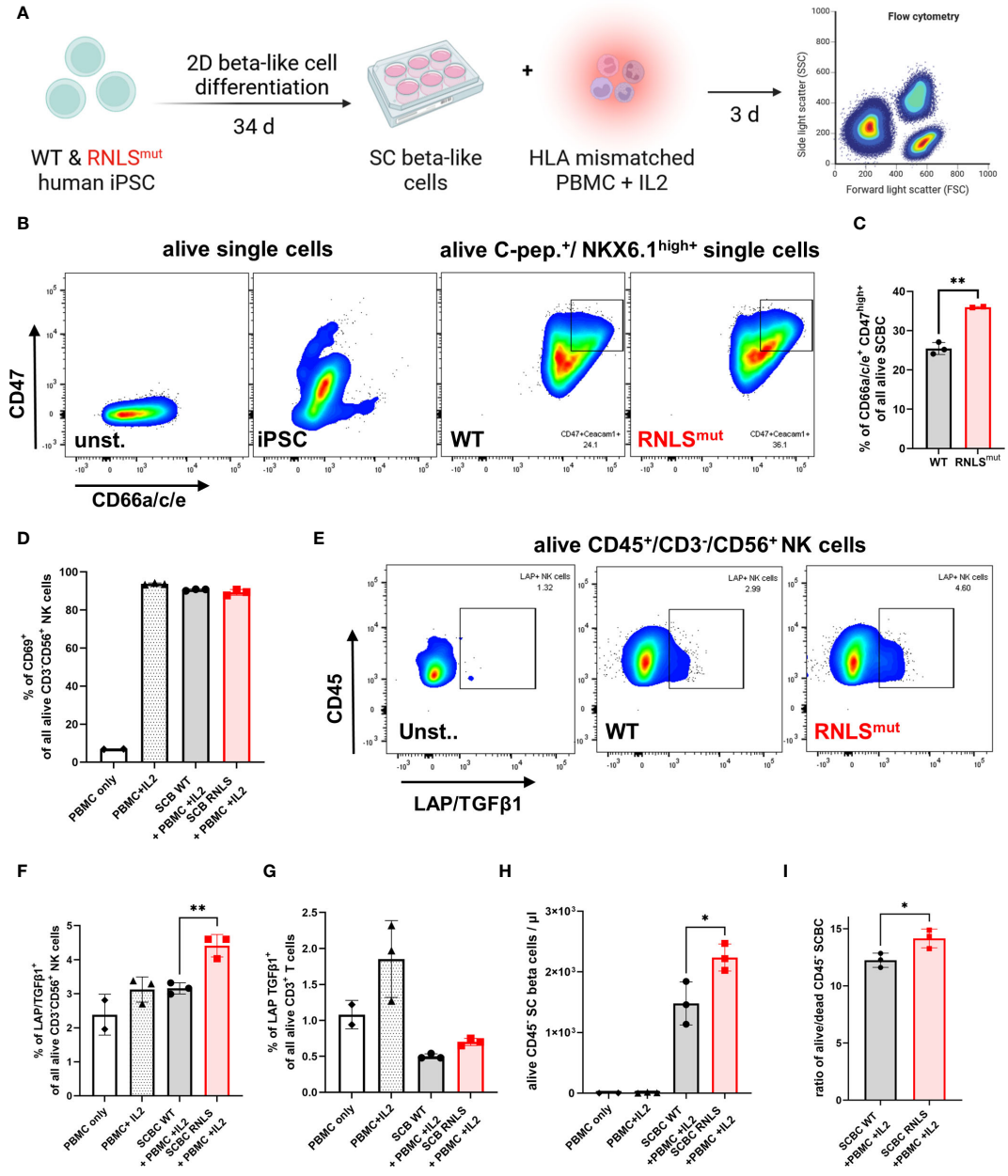

FIGURE 4
Rnls^{mut} stem cell (SC)-derived beta-like cells (SCBC) recapitulate key immune-regulatory features to modulate NK cell activity. **(A)** Schematic representation of the experimental design for SCBC differentiation and for human leukocyte antigen (HLA) mismatched PBMC co-culture approach comparing WT and RNLS-deficient SCBC. **(B, C)** Representative flow cytometry plots or quantification of indicated NK inhibitory ligands on the cell surface of C-peptide (C-pep.)$^{+}$ NKX6.1^{high+} WT and RNLS^{mut} SCBC. **(D)** Quantification of activation marker CD69 on NK cells following 72-h incubation with IL2-stimulated HLA mismatched PBMC in co-culture with WT or RNLS^{mut} SCBC. **(E–G)** Representative flow cytometry plots or quantification of latency-associated peptide (LAP)/TGFβ1 surface expression on indicated immune cell types following IL2-stimulated HLA mismatched PBMC co-culture for 72 h. **(H, I)** Quantification of total numbers of indicated alive CD45^{-} SCBC **(H)** or alive/dead ratio of indicated SCBC **(I)** following IL2-stimulated HLA mismatched PBMC co-culture for 72 h. Results represent the mean ± SD from one out of two independent experiments (n = 2–3). **(C, I)** $^{**}p < 0.01$(unpaired two-tailed t-test). **(D–H)**$^{*}p < 0.05$, $^{**}p < 0.01$ (one-way ANOVA with Dunnett *post hoc* test).

Tgfβ1, reinforcing their role in mitigating autoimmune responses. Dysregulation of Tgfβ1 signaling has been implicated in various autoimmune disorders, highlighting its crucial role in maintaining immune homeostasis and preventing autoimmunity (22). Notably, depletion of NKp46^{+} cells abrogated the protective effects of Rnls^{mut} beta cells against autoimmunity, underscoring the indispensability of innate lymphoid cells, especially NK cells, in mediating immune regulation within the beta cell graft microenvironment.

Our previous work has established that PD-L1 blockade abrogates the survival advantage of Rnls^{mut} beta cells, implicating the PD-1/PD-L1 checkpoint as a crucial mediator of immune regulation in beta cell graft survival. In extension of our previous

finding, our current observations elucidate the role of NKp46^{+} innate lymphoid cells in shaping the phenotype of APCs within the beta cell grafts. We observed a significant reduction in the frequency of mature MHCII^{high+} APCs within Rnls^{mut} beta cell grafts, which was completely dependent on the presence of graft-infiltrating NKp46^{+} immune cells. Enhanced secretion of Tgfβ1 derived from NK cells most likely induces the tolerogenic APC phenotype in Rnls^{mut} beta cell grafts. It is well established that Tgfβ1 prevents dendritic cell maturation including MHCII upregulation, underscoring the intricate cross-talk between NK cells and APCs we have observed here (23). In the context of lung cancer, the interplay between NK cells and APCs has also been noted to have immune-regulatory implications, including impairments in MHCII expression and in modulation of immune checkpoint pathways (24). Of note, and in line with our observations, Tgfβ1 has been described as potent inducer of PD-L1 expression on APCs in pancreatic islet transplantation (11). This suggests that NK cells play a pivotal role in shaping the tolerogenic phenotype of APCs, thereby contributing to the overall immune-regulatory environment conducive for survival of beta cell grafts.

In this study, *in vitro* co-culture experiments provided mechanistic insights into the direct modulation of NK cell activity by Rnls^{mut} beta cells. Beta cells deficient for *Rnls* exhibited the ability to attenuate NK cell activation and cytotoxicity, while concurrently promoting the expression of anti-inflammatory Tgfβ1. Interestingly, Rnls^{mut} beta cells also upregulated inhibitory immune checkpoint molecules such as CD47 and Ceacam1, further corroborating their role in dampening NK cell activity and immune responses (25–29). Overexpression of CD47 on hypo-immunogenic MHCI/II-deficient islets have already been described to play a significant role in the inhibition of NK cells to prevent rejection of engrafted cells in humanized mice (30). However, other surface molecules or immune-modulatory alterations such as cellular metabolism in Rnls^{mut} beta cells may contribute to the inhibition of NK cell activity as well. The results obtained in this study indicate that enhanced glucose metabolism in Rnls^{mut} beta cells contributes to elevated expression of CD47, as the inhibition of glycolysis by 2DG treatment strongly reduced cell surface expression of CD47. It has been shown that PD-L1 expression is regulated by glucose metabolism (20), but it has not been described before that CD47 surface expression is regulated similarly by glucose metabolism. It is known that the expression of PD-L1 and CD47 is often regulated by similar mechanisms such as by stimulation with Ifng (19). However, future studies have to investigate the molecular mechanism how glucose metabolism is linked to the upregulation of CD47 surface expression on beta cells.

Moreover, our findings extend to human beta-like cells derived from induced pluripotent SCs, highlighting the translational relevance of our observations. RNLS^{mut} SCBCs exhibited similar patterns of NK cell regulation and upregulated expression of inhibitory immune checkpoint molecules CD47 and CD66a/c/e. In addition to mouse-derived NIT-1 cells, RNLS^{mut} SCBCs also mediate the induction of TGFβ1 on both human NK cells and T cells (10), further affirming the conservation of immune-modulatory mechanisms across species. This is in line with our previous study where we demonstrated that RNLS^{mut} SCBCs are resistant to stress-induced apoptosis similar to *Rnls*-deficient NIT-1 beta cells (9).

Overall, our study elucidates the intricate interplay between Rnls^{mut} beta cells and NKp46^{+} NK cells in orchestrating protective immune regulation, unveiling potential targets for therapeutic intervention in autoimmune diabetes. By unraveling the mechanisms underlying immune modulation within the beta cell microenvironment, our findings pave the way for the development of novel strategies aimed at preserving beta cell function and improving graft survival in T1D.

Research design and methods

Mice

NOD (nonobese diabetic), NOD.scid (NOD.Cg-Prkdc^{scid}/J), and NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice were purchased from The Jackson Laboratory. Animals were housed in pathogen-free facilities at the Joslin Diabetes Center and all experimental procedures were approved and performed in accordance with institutional guidelines and regulations (IACUC protocol number 2013–03).

Cell lines and induced pluripotent stem cell-derived beta-like cell differentiation

NIT-1 cells were obtained from ATCC (cat # CRL-2055). Cells were maintained in DMEM, high glucose, and pyruvate (cat # 11–995-073; Gibco), supplemented with 2 mM L-glutamine (cat # 25–030-081; Gibco),10% FCS (cat # 10–082-147; Gibco), 50 µM 2-mercaptoethanol (cat # 60–24-2; Sigma-Aldrich), and penicillin/streptomycin (cat # 15140122; Thermo Fisher Scientific) in a 37°C incubator with 5% CO_2. Rnls^{mut} NIT-1 cells were generated previously (10). Human induced pluripotent stem cells (iPSCs) obtained from Local donor 3 with T1D were carefully maintained and differentiated following robust protocols tailored for beta cell differentiation (31). Cultured in planar form using mTeSR medium (cat# 85850; Stem Cell Technologies), iPSCs subjected to daily media changes to sustain optimal growth conditions. Employing a 2-D differentiation protocol (32), iPSCs were seeded at stage 0 onto culture plates at a density of 0.8 × 105 cells/cm^2, supplemented with 10 µM Y-27632 (cat # DNSK-KI-15–02; DNSK International) to enhance cell survival and adherence. Quality control measures were rigorously implemented at each differentiation stage, utilizing immunofluorescence (IF) or flow cytometry analyses with specific differentiation marker to assess differentiation efficiency and cellular identity (data not shown). For effective cell dissociation, TrypLE Express (cat # 12604013; Gibco) treatment was administered at 37° C for 10 min to disrupt cell–cell adhesions, facilitating the generation of single-cell suspensions. Following dissociation, cells were promptly fixed and stained as per established procedures (33) to visualize cellular marker indicative of beta cell differentiation. Quality control at stage 6 involved the use of antibodies targeting C-peptide and NKX6.1 analyzed by flow cytometry (see Table 1).

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TABLE 1 Antibodies used for flow cytometry.

Antibody specificity	Reactivity	Clone	Cat #	Vendor
C-peptide Alexa Fluor 647	Human	U8–424	565831	BD Biosciences
CD3 Brilliant Violet 785	Mouse	17A2	100232	BioLegend
CD3 Brilliant Violet 605	Mouse	17A2	100237	BioLegend
CD3 PE-Cy5	Human	HIT3a	300310	BioLegend
CD4 APC	Mouse	RM4–5	100516	BioLegend
CD8 FITC	Mouse	53–6.7	100706	BioLegend
CD11b APC-Cy7	Mouse	M1/70	101226	BioLegend
CD11c Brilliant Violet 711	Mouse	N418	117349	BioLegend
CD19 Pacific Blue	Mouse	1D3/ CD19	152416	BioLegend
CD44 APC-Cy7	Mouse	IM7	103028	BioLegend
CD44 PE	Human	C44Mab-5	397504	BioLegend
CD45 PE-Cy7	Mouse	30-F11	103114	BioLegend
CD45 PE-Cy7	Human	2D1	368532	BioLegend
CD47 APC	Mouse	Miap301	127514	BioLegend
CD47 PE-Cy7	Human	CC261	323113	BioLegend
CD56 Brilliant Violet 605	Human	HCD56	318334	BioLegend
CD66a	Mouse	Mab-CC1	134540	BioLegend
CD66a/c/e Brilliant Violet 421	Human	ASL-32	342313	BioLegend
CD69 PE-Cy5	Mouse	H1.2F3	104510	BioLegend
CD69 Brilliant Violet 421	Human	FN50	310930	BioLegend
CD107a FITC	Mouse	1D4B	121606	BioLegend
CD200 (OX2) APC	Mouse	OX90	123810	BioLegend
CD200 (OX2) Brilliant Violet 711	Human	OX-104	329222	BioLegend
F4/80 FITC	Mouse	BM8	123108	BioLegend
I-A^k (MHC class II) PE	Mouse	10–3.6	109908	BioLegend
LAP/TGF-β1 PE	Mouse	TW7–16B4	141404	BioLegend
LAP/TGF-β1 FITC	Human	S20006A	300010	BioLegend
Ly-6C Brilliant Violet 421	Mouse	HK1.4	128032	BioLegend
Ly-6G Brilliant Violet 785	Mouse	1A8	127645	BioLegend
NKp46 Brilliant Violet 711	Mouse	9E2	331936	BioLegend
NKp46 PerCP/Cyanine5.5	Mouse	29A1.4	137610	BioLegend
NKX6.1 PE	Human	R11–560	563023	BD Biosciences
PD-L1 PerCP/Cyanine5.5	Mouse	10F.9G2	124334	BioLegend

(Continued)

TABLE 1 Continued

Ethical approval for all human cell experiments was meticulously obtained from the Harvard University Institutional Review Board (IRB) and the Embryonic Stem Cell Research Oversight (ESCRO) committees, underscoring our commitment to ethical research practices and compliance with regulatory guidelines. $RNLS^{mut}$ iPSCs from an individual with T1D (Local donor 3) were obtained, generated, and gifted by the Melton Lab (Harvard University) as described before (9).

Bulk RNA sequencing

Bulk RNAseq of WT and $Rnls^{mut}$ NIT-1 cells were performed previously (10). In brief, triplicates of 2 × 106 NIT-1 cells were used for RNA isolation using Zymo Quick-RNA miniprep plus kit (cat # R1058; Zymo Research), following the manufacturer's protocol. RNA libraries were prepared, and subsequently quality control tested by Novogen Corporation Inc. Sequencing was performed on an Illumina NovaSeq 6000 sequencing system for a >20 million read data output (Novogen Corporation Inc.). For enrichment analysis of immune system-related genes (according to GSEA-MSigDB, https://www.gsea-msigdb.org) the 1,500 most significant upregulated genes in *Rnls* mutant *vs.* WT control are shown (Figure 3A).

NK cell/NIT-1 beta cell co-culture

NK cells were obtained from spleens of 8- to 16-week-old non-diabetic NOD/ShiLtJ mice purchased from The Jackson Laboratory (strain #: 001976; three to four spleens were used per experiment to collect sufficient amount of NK cells) using the $NKp46^+$ NK Isolation Kit (cat # 130–115-818; Miltenyi Biotec). For co-culture experiments, 10^4 NIT-1 cells were seeded in each well of a 96-well flat bottom (trans-well assays) or round-bottom (co-culture assays) plate in 100 µl of NK cell medium (DMEM, high glucose, pyruvate (cat # 11–995-073; Gibco), supplemented with 10% FCS (cat # 10–082-147; Gibco), 2 mM L-glutamine (cat # 25–030-081; Gibco), and penicillin/streptomycin (cat # 15140122; Thermo Fisher Scientific) following incubation for 3 days in a 37°C incubator with 5% CO_2. In trans-well assays, NK cells were fluorescently labeled using *Tag-it Violet* proliferation dye according to the manufacturers' protocol (cat # 425101; BioLegend) and 5 × 10^4 NK cells were added to NIT-1 cell-containing 96-well plates either in direct co-culture or on a

trans-well insert (cat # 3380; Corning). Recombinant mouse (m)IL-2 (cat # 575406; BioLegend) was added to stimulate NK cells with a total of 20–100 ng/mL final concentration as indicated. For trans-well assays, 100 ng/mL mIL2 was freshly added daily to achieve stronger NK cell proliferation. To investigate NK cell proliferation, NK cells were stained with Tag-it Viole Proliferation and Cell Tracking Dye (cat # 425101; BioLegend) according to the manufacturer's instructions. Conditioned media experiments were performed by the addition of medium derived from WT or $Rnls^{mut}$ NIT-1 cells with a ratio of 1:1 (conditioned media:NK cell media). Additional conditioned medium was supplemented following 24 h (final ratio 3:1) and 48 h (final ratio 4:1) each time in combination with repetitive IL2 stimulation (100 ng/mL). Supernatants were collected following centrifugation for 5 min at 300 g and stored at −20°C. ELISA for Ifng (cat # MIF00; R&D Systems) and Tgfβ1 (cat # DB100C; R&D Systems) were performed according to the manufacturer's instructions. In all experiments, cells were co-cultured in a 37°C incubator with 5% CO_2 for 72 h, then collected and stained as described in the "Flow cytometry" section with indicated antibodies.

PBMC/SCBC co-culture

PBMCs collected from allogenic donors (under protocol Joslin CHS # 2013–10) in EDTA tubes were isolated using Lymphoprep density gradient medium (cat # 07801; Stem Cell Technologies). A total of 5×10^6 PBMCs were transferred into each well of a 6-well plate with fully confluent WT or $RNLS^{mut}$ SCBC in 3 mL of HEPES buffered PBMC medium [RPMI-1640 with penicillin/streptomycin, L-glutamine, and HEPES (cat # ABI-Custom American BioInnovations), supplemented with 10% human AB serum (cat # H5667; Millipore Sigma) and 100 ng/mL recombinant human IL2 (cat # 589104; BioLegend)] following incubation at 37°C with 5% CO_2. Following co-culture for 72 h, cells were detached with Trypsin-EDTA (cat # 25–200-056; Gibco) and filtered using a 70-µm pore size strainer twice to obtain a single-cell solution. Collected cells were stained with indicated antibodies listed in Table 1 as described in the "Flow cytometry" section. The quantification of alive $CD45^-$ SCBCs was performed by using Precision Count Beads (cat # 424902; BioLegend). Cells were collected and stained as described in the "Flow cytometry" section with indicated antibodies.

Splenocyte transfer mouse model and graft isolation

WT and $Rnls^{mut}$ NIT-1 beta cells (10^7 each) were injected subcutaneously (s.c.) into opposite flanks of the same immunodeficient NOD.Cg-$Prkdc^{scid}$/J (NOD; strain #: 001303) or NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ (NSG; starin #: 005557) mice purchased from The Jackson Laboratory. Four days later, autoreactive splenocytes from recently diabetic NOD mice were isolated and red blood cells were lysed using 5 mL of ACK lysing buffer (cat # A1049201; Thermo Fisher Scientific) for 4 min at RT. Lysis was stopped using 5 mL of PBS/10% FCS followed by two washing steps using PBS only. Splenocytes were filtered two times in total through a strainer with 70 µm pore size to obtain a single-cell solution. For NK cell depletion, NKp46-expressing splenocytes were removed by using the anti-NKp46 MicroBead kit (cat # 130–095-390; Miltenyi Biotec) according to the manufacturer's instructions. Splenocytes (10^7/mouse) were injected intravenously (i.v.) into NIT-1 cell-bearing NOD.scid/NSG recipient mice to transfer autoimmune beta cell killing for a total of 17 days. NIT-1 beta cell graft isolation was performed as described before (10). In brief, grafts were isolated and scaled on an analytical balance 17 days after splenocyte transfer. Grafts were cut into small pieces and digested using HEPES buffered RMPI 1640 (cat # R4130–10L; Sigma-Aldrich) supplemented with 1 mg/mL Collagenase D (cat # 11088858001; Sigma-Aldrich), 20 µg/mL DNase I (cat # EN0521; Thermo Fisher Scientific), 2% FCS, and 50 µg/mL lipopolysaccharide neutralizing agent Polymyxin B sulfate (cat # 1405–20-5; Sigma-Aldrich) for 45 min at 37°C while shaking by maximum speed (750 rpm) on a heating block. Digested grafts were further disaggregated and filtered through a strainer with 70 µm pore size twice to obtain a single-cell solution. For flow cytometry analysis, cells were stained with the antibodies listed in Table 1 in two separate panels (panel 1: T cells and NK cells; panel 2: myeloid cells and B cells).

Single-cell RNA sequencing

The scRNAseq experiment was performed in our previous study (10). In brief, WT or *Rnls* mutant graft-infiltrating immune cells were FACS sorted ($CD45^+$ PI^-). Individual samples were labeled using hashtag antibodies and scRNAseq was performed on pooled samples using the Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1 (cat # PN-1000213; 10x Genomics) according to the manufacturer's instructions. Samples were super-loaded with 40,000 cells per reaction. The hashtag oligo library (HTO) was generated separately as described previously (https://citeseq.files.wordpress.com/2019/02/cell_hashing_protocol_190213.pdf). Illumina NovaSeq 6000 with approximately 1.1 billion reads was used to sequence the gene expression library, while the HTO library was sequenced separately by Illumina NextSeq with approximately 130 million reads total each. For further details on QC and analysis procedure, please refer to our previous work (10). In this study, gene set enrichment analyses of graft-infiltrating NK cells and ILC1 were performed by using the molecular signatures of hallmark gene sets provided by the MSigDB database (http://www.gsea-msigdb.org/). The indicated number of differential expressed genes within the indicated immune cell population derived from WT or $Rnls^{mut}$ NIT-1 beta cell grafts were used as input.

Flow cytometry

All cell surface staining procedures were performed in PBS/2 mM EDTA/10% FCS. Single-cell suspensions were pre-incubated with FcR-blocking antibody solution (cat # 156604; BioLegend) for 5 min, then stained with antibodies against indicated molecules (Table 1) for 20 min on ice diluted in FcR-blocking solution. Appropriate isotype controls were purchased from BioLegend. Dead cells were excluded using propidium iodide (PI, cat # R37169; Thermo Fisher Scientific) or fixable viability dye eFluor 450 (cat # 65–0863; eBiosciences). For intracellular staining (SCBC QC), the *Transcription Factor Staining Buffer Set* (cat # 00–5523-00; Thermo Fisher Scientific) was used according to the manufacturer's instruction. In brief, following surface staining of CD47, CD66/a/c/e, and CD200 performed as described above using human FcR blocking solution (cat # 422302; BioLegend), cells were washed with PBS/2 mM EDTA/10% FCS following cell fixation with *Fixation/Permeabilization* working solution and pulse vortexing. Cells were incubated for 90 min at 4°C and washed following 2 permeabilization steps using the provided *Permeabilization Buffer*. Cells were pre-incubated in 5% human FcR-blocking antibody solution for 15 min at room temperature (RT). Cells were stained with indicated antibodies against C-peptide and NKX6.1 (working dilution of 1:20) listed in Table 1 for 30 min at RT. Cells were washed twice, kept on ice and analyzed by flow cytometry. For the 2DG experiment to inhibit glucose metabolism in NIT-1 cells, 2×10^5 cells of indicated genotypes were seeded into a 24-well plate for 48 h with or without addition of indicated concentrations of 2DG or sterile dH_2O as control. Cell surface staining of indicated molecules were performed as described above. Prior to analysis, all cell suspensions were filtered using a 35-µm strainer (cat # 352235; Corning) to avoid cell cluster. Data were acquired on an LSRII instrument (BD Biosciences) and analyzed with *FlowJo* software v.10.6.1 (FlowJo LLC). All data are shown using log-scale axes.

Statistical analyses

Statistical analyses were performed by unpaired or paired tests or by one-way ANOVA as indicated using GraphPad Prism v 9.0.2 software. All data are presented as the mean ± standard deviation (SD). $p < 0.05$ was considered statistically significant. Sufficient sample size was estimated without the use of a power calculation. No samples were excluded from the analyses. No randomization was used for the animal experiments. Data analysis was not blinded. All data are representative of two or more similar experiments.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving human materials were approved by Harvard University Institutional Review Board (IRB) and the Embryonic Stem Cell Research Oversight (ESCRO) committees, and Joslin CHS # 2013-10. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Joslin Diabetes Center IACUC 2013-03. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KB: Conceptualization, Formal Analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Data curation. SW: Investigation, Methodology, Writing – review & editing, Formal Analysis. IG: Formal Analysis, Investigation, Methodology, Writing – review & editing. JL: Formal Analysis, Methodology, Writing - review & editing, Data curation. SK: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. PY: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

PY and SK are inventors on a patent application filed by the Joslin Diabetes Center that relates to the targeting of RNLS for the protection of transplanted SC-islets.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1403752/full#supplementary-material

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EDITED BY
Ce Wang,
AbbVie, United States

REVIEWED BY
Siqi Sun,
The State University of New Jersey, United States
Zhangyuan Yin,
Broad Institute, United States

*CORRESPONDENCE
Wenjiao Cai
caiwenjiao2010@163.com

†These authors have contributed equally to this work

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IL6 receptor inhibitors: exploring the therapeutic potential across multiple diseases through drug target Mendelian randomization

Chong Fu[1†], Longquan Wang[2†] and Wenjiao Cai[3*]

[1]Department of Gastroenterology, Anqing Municipal Hospital, Anqing, Anhui, China, [2]Department of Geriatric Medicine, The First Affiliated Hospital of Wannan Medical College, Wuhu, Anhui, China, [3]Department of Nephrology, Anqing Municipal Hospital, Anqing, Anhui, China

Background: High interleukin-6 levels correlate with diseases like cancer, autoimmune disorders, and infections. IL-6 receptor inhibitors (IL-6Ri), used for rheumatoid arthritis and COVID-19, may have wider uses. We apply drug-target Mendelian Randomization (MR) to study IL-6Ri's effects.

Method: To simulate the effects of genetically blocking the IL-6R, we selected single nucleotide polymorphisms (SNPs) within or near the IL6R gene that show significant genome-wide associations with C-reactive protein. Using rheumatoid arthritis and COVID-19 as positive controls, our primary research outcomes included the risk of asthma, asthmatic pneumonia, cor pulmonale, non-small cell lung cancer, small cell lung cancer, Parkinson's disease, Alzheimer's disease, ulcerative colitis, Crohn's disease, systemic lupus erythematosus, type 1 diabetes, and type 2 diabetes. The Inverse Variance Weighted (IVW) method served as our principal analytical approach, with the hypotheses of MR being evaluated through sensitivity and colocalization analyses. Additionally, we conducted Bayesian Mendelian Randomization analyses to minimize confounding and reverse causation biases to the greatest extent possible.

Results: IL-6 inhibitors significantly reduced the risk of idiopathic pulmonary fibrosis (OR= 0.278, 95% [CI], 0.138–0.558; P <0.001), Parkinson's disease (OR = 0.354, 95% CI, 0.215–0.582; P <0.001), and positively influenced the causal relationship with Type 2 diabetes (OR = 0.759, 95% CI, 0.637–0.905; P = 0.002). However, these inhibitors increased the risk for asthma (OR = 1.327, 95% CI, 1.118–1.576; P = 0.001) and asthmatic pneumonia (OR = 1.823, 95% CI, 1.246–2.666; P = 0.002). The causal effect estimates obtained via the BWMR method are consistent with those based on the IVW approach. Similarly, sIL-6R also exerts a significant influence on these diseases.Diseases such as Alzheimer's disease, Crohn's disease, pulmonary heart disease, systemic lupus erythematosus, Type 1 diabetes, Non-small cell lung cancer and ulcerative colitis showed non-significant associations (p > 0.05) and were excluded from further analysis. Similarly, Small cell lung cancer were excluded due to inconsistent results. Notably, the colocalization evidence for asthmatic pneumonia (coloc.abf-PPH4 = 0.811) robustly supports its association with CRP. The colocalization evidence for Parkinson's disease (coloc.abf-PPH4 = 0.725) moderately supports its association with CRP.

Conclusion: IL-6Ri may represent a promising therapeutic avenue for idiopathic pulmonary fibrosis, Parkinson's disease, and Type 2 diabetes.

KEYWORDS

IL-6 receptor, Mendelian randomization, idiopathic pulmonary fibrosis, Parkinson's disease, type 2 diabetes

Introduction

Excessive production of IL-6 is characteristic of many rheumatic diseases, including Rheumatoid Arthritis, Juvenile Idiopathic Arthritis, and Adult-Onset Still's Disease (1). IL-6Ri are increasingly used when traditional treatments with DMARDs, corticosteroids, and non-steroidal anti-inflammatory drugs prove ineffective, particularly in conditions such as RA, systemic Juvenile Idiopathic Arthritis, and Castleman's disease (2). Recent advances in understanding the pathogenesis of rheumatic diseases have expanded the use of IL-6Ri to other rheumatic conditions, including AOSD, Giant Cell Arteritis, Behçet's Disease, and Polymyalgia Rheumatica, thus positioning IL-6 or its receptor blockade as a novel strategy for managing certain rheumatic ailments. IL-6 inhibitors include Tocilizumab, Sarilumab, Sirukumab, Clazakizumab, Olokizumab, MEDI-5117, and ALX-0061 (3). Currently, only Tocilizumab and Sarilumab are commercially available, with Tocilizumab being the most widely used internationally, while other inhibitors are either in development or under research (4).

IL-6 is produced by a variety of cells, including hepatocytes, T cells, B cells, fibroblasts, monocytes, mesangial cells, keratinocytes, endothelial cells, and many tumor cells (5). The IL-6R is an 80 kDa type I transmembrane protein primarily expressed on immune effector cells such as T cells, B cells, monocytes, neutrophils, and macrophages, as well as some non-immune effector cells, including pancreatic and hepatic cells. IL-6R exists in two forms: a membrane-bound version (mIL-6R) and a soluble version (sIL-6R) (6). The binding of IL-6 to mIL-6R can induce homodimerization of gp130, forming a high-affinity IL-6/IL-6R/gp130 complex. The sIL-6Rs are produced either through the cleavage of mIL-6R by the protease ADAM-17 or via selective mRNA splicing. IL-6 can also bind to circulating sIL-6R, forming complexes with gp130 (7). The binding of IL-6 to mIL-6R activates the classical signaling pathway, while its interaction with sIL-6R initiates the trans-signaling pathway, both of which engage with membrane-bound glycoprotein 130 (mgp130) to trigger cascades involving the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and the mitogen-activated protein kinase (MAPK) pathways (8). These complexes induce tyrosine phosphorylation in the cytoplasmic domain of gp130, which promotes the recruitment of STAT-3, leading to the expression of pro-inflammatory genes and suppressor of cytokine signaling proteins (SOCS) (9). The activation of RAS/mitogen-activated protein kinases (MAPKs) mediates the phosphorylation and activation of nuclear factor (NF)-IL-6, which binds to the IL-6 response elements in the promoter regions of acute-phase gene, inducing the production of acute-phase proteins. The IL-6/IL-6R/gp130 complex also activates JAK-1 and JAK-2 kinases, as well as downstream transcription factors such as STAT1, STAT3, and phosphoinositide 3-kinase (PI3K). The activated STATs translocate to the nucleus, regulating several genes, while the activated PI3K in turn activates the serine/threonine-protein kinase (B/AKT). The activation of JAK/STATS/PI3K and MAPK/ERK pathways mediated by IL-6 can induce a broad spectrum of immune responses (10).

MR is an instrumental variable analysis method that employs SNPs from genome-wide association studies (GWAS) as genetic tools to estimate the causal effects of exposures on outcomes. Compared to observational studies, MR's advantage lies in leveraging the random allocation of alleles to circumvent biases from unobserved confounders, such as lifestyle factors and other environmental impacts, as well as issues related to reverse causality (11). This study utilizes drug-target Mendelian Randomization analysis, which employs genetic variants of simulated pharmacological inhibitions as instrumental variables. By conducting regression analysis, this method elucidates the long-term effects of medications and enhances causal inferences about these drug targets' potential impact on diseases. Evidence suggests that drug-target MR effectively identifies targets with up to 70% efficiency (12). We have gathered recently published GWAS summary-level statistics to explore the causal relationships between genetically predicted IL6R inhibition and 15 diseases, including COVID-19, rheumatoid arthritis, asthma, asthmatic pneumonia, cor pulmonale, non-small cell lung cancer, small cell lung cancer, Parkinson's, Alzheimer's disease, ulcerative colitis, Crohn's disease, systemic lupus erythematosus, and types 1 and 2 diabetes through drug-target MR analysis.

Methods

Study design and data resources

Figure 1 summarizes the design of this study. C-reactive protein (CRP) GWAS data was derived from a study encompassing 204,402 Europeans(GWAS ID:ieu-b-35). CRP was selected as a biomarker

because it is well-documented that pharmacological inhibition of IL-6R can reduce CRP levels, a finding substantiated by clinical trials (13, 14). By acquiring instrumental variables that target IL6R to diminish CRP levels, we can simulate the effects of IL6Ri (15). These instrumental variables are SNPs located within ±100kb of the IL6R gene locus, which are associated with CRP levels and meet the genome-wide significance threshold set at p<5×10^-8. To mitigate the impact of strong linkage disequilibrium (LD) on the results, an LD threshold (r^2<0.3) was established. Ultimately, 10 significant IL6R SNPs were retained (Supplementary Material 1: Supplementary Table S2). To verify the absence of weak instrument bias in our selected instrumental variables, we employed the F-statistic, where an F > 10 indicates the absence of weak instrument bias, further validating the associative hypotheses. The formula for the F-statistic is F = [(N - K - 1)/K] × [R^2/(1 - R^2)], where N is the sample size of the exposure, K is the number of instrumental variables, and R^2 is the proportion of exposure variance explained by the instrumental variables.

We utilized fifteen diseases for our drug-target Mendelian Randomization analysis, with rheumatoid arthritis(GWAS ID:finn-b-RHEUMA_NOS,n=217134) and COVID-19(GWAS ID:ebi-a-GCST011077,n=1059456) serving as two positive control datasets, all sourced from European populations. Additionally, we compiled GWAS summary data sets for idiopathic pulmonary fibrosis (GWAS ID:finn-b-IPF,n=198014), asthma (GWAS ID:finn-b-J10_ASTHMA, n=156078),asthmatic pneumonia (GWAS ID:finn-b-ASTHMA_PNEUMONIA_AND_SEPSIS,n=140994), cor pulmonale (GWAS ID:finn-b-FG_PULMHEART,n=218792), non-small cell lung cancer (GWAS ID:finn-b-C3_LUNG_NONSMALL_EXALLC, n=175633), small cell lung cancer (GWAS ID:finn-b-C3_SCLC, n=218792),Parkinson's disease(GWAS ID:finn-b-PDSTRICT, n=218473),Alzheimer's disease (GWAS ID:finn-b-G6_ALZHEIMER,n=156078),ulcerative colitis (GWAS ID:finn-b-K11_ULCER,n=214620),Crohn's disease (GWAS ID:finn-b-K11_CROHN,n=212356),systemic lupus erythematosus (GWAS ID:finn-b-M13_SLE,n=213683), type 1 diabetes (GWAS ID:finn-b-E4_DM1,n=189113), and type 2 diabetes (GWAS ID:finn-b-E4_DM2,n=215654)(Supplementary Material 1: Supplementary Table S1). These principal outcome datasets originated from the Finnish database, accessible via the MR-Base platform (https://www.mrbase.org/). There were no overlapping samples between the exposure and outcome GWAS, and all participants were of European descent.This research complies with the Strengthening the Reporting of Observational Studies in Epidemiology Using Mendelian Randomization(STROBE-MR) guidelines (16), and the corresponding checklist can be found in the Supporting Information (Supplementary Material 1: STROBE-MR checklist).

MR analysis

IL-6Ri have been extensively utilized in the treatment of rheumatoid arthritis and COVID-19. Consequently, we employed GWAS summary data from these diseases as positive controls to validate the efficacy of our instrumental variables. Initially, we harmonized drug-target instrumental variables related to exposure with the outcome datasets. We implemented five MR methods to assess the causal association between these drug-target instrumental variables and the outcome datasets, including MR-Egger regression, Weighted Median Estimator (WME), Inverse Variance Weighted (IVW), Simple Mode, and Weighted Mode. The IVW method was chosen as the primary analytical approach

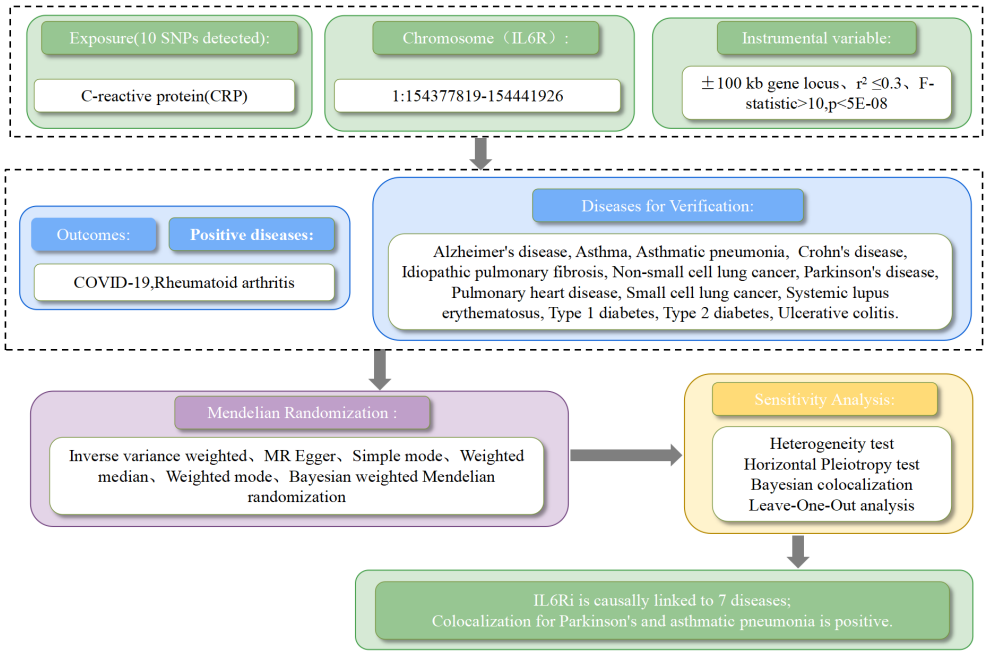

FIGURE 1
The workflow of drug target mendelian randomization study that exhibits causality between IL6 receptor inhibitors and multiple diseases.

because it provides the most precise estimates by analyzing each Wald ratio under the ideal condition that all instrumental variables are valid (17). Given that this represents an ideal scenario, it is crucial to corroborate these findings with other methods to ensure robustness and validity across various analytical conditions. Additionally, a Bayesian weighting strategy was used to simultaneously address measurement errors and pleiotropy, thus enhancing the robustness of our analysis (18).

Sensitivity analyses

Heterogeneity tests were conducted using MR Egger and IVW methods. Cochran's Q value was employed to evaluate the heterogeneity of genetic instruments, with $p > 0.05$ indicating no significant heterogeneity. MR Egger regression and MR PRESSO were used to assess pleiotropy of the genetic instruments, with $p > 0.05$ indicating no pleiotropy (19, 20). These steps help ensure the reliability of our instrumental variables in assessing causal relationships.

Bayesian colocalization analysis is employed to assess the probability that two traits share the same causal variant, facilitated by the 'coloc' package using default parameters (https://github.com/chr1swallace/coloc) (21). Bayesian colocalization offers posterior probabilities for five hypotheses, determining whether two traits share a single variant. This analysis evaluates support for five exclusive hypotheses: 1) associated with neither phenotype; 2) associated only with phenotype 1; 3) associated only with phenotype 2; 4) both phenotypes are associated, but with different causal variants; and 5) both phenotypes are associated with the same causal variant. Strong colocalization support is considered when the posterior probability of a shared causal variant (PH4) exceeds 0.8. Moderate colocalization is defined as a PH4 value between 0.5 and 0.8.

Statistical power calculation

The statistical efficacy of the research was assessed using an online power calculator developed by Burgess (https://sb452.shinyapps.io/power/) (22). This comprehensive evaluation incorporated the total sample size, a significance level of 0.05, the variance explained by instrumental variables concerning exposure, and the ratio of exposure between the case and control groups, as well as the OR from the MR analysis. We established an objective to achieve a statistical power threshold of at least 0.8 to ensure that when the p-value is less than 0.05, the findings achieve statistical significance, thereby validating the scientific rigor of the results.

Results

Positive control analysis

The causal effects of IL6Ri on fifteen diseases were analyzed using Mendelian Randomization (MR), setting a strict p-value threshold of 5×10^{-8}. Through rigorous criteria ($r^2 < 0.3$, kb = 100 KB), ten significant IL6Ri SNPs were identified. The F-statistics for these instrumental variables were all above 10, indicating the absence of weak instrumental bias and confirming the reliability of the results (Supplementary Material 1: Supplementary Table S2). Genetic variations in the IL6R target associated with inhibitors were linked to a reduced risk of rheumatoid arthritis and COVID-19. Specifically, there was a negative causal relationship between IL6Ri and rheumatoid arthritis (OR = 0.567, 95% CI, 0.329–0.977; P< 0.041) and COVID-19 (OR = 0.653, 95% CI, 0.449–0.950; P= 0.026). The causal effect estimates from the BWMR method were consistent with those based on the IVW method (Figure 2, Supplementary Material 1: Supplementary Tables S3, S4).

The causal relationship between gene-simulated IL6Ri and multiple diseases

MR analysis revealed associations between IL6Ri and diseases such as idiopathic pulmonary fibrosis, Parkinson's disease, type 2 diabetes, asthma, and asthmatic pneumonia. Specifically, there was a negative causal relationship between IL6Ri and idiopathic pulmonary fibrosis (OR = 0.278, 95% CI, 0.138–0.558; P <0.001), Parkinson's disease (OR = 0.354, 95% CI, 0.215–0.582; P <0.001), and type 2 diabetes (OR = 0.759, 95% CI, 0.637–0.905; P = 0.002). Conversely, a positive causal relationship was found between IL6Ri and asthma (OR = 1.327, 95% CI, 1.118–1.576; P = 0.001), and asthmatic pneumonia (OR = 1.823, 95% CI, 1.246–2.666; P = 0.002 (Figure 2 and Supplementary Material 1: Supplementary Table S3).The BWMR method were consistent with those based on the IVW method (Figure 3A and Supplementary Material 1: Supplementary Table S4). And the effect of each SNP locus on these diseases is shown in Figures 4, 5. Moreover, the robust statistical power of 100% for all these IVW results emphasizes the findings' reliability (Supplementary File 1: Supplementary Table S4). Diseases such as ulcerative colitis, crohn's disease, systemic lupus erythematosus, and cor pulmonale had p-values greater than 0.05 and were excluded from further analysis. Moreover, non-small cell and small cell lung cancers were excluded due to inconsistent effect directions across the six methods.

Complex pathophysiological mechanisms involving multiple factors in conditions such as cor pulmonale, non-small cell lung cancer, Alzheimer's disease, type 1 diabetes, and inflammatory bowel disease, which includes both ulcerative colitis and Crohn's disease, potentially obscure the efficacy of IL6Ri. In cor pulmonale, factors like chronic obstructive pulmonary disease and pulmonary embolism might interact with or independently influence the disease progression, complicating the role of IL6Ri. Non-small cell lung cancer, driven by mutations in numerous oncogenes and tumor suppressor genes, may diminish the impact of IL6Ri, overshadowed by other predominant genetic factors. The complex etiology of Alzheimer's disease involving amyloid-beta deposition and neuroinflammation suggests that while IL6Ri may participate in controlling inflammation, its overall role in disease progression is

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id.outcome	nsnp	method	pval	orDrug(95% CI)	Q_pval	Pleiotropy_P	Global_Test_P
Alzheimer disease	10	Inverse variance weighted	0.254	0.806 (0.557 to 1.167)	0.757	0.567	
	10	MR Egger	0.357	0.608 (0.224 to 1.649)			
	10	Simple mode	0.983	1.008 (0.490 to 2.076)			
	10	Weighted median	0.440	0.830 (0.518 to 1.330)			
	10	Weighted mode	0.479	0.817 (0.477 to 1.397)			
ASTHMA	10	Inverse variance weighted	**0.001**	1.327 (1.118 to 1.576)	0.425	0.478	0.491
	10	MR Egger	0.101	1.570 (0.975 to 2.529)			
	10	Simple mode	0.207	1.260 (0.903 to 1.759)			
	10	Weighted median	**0.017**	1.324 (1.052 to 1.668)			
	10	Weighted mode	**0.044**	1.330 (1.047 to 1.690)			
ASTHMA_PNEUMONIA_AND_SEPSIS	10	Inverse variance weighted	**0.002**	1.823 (1.246 to 2.666)	0.101	0.244	0.163
	10	MR Egger	**0.047**	3.299 (1.218 to 8.936)			
	10	Simple mode	**0.013**	2.355 (1.373 to 4.039)			
	10	Weighted median	**<0.001**	2.160 (1.450 to 3.219)			
	10	Weighted mode	**0.005**	2.155 (1.437 to 3.231)			
COVID−19	10	Inverse variance weighted	**0.026**	0.653 (0.449 to 0.950)	0.738	0.457	0.820
	10	MR Egger	0.931	0.955 (0.344 to 2.652)			
	10	Simple mode	0.156	0.590 (0.303 to 1.150)			
	10	Weighted median	0.060	0.639 (0.401 to 1.019)			
	10	Weighted mode	0.085	0.605 (0.363 to 1.007)			
Crohn disease	10	Inverse variance weighted	0.168	0.717 (0.448 to 1.150)	0.825	0.942	
	10	MR Egger	0.578	0.686 (0.191 to 2.456)			
	10	Simple mode	0.645	0.806 (0.332 to 1.955)			
	10	Weighted median	0.284	0.724 (0.401 to 1.306)			
	10	Weighted mode	0.549	0.816 (0.430 to 1.547)			
Idiopathic pulmonary fibrosis	10	Inverse variance weighted	**<0.001**	0.278 (0.138 to 0.558)	0.371	0.306	0.475
	10	MR Egger	**0.046**	0.106 (0.016 to 0.682)			
	10	Simple mode	0.447	0.582 (0.153 to 2.211)			
	10	Weighted median	**0.003**	0.269 (0.114 to 0.635)			
	10	Weighted mode	**0.030**	0.310 (0.127 to 0.758)			
Non−small cell lung cancer	10	Inverse variance weighted	0.877	1.043 (0.615 to 1.766)	0.695	0.441	
	10	MR Egger	0.506	0.603 (0.145 to 2.509)			
	10	Simple mode	0.602	1.283 (0.520 to 3.166)			
	10	Weighted median	0.796	1.094 (0.552 to 2.170)			
	10	Weighted mode	0.797	1.104 (0.531 to 2.295)			
Parkinson's disease	10	Inverse variance weighted	**<0.001**	0.354 (0.215 to 0.582)	0.444	0.298	0.494
	10	MR Egger	**0.034**	0.174 (0.045 to 0.669)			
	10	Simple mode	0.230	0.487 (0.163 to 1.457)			
	10	Weighted median	**0.003**	0.366 (0.187 to 0.715)			
	10	Weighted mode	**0.024**	0.361 (0.173 to 0.753)			
Pulmonary heart disease	10	Inverse variance weighted	0.562	1.104 (0.790 to 1.543)	0.871	0.340	
	10	MR Egger	0.280	1.708 (0.690 to 4.224)			
	10	Simple mode	0.915	0.964 (0.500 to 1.859)			
	10	Weighted median	0.554	1.134 (0.747 to 1.722)			
	10	Weighted mode	0.491	1.193 (0.737 to 1.933)			
rheumatoid arthritis	10	Inverse variance weighted	**0.041**	0.567 (0.329 to 0.977)	0.262	0.639	0.341
	10	MR Egger	0.794	0.809 (0.174 to 3.765)			
	10	Simple mode	0.444	0.662 (0.241 to 1.818)			
	10	Weighted median	0.124	0.597 (0.310 to 1.152)			
	10	Weighted mode	0.214	0.620 (0.308 to 1.250)			
Small cell lung cancer	10	Inverse variance weighted	**0.021**	6.269 (1.317 to 29.836)	0.727	0.176	
	10	MR Egger	0.612	0.321 (0.005 to 21.836)			
	10	Simple mode	**0.040**	65.167 (2.126 to 1997.944)			
	10	Weighted median	0.246	3.212 (0.447 to 23.088)			
	10	Weighted mode	0.321	3.321 (0.354 to 31.187)			
Systemic lupus erythematosus	10	Inverse variance weighted	0.450	1.414 (0.576 to 3.475)	0.983	0.888	
	10	MR Egger	0.889	1.196 (0.105 to 13.627)			
	10	Simple mode	0.984	0.983 (0.187 to 5.176)			
	10	Weighted median	0.679	1.282 (0.395 to 4.164)			
	10	Weighted mode	0.749	1.251 (0.331 to 4.730)			
Type 1 diabete	10	Inverse variance weighted	0.195	0.816 (0.601 to 1.109)	0.746	0.228	
	10	MR Egger	0.486	1.362 (0.595 to 3.115)			
	10	Simple mode	0.251	0.657 (0.336 to 1.286)			
	10	Weighted median	0.480	0.866 (0.582 to 1.290)			
	10	Weighted mode	0.895	0.970 (0.626 to 1.504)			
Type 2 diabetes	10	Inverse variance weighted	**0.002**	0.759 (0.637 to 0.905)	0.218	0.873	0.277
	10	MR Egger	0.255	0.730 (0.441 to 1.208)			
	10	Simple mode	0.522	0.884 (0.615 to 1.270)			
	10	Weighted median	**0.020**	0.779 (0.631 to 0.961)			
	10	Weighted mode	0.102	0.801 (0.631 to 1.017)			
Ulcerative colitis	10	Inverse variance weighted	0.334	0.849 (0.610 to 1.183)	0.965	0.655	
	10	MR Egger	0.943	1.034 (0.422 to 2.535)			
	10	Simple mode	0.546	0.824 (0.450 to 1.508)			
	10	Weighted median	0.553	0.881 (0.580 to 1.339)			
	10	Weighted mode	0.695	0.912 (0.583 to 1.426)			

FIGURE 2
Results and forest plot of MR analysis of IL-6Ri's causal relationship with 15 diseases.nsnp: The final number of SNPs used in the analysis. orDrug: The estimated effect of IL6Ri on diseases. Q_pval: The P value of the Cochran Q test. Pleiotropy_P: The P value of the MR-Egger regression intercept hypothesis test. Global_Test_P: The P value of the MR-PRESSO global test.

limited. Similarly, in type 1 diabetes and inflammatory bowel disease, despite the role of IL6R in immune modulation, the effects of IL6Ri are likely moderated by other significant genetic or environmental factors, reducing its singular impact. Thus, even though IL6Ri demonstrates potential therapeutic promise, its actual effectiveness in these complex diseases may be limited by interference from other pathophysiological mechanisms.

Effect of sIL-6R representing the IL-6 trans-signaling pathway on five diseases

Extensive literature suggests that the IL-6 trans-signaling pathway plays a pivotal role in the IL-6 signaling cascade within coronary artery disease (23–26). MR analysis, utilizing rs2228145 as the sole instrumental variable, has demonstrated that attenuated IL-

A

Disease	Drugtarget	nsnp	method	pval	OR(95% CI)
ASTHMA	IL6R	10	BWMR	**0.001**	1.330 (1.581 to 1.119)
ASTHMA_PNEUMONIA_AND_SEPSIS	IL6R	10	BWMR	**0.002**	1.807 (2.606 to 1.253)
COVID−19	IL6R	10	BWMR	**0.026**	0.651 (0.950 to 0.446)
Idiopathic pulmonary fibrosis	IL6R	10	BWMR	**<0.001**	0.273 (0.557 to 0.133)
Parkinson's disease	IL6R	10	BWMR	**<0.001**	0.351 (0.583 to 0.211)
rheumatoid arthritis	IL6R	10	BWMR	**0.040**	0.555 (0.972 to 0.317)
Type 2 diabetes	IL6R	10	BWMR	**0.002**	0.751 (0.897 to 0.629)

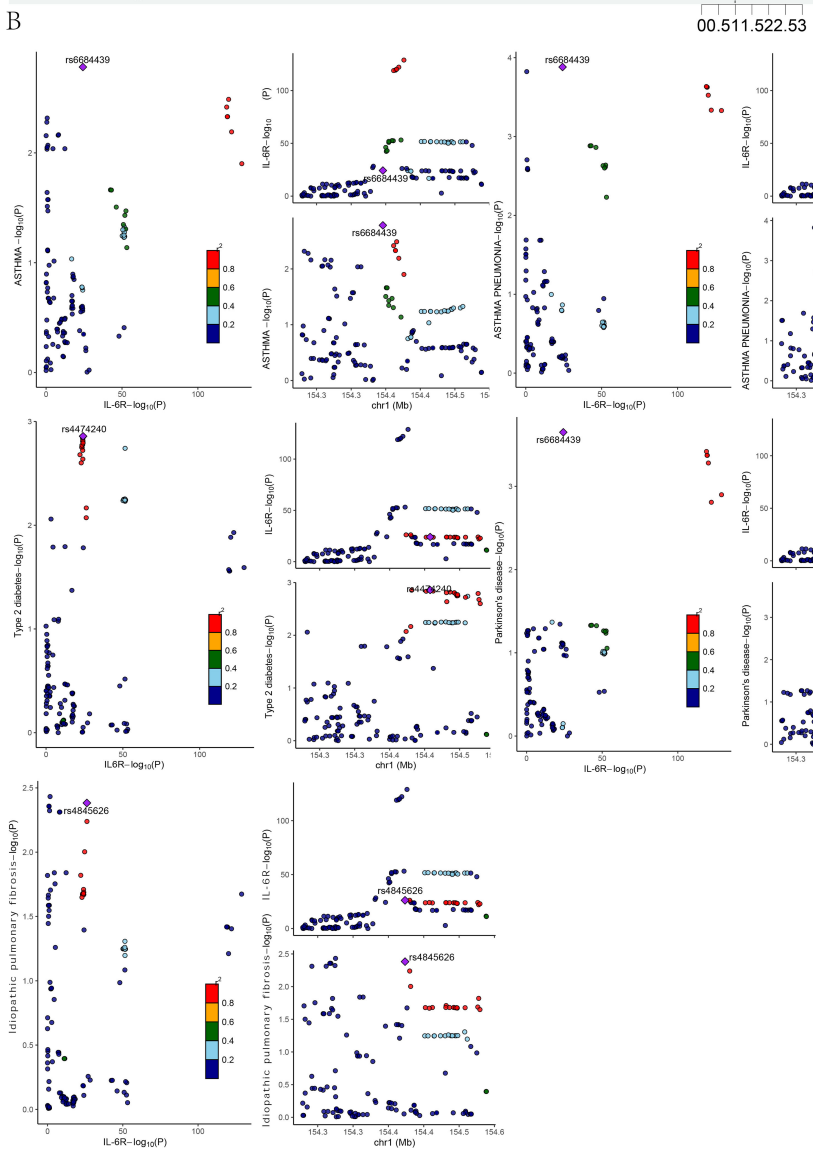

FIGURE 3
(A) Forest plot of BWMR analysis of IL-6Ri's causal relationship with 15 diseases; **(B)** The results of colocalization analysis.

6 signaling can mitigate the risk of coronary artery disease (27). The rs2228145 variant, situated on the IL6R gene, modulates the proteolytic cleavage of membrane-bound IL6R into its soluble form (sIL-6R) (28). Consequently, we selected rs2228145 as an instrumental variable for sIL-6R to investigate the impact of the IL-6 trans-signaling pathway on the disease.

The influence of sIL-6R on Type 2 diabetes (OR: 1.298, 95% CI: 1.025-1.643; P=0.030), Parkinson's disease (OR: 3.570, 95% CI: 1.653-7.709; P= 0.001), idiopathic pulmonary fibrosis (OR: 3.308, 95% CI: 1.178-9.292; P= 0.023), asthma (OR: 0.713, 95% CI: 0.548-0.927; P= 0.011), and asthmatic pneumonia(OR: 0.438, 95% CI: 0.277-0.693; P<0.001), suggests that IL-6 receptor blockade may modulate these five diseases by inhibiting the IL-6 trans-signaling pathway(Supplementary Material 1: Supplementary Table S5).

Sensitivity analyses

Leave-one-out analysis demonstrated consistent risk estimates for idiopathic pulmonary fibrosis, Parkinson's disease, type 2

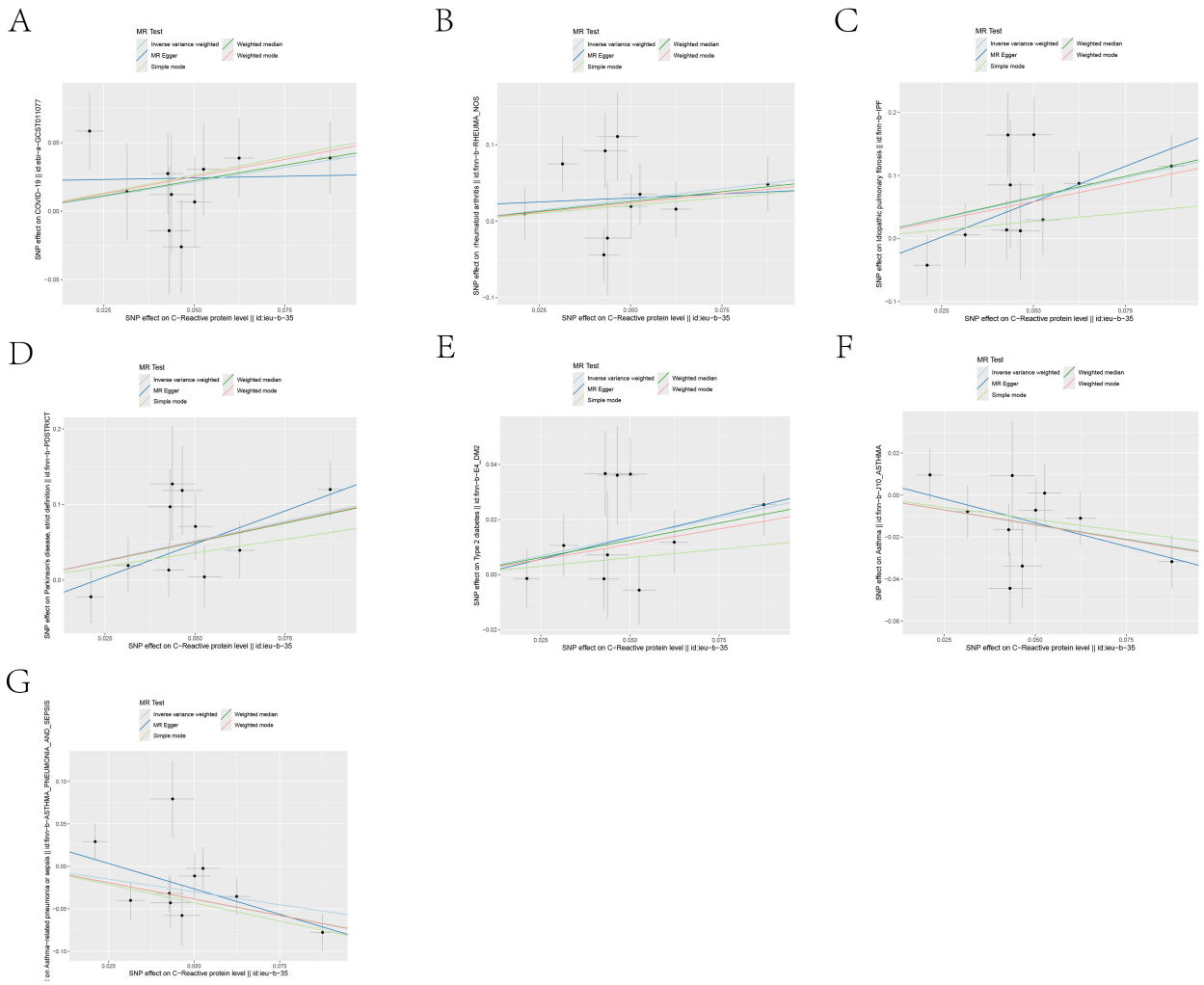

FIGURE 4
Scatter plots of the 5 MR analysis methods. The causal effect of IL-6R inhibitors on seven types of diseases, including: **(A)** COVID-19, **(B)** Rheumatoid arthritis, **(C)** Idiopathic pulmonary fibrosis, **(D)** Parkinson's disease, **(E)** Type 2 diabetes, **(F)** Asthma, **(G)** Asthmatic pneumonia.The vertical axis denotes the influence of single nucleotide polymorphisms on exposure variables,the horizontal axis delineates their impact on outcome variables.

diabetes, asthma, and asthmatic pneumonia after sequentially removing each SNP (Figure 6), and the funnel plots appeared approximately symmetrical(Figure 7).Cochran's Q test did not indicate any signs of heterogeneity. Furthermore, neither MR-Egger regression nor MR-PRESSO detected the presence of horizontal pleiotropy (Figure 2 and Supplementary Material 1: Supplementary Table S3).We further investigated the colocalization of idiopathic pulmonary fibrosis, asthma, asthmatic pneumonia, Parkinson's disease, and type 2 diabetes with CRP. The colocalization evidence for asthmatic pneumonia (coloc.abf-PPH4 = 0.811) strongly supports its association with CRP. For Parkinson's disease, the colocalization evidence (coloc.abf-PPH4 = 0.725) moderately supports a link with CRP. However, the evidence for idiopathic pulmonary fibrosis (coloc.abf-PPH4 = 0.222), asthma (coloc.abf-PPH4 = 0.123), and type 2 diabetes (coloc.abf-PPH4 = 0.062) shows limited colocalization (Figure 3B and Supplementary Material 1: Supplementary Table S6).

Positive results from the colocalization analysis have revealed shared genetic signals within the genetic locus linking CRP with Parkinson's disease and asthmatic pneumonia. These genetic signals are located in the IL6R gene region. These findings suggest that genetic variations in the IL6R region may influence the development of Parkinson's disease and asthmatic pneumonia by modulating inflammation-related biological pathways.

Discussion

In this study, we conducted a systematic analysis of the causal relationships between IL-6Ri and various diseases. To our knowledge, this represents the first large-scale genetic consortium-based MR analysis to establish causal links between IL-6Ri and multiple diseases. Utilizing drug-target Mendelian Randomization and colocalization analyses, we concluded that IL-

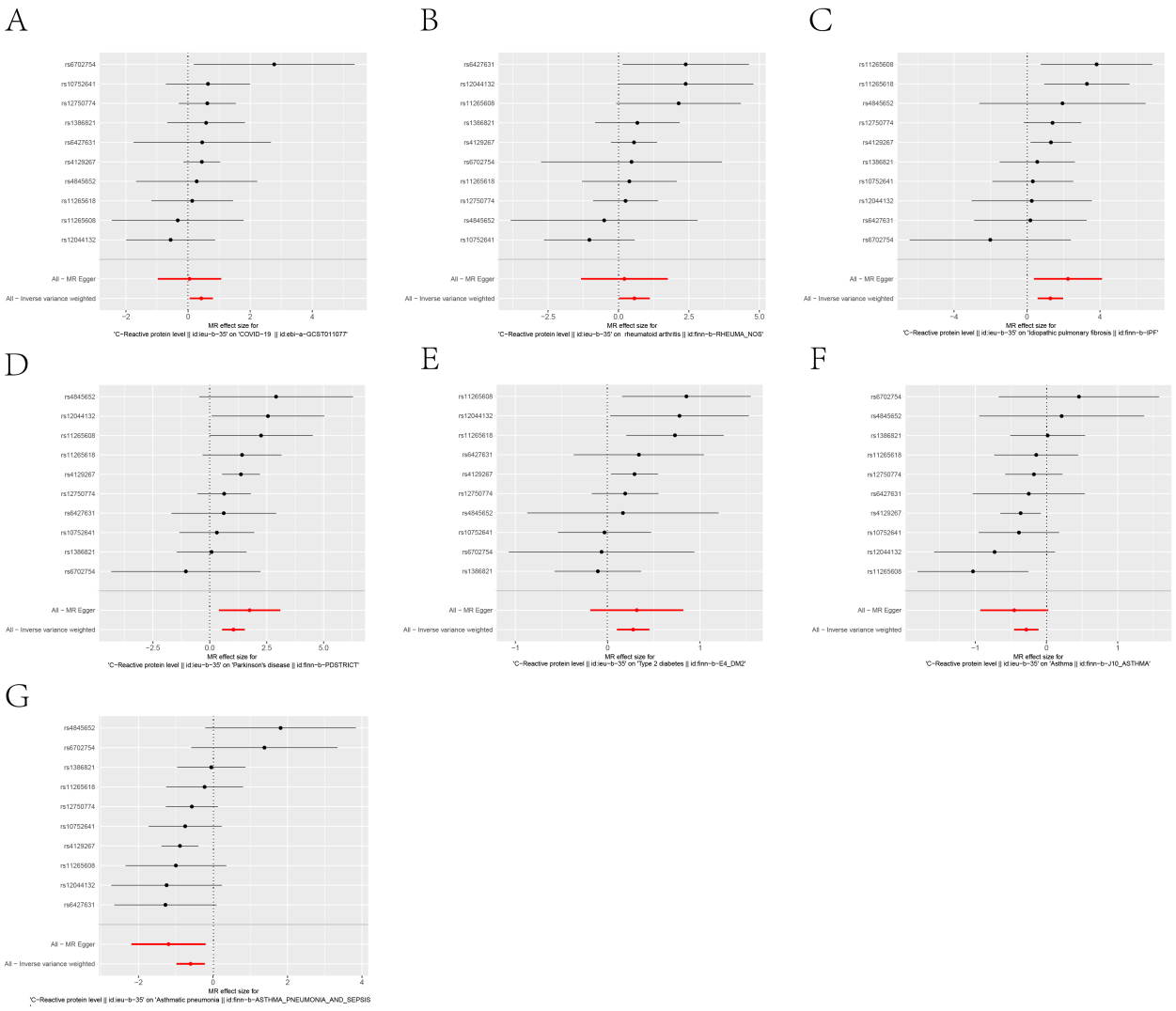

FIGURE 5
Forest plot of each SNP's causal relationship with seven diseases, including: **(A)** COVID-19, **(B)** Rheumatoid arthritis, **(C)** Idiopathic pulmonary fibrosis, **(D)** Parkinson's disease, **(E)** Type 2 diabetes, **(F)** Asthma, **(G)** Asthmatic pneumonia.The black dots represent the mean values,All-MR Egger and All-Inverse variance weighted represent the overall effect of all instrumental variables.

6Ri significantly reduce the incidence risk of Parkinson's disease, idiopathic pulmonary fibrosis, and type 2 diabetes, while potentially increasing the risk of asthma and asthmatic pneumonia.

Parkinson's disease is the most common severe motor disorder and the second most prevalent neurodegenerative disease after Alzheimer's, affecting 400 to 1,900 individuals per 100,000 globally (29). With the aging population, the incidence of Parkinson's disease is expected to double by 2040 (30). In research conducted by Chen et al. (31), an analysis of 84 Parkinson's patients and 165 control samples revealed that plasma inflammation markers—including C-reactive protein, fibrinogen, tumor necrosis factor-alpha, and IL-6—are critical for predicting and diagnosing Parkinson's disease, particularly as elevated IL-6 significantly increases the risk of developing the disease. Studies indicate that IL-6 activates the JAK-STAT pathway by increasing pSTAT3 expression (32). QingQin et al. confirmed that overexpression of α-SYN in a Parkinson's disease model activates the JAK/STAT pathway. Inhibition of the JAK/STAT pathway, demonstrated for the first time, disrupts the circuits of neuroinflammation and neurodegenerative changes, thereby mitigating the pathogenesis of Parkinson's disease (33). In a Parkinson's disease rat model, inhibitors of JAK1 and JAK2, such as AZD1480, reduced microglial proliferation and macrophage infiltration and decreased MHC class II expression. Additionally, treatment with AZD1480 inhibited the activation of STAT1/3/4 and blocked the differentiation of Th1 and Th17 cells, jointly promoting an immune response in the Parkinson's disease model. In this study, we are excited to report that IL6Ri can reduce the risk of Parkinson's disease, and colocalization showed moderate strength. The results of colocalization analysis further confirm that the role of IL6R in the nervous system may be related to its anti-inflammatory properties, providing significant insights into the specific

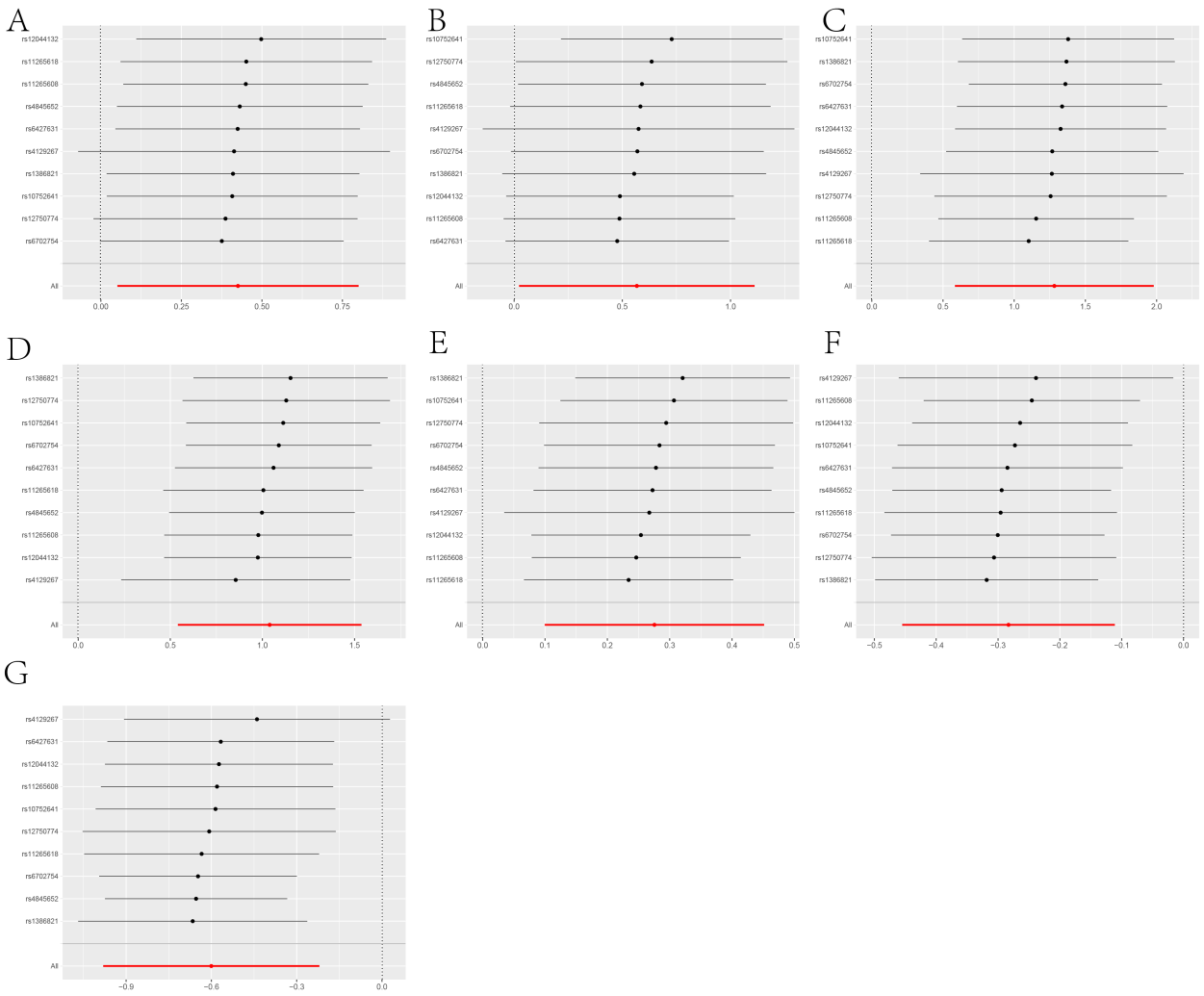

FIGURE 6
A leave-one-out analysis of statistically significant differences in the causal effects of IL-6R inhibitors on seven types of diseases, including: **(A)** COVID-19, **(B)** Rheumatoid arthritis, **(C)** Idiopathic pulmonary fibrosis, **(D)** Parkinson's disease, **(E)** Type 2 diabetes, **(F)** Asthma, **(G)** Asthmatic pneumonia.The Y-axis corresponds to each excluded rsid number and the 'all' condition not excluded by the IVW method. The X-axis corresponds to specific IVW values, with black and red dots representing beta effect values, and the lines indicating the confidence intervals of these beta values.

mechanisms of IL6R in disease. Our research underscores the potential value of targeting the IL6R pathway in the prevention and treatment of Parkinson's disease.

Idiopathic Pulmonary Fibrosis is a chronic fibrosing interstitial pneumonia of unknown origin that predominantly affects the elderly, characterized by progressive respiratory difficulty and continual decline in lung function. Prognosis for patients is poor, with an average life expectancy of about three to five years post-diagnosis (34). According to the Global Burden of Disease study, as of 2019, the incidence rate of interstitial lung diseases and pulmonary sarcoidosis among Chinese men was approximately 65 cases per 100,000 people, while globally, the incidence rates for men and women were 68 and 59 per 100,000 respectively. With the increasing trend of an aging population, the number of individuals with Idiopathic Pulmonary Fibrosis in China is expected to reach at least 500,000. In developed regions like the European Union, the number of Idiopathic Pulmonary Fibrosis patients increases by about 3,500 annually, posing a significant socioeconomic burden and an urgent health care challenge worldwide (35). The JAK/STAT signaling pathway is one of the classic inflammatory pathways closely associated with the development of Idiopathic Pulmonary Fibrosis (36). In this pathway, when the JAK1/STAT1 pathway is activated, JAK1 becomes phosphorylated, which in turn induces the phosphorylation of STAT1. The phosphorylated STAT1 forms heterodimers and translocates to the nucleus, promoting the release of chemokines and pro-inflammatory cytokines such as TNF-α, IL-6, ICAM1, and MCP1 (37). These inflammatory mediators have been identified as potential therapeutic targets for various fibrotic diseases. Furthermore, the SOCS family proteins serve as important negative regulators within the JAK1/STAT1 pathway. As negative regulators of cytokine receptor signaling, they inhibit the overactivation of the JAK/STAT pathway. This feedback inhibition mechanism plays a crucial role in regulating inflammatory responses and preventing excessive tissue fibrosis

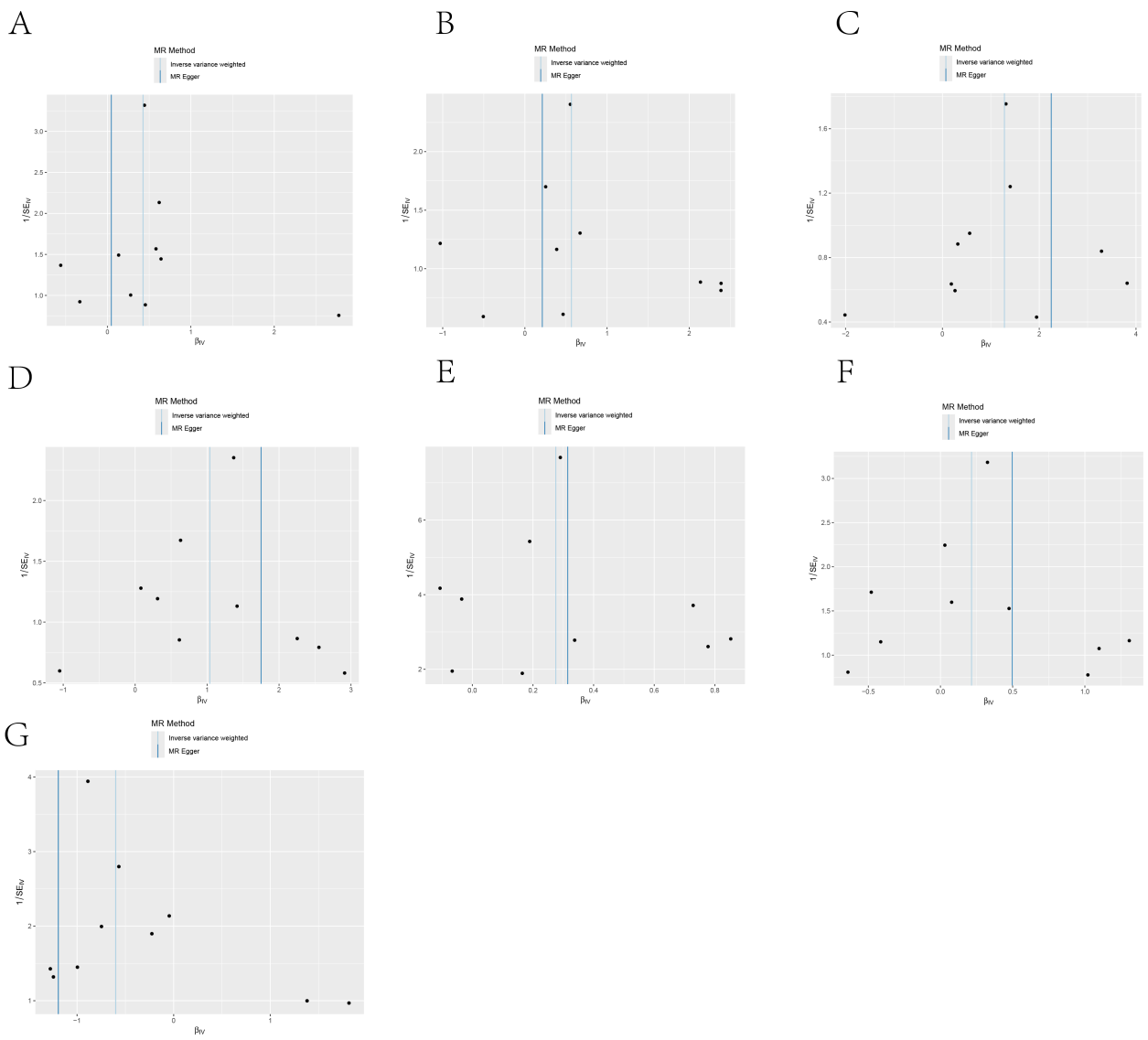

FIGURE 7
Funnel plots showing the symmetry of the causal effect of IL-6R inhibitors on seven cancers, including: **(A)** COVID-19, **(B)** Rheumatoid arthritis, **(C)** Idiopathic pulmonary fibrosis, **(D)** Parkinson's disease, **(E)** Type 2 diabetes, **(F)** Asthma, **(G)** Asthmatic pneumonia.The black dots in the figure represent single nucleotide polymorphisms, with the horizontal axis displaying the β values of these polymorphisms, and the vertical axis showing their standard errors.

(38). Therefore, the potential of IL6Ri to reduce the risk of developing Idiopathic Pulmonary Fibrosis may be attributed to their ability to inhibit the JAK-STAT pathway, though further *in vivo* and *in vitro* studies are necessary to validate this effect.

Diabetes is an endocrine and metabolic disorder characterized by defects in insulin secretion and/or action and chronic hyperglycemia, caused by various factors (39). Epidemiological studies indicate that approximately 537 million people worldwide suffer from diabetes, with projections suggesting this number will rise to 693 million by 2045 (40). The global economic burden of adult diabetes is substantial, with costs estimated at 1.3 trillion USD in 2015, expected to increase to between 2.1 and 2.8 trillion USD by 2030 (41). Since 1999, research has progressively revealed a close association between type 2 diabetes mellitus and inflammation, recognizing the disease as an inflammatory condition mediated by inflammatory cells, their secretory factors, and acute-phase reactants, constituting an innate immune response. Particularly, chronic inflammation of visceral adipose tissue plays a pivotal role in the pathogenesis of type 2 diabetes. Obesity triggers adipocyte hypertrophy and hyperplasia, reduces the anti-inflammatory factor APN, and increases pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α, and chemokines (CCL2, CCL3, and CXCL8), leading to the infiltration of immune cells (M1-type macrophages, CD8+ and CD4+ T lymphocytes, and B lymphocytes) and consequently promoting adipose inflammation (42). Our studies suggest that IL-6 inhibitors can significantly reduce type 2 diabetes, potentially through the inhibition of the JAK-STAT signaling pathway, thereby alleviating insulin resistance.

Common side effects of IL-6Ri include nasopharyngitis, headaches, upper respiratory tract infections, and gastritis. Infections are the most frequently occurring serious side effect, which may lead to gastrointestinal perforations. Typical laboratory abnormalities include neutropenia and elevated liver enzymes (43). Local reactions to injections and infusion reactions are common, but systemic allergic reactions are rare (44, 45). In rheumatoid arthritis patients, the development of resistance antibodies is possible, though they do not affect the efficacy of the treatment. Clinical trials indicate that IL-6Ri increase the risk of infections, consistent with other similar medications, including serious infections such as bacterial pneumonia and atypical infections (46). Interestingly, our research suggests a potential increase in the risk of asthma and asthmatic pneumonia, providing crucial information for clinicians in making therapeutic decisions, especially when considering pre-existing respiratory conditions in patients. Therefore, it is recommended that patients using these inhibitors be closely monitored for potential respiratory complications.

Currently available IL6R inhibitors, such as Tocilizumab and Sarilumab, operate by targeting the IL-6 receptor, effectively obstructing the interaction between IL-6 and its receptor, thus inhibiting both the classical and trans-signaling pathways. These inhibitors not only reduce the availability of membrane-bound IL-6R but also decrease the activity post-binding to sIL-6R, showcasing their suppressive effects across both signaling pathways (28). Conceptually, our genetic tools mirror the actions of these anti-IL6R monoclonal antibodies, although they exhibit a comparatively weaker effect in inhibiting IL6R signaling. Nevertheless, they possess a profound conceptual similarity with IL-6 inhibition. Empirical data supports the variation at this site and its impact on subsequent randomized trial data. Particularly when we employ the "canonical" rs2228145 SNP for MR analysis, despite reduced experimental efficacy, the outcomes align with expectations, exhibiting similar effects. Moreover, Tocilizumab, based on the IgG1 subtype, activates a variety of immune responses, such as antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity. In contrast, Sarilumab, derived from the IgG2 subtype, exhibits lower activity in activating these immune effects. Both Tocilizumab and Sarilumab demonstrate similar therapeutic effects (47); however, Sarilumab presents a reduced risk of side effects (48). Therefore, we remain optimistic about the potential of these inhibitors in treating conditions such as idiopathic pulmonary fibrosis, type 2 diabetes, and Parkinson's disease, anticipating that Sarilumab's side effects, particularly in asthma and asthmatic pneumonia, may be significantly lesser than those of Tocilizumab.

The primary limitations of this study stem from the fact that the causal relationships are based on "genetic predispositions," which can be easily influenced if the instrumental variable data are not properly managed. Due to constraints related to databases and software, this study focused exclusively on Europeans, but the causal conclusions drawn from European populations may not necessarily apply to other groups, such as Asians. Future studies should verify these findings using larger samples across multiple regions and diverse ethnic populations.

Conclusion

In summary, this study assessed the causal relationships between IL-6Ri and various diseases, discovering that IL-6Ri can reduce the risk of three diseases while potentially increasing the risk of asthma and asthmatic pneumonia. This provides preliminary evidence for the potential expansion of therapeutic applications for IL-6Ri.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

CF: Writing – original draft. LW: Data curation, Investigation, Writing – review & editing. WC: Writing – review & editing, Methodology.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1452849/full#supplementary-material

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EDITED BY
Guobao Chen,
AbbVie's Cambridge Research Center,
United States

REVIEWED BY
Anne Cooke,
University of Cambridge, United Kingdom
Karen R. Jonscher,
University of Oklahoma Health Sciences Center, United States

*CORRESPONDENCE
Ivana Stojanović
ivana@ibiss.bg.ac.rs

†These authors share senior authorship

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Novel AHR ligand AGT-5 ameliorates type 1 diabetes in mice through regulatory cell activation in the early phase of the disease

Natalija Jonić[1], Ivan Koprivica[1], Stavroula G. Kyrkou[2], Vasileios-Panagiotis Bistas[2], Christos Chatzigiannis[2], Nataša Radulović[1], Ivan Pilipović[1], Andjelina Jovanović[3], Milan B. Jovanović[3,4], Mirjana Dimitrijević[1], Andreas G. Tzakos[2,5†] and Ivana Stojanović[1*†]

[1]Department of Immunology, Institute for Biological Research "Siniša Stanković" - National Institute of the Republic of Serbia, University of Belgrade, Belgrade, Serbia, [2]Section of Organic Chemistry & Biochemistry, Department of Chemistry, University of Ioannina, Ioannina, Greece, [3]Department of Otorhinolaryngology with Maxillofacial Surgery, Clinical Hospital Center "Zemun", Belgrade, Serbia, [4]Faculty of Medicine, University of Belgrade, Belgrade, Serbia, [5]Institute of Materials Science and Computing, University Research Center of Ioannina (URCI), Ioannina, Greece

Type 1 diabetes (T1D) is an autoimmune disease with a strong chronic inflammatory component. One possible strategy for the treatment of T1D is to stimulate the regulatory arm of the immune response, i.e. to promote the function of tolerogenic dendritic cells (tolDC) and regulatory T cells (Treg). Since both cell types have been shown to be responsive to the aryl hydrocarbon receptor (AHR) activation, we used a recently characterized member of a new class of fluorescent AHR ligands, AGT-5, to modulate streptozotocin-induced T1D in C57BL/6 mice. Prophylactic oral administration of AGT-5 reduced hyperglycemia and insulitis in these mice. Phenotypic and functional analysis of cells in the pancreatic infiltrates of AGT-5-treated mice (at the early phase of T1D) revealed a predominantly anti-inflammatory environment, as evidenced by the upregulation of tolDC and Treg frequency, while $CD8^+$ cell, Th1 and Th17 cells were significantly reduced. Similarly, AGT-5 enhanced the proportion of Treg and tolDC in small intestine lamina propria and suppressed the activation status of antigen-presenting cells through down-regulation of co-stimulatory molecules CD40, CD80 and CD86. The expression levels of Cyp1a1, controlled by the AHR, were increased in $CD4^+$, $CD8^+$ and Treg, confirming the AHR-mediated effect of AGT-5 in these cells. Finally, AGT-5 stimulated the function of regulatory cells in the pancreatic islets and lamina propria by upregulating indoleamine 2,3-dioxigenase 1 (IDO1) in tolDC. These findings were supported by the abrogation of AGT-5-mediated *in vitro* effects on DC in the presence of IDO1 inhibitor. AGT-5 also increased the expression of CD39 or CD73 ATP-degrading ectoenzymes by Treg. The increase in Treg is further supported by the upregulated frequency of IL-2-producing type 3 innate lymphoid cells (ILC3) in the lamina propria. Anti-inflammatory effects of AGT-5 were also validated on human tonsil cells, where *in vitro* exposure to AGT-5 increased the proportion of immunosuppressive dendritic cells and ILC3. These

results suggest that AGT-5, by stimulating AHR, may promote a general immunosuppressive environment in the pancreas and small intestine lamina propria at the early phase of disease, and thereby inhibit the severity of T1D in mice.

KEYWORDS

type 1 diabetes (T1D), aryl hydrocarbon receptor (AhR), T regulatory cell (TREG), insulitis, gut-associated lymphoid tissue (GALT), lamina propria

1 Introduction

Type 1 diabetes mellitus (T1D) is a chronic autoimmune disease with a rising global incidence. This observation highlights a growing influence of environmental factors on T1D onset and development. T1D is characterized by the destruction of pancreatic β-cells, insulin deficiency and hyperglycemia. T1D, and especially its early onset in children, often leads to serious complications including heart disease, renal disease, circulatory problems, blindness, etc. At present, despite the extensive research of various treatment modalities in animal T1D models, the therapy of T1D is limited to multiple daily insulin injections that, often inadequately, prevent severe hyperglycemia and other long-term complications (1).

A breakdown of self-tolerance to pancreatic β-cell antigens is a hallmark of T1D (2). In T1D, β-cells are destroyed by the autoantigen-specific $CD4^+$ and $CD8^+$ T effector cells (Teff) leading to insulin deficiency (3). Regulatory T cells (Treg) maintain immune tolerance and exert immunosuppressive activities toward Teff. The main feature of T1D is the imbalance between the Teff and the $FoxP3^+$ $CD4^+$ Treg (4). Numerous studies have demonstrated that the absence of Treg in mice leads to the development of T1D and that increased activity and number of these cells results in the resolution of T1D (5, 6). Moreover, a reduction in the number or defective function of Treg has been demonstrated in patients with T1D (7, 8).

Key environmental factors associated with T1D susceptibility are the diet and the gut microbiome. A potential mechanistic link between diet, gut microbiome and T1D might be related to the aryl hydrocarbon receptor (AHR), a transcription factor that is activated by many environmental signals (9). The most common biomarker of AHR activity is the expression of Cyp1a1, which belongs to the Cytochrome P450 family of enzymes and is involved in phase I metabolism (detoxification) of numerous endogenous and exogenous compounds. Importantly, AHR activation was found to initiate the transcription of both pro-inflammatory and anti-inflammatory genes in immune cells, depending on the cell type and extracellular environment (9). The anti-inflammatory outcomes of AHR signaling can include, for example, differentiation of type 1 regulatory T cells and $FoxP3^+$ Treg (10), stimulation of IL-10 production by inflammatory macrophages (11), and downregulation of IFN-γ and IL-12 secretion by splenic cells (12).

AHR signaling sustains intestinal barrier integrity through the direct effect on epithelial cells and on the immune cells residing in the gut lamina propria or within the epithelium (13). Dietary AHR ligands modulate the abundance of intestinal bacteria whose metabolites are associated with T1D development (14). Supplementation with AHR activators is very effective in rodent models of inflammation including autoimmune diseases such as T1D. Activation of AHR by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) prevents T1D in NOD mice by increasing Treg population in the pancreatic lymph nodes (15). Also, nanoparticles containing 2-(1' H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), through activation of AHR in tolerogenic dendritic cells (tolDC), prevent T1D in NOD mice (16). Activation of the AHR by certain ligands was shown to prevent insulitis and Teff development independently of $FoxP3^+$ Treg in NOD mice (17).

Having in mind that tolDC, type 3 innate lymphoid cells (ILC3) and Treg exhibit high expression of AHR (9), our aim was to explore the effects of AGT-5, a member of the new class of fluorescent AHR ligands (FluoAHRL) on multiple low dose streptozotocin (STZ)-induced T1D development in mice (18, 19). This compound exerts AHR agonistic effects in both mouse and human cells (20). In addition to emitting in the near-infrared (NIR), enabling bioimaging of AHR, AGT-5 has been demonstrated to stimulate the differentiation, proliferation, and function of Treg *in vitro* (20). This pivotal characteristic positions AGT-5 as a promising candidate for modulating the autoimmune process in T1D.

2 Material and methods

2.1 AGT-5 synthesis

AGT-5 was synthesized according to the protocol published in Jonić et al. (20). Briefly, synthesis was conducted through a Knoevenagel condensation. Specifically, a solution of 2-(2-methyl-4H-chromen-4-ylidene)malononitrile (1eq) and indole-3-carboxaldehyde (1eq) in acetonitrile, with catalytic amount of piperidine, was stirred under reflux for 16 hours. The crude reaction mixture was purified using silica gel column

chromatography with CH_2Cl_2 as the eluent, resulting in a pure red product (yield 64%).^{1}H NMR (250 MHz, DMSO, δ ppm): 11.96 (d, J = 2.9 Hz, 1H), 8.76 (dd, J = 8.4, 1.4 Hz, 1H), 8.29 – 8.24 (m, 1H), 8.10 – 8.01 (m, 2H), 7.92 (ddd, J = 8.5, 7.1, 1.5 Hz, 1H), 7.80 (dd, J = 8.4, 1.3 Hz, 1H), 7.61 (ddd, J = 8.4, 7.1, 1.3 Hz, 1H), 7.52 (dt, J = 8.1, 0.9 Hz, 1H), 7.33 – 7.21 (m, 3H), 7.13 (s, 1H) (Supplementary Figure S1). ^{13}C NMR (63 MHz, DMSO, δ ppm): 160.77, 153.24, 152.57, 138.10, 135.41, 134.98, 133.51, 126.31, 125.19, 125.08, 123.43, 121.65, 121.20, 119.31, 118.48, 117.77, 117.04, 114.03, 113.08, 112.98, 104.99, 57.47 (Supplementary Figure S2).

The product's purity underwent further assessment through Agilent analytical HPLC chromatography employing an InfinityLab Poroshell 120 EC-C18 column, sized at 4.6×150 mm, alongside a diode array detector (DAD). Specific resolution was monitored at 254 nm. A gradient solvent system (A: 5%, B: 95% to A: 100%, B: 0%) composed of A: acetonitrile + 0.1% formic acid and B: H2O + 0.1% formic acid was utilized, maintaining a constant flow rate of 1.0 ml/min over a duration of 10 min. The peak for AGT-5 reached its apex at 8.3 min, revealing a purity level of 96% (Supplementary Figure S3).

A Xevo G2 Q-TOF mass spectrometer, employed for a direct infusion acquisition utilizing full scan MS with a mass scan range of 50–1200 m/z, operated in positive ESI (ElectroSpray Ionisation) mode. Maximum ion intensity was typically achieved with the following source conditions: a capillary voltage of 3.0 kV, sample cone voltage of 40 V, source temperature of 120°C, desolvation temperature of 550°C, cone gas flow rate of 100 L h^{-1}, and desolvation gas (N_2) flow rate of 600 L h^{-1}. MS: m/z for $C_{22}H_{13}N_3O$: calculated 335.11, found 336.11300 $[M+H]^+$ (Supplementary Figure S4).

2.2 Mice and human tissue

C57BL/6 mice were bred and maintained at the Animal Facility at the Institute for Biological Research "Siniša Stanković"- National Institute of the Republic of Serbia, University of Belgrade, with free access to food and water, and hiding structures added for environmental enrichment. All experiments were approved by the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (App. No 119-01-4/11/2020-09) and were in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes. Tonsils were obtained following patient informed consent signing and all procedures were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Clinical Hospital Center "Zemun", Belgrade, Serbia (App. No 14/1, date 27/09/2022).

2.3 Induction of T1D in C57BL/6 mice and AGT-5 treatment

Male 2-month-old C57BL/6 mice were subjected to T1D induction using multiple low doses of STZ, which were applied intraperitoneally for 5 consecutive days. STZ (38 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in cold 0.1 M citrate buffer (pH 6) right before administration. The powder form of AGT-5 was initially dissolved in DMSO (Sigma-Aldrich), and then in sesame oil. AGT-5 was applied orally (with a metal feeding tube inserted into the proximal esophagus) from the first day of T1D induction and treatment continued for 20 days (prophylactic regime). Molecular weight of AGT-5 is 335.37 g/mol. Each day, AGT-5 was freshly dissolved in DMSO to the concentration of 250 mg/ml (2.5 mg in 10 µl DMSO) and then diluted 1:100 with the sesame oil to obtain the final concentration of 2.5 mg/ml. Mice received approximately 100 µl of the final dilution (10 mg/kg body weight). The control group animals were given the same volume of sesame oil containing only DMSO (1% v/v). Number of mice per group for clinical assessment was 8. The occurrence of hyperglycemia, as an indicator of T1D development, was assessed by measuring blood glucose levels, using a glucometer (GlucoSure, Apex Biotechnology Group, Hsinchu, Taiwan). All *ex vivo* analyses of the immune response were performed on day 12 after the beginning of T1D induction on 3-6 mice per group.

2.4 Cell isolation

2.4.1 Lymph nodes and the pancreatic infiltrates

Cells from the pancreatic lymph nodes were obtained by passing the tissue through a cell strainer (40 µm). After removing the pancreatic lymph nodes, the pancreatic tissue was digested to obtain the pancreatic immune cell infiltrates. The pancreas was cut into small pieces (1–2 mm), washed with Hank's balanced salt solution (HBSS; Sigma-Aldrich) containing 10% fetal calf serum (FCS; PAA Laboratories, Pasching, Austria), and incubated with Collagenase type V (2 mg/ml, Sigma-Aldrich) dissolved in HBSS + 10% FCS (7.5 ml per pancreas) for 15 min, with constant shaking (200 S/min) at 37°C, after which the samples were vortexed for 20 s. The digests were then passed through a cell strainer and washed with HBSS + 10% FCS. The samples were then layered onto Histopaque®-1077 gradient media (Sigma-Aldrich) and after centrifugation (700g, 20 min, no rotor brakes) the mononuclear pancreas-infiltrating cells were collected from the interface. The obtained cells were finally resuspended in RPMI 1640 medium containing 5% FCS, 1% penicillin and streptomycin (all from PAA Laboratories), 2 mM L-glutamine and 25 mM HEPES (Sigma-Aldrich).

2.4.2 Small intestine lamina propria cells

Immune cells from the small intestine (SI) lamina propria were isolated according to the amended protocol by Weigmann et al. (21). Briefly, the SI was removed and cut into pieces (approx. 5 cm long), after which the intestinal content and the Peyer's patches were removed. The SI was then opened longitudinally, additionally cut into smaller pieces (approx. 1 cm long) and thoroughly washed three times in cold phosphate-buffered saline (PBS; Sigma-Aldrich). Subsequently, the samples were washed with PBS containing 2% FCS and 2.5 mM Dithiothreitol (DTT, Sigma-Aldrich) in an orbital shaker (250 rpm, 15 min) to reduce the presence of mucus. In the next step, the samples were washed three times in PBS containing 2% FCS and 5 mM EDTA (250 rpm, 15 min) to remove the epithelial cells. The SI pieces were gathered and washed in RPMI

1640 supplemented with 10% FCS (250 rpm, 10 min), and resuspended in a solution of Collagenase D (700 µg/ml) and DNase I (0.1 mg/ml) (both from Roche Diagnostics GmbH, Mannheim, Germany) dissolved in RPMI 1640 + 10% FCS. The samples were incubated for 1 h at 37°C in an orbital shaker (350 rpm). After digestion, the tissue was homogenized through a 70 µm cell strainer and washed twice (550g, 5 min). The pellet was resuspended in a 40% Percoll (Cytiva, Marlborough, MA, USA) gradient solution, layered upon 80% Percoll, and centrifuged at 2000 rpm for 20 min without rotor brakes. Lamina propria cells were collected from the interface between 40% and 80% Percoll, washed twice in PBS and resuspended in RPMI 1640 + 5% FCS for further analyses.

2.4.3 Tonsillar cells

Tonsils were taken during the surgery and transported in sterile saline. The tissue was dissected into smaller pieces and passed through the cell strainer to obtain single cell suspension (in PBS + 3% FCS). After centrifugation at 550g for 5 min, pelleted cells were resuspended in RPMI 1640 + 10% FCS and seeded in 96-well plate. Cells were exposed to AGT-5 dissolved in DMSO (0.75 µM) for 48 h, while the control cells were treated with the same volume of DMSO alone.

2.4.4 Dendritic cells

Dendritic cells (DC) were differentiated from bone marrow cells. The femur was isolated and flushed aseptically with RPMI 1640 + 10% FCS. Cells were then centrifuged, erythrocytes lysed with red blood cell lysis buffer and cells resuspended in RPMI 1640 supplemented with 20% FCS, 2 mM L-glutamine and 1 mM Na-pyruvate (Sigma-Aldrich). Obtained cells were cultivated with 20 ng/ml of GM-CSF (Peprotech, London, UK) for 7 days with medium change every second day. Cells were then washed and collected with accutase (Thermo Fisher Scientific, Waltham, MA, USA) treatment and seeded at 50,000 cells/well in a 96-well U-bottom plate (Sarstedt AG & Co. KG, Nümbrecht, Germany). DC were treated with lipopolysaccharide (LPS; 10 ng/ml, Sigma-Aldrich), LPS + AGT-5 (0.75 µM), or DMSO (the same volume as used for AGT-5 dilution), or 1-methyl tryptophan (1MT) in combination with LPS and AGT-5 (0.75 µM) for 24 h.

2.5 Flow cytometry

Cell surface molecules were detected on isolated cells from different tissues (pancreas, pancreatic lymph nodes and SI lamina propria). All antibodies were dissolved in Flow Cytometry Staining Buffer (eBioscience, San Diego, CA, USA). The list of used antibodies is displayed in the Supplementary Tables S1, S3.

For intracellular cytokine staining, cells were stimulated for 4 h with Cell Stimulation Cocktail (plus protein transport inhibitors) (eBioscience). The cells were then fixed in 2% paraformaldehyde for 15 min at room temperature, permeabilized with Permeabilization buffer (Thermo Fisher Scientific) for 30 min and stained with the fluorescently labelled antibodies (Supplementary Table S2).

Treg were detected by Mouse Regulatory T cell Staining Kit (FoxP3) according to the manufacturer's instructions (eBioscience). RORγt was detected following the same protocol. Each staining was performed for 40 min at 4°C. Isotype-matched controls were included in all experiments (eBioscience). Cell samples were acquired on FACS Aria III (BD Biosciences, Bedford, MA, USA) and analyzed using FlowJo™ 10.10.0 software (BD Life Sciences, Ashland, OR, USA).

2.6 Histological analysis

The pancreata were collected, fixed in 4% neutral buffered formalin, and embedded in paraffin. The embedded tissue was cut into 5 µm thick sections with a microtome, with at least 100 µm between sections. The presence of pancreatic islet inflammatory cell infiltrates and the degree of islet cell destruction were evaluated by staining the tissue sections with Mayer's hematoxylin (Bio-Optica, Milan, Italy) and examined by light microscopy (Leica Microsystems GmbH, Wetzlar, Germany). Insulitis scoring was performed by examining at least 25 islets per pancreas and graded in a blinded fashion: intact islet, without infiltrates; peri-insulitis, with infiltrates in the islet periphery; and insulitis, with infiltrates within the islet. Results are expressed as a percentage of graded islets out of the total number of islets, with three pancreata examined per group.

Insulin expression within the pancreatic islets was evaluated after immunohistochemical staining of the tissue sections with Alexa Fluor® 488-conjugated rabbit anti-mouse insulin antibody (1:400, Cell Signaling Technology, Danvers, MA, USA), with the nuclei counterstained with Hoechst 33342 dye (2 µl/ml, ChemoMetec, Allerød, Denmark). Images were acquired with a Zeiss Axio Imager Z1 florescence microscope (Carl Zeiss Meditec AG, Jena, Germany), at 20× magnification. After converting the images to gray scale, the expression of insulin within the islets was analyzed with the open-source software Fiji (22). Fluorescence intensity was quantified by measuring the mean gray value, which represents the sum of gray values of all pixels in the selected area divided by the total number of pixels. At least 30 islets per pancreas were analyzed, with three pancreata examined per group.

2.7 ELISA assay

Insulin concentration in the serum was determined using an Insulin ELISA kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. IL-10 and TNF concentrations in the supernatants of DC cultures were determined by commercial DuoSet® ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Absorbance was measured by Synergy H1 Hybrid Multi-Mode Reader (BioTek, Swindon, United Kingdom) at 450/570 nm. A standard curve created from the known concentrations of insulin, IL-10 and TNF was used to calculate the concentration values of the tested samples.

2.8 Statistical analysis

Data are presented as mean ± SD. The significance of differences between groups was determined by a two-tailed Student's t-test. Differences are regarded as statistically significant if $p < 0.05$. Statistical analyses were performed using GraphPad Prism 9.0.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

3 Results

3.1 AGT-5 treatment alleviates clinical and histological parameters of T1D in mice

AGT-5 has already been shown to be an effective anti-inflammatory agent as it stimulates proliferation and differentiation of Treg *in vitro* through AHR binding (20). In this study, AGT-5 was administered orally from the first day of T1D induction and treatment continued for 20 days (Figure 1A). Herein we confirmed AGT-5's immunomodulatory properties as it improved T1D clinical signs. In particular, AGT-5 significantly reduced hyperglycemia in T1D mice, and its beneficial effect persisted even after the end of the treatment (on day 20) (Figure 1B). Furthermore, AGT-5-treated mice maintained a similar relative body mass to their untreated counterparts throughout the 32-day observation period (Figure 1C). The initial body weights in STZ and STZ+AGT-5 groups were similar and ranged between 22.9 g and 26.2 g (24.8 ± 1.4 g) for STZ-treated mice and between 23.4 g and 26.6 g for STZ+AGT-5 treated mice (24.5 ± 1.3 g).

Histological analysis of pancreatic islets harvested on day 12 after T1D induction showed that AGT-5-treated mice had a lower percentage of pancreatic islets with insulitis (26.3 ± 7.4%) compared with the control animals (43.8 ± 4.7%) (Figures 1D–F). This was accompanied by the increased production of insulin in the islets (Figures 1G–I), as well as by the increased insulin concentration in the serum (Figure 1J) of AGT-5-treated animals. The AGT-5 treatment continued to have a positive effect on β-cell protection even after the end of treatment, as judged by the reduced number of infiltrated islets and higher number of intact islets in the pancreata of AGT-treated mice (Figures 1K–M), evaluated on day 32.

3.2 AGT-5 treatment promotes an anti-inflammatory response in the pancreas and lymph nodes of mice with T1D

Consistent with the decreased infiltration of immune cells in the pancreas of AGT-5-treated mice, flow cytometric analysis of infiltrates on day 12 after the start of disease induction indicated an anti-inflammatory status in this target tissue. The first cells to infiltrate pancreas, after the initial β-cell injury, are macrophages and DC (23). Although the presence of classical antigen-presenting cells (APC), $CD11b^+$ or $CD11c^+$ was not altered (Figures 2A, B), there was a significant increase in the proportion of anti-inflammatory tolDC ($CD11c^+$ $CD11b^-$ $CD103^+$) in AGT-5-treated mice (Figure 2C). $CD8^+$ cytotoxic lymphocytes, along with the APC, tend to migrate to the pancreatic islets and exert a direct damaging effect on β-cells (23). Treatment with AGT-5 reduced the infiltration of both $CD8^+$ cells and IFN-γ-producing $CD8^+$ cells (Figure 2D). Finally, the infiltration of $CD4^+$ helper cells was also reduced (Figure 2E) and the balance between the Th cell subsets was shifted towards an anti-inflammatory response, as we observed a reduced population of Th1 and Th17 cells (Figures 2F, G) and an increased population of Treg (Figure 2H). As can be seen from the heatmap representation of cell occurrence, AGT-5 had the greatest effect on tolDC and Treg (Figure 2I). Complementary to the *in vivo* findings, *in vitro* exposure of differentiated (bone marrow-derived) DC to the subsequent treatment with LPS, which induces the full maturation of DC, and AGT-5 (0.75 μM), reduced the proportions of tolDC expressing co-stimulatory molecules CD40, CD80 and CD86 (Supplementary Figures 5A–C).

The distribution of immune cell populations in the draining pancreatic lymph nodes was similar to the one observed in the pancreas. AGT-5 efficiently down-regulated the proportion of MHC II^+ $CD11b^+$ APC (Figure 3A) and increased the proportion of tolDC in the pancreatic lymph nodes (Figure 3C), with no observed effect on MHC II^+ $CD11c^+$ cells (Figure 3B). As for the $CD4^+$ cell population, AGT-5 reduced the frequency of pro-inflammatory Th1 cells, while the proportions of Th17 cells and Treg remained unchanged (Figures 3D, E). Importantly, AGT-5 reduced the proportion of $CD8^+$ cells, including those that produce IFN-γ (Figures 3F, G). Analysis of cell composition in the lymph nodes revealed AGT-5's significant impact on $CD11b^+$ cells and tolDC (Figure 3H).

3.3 AGT-5 treatment promotes an anti-inflammatory response in the lamina propria of mice with T1D

As AGT-5 was administered orally and the predominant AHR-expressing immune cells reside within the SI lamina propria (24), we next performed e*x vivo* analysis of cells within this tissue on day 12 after the initiation of T1D induction. The results demonstrated AGT-5's predominant effect on tolDC, ILC3, and Treg. Specifically, AGT-5 treatment down-regulated the frequency of MHC II-expressing $CD11b^+$ APC (Figure 4A), did not change the proportion of MHC II-expressing $CD11c^+$ APC (Figure 4B) and upregulated the proportion of tolDC (Figure 4C). The proportions of $CD11b^+$ and $CD11c^+$ cells and tolDC expressing the co-stimulatory molecules CD80 and CD86 were reduced upon AGT-5 treatment (Figures 4D–I). Overall, the major differences between the treated and control mice were observed in the tolDC compartment (Figures 4J, K).

Another important population of cells that reside in gut-associated lymphoid tissue (GALT) and is dependent on AHR expression is ILC3 population (25). Although ILC3 proportions were reduced in AGT-5-treated mice in comparison to control diabetic mice and (Figure 5A), the frequency of IL-22^+ and IL-2^+ cells in ILC3 were upregulated after AGT-5 treatment (Figures 5B, C). In contrast, AGT-5 down-regulated the ILC3 subpopulation that produces IL-17 (Figure 5D). The heatmap illustrates the primary impact of AGT-5 on IL-2 production in ILC3 (Figure 5E).

AGT-5 influenced the adaptive immune response in the SI lamina propria. While AGT-5 did not affect the proportion of $CD4^+$ cells, it

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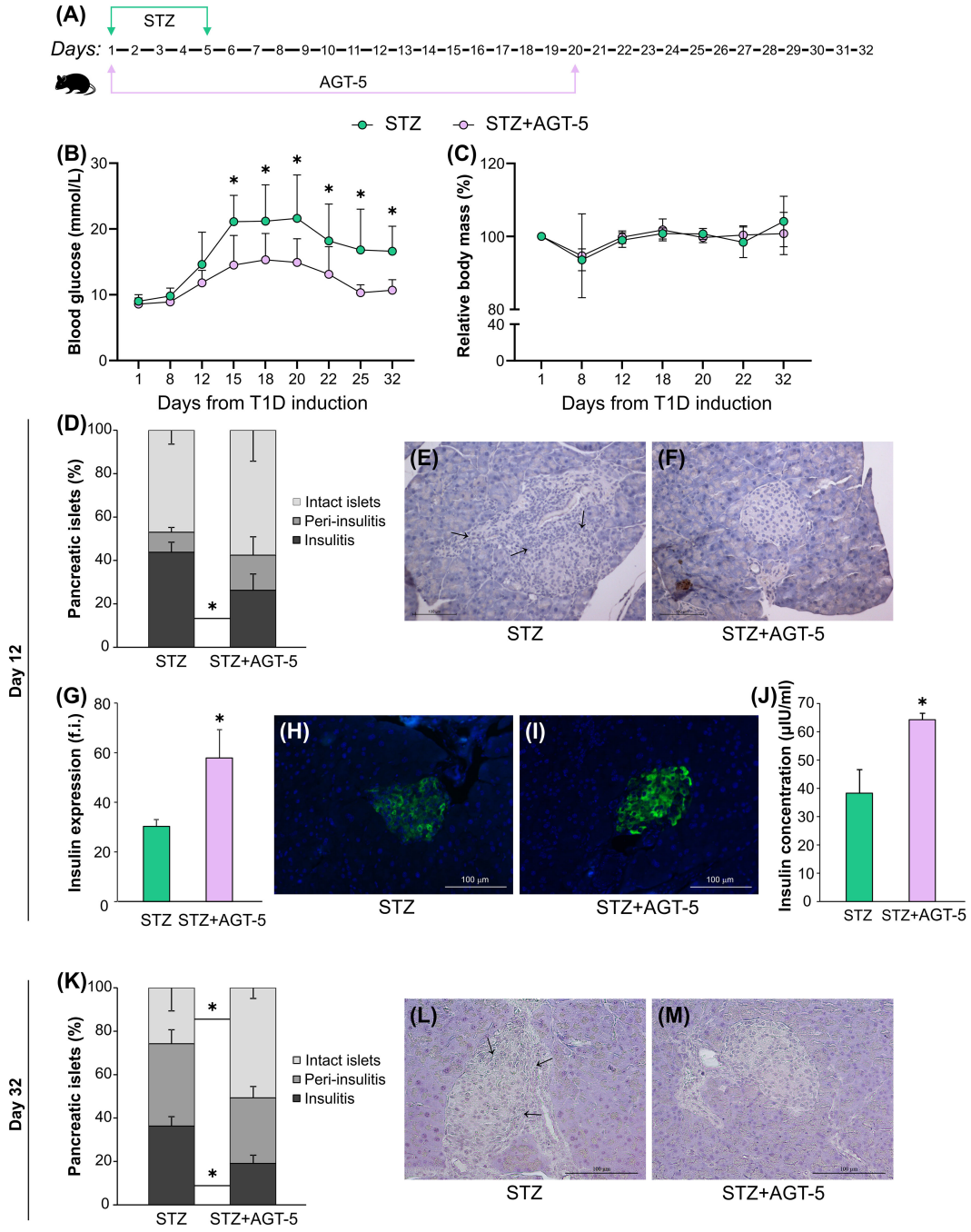

FIGURE 1
AGT-5 reduced the severity of T1D. **(A)** Diagram of T1D induction by STZ and AGT-5 treatment in C57BL/6 mice. Line graphs show **(B)** blood glucose level (mmol/L) and **(C)** relative body mass in relation to the initial body mass (%). Each group consisted of 8 mice. **(D)** Stacked bar graph shows the proportions of islets without infiltration (intact islets), with immune cells surrounding the islet (peri-insulitis) and with immune cells in the pancreatic islet (insulitis, indicated by arrows) on day 12. Representative images of pancreatic islets from the **(E)** STZ group and **(F)** STZ+AGT-5 group, stained with hematoxylin. **(G)** Histogram shows insulin expression (f.i., fluorescence intensity) in the pancreatic islets. Representative images of pancreatic islets from the **(H)** STZ group and **(I)** STZ+AGT-5 group, stained for visualization of insulin (green) and counterstained with Hoechst 33342 (nucleus – blue). **(J)** Histogram shows serum insulin level (µIU/ml). **(K)** Stacked bar graph shows the proportions of islets without infiltration (intact islets), with immune cells surrounding the islet (peri-insulitis) and with immune cells in the pancreatic islet (insulitis, indicated by arrows) on day 32. Representative images of pancreatic islets from the **(L)** STZ group and **(M)** STZ+AGT-5 group, stained with hematoxylin. Data represent results from one out of three independent experiments. *p<0.05 between the STZ and STZ+AGT-5 groups.

increased the proportion of $CD8^+$ lymphocytes (Figure 6A). As a confirmation of its AHR-related effects, AGT-5 significantly upregulated the proportion of Cyp1a1-expressing $CD4^+$ and $CD8^+$ lymphocytes (Figures 6B, C). Also, AGT-5 upregulated the frequency of Th1 cells, but exerted no effect on Th17 cell subset (Figure 6D). In addition to enhancing the proportion of Treg within the SI lamina propria, AGT-5 also increased the frequency of Cyp1a1-expressing Treg, confirming that the observed immunomodulatory effects are

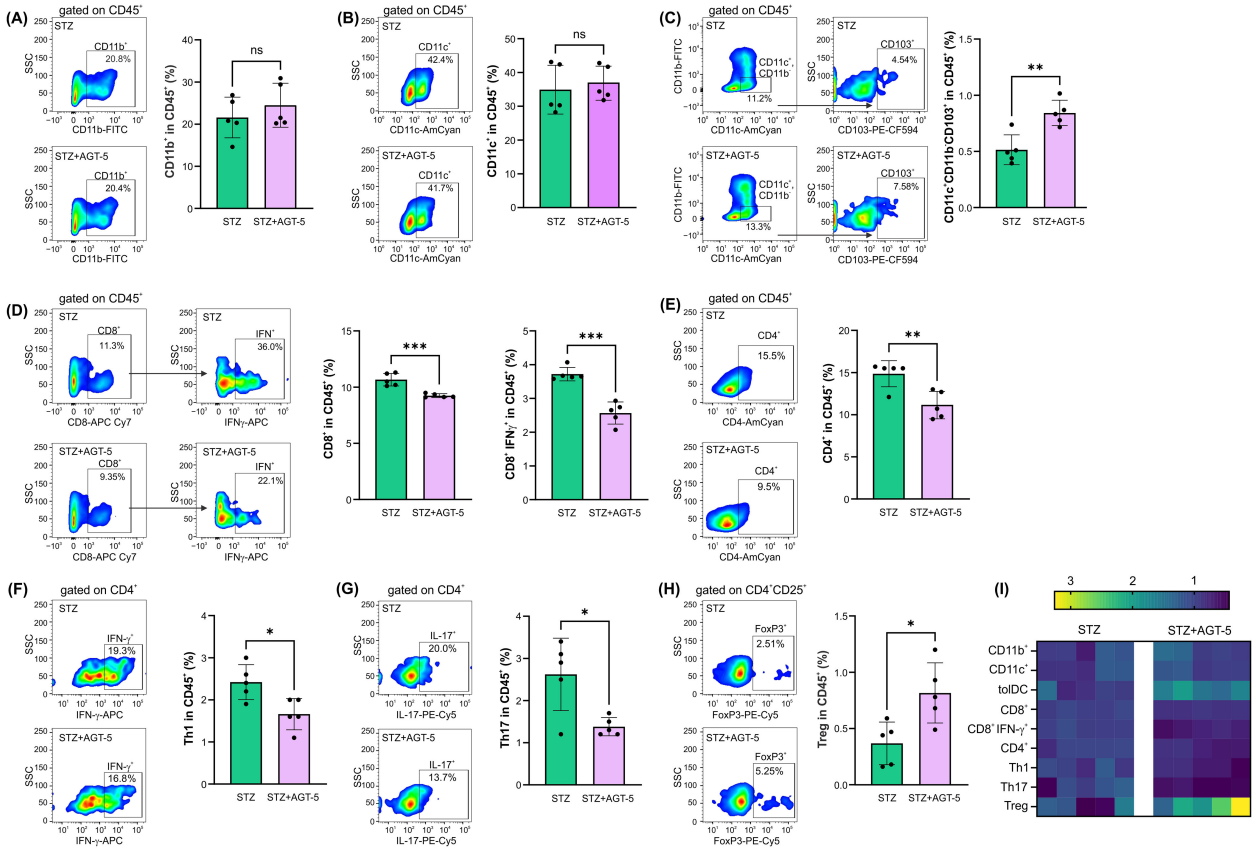

FIGURE 2
The effect of AGT-5 on immune cells in the pancreas. *Ex vivo* analysis was performed on day 12 after the first STZ injection (5 mice per group). Histograms show the proportions of **(A)** $CD11b^+$ cells, **(B)** $CD11c^+$ cells, **(C)** tolDC ($CD11b^-CD11c^+CD103^+$), **(D)** $CD8^+$ cells and IFN-γ-producing $CD8^+$ cells, **(E)** $CD4^+$ cells, **(F)** Th1 ($CD4^+IFN\text{-}\gamma^+$) cells, **(G)** Th17 ($CD4^+IL\text{-}17^+$) cells and **(H)** Treg ($CD4^+CD25^+FoxP3^+$) within $CD45^+$ cells, calculated using FlowJo v10.10.0 software. Corresponding flow cytometry plots show the frequencies of examined cell subsets within the indicated parent gates in the representative samples of STZ and STZ+AGT-5 mice. **(I)** Heatmap of the relative proportion of different cell types in STZ and STZ+AGT-5 groups of mice, normalized to the mean value for each cell type of the STZ group. Data represent results from one out of two independent experiments. *p<0.05, **p<0.01, ***p<0.005 between the STZ and STZ+AGT-5 groups. ns, not significant.

mediated through AHR (Figure 6E). Of note, a similar stimulatory effect of AGT-5 was observed *in vitro* on Cyp1a1-expressing DC (Supplementary Figure S6).

3.4 AGT-5 treatment stimulates tolDC and Treg functions in the pancreas and lamina propria of T1D mice

As AHR activation can directly influence indoleamine 2,3-dioxigenase (IDO1) enzyme expression (26), we have investigated the presence of $IDO1^+$ tolDC within SI lamina propria and pancreas. Within both tissues, AGT-5 increased the proportion of $IDO1^+$ tolDC, indicating their enhanced anti-inflammatory properties and a strong capacity to induce Treg (Figures 7A, B). The effect of AGT-5 on IDO-1 was corroborated by *in vitro* results on mouse bone marrow-derived DC, as AGT-5 exposure enhanced the proportion of $IDO1^+$ DC (Supplementary Figure S7A). IDO1 may represent a primary target molecule for AGT-5 as the presence of IDO1 inhibitor 1-methyl tryptophan (1MT) prevented the down-regulation of activation markers CD40, CD80 and CD86 (Supplementary Figures S7B–D). Additionally, the reduced production of TNF imposed by AGT-5 was increased in the presence of 1MT, while AGT-5-provoked upregulation of IL-10 was absent in 1MT-treated DC, determined by ELISA (Supplementary Figures S7E, F). Alongside the effect on DC, Treg immunosuppressive function in SI lamina propria was also increased after AGT-5 treatment as evidenced by the higher proportion of $CD73^+$ Treg (Figure 7C). However, AGT-5 did not change the proportions of $IL\text{-}10^+$, $CD39^+$ or granzyme B^+ Treg in this tissue (Figures 7D–F). Within the pancreas, AGT-5 increased the proportion of $CD39^+$ cells (Figure 7H), and had no effect on the frequencies $IL\text{-}10^+$, $CD73^+$ or granzyme B^+ Treg (Figures 7G, I, J). These results suggest that AHR activation by AGT-5 enhances the expression of ATP-degrading enzymes (CD39 and CD73) (27).

3.5 The effect of AGT-5 *in vitro* on human DC and ILC3

AGT-5 has previously been shown to upregulate human Treg proliferation *in vitro* (20). Here it is shown that *in vitro* exposure of human tonsil cells to AGT-5 (0.75 μM) increased the presence of

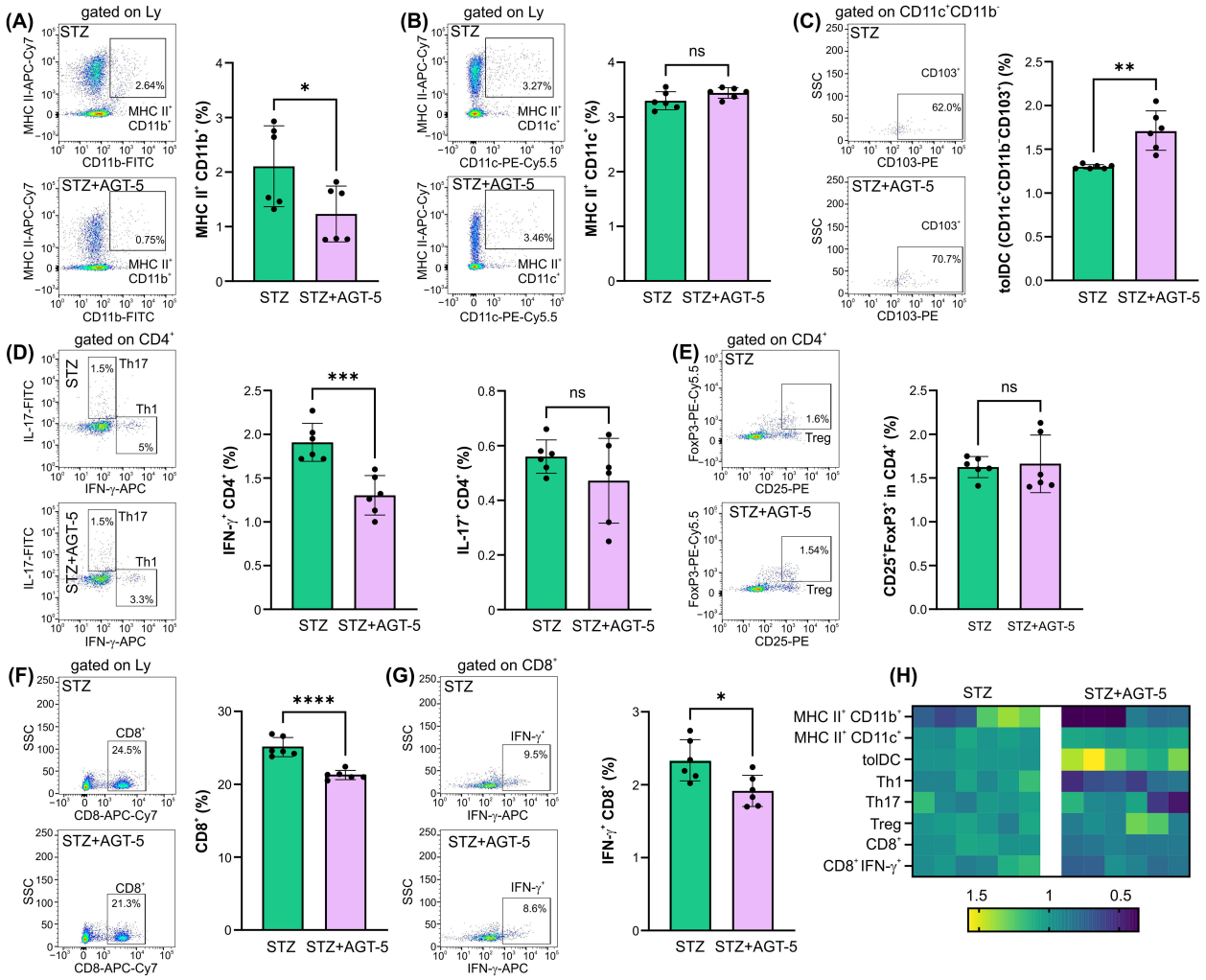

FIGURE 3
The effect of AGT-5 on cells in the pancreatic lymph nodes. *Ex vivo* analysis was performed on day 12 after the first STZ injection (6 mice per group). Histograms show the proportions of **(A)** MHC II$^+$CD11b$^+$ cells, **(B)** MHC II$^+$CD11c$^+$ cells, **(C)** tolDC (CD11b$^-$CD11c$^+$CD103$^+$), **(D)** Th1 (CD4$^+$IFN-γ$^+$) and Th17 (CD4$^+$IL-17$^+$) cells, **(F)** CD8$^+$ and **(G)** CD8$^+$IFN-γ$^+$ cells within lymph node lymphocytes, and **(E)** Treg (CD25$^+$FoxP3$^+$) within CD4$^+$ lymph node cells, calculated using FlowJo v10.10.0 software. Corresponding flow cytometry plots show the frequencies of examined cell subsets within the indicated parent gates in the representative samples of STZ and STZ+AGT-5 mice. **(H)** Heatmap shows the relative proportions of different cell types in STZ and STZ+AGT-5 groups of mice normalized to the mean value for each cell type of the STZ group. Data represent results from one out of two independent experiments. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 between the STZ and STZ+AGT-5 groups. ns, not significant.

immunosuppressive DC that express inhibitory molecule ILT3 (Figure 8A), suggesting that AGT-5 exerts similar effects on mouse and human DC. At the same time, AGT-5 increased the proportion of ILC3 within the whole ILC population in the tonsils (Figure 8B). The gating strategy for ILC3 is displayed in Supplementary Figure S8.

4 Discussion

Type 1 diabetes (T1D) presents a significant unmet clinical need despite advancements in treatment options. Novel approaches targeting the autoimmune component of T1D and aiming to restore immune tolerance or preserve β-cell function are urgently needed to improve outcomes and quality of life for patients with this chronic condition. Herein, we explored the potential to restore immune tolerance via activating the AHR. Along these lines, we evaluated the effect of the oral administration of AGT-5, an AHR-activating compound with fluorescent properties that we recently developed. AGT-5 attenuated the severity of T1D in a murine STZ model of autoimmune diabetes through the blockade of immune cell infiltration into the pancreatic islets, which enabled sufficient insulin production. These beneficial effects on the clinical course of T1D occurred as a consequence of suppression of inflammation, mediated by AHR-driven upregulation of tolDC and Treg compartments that exerted an immunosuppressive function in the GALT and pancreas.

The AHR is a ligand-activated transcription factor that is expressed by many immune cells and can be activated by various environmental factors, dietary components, microorganisms and metabolites. For example, AHR activation by its high-affinity ligand TCDD *in vivo* suppresses the development of experimental

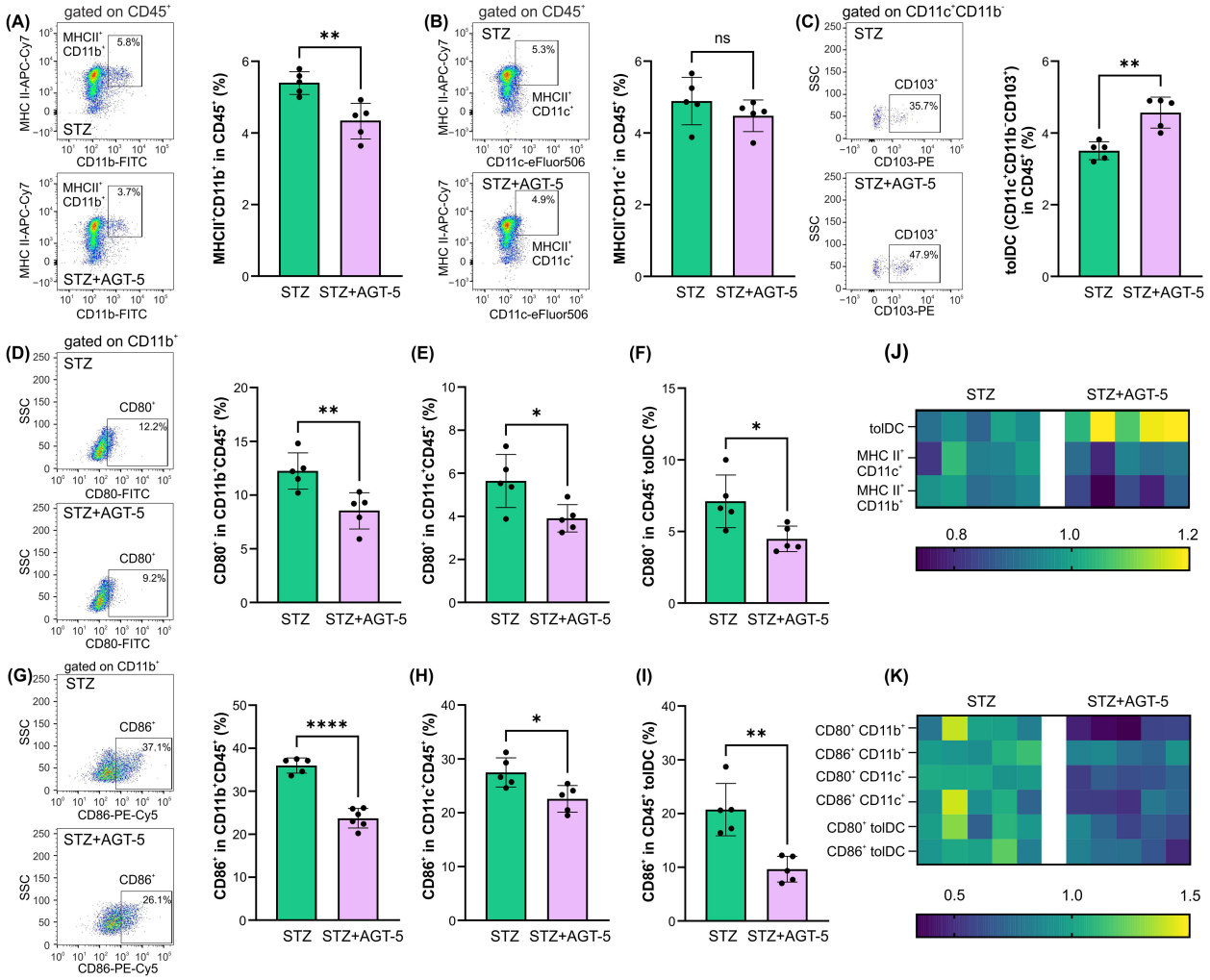

FIGURE 4
The effect of AGT-5 on innate immune cells in the SI lamina propria. *Ex vivo* analysis was performed on day 12 after the first STZ injection (5 mice per group). Histograms show the proportions of **(A)** MHC II$^+$CD11b$^+$ cells, **(B)** MHC II$^+$CD11c$^+$ cells, **(C)** tolDC (CD11b$^-$CD11c$^+$CD103$^+$) within CD45$^+$ cells, and CD80$^+$ cells within **(D)** CD11b$^+$CD45$^+$ cells, **(E)** CD11c$^+$CD45$^+$ cells, **(F)** CD45$^+$ tolDC, or CD86$^+$ cells within **(G)** CD11b$^+$CD45$^+$, **(H)** CD11c$^+$CD45$^+$ cells and **(I)** CD45$^+$ tolDC, calculated using FlowJo v10.10.0 software. Corresponding flow cytometry plots show the frequencies of examined cell subsets within the indicated parent gates in the representative samples of STZ and STZ+AGT-5 mice. **(J, K)** Heatmaps show the relative proportions of different cell types in STZ and STZ+AGT-5 groups of mice normalized to the mean value for each cell type of the STZ group. Data represent results from one out of two independent experiments. *p<0.05, **p<0.01, ****p<0.001 between the STZ and STZ+AGT-5 groups. ns, not significant.

autoimmune encephalomyelitis (EAE), experimental autoimmune uveoretinitis, and spontaneous autoimmune diabetes (15, 28, 29). Although it has strong immunomodulatory properties, TCDD is extremely toxic even in picomolar concentrations and therefore cannot be exploited for human use (30). This feature of TCDD is shared by numerous other AHR ligands. However, several AHR ligands isolated from natural sources (e.g. cruciferous plants) or chemically synthetized ligands (31) exert both low toxicity and a capacity for immunomodulation. AGT-5 belongs to the FluoAHRL family of recently synthetized compounds demonstrating AHR agonistic features and NIR emission. *In vitro* data suggest that AGT-5 preferentially stimulates Treg proliferation and differentiation, and increases Treg function (20). In addition to being a potent pro-Treg driver, AGT-5 showed no signs of toxicity at micromolar concentrations in zebrafish embryos or when administered to mouse macrophages *in vitro* (20). Therefore, this compound was selected as a suitable candidate for the modulation of the inflammatory autoimmune process during T1D development in the STZ animal model.

As expected, according to its effects *in vitro*, AGT-5 successfully attenuated T1D clinical symptoms in mice through interference with both the innate and adaptive immune responses in the pancreas, the draining lymph nodes and SI lamina propria. Initial events related to the pathogenesis of T1D in the STZ model encompass APC infiltrating the pancreas. These cells aim to repair the damage imposed by STZ, but also serve to present engulfed autoantigens and thereby initiate the autoimmune response. AGT-5 successfully reduced the relative number of classical MHC II$^+$ CD11b$^+$ APC in pancreatic draining lymph nodes, although it did not affect the prevalence of CD11b$^+$ APC

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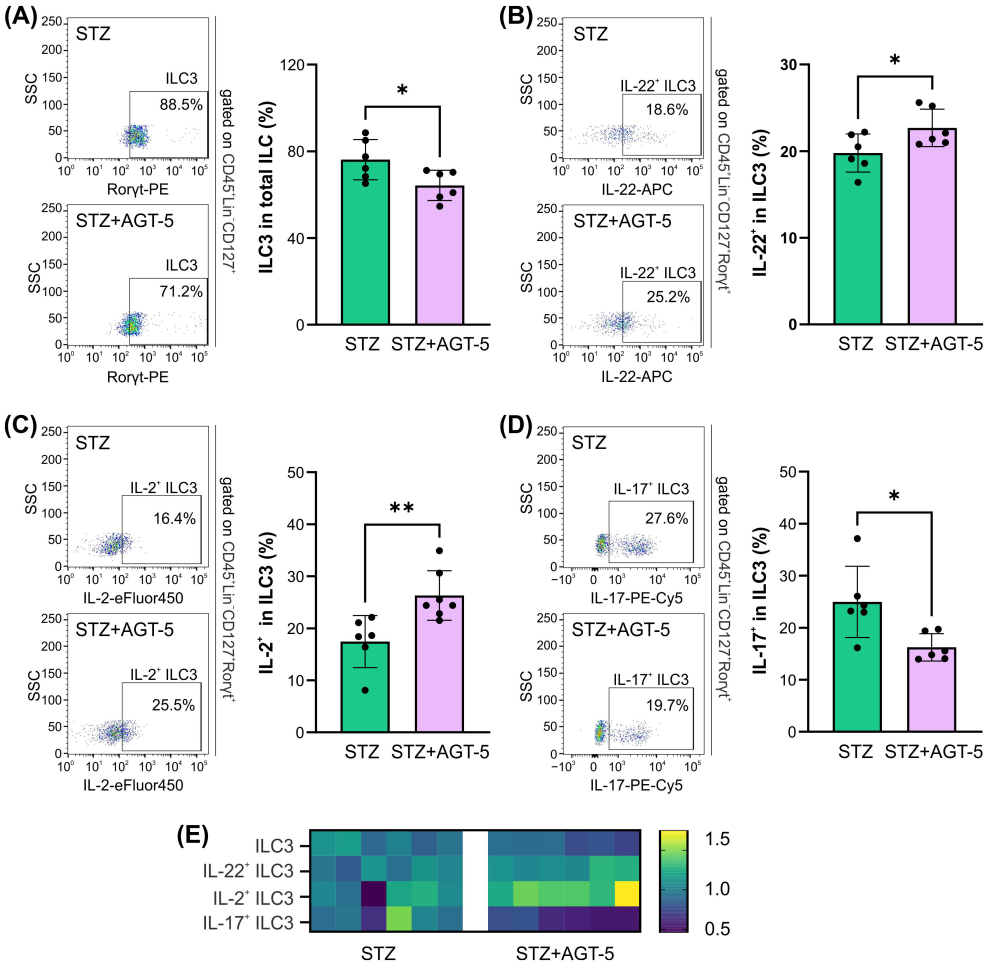

FIGURE 5
The effect of AGT-5 on ILC3 in the SI lamina propria. Histograms depict the proportions of **(A)** ILC3 (RORγt$^+$) within total ILC (CD45$^+$Lin$^-$CD127$^+$), **(B)** IL-22-producing cells within ILC3, **(C)** IL-2-producing cells within ILC3 and **(D)** IL-17-producing cells within ILC3, calculated using FlowJo v10.10.0 software (6 mice per group, day 12 after the initiation of the experiment). Corresponding flow cytometry plots show the frequencies of examined cell subsets within the indicated parent gates in the representative samples of STZ and STZ+AGT-5 mice. **(E)** Heatmap shows the relative proportions of different cell types in STZ and STZ+AGT-5 groups of mice normalized to the mean value for each cell type of the STZ group. *p<0.05, **p<0.01 between the STZ and STZ+AGT-5 groups.

within the pancreatic infiltrates. In addition to interference with the proportions of macrophages and DC, AGT-5 down-regulated the frequency of cells expressing co-stimulatory molecules CD40, CD80 and CD86 within CD11b$^+$, CD1c$^+$ and tolDC suggesting the reduced ability of APC to trigger T cell response. Cells that counteract classical APC are tolDC, and their abundance was consistently increased along the SI lamina propria-pancreatic lymph node-pancreas axis after the treatment with AGT-5. The potential for increasing the tolDC response is not uniquely attributed to AGT-5, as several other AHR ligands have shown similar properties. For example, AHR activation with the AHR ligand ITE (16, 32) or with the synthetic agonist laquinimod induces a tolerogenic phenotype in mouse and human DC (33–35). Also, nanoparticles loaded with ITE exert similar pro-tolerogenic effects and suppress the development of EAE (32).

The main feature of T1D is the imbalance between CD4$^+$ or CD8$^+$ Teff and the FoxP3$^+$CD4$^+$ Treg which leads to the destruction of pancreatic β-cells, ultimately causing insulin deficiency (4). According to our data, AGT-5 successfully attenuated the severity of T1D by favoring immunosuppressive Treg at the expense of pathogenic Th1 and Th17 cells in the pancreatic infiltrates. The interference with the gut-associated immune response is extremely important for the modulation of T1D as it has been demonstrated that patients with T1D exhibit subclinical intestinal immune activation that may be an indirect proof of the relation between gut inflammation and pancreatic islet-directed autoimmunity (36). Moreover, the activation of insulin-specific T cells can occur in the GALT and their presence was confirmed in Peyer's patches and mesenteric lymph nodes almost at the same frequency as in the pancreatic lymph nodes (37). In line with these findings, a recent study demonstrated that the disruption of gut barrier continuity leads to the activation of islet-reactive T cells in the intestine, ultimately contributing to the development of autoimmune diabetes (38). In our previous study it was found that AGT-5 can diffuse into the SI lamina propria where it can bind to AHR in immune cells (20). This study confirms that AGT-5 activates AHR in lamina propria, as higher proportions of CD4$^+$, CD8$^+$ cells and Treg that

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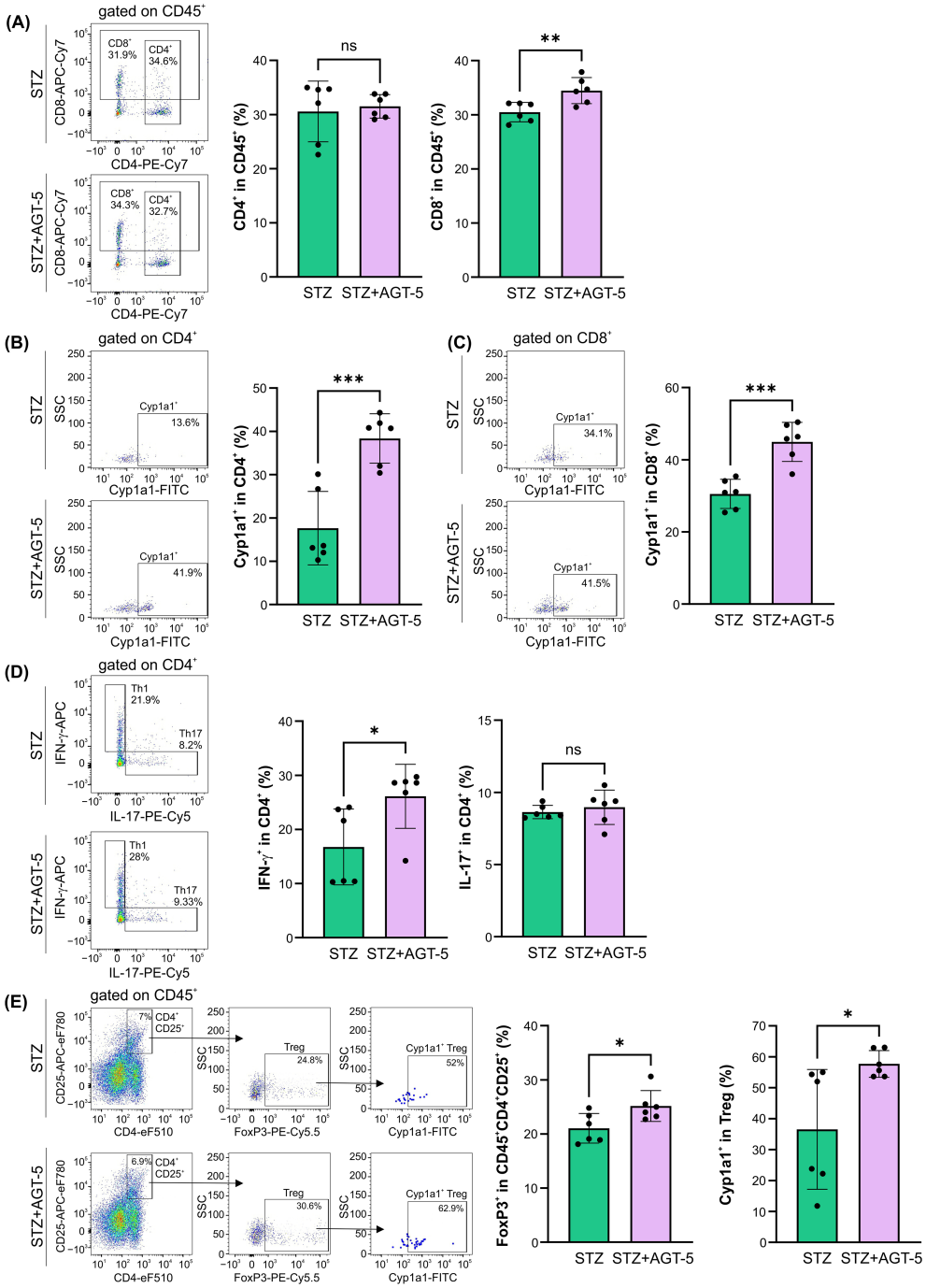

FIGURE 6
The effect of AGT-5 on the adaptive immune cells in the SI lamina propria. *Ex vivo* analysis was performed on day 12 after the first STZ injection (6 mice per group). Histograms show the proportions of **(A)** $CD4^+$ and $CD8^+$ cells within $CD45^+$ cells, **(B, C)** $Cyp1a1^+$ cells within **(B)** $CD4^+$ and **(C)** $CD8^+$ cells, **(D)** Th1 ($IFN\text{-}\gamma^+$) and Th17 ($IL\text{-}17^+$) cells within $CD4^+$ cells, and **(E)** $FoxP3^+$ Treg within $CD4^+CD25^+CD45^+$ cells and $Cyp1a1^+$ cells among them, calculated using FlowJo v10.10.0 software. Corresponding flow cytometry plots show the frequencies of examined cell subsets within the indicated parent gates in the representative samples of STZ and STZ+AGT-5 mice. Data represent results from one out of two independent experiments. *p<0.05, **p<0.01, ***p<0.005 between the STZ and STZ+AGT-5 groups. ns, not significant.

expressed Cyp1a1 (the primary target gene for AHR) were detected. AGT-5 can actually stimulate *de novo* differentiation of Treg as it was shown that AHR enhances the expression of *Foxp3* mRNA (39). According to the results by Guo et al. (40), targeting and possible manipulation of Treg in the intestinal lamina propria may be beneficial for achieving tolerance and suppressing the autoimmune response. Therefore, AGT-5 can directly, through AHR-driven events, stimulate Treg within the SI lamina propria and this anti-inflammatory environment can be carried over further to the target tissue.

In addition to the direct activation of Treg, AGT-5 can also stimulate Treg through modulation of other cells. For example, ILC3 express high levels of AHR and their differentiation and

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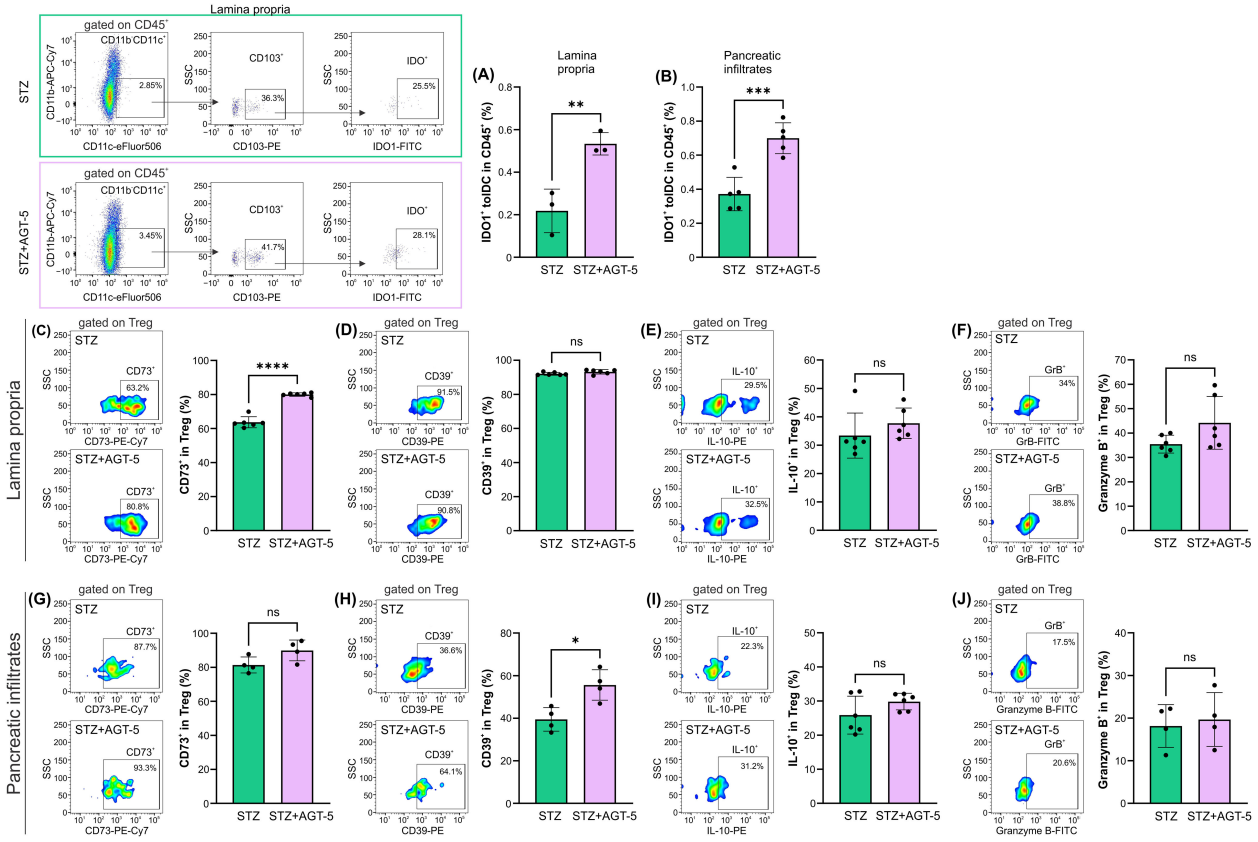

FIGURE 7
The effect of AGT-5 on tolDC and Treg populations in SI lamina propria and pancreatic infiltrates. *Ex vivo* analysis was performed on day 12 after the first STZ injection (3-6 mice per group). Histograms show the proportions of **(A, B)** IDO1$^+$ tolDC within CD45$^+$ cells in the **(A)** lamina propria and **(B)** pancreatic infiltrates; **(C, G)** CD73$^+$ cells, **(D, H)** CD39$^+$ cells, **(E, I)** IL-10$^+$ cells and **(F, J)** Granzyme B$^+$ cells within Treg in the **(C–F)** lamina propria and **(G–J)** pancreatic infiltrates. Corresponding flow cytometry plots show the frequencies of examined cell subsets within the indicated parent gates in the representative samples of STZ and STZ+AGT-5 mice. The frequencies of IDO1$^+$ tolDC within CD45$^+$ cells were calculated using FlowJo v10.10.0 software. Data represent results from one out of two independent experiments. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 between the STZ and STZ+AGT-5 groups. ns, not significant.

viability is completely dependent upon AHR (25, 41, 42). In this study, AGT-5 down-regulated the proportion of IL-17$^+$ within ILC3 (responsible for anti-microbial response), and increased the frequency of ILC3 that produce IL-2. As IL-2 is a well-recognized Treg growth factor, it would be reasonable to assume that IL-2$^+$ ILC3 participated in the upregulation of Treg after AGT-5 treatment. Indeed, there are studies suggesting that IL-2, derived from ILC3, is responsible for proper Treg activation in the lamina propria (43, 44). Also, ILC3 importance for T1D development is reflected by the reduced numbers of IL-2$^+$ ILC3 in the lamina propria that preceded the occurrence of insulitis in NOD mice and the appearance of STZ-induced T1D (44). In addition, AGT-5 increased the number of ILC3 that produced IL-22 and thereby may have improved epithelial barrier stability that is usually disturbed during T1D in both mice and humans (44–47).

One of the possible indirect Treg-stimulatory actions of AGT-5 can be exerted through the AGT-5-mediated influence on APC. More specifically, Treg can be activated through the action of tolDC. Activation of AHR was shown to induce the expression of tolerogenic markers of DC – enzymes IDO1 and 2 (26), which, through depriving tryptophan and increasing kynurenine production, promote FoxP3$^+$ Treg differentiation. This is in line with our data, as AGT-5 increased the proportion of IDO1$^+$ tolDC in both SI lamina propria and the pancreas. *In vitro* results on bone marrow-derived DC support *ex vivo* data since AGT-5 efficiently blocked differentiation of DC into the fully mature APC through down-regulation of co-stimulatory molecules CD80, CD86 and CD40. IDO1 is probably responsible for these effects, as the inhibition of IDO1 by 1MT abrogated the AGT-5-mediated reduction of co-stimulatory molecules in DC.

AGT-5 changes the proportion of Treg, but also affects their function. Treg function is exerted through secretion of IL-10, ATP depletion, expression of inhibitory (CTLA-4, PD-1) or cytotoxic (granzyme B) molecules that inhibit Teff (4). Seemingly, AGT-5 did not stimulate IL-10 production in the T1D setting, which is in contrast to the results obtained from healthy murine SI lamina propria cells (20). In this inflammatory setting, AGT-5 predominantly influenced the ATP depletion machinery in Treg as it increased the proportion of CD73$^+$ Treg in the pancreas and CD39$^+$ Treg in the SI lamina propria. It is reasonable to assume that these are AHR-related events as it was shown that AHR drives the expression of the ectoenzyme CD39, which cooperates with CD73

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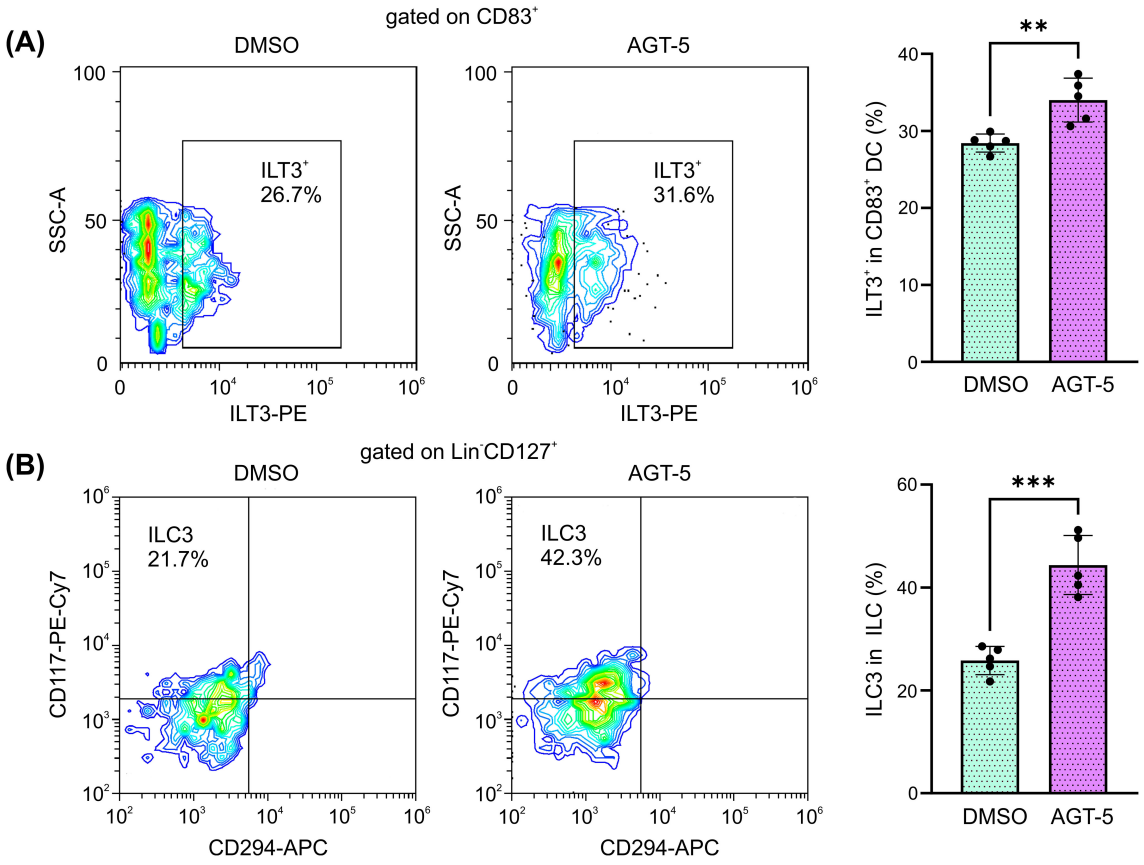

FIGURE 8
AGT-5 increases the proportion of immunosuppressive DC and ILC3 that originate from human tonsils. Tonsil cell suspension was incubated with AGT-5 (0.75 μM) for 48 h, and flow cytometry was used to detect **(A)** ILT3$^+$ DC and **(B)** Lin$^-$CD127$^+$CRTH2(CD294)$^-$CD117$^+$ ILC3. Histograms show the proportions of **(A)** ILT3$^+$ cells within CD83$^+$ DC, and **(B)** CRTH2$^-$CD117$^+$ ILC3 within total ILC. Representative flow cytometry plots are shown. Representative experiment out of two performed is displayed. **p<0.01, ***p<0.005 between the DMSO-treated and AGT-5-treated cells.

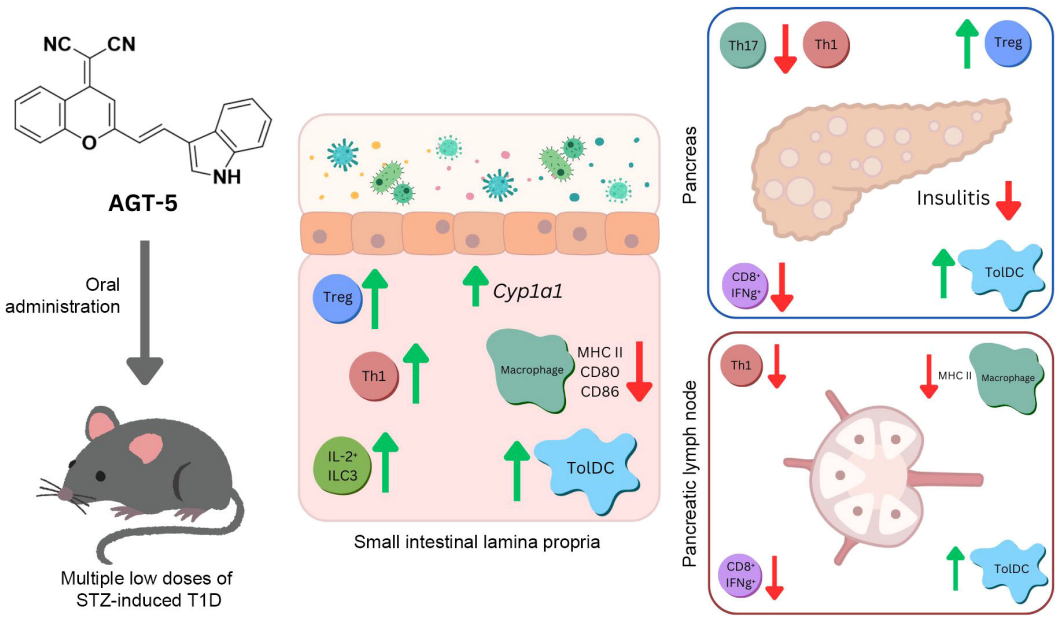

FIGURE 9
Schematic presentation of AGT-5 impact on T1D pathogenesis.

to deplete the pro-inflammatory extracellular ATP and catalyze its conversion into anti-inflammatory adenosine (27).

While the present study suggests potential cellular and molecular targets of AGT-5 in the treatment of T1D, it comes with several limitations. First, to identify specific molecular targets and determine precise mechanism of AGT-5 action, RNA sequencing may be used. Second, this study does not cover the possible effect of AGT-5 on the phenomenon termed "Treg resistance". This represents the lack of sensitivity of Teff towards Treg-mediated suppression, and it has been described in multiple sclerosis, rheumatoid arthritis and T1D patients (48–50). Finally, for the purpose of conducting translational research, pharmacokinetic status of AGT-5 should be explored in future studies.

In conclusion, our study demonstrates that the novel nontoxic fluorescent AHR ligand AGT-5, when administered orally, acts on tolDC, ILC3 and T cells to promote the induction of functional FoxP3$^+$ Treg in the GALT and pancreatic islets, thereby inhibiting insulitis and preserving insulin production (Figure 9). In addition to its potential as an immunomodulatory therapeutic, AGT-5, due to its fluorescent properties, can be used for *in vivo* imaging. This allows for the visualization of AHR activation and distribution within living organisms. The fluorescent capability of AGT-5 can facilitate and accelerate research in targeting AHR-related pathways for potential therapeutic interventions.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee of Clinical Hospital Center "Zemun", Belgrade, Serbia (App. No 14/1, date 27/09/2022). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Veterinary Administration, Ministry of Agriculture, Forestry and Water Management, Republic of Serbia (App. No 119-01-4/11/2020-09). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NJ: Investigation, Writing – review & editing, Methodology, Visualization. IK: Investigation, Methodology, Visualization, Writing – review & editing. SK: Investigation, Methodology, Writing – review & editing, Formal analysis, Validation. V-PB: Formal analysis, Investigation, Methodology, Writing – review & editing. CC: Investigation, Methodology, Validation, Writing – review & editing. NR: Formal analysis, Investigation, Methodology, Writing – review & editing. IP: Formal analysis, Investigation, Methodology, Writing – review & editing. AJ: Investigation, Writing – review & editing, Resources. MJ: Resources, Writing – review & editing. MD: Writing – review & editing, Data curation, Formal analysis. AT: Writing – review & editing, Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft. IS: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing, Formal analysis, Investigation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1454156/full#supplementary-material.

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EDITED BY
Feng Dong,
AbbVie, United States

REVIEWED BY
Saeed Mohammadi,
Golestan University of Medical Sciences, Iran
Virginia Flores-Morales,
Universidad Autonoma de Zacatecas, Mexico

*CORRESPONDENCE
Yan-bin Li
13864006933@163.com
Guan-qing Wang
wangguanqing2021@126.com

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Therapeutic potential of natural coumarins in autoimmune diseases with underlying mechanisms

Yan Li[1,2], Guan-qing Wang[1*] and Yan-bin Li[1*]

[1]Department of Neurology, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, Shandong Institute of Neuroimmunology, Shandong Provincial Key Medical and Health Laboratory of Neuroimmunology, Jinan, China, [2]College of First Clinical Medicine, Shandong University of Traditional Chinese Medicine, Jinan, China

Autoimmune diseases encompass a wide range of disorders characterized by disturbed immunoregulation leading to the development of specific autoantibodies, which cause inflammation and multiple organ involvement. However, its pathogenesis remains unelucidated. Furthermore, the cumulative medical and economic burden of autoimmune diseases is on the rise, making these diseases a ubiquitous global phenomenon that is predicted to further increase in the coming decades. Coumarins, a class of aromatic natural products with benzene and alpha-pyrone as their basic structures, has good therapeutic effects on autoimmune diseases. In this review, we systematically highlighted the latest evidence on coumarins and autoimmune diseases data from clinical and animal studies. Coumarin acts on immune cells and cytokines and plays a role in the treatment of autoimmune diseases by regulating NF-κB, Keap1/Nrf2, MAPKs, JAK/STAT, Wnt/β-catenin, PI3K/AKT, Notch and TGF-β/Smad signaling pathways. This systematic review will provide insight into the interaction of coumarin and autoimmune diseases, and will lay a groundwork for the development of new drugs for autoimmune diseases.

KEYWORDS

coumarins, autoimmune diseases, anti-inflammatory, MAPKs, NF-κB, epigenetic modulation

1 Introduction

Autoimmune diseases (AIDs) are inflammatory disorders caused by immune dysfunction and loss of immune tolerance, leading to the recognition of self-antigens by the body's immune system (1, 2). Currently, more than 80 AIDs have been identified, including rheumatoid arthritis, type 1 diabetes mellitus, and psoriasis (3). AIDs can occur at any age and are particularly more prevalent in women than in men. It is estimated that 8% to 10% of population worldwide is afflicted by AIDs (4). Autoimmunity and autoimmune diseases have been increasing dramatically in many parts of the world in

recent years, possibly due to changes in our exposure to environmental factors. Current evidence suggests that major changes in our food, exogenous substances, air pollution, infections, personal lifestyles, stress, and climate change are responsible for these increases (5). Autoimmune diseases have a devastating impact on individuals and caregivers in our society, and a large amount of healthcare utilization leads to high public and private costs, and current projections suggest that they will become more prominent diseases in the future (6). In particular, AIDs pose a major challenge to the public health system, which is second only to cancer and cardiovascular diseases, due to their long cycle and susceptibility to relapse (7–10). Therefore, it is of great importance and urgency to find effective methods for the prevention and treatment of AIDs. Coumarins are a class of aromatic natural products with benzene and alpha-pyrone as its basic structure, which are widely found in Umbelliferae, Brassicaceae, Asteraceae, Leguminosae, Orchidaceae (11). Coumarins can be divided into simple coumarins, furanocoumarins, pyranocoumarins and others based on the chemical structures (12). Accumulating studies have shown that coumarins possess a variety of pharmacological activities such as anti-tumor, anti-inflammatory, and anti-osteoporosis (13). Nowadays, coumarins have been gaining more attention from investigators due to its excellent biological activities in AIDs. Here, we review the latest research data on coumarins for the treatment of AIDs with the aim of understanding the pharmacological mechanisms of coumarins and developing novel agents for the treatment of AIDs.

2 Pathophysiology of autoimmune diseases

The mechanism by which the immune system prevents pathogens from attacking the organism is very complex. it can remove senescent cells and immune complexes from the body through various immune cells (such as macrophages, dendritic cells (DCs), T-lymphocytes, B-lymphocytes, etc.), and at the same time, it can recognize its own tissues and cells as its "self", thus forming immune tolerance. Immune tolerance is defined as a state in which immunologically active cells are unable to produce specific immune effector cells and specific antibodies when exposed to antigenic substances, thus failing to execute a normal immune response (9, 14). In some cases, autoimmune tolerance is disrupted and the absence of immune tolerance induces the immune system to produce autoantibodies in response to self-antigens. Antigen presenting cells (APCs) present autoantigens to T cells with the participation of major histocompatibility complex (MHC) molecules (15). T helper cells are stimulated by MHC II and release different cytokines that can directly trigger macrophages (MP), monocytes, and B cells (16). T cells control the immune response by influencing the mixture of interleukins produced. B cells produce antibodies against their own molecules that react with accessible cells and directly or indirectly mediate damage (17). When the immune system produces a strong and sustained immune response against its own tissues and cells, leading to cellular destruction or tissue damage and clinical symptoms, it can lead to AIDs (18). Broadly speaking, AIDs are diseases caused by the immune response of the immune system against its own components. All diseases caused by dysfunctions of the autoimmune system can be referred to as AIDs.

Genetic, epigenetic, and environmental factors (hormones, nutrition, drugs, microbiota, apoptosis, and others) are predisposing factors for autoimmunity (19). Although AIDs are considered rare, epidemiologic data show that nearly 3-5% of the population suffers from type 1 diabetes (T1D) and autoimmune thyroid diseases (20). According to clinical manifestations, AIDs can be categorized into two categories: systemic AIDs and organ-specific AIDs (3, 21). Systemic AIDs are those in which immune response causes pathological damage to multiple organs and tissues throughout the body, mainly including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren′s syndrome (SS) and others. Organ-specific AIDs refer to patients whose lesions are generally confined to a specific organ and caused by an autoimmune response against the particular organ. It mainly includes Hashimoto's thyroiditis (HT), Graves' Disease (GD), myasthenia gravis (MG) and others.

The imbalance of immune cells activation and regulation caused by the failure of lymphocyte self-tolerance mechanisms is considered to be a major driver of the progression of human AIDs (22). The production of autoantibodies is a key event in the development of AIDs. Under the influence of T cells or innate triggers, self-tolerance is first interrupted, and the B-cell response leads to systemic autoimmunity and the production of pathogenic autoantibodies, which are the main immune abnormality in AIDs (23). Expansion of self-reactive T cells is a biomarker of many AIDs, which is essential in the orchestration of innate and adaptive immune responses and in the induction of tissue damage. Among them, CD4+ T cells make important contributions by secreting various cytokines, chemokines and cell-cell interactions. IL-17-producing CD4+ T cells (Th 17 cells) are the core of the disease pathogenesis. When activated by antigen presenting cells (APCs), CD4+ T cells differentiate into different cell lines with unique functions, including helper T (Th) 1, Th2, Th17, and regulatory T (Treg) cells, each of which secretes its own set of cytokines (24). A balance is required to maintained between Th cell activation and Treg cells-mediated inhibition to maintain effective immune homeostasis. Disruption of this balance lead to lymphocytes generating an immune response and/or producing antibodies against their own cells and tissues (25).

The T cell subsets involved in the inflammatory response are mainly Th1 and Th17. Th1 cells are generated from CD4+ T lymphocytes activated by interleukin (IL)-12 through the STAT4 signaling pathway and the transcription factor T-bet, mainly secreting cytokines, such as IL-2, IL-12, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), and participate in the cellular immune response (26). Th2 cells are induced by IL-4 through the STAT6 signaling pathway and the transcription factor GATA-3, mainly secreting IL-4, IL-5, IL-6, IL-10 and IL-21 to participate in the immune response. Th1 plays a certain role in inhibiting the activation of Th2, and the two regulate and constrain each other, putting the body in a dynamic balance of cellular immunity and humoral immunity (27). Th 17 cells are one of the

most predominant pathogenic cells among Th cells, and their main function is to secrete cytokines IL-17A, IL-17F, and IL-22. Their activation and proliferation require multiple transcription factors (such as NF-κB, STAT3) and specific cytokines (such as transforming growth factor-β (TGF-β), IL-6, IL-23) (28, 29). Treg cells, as an important factor in the maintenance of immune tolerance by the organism, can regulate the stable state of lymphocytes. Under the induction of the specific transcriptional regulator Forkhead box protein P3 (Foxp3), they exert anti-inflammatory effects by releasing anti-inflammatory cytokines such as IL-10 and TGF-β (30).

Recent studies have confirmed the role of immune cells and cytokines in AIDs. For example, multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system, characterized by a positive correlation between the imbalance of the Th17/Treg ratio and the severity of MS symptoms (31, 32). Similarly, inflammatory bowel disease (IBD) and SLE are AIDs characterized by elevated levels of pro-inflammatory cytokines IL-1 and LTB4 (33). Furthermore, it is known that psoriasis is caused by activation of the IL-23/Th17 cytokine axis (34, 35). In UC patients, the increasement of IL-1, IL-6 and TNF-α are observed. In addition, elevated levels of IL-6 can also be observed in patients with T1D, RA and psoriasis (36). Studies have confirmed that Th17 cells are critical for the severity of collagen-induced arthritis and RA (37). Similarly, IL-17 and TNF-α-induced increase in intestinal barrier permeability can promote the development of Crohn's disease (CD), ulcerative colitis (UC), and MS (38, 39).

AIDs are the result of a combination of genetic predisposition and environmental factors. Genome-wide association studies (GWASs) has been widely used to identify susceptibility genes for AIDs and has identified many relevant mutations in T cells, including Single Nucleotide Polymorphism (SNPs) in IL-23R, IL-17A/F, IL-21, JAK2, STAT2, CARD9, CCR6, and others (40). In addition, epigenetic mechanisms influence the development of many AIDs under the influence of environmental factors. M6A-modified regulatory factors can be involved in T cell-mediated autoimmune diseases. It was found that the m6A-modifying demethylase ALKBH5 promotes IFN-γ and CXCL2 mRNA stability in CD4+ T cells, which in turn enhances CD4+ T cell pathogenicity in experimental autoimmune encephalomyelitis (EAE), whereas the demethylase FTO does not function (41). In experimental autoimmune uveitis, the presence of METTL3 in autoreactive Th17 attenuates Th17 pathogenicity by enhancing ASH1L mRNA stability to reduce IL-17 and IL-23 receptor expression (42). However, in psoriasis, T cell-specific deletion of ALKBH5 instead exacerbates skin inflammation (43). Epigenetic modifications regulate the body's inflammatory response and immune response at multiple levels through DNA methylation, histone acetylation, and microRNAs, while the DNA sequence remains unchanged (44). For example, due to the reduced expression of H3K4 methyltransferase Ash1L, Tregs in RA patients express low levels of Foxp3 while Ash1L can enhances TGF-β/Smad signaling promotes Treg differentiation, inhibits histone deacetylase 1 (HDAC1), and reduces histone deacetylation of Foxp3 (45).

3 Clinical status of coumarins in autoimmune disorders

Coumarins are a class of natural compounds widely found in nature (46). Modern pharmacological and clinical studies have shown that coumarins have pharmacological effects such as anti-tumor (47), anti-inflammatory (48), anti-osteoporosis (49), cardiovascular and neuroprotection (50), anti-bacterial (51), anti-tuberculosis (52), and photosensitization (53). In addition, coumarins are effective in the treatment of several AIDs in studies. Therefore, coumarins need more attention. The chemical structures of the constituents were screened using the PubChem database (http://pubchem.ncbi.nlm.nih.gov) and the structures of the most widely studied coumarins are given in Figure 1.

Given its immunomodulatory activity, coumarin has become a pharmacological tool for the treatment of various AIDs. Currently, coumarin combinations are used in the treatment of skin and AIDs such as psoriasis (54). Furanocoumarins are a class of natural plant photosensitizers. Studies have confirmed that furanocoumarins can increase the body's sensitivity to long-wave ultraviolet light (53). PUVA, a combination of psoralen (P) and ultraviolet A (UVA), is increasingly being used to treat chronic plaque-type psoriasis and chronic palmoplantar psoriasis, and has become a second-line therapy for patients with moderate to severe psoriasis (55–61). In the treatment of severe chronic atopic dermatitis, PUVA therapy provides better short- and long-term efficacy than UV therapy alone (62–64). PUVA is also safe and effective in the treatment of cutaneous T-cell lymphoma (65). And, PUVA therapyis effective in patients with cutaneous T-cell lymphoma (CTCL) complicated by ankylosing spondylitis (AS) (66). In patients with alopecia areata (AA), dilutions of psoralen were applied to the patient's scalp, and hair regrowth was observed in 6 of 9 patients after up to 10 weeks of treatment (67). Also, PUVA is effective in patients with AA (68, 69). In clinical practice, coumarins have been used regularly in the treatment of vitiligo. Psoralen and bergapten can increase the tolerance of human skin to radiation and produce hyperpigmentation when exposed to ultraviolet light (70). In addition, in a clinical study evaluating the photochemotherapeutic properties of bergapten microcrystalline formulations, the data results showed that bergapten was almost completely free of phototoxic and drug intolerance reactions, and that other side effects, such as severe erythema, itching, and nausea, were seen only rarely. Bergapten may be used as a photochemical therapy (PUVA) as an important alternative therapy (71). A study investigated the distribution of bergapten in the skin following oral administration of the drug. Bergapten concentrations in the skin following single and multiple oral doses of the drug were measured at healthy and psoriatic sites in 10 patients with psoriasis. The results showed that after oral administration of bergapten, accumulation levels were higher in the more external layers of the skin, the drug had a high affinity for the stratum corneum, and drug concentrations were similar in healthy and psoriasis sites, suggesting that lesions did not affect the distribution of the drug in the skin (72).

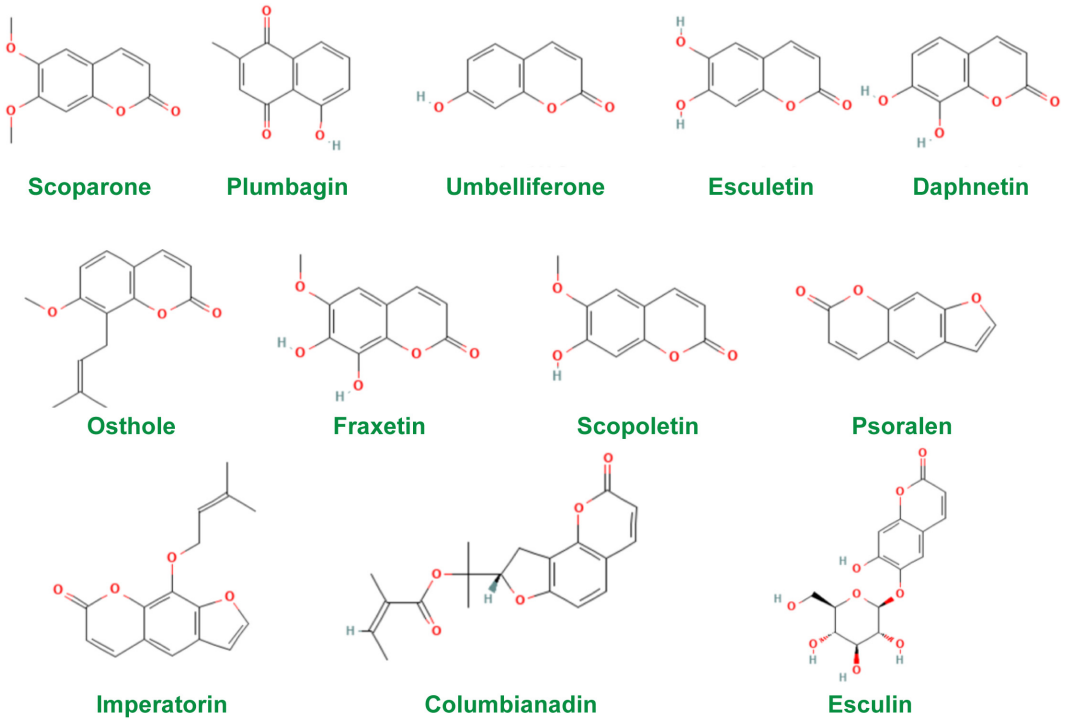

FIGURE 1
Chemical structures of coumarins used in studies.

4 Coumarins act on immune cells and cytokines in autoimmune disorders

AIDs have been categorized into several types and more than 80 such diseases have been identified. Coumarins can act on immune cells and cytokines to exert beneficial effects on AIDs. The regulatory effects of coumarins on significant immune cells and cytokines are shown in Figure 2.

RA is a chronic, systemic autoimmune disease with symmetrical, erosive, inflammation occurring in multiple joints as its main clinical manifestation. CD83 is a dendritic cell marker that belongs to the immunoglobulin superfamily and is closely associated with autoimmune diseases (73). TNF-β, which mediates a variety of inflammatory and immunostimulatory responses (74). Sterol regulatory element-binding protein 1 (SREBP1), a transcription factor, is a major regulator of genes that control cellular lipid homeostasis (75). Synergistic treatment with Imperatorin and β-sitosterol significantly up-regulated the expression levels of TNF-β, CD83, and SREBP1 in peripheral blood CD4+ T cells, which improved the severity of arthritis in collagen-induced arthritis (CIA) rats (76). In addition, imperatorin inhibits cell proliferation and induces apoptosis in RA fibroblast-like synoviocytes (RA-FLSs) cells through mitochondrial/caspase-mediated signaling pathways (77). Migration inhibitory factor (MIF) is a pleiotropic inflammatory cytokine important in both innate and adaptive immune responses, and studies have demonstrated that elevated levels of MIF expression are observed in synovial tissues of RA patients compared to healthy individuals (78). Isopsoralen has been shown to ameliorate RA by targeting macrophage MIF, as evidenced by a significant decrease in serum production of IL-6, IL-1β, and cartilage oligomeric matrix protein (COMP) but an increase in IL-10 production in CIA mice (79).

SLE is a chronic autoimmune disease in which 75% to 80% of patients have skin manifestations, such as erythema of the cheeks, rashes, and skin ulcers (80). Double-negative (DN) T cells are defined by the lack of CD4 and CD8 and the ability to produce pro-inflammatory cytokines, such as IFN-γ, which have been implicated in the pathogenesis of SLE in humans and mice (81). Umbelliferone reduced DN T cells, plasma cells, IFN-γ+CD4+ T cells, and T follicular helper cells (CD3+TCRβ+CD4+CXCR5+PD1+) and increased the percentage of Treg cells in lupus nephritis MRL/lpr mice (82).

MS is a common clinical neuroimmune disease, which occurs in young and middle-aged people between the ages of 20 and 40, with more female patients than male patients, strong relapses, and a high disability rate (83). EAE is the classical animal model of MS. Different coumarins including daphnetin, plumbagin, umbelliferone, and osthole can treat EAE. Daphnetin attenuates EAE by up-regulating Th2 and Treg cells and inhibiting Th1 and Th17 cells, as evidenced by increased expression of anti-inflammatory cytokines and transcription factors (IL-4, IL-10, IL-33, GATA3, Foxp3), and decreased pro-inflammatory cytokines and transcription factors (IL-17, TNF-α, IFN-γ, STAT4, T-bet, STAT3, ROR-γt) production (84). In addition, daphnetin reduced pro-inflammatory cytokines, including IL-17, IFN-γ, IL-6, IL-12a, and IL-23a, in brain tissues of EAE mice. Heme oxygenase-1 (HO-1) is a typical antioxidant and anti-inflammatory factor. The study confirmed the ability of daphnetin to inhibit IL-1β, IL-6, and

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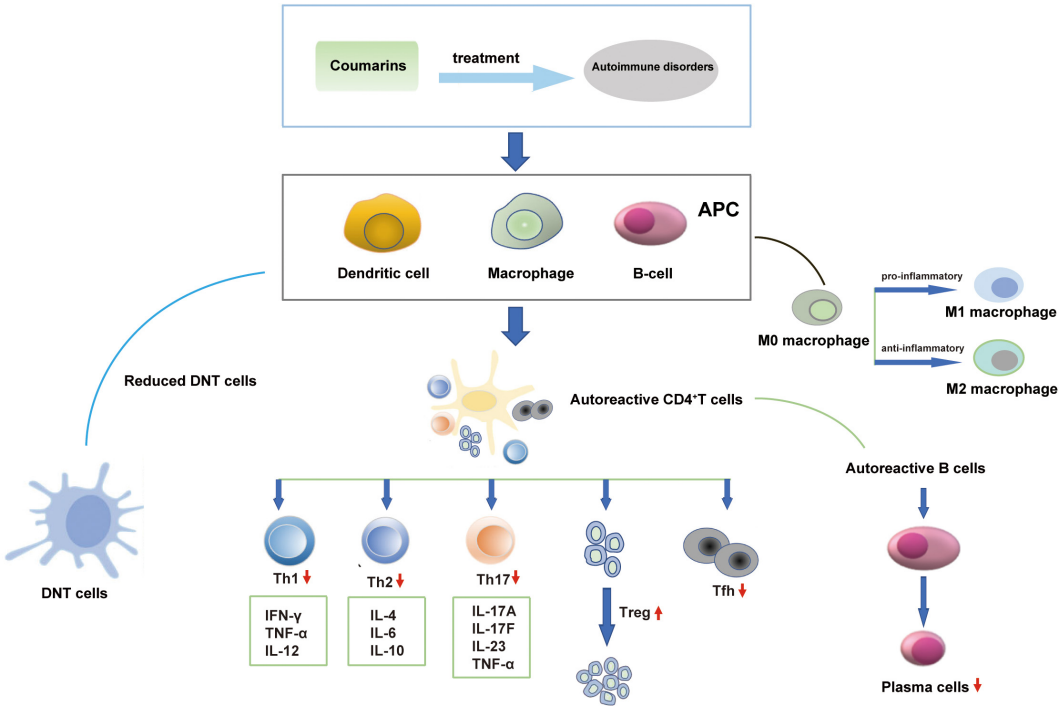

FIGURE 2
Coumarins act on immune cells and cytokines in autoimmune disorders.

TNF-α production and significantly elevate HO-1 levels in lipopolysaccharide (LPS)-stimulated mouse BV2 microglial cells (85). A study looking at the effects of plumbagin on EAE found that plumbagin inhibited the differentiation, maturation, and function of human monocyte-derived DCs, as well as inhibited Th1 and Th17 cell polarization (decreasing the expression levels of IL-6, IL-1β, and IL-23), and promoted Th2 cell polarization (up-regulating the expression level of IL-4) (86). Umbelliferone attenuates clinical symptoms in EAE mice by inhibiting the activation of autoreactive T cells, suppressing Th1 cell polarization, and increasing the level of Foxp3+ regulatory T cells (87). The effects of osthole on EAE have also been reported. Osthole augments the therapeutic efficiency of neural stem cells and inhibits the reduction of nerve growth factor (NGF) and the elevation of IFN-γ in EAE mice (88, 89).

Psoriasis is primarily a refractory disease mediated by T-lymphocytes with a combination of genetic and environmental effects (90). Daphnetin treatment inhibits the proliferation and inflammatory response of human HaCaT keratinocytes and ameliorates imiquimod (IMQ)-induced psoriasis-like skin injury in mice and attenuates the IMQ-induced upregulation of inflammatory cytokines, including IL-6, IL-23A, and IL-17A (91). Treatment of psoriasis mice with an ointment containing osthole was found to reduce the secretion of TNF-α, IL-12, IL-17, and IL-23 in the skin of mice (92).

UC is a chronic non-specific inflammatory bowel disease, with lesions mainly located in the mucous membrane and submucosa. The main clinical manifestations include abdominal pain, diarrhea, and bloody stools (93). Treatment with decursin and decursinol inhibitthe production of IL-6, TNF-α, cyclooxygenase (COX)-2, hypoxia inducible factor (HIF)-1a, and prostaglandin E2 (PGE2) in colonic tissues of UC mice induced by dextran sulfate sodium (DSS) (94). Decursinol angelate ameliorates DSS-induced colitis by modulating Th17 cell responses, which is reflected in its ability to reduce the mRNA level of RORγt in Th17 cells and the expression of IL-17 in CD4+ T cells (95). In addition, plumbagin reduces the expression of circulating inflammatory monocytes (CD14+/CD16+) and cytokines (TNF-α and IFN-γ) in UC mice (96). Osthole treatment down-regulated the levels of pro-inflammatory Th1-associated cytokines (TNF-α) and Th17-associated cytokines (IL-17) and up-regulated the levels of anti-inflammatory Th2-associated cytokines (IL-4 and IL-10) (97). Another study confirmed that daphnetin can improve UC by regulating Treg/Th17 balance (98). In addition, the therapeutic effects of esculin, bergapten, esculetin and scoparone have been reported in UC (99–102).

Type 1 diabetes mellitus (T1DM) is an autoimmune disease in which pancreatic β cells are destroyed, resulting in an absolute lack of insulin (103). Umbelliferone increases the number of Foxp3+ regulatory T cells, thereby alleviating the severity of type 1 diabetes (104). In addition, imperatorin acts as a Takeda G-protein-coupled receptor 5 (TGR5) and G-protein-coupled receptor 119 (GPR119) agonist, inducing glucagon-like peptide (GLP-1) secretion and lowering blood glucose levels in type 1 diabetic rats through activation of TGR5 and GPR119 (105).

Chronic prostatitis (CP) is a common and intractable genitourinary chronic inflammatory disease in young and middle-aged men, characterized by slow onset, stubbornness, recurrent episodes, and intractability. The autoimmune response caused by

the imbalance of CD4+ T cell differentiation was found to be an important etiological factor of CP. 4-methylumbelliferone could reduce the severity of experimental autoimmune prostatitis (EAP) in experimental EAP mice by significantly decreasing the proportion of Th1 cells (106).

5 Coumarins targeting various signaling pathways

Coumarin-like chemicals are notable for their anti-tumor properties. In recent years, accumulated studies have shown that coumarins exhibit promising immune regulatory effects in AIDs. Each type of coumarin targets different immune cells, thus triggering a large number of different intracellular signaling pathways, ultimately regulating the host's immune response. The modulation of several signaling pathways leads to alterations in the expression of pro-inflammatory genes, which ultimately lead to an improvement in immune environment. To date, most of the mechanistic studies have been conducted in animal experiments. Many mechanistic studies have been conducted in animal and cell experiments. The action of coumarins in AIDs are summarized in Figure 3 and Table 1.

5.1 Mitogen-activated protein kinases (MAPKs) pathway

The family of MAPKs includes several subfamilies such as c-Jun n-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinases (ERK), which can regulate proliferation, differentiation, apoptosis, or survival, cellular activities such as inflammation and innate immunity (107, 108). There is connectivity and relative independence between different signaling pathways in the MAPK family. The c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 kinase are activated by environmental stress and inflammatory signals, whereas extracellular signal-regulated kinase (ERK1/2/5) is mainly activated by growth factor receptors and some cytokine receptors. Once activated, MAPK will phosphorylate different proteins, acting as other kinase translation regulators and transcription factors, leading to cellular responses (109). Signals can be transmitted from the cell surface to the nucleus by activated MAPK to increase the expression of relevant inflammatory genes and promote the secretion of a variety of inflammatory factors, such as COX-2, PGE2, Monocyte chemoattractant protein-1 (MCP-1), IL-1β, IL-6, and TNF-α (110). Several studies have reported the inhibitory effects of coumarins on JNK, ERK1/2, and p38. These inhibitory effects lead to a reduction in the expression and release of pro-inflammatory mediators (IL-1β, IL-6, COX-2, MCP-1, e.g.). For example, osthole inhibited the expression of p38 MAPK, COX-2, inducible nitric oxide synthase (iNOS), and IκB α in LPS-induced RAW 264.7 cells and decreased the levels of NO, PGE2, TNF-α, and IL-6. On this basis, in DSS-induced UC mice, osthole decreased the expression of NF-κB p65 and p-IκB α in colonic tissues (111). Similarly, osthole significantly inhibited the phosphorylation of p38, which was induced by 2,4,6-Trinitrobenzenesulfonic acid (TNBS) in mice or by LPS in Raw264.7 cells, and strongly inhibited IL-1β, IL-6, COX-2, and MCP-1. Interestingly, the inhibition by protein kinase A (PKA) partially reversed the suppressive effects of osthole on p38 phosphorylation in LPS-stimulated cells (112). In endometriotic animal models and cells (End1/E6E7 and VK2/E6E7), fraxetin reduced endometriotic lesions by inhibiting P38/JNK/ERK phosphorylation, inducing apoptosis, and generating

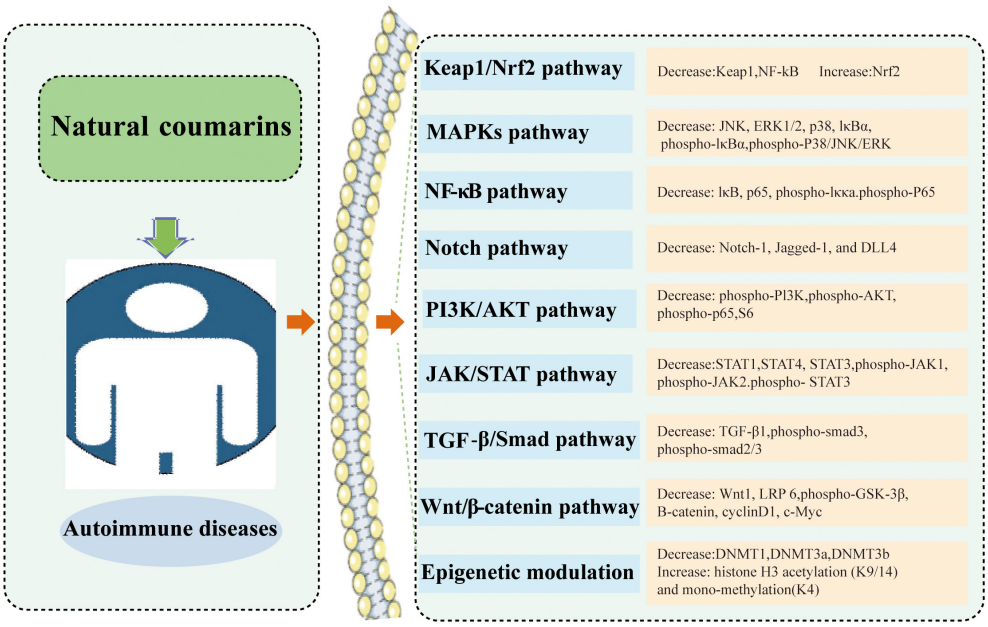

FIGURE 3
The main point of action of coumarins in autoimmune diseases.

TABLE 1 Coumarins targeting signaling pathways.

Signaling Pathways	Coumarins	Actions	References
MAPKs pathway	Osthole	Inhibiting the phosphorylation of IκB α and p38 pathway proteins alters pro-inflammatory cytokine production and expression	(111, 112)
	Fraxetin, imperatorin, plumbagin, daphnetin, esculetin	Suppressed the phosphorylation of ERK, JNK, AKT and p38 pathway proteins	(113–115, 117–119)
	4-methylumbelliferone	inhibiting ERK1/2 signaling and increasing the number of $Foxp3^+$ T cells in an ERK1/2-dependent manner	(116)
	Osthole	Regulating the NLRP 3 inflammasome by activation of AMPK	(120)
TGF-β/Smad signaling pathway	Umbelliferone, esculetin	downregulated TGFβ1 and p-smad2/3 levels, while inhibiting nuclear translocation of NF-κB p65 and increasing Nrf 2 protein levels	(127, 128, 130)
	Esculetin	Reduced levels of TGF-β 1 and fibronectin	(129)
NF-κB signaling pathway	Umbelliferone, plumbagin, daphnetin, scopoletin, osthole, imperatorin, esculetin	Modulates NF-κB signaling pathway and reduces levels of pro-inflammatory cytokines, dendritic cells, and NLRP3 inflammasome.	(136–142, 147–149)
PI3K/AKT signaling pathway	Imperatorin	inhibits activation of the PI3K/AKT/NF-κB pathway	(153)
	Fraxetin	reduced phosphorylation of AKT and S6 levels and S6 protein levels	(113)
	Umbelliferone	inhibits AKT phosphorylation to prevent bone loss and suppresses osteoclastogenesis	(154)
Keap1/Nrf2 signaling pathway	Columbianadin	regulates the Keap1/Nrf2 signaling pathway and inhibits NF-κB activation	(157)
	Imperatorin	regulates the expression of Nrf-2, ARE and HO-1, thereby inhibiting pro-inflammatory cytokine secretion	(158)
	Esculetin	inhibiting complement activation and enhancing Nrf2 signaling pathway	(128)
	Umbelliferone	activating Nrf2 signal transduction	(159)
	Esculetin	decreases the expression of Keap1	(160)
Wnt/β-catenin signaling pathway	Umbelliferone	reduces the levels of pathway-associated proteins (Wnt1, LRP6, p-GSK-3β, β-catenin, cyclin D1, and c-Myc) and inhibits β-catenin nuclear translocation	(163, 164)
JAK/STAT signaling pathway	Plumbagin, daphnetin	inhibits the phosphorylation of STAT1, STAT3, and STAT4, as well as the upstream kinases JAK 1 and JAK 2	(170, 171)
	Colombianadin	inhibited the JAK1/STAT3 pathway to attenuate the inflammatory response and regulated the NF-κB pathway and Keap1/Nrf2 pathway	(157)
Epigenetic modulation	Umbelliferone	Activation of SIRT1 resulting in the inhibition of NF-κB, TLR4 and iNOS	(177)
	Osthole	Downregulates n6 -methyladenosine-modified TGM2 and attenuates the NF-κB signaling pathway	(179)
	Daphnetin	reduces gene expression of the methyltransferases DNMT1, DNMT3a and DNMT3b and demethylates the proapoptotic genes PDCD5, FasL, DR3 and p53	(178)
	Esculetin	restores histone H3 acetylation (K9/14) and mono-methylation (K4)	(129)
	Osthole	increases miR-1224-3P expression and decreases AGO1 expression and inhibits pro-inflammatory cytokine levels	(181)
Notch signaling pathway	Agrimonolide	decreasing the mRNA and protein levels of Notch-1, Jagged-1, and DLL4 and inhibiting the phosphorylation of JAK2 and STAT3	(186)

reactive oxygen species (ROS) (113). Imperatorin was able to attenuate symptoms associated with a mouse model of psoriasiform dermatitis by inhibiting the phosphorylation of ERK, JNK, and AKT. Meanwhile, the inhibitory effects of imperatorin on cell responses and signaling could be reversed by a PKA inhibitor, suggesting that cAMP/PKA is involved in the anti-inflammatory effects of imperatorin (114). In addition, inhibition of p38 activation by imperatorin has been reported (115). 4-methylumbelliferone (4-MU) inhibits ERK 1/2 signaling and increases the number of Fox P3+ T cells in an ERK1/2-dependent manner, thereby inhibiting hyaluronan synthesis to restore immune tolerance in autoimmune insulitis (116). Additionally, other coumarins, such as plumbagin, daphnetin, and esculetin have been shown to reduce inflammation by interfering with the MAPKs pathway (117–119).

It is noteworthy that the beneficial effects of coumarins may also be associated with increased signaling in the AMPKs pathway. For example, the anti-RA activity of osthole requires the involvement of AMPK phosphorylation activation. Osthole can regulate NLRP3 inflammasome by activating AMPK. This result was also reverse-validated by the experimental application of the AMPK inhibitor compound C, which blocked the activation of AMPK by osthole and also attenuated the positive effect of osthole on inflammasome activation, which was manifested as increased protein levels of NLRP3, CAS1, ASC and IL-1β (120).

5.2 Transforming growth factor beta/small mother against decapentaplegic (TGF-β/Smad) signaling pathway

The TGF-β/Smad signaling pathway is involved in many cellular processes (121). TGF-β is a multifunctional cytokine consisting of three isoforms, TGF-β1, TGF-β2, and TGF-β3, which is widely expressed in different types of cells and tissues, with TGF-β1 being the major isoform. TGF-β can negatively regulate immune cell proliferation, differentiation, and activation and plays an important role in suppressing immunity and inflammation (122, 123). Smad proteins, on the other hand, are signal transducers of intracellular TGF-β and mediate most of the functions of TGF-β (124). One of the mechanisms by which coumarins can modulate the immune response is through direct inhibition of the TGF-β/Smad signaling pathway. During diabetes, the expression of TGF-b is increased in the kidney, which leads to further deterioration of nephropathy (125). The circulating level of TGF-b1 is one of the important markers for predicting diabetes-related renal injury (126). Umbelliferone reduces Renal damage in type 1 diabetic rats by decreasing the levels of TGF-β1 in Renal tissue and circulation (127). In the MRL/lpr mouse model, esculetin significantly down-regulated the levels of TGFβ1 and p-smad3 in renal tissues, as well as significantly inhibited the nuclear translocation of NF-κB p65 and increased the level of Nrf2 protein in the nucleus, which had a significant therapeutic effect on murine lupus nephritis (128). In addition, esculetin treatment protects against the increase in expression of TGF-β1and fibronectin in type I diabetic rat kidney and hence shows efficacy in attenuating glomerulosclerosis (129). Another study reported that umbelliferone and esculetin could inhibit the activation of TGF-smad signal, which showed that they could down-regulate the secretion of fibronectin in HK2 cells stimulated by TGF-β1 and inhibit smad2/3 phosphorylation, thus playing a beneficial role in rats with type 1 diabetic nephropathy (130).

5.3 Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway

NF-κB is an important intracellular transcription factor that regulates the expression of a wide range of genes and plays a key regulatory role in a variety of biological processes including inflammatory response, cell proliferation, apoptosis, and cell infiltration (131). NF-κB is phosphorylated by IκB kinase upon cellular stimulation by chemical or mechanical signals and subsequently degraded via the ubiquitin-proteasome system. After IκB degradation, NF-κB dimers detached from IκB are activated by translation and enter the nucleus to participate in transcription (132–134). Activated NF-κB regulates the production of inflammatory factors such as TNF-α, COX-2, and PGE2 in the nucleus, and participates in and mediates a variety of immune responses and inflammatory reactions in the body (135). Different coumarins including umbelliferone, plumbagin, daphnetin, scopoletin, osthole, imperatorin, and esculetin all inhibit NF-κB pathways. In FLS of RA, umbelliferone and scopoletin counteract RA by binding to and inhibiting tyrosine kinases in RA-FLS and subsequently inhibiting NF-κB (136). Furthermore, umbelliferone ameliorates RA induced by complete Freund's adjuvant by inhibiting the NF-κB signaling pathway in osteoclast differentiation (137). In a mouse model of EAE, scopoletin attenuates DCs activation through inhibition of the NF-κB signaling pathway and significantly reduces central nervous system (CNS) inflammation and demyelination in EAE mice (138). The inhibitory effect of daphnetin on NF-κB activation has been reported in various autoimmune disease models (psoriasis mice, EAE mice, NZB/WF1 SLE mice) (139–141). Plumbagin has been shown to reduce the levels of TNF-α, IL-6 and matrix metalloproteinases (MMPs) in RA mouse cells by inhibiting NF-κB activation, and its mechanism of action is related to the inhibition of IκB and NF-κB activation as well as the entry of p65 into the cell nucleus (142). Furthermore, in patients, IL-1β plays a pathogenic role in the evolution of IgA nephropathy (143), and serum levels of IL 18 are elevated in IgA nephropathy patients (144). Mature IL-1β and IL-18 are produced by active caspase-1 from NLRP3 inflammasome from their respective precursors pro- IL-1β and pro IL-18 (145, 146). In a mouse model of progressive IgA nephropathy, osthole blocked the activation of NF-kB and NLRP3 inflammasome, thereby improving renal function and blocking progressive renal lesions (147). IL-1β A study observing the effects of esculetin on skin inflammation in psoriasis mice found that esculetin inhibited the activation of the NF-κB signaling pathway, including inhibiting the phosphorylation of IKKα and P65 in psoriatic skin (148). In addition, the inhibitory effect of imperatorin on NF-κB has been reported in mice with UC (149).

5.4 Phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway

The PI3K/AKT pathway is an important signaling pathway in the body, consisting of two protein kinases, PI3K and AKT, which are involved in the phosphorylation of NF-κB p65 and nuclear translocation, and contribute to the production of inflammatory mediators (150, 151). Overall, the PI3K/AKT pathway activates the signaling pathway upon stimulation of the corresponding upstream signals, which in turn directs the downstream signaling substances as well as the cytosolic nucleus to make the corresponding response, and further regulates the phenomena of cell autophagy, apoptosis, and inflammation release, which ultimately affects the development of diseases (152). It is hypothesized that the beneficial effects of coumarins may be related to the inhibition of signaling pathways in the PI3K/AKT pathway. It was confirmed that imperatorin significantly inhibited the activation of the PI3K/AKT/NF-κB pathway by inhibiting the phosphorylation levels of PI3K, AKT, and p65 in the ectopic endometrium tissue, thereby significantly inhibiting the growth and ameliorate the histopathological features of ectopic endometrium in experimental endometriosis rats (153). Another study reported that fraxetin significantly reduced phosphorylation of AKT and S6 levels and S6 protein levels in End1/E6E7 and VK2/E6E7 cells (endometriotic epithelial cell lines) (113). Furthermore, umbelliferone prevents LPS-induced bone loss and inhibits RANKL-induced osteoclastogenesis by inhibiting AKT phosphorylation (154).

5.5 Kelch-like-ech-associated protein 1-nuclear factor E2-related factor 2 (Keap1/Nrf2) signaling pathway

Kelch-like-ech-associated protein 1 (Keap1)-nuclear factor E2-related factor 2 (Nrf2) pathway is closely related to oxidative stress and inflammation in various organs and systems of the body, and is considered as the therapeutic target of many organ protection. Nrf2 is the main regulator of cell antioxidant response, and its activity is precisely regulated by the negative regulatory protein Keap1. The antioxidant effect of Nrf2 was inhibited by the interaction with Keap1 (155, 156). The imbalance of Keap1/Nrf2 transcription activity is related to the pathogenesis of many diseases. Keap1/Nrf2 axis has become the most important regulator of intracellular homeostasis and plays an important role in the occurrence and development of many chronic diseases. Some studies have reported the regulatory effects of coumarin on Nrf2 and Keap1. For example, in the collagen-induced RA mouse model, columbianadin can play an anti-RA role by regulating inflammation and oxidative stress, and its mechanism includes inhibiting the expression of Keap1 at mRNA and protein levels, increasing the expression of Nrf2 mRNA in CIA mice, regulating Keap1/Nrf2 signaling pathway in CIA mice, and inhibiting the activation of NF-κB (157). In addition, imperatorin has been proven to interfere with the expression of Nrf2 in the colon of rats with UC induced by TNBS, and inhibit the secretion of TNF-α and IL-6 by regulating the expressions of Nrf-2, ARE and HO-1, thus alleviating the symptoms of UC (158). Umbelliferone can also alleviate UC induced by DSS by inhibiting inflammation, which is related to activating Nrf2 signal transduction (159). Other studies have reported that esculetin can treat lupus nephritis in mice by inhibiting complement activation and enhancing Nrf2 signaling pathway (128). In addition, the inhibition of esculetin on Keap1 activation has also been reported. In a study, it was reported that esculetin can reduce the expression of Keap1 in aorta of hyperinsulinemia combined with T1DM rats, and has a protective effect on vascular function (160).

5.6 Wngless-type/beta-catenin (Wnt/β-catenin) pathway

The Wnt/β-catenin signaling pathway, also known as the Canonical Wnt signaling pathway, is a conserved signaling axis (161). The Wnt/β-catenin pathway consists of four segments: the extracellular signaling, membrane segment, cytoplasmic segment, and nuclear segment. Extracellular signaling is mainly mediated by Wnt proteins, among which are Wnt3a, Wnt1, and Wnt5a. The cytosolic fragment mainly contains the Wnt receptor Frizzled and low-density lipoprotein receptor-related protein (LRP5/6). The cytoplasmic fraction mainly consisted of β-catenin, Dishevelled (DVL), glycogen synthase kinase-3β (GSK-3β), AXIN, adenomatous polyposis coli (APC) protein, and casein kinase-1 (CK-1). Nuclear segments mainly include β-catenin translocated to the nucleus, T-cell factor/lymphoid enhancer factor family (TCF/LEF), and β-catenin downstream target genes such as MMPs and c-Myc (162). Coumarins can alter the Wnt/β-catenin pathway along multiple steps in the signaling cascade. Umbelliferone reduces Wnt1 protein levels, activates GSK-3β kinase by blocking GSK-3β (Ser9) phosphorylation, and reduces the protein level and nuclear translocation of β-catenin (163). Furthermore, in FLS from RA rats, Umbelliferone could reduce the activation of the Wnt/β-catenin pathway by restoring GSK-3β activity, reducing the levels of pathway-associated proteins (e.g., Wnt1, LRP6, p-GSK-3β (Ser 9), β-catenin, cyclin D1, and c-Myc), and inhibiting β-catenin nuclear translocation (164).

5.7 Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway

The JAK-STAT pathway is a signaling pathway from the cell membrane to the nucleus and is critical in apoptosis, proliferation and differentiation, body immune function, and inflammatory response (165, 166). The JAK-STAT pathway consists of JAK-associated receptors, JAK, and STAT (167). Among them, the Janus kinase family is a class of non-receptor-type protein tyrosine kinases including JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2) (168). STAT is a class of cytosolic proteins, located downstream of JAK, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 (169). These molecules contribute to the inflammatory process and, by inference, their inhibition represents a therapeutic target for the reduction of inflammation. Thus, the mechanism by which coumarins can exert

immunomodulatory effects may be the inhibition of these molecules. Plumbagin significantly inhibited the phosphorylation of STAT1, STAT4, and STAT3, as well as the upstream kinases JAK1 and JAK2, resulting in a reduction in the number of CD4+ T-lymphocytes and pro-inflammatory cytokines in mice with experimental autoimmune encephalomyelitis, which ameliorated the locomotor dysfunction and body weight loss of mice (170). Similar results were observed in LPS-induced Caco-2 cells, where daphnetin inhibited the phosphorylation of JAK2 and STAT3 proteins (171). In addition, in mice models of CIA, colombianadin was able to exert anti-RA effects by modulating immune and inflammatory responses, and its mechanism of action included decreasing the phosphorylation levels of JAK1 and STAT3 in the ankle joints of mice with CIA as well as the STAT3 mRNA expression, suggesting that colombianadin attenuates inflammatory responses by inhibiting the JAK1/STAT3 pathway. It is worth mentioning that columbianadin also inhibited the protein expression of P65, P50, and phosphorylated IκBα in the ankle joints of mice, inhibited the expression of Keap1 at the mRNA and protein levels, and increased the expression of Nrf2 at the mRNA level in CIA mice (157).

5.8 Epigenetic modulation

More and more studies show that epigenetic modification can regulate the inflammatory response and immune response through DNA, histone, transcriptional, and post-transcriptional levels (44, 172). Indeed, a series of studies have reported the existence of coumarin-induced epigenetic modifications leading to gene activation or silencing in the absence of changes in DNA sequence (173–176). A novel point of coumarins in cellular control is their ability to modulate modular epigenetic mechanisms such as DNA methylation, histone modifications, and posttranscriptional regulation of microRNAs, thereby regulating immune cell activation and differentiation. Among various coumarins, umbelliferone has been shown to be a strong activator of Silent information regulator 1 (SIRT1), leading to down-regulation of gene and protein expression of TLR4, NF-κB, and iNOS signaling factors, as well as decreasing the levels of TNF-α, IL-6, MPO, and VCAM-1 in the colon, resulting in a potent anti-inflammatory effect in acetic acid-induced UC rats (177). It is reported that esculetin can attenuate the decrease in histone H3 acetylation (K9/14) and mono-methylation (K4) in the kidney of rats with type I diabetic nephropathy induced by streptozotocin (STZ) (129). In addition, daphnetin had a demethylating effect on the proapoptotic genes PDCD5, FasL, DR3, and p53 in CIA rat synovial cells, and decreased the gene expression of the methyltransferases DNMT1, DNMT3a, and DNMT3b (178). In a study, osthole downregulated n6-methyladenosine-modified TGM2 to exert its additive effect with methotrexate and suppress the proliferation, migration, and invasion of RA-FLSs by attenuating NF-κB signaling pathway, resulting in the suppression of RA progression (179).

MicroRNAs are small and non-coding regulatory RNAs that can regulate the translocation and/or degradation of messenger RNAs (180). The regulatory effect of coumarin on microRNAs was also reported. It is reported that osthole can increase the expression of microRNA-1224-3p (miR-1224-3p) and decrease the expression of AGO1 in HUM-iCell-s010 RA cells, and decrease the levels of IL-6 and IL-1β in these cells. This discovery suggests that osthole may have the potential to treat RA by regulating the expression of miR-1224- 3 P and AGO 1 and reducing the level of proinflammatory cytokines (181).

5.9 Other pathways

The Notch signaling pathway is a conserved and important mechanism for maintaining immune homeostasis by regulating cell differentiation and modulating inflammation (182). In mammals, the pathway includes ligands (e.g., Jagged1, Jagged2, Delta1, Delta3, and Delta4), Notch receptors (Notch1-4), and downstream signaling components (183, 184). Aberrant activation of the Notch signaling pathway disrupts Th17/Treg cell homeostasis (185). Agrimonolide was able to correct the imbalance of Th17/Treg cells by significantly decreasing the mRNA and protein levels of Notch-1, Jagged-1, and DLL4, as well as inhibiting the phosphorylation of JAK2 and STAT3, which effectively attenuated the symptoms of weight loss and hematochezia, decreased the expression of inflammatory cytokines, and repaired intestinal mucosal barrier in UC mice (186). The Hedgehog (HH) pathway is critical for embryonic development and homeostatic maintenance of many adult tissues and organs. It is also associated with certain functions of the innate and adaptive immune system (187). HH, including sonic hedgehog (SHH), Indian hedgehog (IHH), and desert hedgehog (DHH) (188). FLSs are the main effector cells responsible for synovitis and joint destruction in RA. Studies have shown that the SHH signaling pathway is involved in the aberrant activation of RA-FLSs, and inhibition of the SHH pathway reduces the proliferation and migration of RA-FLSs (189). Therefore, the Hedgehog signaling pathway may be one of the pathways of coumarins for the treatment of autoimmune diseases, and it also provides a reference for the further development and utilization of coumarins.

6 Conclusions

Therapeutic agents available for the treatment of AIDs are limited, and there are certain shortcomings, such as dosage, route of administration, and bioavailability. Some therapeutic drugs have varying degrees of side effects. Given these limitations, we reviewed various coumarins that shown promising efficacy in AIDs such as T1DM, UC, SLE, RA, MS. Coumarin can regulate inflammatory cytokines such as IL-4, IL-6, IL-10, IL-17, IL-23, TNF-α, and IFN-γ, as well as related signaling pathways in immune cells, including JAK-STAT, Wnt/β-catenin, PI3K-AKT, TGF-β/Smad, MAPKs, Keap1/Nrf2, Notch and NF-κB pathways. The review provides new evidence for the discovery of effective and safe new drugs for AIDs.

Author contributions

YL: Visualization, Writing – original draft. G-QW: Resources, Supervision, Writing – review & editing. Y-BL: Funding acquisition, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Ce Wang,
AbbVie, United States

REVIEWED BY
Takashi Yamashita,
The University of Tokyo, Japan
Siqi Sun,
Rutgers, The State University of New Jersey, United States

*CORRESPONDENCE
Yunlu Gao
gaoyunlu@hotmail.com

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Case report: Abrocitinib: a potential therapeutic option for lichen amyloidosis associated with atopic dermatitis

Yizhen Zhang[1,2], Dawei Huang[1,2] and Yunlu Gao[1,2*]

[1]School of Medicine, Tongji University, Shanghai, China, [2]Department of Dermatology, Shanghai Skin Disease Hospital, Shanghai, China

Lichen amyloidosis (LA) is a predominant type of primary cutaneous amyloidosis that is characterized by persistent and intense skin itching. Although multiple therapeutics strategies are available for its treatment, there is no standard treatment so far. Abrocitinib, an oral small-molecule Janus kinase 1 inhibitor, has been authorized for the treatment of severe atopic dermatitis (AD) and can also provide rapid relief from pruritus. Here, we discuss the case of a 32-year-old man who was diagnosed with LA with severe AD based on the presence of multiple, discrete, and hyperpigmented papules and pruritic, erythematous macules with lichenification of the limbs, trunk, and buttocks. Given the inefficacy of conventional therapy, abrocitinib treatment was recommended in this patient. After 1 month of treatment, the patient's Eczema Area And Severity Index score decreased significantly from 48 to 15 points, accompanied by a notable reduction in pruritus symptoms. Furthermore, significant improvements were observed in the thickness and pigmentation of the hyperkeratotic papules. Thus, abrocitinib exhibited excellent effectiveness and safety in the treatment of severe AD with LA and warrants further investigation for its potential therapeutic benefits.

KEYWORDS

abrocitinib, atopic dermatitis, lichen amyloidosis, small molecule drugs, case report

1 Introduction

Lichen amyloidosis (LA) is a form of primary cutaneous amyloidosis that is characterized by numerous, itchy, hyperpigmented, and hyperkeratinized papules that appear on the anterior shins, lateral arms, and lower back (1). LA is a feature of a variety of skin diseases that eventually lead to pruritus, including atopic dermatitis (AD) (2). Although several therapeutic approaches have been proposed for the treatment of LA, such as topical and intralesional

corticosteroids, oral antihistamines, systemic cyclosporine, and UV radiation, none of them have demonstrated consistency in their effectiveness (2–4).

Abrocitinib, a small-molecule inhibitor of Janus kinase 1 (JAK1), modulates multiple cytokine pathways involved in several inflammatory cutaneous diseases. It is authorized for the treatment of moderate to severe AD and also provides rapid relief from pruritus (5, 6). Because LA might be related to the onset of AD, small-molecule drugs for AD are currently being used as novel therapeutic agents for the treatment of LA with promising results. Here, we describe a case of LA associated with severe AD in which the patient responded well to treatment with abrocitinib after conventional therapy was ineffective.

2 Case report

A 32-year-old man with a 20-year history of refractory AD presented to our department with severe, widespread uncontrollable pruritus. Physical examination showed typical chronic eczema-like changes in the form of numerous, discrete, and hyperpigmented papules on the back, neck, and lower legs that were particularly prominent on the lower legs (Figures 1A, C, E). Dryness, scaling, pigmentation, and irregularly shaped lichenoid plaques due to repeated scratching were observed on the neck and upper back, areas that are easily accessible to the patient (Figure 1A). A punch biopsy of the right arm revealed the presence of a uniform eosinophilic substance in the papillary dermis that covered the hyperplastic epidermis, and Congo red staining demonstrated a substantial accumulation of fibrous amyloid deposits located beneath the dermal-epidermal junction, representing a characteristic manifestation of LA (Figures 2A, B). Additionally, immunofluorescence analysis indicated that the expression of Interleukin-31 (IL-31) in the skin of this patient was significantly elevated compared to that in a pathological section of normal skin (Figures 2C, D). Microscopic examination of the underlying causes of itching, including scabies, yielded negative results. According to the history and typical clinical features, the patient was diagnosed with LA with severe AD [Eczema Area and Severity Index (EASI) = 48]. Topical corticosteroids and antihistamines were not considered because they had previously failed to alleviate patient's discernible clinical manifestations of AD and LA. In addition, the use of duplizumab was contraindicated because of the patient's history of allergic conjunctivitis. Therefore, treatment with abrocitinib was recommended. The patient was administered 100 mg of abrocitinib orally once daily, which resulted in alleviation of intractable pruritus within 2 days of treatment. Within 2 weeks, the patient experienced relief from unbearable itching, and the severity of pruritus decreased from 10 points on the Numeric Rating Scale to only 1 point. After 1 month of treatment, the patient's EASI score significantly decreased from 48 to 15. This was accompanied by a decrease in the size and number of plaques on the back and lower legs, significant flattening of the LA papules, and gradual easing of the desquamation (Figures 1B, D, F). So far, this patient has been on continuous 3-month course of abrocitinib with no recurrence of pruritus and sustained clinical benefit. The patient has not exhibited any of the known side effects of abrocitinib, such as infection, transaminase abnormalities, cardiotoxicity, and skeletal dysplasia.

3 Discussion

LA is a chronic pruritic type of cutaneous amyloidosis that results from extracellular deposition of amyloid-derived intermediate filaments in the dermis, and the itch-scratch cycle plays a crucial role in its pathogenesis. Although various conventional treatments, such as topical corticosteroids, oral antihistamines, and systemic cyclosporine, have been used for LA with AD, these treatments are frequently unsatisfactory or provide

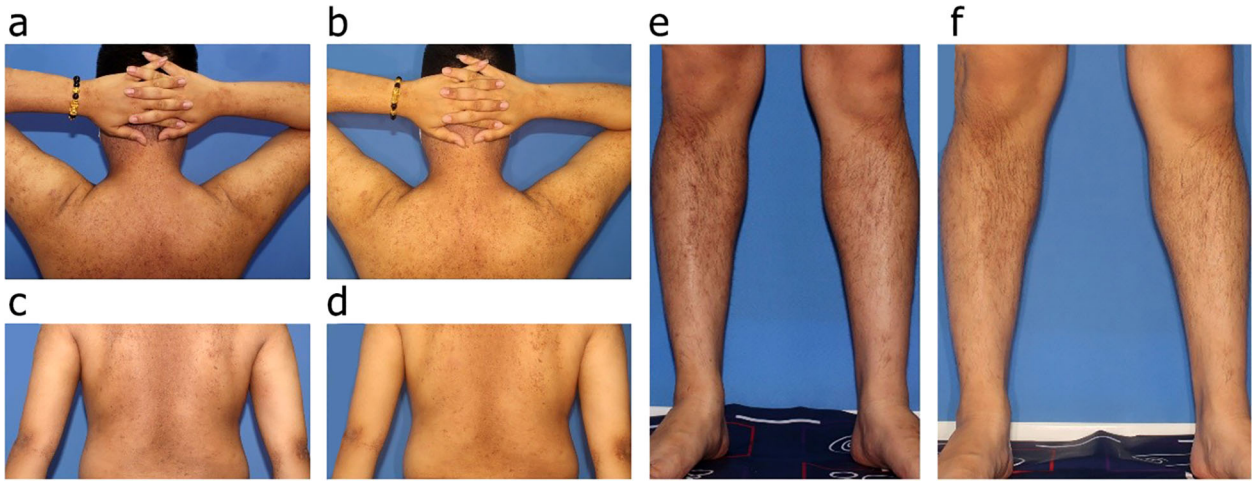

FIGURE 1
(A, C, E) Clinical images of the patient with LA associate with AD before abrocitinib treatment, illustrating multiple, discrete, firm, and hyperpigmented papules present on the extensor surfaces of the limbs, trunk, and buttocks. **(B, D, F)** Clinical images of the patient 4 weeks after treatment with abrocitinib, demonstrating that all papules had flattened and exhibited a reduction in both size and number.

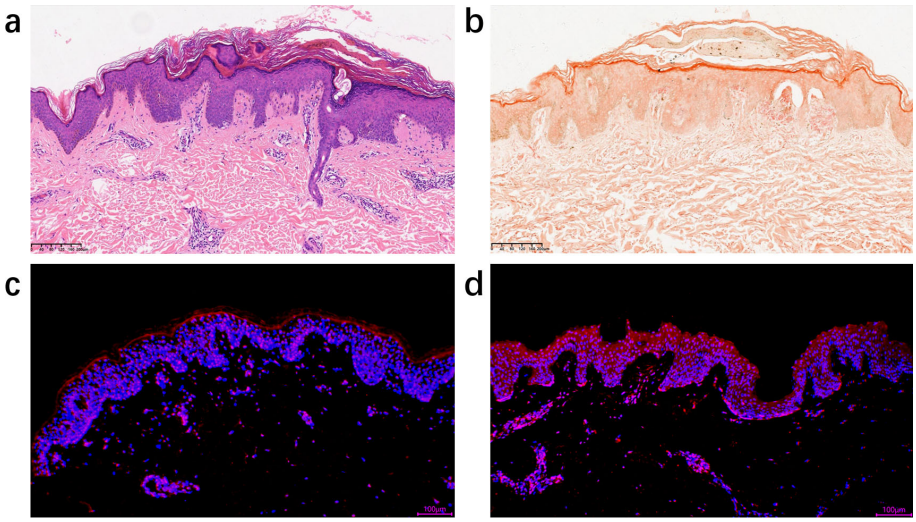

FIGURE 2
(A) Histopathological changes consistent with LA, uniform eosinophilic substance in the papillary dermis that covered the hyperplastic epidermis (hematoxylin-eosin; 10×). **(B)** A substantial accumulation of fibrous amyloid deposits is observed beneath the dermal-epidermal junction, which stain orange with Congo red. Notably, distinct fissures are present between these deposits (10×). **(C)** Immunofluorescence staining of IL-31 expression in a pathological section of normal skin (10×). IL-31 were stained in red, whereas nuclei were stained in blue 4',6-diamidino-2-phenylindole (DAPI). **(D)** Immunofluorescence staining of IL-31 expression in a pathological section of the patient in this case. IL-31 infiltrated in the epidermis of the patient and expressed at significantly higher levels compared to that in normal human skin tissues.

relief from the itching without regression of the lesion (7). Recently, successful treatment of a few cases of LA has been reported with small-molecule drugs and biological agents such as dupilumab, upadacitinib, and baricitinib (8–10). For example, Dengmei et al. (10) reported that, in a patient with refractory LA coexisting with AD, the LA lesions significantly improved and eczema-like lesions and pruritus rapidly resolved after 16 weeks of baricitinib treatment. Further, Qingzhu et al. (11) reported four cases of refractory LA with AD that were treated with dupilumab after conventional therapy had proved ineffective. After 16 weeks of treatment with the biologic agent, reduction in the number of skin lesions and improved quality of life were observed in all the patients, with no significant adverse effects. In contrast to the previous case, in our case, the use of the biological agent dupilumab was contraindicated because of the patient's history of allergic conjunctivitis. Therefore, abrocitinib, a small-molecule drug also approved for the treatment of AD, was used in this case, with excellent therapeutic results.

Abrocitinib, a selective JAK1 inhibitor, affects epidermal barrier modulation and peripheral nerve modulation involved in pruritus transduction by targeting the JAK- signal transducer and activator of transcription (STAT) signaling pathway (12). Although the pathogenesis of LA is unclear, IL-31, one of the cytokines involved in the JAK-STAT signaling pathway, is considered to be a central mediator of T-cell–mediated pruritus. Furthermore, several articles have indicated that pruritus in LA may be correlated with the hypersensitivity of dermal nerve fibers, which are linked to IL-31 receptors in the epidermis (13). JAK1 inhibitors are known to directly affect T-cell function; moreover and importantly, blockade of IL-31 and IL-4 signaling by these inhibitors in primary afferent sensory neurons can influence inflammation and neurosensory pathways (14, 15). In our case, significant increases in the expression of IL-31 associated with pruritus were observed in the patient's skin, with pruritus being observed in the patient's skin, with rapid relief of itching following treatment with abrocitinib. Thus, the mechanism of abrocitinib in LA may involve reduction in the levels of the inflammatory cytokine IL-31 and repair of the damaged skin barrier.

The observations of this case report indicate that abrocitinib could be a promising therapeutic agent for the treatment of LA in patients with AD. However, more cases are required to assess the long-term efficacy of small-molecule drugs and their sustained effects after withdrawal. In addition, future studies need to focus on the mechanism of abrocitinib in relieving the pruritus associated with LA, as this may provide deeper insight into LA therapy.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee of Shanghai skin disease hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YZ: Writing – original draft. DH: Writing – review & editing. YG: Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Asif Amin Dar,
Children's Hospital of Philadelphia,
United States

REVIEWED BY
Gerson D. Keppeke,
Universidad Católica del Norte, Chile
Saeed Mohammadi,
Golestan University of Medical Sciences, Iran

*CORRESPONDENCE
Maria Grazia Roncarolo
mgroncarolo@tr1x.bio

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From promise to practice: CAR T and Treg cell therapies in autoimmunity and other immune-mediated diseases

Yannick Bulliard[1], Robert Freeborn[1], Molly Javier Uyeda[1], Daryl Humes[1], Ryan Bjordahl[1], David de Vries[1] and Maria Grazia Roncarolo[1,2]*

[1]Department of Research and Development, Tr1X, Inc., San Diego, CA, United States, [2]Division of Hematology, Oncology, Stem Cell Transplantation, and Regenerative Medicine, Department of Pediatrics, Stanford University School of Medicine, Stanford, CA, United States

Autoimmune diseases, characterized by the immune system's attack on the body's own tissues, affect millions of people worldwide. Current treatments, which primarily rely on broad immunosuppression and symptom management, are often associated with significant adverse effects and necessitate lifelong therapy. This review explores the next generation of therapies for immune-mediated diseases, including chimeric antigen receptor (CAR) T cell and regulatory T cell (Treg)-based approaches, which offer the prospect of targeted, durable disease remission. Notably, we highlight the emergence of CD19-targeted CAR T cell therapies, and their ability to drive sustained remission in B cell-mediated autoimmune diseases, suggesting a possible paradigm shift. Further, we discuss the therapeutic potential of Type 1 and FOXP3$^+$ Treg and CAR-Treg cells, which aim to achieve localized immune modulation by targeting their activity to specific tissues or cell types, thereby minimizing the risk of generalized immunosuppression. By examining the latest advances in this rapidly evolving field, we underscore the potential of these innovative cell therapies to address the unmet need for long-term remission and potential tolerance induction in individuals with autoimmune and immune-mediated diseases.

KEYWORDS

Tr1, Treg, autoimmunity, CAR T, cell therapy, Tr1 cells

1 Introduction to autoimmune and other immune-mediated diseases

1.1 Overview of autoimmune diseases and current treatment approaches

Autoimmune and inflammatory diseases, which encompass a wide range of disorders with complex pathology and etiology, affect between 5-10% of the population in industrialized regions, and their burden continues to increase (1). Although heterogeneous, autoimmunity generally arises from a combination of genetic predisposition and environmental co-factors leading to initiation and potentiation of attacks by the body's own immune cells, specifically autoreactive T and B cells (2). Conventional first-line approaches for the treatment of autoimmune diseases have historically involved either the suppression of general immune function to modulate uncontrolled inflammation via corticosteroids or immunosuppressants such as azathioprine and methotrexate, or the use of disease-modifying anti-rheumatic drugs (DMARDs). This broad, non-specific efficacy, combined with lifelong treatment requirements, leads to frequently intolerable side effects, including increased risk of infection due to continued immunosuppression (3). More recently, biologics focused specifically on certain components of the immune system, such as cytokines or cell surface proteins, have been applied successfully to these types of diseases. Similar to steroids and DMARDs, these newer biologics and small molecules, which include TNF-alpha inhibitors, other monoclonal antibodies, and JAK or TYK2 inhibitors, target downstream inflammatory mediators but generally do not address the underlying disease drivers. Unfortunately, many patients eventually experience primary treatment failure or lose response to these therapies over time due to the development of anti-drug antibodies (4).

Autoimmune diseases are largely characterized by an aberrant immune response directed against self-antigens, often mediated by dysregulated T and B lymphocytes. T cells can inflict direct tissue damage or orchestrate inflammatory cascades via cytokine release (5), while B cells contribute to pathology through both production of pro-inflammatory cytokines and through production of autoantibodies, which can bind to self-antigens on tissues or form immune complexes leading to complement activation and inflammation (6). Therapeutic strategies aimed at depleting these pathogenic lymphocytes seek to interrupt the self-enhancing damage they cause and have demonstrated clinical success across several indications.

1.2 Dual B and T cell targeting: mechanistic rationale to treat autoimmunity

While not curative, lymphocyte depletion or suppression has demonstrated efficacy in managing a range of autoimmune conditions, albeit with a concomitant risk of opportunistic infections due to transient generalized immunosuppression. Rituximab, a chimeric monoclonal antibody targeting the CD20 antigen on B cells, exemplifies the clinical utility of B cell depletion in autoimmune disease management. It has been shown to reduce disease activity in rheumatoid arthritis, systemic sclerosis and multiple sclerosis, among other diseases (7). Conversely, abatacept, an IgG1/CTLA4 fusion protein, works to inhibit T-cell activation by binding to CD80 and CD86 on antigen-presenting cells, preventing their interaction with CD28 on effector T cells and is approved in several autoimmune indications including rheumatoid arthritis and psoriatic arthritis. Combination therapies, aiming to target both cell subsets, including using rituximab and abatacept simultaneously have been attempted in autoimmune diseases such as rheumatoid arthritis. The results have demonstrated good efficacy but a mixed safety profile due to broad immunosuppression and associated increased infection rate (8). This dual targeting approach is also being explored in trials evaluating anti CD20 therapies in combination with different cell therapies, including NK cells, as further described below.

Newer agents have sought to improve on the specificity and safety profile of previous generation biologics by focusing on the mechanisms and signaling driving inflammation, tissue damage and autoantibody production, rather than broadly depleting B cells or suppressing T cell proliferation. Specifically, antibodies targeting more mature B cells than anti-CD20 (9) or both mature B and T cell subsets (e.g. anti-CD52 antibodies) (10) are being investigated in a number of autoimmune disease settings. Further studies are ongoing to identify bi-specific antibodies capable of an even more robust and precise B cell depletion than traditional monoclonal antibodies, by driving improved lymph node and tissue penetration (11). Beyond depletion, therapeutic antibodies and small molecules that can act on B cell and T cell signaling have also gained traction in treating a wide range of autoimmune disorders, such as those focusing on intracellular pathways (e.g complement inhibitors, S1PR modulators, TYK2 inhibitors, IL-12/23p40 inhibitors, and JAK inhibitors, among others) (12–14). In particular, JAK inhibitors have been successful in tackling a number of autoimmune and inflammatory diseases across rheumatological, dermatological, gastrointestinal and transplant-related disorders. Their efficacy in these multiple settings is somewhat hindered by their less-than-ideal toxicity profile, including the increased risk of infections, cardiovascular risk, hematological effects and malignancies, resulting in a boxed warning from the FDA.

Crucially, not all patients respond equally well, both in terms of depth and duration of response within the same indication to the same class of drug. This highlights the importance of understanding both the unique pathophysiology of disease in each individual, and the need for identification of biological markers predictive of response in heterogeneous patient populations. A recent example is the use of a companion diagnostic to identify likely responders to anti-tumor necrosis factor-like cytokine 1A (TL1A) antibodies for patients with inflammatory bowel disease (15)

Yet, despite these improvements in precision and reduction in overall toxicity profile, these novel therapies still only serve to manage, rather than cure autoimmune and immune-mediated diseases. Cell therapies, in particular CAR-T cells, which involve the manipulation and administration of engineered T cells to treat disease, have revolutionized the treatment landscape in oncology and are now being adapted for autoimmune and inflammatory

disorders. In addition to conventional $CD3^+$ T cells, other cell therapies, including hematopoietic stem cell transplant (HSCT), regulatory T (Treg) cells (16), natural killer (NK) cells and mesenchymal stem cells (MSCs) are being investigated for their ability to modulate immune responses and promote tolerance. These therapies aim to restore immune homeostasis by either suppressing autoreactive immune cells or promoting regulatory pathways that prevent tissue damage (17). The goal of these therapies is to induce long-term remission via the restoration of immune tolerance.

2 Cell therapies in autoimmunity and immune-mediated diseases: a new frontier

2.1 HSCT: the last line of defense

The use of cell therapy to treat autoimmune diseases traces back to the 1990s with the first confirmed use of hematopoietic stem cell transplantation (HSCT) for connective tissue disease and severe pulmonary hypertension (18). Since those early cases, HSCT has evolved to become a viable option for various severe, treatment-resistant autoimmune conditions, including multiple sclerosis, systemic sclerosis, and Crohn's disease (19). HSCT involves the replacement of a patient's hematopoietic and immune system with stem cells from a healthy donor (allogeneic HSCT), or more commonly in autoimmune diseases, from the patient themselves (autologous HSCT).

The mobilization and collection of peripheral stem cells for HSCT is a multi-step process that requires careful monitoring and management. Mobilization of stem cells from the bone marrow of patients into the peripheral blood is achieved primarily through the administration of granulocyte colony-stimulating factor (G-CSF), or in combination with cyclophosphamide as pre-treatment (20). Once mobilized, the stem cells are collected from the peripheral blood via leukapheresis. After collection, these cells can undergo further processing or manipulation based on the specific HSCT protocol being followed. Various protocols are currently used, and the optimal protocol for maximum therapeutic benefit has not been definitively established. For instance, in patients with systemic sclerosis treated with autologous HSCT, enrichment of CD34+ progenitor cells does not appear to provide additional clinical benefit (21).

The rationale behind HSCT in autoimmune diseases lies on the potential to 'reset' the aberrant immune system, fostering the regeneration of a self-tolerant immune repertoire (22). The precise mechanisms by which HSCT orchestrates immunological reset remain an area of active investigation. The process involves a multi-faceted interplay of immune cell depletion via lymphoablative conditioning, lymphopenia-induced proliferation, differentiation of new T cell progenitors in the thymus, and the modulation of Treg cells (23). Importantly, Treg cells, key players in immune tolerance, undergo significant changes following HSCT, with studies reporting an increase and more diverse Treg cell repertoire after autologous HSCT. This is associated with greater suppressive activity in immune mediated diseases such as Crohn's, multiple sclerosis, systemic lupus erythematosus (SLE), and systemic sclerosis (24–28). In the context of allogeneic HSCT, a high frequency of donor Treg cells has been associated with a lower risk of Graft-versus-Host Disease (GvHD) mediated by donor effector T cells, which remains a problematic and frequently lethal side effect of HSCT (29).

Importantly, the procedure is not without risks. HSCT is an onerous process for patients that involves multiple complexities, including the need for taxing immunoablative pre-conditioning regimens, which increases treatment-related morbidity and mortality due to severe infections after immunosuppression and cytopenia (30). In the last decade, the safety of the procedure has shown remarkable improvement, thanks to better selection of the most appropriate patients to transplant, better donor matching, and decreased intensity of conditioning regimens, including use of novel agents. For patients with a relapse-remitting form of multiple sclerosis for instance, treatment related mortality due to autologous HSCT has decreased to <1% (31). For systemic sclerosis, where treatment-related mortality mostly stemmed from cardiac complications, careful patient selection has enabled a decrease mortality rate to <6% (19). While less frequently used in autoimmune disease compared to oncology, HSCT can be with is only really considered as a treatment option for life-threatening forms of these diseases or relapsing courses of disease occurring after autologous transplantation. Allogeneic HSCT carries a greater risk of death and complications compared to autologous HSCT (23). Thus, though HSCT has demonstrated benefit in treating conditions including multiple and systemic sclerosis, its use remains restricted to a limited number of patients with severe disease who have not responded to conventional or approved therapies.

2.2 Mesenchymal stem cell therapies

Research into the immuno-modulating and regenerative properties of MSCs has sparked interest in this primarily allogeneic stem cell-based modality for the treatment of autoimmune diseases (32). MSCs are believed to exert their therapeutic effects through paracrine signaling, releasing cytokines that can suppress pro-inflammatory responses, promote tissue repair, and modulate immune cell function (33). MSCs can also be engineered to deliver a therapeutic payload. For instance, IL-10-expressing MSCs have been shown to decrease inflammation and improve fine motor function after traumatic brain injury in rats (34). Clinical trials investigating the use of MSCs in autoimmune diseases such as Crohn's disease and ulcerative colitis (35–37), GvHD (38), multiple sclerosis (39, 40) and severe idiopathic nephrotic syndrome (41) have so far reported encouraging, albeit mixed results. Likely contributors to these mixed results include the heterogeneity of MSC populations across tissues (42), the variability in isolation and expansion protocols (43), their limited persistence and trafficking (44), and uncertainty regarding the optimal route and dosing regimens to use across indications (45).

2.3 Moving beyond stem cells

The utilization of stem cells of hematopoietic or mesenchymal origin in autoimmune diseases, while groundbreaking, has encountered limitations such as high toxicity and inconsistent efficacy in a limited number of patients. Moreover, these therapies are not specifically tailored to target or eliminate disease-inducing immune cell subsets, such as autoreactive B or T lymphocytes. These limitations have spurred the exploration of alternative cell-based therapies that offer a more targeted and potentially safer approach to modulating the immune system in Treg cells and conventional T cells armored with a B-cell targeting moiety, such as a CD19-targeting chimeric antigen receptor (CAR).

While outside the scope of this review, additional differentiated cell subsets are also in development, such as NK cell therapy, an emerging modality in cancer immunotherapy and autoimmune disease. NK cells are innate immune effector cells that possess potent cytolytic function while exhibiting minimal risk of GvHD in the context of an allogeneic, donor-derived therapeutic due to both low allo-reactivity and limited persistence. Thus, NK cells do not have the need for strict HLA matching, a limitation of allogeneic effector T cell-based therapies. Engineering NK cells to express CARs targeting autoantigens or B-cell specific receptors enable precise targeting and elimination of pathogenic cell populations (46, 47). The efficacy of NK-based cell therapy, both with and without CAR modifications, in treating autoimmune diseases like SLE is currently being investigated in ongoing clinical trials both as a standalone therapy and in combination with more traditional B cell depleting agents. The results of these trials will provide valuable insights into the potential of this therapeutic approach for managing immune-mediated conditions.

3 CAR T cells to treat autoimmunity

3.1 CAR and TCR T cell therapy in oncology

The development of CAR T cells engineered to target the CD19 cell-surface antigen has revolutionized the treatment of B cell malignancies. Clinical trials have demonstrated their remarkable efficacy, with complete response rates ranging from 40-54% in multiple B cell lymphoma subtypes (48–50), 71-81% in B cell acute lymphoblastic leukemia (51, 52) and 18% in chronic lymphocytic leukemia (53). The durability of these responses suggests curative potential for some patients receiving these cells. The success of CD19-targeted CAR T cells has led to their approval by the FDA, either as a standalone therapy or as a bridge to HSCT (Axicabtagene ciloleucel, Tisagenlecleucel, Lisocabtagene maraleucel, Brexucabtagene autoleucel). In addition, CAR T cells targeting BCMA (Idecabtagene vicleucel, Ciltacabtagene autoleucel) have demonstrated efficacy in difficult to treat multiple myeloma, further expanding the therapeutic landscape for CAR T cell therapy among hematological cancers. Development of TCR-engineered T cells and tumor-infiltrating lymphocyte (TIL)-derived therapeutics have followed closely behind CAR T cell development. Recently, a MAGE-A4-targeted TCR-T cell therapy was granted accelerated approval by the FDA for the treatment of synovial sarcoma (uzatresgene autoleucel; NCT03132922) (54). Likewise, in 2024, approval was granted to a patient-derived TIL cell therapy for the treatment of melanoma (lifileucel; NCT02360579) (55).

3.2 CAR T cell therapy in autoimmunity

Application of CAR T cells in autoimmunity has largely followed the path laid out by hematological malignancies, namely using autologous CAR T cells targeting BCMA and CD19 antigens on the surface of B cells. The rationale for employing B-cell targeted CAR T cell therapy in autoimmune diseases is grounded in the critical role played by pathogenic B cells and autoreactive antibodies in the pathogenesis of many such conditions. The aim of B-cell targeted CAR T cell therapy is to disrupt these pathogenic processes and restore immune balance by depleting both circulating B cells, as well as those in the tissues and lymph nodes that are not reached by traditional anti-CD20 therapies. Early clinical trials have yielded promising results, demonstrating the safety and efficacy of B-cell targeted CAR T cell therapy in various autoimmune diseases, including SLE, myasthenia gravis, idiopathic inflammatory myopathy, systemic sclerosis, neuromyelitis optica spectrum disorder, and multiple sclerosis (56) (Tables 1, 2) (56). In a study involving five patients with refractory SLE who received anti-CD19 CAR T cell therapy, all patients achieved durable drug-free remission and cessation of nephritis (61). Mild cytokine release syndrome (CRS) occurred in all patients, with no concomitant immune effector cell-associated neurotoxicity syndrome (ICANS). Importantly, the B cell compartment reconstituted in these patients over a median of 8 months without disease flare or relapse. A follow-up study evaluating this product in fifteen severely ill patients with various autoimmune diseases, including eight with SLE, four with systemic sclerosis and three with inflammatory myopathies further confirmed these preliminary results. At a median follow-up of fifteen months, all the SLE patients were in drug-free remission without symptoms (62). The three treated patients with myositis also showed a major clinical response at six months, while the four patients with systemic sclerosis experienced decreases in disease severity scales and successfully discontinued all immunosuppressive medications during the follow-up period. Most autoantibodies were undetectable during up to twenty nine months of follow-up. Separately, in a Phase 1b/2a clinical trial of an anti-BCMA mRNA CAR T therapy in patients with generalized myasthenia gravis, eleven patients received six outpatient cell infusions (70). Patients receiving weekly infusions showed clinically meaningful benefits across three established clinical scoring systems and responses were maintained through the six-month follow-up. Serum titers of anti-AChR autoantibodies decreased by 22% at week 8 in patients with anti-AChR+ disease. Confirming these initial promising datasets, another anti-BCMA CAR T therapy was studied in twelve patients with neuromyelitis optica spectrum disorder (68). Eleven of twelve patients exhibited

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TABLE 1 Ongoing clinical trials using CAR T Cell therapies in autoimmune diseases.

Sponsor	Product Name	Product Class	Product Type	Indication	Phase	Clinical Trial Number
Adicet Bio	ADI-001	Allogeneic	CD20 γδ CAR T	LN & SLE	Phase 1	NCT06375993
Atara Bio	ATA3219	Allogeneic	EBV+ CD19-1XX CAR T	LN, SLE	Phase 1	NCT06429800
Autolus Tx	Obe-cel	Autologous	CD19 CAR T	SLE	Phase 1	NCT06333483
Bioray Lab	BRL-301 TyU19	Allogeneic	CD19 CART + HLA-A/B, HLA-II, TRAC, PD1 KO	SLE	Phase 1	NCT05988216
				SLE; SSc; IM; Sjogren's Syndrome	Phase 1	NCT05859997
BMS	CC-97540	Autologous	CD19 NEX T	Multiple Sclerosis	Phase 1	NCT05869955
		Autologous	CD19 NEX T	SLE, IIM, SSc	Phase 1	NCT05869955
Cabaletta Bio	CABA-201	Autologous	CD19 CAR T	MG	Phase 1	NCT06359041
				Myositis	Phase 1	NCT06154252
				SLE	Phase 1	NCT06121297
				Systemic Sclerosis	Phase 1	NCT06328777
				Pemphigus Vulgaris	Phase 1	NCT04422912
	DSG3-CAART	Autologous	Chimeric Auto-Antibody Receptor T cells	Pemphigus Vulgaris	Phase 1	NCT04422912
	MuSK-CAART	Autologous	Chimeric Auto-Antibody Receptor T cells	MG	Phase 1	NCT05451212
Caribou Bio	CB-010	Allogeneic	CD19 CAR T	LN, ERL	IND cleared	n.a.
Cartesian Tx	Descartes-08	Autologous	BCMA mRNA CAR T	Myasthenia Gravis	Phase 2	NCT04146051
				SLE	Phase 2	NCT06038474
Essen Biotech	N/A	Autologous	CD19 BCMA CAR T	Sjogren's Syndrome, SLE	Phase 1/2	NCT06350110
Fate Tx	FT819	Allogeneic	CD19 CAR T	SLE	Phase 1	NCT06308978
Friedrich-Alexander University	N/A	Autologous	CD19 CAR T	SLE	Phase 2	n.a.
				SSc	Phase 1	n.a.
				IIM (Antisynthetase syndrome)	Phase 1	n.a.
Gracell Bio/AstraZeneca	GC012F	Autologous	CD19/BCMA CAR T	SLE	Phase 1	NCT05846347
IASO/Innovent	CT103A	Autologous	BCMA CAR T	NMOSD, MG, IMNM	Phase 1	n.a.
				NMOSD, MG, CIDP, IIM, MS, AE, MOGAD, POEMS Syndrome	Phase 1	NCT04561557
iCell Gene Tx	BCMA-CD19 CAR T	Autologous	CD19/BCMA CAR T	SLE	Phase 1	NCT05474885
Immpact Bio	IMPT-514	Autologous	CD19/CD20 CAR T	SLE, LN	Phase 1/2	NCT06153095
				SLE, AAV, IIM	Phase 1	NCT06462144
				MS	IND cleared	n.a.
JW Tx/BMS	Relma-cel	Autologous	CD19 CAR T	SLE	Phase 1	NCT05765006
Kyverna Tx	KYV-101	Autologous	CD19 CAR T	MS	Phase 2	NCT06384976
				LN	Phase 1/2	NCT06152172
				IIM, SSc, SLE, AAV	Phase 1	NCT06152172
				MG	Phase 2	NCT06193889

(Continued)

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TABLE 1 Continued

Sponsor	Product Name	Product Class	Product Type	Indication	Phase	Clinical Trial Number
				Myositis and myopathies	Phase 1	NCT06152172
				Stiff Person Syndrome	Phase 2	NCT06588491
				SSC	Phase 1/2	NCT06152172
Luminary Tx	LMY-920	Autologous	BAFF CAR T	SLE	Phase 1	NCT06340750
Miltenyi Biomedicine	MB-CART19.1	Autologous	CD19 CAR T	SLE	Phase 1	NCT06189157
Novartis	YTB323 (Rap-cel)	Autologous	CD19 CAR T	SLE	Phase 1/2	NCT05798117
Renocell	RY_SW01	Allogeneic	MSC	LN	Phase 2	NCT06058078
				SSC	Phase 1/2	NCT06489652
Sana Bio	SC291	Allogeneic	CD19 CAR T	LN, SLE, AAV	Phase 1	NCT06294236
Synthekine	SYNCAR-001	Autologous	CD19 CAR T + orthogonal IL2	SLE, LN	Phase 1	NCT06544330

SLE, systemic lupus erythematosus; LN, lupus nephritis; SSc, systemic sclerosis; IM, inflammatory myopathy; IIM, idiopathic inflammatory myopathy; ERL, extrarenal lupus; NMOSD, Neuromyelitis optica spectrum disorder; MG, myasthenia gravis; IMNM, immune-mediated necrotizing myopathy; CIDP, chronic inflammatory demyelinating polyneuropathy; AE, autoimmune encephalitis; MOGAD, myelin oligodendrocyte glycoprotein antibody disease; AAV, ANCA-associated vasculitis.

drug-free remission with no relapse during a median 5.5 months of follow-up. It is important to note that in these trials, all patients received lymphodepletion chemotherapy before CAR T infusion, and all experienced Grade 1 or 2 CRS.

Of note, CD19-targeted CAR T cells appear to be more effective compared to other systemically administered B cell depleting agents like rituximab and obinutuzumab, in SLE and lupus nephritis (9, 71, 72). This difference in efficacy could be attributed to several factors: CD20, the target of rituximab and obinutuzumab, has a more restricted expression pattern along the B cell development axis compared to CD19 (73). Thus, the broader expression of CD19 allows CD19-targeted CAR T cells to deplete a wider range of B-cell populations, including CD19+CD20- plasma cells. These cells may play an important role in the pathogenesis of certain diseases, as they have been implicated in increased disease severity in preclinical models of multiple sclerosis (74). Additionally, the ability of CAR T cells to expand upon antigen-encounter, infiltrate tissues and induce deep *in situ* B-cell depletion, including tissue-resident B cells that are less sensitive to anti-CD20 antibody-mediated depletion, may contribute to their enhanced efficacy (75–77). The use of a lymphodepleting conditioning regimen prior to CAR T infusion may also contribute to deeper B cell depletion than antibodies alone.

Although CD19-targeted CAR T cell therapy effectively eliminates most autoantibodies against DNA and nucleosomal structures in SLE, certain autoantibodies, like those against Ro60 and Scl-70, persist (62). A possible hypothesis in need of confirmation is that B cell subsets responsible for their production do not express CD19. Despite a broader range of CD19 expression across B cell lineages compared to CD20, CD19-targeted CAR T cell therapies preserve the protective effect of prior vaccination in patients (62), possibly due to the preservation of long-lived, CD19-negative plasma cells responsible for vaccine-induced humoral immunity (78).

BCMA, expressed on mature B cells and plasma cells, presents another promising target for conditions where autoantibody production is driven by CD19-negative, $BCMA^+$ plasma cells. Depleting BCMA-expressing cells with CAR T cell therapy has shown encouraging efficacy in neuromyelitis optica spectrum disorder, anti-signal recognition particle (SRP) necrotizing myopathy, and SLE (67, 68, 79). BCMA-targeted therapies may lead to a more extensive B-cell depletion and a greater alteration of the autoantibody repertoire, possibly increasing clinical benefit compared to CD19 CAR T (80). However, this broader targeting also results in a more significant depletion of protective antibodies, increasing the rate of infections and impairing pre-existing vaccine responses (81–83). BCMA CAR T cell therapy recipients have lower seroprotection to vaccine-preventable infections compared to CD19 CAR T-cell recipients, likely due to depletion of antibody-producing plasma cells (82). In a study investigating the impact of teclistamab, a BCMA-targeted T-cell engager antibody, on humoral immunity, the therapy was found to reduce the levels of polyclonal immunoglobulins and impair humoral immune response after vaccination (81). The high rate of serious infections in this patient group was attributed, at least in part, to the development of hypogammaglobulinemia and failure to generate new humoral immune responses. The use of IVIG supplementation was associated with a significantly lower risk of serious infections, supporting the use of immunoglobulin supplementation as primary prophylaxis in patients receiving a BCMA-targeting therapies. The

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TABLE 2 Published clinical results for CAR T cell therapies in autoimmunity.

Institution	Target Antigen	Indication(s)	Pts #	Key Clinical Results	Reference
Ruhr-University Bochum	CD19	Myasthenia Gravis, Lambert Eaton Myasthenic Syndrome	2	Full mobility restored at 4- and 6-months post-CAR T	(57)
Otto-von-Guericke University	CD19	Myasthenia Gravis	1	Restoration of mobility at 62 days post-CAR T	(58)
Friedrich-Alexander University	CD19	Antisynthetase syndrome	1	Drug-free remission at 150 days follow up post-infusion	(59)
University Medical Center Hamburg-Eppendorf	CD19	Multiple Sclerosis	2	EDSS score decreased from 4.5 to 4 at 100 days follow up (n=1) or remained stable at day 28 follow up (n=1)	(60)
Friedrich-Alexander University	CD19	SLE	5	Drug-free remission maintained to median 12 month follow up in all patients	(61)
	CD19	SLE	8 (follow-up study)	100% of patients reached complete remission, with disease activity absent up to 29 months post-CAR T	(62)
	CD19	Idiopathic inflammatory myositis	3	Normalized muscular function	(62)
	CD19	Systemic Sclerosis	4	EUSTAR activity index decreased at 6 months post CAR T	(62)
	CD19	Systemic Sclerosis	1	Reduced disease activity indicated by EUSTAR activity index maintained through 6 month follow up	(63)
University Hospital Tübingen	CD19	Anti-synthetase Syndrome	1	Significant decrease in physician's global assessment of disease activity at 150 days follow up.	(64)
Shanghai Jiao Tong University	CD19	Sjogren's Syndrome with concurrent diffuse large B-cell lymphoma	1	Normal levels of ANA, anti-Ro-52, and cytokines and the improvement of dry mouth symptoms, without the use of glucocorticoids or tocilizumab	(65)
Shanghai Changzheng Hospital	CD19 (allogeneic)	Myositis and systemic sclerosis	3	Complete remission at 6 months follow-up in 3/3 pts	(66)
Huazhong University of Science and Technology	BCMA	Anti-SRP necrotizing myopathy	1	Disease improvement maintained at 18 months	(67)
	BCMA	Neuromyelitis optica spectrum disorder	12	92% drug-free remission with 11/12 patients relapse-free at median follow-up of 5.5 months	(68)
Zhongshan City People's Hospital	BCMA-CD19	SLE + lupus nephritis	13	Symptom and medication-free remission maintained at 46 months for 10/10 evaluable patients	(69)

EDSS, expanded disability status scale; EUSTAR, European scleroderma trials and research group. Pts #, number of patients reported.

development of bi-specific CAR T cells capable of targeting both CD19 and BCMA aims to further enhance B-cell depletion and prevent antigen escape (69, 84), but likewise might further elevate the risk of severe infections due to profound B cell depletion and suppressed vaccine-induced immunity.

To improve specificity, chimeric autoantigen receptor (CAAR) T cells have also been developed, intending to directly target autoantibody-producing pathogenic B cells. This is achieved by replacing the CAR's targeting moiety with the autoantigen itself, while keeping the architecture of the CAR intracellular domain intact, in theory leading to the specific elimination of those B cells (85). CAAR T cell therapy is particularly relevant for diseases driven by well-defined autoantigens, such as muscle-specific tyrosine kinase (MuSK) in myasthenia gravis or desmoglein 3 (DSG3) in pemphigus vulgaris (86, 87). This approach is currently being tested in two clinical trials (NCT04422912, NCT05451212). It is important to note, however, that CAAR T cells primarily target B cells expressing a membrane-bound B-cell receptor (BCR) and may not be effective against diseases driven by long-lived plasma cells devoid of a BCR (86).

(85) In contrast to autoimmune diseases with a dominant pathogenic B cell component, chronic inflammatory diseases such as psoriasis and other inflammatory skin diseases, inflammatory bowel disease, and spondyloarthritis are unlikely to respond to B cell-targeted CAR T cell therapies. Indeed, these diseases are instead characterized

by dysregulated T cell activation and IL-23/IL-17-mediated inflammation (88–90). Therefore, T cell-directed CAR T cells, such as those targeting the T cell-specific antigens CD5 and CD7, represent a promising alternative for patients with these predominantly T-cell mediated diseases. Several such products are currently undergoing clinical evaluation for the treatment of T cell malignancies with encouraging results (91–94), while their efficacy in autoimmune and chronic inflammatory diseases remains to be demonstrated.

A key limitation of current CAR and CAAR T cell therapies is their inability to simultaneously target multiple pathogenic cell types. This constraint poses a challenge in treating autoimmune diseases where the underlying pathophysiology involves a complex interplay of dysregulated B and T lymphocytes, along with inflammation driven by myeloid-derived innate immune cells. For example, while ANCA-associated vasculitis and myasthenia gravis both have a clear B cell component, neutrophils and thymus-derived T cells also play critical roles in their pathogenesis (95, 96). Similarly, in multiple sclerosis, while autoreactive B cells contribute to disease pathology, as evidenced by the efficacy of B cell-depleting antibodies (97, 98), definitive disease-causing autoantibodies have not been identified. Instead, a complex interplay between pathogenic B cells, autoreactive T cells, and CNS-localized inflammation is believed to drive demyelination, neurotoxicity, and disease progression (99–101). As discussed in the next section, FOXP3$^+$ and Tr1-based Treg cell therapies are well-suited to dampen T cell-driven autoimmunity and inflammation, but lack the ability to specifically deplete B cells. Combining these therapies with B cell-modulating agents, such as anti-CD20 antibodies or Bruton tyrosine kinase (BTK) inhibitors, may represent the next step in the evolution of these treatments, conferring multimodal activity for complex autoimmune diseases. Crucially, any combination therapy must maintain an acceptable safety and tolerability profile without inducing generalized immunosuppression that could increase the risk of opportunistic infections.

4 Regulatory T cells to treat immune-mediated diseases

Regulatory T cells represent a diverse subset of lymphocytes responsible for maintaining immunological tolerance and homeostasis to both self and foreign peptides in healthy individuals. Treg cells are frequently underrepresented or dysregulated in patients with autoimmune conditions. Here we highlight the immunomodulatory mechanisms of two major CD4$^+$ regulatory T cell types: FOXP3$^+$ Treg cells and Tr1 Treg cells, and how their functionality can be harnessed to treat autoimmunity (Figure 1).

4.1 Immunosuppressive functionalities of FOXP3$^+$ and Tr1 Treg cells

FOXP3$^+$ Treg cells are primarily identified by their robust expression of the transcription factor Forkhead Box Protein 3 (FOXP3) alongside high levels of the high-affinity IL-2 receptor α (CD25) and low expression of the IL-7 receptor (CD127) (102). Additional markers associated with FOXP3$^+$ Treg cells include elevated expression of the transcription factor Helios (103), high levels of the co-inhibitory surface molecule CTLA-4 (104), and specific demethylation patterns within the Treg-specific demethylation region (TSDR) (105). These phenotypic attributes underpin the potent immunomodulatory functions of FOXP3$^+$ Treg cells, which are mediated through a combination of indirect and direct mechanisms (106) and include:

1. IL-2 deprivation: FOXP3$^+$ Treg cells express high levels of CD25, enabling them to efficiently consume IL-2, thereby limiting the availability of this cytokine for effector T cells and curbing their proliferation (107).
2. Co-inhibitory signaling: FOXP3$^+$ Treg cells express co-inhibitory receptors such as CTLA-4, which can modulate APCs and alter their antigen-presentation functionality (108).
3. Adenosine production: Through the sequential action of the ectoenzymes CD39 and CD73, FOXP3$^+$ Treg cells convert extracellular ATP to adenosine, an immunosuppressive molecule that promotes a tolerogenic environment (109).
4. Inhibitory cytokine secretion: FOXP3$^+$ Treg cells secrete a range of inhibitory cytokines, especially TGF-β and IL-35, which suppress immune cell activation and foster anti-inflammatory responses (110, 111).

The critical role of FOXP3$^+$ Treg cells in immune regulation is underscored in disease states where their function or presence is compromised. For instance, patients harboring mutations in the FOXP3 gene are diagnosed with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (112). These individuals often exhibit a constellation of autoimmune manifestations, including enteropathy, type 1 diabetes, and dermatitis (113). In other autoimmune diseases with more complex etiologies, such as SLE (114) and rheumatoid arthritis, the relationship between FOXP3$^+$ Treg cells and disease pathogenesis is less clear. Studies have reported inconsistent findings regarding FOXP3$^+$ Treg cell phenotype and frequency in these conditions, with observations ranging from increased to decreased numbers, to no discernible changes compared to healthy donors or in relation to disease severity (115), though there is clear evidence of FOXP3$^+$ Treg dysregulation (116). Whether FOXP3$^+$ Treg defects are a causative factor in these systemic autoimmune diseases, a consequence of the ongoing inflammatory process, or a combination of both, remains an area of active investigation (115, 117).

Tr1 Treg cells are a distinct subset of regulatory T cells that do not constitutively express high levels of the transcription factor FOXP3. These cells are induced in the periphery and play a crucial role in maintaining tolerance to non-pathogenic antigens. Tr1 Treg cells are characterized by the co-expression of the surface markers CD49b and LAG3, and the production of high levels of IL-10 and IL-22, with low levels of IL-2 and IL-4 (118). IL-10, a key immunoregulatory cytokine produced by Tr1 Treg cells, can suppress the activation and proliferation of effector T cells directly, or indirectly by downregulating the function of antigen-presenting cells (119–121). Furthermore, high IL-10 secretion by Tr1 Treg cells has been shown to downregulate the NLRP3 inflammasome in monocytes, reducing the production of pro-inflammatory cytokines such as IL-1β (122). IL-22 secretion by Tr1 cells contributes to the protection of epithelial barrier integrity from inflammatory damage (123). In addition to cytokine-mediated suppression, Tr1 Treg cells also exert cell-to-cell

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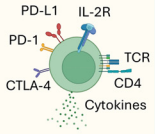
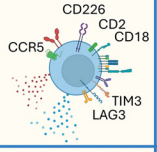
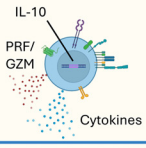
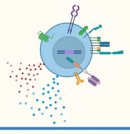
	Effector CAR-T Cell	FOXP3⁺ Treg	Engineered FOXP3⁺ Treg	Engineered Ag-specific FOXP3⁺ Treg	Tr1	Engineered Tr1	Engineered Ag-specific Tr1
							
Cell Source	CD3⁺ T cells	CD127^{low} CD25^{bright} CD4⁺ T cells	CD127^{low} CD25^{bright} CD4⁺ T cells or conventional CD4⁺ T cells	CD127^{low} CD25^{bright} CD4⁺ T cells or conventional CD4⁺ T cells	*Ex vivo* differentiated Tr1 cells	Conventional CD4⁺ T cells	Conventional CD4⁺ T cells
Therapeutic Mechanisms	Killing of pathogenic B cells	T cell suppression, modulation of APCs, infectious tolerance	T cell suppression, modulation of APCs, infectious tolerance	Ag-specific T cell suppression, modulation of APCs, infectious tolerance	T cell suppression, modulation of APCs, infectious tolerance	T cell suppression, modulation of APCs, infectious tolerance	Killing of pathogenic B cells, T cell suppression, modulation of APCs, infectious tolerance
Product Purity	Medium	Medium	High due to selection	High due to selection	Low	High due to selection	High due to selection
Clinical Status	Early trials underway	Several Phase I trials complete; more early trials underway	Early trials underway	Early trials underway	Several Phase I trials complete; more early trials underway	Early trials underway	Pre-clinical

FIGURE 1
Different types of T-cell based cell therapies for the treatment of autoimmune diseases. Highlighted are some of the hallmark features of each cell therapy subset. FOXP3⁺ Tregs secrete immuno-regulatory cytokines like TGF-β and IL-35, while Tr1 cells produce high levels of IL-10 and TGF-β, along with moderate amounts of IFN-γ, IL-22, and IL-5. FOXP3⁺ Tregs are identified by elevated expression of the high-affinity IL-2 receptor and co-inhibitory receptors (PD-L1, PD-1, CTLA-4). Tr1 cells exhibit higher levels of CCR5, CD2, CD18, CD226, and co-inhibitory receptors (LAG-3, PD-1, TIM-3, CTLA-4, PD-L1). In therapies lacking an engineered surface marker (e.g., CAR, truncated-non-signaling forms of NGFR or EGFR), a combination of markers is used to define product purity. PRF, perforin; GZM, granzymes.

contact-mediated suppression through the engagement of co-inhibitory receptors such as PD-1, CTLA-4, and TIM-3 (124).

Tr1 cells were first identified in patients with severe combined immunodeficiency (SCID) receiving mismatched HSCT (125). These patients showed long term engraftment of donor-derived T cells with no GvHD, despite complete HLA-mismatch, providing evidence for peripherally-induced tolerance. Subsequent studies on patient-derived material identified antigen-specific IL-10-producing T cells, later termed Tr1 Treg cells, as crucial in achieving transplant tolerance (126, 127). Dysregulated Tr1 Treg cell function has been strongly associated with autoimmune and inflammatory diseases such as multiple sclerosis, Crohn's Disease, and type 1 diabetes (128). Studies characterizing the immune systems of patients with these diseases consistently show a reduced frequency or impaired suppressive function of Tr1 Treg, or IL-10-producing CD4⁺ T cells, compared to healthy individuals (128).

4.2 Ex-vivo expanded FOXP3⁺ Treg cells as therapy to treat immune-mediated diseases

The immunomodulatory properties of regulatory T cells have generated considerable interest in their use as a therapeutic. However, harnessing their potential faces challenges, including the low frequency of FOXP3⁺ Tregs in the blood, which complicates their isolation and expansion for therapeutic use. Manufacturing polyclonal FOXP3⁺ Treg-based cell therapies typically involves a multi-step process: first, Treg cells are isolated from peripheral blood based on surrogate cell surface markers such as CD4, CD127, and CD25. This step may also include the depletion of CD8⁺ T or CD19⁺ B lymphocytes. Next, the isolated FOXP3⁺ Treg cells are activated and expanded *in vitro*, typically using anti-CD3 and anti-CD28 antibodies in the presence of high-dose IL-2 (129). Rapamycin, an mTOR inhibitor, which has been shown to promote the expansion of FOXP3⁺ Tregs, is often added to selectively promote Treg expansion while limiting the growth of contaminating non-Treg cells (130, 131).

Early clinical trials have employed *ex-vivo* expanded polyclonal FOXP3⁺ Treg cells for the prevention or treatment of GvHD in the context of allogeneic HSCT, or graft rejection in the context of solid organ transplants. In 2009, a first of its kind clinical study used ex-vivo expanded polyclonal FOXP3⁺ Treg cells for the treatment of GvHD (132). Another study of polyclonal FOXP3⁺ Treg cells in kidney transplant proved safe, with increased levels of circulating Treg cells following the cell therapy's administration (NCT02145325) (133). In the context of solid organ transplantation, donor-antigen-specific FOXP3⁺ Treg cells, generated by activation with donor-derived antigen-presenting cells, have shown superior efficacy in promoting allograft survival in preclinical models compared to polyclonal FOXP3⁺ Treg cells (134–137). A clinical study involving liver transplant patients receiving donor-specific Treg cells demonstrated the safety of this approach, with some patients able to discontinue immunosuppressive drugs for over 24 months (138). An alternative method of activation, distinct from CD3 or APC stimulation, utilizes the HIV envelope glycoprotein gp120's ability to stimulate these cells (139). The interaction of gp120 with CD4 on Treg cells triggers their activation and boosts their suppressive function, demonstrating protection against GvHD in humanized mice. This approach (Actileucel) has progressed to clinical trials,

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TABLE 3 Ongoing clinical trials using non-engineered Treg and Tr1 cells in autoimmunity.

Sponsor	Product Name	Product Class	Product Type	Indication(s)	Stage	Clinical Trial Number
ActiTrexx	Actileucel	Allogeneic	gp120-stimulated FOXP3 Tregs	GvHD	Phase 1	n.a.
Cellenkos	CK0803	Allogeneic	CXCR4 UCB FOXP3 Tregs	ALS	Phase 2	NCT05695521
	CK0801	Allogeneic	CXCR4 UCB FOXP3 Tregs	Bone marrow failure	Phase 1	NCT03773393
	CK0802	Allogeneic	CXCR4 UCB FOXP3 Tregs	COVID-19 ARDS	Phase 2	NCT04468971
	CK0804	Allogeneic	CXCR4 UCB FOXP3 Tregs	Myelofibrosis	Phase 1	NCT05423691
Fundacion Publica Andaluza	N/A	Autologous	FOXP3 Tregs	GvHD	Phase 1	NCT05095649
ORCA Bio	Orca-Q	Autologous	Freshly sorted Treg enriched graft; haplo	GvHD	Phase 1	NCT03802695
	Orca-T	Autologous	Freshly sorted Treg enriched graft; full match required	GvHD	Phase 3	NCT04013685 and NCT05316701
PolTreg	PTG-007	Autologous	FOXP3 Tregs	Multiple sclerosis	Phase 1	n.a.
				T1D	Phase 2	n.a.
Stanford University	T-allo10	Allogeneic	Tr1	GvHD	Phase 1	NCT04640987
Tract Tx	TRACT	Autologous	FOXP3 Tregs	Kidney transplant	Phase 2	NCT02145325

UCB, umbilical cord blood; haplo, haploidentical; ALS, amyotrophic lateral sclerosis; ARDS, acute respiratory distress syndrome; T1D, type-1 diabetes; GvHD, GvHD prevention studies.

with the first patient treated earlier this year in a GvHD prevention trial. Beyond GvHD, multiple trials have assessed or are actively investigating the safety and efficacy of polyclonal FOXP3$^+$ Treg cell therapy in various autoimmune disease settings, including type 1 diabetes (NCT02691247) (140–143), lupus (144), multiple sclerosis (145), Crohn's (146, 147), and Pemphigus vulgaris (NCT03239470), among others. These trials have collectively demonstrated the safety of FOXP3$^+$ Treg cell therapy, while additional research is ongoing to further establish their efficacy (Table 3).

Umbilical cord blood has been used as an alternative source of FOXP3$^+$ Treg cells for cell therapy. A small-scale trial for GvHD prevention investigated the use of partially matched cord-blood FOXP3$^+$ Treg products in five patients undergoing stem cell transplantation (NCT02423915). Although GvHD occurred in all four surviving patients, symptoms resolved, and patients were able to discontinue immunosuppressive medications after a median follow-up of 25 months (148). The safety and potential efficacy of allogeneic cord-blood-derived FOXP3$^+$ Treg cells are also being evaluated in an ongoing clinical trial involving six patients with amyotrophic lateral sclerosis (ALS) without HLA matching (NCT05695521). A subsequent Phase 1b trial is currently underway, aiming to assess 60 additional patients in a randomized, controlled study.

A key challenge of natural FOXP3$^+$ Treg cell manufacturing has been the potential for these cells to lose FOXP3 expression, particularly when exposed to inflammatory conditions (149), and convert to an undesired effector T cell phenotype (150–153). Conventional CD4$^+$ T cells engineered to constitutively express FOXP3 present a compelling alternative to ex-vivo expanded natural FOXP3$^+$ Treg cells. Enforced expression of FOXP3 effectively establishes a stable FOXP3$^+$ Treg phenotype and associated suppressive functions, even under inflammatory conditions (154–156). The efficacy of these cells, generated using lentiviral-mediated transduction of FOXP3, has been demonstrated in preclinical models of GvHD and IPEX, and is currently being evaluated in a clinical study of patients with IPEX syndrome (NCT05241444) (154, 157). Gene editing technologies, such as CRISPR or TALEN, have also been employed to drive FOXP3 expression and generate engineered FOXP3$^+$ Treg cells with a stable phenotype. In the context of IPEX patients, these techniques have been employed to restore functional FOXP3 expression by substituting the mutated and deficient endogenous sequence with a functional version of the gene (155). Alternative gene editing techniques include insertion of a robust enhancer/promoter near the FOXP3 locus (156), or utilization of a dual knock-in/knock-out approach to insert FOXP3 into a specific locus, while simultaneously disrupting expression of the targeted gene (158).

4.3 Tr1 Treg cell therapies to treat immune-mediated diseases

In a 2009 study, antigen-specific, clinical-grade Tr1 Treg cell clones were successfully isolated and expanded *in vitro*, and used to treat patients with refractory moderate-to-severe Crohn's disease (159). This Phase 1/2a trial evaluated the safety and efficacy of autologous, ovalbumin-specific Tr1 Treg cell clones in patients fed with an ovalbumin-rich diet, to ensure cell activation specifically in the gut (160). The therapy was well-tolerated, and early efficacy signals were noted despite documented product impurities and cell exhaustion following an extensive and time-consuming expansion and activation

protocol. Other allo-antigen-specific Tr1-enriched cell therapies have been developed and tested in the prevention of GvHD. $CD4^+$ T cells co-cultured with CD3-depleted allogeneic PBMCs were manufactured in presence of IL-10, resulting in a product containing 3-5% of Tr1 Treg cells. This cell product was infused in patients undergoing haploidentical HSCT to facilitate immune reconstitution without GvHD (161). The treatment resulted in long term tolerance and cure (follow up > 7 years) in a subset of patients, without inducing general immune suppression, as tolerized patients mounted normal immune responses against microbes and vaccines. More recently, another host alloantigen-specific donor-autologous Tr1-enriched cell product containing 9-13% of Tr1 cells, was used to treat children and adults with hematologic malignancies receiving unmanipulated (NCT03198234) or $TCR\alpha\beta^+$ T cell and $CD19^+$ B cell depleted (NCT04640987) HSCT from mismatched unrelated or haploidentical donors (162). In these two clinical trials, no dose limiting toxicity has been observed in any of the patients treated so far. Patients displayed good T-cell immune reconstitution without increased risk of GvHD. Preliminary analyses suggested a reduced incidence of severe infections compared to historical controls. Furthermore, Tr1 Treg cells were detectable in the peripheral blood shortly after product infusion, reaching the highest levels at day +7 post-infusion and persisting for up to one year after administration (163).

While clinical trials have demonstrated the safety of patient- or donor-derived Tr1 Treg cell therapies, just like with natural $FOXP3^+$ Treg cells, generating large numbers of highly pure Tr1 Treg cells from a low-frequency population remains a challenge. To address this limitation, recent efforts have focused on engineering allogeneic $CD4^+$ T cells to express IL-10 and inducing cellular reprogramming through specific activation and expansion protocols. The resulting engineered cells effectively recapitulate many aspects of natural Tr1 cell biology, including high IL-10 secretion, low IL-4 production, *in vitro* suppression of T cells, and *in vivo* protection from GvHD in humanized mouse models (164–166). This efficient manufacturing of Tr1-like cells has enabled the development of novel polyclonal, allogeneic, IL-10-engineered Tr1 Treg cell therapies. These therapies are currently in clinical development for the prevention of GvHD following mismatched HSCT (NCT06462365), as well as for the treatment of refractory Crohn's disease (167).

4.4 Opportunities for antigen-targeted, engineered $FOXP3^+$ Treg and Tr1 Treg cell and Tr1 cell therapies

To achieve localized immune modulation, improve efficacy, and avoid the risk of systemic, generalized immunosuppression, engineered regulatory T cells can be equipped with a targeting moiety to focus their activity on specific tissues or cell types. The advantage of this approach is supported by evidence that antigen-specific Treg cells exhibit greater potency compared to their polyclonal counterparts (134–137). However, an inherent challenge lies in the typically low frequency of antigen-specific Treg cells, which can complicate the manufacturing process and limit its scalability.

As an alternative, antigen-specific regulatory T cells can be generated by incorporating a CAR or antigen-specific TCR, which facilitates the generation of large numbers of cells. For instance, conventional $CD4^+$ T cells have been reprogrammed to co-express FOXP3, an engineered IL-2 receptor responsive to rapamycin, and a pancreatic islet antigen-specific TCR (168). This TCR-$FOXP3^+$ Treg cell therapy product is being developed for the treatment of type 1 diabetes, aiming to prevent T cell-mediated destruction of pancreatic islet β cells. Another example comes from HLA-A2-directed CAR $FOXP3^+$ Treg cells, which have demonstrated superior potency compared to polyclonal $FOXP3^+$ Treg cells, particularly in solid organ transplantation (169–173). Clinical trials are currently underway to evaluate autologous HLA-A2-specific CAR Treg cell therapy in kidney and liver transplantation, aiming to induce immunological tolerance to the transplanted organ and eliminate the need for lifelong immunosuppression (NCT04817774; NCT05234190) (174). An alternative target, CD6, is a cell surface antigen expressed on T lymphocytes and NK cells. An allogeneic, CD6-CAR $FOXP3^+$ Treg cell therapy is in development for the treatment of chronic GvHD after HSCT (NCT05993611). Beyond transplantation and GvHD, several other autologous CAR- and TCR-redirected $FOXP3^+$ Treg cell therapies are being developed for various autoimmune diseases, including multiple sclerosis (NCT06566261), SLE and lupus nephritis (175, 176), rheumatoid arthritis (NCT06201416), and hidradenitis suppurativa (NCT06361836). Several donor-derived, allogeneic CAR-Treg cell therapies are also in earlier stages of development and are anticipated to enter clinical trials soon. Tr1 Treg cells possess both regulatory and cytotoxic functions (66, 165). Another tantalizing, yet theoretical strategy would be to equip Tr1 Treg cells with a B cell-targeting CAR, enabling them to eliminate autoreactive B cells, while simultaneously suppressing pathogenic T cells and inflammation.

A comprehensive overview of clinical trials involving antigen-targeted, engineered regulatory T cells in autoimmune and immune-mediated diseases is presented in Table 4. The relatively small, but growing number of these efforts highlight an increasing interest in harnessing the therapeutic potential of autologous or allogeneic engineered regulatory T cells to treat a wider range of immune disorders.

5 Limitations and opportunities

5.1 Moving beyond lymphodepleting conditioning regimens

Lymphodepletion, commonly achieved through the combined administration of fludarabine and cyclophosphamide, is a standard conditioning regimen employed in CAR T cell therapies for both cancer and autoimmune diseases. This procedure is crucial for enhancing the engraftment, expansion, and persistence of the infused CAR T cells. However, it is associated with significant toxicity, including prolonged cytopenia and increased susceptibility to infections (177–183). While lymphodepletion is generally well-tolerated in the context of oncology, its application in autoimmune

diseases raises concerns due to the potential for exacerbating the underlying condition through severe side effects. Interestingly, a case study involving a patient with SLE suggests that CAR T cell therapy may be effective in autoimmune diseases even with reduced doses of lymphodepleting agents (184). This observation may indicate that the threshold for achieving therapeutic efficacy in autoimmune diseases might be lower than in oncology, opening the door for lower, less aggressive lymphodepletion regimens. Reducing the intensity of conditioning regimens may necessitate strategies to enhance T cell expansion and compensate for lower peak CAR T cell numbers, a factor strongly correlated with efficacy in oncology (181, 183). Various engineering strategies aimed at augmenting T cell expansion and function will be explored below.

For Treg-based cell therapies, the IL-2 signaling pathway plays a critical role in the homeostasis and function of FOXP3$^+$ Treg cells, therefore, augmenting IL-2 signaling represents a promising strategy for enhancing this type of cell therapy. Early clinical investigations employing IL-2 as an adjuvant for CAR T cell therapy in non-Hodgkin lymphoma demonstrated an increase in the frequency of FOXP3$^+$ Treg cells (185, 186). This observation is consistent with the well-established role of IL-2 in promoting the survival, stability, and suppressive function of FOXP3$^+$ Tregs (187, 188). Furthermore, clinical trials exploring low-dose IL-2 administration in patients with autoimmune diseases have consistently reported an expansion of FOXP3$^+$ Treg populations, although the overall clinical responses have been variable (189). However, a significant limitation of systemic IL-2 administration, even at low doses, is its potential to activate other immune cell subsets and induce severe side effects, thus restricting its utility as an adjuvant for cell therapy (190). To circumvent this challenge, innovative orthogonal IL-2 systems have been developed. These systems utilize a modified IL-2 molecule that selectively binds to an engineered IL-2 receptor, enabling the targeted expansion of cells expressing this artificial receptor (191, 192). Preclinical studies have demonstrated the efficacy of this approach. FOXP3$^+$ Tregs engineered to express the orthogonal IL-2 receptor can be selectively expanded *in vivo* using orthogonal, soluble IL-2, leading to improved donor hematopoietic stem cell engraftment and organ transplant tolerance (193). While this orthogonal IL-2 technology is currently undergoing clinical development in the field of oncology (NCT05665062), its therapeutic potential for autoimmune diseases warrants further investigation.

Beyond IL-2, other cytokine pathways offer promising targets for enhancing the *in vivo* proliferation and persistence of therapeutic effector and regulatory T cells. To mitigate the risk of exacerbating autoimmune pathology through systemic cytokine exposure, a favored strategy is to engineer T cells to co-opt the pathway in a cell-intrinsic fashion (194). Homeostatic cytokines such as IL-7 and IL-15 are well-known for their ability to augment the persistence and function of effector T cells. Notably, the efficacy of lymphodepletion is partially attributed to the increased availability of these cytokines (195). Preclinical studies have demonstrated that engineering CAR T cells to express a constitutively active form of the IL-7 receptor enhances their proliferation and persistence *in vivo* (196, 197). Similarly, co-expressing CAR with membrane-bound IL-15 has been shown to improve CAR T cell expansion and persistence in murine models (198), with encouraging clinical results reported in an oncology setting (199). Given the critical role of IL-15 in the maintenance and expansion of Tr1 Treg cells (200), IL-15 overexpression could also prove beneficial for Tr1-based therapies. While IL-12 and IL-18 have demonstrated potential in promoting CAR T cell proliferation and persistence without preconditioning in murine models (201, 202), their pro-inflammatory nature poses a significant challenge for their application in autoimmune diseases.

The pleiotropic nature of IL-10 presents a unique advantage for CAR T cell therapy in autoimmune diseases. IL-10 can enhance the persistence of engineered T cells through autocrine signaling while

TABLE 4 Ongoing clinical trials using engineered Treg and Tr1 Cell therapeutics in autoimmunity.

Sponsor	Product Name	Product Class	Product Type	Indication	Phase	Clinical Trial Number
Abata Tx	ABA-101	Autologous	DRB1*15:01-specific TCR FOXP3 Treg	Multiple sclerosis	Phase 1	NCT06566261
City of Hope	CD6 CAR Treg	Autologous	CD6 CAR FOXP3 Treg	GvHD	Phase 1	NCT05993611
Quell Tx	QEL-001	Autologous	HLA-A2-specific CAR FOXP3 Treg	Liver transplant	Phase 1	NCT05234190
Sangamo Tx	TX200-TR101	Autologous	HLA-A2-specific CAR FOXP3 Treg	Kidney transplant	Phase 1	NCT04817774
Sonoma Biotherapeutics	SBT-77-7101	Autologous	CAR FOXP3 Treg	Rheumatoid Arthritis	Phase 1	NCT06201416
				Hidradenitis suppurativa	Phase 1	NCT06361836
Stanford University	$CD4^{LVFOXP3}$	Autologous	FOXP3 Treg	IPEX	Phase 1	NCT05241444
Tr1X	TRX103	Allogeneic	IL10-engineered Tr1	GvHD	Phase 1	NCT06462365
				Crohn's	IND cleared	n.a.

GvHD, GvHD prevention study; IPEX, Immune Dysregulation Polyendocrinopathy Enteropathy X-linked syndrome.

simultaneously promoting an anti-inflammatory response via paracrine signaling. Studies have shown that IL-10 improves CAR T cell proliferation and mitochondrial health, a critical determinant of T cell fitness (203). Moreover, IL-10 contributes to memory $CD4^+$ T cell homeostasis and has been linked to the prolonged persistence of $CD4^+$ T cells in the central nervous system during viral infections (204, 205). Importantly, Tr1 Treg cells, which naturally produce IL-10, can transfer a Tr1-like phenotype to bystander $CD4^+$ T cells, a positive feedback loop known as infectious tolerance (206, 207). Through these combined mechanisms, it is plausible that Tr1-based therapies may require less expansion *in vivo* compared to effector CAR T cells to achieve comparable clinical benefit.

5.2 Long-term toxicity/safety of B-cell targeting effector CAR T and CAR Treg cells

Beyond the immediate risks associated with lymphodepletion, careful consideration must be given to the long-term safety of B-cell targeting CAR T cell therapies, particularly the potential for prolonged B cell aplasia and its impact on humoral immunity. In the context of cancer treatment, extended B cell depletion can increase the risk of infections and compromise B cell memory responses, with studies reporting a significant incidence of pneumonia requiring hospitalization months after CAR T cell infusion (208, 209). However, current treatment protocols for autoimmune diseases appear to result in the rapid clearance of CAR T cells, potentially mitigating this risk (61, 62). This observation is supported by clinical data demonstrating a low incidence of serious infections following CAR T cell therapy, despite profound and prolonged B cell depletion, lasting more than 100 days in some patients (62). It is crucial to recognize that these risks may be more pronounced with BCMA-targeting CARs compared to CD19-targeting CARs, due to the distinct B cell subpopulations expressing these antigens (81–83). Additionally, the risk of excessive immunosuppression warrants particular attention for non-targeted regulatory T cell therapies, as $FOXP3^+$ Treg cells are known to suppress immune responses to various pathogens and malignancies (210, 211). Therefore, as the field progresses, it will be essential to develop strategies for controlling the proliferation of therapeutic $FOXP3^+$ T cells, particularly those of autologous origin. Incorporating "kill switch" or suicide gene technology may enable the controlled elimination of these cells once the therapeutic objective is achieved.

One widely employed approach is the inducible caspase 9 (iCas9) system, which has been utilized in numerous clinical trials (212). Activation of iCas9 triggers apoptosis, leading to the elimination of the engineered T cells. Another strategy involves the herpes simplex virus thymidine kinase (HSV-tk) suicide gene, which converts the prodrug ganciclovir into a toxic metabolite, disrupting DNA synthesis and inducing cell death (213). However, the potential immunogenicity of HSV-tk can lead to premature elimination of the therapeutic cells by the host immune cells (214). Alternatively, co-expressing a truncated, inactive form of EGFR (EGFRt) or CD20 on the surface of engineered cells allows for their targeted elimination through antibody-dependent cellular cytotoxicity or complement activation upon administration of clinically approved monoclonal antibodies such as cetuximab or rituximab (215, 216).

While these safety mechanisms have primarily been explored in CAR T cell therapies for cancer, there is growing interest in incorporating them into engineered Treg cell therapies as well (217). This is exemplified by an ongoing Treg-based clinical trial employing an undisclosed safety switch technology (NCT05234190).

While suicide switch technologies offer effective elimination of engineered T cells, complete eradication may be disadvantageous for CAR Treg cell therapies, where maintaining a reservoir of potentially beneficial cells could be desirable. Positive regulators of CAR activity provide an alternative approach, enabling future reactivation of the therapeutic cells if needed. These systems utilize small molecules or drugs to control the expression or activity of the CAR transgene. This control can be implemented at the genetic level, using inducible promoters to regulate CAR expression, or at the protein level, through various mechanisms (comprehensively reviewed in (218, 219)). An example of such an inducible system is currently being evaluated in a clinical trial for acute myeloid leukemia (NCT05105152), where rapamycin acts as a dimerization agent to activate CAR functionality (220, 221). These inducible systems provide an "on-off" switch for CAR T cell activity, allowing for treatment to be tailored to the patient's disease activity and offering greater control and flexibility. It is important to note that donor-derived allogeneic T cell therapies are naturally subject to rejection by the host immune system over time. Therefore, this class of therapeutics is expected to pose a lower risk of long-term adverse effects.

5.3 Scaling the production of cell therapies for autoimmunity

The scalability and cost of manufacturing autologous T cell therapies, including Treg, Tr1, and CAR T cell therapies, has been a major obstacle to their widespread application beyond rare diseases like acute lymphoblastic leukemia or multiple myeloma. The prospect of treating more prevalent autoimmune diseases, such as lupus and multiple sclerosis, which affect millions of patients worldwide, poses significant manufacturing, logistical, and financial hurdles. Furthermore, patients with autoimmune diseases often require long-term treatment with glucocorticoids or other immunosuppressive agents, particularly when their disease is poorly controlled. These medications can negatively impact T cell number and function, potentially compromising the collection, manufacture, and quality of the final autologous T cell product. Allogeneic, or "off-the-shelf", cell therapies, where cells from universal donors are engineered for multiple patients, offer a potential solution to the inherent limitations of autologous cell therapy. However, allogeneic T cell therapy faces significant

challenges, including the risk of GvHD due to the alloreactive TCR repertoire of the infused cells, and the potential for rapid rejection mediated by host CD8+ T cells and NK cells (222).

The advent of gene editing technologies, particularly CRISPR-Cas9 and its derivatives, has ushered in a new era for allogeneic T cell therapies. These tools enable precise modifications of T cells, mitigating the risk of GvHD by directly eliminating TCR expression and enhancing their persistence in non-HLA identical recipients by ablating HLA expression, thereby reducing the likelihood of rejection by the host immune system. The clinical potential of multiplexed gene editing with CRISPR-Cas9 has been demonstrated in several effector CAR T cell clinical trials in oncology (NCT04502446) (223–226). Consequently, numerous allogeneic CD19-targeting CAR T cell therapies are being actively developed for the treatment of autoimmune diseases (Table 1). One such product utilizes CRISPR-Cas9 to simultaneously knock-out the TCR α chain to prevent GvHD, as well as HLA-A/B, CIITA, and PD-1 to evade host-mediated immune rejection and enhance persistence (66). This therapy is currently in clinical development for myositis and systemic sclerosis, where it has shown promising results, achieving sustained remission in three out of three patients (NCT05859997). Other clinical studies with gene-edited CAR T cells are also underway in lupus and ANCA-associated vasculitis (NCT06308978, NCT06294236). It is worth noting that multiplexed gene editing using CRISPR-Cas9 may come with risks due to unpredictable translocation events between double-stranded breaks (227). Future strategies using Cas9-derived "base editors" hold promise to greatly improve the safety profile of edited allogenic products. These variants may enable the targeted editing of specific genetic loci without inducing genotoxic breaks in the DNA (228). The improved safety profile and efficacy of this approach has been demonstrated preclinically and in a Phase 1 study with quadruply-edited allogeneic CAR-T cells for relapsed leukemia showing undetectable translocation events (229, 230). As an alternative to using genetic editing to knock-out TCR expression, generating allogeneic CAR T cells with a virus-specific, non-alloreactive TCR may reduce the risk of GvHD and simplify the manufacturing process, an approach being tested clinically for patients with SLE (NCT06429800).

Unlike allogeneic effector CAR T cells, which require extensive genetic engineering to prevent GvHD and rejection, Tr1 and FOXP3$^+$ Treg cells possess inherent suppressive properties that naturally mitigate their immunogenicity and potential to induce toxicity via GvHD. To date, there is no evidence to suggest that the transfer of allogeneic Tr1 or FOXP3$^+$ Treg cells induces GvHD in humans. For instance, a recent Phase 1 study of HLA-mismatched, cord-blood derived Treg cells used to treat nine patients with bone marrow failure syndrome showed no signs of GvHD or other serious side effects (231). Moreover, IL-10 production by Tr1 cells can directly and indirectly suppress alloantigen-specific T cell responses (232), potentially enhancing their persistence *in vivo* (NCT06462365). Development of an allogeneic cell therapy that does not necessitate extensive gene editing would greatly simplify manufacturing complexities and dramatically lower its costs. This opens the door for broader development of allogeneic Treg-based cell therapies as a way to obviate manufacturing and scalability challenges.

6 Discussion

Over the last thirty years, the treatment of autoimmune and inflammatory diseases has undergone a remarkable transformation, progressing from broadly immunosuppressive drugs to targeted biologics and now to the promising field of cell therapies. HSCT, while effective in severe cases, carries significant risks and has limited applicability. MSCs offer a less invasive option but face challenges in standardization, persistence and in achieving consistent clinical outcomes.

The success of CAR T cell therapy in oncology has paved the way for its exploration in autoimmunity, with promising early clinical trials showcasing the safety and efficacy of B-cell-targeted CAR T cells in various conditions. The prospect of inducing long-term remission through an immune system reset offers hope for a long-lasting, if not curative, outcome. However, further validation is needed through larger-scale clinical trials with extended follow-up periods. These studies will be crucial for confirming the long-term safety and efficacy of this approach in a broader patient population and for assessing the durability of treatment responses. Treg and CAR Treg cell therapies, which suppress pathogenic T cells and inflammation, offer a potentially safer and more effective approach for treating diseases characterized by dysregulated T cell activation and inflammation, such as psoriasis or rheumatoid arthritis. Furthermore, combining the immunosuppressive properties of Treg cell therapies with a B-cell suppressing modality may offer a promising new avenue for treating diseases caused by both pathogenic B and T lymphocytes, such as multiple sclerosis.

The path forward for these promising therapies involves addressing challenges such as minimizing the need for lymphodepletion, ensuring long-term safety, and enhancing scalability, lower cost and increase accessibility. Onerous autologous approaches continue to pose a significant hurdle for their broader application in autoimmune diseases with higher prevalence than subtypes of cancer. Allogeneic therapies, coupled with gene engineering or gene editing technologies like CRISPR-Cas9, offer a potential solution by overcoming the limitations of personalized treatment and the risk of GvHD or rejection, although achieving sufficient cell persistence remains a concern.

The future of cell therapies in autoimmune and immune-mediated diseases holds immense promise in the quest to alleviate patients' lifelong reliance on immunosuppressants. Ongoing research and clinical trials are laying the groundwork for the development of next-generation therapies that are safer, more effective, and scalable. The potential to achieve long-term remission or even cures, coupled with a deeper understanding of the complex mechanisms governing immunological tolerance offers hope for a transformative shift in the treatment of these diseases.

Author contributions

YB: Writing – original draft, Writing – review & editing. RF: Writing – original draft. MU: Writing – original draft. DH: Writing – original draft. RB: Writing – original draft. DdV: Writing – original draft, Writing – review & editing. MR: Writing – review & editing.

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EDITED BY
Guobao Chen,
AbbVie's Cambridge Research Center,
United States

REVIEWED BY
Salvador Iborra,
Inmunotek SL, Spain
Krzysztof Bryniarski,
Jagiellonian University Medical College,
Poland

*CORRESPONDENCE
Cody Deumont Moorman
cmoorman@amgen.com

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A comprehensive overview of tolerogenic vaccine adjuvants and their modes of action

Sabine Arve-Butler[1,2] and Cody Deumont Moorman[2]*

[1]Amgen R&D Postdoctoral Fellows Program, Amgen Inc, South San Francisco, CA, United States, [2]Amgen Research, Amgen Inc., South San Francisco, CA, United States

Tolerogenic vaccines represent a therapeutic approach to induce antigen-specific immune tolerance to disease-relevant antigens. As general immunosuppression comes with significant side effects, including heightened risk of infections and reduced anti-tumor immunity, antigen-specific tolerance by vaccination would be game changing in the treatment of immunological conditions such as autoimmunity, anti-drug antibody responses, transplantation rejection, and hypersensitivity. Tolerogenic vaccines induce antigen-specific tolerance by promoting tolerogenic antigen presenting cells, regulatory T cells, and regulatory B cells, or by suppressing or depleting antigen-specific pathogenic T and B cells. The design of tolerogenic vaccines vary greatly, but they all deliver a disease-relevant antigen with or without a tolerogenic adjuvant. Tolerogenic adjuvants are molecules which mediate anti-inflammatory or immunoregulatory effects and enhance vaccine efficacy by modulating the immune environment to favor a tolerogenic immune response to the vaccine antigen. Tolerogenic adjuvants act through several mechanisms, including immunosuppression, modulation of cytokine signaling, vitamin signaling, and modulation of immunological synapse signaling. This review seeks to provide a comprehensive examination of tolerogenic adjuvants currently utilized in tolerogenic vaccines, describing their mechanism of action and examples of their use in human clinical trials and animal models of disease.

KEYWORDS

tolerogenic adjuvant, tolerogenic vaccine, autoimmune disease, immune tolerance, regulatory T cells, tolerogenic dendritic cells, immunoregulation, adjuvant

1 Introduction

Immunological tolerance is a state of unresponsiveness or an anti-inflammatory response that promotes immune homeostasis and prevents detrimental immune reactions directed toward self-antigens and tissues (1). Tolerogenic vaccines aim to induce antigen-specific tolerance in conditions where tolerance has failed, or where there is an aberrant inflammatory response toward antigens not associated with danger. Tolerogenic vaccines differ from general immunosuppression or immunomodulation in

that they aim to induce specific immune tolerance to the disease-relevant antigens, thereby suppressing the autoimmune response without affecting the immune system as a whole (2, 3). Autoimmune disease, transplantation rejection, anti-drug antibody responses, and hypersensitivity all represent conditions where tolerogenic vaccines are promising new therapeutic regimens (2, 3).

Tolerogenic vaccines can induce antigen-specific tolerance via effects on key players in peripheral tolerance. Mechanisms of immune tolerance are reviewed extensively elsewhere (1, 4, 5). In brief, master regulators of peripheral tolerance toward specific antigens are regulatory T cells (Tregs) and tolerogenic dendritic cells (tolDCs) (1, 3–6). Tregs exert their immunoregulatory effects via anti-inflammatory cytokines, direct suppression of conventional T cell (Tcon) proliferation, and by modulating DC maturation and function. TolDCs act by promoting Treg development, suppressing effector T cell responses, and by inducing antigen-specific T cell anergy. Many other cell types can contribute to immunological tolerance, of note are B cells producing anti-inflammatory cytokines (7) and type 1 regulatory T cells (Tr1 cells) (8).

Adjuvants are molecules used to enhance the effect of pharmacological treatments (9), therefore tolerogenic adjuvants aim to increase anti-inflammatory responses and enhance vaccine efficacy. A schematic overview of the main mode(s) of action for tolerogenic adjuvants described in this review are depicted in Figure 1. Tolerogenic vaccines use adjuvants in different ways depending on vaccine type. Antigen and adjuvants can be administered either separately or co-delivered, where the co-delivery can range from simultaneous administration of free antigen and adjuvant to intricate delivery systems, fusion molecules, or DNA vectors (Figure 2). Tolerogenic adjuvants can also be used to differentiate tolDCs for cell transfer of antigen-loaded tolDCs (Figure 2).

The tolerogenic adjuvants described in this review are grouped in five categories based on their properties and mechanism of action: general immunosuppressive agents (Table 1), cytokines and neuropeptides (Table 2), vitamins and vitamin derivatives (Table 2), modulators of contact-dependent immune cell signaling (Table 3), and other (Table 4). In tolerogenic vaccines using a combination of adjuvants, tolerance can be mediated by multiple mechanisms (Table 5). Tolerogenic vaccines in human clinical trials are discussed for each category and summarized in Table 6.

General immunosuppressive agents include molecules with broad immunosuppressive effects on multiple aspects of the immune system, often by suppressing intracellular signaling pathways to reduce inflammation-induced gene expression (Figure 1B). General immunosuppressive tolerogenic adjuvants are used both to promote tolerogenic features on antigen presenting cells (APCs) and to suppress effector T and B cell function.

Cytokines orchestrate the balance between immune activation and regulation, making them valuable tools as tolerogenic adjuvants to shape antigen-specific immune responses. Classical anti-inflammatory cytokines like transforming growth factor β (TGF-β), and interleukin (IL)-10 exert potent immunosuppressive functions affecting many aspects of the immune system, including the capacity to induce tolDCs and Tregs (Figures 1C, D). In addition to classical anti-inflammatory cytokines, cytokines affecting specific immune cell subsets or skewing immune balance can also be harnessed as tolerogenic adjuvants.

Many vitamins are important for maintaining a healthy immune system. Vitamins A and D signal in a hormone-like manner via nuclear receptors and are potent immunoregulators (10). Some of their immunomodulatory effects are inhibition of T effector cell activation and promotion of tolerogenic APCs (Figures 1C, D).

Modulating the immune synapse, the dynamic interface between APCs and T cells during antigen recognition, offers a promising approach to inducing immune tolerance (Figure 1D). By manipulating the immune synapse, one can modify T cell receptor (TCR) signaling strength to skew T cell responses or induce anergy or apoptosis (11). In addition to TCR signaling, modulation of APC co-stimulatory or co-inhibitory molecules in the immunological synapse can also be utilized to modify the outcome of APC-T cell interactions (12). (Figures 1D, E).

In addition to the adjuvant types described above, there are several other types of adjuvants. Described here are apoptotic remnant mimics (Figure 1F), Toll-like receptor (TLR) agonists and glycans and glycan-binding proteins which may promote tolerogenic immune responses.

Depot adjuvants and delivery systems including cell targeting moieties and nanoparticles/microparticles inherently act as adjuvants since they enhance the immune responses to incorporated antigens (2, 3, 13, 14). Cell targeting strategies which promote antigen delivery to dendritic cells often have tolerogenic properties (3, 14) and can be considered tolerogenic adjuvants. However, in this review we specifically explore adjuvants with tolerogenic properties that actively modify the immune milieu to facilitate shifts in immune cell subsets and/or phenotypes.

2 Tolerogenic adjuvants

2.1 Immunosuppressive agents

2.1.1 Dexamethasone

Glucocorticoids are potent anti-inflammatory and immunosuppressive agents used for treatment of autoimmune and inflammatory conditions (15–17). They mediate their effects through engagement with the nuclear glucocorticoid receptor inducing transcriptional regulation or rapid non-genomic effects (15, 17). In immune cells, the synthetic glucocorticoid dexamethasone suppresses production of most cytokines while increasing production of IL-10, inhibits lymphocyte activation and promotes lymphocyte apoptosis (15). Glucocorticoids dampen overall T cell activation by interfering with TCR signaling, and evidence suggests that glucocorticoids preferentially suppresses Th1 and Th17 T cells. Glucocorticoid treatment is also associated with increased circulating Tregs (15). In APCs, glucocorticoids induce tolerogenic features including attenuated DC maturation and reduced expression of MHC class II and co-stimulatory molecules (15, 18).

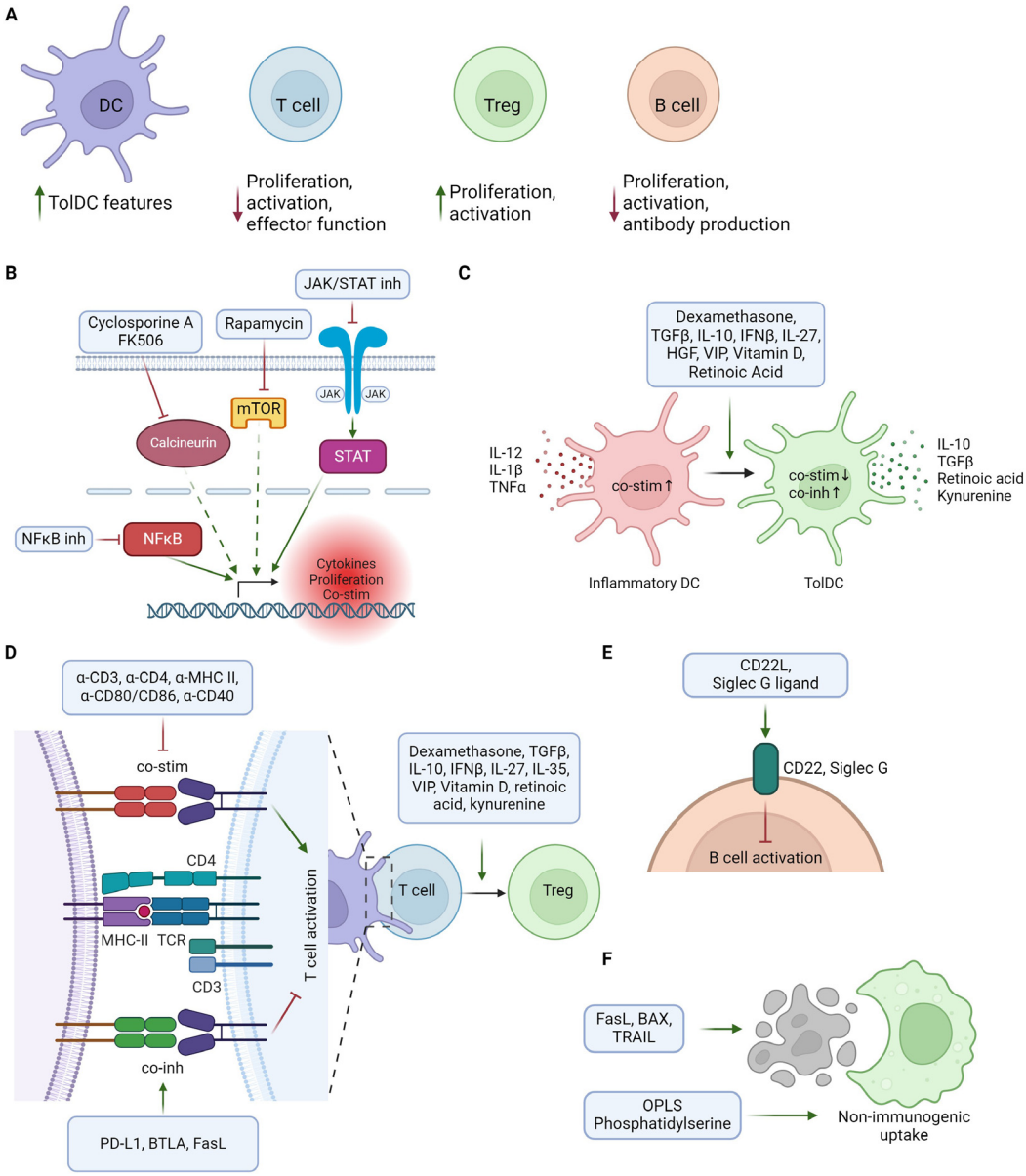

FIGURE 1
Schematic overview of tolerogenic adjuvants mechanisms of action. **(A)** Overview of main tolerogenic effects of adjuvants on different immune cell types, including suppression of immune activation and promotion of tolerogenic/immunoregulatory cells. **(B)** Immunoregulation through direct or indirect suppression of pro-inflammatory gene expression. **(C)** Adjuvants supporting differentiation from inflammatory DCs (red) to tolerogenic DCs (green). TolDCs are characterized by lower levels of co-stimulatory and increased levels of co-inhibitory molecules, together with release of Treg-promoting cytokines and metabolites. **(D)** Regulation of T cell responses by modulation of the immunological synapse or Treg promoting factors. Tolerogenic adjuvants can suppress T cell activation by inhibiting co-stimulation and/or promoting co-inhibition in the interaction between T cells and antigen presenting cells. Treg differentiation can be enhanced both by APC-T cell interactions and by Treg promoting factors acting on T cells. **(E)** Suppression of B cell activation by activation of inhibitory receptors. **(F)** Promoting non-immunogenic uptake of tolerogenic vaccine by mimicking or inducing apoptotic cells. Created in BioRender.com.

Tolerogenic vaccines consisting of antigen delivered with dexamethasone have been successful in murine models of experimental autoimmune encephalitis (EAE) (19–22), atherosclerosis (23), antigen-induced arthritis (AIA) (24) and type 1 diabetes (T1D) (25). Other tolerogenic vaccines use dexamethasone to induce tolDCs, which when loaded with disease relevant antigens and injected reduced disease in models of arthritis (26, 27), dust mite allergy (28) and T1D (29). The disease inhibition was often accompanied by an increase in Tregs over effector T cells and increase in anti-inflammatory cytokines. However, tolDC-mediated disease suppression in one arthritis model was independent of antigen (27) and antigen-loaded dexamethasone-derived tolDCs exacerbated a model of T1D while unloaded tolDCs suppressed disease (30) (Table 1).

Dexamethasone is often used together with other adjuvants to induce tolDCs. TolDC vaccines of dexamethasone in combination

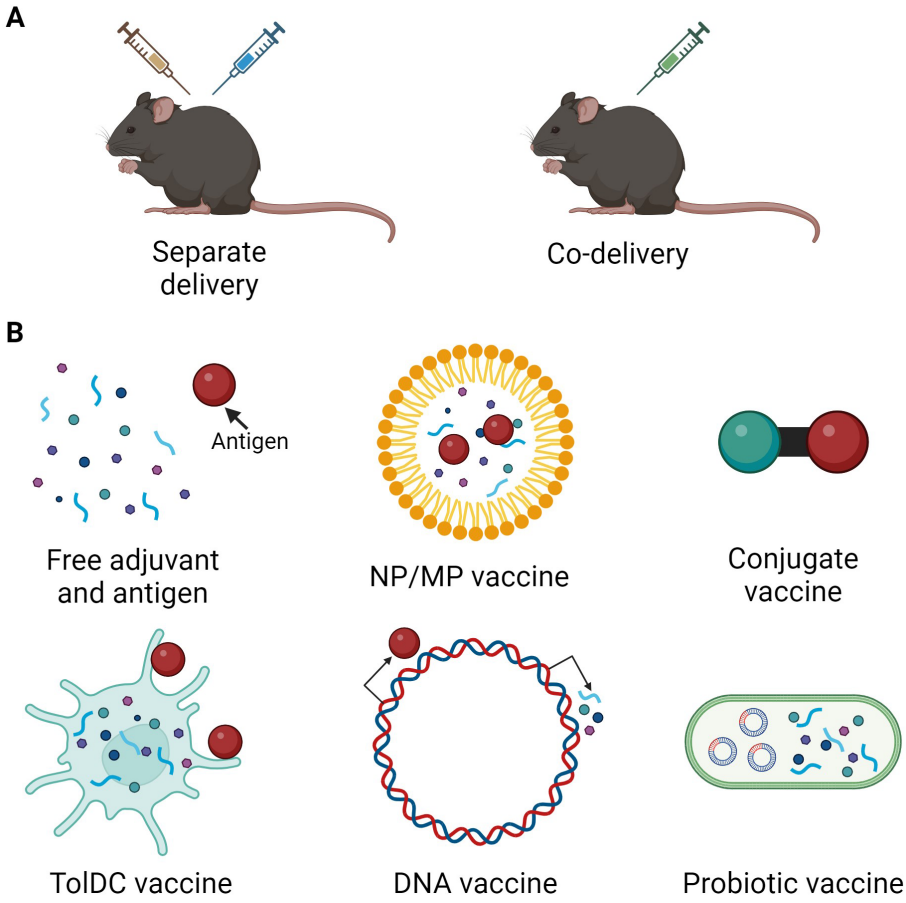

FIGURE 2
Overview of tolerogenic vaccine design and delivery. **(A)** Tolerogenic vaccine delivery types. **(B)** Different types of tolerogenic vaccines include administration of free adjuvant and antigen, incorporation of antigen and/or adjuvant in nanoparticles (NP) or microparticles (MP), conjugates or fusion molecules of antigen and adjuvant, antigen-loading of adjuvant-treated TolDCs, delivery of DNA vectors encoding antigen and/or adjuvant, and probiotic bacteria engineered to express antigen and/or adjuvant. Created in BioRender.com.

with one or more other adjuvants suppressed disease in models of EAE (31, 32) and systemic lupus erythematosus (SLE) (33) (Table 5).

Dexamethasone-containing tolerogenic vaccines have been investigated for human use. A phase I clinical trial investigated transfer of antigen-loaded dexamethasone-treated monocyte-derived DCs for treatment of multiple sclerosis and neuromyelitis optica spectrum disorders, by intravenous injection of tolDC every two weeks for a total of three doses. The tolDC vaccine was well tolerated and there was an increase of regulatory Tr1 cells at 12 weeks of follow-up (34). In another phase I trial, antigen-loaded autologous monocyte-derived DCs treated with a combination of dexamethasone, the vitamin D derivative calcitriol, and the TLR4 agonist MPLA were administered into inflamed knee joints. The treatment was well tolerated but did not result in reduction in disease severity or consistent immunoregulatory features (35) (Table 6).

2.1.2 Rapamycin

Rapamycin is a small molecule inhibitor of mTOR, a kinase that regulates cell growth and metabolism. Immunosuppressive effects of rapamycin includes suppression of the activation and proliferation of conventional T cells, promotion of T cell anergy or deletion, and enhancement of the development and function of Tregs (36). Additionally, rapamycin can inhibit the differentiation and maturation of DCs and promote tolDC features (37). Rapamycin is clinically used as an immunosuppressant for prevention of organ transplant rejection, as well as an anti-cancer drug.

Tolerogenic nanoparticle vaccines with antigen and rapamycin have successfully reduced disease in multiple models of autoimmunity including arthritis (38), Alzheimer's disease (39), vitiligo (40), allergic airway disease (41, 42), T1D (43), and EAE (44, 45). Rapamycin-containing nanoparticle vaccines also prevented anti-drug antibody responses toward coagulation factor VIII (FVIII) (45, 46), uricase (47), adalimumab (anti-TNFα) (47), and adenoviral vectors used in gene therapy (48–50). Administration of rapamycin without nanoparticle carrier suppressed OVA-induced skin graft rejection (51) and anti-FVIII anti-drug antibodies in mice (52). Observed immunological alterations in studies of tolerogenic vaccines with rapamycin included increased Tregs, decreased levels of inflammatory cytokines, and/or decreased co-stimulatory molecules on DCs (Table 1).

Two phase Ia and phase Ib clinical trials were conducted investigating administration of rapamycin together with

TABLE 1 Immunosuppressive adjuvants.

Adjuvant	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
Dexamethasone	$PLP^{139-151}$	Conjugate Vaccine: Adj-Ag	Prophylactic: s.c.	EAE	↓ Disease Incidence ↓ IL-2+ Cells	(19)
Dexamethasone	MOG^{35-55}	Conjugate Vaccine: Adj-Ag	Prophylactic: s.c.	EAE	↓ Disease Score ↓ T Cell Responses ↓ Ag-Th17 ↑ TolDC	(20)
Dexamethasone	$HSP60^{292-308}$	Separate Delivery Vaccine: Adj and Ag	Prophylactic: Adj i.m. Ag s.c.	Atherosclerosis	↓ Disease Severity ↓ T Cell responses ↑ Ag-Tregs ↑ B-1	(23)
Dexamethasone	$InsB^{9-23}$	Separate Delivery Vaccine: Adj and Ag	Prophylactic: foot pad injection	T1D	↓ Disease Incidence ↑ TolDC ↑ Tregs, IL-10 Tregs	(25)
Dexamethasone	MOG^{35-55}	MP Vaccine: MPs containing Adj and Ag	Therapeutic: s.c.	EAE	↓ Disease Incidence ↓ IL-17, GM-CSF ↑ TolDC	(22)
Dexamethasone	Human proteoglycan	MP Vaccine: MPs containing Adj and Ag	Prophylactic: i.v. Therapeutic: i.v.	AIA	↓ Disease Incidence ↓ Disease Score ↓ Ag-antibodies ↑ Tr1 ↑ TolDC	(24)
Dexamethasone	B. tropicalis	TolDC Vaccine: BMDC treated with Adj, loaded with Ag	Prophylactic: i.p.	Allergy	↓ Cell Infiltration ↓ Eosinophils ↓ Ag-Antibodies, IgE ↓ IFN-γ, IL-4 ↑ Tregs	(28)
Dexamethasone	MOG^{35-55}	NP Vaccine: NPs containing Ag-Adj conjugates	Therapeutic: i.v.	EAE	↓ Disease Score (with and without Dexamethasone) ↑ TolDC ↑ Tregs	(21)
Rapamycin	Amyloid β	NP Vaccine: NPs containing Adj and Ag	Therapeutic: i.v.	Alzheimer's	↓ Cognitive Decline ↓ IFN-γ ↑ TGF-β, IL-10, Arg1 ↑ Tregs ↑ TolDC	(39)
Rapamycin	Citrullinated proteins	NP Vaccine: NPs containing Adj and Ag	Therapeutic: i.v Prophylactic: i.v.	CIA, AIA	↓ Disease Score ↓ IFN-γ, IL-17, TNF, IL-1β ↑ TGF-β, IL-10 ↑ Tregs	(38)
Rapamycin	HEL^{46-61}	NP Vaccine: NPs containing Adj and Ag	Prophylactic: i.v.	Vitiligo	↓ Disease Score ↓ Ag-T Responses ↓ IL-6, IFN-γ ↑ IL-10 ↑ Tregs ↑ TolDC	(40)
Rapamycin	OVA	NP Vaccine: NPs containing Adj and Ag	Prophylactic: i.v.	Anaphylaxis, Allergy	↓ Disease Score ↓ Ag-Antibody ↓ Eosinophils, Neutrophils ↓ IL-4, IL-5 ↑ Tregs ↑ TGF-β	(41)
Rapamycin	P31	NP Vaccine: NPs containing Adj and Ag	Prophylactic: s.c.	T1D	↓ Disease Incidence ↑ Tregs	(43)
Rapamycin	$PLP^{139-151}$ OVA FVIII	NP Vaccine: NPs containing Adj and Ag	Therapeutic: i.v. and s.c. Prophylactic: i.v. and s.c.	EAE, Anaphylaxis, ADA	↓ Disease Score ↓ Ag-Antibody ↓ Eosinophils ↑ Ag-Tregs	(44, 45)

(Continued)

TABLE 1 Continued

Adjuvant	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
Rapamycin	FVIII	NP Vaccine: NPs containing Adj and Ag	Therapeutic: i.v. Prophylactic: i.v	ADA	↓ Ag-Antibody	(46)
Rapamycin	Uricase Adalimumab (anti-TNFα)	Co-Delivery Vaccine: NPs containing Adj and free Ag	Prophylactic: s.c.	ADA	↓ Ag-Antibody ↓ Disease Score	(47)
Rapamycin	$OVA^{323-339}$	Separate Delivery Vaccine: Adj and Ag	Prophylactic: i.p.	Graft rejection	↓ Graft Rejection ↑ Ag-Tregs	(51)
Rapamycin	FVIII	Separate Delivery Vaccine: Adj and Ag	Prophylactic: i.v.	ADA	↓ Ag-Antibody ↓ IL-6, IL-2, IL-4 ↑ Tregs ↑ CTLA-4 ↑ TGF-β	(52)
Rapamycin	OVA	NP Vaccine: NPs containing Adj and Ag	Prophylactic: s.c.	Allergy	↓ Ag-Antibody ↓ Eosinophils, Cell Infiltration ↓ Th17, Th2 ↑ Tregs ↑ IL-10+ T Cells	(42)
Rapamycin	AAV8	NP Vaccine: NPs containing Adj, separate Ag vector	Prophylactic: i.v.	ADA	↓ Ag-Antibody ↓ B and T Cell Activation	(48)
Cyclosporine A	$GAD65^{206-220}$ $GAD65^{536-55}$ $InsB^{9-23}$ $InsC^{17-A1}$	Co-Delivery Vaccine: Free Ag and Adj	Prophylactic: s.c.	T1D	↓ Disease Incidence ↓ T Cell Responses ↓ TNF-α+, IL-2+ Cells ↓ Th1 ↑ Ag-Tregs ↑ IL-10 ↑ TolDC	(56)
FK506	CII	TolDC Vaccine: MoDC treated with Adj, loaded with Ag	Therapeutic: i.p.	CIA	↓ Disease Score ↓ T Cell Responses ↓ Th17	(58)
FK506	MOG^{35-55}	DNA Vaccine/Co-delivery Vaccine: Adj and separate DNA Ag construct	Prophylactic: i.m.	EAE	↓ Disease Score ↓ T Cell Infiltration ↓ Th17, ↓ IFN-γ ↑ Tregs ↑ IL-4	(57)
Kynurenine	GAD65	Co-Delivery Vaccine: Adj and Ag phage vaccine	Prophylactic: s.c.	T1D	↓ Disease Incidence ↓ Ag T Cell Responses ↓ DC Activation ↓ IFNγ, IL-2 ↑ Tregs ↑ IL-10, TGF-β	(70)
ITE	MOG^{35-55} $PLP^{139-151}$ Proinsulin	NP Vaccine: NPs containing Adj and Ag	Therapeutic: i.p. Prophylactic: i.p.	EAE	↓ Disease Score ↓ Ag-Response ↓ IFN-γ, IL-17 ↓ Th17 ↑ TolDC, Tregs	(67–69)
Andrographolide	FVIII	TolDC Vaccine: BMDC treated with Adj, loaded with Ag	Prophylactic: i.v.	ADA	↓ Ag-Antibody ↓ IL-4, IFN-γ ↑ Tregs	(61)
A20	OVA	NP Vaccine: Adj and Ag	Therapeutic: nasal	Asthma	↓ Disease Score ↓ Ag- IgE ↓ IL-4, IL-5, IL-13 ↑ Foxp3	(63)
BAY 11-7082	mBSA	TolDC Vaccine: BMDC treated with Adj, loaded with Ag	Therapeutic: s.c.	AIA	↓ Disease Score ↓ Ag-Antibody	(60)

(Continued)

TABLE 1 Continued

Adjuvant	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
Tofacitinib	MOG^{35-55}	TolDC Vaccine: BMDC treated with Adj, loaded with Ag	Prophylactic: i.v.	EAE	↓ Disease Score ↓ Ag-T Cell Responses ↓ Th17, Th1 ↑ Tregs	(72)
BD750	MOG^{35-55}	TolDC Vaccine: BMDC treated with Adj, loaded with Ag	Prophylactic: i.v.	EAE	↓ Disease Score ↓ T Cell Responses ↓ Th17, Th1 ↓ IFN-γ, IL-17 ↑ Tregs ↑ IL-10	(73)
Rosiglitazone	CII	TolDC Vaccine: BMDC treated with Adj, loaded with Ag	Therapeutic: s.c.	CIA	↓ Disease Score ↓ Th1 ↑ Tregs	(75)
K313	MOG^{35-55}	TolDC Vaccine: BMDC treated with Adj, loaded with Ag	Therapeutic: i.v.	EAE	↓ Disease Score ↓ Th17, Th1 ↑ Tregs	(77)
Iloprost	$OVA^{323-339}$	TolDC Vaccine: BMDC treated with Adj, loaded with Ag	Prophylactic: intrathecally	Allergy	↓ Disease Score ↓ Infiltrating Cells ↓ Eosinophils ↓ IL-5, IL-4, IL-13, IFN-γ ↑ Tregs	(80)

AAV8, Adeno-associated virus 8; ADA, Antidrug antibody; Adj, Adjuvant; Ag, antigen; AIA, antigen-induced inflammatory arthritis; BMDC, bone marrow-derived dendritic cells; CD, cluster of differentiation; CIA, collagen-induced arthritis; CII, type II collagen; DC, dendritic cell; DNA, deoxyribonucleic acid; EAE, experimental autoimmune encephalomyelitis; FOXP3, forkhead box P3; FVIII, Factor VIII; GAD65, glutamic acid decarboxylase; HEL, Hen egg lysozyme; HSP60, Heat shock protein 60; IFN-γ, interferon-γ; i.m., intramuscular; ITE, 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester; IL, interleukin; Ins, Insulin; i.p., intraperitoneal; i.v., intravenous(ly); mBSA, Methylated bovine serum albumin; MOG, myelin oligodendrocyte glycoprotein; MP, microparticle; NP, nanoparticle; OVA, ovalbumin; PBMCs, peripheral blood mononuclear cells; PLP, proteolipid protein; s.c., subcutaneous(ly); T1D, type 1 diabetes; Th; T helper; TGF-β, Transforming Growth Factor-β; TNF, tumor necrosis factor; Tr1, type 1 regulatory T cell; Treg, regulatory T cell; TolDC, tolerogenic dendritic cell.
↓, decrease; ↑, increase.

TABLE 2 Cytokine and vitamin adjuvants.

Adjuvant	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
TGF-β	PLP	NP Vaccine: Adj and Ag	Therapeutic: i.v. Prophylactic: i.v.	EAE	↓ Disease Score ↓ T Cell Responses ↓ DC Activation ↓ IL-6, IL-12 ↑ Tregs ↑ TGF-β	(83)
TGF-β	MOG	DNA Vaccine: Adj and Ag constructs	Prophylactic: i.d.	EAE	↓ Disease Score ↓ T Cell Infiltration ↓ Th17, Th1 ↓ INF-γ, IL-17	(94)
TGF-β	FVIII	TolDC Vaccine: BMDCs treated with Adj, loaded with Ag	Prophalctic: i.v.	ADA	↓ Ag-Antibody ↓ IL-2 ↑ Tregs ↑ IL-10	(85)
TGF-β signaling agonist (T74)	CII	TolDC Vaccine: BMDC treated with Adj, loaded with Ag	Therapuetic: s.c.	CIA	↓ Disease Score ↓ INF-γ ↓ Th1, Th17 ↑ Tregs ↑ IL-10	(84)
IL-10	MBP^{68-86}	DNA Vaccine: Plasmid encoding Adj and plasmid encoding Ag	Therapeutic: i.p. Prophylactic: i.p.	EAE	↓ Disease Score ↓ Ag-T Cell ↑ IL-10	(103)

(Continued)

TABLE 2 Continued

Adjuvant	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
IL-10	MOG^{35-55}	NP Vaccine: Separate NPs with Adj and Ag	Prophylactic: s.c.	EAE	↓ Disease Score ↓ T Cell Infiltration ↓ IFN-γ, IL-17	(101)
IL-10	OVA	TolDC Vaccine: BMDCs retrovirally transduced with IL-10 construct, loaded with Ag	Prophylactic: i.t. Therapeutic: i.t.	Asthma	↓ Disease Score ↓ Cell Infiltration ↓ IFN-γ, IL-4 ↓ Autoantibodies ↑ Tregs	(98)
IL-10	$GAD65^{190-315}$	DNA Vaccine: Adj and Ag construct	Prophylactic: i.m.	T1D	↓ Disease Incidence ↑ IL-10, IL-4 ↑ Th2 ↑ Ag-Tregs	(102)
IL-10	MOG^{35-55}	TolDC Vaccine: BMDCs treated with adj, loaded with Ag	Prophylactic: i.v.	EAE	↓ Disease Score ↓ Ag-T Cell Responses	(100)
IL-10	$InsB^{4-29}$ BDC2.5mi	TolDC Vaccine: BMDCs transduced with IL-10 construct, loaded with Ag	Prophylactic: i.p.	T1D	↓ Disease Incidence ↑ Tr1	(99)
IL-2	IRBP	Separate Delivery Vaccine: Free Ag and Adj	Prophylactic: Ag orally adj i.p.	EAU	↓ Disease Score ↑ TGF-β, IL-10, IL-4	(111)
IL-2	MBP^{69-88}	Conjugate Vaccine: Adj-Ag fusion protein	Therapeutic: s.c. Prophylactic: s.c.	EAE	↓ Disease Score	(109)
IL-2/anti-IL-2 complex	OVA	Separate Delivery Vaccine: Free Ag and Adj	Prophylactic: i.v.	DTH	↓ Disease Score ↑ Tregs	(112)
IL-2/anti-IL-2 complex	BDC2.5mi	Separate Delivery Vaccine: Free Ag and Adj	Prophylactic: i.p.	T1D	↓ Disease Incidence ↑ Ag-Tregs ↑ Treg Function	(110)
IL-2/anti-IL-2 complex	FVIII	Separate Delivery Vaccine: Free Ag and Adj	Prophylactic: Adj i.p., Ag i.v.	ADA	↓ Ag-Antibodies ↓ Ag-T Cell Responses ↑ Tregs	(113)
GM-CSF	MOG^{35-55} $PLP^{139-151}$ MBP^{68-87}	Conjugate Vaccine: Adj-Ag fusion protein	Therapeutic: s.c. Prophylactic: s.c.	EAE	↓ Disease Incidence ↑ Ag-Tregs	(125–129)
GM-CSF	$IRBP^{161-180}$	TolDC Vaccine: BMDCs treated with adj, loaded with Ag	Prophylactic: s.c.	EAU	↓ Disease Score ↓ IL-2, IFN-γ ↑ IL-4, IL-5	(130)
IFN-β	MOG^{35-55} $PLP^{178-191}$	Co-Delivery Vaccine: Adj and Ag in Alum or Adj-Ag fusion protiens	Therapeutic: s.c. Prophylactic: s.c.	EAE	↓ Disease Score ↑ Ag Tregs	(120, 121)
IL-35	MOG^{35-55}	TolDC Vaccine: Transformed DC line transduced with IL-35 construct, loaded with Ag	Prophylactic: i.v.	EAE	↓ Disease Score ↓ Th1 ↓ T Cell Responses	(133)
IL-35-Ig	HY peptide	TolDC Vaccine: Splenic DCs transduced with IL-35-Ig construct, loaded with Ag	Prophylactic: i.v.	DTH	↓ Disease Score ↑ $CD39^+$ Tregs ↑ Arginase 1	(134)
IL-27	MOG^{35-55} $PLP^{178-191}$	TolDC Vaccine: BMDCs treated with adj, loaded with Ag	Therapeutic: i.v. Prophylactic: i.v.	EAE	↓ Disease Score ↓ Ag-T Cell Responses ↓ IFN-γ, IL-17 ↑ Tregs ↑ IL-10, TGF-β	(136)
IL-4	GAD^{65}	DNA Vaccine: Transgenic plant leaf expressing Ag + Adj	Prophylactic: oral	T1D	↓ Disease Incidence ↓ IFN-γ ↑ Regulatory Cells ↑ IL-4	(141)

(Continued)

TABLE 2 Continued

Adjuvant	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
IL-4	GAD^{65}	DNA Vaccine: Ag-IgGFc, fusion and Adj constructs	Prophylactic: i.m.	T1D	↓ Disease Incidence ↓ IFN-γ ↑ IL-4, IL-5	(142)
IL-4	PLP$^{139-151}$ MOG	DNA Vaccine: Adj and Ag construct	Therapeutic: i.m. Prophylactic: i.m.	EAE	↓ Disease Score ↓ Ag-T Cell Response ↓ IFN-γ ↑ IL-4, IL-10	(139)
IL-4	CII	DNA Vaccine: Adj + Ag construct	Prophylactic: i.m.	CIA	↓ Disease Score ↓ TNF, IFN-γ	(140)
HGF	MOG$^{35-55}$	TolDC Vaccine: Primary DCs treated with Adj, loaded with Ag	Therapeutic: i.v.	EAE	↓ Disease Score ↓ Infiltrating Cells ↓ Th17, Th1 ↓ IFN-γ, IL-17 ↑ Tregs ↑ IL-10, TGF-β	(144)
VIP	CII MOG	TolDC Vaccine: BMDCs treated with adj, loaded with Ag	Therapeutic: i.v.	CIA EAE	↓ Disease Score ↓ Ag-response, Ag-antibodies ↓ IFN-γ ↑ Tr1 ↑ IL-10	(146)
TRAIL	MOG$^{35-55}$	TolDC Vaccine: BMDC treated with Adj plasmid and Ag plasmid	Therapeutic: i.v.	EAE	↓ Disease Score ↓ Ag-Response ↓ Cell Infiltration ↑ Tregs	(148, 149)
BAFF-siRNA	CII	TolDC Vaccine: BMDC transduced with Adj construct, loaded with Ag	Therapeutic: i.v.	CIA	↓ Disease Score ↓ Ag-antibody ↓ Rorγt ↓ IL-17, IL-1β, IL-6, IL-12 ↑ Foxp3 ↑ IL-10	(151)
VitD	MOG MOG$^{35-55}$	TolDC Vaccine: BMDC treated with Adj, loaded with Ag or Ag-mRNA	Therapeutic: i.v.	EAE	↓ Disease Score ↓ Ag-T Response	(156)
VitD	MOG$^{40-55}$	TolDC Vaccine: BMDC treated with Adj, loaded with Ag	Therapeutic: i.v. Prophylactic: i.v.	EAE	↓Disease Score ↓ Ag-response ↑ IL-10 ↑ Treg ↑ Bregs	(153)
VitD	MOG$^{35-55}$	Separate Delivery Vaccine: Adj and Ag	Prophylactic: i.p.	EAE	↓Disease Score ↓ IL-6, IL-17, TNF, IFN-γ ↑ TGF-β ↑ TolDC	(159)
VitD	MOG$^{35-55}$	TolDC Vaccine: BMDC treated with Adj, loaded with Ag	Therapeutic: i.v.	EAE	↓ Disease Score ↓ Th1, Th17 ↑ Bregs ↑ IL-10^{+} T Cells ↑ Tregs ↑ TolDC	(155)
VitD analog	IGRP$^{206-214}$	NP Vaccine: Adj and Ag	Prophylactic: s.c.	T1D	↓ Disease Incidence ↓ TNF, IFN-γ ↑ TolDC	(158)
VitD analog	OVA	Separate Delivery Vaccine: Adj and Ag	Prophylactic: Topical Adj, epicutaneous Ag	DTH	↓ Swelling ↓ Ag-T Response ↓ IFN-γ ↑Tregs	(161)

(Continued)

TABLE 2 Continued

Adjuvant	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
VitD analog	BDC2.5 mimotope	NP Vaccine: Adj + Ag	Prophylactic: s.c.	T1D	↓ Disease Incidence ↓ INF-γ ↑ Ag-Tregs	(157)
Retinoic acid	MOG	NP/MP Vaccine: Adj + Ag	Prophylactic: s.c	EAE	↓ Disease Score ↓ IL-17A, ↑ Ag-Tr1	(167)

ADA, Antidrug antibody; Adj, Adjuvant; Ag, antigen; BMDC, bone marrow-derived dendritic cells; CD, cluster of differentiation; CIA, collagen-induced arthritis; CII, type II collagen; DTH, Delayed type hypersensitivity; DC, dendritic cell; DNA, deoxyribonucleic acid; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; FOXP3, forkhead box P3; FVIII, Factor VIII; GAD65, glutamic acid decarboxylase; HEL, Hen egg lysozyme; i.d., intradermal; IFN-γ, interferon-γ; IGRP, Islet-specific glucose-6-phosphatase catalytic subunit-related protein; i.m., intramuscular; IRBP, Interphotoreceptor retinoid-binding protein; IL, interleukin; Ins, Insulin; i.p., intraperitoneal; i.v., intravenous(ly); MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MP, microparticle; NP, nanoparticle; OVA, ovalbumin; PLP, proteolipid protein; s.c., subcutaneous(ly); T1D, type 1 diabetes; Th; T helper; TGF-β, Transforming Growth Factor-β; TNF, tumor necrosis factor; Tr1, type 1 regulatory T cell; Treg, regulatory T cell; TolDC, tolerogenic dendritic cell.
↓, decrease; ↑, increase.

TABLE 3 Modulators of contact-dependent immune cell signaling.

Adjuvant	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
α-CD3	$InsB^{9-23}$	DNA Vaccine: Adj + Ag vector	Prophylactic: i.v.	T1D	↓ Disease Incidence	(170)
α-CD4 (non-depleting)	FVIII	Separate Delivery Vaccine: Injection of Ag in Alum + Adj	Prophylactic: Anti-CD4 i.p. or i.v. and FVIII s.c. or i.p	ADA	↓ Ag-Antibody	(172)
α-CD4 (depleting)	$IRBP^{1-20}$ arrestin MOG^{35-55}	Separate Delivery Vaccine	Therapeutic: i.p.	EAU	↓ Disease Score ↓ Ag-Response ↓ Th1, Th17 ↓ IL-17, IFN-γ ↑ TGF-β, IL-10 ↑ Tregs	(173)
Tregitope	Preproinsulin	NP Vaccine: Adj + Ag	Prophylactic: i.p.	T1D	↓ Disease Incidence	(174)
Tregitope	Preproinsulin	NP Vaccine: Adj-albumin fusion protein + Ag	Therapeutic: s.c.	T1D	↓ Severe Disease Incidence ↑ Mild Disease Reversal	(175)
α-GalCer	$InsB^{9-23}$	NP Vaccine: Adj + Ag	Prophylactic: i.p.	T1D	↓ Disease Incidence ↑ Foxp3	(177)
α-CD40L	FVIII	Co-Delivery Vaccine: Adj + Ag	Prophylactic: i.v.	ADA	↓ Ag-Antibody ↓ Ag-T Cell Responses ↓ IL-2, IL-4, IIFN-γ	(182)
mutant B7.1/CD40L	Proinsulin	DNA Vaccine: encoding membrane bound Ag and Adj fusion protein	Prophylactic: i.m.	T1D	↓ Disease Incidence	(184)
CD40, CD80 and CD86 knockdown	BDC2.5 mimotope	NP Vaccine: encapsulating pCAS9 DNA and CD80, CD86 and CD40 gRNA	Prophylactic: i.v.	T1D	↓ Disease Incidence ↓ IL-17,IFN-γ, IL-6 ↑ IL-10 ↑ Ag-Tregs ↑ TolDC	(183)
LFA-1 peptide (ICAM-1 Inhibitor)	$PLP^{139-151}$	Co-Delivery Vaccine: Adj + Ag on hyaluronic acid backbone	Prophylactic: s.c.	EAE	↓ Disease Score	(186–188)
LFA-1 peptide (ICAM-1 Inhibitor)	$PLP^{139-151}$ MOG^{38-50} GAD65	Conjugate Vaccine: Adj -Ag	Prophylactic: s.c. or i.v.	EAE T1D	↓ Disease Score ↓ Ag-Response ↓ IFN-γ, IL-6 ↓ Th17 ↑ TolDC ↑ Tregs	(189, 190)

(Continued)

TABLE 3 Continued

Adjuvant	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
α-OX40 (Agonist)	$InsB^{9-23}$	Co-Delivery Vaccine: Adj and Ag	Prophylactic: i.n.	T1D	↓ Disease Incidence ↑ Tregs ↑ IL-10	(192)
PD-L1	MOG^{35-55}	TolDC Vaccine: BMDC treated with Adj plasmid and Ag Plasmid	Prophylactic: i.v.	EAE	↓ Disease Score ↓ Ag-Response ↓ Cell Infiltration	(148, 149)
BTLA	MOG^{35-55}	TolDC Vaccine: BMDC treated with Adj plasmid treated with Ag load NP	Prophylactic: i.p.	EAE	↓ Disease Score ↓ IFN-γ, IL-2 ↑ Tregs ↑ IL-10, TGF-β	(196)
CD22	FVIII	NP Vaccine: Incorporating Adj +Ag	Prophylactic: i.p.	Hemophilia	↓Bleeding ↓Ag-antibody	(201)
Siglec-GL	OVA HEL	NP Vaccine: incorporating Adj + Ag	Prophylactic: i.v.	Autoantibody	↓ Ag-antibody ↑ Ag-B Cell Responses	(202)

ADA, Antidrug antibody; Adj, Adjuvant; Ag, antigen; BMDC, bone marrow-derived dendritic cells; BTLA, B- and T-lymphocyte attenuator; CD, cluster of differentiation; DTH, Delayed type hypersensitivity; DC, dendritic cell; DNA, deoxyribonucleic acid; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; FOXP3, forkhead box P3; FVIII, Factor VIII; GAD65, glutamic acid decarboxylase; HEL, Hen egg lysozyme; IFN-γ, interferon-γ; IGRP, Islet-specific glucose-6-phosphatase catalytic subunit-related protein; i.m., intramuscular; IRBP, Interphotoreceptor retinoid-binding protein; IL, interleukin; Ins, Insulin; i.p., intraperitoneal; i.v., intravenous(ly); LFA-1, Lymphocyte function-associated antigen 1; MOG, myelin oligodendrocyte glycoprotein; MP, microparticle; NP, nanoparticle; OVA, ovalbumin; PLP, proteolipid protein; s.c., subcutaneous(ly); T1D, type 1 diabetes; Th; T helper; TGF-β, Transforming Growth Factor-β; TNF, tumor necrosis factor; Treg, regulatory T cell; TolDC, tolerogenic dendritic cell.
↓, decrease; ↑, increase.

TABLE 4 Other adjuvants.

Adjuvant	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
O-phospho-L-serine (OPLS)	FVIII	Co-Delivery Vaccine: Adj + Ag	Prophylactic: s.c.	ADA	↓ Ag-antibody ↑ TolDC	(207)
Phosphatidylserine	$InsA^{21}$ $InsB^{30}$	NP Vaccine: Phosphatidylserine-Liposomes with Ag	Prophylactic: i.p.	T1D	↓ Disease Incidence ↓ T Cell Responses ↑ Ag-CD4 T cells	(209)
Phosphatidylserine	FVIII	NP Vaccine: Phosphatidylserine-Liposomes with Ag	Prophylactic: s.c.	ADA	↓ Ag-Antibody	(208)
BAX	GAD55	Co-Delivery DNA Vaccine: DNA-Ag + DNA-Adj	Prophlactic: i.m. Therapeutic: i.d.	T1D	↓Disease Incidence ↓IFN-γ, TNF ↑Tregs	(211) (212)
LPS	MOG^{35-55}	TolDC Vaccine: BMDC treated with Adj and loaded with Ag	Therapeutic: i.v.	EAE	↓ Disease Score ↑ $CD127^{+}$ Tregs	(215)
Flagellin B	OVA Der p 2	Co-Delivery Vaccine: Adj + Ag	Prophylactic: i.n.	Allergy	↓ Disease Score ↓ Ag-Antibody ↓ Eosinophils ↓ IL-5, IL-4, IL-13, IFN-γ ↑ IL-10, TGF-β ↑ Tregs ↑ TolDC	(218, 219)
Flagellin B	Der p 2	Fusion Protein Vaccine: Adj-Ag fusion protein	Prophylactic: i.n.	Allergy	↓ Disease Score ↓ Ag-IgE Antibody	(220)
Flagellin A	OVA	Conjugate Vaccine: Adj-Ag	Prophylactic: i.p.	Allergy	↓ Disease Score ↓ Ag-Antibody ↓ IL-6, IL-4, IL-5, IFN-γ ↑ IL-10	(216, 217)

(Continued)

TABLE 4 Continued

Adjuvant	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
β-glucan	β-cell-Ag	Co-Delivery Vaccine: Adj + Ag	Prophylactic: i.v.	T1D	↓ Disease Incidence ↑ TolDC, Tregs	(224)
Mannan	Grass pollen Ag	Conjugate Vaccine: Adj-Ag	sublingual	Allergy	↑ IgG/IgE ratio ↑ IFN-γ/IL-4 ratio ↑ IL-10 ↑ Tregs	(226)
Mannan	Grass pollen Ag	Conjugate Vaccine: Adj-Ag	Skin prick test (human) Sublingual (mouse)	Allergy	↓ Skin Prick Test Area ↑ IL-10 ↑ Tregs	(225)
Galectin-1	MOG^{35-55}	TolDC vaccine: BMDC treated with Adj and loaded with Ag	Therapeutic: i.p.	EAE	↓ Disease Score ↓ IL-17, IFN-γ ↑ IL-10, IL-27	(230)

ADA, Antidrug antibody; Adj, Adjuvant; Ag, antigen; BMDC, bone marrow-derived dendritic cells; CD, cluster of differentiation; DC, dendritic cell; Der p 2, Dermatophagoides pteronyssinus; EAE, experimental autoimmune encephalomyelitis; FOXP3, forkhead box P3; FVIII, Factor VIII; GAD65, glutamic acid decarboxylase; IFN-γ, interferon-γ; i.n., intranasal; i.m., intramuscular; IL, interleukin; Ins, Insulin; i.p., intraperitoneal; i.v., intravenous(ly); MOG, myelin oligodendrocyte glycoprotein; NP, nanoparticle; OVA, ovalbumin; PLP, proteolipid protein; s.c., subcutaneous (ly); T1D, type 1 diabetes; Th; T helper; TGF-β, Transforming Growth Factor-β; TNF, tumor necrosis factor; Treg, regulatory T cell; TolDC, tolerogenic dendritic cell.
↓, decrease; ↑, increase.

TABLE 5 Multiple adjuvants.

Adjuvants	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
Dexamethasone + VitD	GAD65	TolDC Vaccine: Monocyte-derived DC treated with Adj and loaded Ag	Prophylactic: i.p.	T1D	↓ Disease Incidence ↓ IL-17, IFN-γ, IL-9, TNF ↑ Il-10 ↑ Tregs	(29)
Dexamethasone + Minocycline	MOG35-55	TolDC Vaccine: BMDC treated with Adj and loaded with Ag	Prophylactic: i.v.	EAE	↓ Disease Score ↓ Ag-T Response ↑ IL-10	(31)
Dexamethasone + abatacept	MOG^{35-55}	NP Vaccine: Particles carrying adj + Ag	Therapeutic: s.c. Prophylactic: s.c.	EAE	↓ Disease Score ↓ Cell Infiltration ↓ Th1, Th17 ↑ Tregs	(180)
Dexamethasone + anti-MHC II	MOG^{35-55}	Co-Delivery Vaccine: Adj + anti-MHC II nanobody-Ag fusion protein	Therapeutic: i.v.	EAE	↓ Disease Score	(178)
Rapamycin + CD22L	OVA	Co-Administration NP vaccine: LNP with CD22L and AG and separate PGLA NP with Rapamycin or combined	Prophylactic: i.v.	AIA	↓ Disease Incidence ↓ Ag-Antibody ↓ Ag-Plasma Cells ↑ Tregs	(205) (205) (204)
Rapamycin + IL-2/α-IL2 IC	BDC2.5mi	Co-Delivery Vaccine: Adj + Ag	Prophylactic: i.v.	T1D	↓ Disease Incidence ↑ Tregs ↑ IL-10, IL-4	(115)
Rapamycin + IL-2 mutein	HIP6.9 PDC-E2	NP Vaccine: containing Adj + Ag	Prophylactic: i.v.	T1D PBC	↓ Disease Incidence ↓ Disease Score ↓ Pathogenic T Cells ↓ IFN-γ, IL-6 ↑ Tregs ↑ IL-10, IL-4	(114)
Rapamycin + IL-2 fusion protein	MOG	NP Vaccine: containing rapamycin IL-2-α–IL2 fusion proteins and MHC class II/Ag	Therapeutic: i.v. or intra lymph node Prophylactic: i.v. or intra lymph node	EAE	↓Disease Score ↑ Tregs	(116)

(Continued)

TABLE 5 Continued

Adjuvants	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
IL-2 + Retinoic acid	MOG IRBP	Co-Delivery Vaccine: Adj + Ag	Prophylactic: s.c.	EAE EAU	↓ Disease Incidence ↑ Tr1 Cells ↓ Th17 ↓ IL-17, IFN-γ ↑ CTLA-4^{+} T Cells, IL-10^{+} T Cells	(117)
IFN-β + VitD	MOG$^{35-55}$	TolDC Vaccine: BMDCs treated with VitD and Ag and treatment of mice with IFN-β	Therapeutic: s.c. IFN-β and i.v. TolDCs	EAE	↓ Disease Score ↓ Th17 ↑ IL-10 ↑ Th2	(122)
IL-4 + Retinoic acid	MOG$^{35-55}$	Separate Delivery Vaccine: Free Ad and Adj	Prophylactic: Adj s.c., Ag i.p.	EAE	↓ Disease Score ↓ IL-17, IFN-γ	(168)
IL-10 + α-CD3	Proinsulin GAD65	Probiotic vaccine: L. lactis genetically modified to secrete IL-10 and proinsulin coadministered with α-CD3	Therapeutic: α-CD3 i.v. and intragastric inoculation of probiotic	T1D	↓ Reversed Disease ↓ IFN-γ ↑ IL-10 ↑ CTLA-4^{+} Tregs ↑ Tregs	(104–106)
IL-10 + TGF-β	CII	TolDC Vaccine: BMDC treated with Adj and loaded with Ag	Therapeutic: i.v.	CIA	↓ Disease Score ↓ Autoantibody ↓ IL-17, IFN-γ, IL-6, TNF ↑ TGF-β, IL-10 ↑ Tregs	(86)
IL-10 + TGF-β	FVIII	TolDC Vaccine: BMDC treated with Adj and loaded with Ag	Prophylactic: i.v.	ADA	↓ Ag-antibody	(87)
TGF-β + Retinoic acid	InsB$^{9-23}$	NP Vaccine: Containing Adj + Ag	Prophylactic: s.c.	T1D	↓ Disease Incidence	(93)
GM-CSF + CpG	Ins	MP Vaccine: Co-Delivered MP loaded with Ag and hydrogel loaded with Adj	Prophylactic: s.c.	T1D	↓ Disease Incidence ↓ Protein Urea ↑ IL-10	(131)
FasL + MCP-1	MOG$^{35-55}$ GAD$^{524-543}$	MP Vaccine: loaded with MCP-1, surface FasL and Ag	Therapeutic: i.v.	T1D EAE	↓ Disease Incidence ↓ Disease Score ↓ Pathogenic T Cells ↓ IFN-γ, IL-17, TNF, IL-6 ↑ Ag-Tregs	(197)
Leflunomide + phosphatidylserine	CII$^{250-270}$	NP Vaccine: Co-Delivery of Leflunomide Ag in Phosphatidylserine Lipid NPs.	Therapeutic: i.v.	CIA	↓Disease Score ↑Tregs	(210)
Dexamethasone + GM-CSF + VitD	Human proteoglycan	TolDC Vaccine: BMDCs treated with adj and Loaded with Ag	prophalactic: i.v.	AIA	Disease reducetion was antigen independent.	(27)
Dexamethasone + cobalt (III) protoporphyrin (CoPP) + Rosiglitazone	Histone	TolDC Vaccine: BMDC were treated with adjuvants and loaded with ag	Prophylactic: i.v.	SLE	↓ Disease Score ↓ Protein Urea ↓ Ag-Antibody	(33)
Dexamethasone + MPLA + VitD	GAD65	TolDC Vaccine: BMDC treated with Adj + Ag	Prophylactic: i.p.	T1D	↓ Disease Incidence *antigen-loading reduced TolDC suppressive effects	(30)
GM-CSF + TGF-β + VitD	MOG$^{35-55}$	MP Co-Delivery Vaccine: 1μM MP loaded with Ag or VitD and 30 μM particles loaded with GM-CSF or TGF-β.	Therapeutic: s.c. Prophylactic: s.c.	EAE	↓ Disease Score ↓ IL-17, GM-CSF, IL-6, IL-12, TNF ↑ TolDC	(89, 90)
GM-CSF + TGF-β + VitD	Ins	MP Co-Delivery Vaccine: 1μM MP loaded with Ag or VitD and 30 μM particles loaded with GM-CSF or TGF-β.	Therapeutic: s.c. Prophylactic: s.c.	T1D	↓ Disease Incidence ↑ Tregs ↑ TolDC	(88, 91, 92)

(Continued)

TABLE 5 Continued

Adjuvants	Antigen	Formulation	Administration	Disease Model	Major Results	Ref
IL-10 + IL-2 + TGF-β	Preproinsulin	DNA Vaccine: Single plasmid encoding Ag and Adj	Prophylactic: i.m.	T1D	↓ Disease Incidence ↓ IL-6 ↑ IL-10	(95)
Dexamethasone + CpG + SC-514 + Simvastatin	MOG^{35-55}	NP Vaccine: containing Adj + Ag	Prophylactic: i.p.	EAE	↓ Disease Score ↓ Ag-T Cells ↓ Ag-Antibody ↑ Ag-Tregs ↑ TolDC	(32)
TGF-β + α-Fas + CD47Fc+ PD-L1Fc	MOG^{40-54} MOG^{35-55}	NP Vaccine: MOG40–54/H-2Db-Ig dimer, MOG35–55/I-Ab multimer, anti-Fas, PD-L1-Fc and CD47-Fc and encapsulating transforming growth factor-β1	Therapeutic: i.v.	EAE	↓ Disease Score ↓ T Cell Infiltration ↓ Th1, Th17 ↑ Tregs ↑ IL-10, TGF-β ↑ Apoptotic T Cells	(198, 199)

ADA, Antidrug antibody; AIA, antigen-induced arthritis; Adj, Adjuvant; Ag, antigen; BMDC, bone marrow-derived dendritic cells; CD, cluster of differentiation; CIA, collagen-induced arthritis; CII, type II collagen; DC, dendritic cell; DNA, deoxyribonucleic acid; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; FOXP3, forkhead box P3; FVIII, Factor VIII; GAD65, glutamic acid decarboxylase; HEL, Hen egg lysozyme; HIP, Hsp70-interacting protein; IFN-γ, interferon-γ; i.m., intramuscular; IL, interleukin; Ins, Insulin; i.p., intraperitoneal; i.v., intravenous(ly); IRBP, Interphotoreceptor retinoid-binding protein; MOG, myelin oligodendrocyte glycoprotein; MP, microparticle; NP, nanoparticle; OVA, ovalbumin; s.c., subcutaneous(ly); PDC-E2, E2 component of the pyruvate dehydrogenase complex; SLE, systemic lupus erythematosus; T1D, type 1 diabetes; Th; T helper; TGF-β, Transforming Growth Factor-β; TNF, tumor necrosis factor; Tr1, type 1 regulatory T cell; Treg, regulatory T cell; TolDC, tolerogenic dendritic cell.
↓, decrease; ↑, increase.

pegadricase, a biological drug used for the treatment of gout, finding that rapamycin suppressed the development of anti-drug antibodies in a dose dependent manner (53). Their following phase III trial demonstrated high response rate, safety, and clinically meaningful reduction in serum urate (54). Another phase I clinical trial administered rapamycin-containing nanoparticles together with AAV8 capsid used in adenoviral gene therapies, leading to reduced development of anti-AAV8 antibodies compared to subjects receiving capsid without rapamycin (press release) (Table 6).

2.1.3 Calcineurin inhibitors

Cyclosporine A and FK506 (tacrolimus) are calcineurin inhibitors which suppress downstream nuclear factor of activated T cell (NFAT) signaling and IL-2 production (55). Tolerogenic vaccination with cyclosporine A and autoantigens prevented the development of T1D in pre-diabetic mice and promoted Tregs and tolDCs (56). Likewise, tolerogenic vaccination with FK506 co-delivered with DNA encoding autoantigen suppressed EAE (57), and adoptive transfer of antigen-loaded FK506-induced tolDCs reduced disease in a model of CIA (58), in both studies with a reduction in Th17 responses (Table 1).

2.1.4 NFκB inhibitors

Nuclear factor kappa B (NFκB) is a transcription factor inducing expression of pro-inflammatory genes including cytokines, cell adhesion molecules, and immunoreceptors. Inhibition of NFκB suppresses T cell responses and induces immunoregulatory features in APCs (59).

Tolerogenic vaccination with antigen-loaded tolDCs treated with NFκB inhibitor BAY 11-7082 suppressed disease in AIA (60), and antigen-loaded tolDCs treated with NFκB inhibitor andrographolide reduced anti-drug antibodies in hemophilia A mice (61). A nanoparticle vaccine loaded with antigen and A20, an anti-inflammatory protein inhibiting NF-κB activation (62), suppressed Th2 responses and reduced disease in an asthma model (63) (Table 1).

A phase I clinical trial investigated antigen-loaded autologous Bay11-7082-treated tolDCs in rheumatoid arthritis. The vaccine reduced effector T cells and increased Tregs, together with a reduction in inflammatory cytokines. The treatment did not induce disease flares and lead to decreased rheumatoid arthritis DAS28 score (64) (Table 6).

2.1.5 Kynurenine and AhR agonists

Kynurenine is an immunoregulatory tryptophan metabolite signaling via the aryl hydrocarbon receptor (AhR). AhR signaling can influence T cell differentiation and function of APCs to favor expansion of Tregs (65, 66). Tolerogenic vaccination with nanoparticles loaded with autoantigen and the AhR agonist ITE (2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester), suppressed disease in models of EAE (67, 68) and T1D (69), and tolerogenic vaccination with kynurenine and antigen-expressing phages prevented hyperglycemia in a model of T1D (70) (Table 1).

2.1.6 Other immunosuppressive agents

Janus kinase (JAK) and signal transducer and activator of transcription proteins (STAT) signaling induces immune cell activation and cytokine production (71). Tolerogenic vaccination with autoantigen-loaded tolDCs treated with JAK/STAT inhibitors tofacitinib and BD750 suppressed disease, reduced Th1 and Th17 responses, and increased Tregs in models of EAE (72, 73) (Table 1).

Rosiglitazone is an anti-diabetic drug that activates peroxisome proliferator–activated receptor gamma (PPARγ). In immune cells, rosiglitazone can inhibit inflammatory cytokines and promote tolerogenic APCs (74). TolDC vaccination with antigen-loaded,

TABLE 6 Human clinical trials.

Adjuvant	Antigen	Formulation	Rout	Disease	Phase	Major Results	PMID/Trial Number
Dexamethasone	$MBP^{13–32}$ $MBP^{83–99}$ $MBP^{11–129}$ $MBP^{146–170}$ $MOG^{1–20}$ $MOG^{35–55}$ $PLP^{139–154}$	TolDC Vaccine: Monocyte-derived DC treated with Adj and loaded with Ag	i.v.	MS	Phase I	↑Tr1	(34) NCT02283671
Dexamethasone + VitD	Proinsulin (C19-A3)	TolDC Vaccine: Monocyte-derived DCs treated with Adj and loaded with Ag	i.d.	T1D	Phase I	↓ Ag-CD8+ T Cells ↓ INF-γ ↑ $ICOS^+CCR4^+TIGIT^+$ Tregs No change in Autoantibody ↑ Uricase Activity	(164) 2013-005476-18
Dexamethasone + VitD + MPLA	autologous Synovial Fluid	TolDC Vaccine: Monocyte-derived DC treated with Adj and loaded with Ag	intra-articular	RA	Phase I	- No clinical effects were detectable.	(35) NCT01352858
Rapamycin	Pegadricase	NP/Co-Delivery Vaccine: NP with rapamycin alongside IV pegadricase	i.v.	Gout	Phase Ia, Ib and III	↓ Ag-antibody ↓ Uric Acid Levels ↑ Uricase Activity	(53, 54) NCT02464605 NCT04513366 NCT02648269 NCT04596540
Rapamycin	AAV8	NP/Co-Delivery Vaccine: Rapamycin NP given alongside IV AAV8 vector	i.v.	ADA	Phase I	↓Ag-antibody	press release
Bay11-7082	Citrullinated aggrecan, vimentin, CII, and fibrinogen peptides	TolDC Vaccine: Monocyte-derived DC treated with Adj and loaded with Ag	i.d.	RA	Phase I	↓ T Effector ↓ IL-15 ↓ IL-6 ↓ IL-29 ↓ CX3CL1 ↓CXCL11 ↑ Tregs	(64) CTRN12610000373077
VitD	GAD65	Separate Vaccine: Ag in alum and oral VitD	Ag-alum intra-lyphatic, oral vitamin D	T1D	Phase I-III	- Stable Beta Cell Function - Stable Metabolic Control	(162) NCT04262479NCT050 18585NCT03345004NCT02352974
VitD	MBP^{13-32} $MBP^{111-129}$ $MBP^{154-170}$ $PLP^{139-154}$ MOG^{1-20} MOG^{35-55} MBP^{83-99}	TolDC Vaccine: Monocyte-derived DC treated with Adj and loaded with Ag	i.d.	MS	Phase I	N/A	(163) NCT02618902 and NCT02903537
VitD analog	$CII^{259-273}$	NP Vaccine: LNP encapsulating Adj + Ag	s.c	RA	Phase I	↓ Ag T Cells ↓ Memory B Cells ↑ $PD1^+$ T Cells	(165) ACTRN12617001482358
TGF-β1 + IL-10 + IL-2	Preproinsulin	DNA Vaccine: Adj + Ag plasmid,	s.c.	T1D	Phase I	N/A	NCT04279613
IL-10 + α-CD3	Preproinsulin	Probiotic Vaccine: L. lactis bacteria carrying encoding Ag + Adj	oral probiotic and i.v. infusions of teplizumab (α-CD3)	T1D	Phase Ib and IIa	↑ Metabolic Improvement ↑ Exhausted $CD8^+$ T Cells ↓ Ag-$CD8^+$ T Cells	NCT03751007 (107)

(Continued)

TABLE 6 Continued

Adjuvant	Antigen	Formulation	Rout	Disease	Phase	Major Results	PMID/Trial Number
Mannan	Grass pollen Ag	Conjugate Vaccine: Adj-Ag	s.c. & sublingual	Allergy	Phase II	↓ Nasal Provocation	(227) NCT02654223
Mannan	Dust mite pollen Ag	Conjugate Vaccine: Adj-Ag	s.c. & sublingual	Allergy	Phase II	↓ Nasal Provocation	(228) NCT02661854

ADA, Antidrug antibody; Adj, Adjuvant; Ag, antigen; CD, cluster of differentiation; DC, dendritic cell; DNA, deoxyribonucleic acid; GAD65, glutamic acid decarboxylase; i.d., intradermal; IFN-γ, interferon-γ; i.m., intramuscular; IRBP, Interphotoreceptor retinoid-binding protein; IL, interleukin; Ins, Insulin; i.v., intravenous(ly); MOG, myelin oligodendrocyte glycoprotein; NP, nanoparticle; PLP, proteolipid protein; s.c., subcutaneous(ly); RA, rheumatoid arthritis; T1D, type 1 diabetes; Th, T helper; TGF-β, Transforming Growth Factor-β; TNF, tumor necrosis factor; Treg, regulatory T cell; TolDC, tolerogenic dendritic cell.
↓, decrease; ↑, increase.

rosiglitazone-treated tolDCs suppressed disease in CIA (75) (Table 1), and autoantigen-loaded tolDCs treated with rosiglitazone in combination with dexamethasone and cobalt (III) protoporphyrin suppressed a murine model of SLE (33) (Table 5).

Inhibition of the protein kinase glycogen synthase kinase 3 (GSK-3) in immune cells leads to reduced inflammatory cytokine production and increased IL-10 (76). Vaccination with antigen-loaded tolDCs treated with GSK-3β inhibitor K313 suppressed disease in EAE (77) (Table 1).

Prostaglandin I2 (PGI2) is a lipid signaling mediator most known for its vasodilating and anti-thrombotic effects. PGI2 also has anti-inflammatory properties and protective effects in allergy and asthma (78, 79). Tolerogenic vaccination with antigen-loaded tolDCs treated with PGI2 analog iloprost reduced disease in ovalbumin-induced asthma (80). Additionally, direct delivery of iloprost and antigen using a hydrogel suppressed antigen-induced lung inflammation and increased the frequency of antigen-specific Tregs (80) (Table 1).

2.2 Cytokines and chemokines

2.2.1 TGF-β

TGF-β is a strongly immunosuppressive cytokine, because genetic deficiency of TGF-β leads to fatal autoimmunity (81). TGF-β is an immunosuppressive cytokine with multiple effects on the immune system: it promotes Treg development and function, inhibits B and T cell proliferation, suppresses differentiation of Th1 and Th2 cells, and induces tolDCs (82). However, when combined with specific other cytokines, TGF-β may trigger T cells to differentiate into non-regulatory phenotypes such as Th17 effectors in presence of IL-6 and Th9 in presence of IL-4 (82).

A tolerogenic nanoparticle vaccine containing autoantigen and TGF-β reduced disease and immune cell activation in EAE (83). TolDC vaccination with autoantigen-loaded tolDCs cultured in presence of TGF-β or TGF-β receptor agonist suppressed disease in a CIA model (84) and reduced anti-drug antibodies toward FVIII (85), together with increases in Tregs and IL-10 (Table 2).

In combination with other adjuvants, tolerogenic vaccines using both TGF-β and IL-10 suppressed disease in CIA (86) and reduced anti-drug antibodies toward FVIII (87). Tolerogenic vaccination with microparticles containing GM-CSF and TGF-β1 alongside nanoparticles with antigen and vitamin D suppressed EAE and T1D (88–92) and microparticles loaded with TGF-β, retinoic acid, and autoantigens suppressed T1D (93) (Table 5).

A tolerogenic DNA vaccination with autoantigen-encoding plasmids in combination with plasmids for either TGF-β, IL-10, and or IL-2 suppressed disease in EAE (94) and T1D (95) (Table 5). A phase I clinical trial is registered for this tolerogenic DNA vaccine to evaluate vaccine safety in patients with T1D (Table 6).

2.2.2 IL-10

IL-10 is a powerful immunosuppressive and anti-inflammatory cytokine, absence of which causes spontaneous colitis in mice (96). IL-10 suppresses antigen presentation and inflammatory cytokine production by APCs and simultaneously increases their release of anti-inflammatory mediators. In $CD4^+$ T cells, IL-10 inhibits proliferation and cytokine production and promotes the development of regulatory Tr1 cells (97).

Tolerogenic vaccination with antigen-loaded tolDCs engineered to express IL-10 suppressed disease in mouse models of T1D and asthma (98, 99), and antigen-loaded tolDCs cultured in presence of IL-10 reduced disease in EAE (100). Tolerogenic vaccination with nanoparticles containing IL-10 and antigen suppressed disease in EAE (101), and DNA vaccines encoding IL-10 and antigen suppressed disease in EAE and T1D models (102, 103) (Table 2).

A probiotic vaccine of *Lactococcus lactis (L. lactis)* genetically engineered to secrete IL-10 and pro-insulin administered together with anti-CD3 ameliorated disease and increase Tregs in models of T1D (104–106) (Table 5). The *L. lactis* probiotic vaccine has been studied in human clinical trials where results from phase Ib and IIa studies demonstrated treatment to be safe, metabolic variables were either stabilized or improved, and antigen-specific $CD8^+$ T cells were reduced (107) (Table 6).

2.2.3 IL-2

IL-2 mediates T cell survival, differentiation, and proliferation. IL-2 is specifically required for Treg homeostasis and suppression of autoimmunity and genetic deletion results in systemic autoimmunity in mice (108). In addition, recent studies showed that low-dose IL-2 treatment induces the expansion of Treg cells and had efficacy in numerous mouse models and some early efficacy in clinical trials of T1D, graft-vs-host disease and SLE. Different types of tolerogenic vaccination with IL-2 treatment in combination with antigen exposure suppressed disease in models of EAE (109), experimental autoimmune uveitis (EAU) (110, 111), T1D (112),

delayed-type hypersensitivity (DTH) (112), and reduced development of anti-drug antibodies toward FVIII in hemophilia A (113). Overall, the vaccines led to increased Tregs and anti-inflammatory cytokines (Table 2).

Tolerogenic vaccines using IL-2 in combination with rapamycin expanded Tregs and suppressed disease in models of T1D (114, 115), EAE (116), and primary biliary cholangitis (114). Furthermore IL-2 in combination with Retinoic Acid suppressed EAE and EAU (117). These combination vaccines expanded Tregs or induced antigen-specific Tr1 cells (Table 5).

2.2.4 IFN-β

Interferon beta (IFN-β) is a type I interferon with immunomodulatory properties, used therapeutically for multiple sclerosis (MS). IFN-β reduces T cell activation, promotes Tregs and induces tolDCs (118, 119). Tolerogenic vaccines comprised of autoantigen and IFN-β suppressed murine and rat models of EAE via the induction of neuroantigen-specific, suppressive $CD25^+$ Tregs (120, 121) (Table 2). In addition, mice treated with IFN-β while receiving autoantigen-loaded vitamin D-treated tolDCs, further suppressed disease in a model of EAE (122) (Table 5).

2.2.5 GM-CSF

In addition to being a growth factor and chemokine, GM-CSF possesses anti-inflammatory effects. Administration of GM-CSF leads to a reduction in disease severity in several animal models of autoimmune disease (123), and GM-CSF promotes development and function of both tolDCs and Tregs (123, 124).

Tolerogenic vaccines with antigen-GM-CSF conjugates using neuropeptide autoantigens have been used to treat EAE in mice and rats, accompanied by increased Tregs (125–129). Additionally, GM-CSF is used for differentiation of DCs for most tolDC vaccines. Although most tolDC vaccines use additional adjuvants, also without other adjuvants transfer of antigen-loaded GM-CSF differentiated tolDCs suppressed murine EAU (130) (Table 2). A hydrogel/microparticle vaccine incorporating GM-CSF and TLR9 agonist CpG with autoantigen suppressed disease in a model of T1D (131) (Table 5).

2.2.6 Other cytokines and chemokines

IL-35 is a potent inducer of Tregs and regulatory B cells, and it can inhibit the proliferation and function of effector Th1 and Th17 cells. IL-35 has been shown to be protective against autoimmune disease and IL-35 treatment have been able to suppress disease in multiple models of autoimmunity and chronic inflammation (132). In tolerogenic vaccines, tolDCs engineered to overexpress IL-35 and loaded with disease relevant antigen suppressed EAE and DTH (133, 134) (Table 2).

IL-27 is an immunoregulatory cytokine which can promote tolerance by supporting development of Tregs and Tr1, antagonizing development of Th2 and Th17 cells, and by increasing co-inhibitory receptor expression on APCs (135). Tolerogenic vaccination by adoptive transfer of IL-27-conditioned antigen-loaded DCs led to a significant amelioration of disease and reduction in Th1 and Th17 cells in murine EAE (136) (Table 2).

IL-4 promotes type 2 immunity and suppresses Th1 polarization. Treatment with IL-4 suppressed disease severity in models of EAE and arthritis (137, 138). Tolerogenic DNA vaccines encoding IL-4 and antigen suppressed murine models of CIA, EAE, and T1D (139–142) (Table 2).

Hepatocyte growth factor (HGF) is a cytokine with pleiotropic effects, including the promotion of tolDCs (143). In a model of EAE, systemic HGF ameliorated disease and tolerogenic vaccination with HGF-treated antigen-loaded DCs mediated functional recovery in mice with established EAE and suppressed T cell mediated inflammation (144) (Table 2).

Vasoactive intestinal peptide (VIP) is a peptide functioning as a neurotransmitter in the central and peripheral nervous systems and has multiple effects, including immune modulation. VIP reduces the release of inflammatory cytokines, stimulates production of IL-10 and TGF-β, and decreases the co-stimulatory activity of APCs (145). Tolerogenic vaccination with antigen-loaded VIP-treated tolDCs led to amelioration of CIA and EAE, accompanied by increased levels of Tr1 cells (146) (Table 2).

TNF-related apoptosis-inducing ligand (TRAIL) is a cytokine that induces apoptosis and activation of NFκB. TRAIL has immunoregulatory effects demonstrated by the exacerbated development of autoimmunity in TRAIL-deficient mice (147). Tolerogenic vaccination with DCs engineered to co-express TRAIL and antigen reduced antigen-specific T cell responses and disease symptoms in models of EAE (148, 149) (Table 2).

2.2.7 Cytokine silencing

Just as the addition of anti-inflammatory or immunoregulatory cytokines can promote tolerogenic responses, silencing of inflammatory cytokines can also be effective. Silencing of B cell activating factor (BAFF), an essential cytokine for both T and B cell activation (150), in TolDCs using siRNA suppressed murine CIA and promoted Tregs (151) (Table 2).

2.3 Vitamins and vitamin derivates

2.3.1 Vitamin D

Vitamin D is primarily known for its role in calcium homeostasis and bone health, but it is also immunoregulatory. Having low levels of vitamin D is associated with increased susceptibility to a variety of infectious and autoimmune diseases. Vitamin D suppresses T cell activation, skews T cell differentiation away from Th17 while promoting Tregs and Th2 responses, and promotes tolerogenic DC features, including low surface expression of co-stimulatory molecules and decreased production of inflammatory cytokines (10, 152). Vitamin D signals via a nuclear receptor and exert immunoregulatory effects by regulating gene expression (10).

Many tolerogenic vaccines using vitamin D are tolDC vaccines. Vitamin D-treated tolDCs loaded with antigen or antigen-encoding mRNA reduced disease and promoted immunoregulatory cells and cytokines in murine models of EAE (153–156). The vitamin D-treated tolDCs had reduced expression of MHC class II, co-

stimulatory molecules, and pro-inflammatory cytokines, and induced less T cell proliferation compared to DCs that were untreated (153, 154) (Table 2). TolDC vaccines using a combination of vitamin D and dexamethasone suppressed murine models of arthritis (26) and T1D (29) (Table 5).

Tolerogenic nanoparticle vaccines with vitamin D have been examined in T1D (157, 158), where the nanoparticles reduced disease incidence and increased Tregs or tolDCs *in vivo*. Separate delivery studies of vitamin D and antigen have been studied in EAE (159, 160) and DTH (161), in all studies reducing disease severity and inflammation (Table 2).

Vitamin D-containing tolerogenic vaccines have been investigated in human clinical trials. In a phase IIa trial of latent autoimmune diabetes in adult patients were treated with daily oral Vitamin D and monthly injections of antigen-alum. The trial demonstrated safety and stable β-cell function and metabolic control at 5 months follow-up (162). Two phase I clinical trials have been registered to test tolerogenic vaccination with antigen-loaded vitamin D-treated monocyte-derived DCs in multiple sclerosis (163). Likewise, a phase I clinical trial tested monocyte-derived DC loaded with proinsulin and treated with Vitamin D and dexamethasone in T1D. The study showed the treatment was safe and lead to reduce proinsulin specific CD8+ T cells and increased $ICOS^+$ $CCR4^+$ $TIGIT^+$ Tregs (164). Another phase I trial investigated liposomes containing collagen II and calcitriol, the active form of vitamin D, for the treatment of rheumatoid arthritis. The calcitriol-antigen-liposomes led to reduced pathogenic T cells and expansion of antigen-specific $PD1^+$ T cells (165) (Table 6).

2.3.2 Retinoic acid

Retinoic acid is an immunoregulatory vitamin A metabolite, which like vitamin D signals via a nuclear receptor (10). Retinoic acid promotes the development and function of Tregs while inhibiting the differentiation and activation of effector Th1 and Th17 cells. DCs and macrophages can produce retinoic acid to support Treg induction and maintenance, and the retinoic acid-producing capacity of DCs is further enhanced upon retinoic acid exposure (10, 166).

Tolerogenic vaccination with liposomes incorporating retinoic acid and autoantigen converted pathogenic autoantigen specific Th17 cells to Tr1 cells and suppressed disease in EAE (167). Tolerogenic vaccination with retinoic acid, IL-2, and autoantigen suppressed EAE and EAU and pathogenic Th17 and Th1 responses (117). Prophylactic tolerogenic vaccination with retinoic acid, TGF-β and autoantigen inhibited the incidence of T1D in mice (93), and in another study treatment of mice with autoantigen and retinoic acid in combination with IL-4 suppressed EAE (168) (Tables 2, 6).

2.4 Modulators of contact-dependent immune cell signaling

2.4.1 T cell modulation

CD3 is the invariant chain of the TCR. Anti-CD3 monoclonal antibodies suppresses disease in numerous animal models of autoimmunity, and anti-CD3 is an FDA approved treatment to delay early onset type 1 diabetes. The exact mechanism of anti-CD3-mediated immune suppression is unclear but proposed mechanisms include prevention of T cells from recognizing their antigens and the induction of anergy or apoptosis in activated T cells while sparing Tregs (169). Tolerogenic vaccination with anti-CD3 treatment in combination with an antigen-expressing lentiviral vector suppressed T1D and induced autoantigen-specific Tregs (170). Anti-CD3 is also a component of the previously described probiotic vaccine (104–106) (Tables 3, 5).

CD4 is a glycoprotein on helper T cells which primarily functions as a TCR co-receptor. Non-depleting anti-CD4 therapy has been shown to suppress autoimmunity and graft rejection by modulating the function of $CD4^+$ T cells by blocking T cell activation and promoting Treg differentiation and suppressor functions (171). A tolerogenic vaccine comprised of aluminum hydroxide (alum), FVIII, and non-depleting anti-CD4 prevented development of anti-drug antibodies in mice (172). Another tolerogenic vaccine using treatment with depleting anti-CD4 antibodies followed by antigen administration suppressed disease in a murine model of EAU via the induction of antigen-specific Tregs (173) (Table 3).

"Tregitopes" are peptides derived from IgG that are recognized by a subset of natural Tregs. When presented in MHCII, these peptides activate Tregitope-specific Tregs and suppression of effector T cell responses to co-delivered antigens. Administration of nanoparticles with antigen and tregitopes decreased incidence and severity of T1D in mice (174). Likewise, co-administration of autontigen with Tregitope-albumin fusion proteins decreased incidence and reverse mild T1D (175) (Table 3).

Invariant natural killer T cells (iNKT cells) are immunoregulatory T cells important for preventing autoimmune reactions. The glycolipid α-galactosylceramide (α-GalCer) is a strong inducer of iNKT cells and has been shown to suppress disease in multiple animal models of autoimmunity (176). A tolerogenic vaccine comprised of lipid nanoparticles carrying autoantigen and α-GalCer prevented the development of diabetes in prediabetic mice (177) (Table 3).

2.4.2 Modulation of the immunological synapse

Including MHC class II-targeting molecules in a tolerogenic vaccine ensures delivery to APCs and may disrupt the immunological synapse. A tolerogenic vaccine with antigen conjugated to antibody fragments (nanobodies) targeting MHC class II suppressed disease in EAE. When combined with dexamethasone, the vaccine also overcame the inflammation associated with antigen exposure (178) (Table 5).

Co-stimulatory signals, such as CD80 and CD86, are necessary for T cell activation by APCs. T cell recognition of antigen on MHC II without co-stimulation results in anergy or apoptosis. Abatacept, a cytotoxic T-lymphocyte associated protein 4 (CTLA-4)-Fc fusion protein blocks CD80 and CD86 and is FDA approved for the treatment of autoimmune arthritis (179). Tolerogenic vaccination with nanoparticles displaying abatacept and carrying autoantigen and dexamethasone suppressed EAE (180) (Table 5).

Signaling via CD40-CD40L induces activation and pro-inflammatory cytokine production in both B cells, T cells, and

APCs, and CD40L-blockade reduced disease in numerous animal models of autoimmunity (181). Tolerogenic vaccination with FVIII and anti-CD40 prevented subsequent development of anti-FVIII antibodies during rechallenge (182) (Table 3).

Other approaches include genetic modification of co-stimulatory signals. Antigen delivered with a CRISPR-Cas9 plasmid and guide RNAs toward CD80, CD86, and CD40 disrupted co-stimulation by DCs, reduced inflammatory cytokines, increased Tregs, and suppressed disease in a model of T1D (183) (Table 5). Administration of a DNA vector encoding membrane-bound autoantigen together with a B7.1/CD40L mutant fusion protein binding to CTLA-4 but not CD28, providing co-inhibitory but not co-stimulatory signals, reduced disease incidence in murine model of T1D (184) (Table 3).

Intercellular adhesion molecule 1 (ICAM-1) is a cell surface glycoprotein most known for its role in leukocyte migration. Inhibition of ICAM-1 can block T cell activation and induce tolerance by disrupting T cell-APC interactions, inhibiting co-stimulation, promoting PD-L1 expression, and by inducing T cell anergy or exhaustion (185). A tolerogenic vaccine comprised of hyaluronic acid with autoantigen and an ICAM-1 inhibitory peptide suppressed disease in EAE (186–188). In another tolerogenic vaccine, fusion molecules of antigen and ICAM-1 inhibitory peptide prevented the development of T1D (189) and suppressed EAE (190) (Table 3).

OX40 is a TNF receptor superfamily member expressed by activated T cells and resting Tregs, which acts as a co-stimulatory molecule promoting cell proliferation (191). Tolerogenic vaccination of prediabetic mice with antigen and an OX40 agonistic antibody reduced diabetes incidence and increased antigen specific Tregs (192) (Table 3).

2.4.3 Activation of inhibitory receptors

Programmed cell death protein 1 (PD-1) is an immune checkpoint molecule that induces T cell apoptosis and suppresses conventional T cell activation and favors development of Tregs (193). Treatment with tolDCs engineered to express PD-L1 and MOG reduced antigen-specific T cell responses and suppressed MOG induced EAE but not MBP induced EAE (148, 149) (Table 3).

B and T lymphocyte attenuator (BTLA) is an inhibitory receptor structurally related to PD-1, activation of which leads to the suppression of T cell activation (194), and BTLA-expressing DCs promoted Treg development (195). Adoptive transfer of bone marrow derived DCs treated with a nanoparticle containing antigen and a BTLA-encoding plasmid suppressed EAE (196) (Table 3).

Fas is a death receptor inducing apoptosis upon binding to Fas ligand (FasL). A tolerogenic vaccine using FasL-conjugated microparticles containing monocyte chemotactic protein-1 (MCP-1) recruited T cells and then induced their apoptosis. When coupled with respective antigens, the microparticles could suppress EAE and prevented development of T1D in pre-diabetic mice (197). (Table 5) A tolerogenic vaccine aiming for engagement of multiple inhibitory receptors for immune suppression used microparticles displaying surface PD-L1-Fc, anti-Fas, and self-marker CD47, and containing TGF-β. The vaccine led to reduced T cell infiltration and EAE suppression (198, 199) (Table 5).

CD22 and siglec G are inhibitory receptors inhibiting B cell receptor (BCR) signaling, thus suppressing B cell responses (200). Tolerogenic vaccination with liposomes displaying antigen and CD22/siglec G ligands induced antigen-specific tolerance in mice and reduce development of anti-drug antibodies toward FVIII in a model of hemophilia A (201) and reduced antigen-specific antibody production (202) (Table 3). Encapsulation of rapamycin in CD22L autoantigen liposomes suppressed arthritis in mice (203–205) (Table 5).

2.5 Other adjuvants

2.5.1 Modulators of apoptotic pathway signaling

Apoptotic cells are cleared by phagocytic cells via anti-inflammatory mechanisms, which in part is mediated by phosphatidylserine exposed on the apoptotic cell surface (206). Therefore, phosphatidylserine liposomes, O-phospho-L-serine (OPLS), or pro-apoptotic factors have been used as tolerogenic adjuvants to reduce antigen immunogenicity.

In hemophilia A, both co-delivery of FVIII with OPLS and tolerogenic nanoparticle vaccination with FVIII encapsulated in phosphatidylserine liposomes led to a reduction in anti-drug antibodies toward FVIII in mice (207, 208), and antigen-containing phosphatidylserine liposomes reduced disease incidence in a model of T1D (209). In addition, phosphatidylserine liposomes loaded with collagen peptide and the immunomodulator leflunomide, which inhibits the mitochondrial enzyme dihydroorotate dehydrogenase preventing uridine synthesis, suppressed CIA in mice (210). Furthermore, a DNA vaccine encoding antigen and the pro-apoptotic protein BAX, promoting apoptosis in cells expressing antigen, suppressed T1D via modulation of APC function and promotion of Treg development (211, 212) (Table 4).

2.5.2 TLR agonists

TLR agonists are well known inflammatory stimuli and often used as adjuvants in immunogenic vaccines to enhance the immune response to the target antigen (213). However, signaling through microbial pattern recognizing receptors might also protect from development of autoimmunity. Dose and administration of the TLR agonist affects if the response is immunogenic or tolerogenic, it is believed that a short-term, high-dose stimulation will result in immunogenic responses whereas low-dose, repeated stimulation results in tolerance (214).

TLR4 agonist LPS is often used in cultures of DCs to induce maturation and enhance their antigen presenting capacity. Antigen-loaded LPS-treated DCs suppressed EAE while non-treated DCs did not (215). A recombinant fusion protein of autoantigen and flagellin A, TLR5 agonist, induced production of IL-6 and IL-10 in DCs and reduced T cell-driven inflammation in a murine model of intestinal allergy (216, 217). Co-delivery of flagellin B and antigen reduced disease in models of allergy (218, 219) and a fusion protein

of antigen and flagellin B reduced disease and IgE responses in allergy (220) (Table 4).

TLR9 agonist CpG DNA has been used in different tolerogenic vaccines in combination with other adjuvants. Treatment with a hydrogel vaccine containing CpG DNA, antigen, and GM-CSF prevented and delayed disease onset in pre-diabetic mice, and the inclusion of CpG DNA enhanced efficacy compared to GM-CSF alone (131). Tolerogenic vaccination with antigen, CpG DNA, and heat shock protein 60 induced an antigen-specific increase in IL-10 production and reduced disease severity in a model of arthritis (221) (Table 5).

2.5.3 Glycans and glycan-binding proteins

The use of glycans in tolerogenic vaccine design can mediate tolerance by targeting antigen to APCs expressing receptors for these glycans, promoting its uptake and processing, while concurrently inducing immunoregulatory effects in DCs (14, 222, 223).

β-glucan is a polysaccharide naturally occurring in cell walls of plants, bacteria, and fungi, and binds to Dectin-1 on myeloid cells. In a model of T1D, treatment with β-glucan and antigen led to increased protection from disease compared to treatment with β-glucan or antigen alone, and promoted tolDC features and increased Tregs (224). Conjugates of allergens to mannan, targeted these antigens to APCs expressing mannose and C-type lectin receptors, and promoted a tolerogenic response in comparison to native allergens *in vitro* and *in vivo* (222, 223). Skin-prick tests with mannan-conjugated grass pollen allergoids caused less inflammation than native allergens in patients with grass pollen allergy (225), and immunization of mice with mannan-allergoid conjugates led to tolerogenic responses and increase in $Foxp3^+$ Tregs compared to native antigen (225, 226) (Table 4). Mannan-allergoid conjugates have been tested for dust mite and grass pollen allergens in two phase II clinical trials, showing improvement in nasal provocation test (227, 228) (Table 6).

Galectin-1 is a glycan-binding protein with diverse functions, including modulation of DCs and T cell responses (229). In a study of EAE, treatment of DCs with galectin-1 led to tolDC differentiation that suppressed EAE when loaded with relevant autoantigen (230). The disease suppression was dependent on IL-27 and IL-10 induced by galectin-1 (230) (Table 4).

3 Discussion

Tolerogenic vaccines are promising experimental treatments for a wide range of conditions, including autoimmune disease, anti-drug antibody responses, transplantation rejection, and hypersensitivity (3, 6). Successful reintroduction of immune tolerance via tolerogenic vaccination would have numerous benefits over traditional immunosuppression or immune modulation. First, tolerance could be durable as tolerogenic vaccines may deplete or inactivate pathogenic cells, while concurrently inducing long lived suppressive Tregs and/or regulatory B cells which can self-renew and persist (231, 232). Second, tolerogenic vaccines engaging antigen-specific Treg responses may engender bystander suppression and infectious tolerance to suppress autoimmune responses to unknown antigens (3, 233). Third, tolerogenic vaccines may have efficacy with minimized toxicity as they modulate the antigen-specific response, leaving the rest of the immune system intact. Together these characteristics could constitute a functional cure.

Although tolerogenic adjuvants are not always necessary in tolerogenic vaccines (3, 234, 235) addition of tolerogenic adjuvants have the potential to greatly enhance the efficacy of tolerogenic vaccines by several mechanisms. Immunosuppressive or anti-inflammatory tolerogenic adjuvants promote an anti-inflammatory environment upon antigen encounter, thereby reducing the risk of unwanted inflammatory responses, anaphylaxis or disease exacerbation when re-introducing disease-relevant antigens. Immunomodulatory tolerogenic adjuvants can steer the antigen-specific immune response in desired direction, and cell-targeting adjuvants ensure vaccine delivery to intended cell types and minimizes off-target effects. Additionally, tolDC transfer is a common tolerogenic vaccine modality, but this type of tolerogenic vaccine is associated with high costs and difficulties of standardization across patients. Therefore, tolerogenic vaccines using adjuvants to deliver antigen to and modulate DCs *in vivo* may represent a more feasible treatment option.

A major limitation to the development of tolerogenic vaccines is a lack of understanding of the autoantigen pools that drive autoimmune diseases. Few autoimmune diseases have limited and well-defined antigen pools, while most autoimmune disease have numerous, poorly defined or undefined antigen pool, and disease antigens may change through time or differ across patients. Therefore, initial clinical trials using tolerogenic vaccines have focused on conditions which have relatively defined antigen pools such as celiac disease, pemphigus vulgaris, T1D and anti-drug antibody responses. However, preclinical data suggest that induction of tissue-specific Tregs may circumvent the need-to-know exact antigens involved in disease as these vaccine-induced tissue-specific Tregs can traffic to the inflamed tissue and exert suppressive functions via bystander suppression or infectious tolerance to suppress immune responses to unknown antigens involved autoimmune disease (3, 233). Utilizing adjuvants to expand or enhance Treg responses, may therefore enable further application of tolerogenic vaccines also in autoimmune diseases with complex autoantigen pools.

Another unknown is if tolerogenic vaccines that induce tolDCs and/or Tregs can suppress preexisting pathogenic B cell that were licensed by CD4 T cell and have limited ongoing interactions with either Tregs or TolDCs. Therefore, tolerogenic vaccines might have to address B cells separately. This could be achieved by B cell targeting or B cell suppression, such as by adjuvants signaling via Siglec G and CD22 or using adjuvants with effects on both B and T cell responses. This approach could be combined with a tolerogenic vaccine design acting on tolDC and/or T cells to prevent further activation of novel pathogenic B cell clones.

In conclusion, tolerogenic vaccines may be the therapeutics of the future for autoimmune and inflammatory conditions. Tolerogenic adjuvants are powerful tools with capacity to both enhance antigen-specific tolerance as well as reduce the risk of unwanted inflammatory responses or off-target effects.

Author contributions

CM: Writing – original draft, Writing – review & editing. SA-B: Writing – original draft, Writing – review & editing, Visualization.

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EDITED BY
Ce Wang,
AbbVie, United States

REVIEWED BY
Marc Schmalzing,
University Hospital Würzburg, Germany
Baiyi Cai,
AbbVie, United States
Shiyu Xiao,
Northwestern University, United States

*CORRESPONDENCE
Graham E. Holder
ophgeh@nus.edu.sg

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Hematopoietic stem cell transplantation as rescue therapy for refractory autoimmune retinopathy: a case report

Wendy Meihua Wong[1,2], Yvonne Loh[3], Hwei Wuen Chan[1,2], Warren Fong[4], Soon-Phaik Chee[1,5], Adrian Koh[5] and Graham E. Holder[1,2]*

[1]Department of Ophthalmology, National University Hospital, National University Health System, Singapore, Singapore, [2]Centre for Innovation and Precision Eye Health, Department of Ophthalmology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore, [3]Curie Oncology and Haematology, Mt Elizabeth Medical Centre, Singapore, Singapore, [4]Department of Rheumatology and Immunology, Singapore General Hospital, Singapore, Singapore, [5]Eye and Retina Surgeons, Camden Medical Centre, Singapore, Singapore

Autoimmune retinopathy (AIR) is a rare, potentially blinding retinal disease that remains a challenging condition to manage when resistant to conventional immune-modulatory approaches. We report clinical and electrophysiological improvement in a 49-year-old patient who underwent an autologous hematopoietic stem cell transplant (aHSCT) for thymoma-associated AIR after experiencing progressive disease despite receiving periocular and systemic steroids, mycophenolate mofetil, baricitinib, tacrolimus, bortezomib, rituximab, plasmapheresis, and intravenous immunoglobulin. The aHSCT had two stages: (i) peripheral blood stem cell harvest following mobilization with cyclophosphamide and granulocyte colony-stimulating factor, and (ii) conditioning regimen with plasmapheresis, rituximab, cyclophosphamide, and anti-thymocyte globulin high-dose therapy, followed by autologous hematopoietic cell infusion of 5.74 million cells. Symptoms of photopsia rapidly abated after undergoing aHSCT, and objective investigations of structure and function similarly demonstrated improvement. At 22 months' follow-up, she continued to demonstrate the durability of the clinical response. The present report suggests that in judiciously selected patients, HSCT may provide a rescue option for refractory AIR. Further cases are needed to confirm these results.

KEYWORDS

antiretinal antibodies, autoimmune retinopathy, electroretinogram, hematopoietic stem cell transplant, plasmapheresis, thymoma, retinal vasculitis

Introduction

Autoimmune retinopathies (AIRs) are a group of rare immune-mediated diseases characterized by progressive painless visual deterioration, visual field defects, and electroretinographic abnormalities, in association with circulating anti-retinal antibodies (ARAs) (1, 2). The pathophysiology is postulated to involve the circulating ARAs targeting retinal antigens, causing damage to photoreceptor cells, bipolar cells, or retinal ganglion cells. The two main types are paraneoplastic and non-paraneoplastic AIR; the distinction is important due to the systemic implications (3).

The essential diagnostic criteria for AIR are the presence of serum ARAs and electroretinogram (ERG) abnormalities (4). However, ARAs can be present in other degenerative retinal disorders, systemic autoimmune diseases (ADs), and some healthy individuals, and are not pathognomonic for AIR (5). Whether ARAs initiate the pathogenesis of AIR or represent an epiphenomenon consequent upon retinal dysfunction remains unclear. Electrophysiology is important as ERGs provide objective evidence of the nature, severity, and localization of disease (cone and/or rod photoreceptors, bipolar cells, etc.). In addition, ERG abnormalities may precede the development of structural changes on fundus examination or imaging.

Various immunomodulatory approaches have been employed to alter the disease course in AIR given the presumed immune-mediated nature of the disease. These include local or systemic steroids, antimetabolites (azathioprine and mycophenolate mofetil), T-cell inhibitors (cyclosporin), biologics (rituximab, bortezomib, and IL-6 antagonists), intravenous immunoglobulin (IVIG), and plasmapheresis (6). There is a single case reporting the benefit of nonmyeloablative unmanipulated autologous hematopoietic stem cell transplant (aHSCT), preceded by immune ablation (7). No large-scale randomized controlled trials are available due to the rarity of the condition and the variable natural history. Current available evidence for therapeutic intervention primarily relies on retrospective case series and case reports, and there is no consensus on the most effective treatment regimen (2, 6, 8).

Registry data from the Autoimmune Disease Working Party recorded 3,502 patients undergoing aHSCT, between 1994 and 2021, for various severe ADs (9). The rationale of using aHSCT for AD is to restore immune self-tolerance by eradicating autoreactive immune cells with a conditioning regimen. This is followed by immune reconstitution from myeloid or lymphoid progenitor cells to "reset" the immunological memory (10, 11). There are myeloablative and non-myeloablative conditioning regimens. For AD, the conditioning regimen has a greater emphasis on safety than the regimens designed for malignancies, and the goal of conditioning is immune ablation, not myeloablation. Therefore, a non-myeloablative conditioning regimen with reduced regimen-related toxicity is deemed more suitable for AD (12, 13). In certain ADs, such as relapsing–remitting multiple sclerosis, aHSCT is considered a standard treatment because it achieves sustained remission and neurological improvement with an acceptable safety profile (9, 11, 14). Other major indications for aHSCT are systemic sclerosis and Crohn's disease, and there is growing evidence for rarer indications such as systemic lupus erythematosus, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, Takayasu arteritis, and Behcet's disease (9, 15–17). The present report provides the outcomes of aHSCT in a patient whose central vision was under threat from refractory AIR.

Case report

Medical history prior to autologous stem cell transplant

A 49-year-old female patient presented to our institution after receiving treatment at two other healthcare facilities over the previous 18 months. She had a history of thymoma, diagnosed following onset of unilateral ptosis from ocular myasthenia gravis, and had undergone a maximal thymectomy. Histopathological evaluation revealed a stage 1A Masaoka Type B thymoma. A month after surgery, although the ptosis had improved, she developed bilateral photopsia, more prominent in her right eye (RE). At that stage, uncorrected visual acuity was 20/30 bilaterally with normal intraocular pressures and anterior segment biomicroscopy. Posterior segment examination showed bilateral disc hyperemia and peripheral retinal vasculitis, confirmed on fundus fluorescein angiography (FFA). Optical coherence tomography (OCT) showed disruption of the ellipsoid zone in the peripheral macula, and it was worse in her RE.

ARA testing had been positive for antibodies directed towards carbonic anhydrase II, Rab6, aldolase, enolase, HSP60, and TULP1, and a diagnosis of thymoma-associated AIR has been made. There were elevated anti-nuclear antibody titers (>1:640 with a homogeneous pattern) and an inverted CD4/CD8 ratio of 0.61. C-reactive protein, erythrocyte sedimentation rate, extractable nuclear antigen profile, ANCA profile, T-spot TB, TB quantiferon, treponema pallidum particle agglutination assay, and VDRL were normal.

The patient had received numerous immune-modulatory treatments prior to presenting to our center (Figure 1). She had initially been treated with periocular triamcinolone acetonide and commenced on 50 mg of oral prednisolone (dose of 1 mg/kg) with gradual tapering over 10 weeks. Her disease had continued to be active despite treatment with mycophenolate mofetil, baricitinib, tacrolimus, bortezomib, rituximab, IVIG, and plasmapheresis. FFA, repeated after 4 months of immunosuppression, continued to show similar retinal vascular leakage in both eyes. Antibody testing, repeated at the same time, was positive for several ARAs (30-kDA, 35-kDA, 40-kDA, 44-kDA, 46-kDA, 76-kDA, and 123-kDA proteins) and anti-optic nerve antibodies (40kDA,35kDA, and 123kDA).

The fundus appearance and OCT imaging on presentation to our center are shown in Figure 2. As there continued to be features of disease activity despite 6 months of immunosuppressive treatment, electrophysiological examination was performed at presentation (Figure 3, row 1), and repeated 2 months later (Figure 3, row 2). There was generalized retinal dysfunction bilaterally, with marked cone system involvement, worse in the

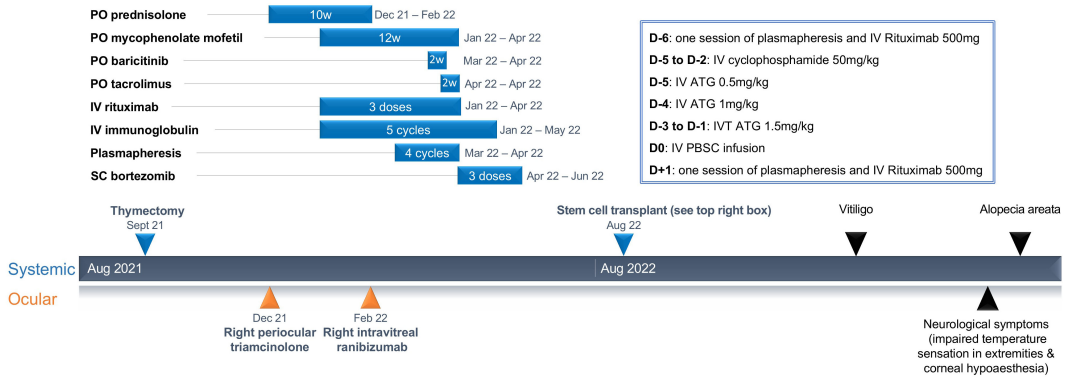

FIGURE 1
Summary of treatment and duration of treatment leading up to stem cell transplant, and secondary autoimmune diseases after.

RE. Photopic 30-Hz flicker ERG, which reflects generalized retinal cone system function, was markedly delayed in her RE at 42 ms (41 ms on repeat testing) and mildly delayed in her left eye at 33 ms (32 ms on repeat testing).

There was no known family history of vision impairment. The patient was well-informed about her condition and proactively researched her condition, the immune system, and the mechanism of action of her different treatments. She was acutely aware of her ongoing photopsias and that it was approaching her central vision. Additionally, the patient kept a meticulous record of all her test results and tracked the progression evident on structural and functional testing. As her condition continued to deteriorate despite trying multiple lines of immunosuppression, she elected to undergo an aHSCT. The patient was counseled about the risks of aHSCT, and understood the attendant risks including mortality.

Methods

The patient underwent medical evaluation with an echocardiogram and pulmonary function test before undergoing aHSCT. The aHSCT had two stages: (i) mobilization and peripheral blood stem cell (PBSC) harvest, and (ii) high-dose therapy with autologous hematopoietic cell infusion. The patient received a combination of IV cyclophosphamide 2 g/m^2 and subcutaneous filgrastim, a recombinant human granulocyte colony-stimulating factor (G-CSF). This regimen is widely used in both cancer and ADs for mobilization of PBSCs (18, 19). A combination regimen was selected because the use of G-CSF alone may trigger flares of AD (20, 21). Cyclophosphamide aids stem cell mobilization by inducing mild cytopenia, which stimulates the marrow to increase the division of stem cells. The PBSCs harvested via apheresis on day 10 after cyclophosphamide had a yield of 5.74×10^6 CD34+ cells/kg body weight and were cryopreserved.

The conditioning regimen was one session of plasmapheresis (on day −6); IV rituximab 500 mg (on day −6 and day +1); IV cyclophosphamide 50 mg/kg (on day −5 to day −2); and IV rabbit anti-thymocyte globulin 0.5 mg/kg (on day −5), 1 mg/kg (on day −4), and 1.5 mg/kg on (day −3 to day −1). This conditioning regimen has been used in aHSCT of patients with neuromyelitis optica (NMO), another antibody-mediated AD that affects the eye (22). Plasmapheresis reduces pre-formed antibodies, while rituximab and anti-thymocyte globulin are postulated to deplete B and T cells (23). IV PBSCs were infused on day 0. She achieved neutrophil engraftment on day +10. Platelets recovered to >20 on day +10 and >50 on day +11. Her transplant course was complicated by neutropenic fever that settled promptly with empirical antibiotics, and she was discharged well by day +11. She also had asymptomatic low-level CMV viremia detected on PCR that resolved without treatment.

Results

Clinical evaluation

Shortly after undergoing the transplant, the patient reported rapid resolution of her photopsia. Fundus examination showed resolution of the retinal vasculitis-related vascular sheathing (Figure 2).

Optical coherence tomography

There was restoration of reflectivity in the band corresponding to the ellipsoid zone of the photoreceptors on structural imaging and reduction in the likely inflammation-related intra-retinal hyperreflective foci (Figure 2).

Static automated perimetry

Visual field testing repeated 3 months after the transplant showed increase in mean deviation scores bilaterally (RE −8.86

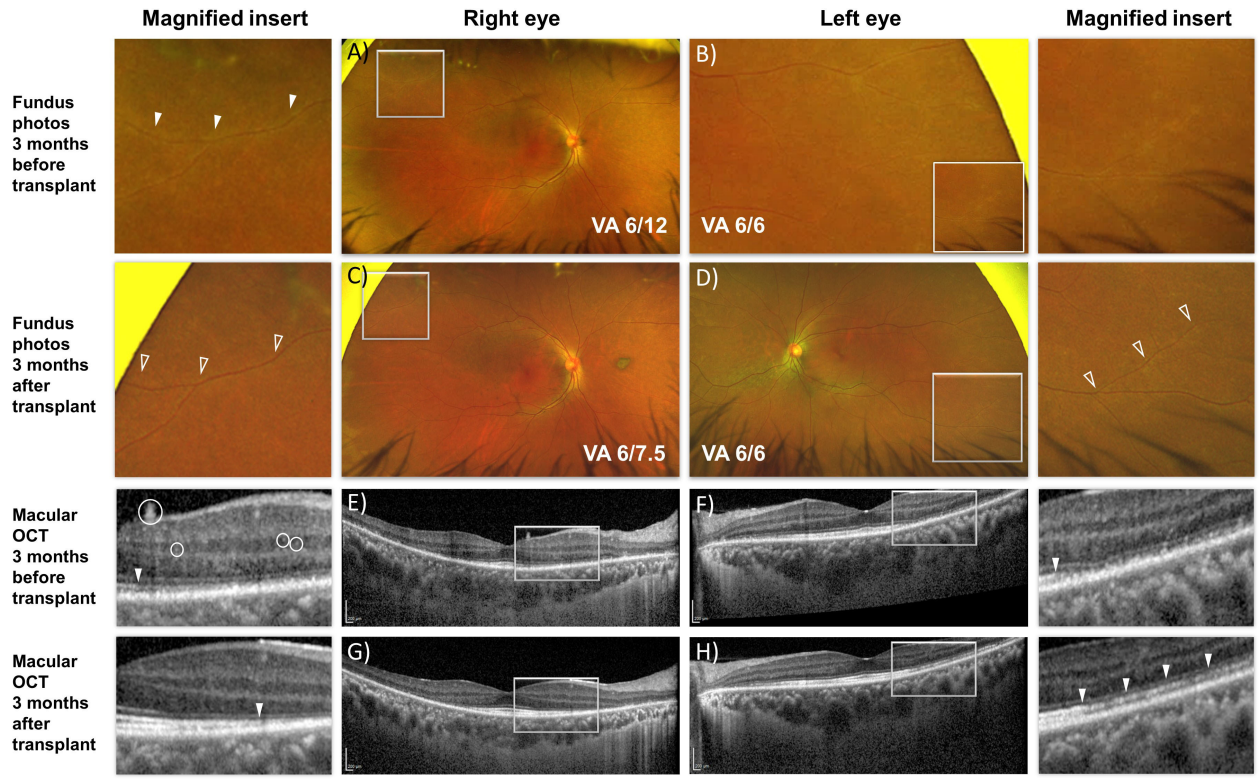

FIGURE 2
En face fundus images **(A–D)** and cross-sectional optical coherence tomography b-scan **(E–H)** of the macula of both eyes before and after autologous hematopoietic stem cell transplant. **(A, B)** Peripheral vascular sheathing was present in both eyes (magnified insert). **(C, D)** Resolution of the peripheral vascular sheathing 3 months after transplant. **(E, G)** Hyper-reflective dots within the retina and on the retinal surface (white circles) resolved after transplant accompanied by improvement in reflectivity from the ellipsoid zone corresponding to the photoreceptors (solid arrowhead). **(F, H)** Improvement in reflectivity from the ellipsoid zone was similarly present in the left eye 3 months after transplant.

dB from −13.91 dB; LE −7.43 dB from −11.51 dB) and reduction in the size of the scotoma in her RE (Figure 4).

Electroretinography

Electroretinography showed profound improvement in RE cone system function and significant improvement in the left eye. There was also rod system improvement with reversion to a normal waveform of the dark-adapted bright flash b-wave (Figure 3). See legend for further details.

Follow-up

Cutaneous manifestations of generalized vitiligo and alopecia areata developed at 6 and 12 months, respectively, after HSCT. Baricitinib was restarted by the dermatologist, but because of incomplete resolution of vitiligo following 4 months of treatment, this was switched to tofacitinib. Improvement in the alopecia was observed.

The patient also experienced neurological symptoms, including impaired temperature sensation in her extremities and bilateral corneal hypoesthesia at 10 and 12 months, respectively, after HSCT, which were managed conservatively.

Discussion

This report demonstrates a clear response following HSCT in a patient with AIR, in whom a variety of immunomodulatory therapies had been unsuccessful. The criteria for response were objective electroretinographic demonstration of improved retinal function, psychophysical improvement in visual fields, and morphological improvement on OCT imaging. The patient also reported rapid cessation of photopsia. One notable feature in this patient was the resolution of an abnormal electroretinographic b-wave appearance (see Figure 3), which has been associated with progressive AIR (Mantel et al., LE, Figure 4B) (24).

HSCT is a complex procedure that involves ablation of the host hematopoietic system through chemotherapy and immunotherapy followed by reconstruction of a new system by transplanting hematopoietic stem cells (25). Although its first and most common application is the treatment of onco-hematological disease, HSCT is increasingly being explored as a potential treatment for aggressive ADs (26, 27). While it is challenging to establish that ARAs are the cause of retinal damage in AIR rather than an epiphenomenon, it is suspected

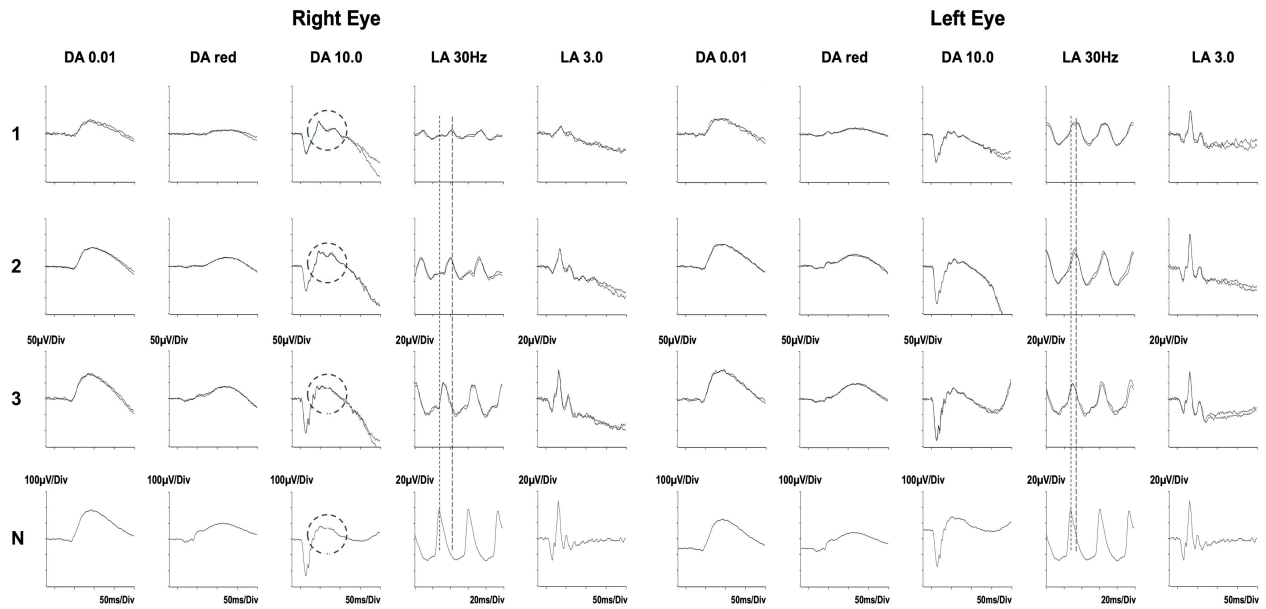

FIGURE 3
Serial electrophysiological tests performed before (rows 1 and 2) and after the autologous stem cell transplant (row 3). Shown are Standard ERGs as recommended by the International Society for Electrophysiology of Vision. DA = dark adapted; LA = light adapted; the numbers indicate stimulus strength in cd.s/m^2.The ERGs after 6 months of immunosuppression (24 May 2022, row 1) and repeated 10 days prior to her transplant (12 Aug 2022, row 2) show bilateral generalized retinal cone system dysfunction and severe delay (LA 30 Hz, peak time delay indicated by the interrupted lines). There had been minimal improvement during that time despite ongoing treatment. In addition, in the right eye, the b-wave of the DA 10.0 response showed an abnormal bifid waveform (circled). The LA 30 Hz ERGs 3 months after her transplant (15 November 2022, row 3) show substantial improvement in the delay in the right eye, and normalization in the left eye. There is additional resolution of the abnormal bifid waveform in the DA10.0 of the right eye (circled) to the waveform present in the normal subject (N). Data from a representative normal subject appear for comparison in the lower row (N).

that the initiating event was thymic dysfunction, with loss of self-tolerance and resultant production of autoantibodies leading to both myasthenia gravis and AIR (2). A conditioning regimen was therefore selected to target both self-reactive B and T cells. Rituximab and plasmapheresis were utilized to target pre-formed antibodies and B cells, and anti-thymocyte globulin targeted the T cells (28). The safety profile of this regimen has been documented for other immune-related disorders, such as NMO (26). A prospective open-label cohort study of NMO enrolled 13 patients, of whom 12 did not have any active coexisting ADs. In this group, where 11 patients were more than 5 years post-transplant, 80% remained relapse-free off all immunosuppression (22).

A study to evaluate hematopoietic stem cell transplantation in autoimmune-related retinopathy and optic neuropathy (ARRON)

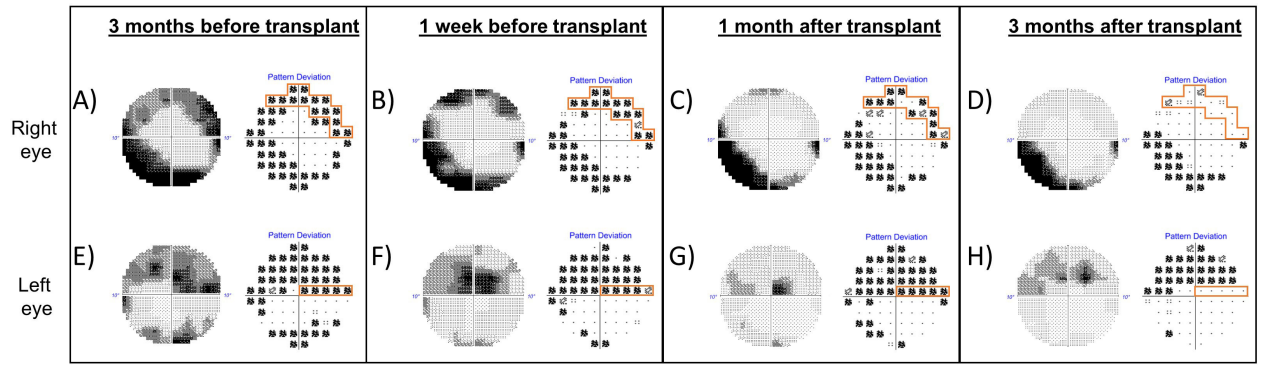

FIGURE 4
Grayscale map and pattern deviation plot of Humphrey visual fields (10-2 strategy) of the right eye (top row) and left eye (bottom row), before and after autologous stem cell transplant. A reproducible superior scotoma present in the right eye prior to transplant (**A, B**, outlined in orange) demonstrated improvement 1 month after transplant **(C)** and near-complete resolution of the defect 3 months after transplantation **(D)**. **(A–D)** The inferior arcuate defect in the right eye was persistent. There was a reproducible superior visual field defect in the left eye, which threatened fixation before transplant and persisted even 1 month following transplant **(E–G)**. Three months after transplant, there was improvement in the scotoma close to fixation **(H)**.

syndrome was initiated in 2004 and terminated 8 years later after recruiting two subjects (clinicaltrials.gov identifier NCT00278486). Although no publications were specifically linked, there is a single case report of a patient with ARRON who had been refractory to treatment with steroids, methotrexate, plasma exchange, IVIG, and cyclophosphamide between 2001 and 2003 (7). Improvement was suggested to be present in the ERGs of that case, but the reported changes were minor and the values stated may fall within test–retest variability. Our patient had several notable differences from the previously reported case with a different clinical phenotype, in particular the absence of any clinical or electrophysiological evidence of optic nerve dysfunction, notwithstanding the presence of anti-optic nerve antibodies; the presence of ARAs is not uncommon in the sera of normal humans and the same may apply to anti-optic nerve antibodies (29). There were further differences in disease severity at the time of HCST; our patient had not developed irreversible structural damage leading to central vision loss. Finally, while intravenous cyclophosphamide was used in both cases, the only other immune-ablative drug used in the published case was CAMPATH-1H. CAMPATH-1H is also known as alemtuzumab, and is a humanized monoclonal antibody targeting CD52 on surfaces of lymphocytes, eosinophils, and monocytes, facilitating cell destruction through complement activation and antibody-dependent cellular cytotoxicity (30, 31). The regimen used for the present case, along with its rationale, is detailed above.

Despite access to multiple modern immune-modulating therapeutic agents, the present patient had failed to show significant improvement prior to the HSCT. At the time of writing, the patient has shown durable clinical response for 22 months. Of note, secondary ADs are reported to complicate between 2% and 14% of aHSCTs performed for an AD (28). The postulated mechanisms for a secondary AD to develop include a loss of peripheral tolerance after conditioning (perhaps by deletion of regulatory cells), proliferation of autoreactive cells after HSCT by homeostatic expansion, a failure of negative selection during *de novo* thymic ontogenesis of T lymphocytes, or accumulation of mutations during increased proliferation of lymphocytes (32). During the informed consent process, it is important to have a candid discussion about the potential risks of secondary ADs, as well as the risk of transplant-related mortality, which is estimated to be approximately 1.3% with incorporation of less myeloablative regimens, center experience, and center accreditation (33). Cardiovascular complications can occur acutely, with the most frequent being arrhythmias such as atrial fibrillation and flutter and long-term complications include heart failure; a cardiovascular evaluation prior to aHSCT is thus of paramount importance (34). Patients of child-bearing potential need to also be aware of the risks of temporary or permanent ovarian/testicular failure and infertility following aHSCT (14). In view of the risks of aHSCT, judicious patient selection is important and should be considered where the disease is sight-threatening, standard treatments have failed, and there are no alternative safer therapeutic options. Although randomized controlled trials would be ideal to establish the safety and efficacy of autologous HSCT, it is important to recognize that the inherent challenge is the rarity of IRDs, which is estimated to account for under 1% of all cases even at a tertiary uveitis and ocular immunology clinic (1).

In conclusion, this case report demonstrates that escalation to HSCT in a patient with aggressive AIR that had failed to respond to advanced immunotherapy and threatened central vision was successful in abating the symptoms and led to objective improvement in both structural and functional investigations. Those improvements have been sustained over a 22-month follow-up period. Other symptoms, which may or may not be a direct consequence of the HSCT, have responded to appropriate treatment. Further cases are needed to confirm HSCT as a viable rescue option for refractory AIR.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

WW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. YL: Methodology, Writing – review & editing. HC: Writing – review & editing. WF: Data curation, Methodology, Writing – review & editing. SC: Investigation, Methodology, Validation, Writing – review & editing. AK: Writing – review & editing. GH: Conceptualization, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Guobao Chen,
AbbVie's Cambridge Research Center,
United States

REVIEWED BY
Xianliang Rui,
Boston Children's Hospital and Harvard Medical School, Boston, United States
María Tristán-Manzano,
Granada, Spain

*CORRESPONDENCE
Hui Xie
huixie@vip.163.com
Dongze Wu
dongze_wu@163.com

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Immune mediated inflammatory diseases: moving from targeted biologic therapy, stem cell therapy to targeted cell therapy

Zhenguo Liang[1], Hui Xie[2*] and Dongze Wu[3*]

[1]Department of Rheumatology and Immunology, The Fifth Affiliated Hospital, Sun Yat-Sen University, Zhuhai, Guangdong, China, [2]Department of Clinical Research and Development, Antengene Corporation, Shanghai, China, [3]Department of Rheumatology and Immunology, Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China, Chengdu, China

Despite the advancements in targeted biologic therapy for immune-mediated inflammatory diseases (IMIDs), significant challenges persist, including challenges in drug maintenance, primary and secondary non-responses, and adverse effects. Recent data have strengthened the evidence supporting stem cell therapy as an experimental salvage therapy into a standard treatment option. Recent preclinical and clinical studies suggested that chimeric antigen receptor T cell (CAR-T) therapy, which depleting tissue and bone marrow B cells, may lead to improvement, even inducing long-lasting remissions for patients with IMIDs. In this review, we address the unmet needs of targeted biologic therapy, delineate the critical differences between stem cell transplantation and CAR-T therapy, evaluate the current status of CAR-T therapy for IMIDs and explore its potential and existing limitations.

KEYWORDS

CAR-T, immune mediated inflammatory disease, stem cell therapy, biologic therapy, cell therapy

1 Introduction

Immune-mediated inflammatory diseases (IMIDs) comprise both B-cell-mediated autoimmune conditions—including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), type 1 diabetes (T1DM), pemphigus, and multiple sclerosis (MS)—as well as B-cell-independent chronic inflammatory disorders like psoriasis and other inflammatory diseases (1). The past three decades have witnessed an upsurging new case of IMIDs despite the incidence patterns of IMIDs varied considerably across the world (2). The co-occurrence of 19 IMIDs aligns with shared underlying pathogenetic mechanisms and predisposing factors that interact and vary in influence across these diseases, while also exhibiting distinct dynamic regulatory elements that shape clinical phenotypes and therapeutic responses (3, 4). The advent of targeted biologic therapies, alongside stringent inflammation control, has significantly improved both quality of life and

longevity for the clinically diverse spectrum of IMIDs. However, these treatments require continuous administration, rarely restore organ function or reverse disability, and remain non-curative (5). Emerging evidence indicates that stem cell-based therapies, such as mesenchymal stromal cells (MSCs) and hematopoietic stem cell transplantation (HSCT), may achieve sustained, drug-free, and symptom-free remission in multiple refractory IMIDs (6–8).

More importantly, recent data suggest that CD19-targeted chimeric antigen receptor (CAR) T cells therapy brings a ray of healing light to severe refractory IMIDs (9, 10). Beyond HSCT, the adoption of CAR-T cells as a new second-line treatment for patients with refractory or early-relapsing large B-cell lymphoma prompts a reevaluation of CAR-T therapy advancements in IMIDs (11–13).

2 Unmet clinical need of targeted biologic therapies for immune mediated inflammatory disease

Current targeted therapies for IMIDs, including biologic agents and small molecule drugs, can achieve sustained low disease activity or remission (14–16). However, unmet needs persist, such as primary and secondary non-response, drug resistance, adverse events, and increased risks of infections, potential malignancies and new onset of another IMIDs. Primary non-response may result from mechanistic failure, while secondary non-response often involves immunogenicity and antidrug antibody (ADAb) formation, which vary across agents (17, 18). ADAbs contribute to therapeutic failure and reduced drug survival rates (19, 20). Furthermore, targeted therapies may trigger new IMIDs or exacerbate coexisting conditions (21). They also heighten infection risks, with serious infections occurring in 3-4% of patients, and may slightly increase malignancy risks, particularly in older patients or with prolonged anti-TNF use (22). Infection, malignancy, risk of VTE, herpes zoster are key points to consider for the treatment of IMID with JAK inhibitors (23, 24).

Although the B cell depletion antibody (BCDA), rituximab, failed to meet primary endpoints in randomized controlled trials for SLE, favorable clinical experience has led to its frequent off-label use. Compared to rituximab, deeper B cell depletion is being explored with antibodies targeting CD20 (obinutuzumab), CD19 (inebilizumab), CD38 (daratumumab), the BAFF receptor (ianalumab), and TACI (telitacicept), as well as proteasome inhibition with bortezomib, all showing promise in IMIDs (25, 26). The SLAMF7-targeting monoclonal antibody elotuzumab, used in multiple myeloma, may also be relevant for certain IMIDs (27). In contrast, monoclonal antibodies targeting CD19 and FcγRIIb (obexelimab) and CD22 (epratuzumab) have not demonstrated efficacy in SLE (28, 29).

A key limitation of BCDA is the increased infection risk due to prolonged B-cell depletion, concurrent immunosuppressive therapy, and hypogammaglobulinemia (30). Additionally, some IMIDs, such as SLE, respond poorly to BCDA, as short-lived autoreactive plasma cells initially susceptible to depletion become resistant once they differentiate into long-lived plasma cells (31, 32). Another possible failure mechanism is the preferential depletion of regulatory B cells or phagocytic defects in macrophages (33). Crucially, the depth of B-cell depletion may determine clinical efficacy in IMIDs. While BCDA effectively depletes circulating B cells, it is less effective in the bone marrow and secondary lymphoid organs, potentially preventing a full 'reset' of IMIDs (25).

Despite a plethora of successful targeted biologic therapies with different mechanisms of action, most patients with IMID still do not achieve remission, let alone drug-free remission (34). The durability of biologic treatments remains suboptimal, often necessitating medication switches, which generally result in diminished therapeutic responses. Moreover, biologic drugs have largely failed to cure IMIDs or prevent their onset in most cases (15).

3 From stem cell therapy to targeted cell therapy for immune mediated inflammatory diseases

3.1 Transition from stem cell therapy to chimeric antigen receptor T cell therapy

In contrast to the chronic immune suppression associated with targeted biologic therapies, stem cell treatments, including both mesenchymal stem cell therapy (MSCT) and hematopoietic stem cell therapy (HSCT)—present a promising approach for potentially curing IMIDs by reinducing self-tolerance. This is supported by evidence from HSCT in MS, SSc, and Crohn's disease (6), as well as significant symptom improvement in patients with SLE and IBD following MSCT (35). These therapies target the long-term and extensive depletion of immune cells, particularly within the B and T cell populations (6, 36, 37). HSCT offers the advantage of complete eradication of autoreactive cells and broad immune system regeneration, promoting tolerance to autoantigens and potential long-term remission in MS, SSc, and SLE (6). However, its major drawbacks include high toxicity from conditioning regimens, increased infection risk, potential relapses, GVHD risk in allo-HSCT, and relatively high treatment-related mortality (38, 39). MSCT provides potent immunomodulatory and anti-inflammatory effects with no need for chemotherapy, no GVHD risk, and fewer severe side effects than HSCT. However, its variable efficacy and uncertain long-term durability remain key drawbacks (37, 40). While HSCT and MSCT have been valuable in immune modulation, their limitations—toxicity, incomplete immune reset, and variability in efficacy—have paved the way for targeted cell therapy as a more precise, effective, and durable treatment for IMIDs. Unlike stem cell therapy, targeted cell therapy leverages immunoengineering to program immune cells to eliminate pathogenic cells directly within diseased tissues, precisely where they drive systemic autoimmunity (41). The application of immunoengineering has enabled T cells to express a chimeric antigen receptor (CAR) specifically targeting B cell surface antigens, revolutionizing the treatment of hematological malignancies and IMIDs (41, 42). CAR-T cells and T cell engineering to express a chimeric autoantibody receptor (CAAR) have emerged as a potentially curative strategy for autoimmunity,

with the goal of achieving profound, short-term depletion of the B-cell compartment (43). CAR-T therapy offers selective depletion of pathogenic cells, deep and sustained B-cell depletion in tissues, high personalization, and the potential for long-term remission, but its main drawback is moderate toxicity, including risks of cytokine release syndrome (CRS) and neurotoxicity. Differences between stem cell therapy and CAR-T cell therapy have been identified in terms of composition, structure, mechanisms, preparation, pretreatment, and post-infusion monitoring (Figure 1).

3.2 Structure, generation and type CAR based therapy

The evolution of CAR-T cell generations has focused on enhancing signal transduction and immune response. First-generation CAR-T cells contain a CD3ζ domain for activation, while second-generation CAR-T cells add a costimulatory domain (e.g., CD28, ICOS, or 4-1BB) to improve efficacy. Third-generation CAR-T cells further enhance activation by incorporating two

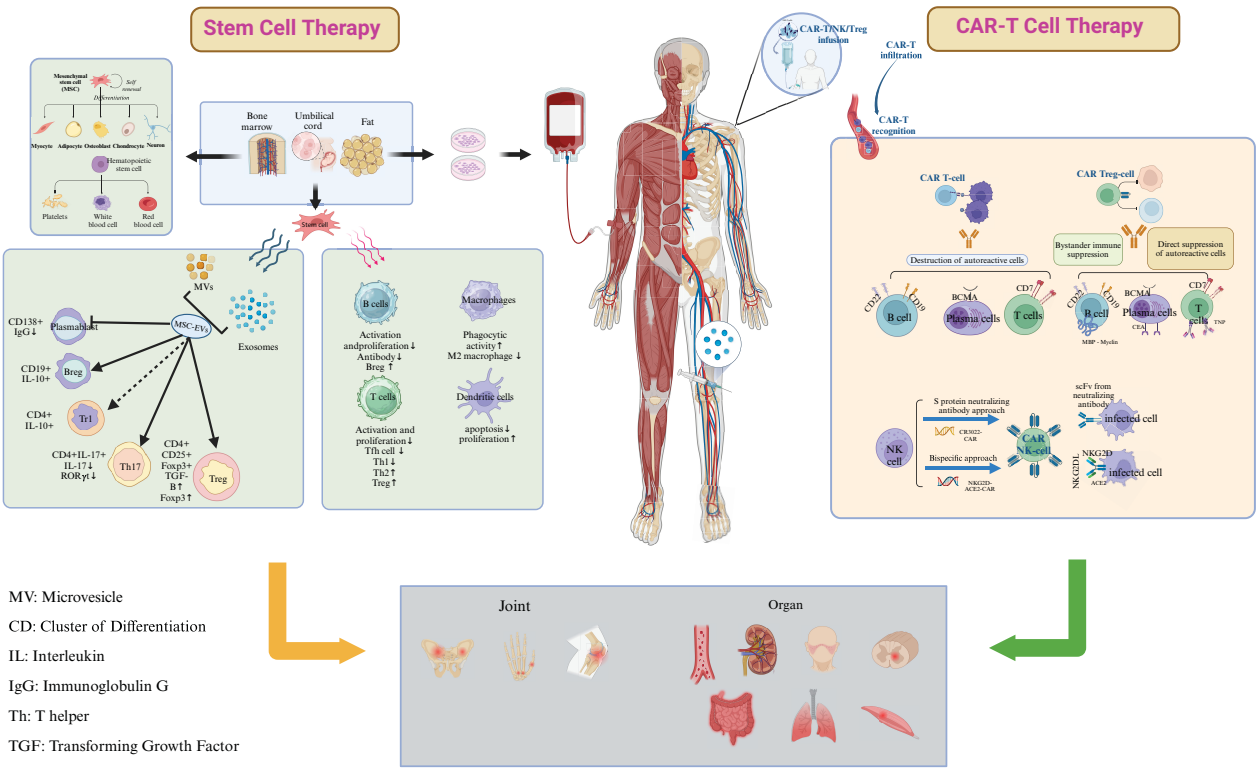

FIGURE 1
Key differences between stem cell therapy and chimeric antigen receptor T cell therapy. Composition and Structure: Stem cell therapy encompasses both mesenchymal stromal cells (MSCT) and hematopoietic stem cells (HSCT). Both types of stem cells can originate either from the patient (autologous) or a donor (allogeneic). Engineered chimeric antigen receptor (CAR) molecules are hybrid constructs derived from antibodies and T-cell receptors; they include an antigen-binding motif, a hinge domain, a transmembrane domain, one or more costimulatory domains, and a constitutive activation domain (CD3ζ signaling domain). Mechanism: Both stem cell therapy and CAR-T therapy can reboot the immune system and induce *de novo* tolerance in IMIDs. The immunomodulatory and trophic functions of MSCs are likely mediated through the production of soluble factors and the secretion of neurotrophic growth factors. The efficacy of HSCT may stem from the depletion of autoreactive immunologic memory and the generation of a rejuvenated and tolerant immune system. Engineered CAR-T cells specifically target and eliminate pathogenic immune cells, such as autoreactive B cells, thus mitigating the symptoms of such diseases. Furthermore, CAR-T cells exhibit potent depletive effects, enhanced by their superior tissue penetration, intrinsic lethality, and direct cytotoxicity towards plasmablasts and plasma cells. Preparation: MSCT: MSCs are isolated using density gradient centrifugation and adherence methods, cultured, and expanded ex vivo from bone marrow stem cell niches and other sources such as adipose tissue and umbilical cords. HSCT: peripheral blood stem cells are mobilized using cyclophosphamide; peripheral leukocyte counts are monitored, and cells are harvested when the white blood cell level rebounds. CAR-T therapy involves isolating T cells from the patient's peripheral blood mononuclear cells, engineering these cells to express CARs that recognize specific antigens, expanding the CAR-T cells *in vitro*, and then infusing the engineered cells back into the patient to target and destroy disease-causing cells. Pretreatment: The HSCT pretreatment regimen includes intravenous rabbit antithymocyte globulin and methylprednisolone, administered in a 100-grade laminar flow ward. Cyclophosphamide pretreatment is not recommended before MSCT. Prior to CAR T-cell infusion, patients undergo a conditioning chemotherapy regimen in a standard ward, which includes fludarabine and cyclophosphamide to achieve lymphocyte depletion. Post-infusion Monitoring: For stem cell therapy, post-infusion monitoring encompasses evaluating the success of stem cell implantation, blood cell recovery, occurrence of graft-versus-host disease, and the long-term function and potential complications of the immune system. In the case of CAR-T therapy, post-infusion monitoring focuses on detecting cytokine release syndrome, neurotoxicity syndrome, the persistence and activity of CAR-T cells *In vivo*, and the potential development of secondary malignancies.

costimulatory domains alongside CD3ζ. Fourth-generation CAR-T cells, known as TRUCK CARs, build on second-generation designs by introducing inducible cytokine expression, such as IL-12, to enhance the immune response. Fifth-generation CAR-T cells refine previous designs by integrating the IL-2R beta-chain, which activates the JAK/STAT pathway to optimize antigen-dependent T cell responses (44, 45).

In addition, multiple types of CAR-T cells have been engineered to address the complex immunopathology of hematologic malignancy and IMID. CAR-T cells target specific antigens on pathological cells, while CAAR-T cells recognize the B cell receptor (BCR) on self-reactive B cells to eliminate those involved in autoimmune diseases without requiring HLA or TCR recognition. In contrast, CAR-Treg therapy utilizes regulatory T cells to restore immune balance by suppressing excessive immune responses. CAR-NK cell therapies, with favorable safety and diverse sourcing of NK cells, may offer a cost-effective, "off-the-shelf" alternative that improves accessibility (46).

A key consideration in using CAR-T cells for IMID is determining how long they must persist for therapeutic effects. Unlike hematological malignancy, which may require long-term engraftment, a short burst of CAR-T cells might be enough to trigger an "immune reset" (47). Although the CD28 or 4-1BB co-stimulatory domains in CARs enhance the function T cells activation and anti-cancer activity, the ideal co-stimulatory domain for IMID remains unclear. However, minimizing toxicity will likely be a key consideration in the design of CAR therapies specifically for IMID (48).

3.3 Mechanisms and dynamics and of CAR-T cell therapy in IMIDs

T-cell-mediated autoimmunity in IMIDs leads to B-cell activation and autoantibody formation. Complete B-cell depletion may not be effective for refractory IMIDs, as residual autoreactive T cells and tissue-resident T cells could still sustain the disease (49). Engineered CAR-T cells can selectively target autoantigens such as CD19, CD7, and BCMA, eliminating autoreactive B cells, T cells, and plasma cells, to modulate the immune microenvironment and alleviate IMID symptoms (50, 51). In consistent with evidence from tumor studies showing that CAR-T cell residency in the bone marrow is more effective than peripheral persistence for tumor control (52, 53), CAR-T therapies not only deplete circulating B cells but also tissue-resident B cells, particularly in secondary lymphoid tissues, although memory B cells in these tissues may be resistant to depletion (49). However, whether CAR-T cell infiltration into tissues is insufficient or limited to certain subsets of CAR-T cells remains unclear. Furthermore, CAR-T cells derived from SLE patients can induce CD19-dependent activity against autologous primary B cells, accompanied by reduced inflammatory cytokine production (54).

Single-cell profiling and in-depth immunophenotyping in hematologic malignancy have demonstrated that long-lasting CD19-redirected CAR-T cells exhibit cytotoxic characteristics, along with sustained functional activation and proliferation, lasting for more than a decade (55, 56). Dynamic tracking of CAR-T cells in patients with anti-SRP necrotizing myopathy or myasthenia gravis suggests that anti-BCMA CAR-T cells may normalize the immune microenvironment, including the reconstitution of B-cell lineages with sustained reductions in pathogenic autoantibodies, inhibition of proliferative activity in CD8 + CAR-T cells, replacement of T cell subpopulations, and suppression of overactivated immune cells (57, 58). *In vivo* dynamic changes of CAR-T cells post-infusion revealed that the infused CAR-T cells were in a highly metabolically active state, with elevated glycolysis and biosynthetic gene expression. They gradually transitioned from a highly proliferative state to a highly cytotoxic state along a developmental trajectory, reaching a late remission phase characterized by a loss of proliferative activity but sustained cytotoxicity. Eventually, both proliferative and cytotoxic signatures in hematologic malignancy declined during the remission phase (59, 60). Longitudinal tracking of clones in blood and cerebrospinal fluid from five neuromyelitis optica spectrum disorder (NMOSD) patients treated with anti-BCMA CAR-T cell therapy suggests that CD8+ cycling CAR-T cells play a predominant role in autoimmunity, primarily derived from CD8+ effector T cells at baseline prior to manufacturing (61). Longitudinal analysis of peripheral blood mononuclear cells from an Scl70+ SSc patient suggested that CD19-CAR-T cell therapy reverses adaptive autoimmunity and restores Fcγ-receptor IIIA-expressing innate immune cells by reducing immune complex activity (62).

4 Preclinical studies of CAR-T therapy for immune mediated inflammatory diseases

The preclinical studies of CAR-T therapy for IMIDs were summarized in Table 1.

4.1 Mouse models of lupus

In two lupus mouse models (NZB/W and MRL-lpr strains), CD19 CAR-T cell transfer induced efficient and stable B cell depletion, elimination of circulating anti-DNA antibodies, and improvements in kidney function, spleen size, lymphocyte subset ratios, and skin inflammation, as well as a significant increase in lifespan (63). Furthermore, in the MRL-lpr lupus model, transfer of syngeneic anti-CD19 CAR-T cells resulted in a more sustained B-cell depletion effect than antibody treatment, prevented disease pathogenesis before the onset of symptoms, and exhibited therapeutic benefits at later stages of disease progression (64). Moreover, multiple preclinical models show that B cell activating factor (BAFF) CAR-T cell therapy effectively depletes autoreactive B cells, offering potential benefits such as avoiding B cell aplasia and preventing long-lived plasma cell escape, though it may also deplete normal mature B cells (65).

4.2 ANCA-induced acute kidney injury

In a preclinical model of myeloperoxidase-anti-neutrophil cytoplasmic autoantibody (MPO-ANCA)-associated vasculitis (AAV),

TABLE 1 Preclinical studies of CAR-T therapy for immune mediated inflammatory diseases.

First author	Year	Recognition domain	Carrier cells	Target cells	Dosage	Persistence
Mouse model of lupus						
Rita Kansal (63)	2021	CD19 scFv	CAR- T	CD19+B cells	1.2*10^7cells	(63)
Xuexiao Jin (64)	2021	CD28 or 4-1BB	CAR- T	CD28+B cells	6.7*10^6cells	
Vinayak Uppin (65)	2024	CD19 scFv	BAFF CAR-T	CD19+B cells	1 × 10^6cells	3\8\12\ 22 weeks
ANCA-induced acute kidney injury						
Dörte Lodka (66)	2024	CD3ζ	CAR- T	SP6 or CD19	2.0*10^6 cells	2\5\8 weeks
Multiple sclerosis						
Moa Fransson (67)	2012	CAR/FoxP3_Tregs	CAR/FoxP3_Tregs	Myelin oligodendrocyte glycoprotein	1.0*10^5cells	7 days
Meike Mitsdoerffer (68)	2021	CD19 scFv	T cell-conditional α4 integrin	CD19+ B cells	1.0–2.0*10^6 cells	4 days
Sasha Gupta (69)	2023	CD19 scFv	CAR- T	CD19+ B cells	2.0*10^6cells	
Autoimmune encephalitis						
S Momsen Reincke (71)	2023	CD8	NMDAR-CAART	B cell receptors (BCRs)	1,0*10^6cells	13 days
Cody D Moorman (72)	2023	anti-human myostatin scFv	CAR-T/CAR-Tregs	XCR1	3 ng/mL/ 1.0*10 ^6cells	7-21days
Autoimmune cholangitis						
Hao-Xian Zhu (73)	2024	CD28 and CD3ζ moieties	CAR-T	CD8+ T Cells	1.0*10^6 cells	
Graves' disease						
Honghong Duan (74)	2023	Signal domain (4-1BB)	TSHR-CAR-T	B cells	2.0*10^6 cells	
Type 1 diabetes						
Sigal Fishman (75)	2017	N terminus of β2m/CD3-ζ,	TCR	CD8+ T Cells	6–10*10^6 cells	
Li Zhang (78)	2018	GFP+CD8+ T cells	287-CAR T	CD4+ T cells	5.0*10^6 cells	
Michel Tenspolde (79)	2019	insulin-specific scFvs	CAR-Tregs	Insulin	2.5*10^6 cells	
Ilian A Radichev (77)	2020	Anti-HPi2 scFv	HPi2-CAR	CD98	1.0*10^6 cells	48 hours
Shio Kobayashi (76)	2020	I or II MHC molecules (pMHCI/II)	5MCAR	Antigen-specific T cells		
Justin A Spanier (80)	2023	CD28 and CD3ζ signaling domains.	CAR Tregs	CD4+ T Cells	1.5*10^6 cells	20weeks
Hemophilia A						
Kalpana Parvathaneni (81)	2018	CAR scFv	BAR	CD8+ T cells	1.0*10^6 cells	
Asthma						
Jelena Skuljec (82)	2017	CEA	CAR Tregs	Tregs	5.0*10^4 cells/well	
Gang Jin (84)	2024	5TIF4	IL-5 CAR T cells	BCOR and ZC3H12A	1 mg/kg	5 days a week
Cardiac fibrosis						
Haig Aghajanian (85)	2019	CD3ζ and CD28 cytoplasmic domains	CAR T	MHC I	1.0*10^7 cells	1 week
Joel G Rurik (86)	2022	CD5	CAR T	Lymphocytes		

(Continued)

TABLE 1 Continued

First author	Year	Recognition domain	Carrier cells	Target cells	Dosage	Persistence
Myasthenia gravis						
Sangwook Oh (87)	2023	CD137-CD3ze	MuSK-CAART	Anti-MuSK autoantibody or B cell receptor (BCR)	1.0*10^7 cells	
Pemphigus vulgaris						
C M Proby (88)	2000	Terminal EC1 and part of EC2	Dsg3-GFP	B cells	5 ug	12 weeks
Christoph T Ellebrecht (89)	2017	Dsg3 EC1-3/EC1-4/EC1-5	Dsg3 CAAR-T	B cell receptor (BCR)		
Systemic sclerosis						
Jérôme Avouac (90)	2023	CD19 scFv	CAR T	CD19+B cells	10 - 20*10^6 cells	6 weeks
IgG4-related disease						
Yeting Sun (93)	2024	CD28 or 4-1BB	CAR-T	CD19+B cells	1.5 × 10^6 cells	24hours/9 weeks
Ulcerative colitis						
Dan Blat (94)	2014	CD28 and CD3ζ moieties	CAR Tregs	Colorectal tumor	1.0*10^6 cells	

CD19-targeted CAR T cells effectively depleted B cells and plasmablasts, promoted a decline in MPO-ANCA levels, and, crucially, provided protection against necrotizing crescentic glomerulonephritis (66).

4.3 Multiple sclerosis

The CD4+ T cells were genetically engineered to express a CAR targeting myelin oligodendrocyte glycoprotein (MOG) in combination with the murine FoxP3 gene, resulting in a reduction of disease symptoms and a decrease in IL-12 and IFN-γ mRNA levels in brain tissue from a murine model of MS (67). In a mouse model of MS, treatment with anti-CD19 CAR-T cells resulted in the elimination of meningeal B cell aggregates and exacerbated clinical disease; however, in another B-cell-dependent MS model, anti-CD19 CAR-T cells effectively depleted B cells in both peripheral tissues and the central nervous system (CNS), leading to a reduction in clinical scores and lymphocyte infiltration (68, 69). These contrasting findings in two MS models highlight the diverse autoimmune mechanisms that drive tissue pathology, emphasizing the importance of careful model selection aligned with the primary objectives of the study when evaluating CAR-T therapies (70).

4.4 Autoimmune encephalitis

Anti-N-methyl-d-aspartate receptor chimeric autoantibody receptor (NMDAR-CAAR) T cells, which consist of an extracellular multi-subunit NMDAR autoantigen and intracellular 4-1BB/CD3ζ signaling domains, effectively depleted engineered B cells expressing an NMDAR-reactive antibody, resulting in a reduction of serum and brain autoantibodies, with no evidence of off-target toxicity or adverse events (71). X-C motif chemokine receptor 1-specific chimeric antigen receptor T cells (XCR1-CAR-T) and CAR-Tregs led to the depletion of DC1, modestly suppressing the onset of Th1-driven experimental autoimmune encephalomyelitis (72).

4.5 Autoimmune cholangitis

Primary biliary cholangitis is characterized by an autoreactive T cell response targeting intrahepatic small bile ducts. CD8+ tissue-resident memory T cells induce apoptosis of bile duct epithelial cells and upregulate PD-1 expression. Treatment of DKO mice with PD-1 CAR-T cells selectively depleted liver CD8+ tissue-resident memory T cells and alleviated autoimmune cholangitis (73).

4.6 Graves' disease

Graves' disease (GD) is caused by stimulating antibodies that target the thyroid-stimulating hormone receptor (TSHR), referred to as TSH receptor antibodies (TRAb). TSHR-CAR-T was constructed by incorporating the first extracellular domain (21-413aa) of TSHR, fused with the CD8 transmembrane and intracellular signaling domain (4-1BB), which can recognize and effectively eliminate TRAb-producing B lymphocytes both *in vitro* and *in vivo* (74).

4.7 Type 1 diabetes

First-generation CD3-ζ-CAR T cells, expressing the β2-microglobulin component of MHC I and targeting the anti-

InsB15-23-H-2Kd TCR, significantly reduced insulitis and protected non-obese diabetic (NOD) mice from T1DM (75). Another first-generation CAR cytotoxic T lymphocytes, equipped with five modules targeting clonotypic TCRs expressed by CD4+ T cells, could eliminate pathogenic CD4+ T cells, neutralize their impact on T1DM, and reduce insulitis in the pancreas of treated NOD mice (76). Two types of CD28-CAR Tregs targeting the human pancreatic endocrine marker Hpi2 demonstrated no immunologic function against the Hpi2 antigen *in vitro*, due to the broad expression of the Hpi2 antigen across multiple cell types. This underscores the importance of careful selection of the CAR recognition target (77). None of the 287 NOD mice treated with second-generation CD28-CAR and 4-1BB-CAR CD8+ T cells targeting the I-Ag7-B:9-23(R3) complex developed T1DM before 18 weeks of age; however, protection declined over time, indicating that these CAR-T cells can only delay, but not prevent, the development of T1D (78). The second-generation CD28-CAR Tregs targeting soluble insulin were unable to prevent T1DM development in NOD mice but remained present even after four months (79). Another second-generation CD28-CAR Tregs targeting the insulin B-chain 10-23 peptide MHC complex were constructed and co-transferred, preventing adoptive transfer diabetes induced by BDC2.5 T cells in immunodeficient NOD mice. Additionally, these Tregs prevented spontaneous diabetes in WT NOD mice (80). The use of InsB-g7 CAR Tregs for the reversal of autoimmune diabetes following islet transplantation may be more amenable to clinical translation than disease prevention.

4.8 Hemophilia A

As 20% to 30% of patients receiving factor VIII (FVIII) gene therapy (F8) produce neutralizing anti-FVIII antibodies, an engineered B-cell antibody receptor (BAR) targeting the immunodominant FVIII domains (A2 and C2) was developed. This receptor could effectively eliminate FVIII-reactive B-cell hybridomas both *in vitro* and *In vivo*, significantly reducing anti-FVIII antibody formation in hemophilic mice (81).

4.9 Asthma

Allergic airway inflammation is characterized by airway hyper-reactivity (AHR) and a chronic, T helper-2 (Th2) cell-dominated immune response to allergens. Carcinoembryonic antigen (CEA), which is expressed on the surface of adenoepitheliomas in the lung and gastrointestinal tract, was targeted by the development of an anti-CEA chimeric antigen receptor (CAR). Subsequently, CD4+CD25+ regulatory T cells (Tregs) and CD4+CD25− effector T cells (Teffs) expressing the anti-CEA CAR were isolated from the spleens of anti-CEA CAR-transgenic (CARtg) mice (82). The targeted CD4+CD25+ regulatory T cells (Tregs) significantly reduced airway hyper-reactivity, airway eosinophilia, mucus hypersecretion, Th2 cytokine production, and allergen-specific IgE after sensitization with a model allergen. Furthermore, they efficiently suppressed the proliferation of CAR-expressing effector T cells (Teffs) *in vitro*, either upon stimulation through their T cell receptor (TCR) or via CAR binding to the cognate antigen (82). Given that IgE is a key mediator of allergic responses, IgE-expressing cells can be targeted by T cells through the recognition of the transmembrane form of IgE (mIgE), via the extracellular domain of the high-affinity IgE receptor, FεRIα. Low-affinity FcεRIα-based CARs are capable of mediating potent and specific T cell responses against mIgE-expressing target cells, even in the presence of secreted IgE (83). Type 2-high asthma, characterized by IL-5-driven eosinophilia, prompted the development of long-lived CAR T cells engineered with IL-5 as the targeting domain. These cells deplete BCOR and ZC3H12A, leading to sustained repression of lung inflammation and alleviation of asthmatic symptoms in asthma models (84).

4.10 Cardiac fibrosis

Excessive cardiac fibrosis is associated with various forms of cardiac disease and heart failure; however, limited clinical interventions are available to address cardiac fibrosis. Adoptive transfer of T cells expressing CARs targeting fibroblast activation protein (FAP), an endogenous marker of cardiac fibroblasts, results in a significant reduction in cardiac fibrosis and the restoration of function after injury in mice (85). Transient antifibrotic CAR-T cells were generated *In vivo* by delivering modified messenger RNA (mRNA) encapsulated in T cell–targeted lipid nanoparticles (LNPs). Treatment with modified mRNA-targeted LNPs reduced fibrosis and restored cardiac function after injury, highlighting the promising potential of *In vivo* generation of CAR T cells as a therapeutic platform for treating various diseases (86).

4.11 Myasthenia gravis

T cells were genetically engineered to express a MuSK chimeric autoantibody receptor (MuSK-CAART) containing CD137-CD3ζ signaling domains, which demonstrated comparable efficacy to anti-CD19 chimeric antigen receptor T cells in depleting anti-MuSK B cells and retained cytolytic activity even in the presence of soluble anti-MuSK antibodies (87).

4.12 Pemphigus vulgaris

A chimeric autoantigen-toxin molecule, consisting of the immunoreactive portion of the extracellular domain of desmoglein 3 and Pseudomonas exotoxin, was constructed to specifically recognize and eliminate autoimmune B cells in pemphigus vulgaris (PV). The chimeric molecules have been

shown to bind and partially kill B cell hybridomas producing anti-desmoglein 3 monoclonal antibodies. They can also partially eliminate antigen-specific B cells from the spleens of mice immunized with desmoglein 3 (88). A CAAR, consisting of the PV autoantigen desmoglein (Dsg) 3 fused to CD137-CD3z signaling domains, exhibited specific cytotoxicity against cells expressing anti-Dsg3 BCRs *in vitro*. It also expanded, persisted, and specifically eliminated Dsg3-specific B cells *In vivo* (89). DSG3 CAAR-T cells selectively kill DSG3-reactive B cells while sparing non-affected B cells, and they do not affect keratinocytes either *in vitro* or *in vivo*.

4.13 Systemic sclerosis

In the Fra-2 transgenic (Tg) mouse model, depleting circulating B cells with anti-CD20 mAb infusion three days before CAR-T cell injection resulted in deeper B-cell depletion in both peripheral blood and lesional lungs compared to anti-CD20 mAb alone. However, CAR-T cell infusion significantly increased lung collagen content, histological fibrosis scores, and right ventricular systolic pressure, while also worsening clinical outcomes and increasing mortality in Fra-2 Tg mice (90). While anti-CD20 mAb pre-treatment may enhance CAR-T cell engraftment and persistence (91), it could also lead to unpredictable adverse effects. The persistence and accumulation of CD19-targeted CAR-T cells in lesional lung tissue may be exacerbated by severe lung inflammation. Given the extensive lung involvement in the Fra-2 model compared to murine lupus (92), these findings suggest that CD19-targeted CAR-T cells may not be suitable for initial use in SSc patients with a high lung inflammatory burden.

4.14 IgG4-related disease

Dysregulated B-cell activation drives IgG4-related disease (IgG4-RD). In a LatY136F knock-in mouse model, CD19-targeted CAR-T cell infusion depleted B-cells and reduced CD138+ plasma cells, with CD28 co-stimulatory domain CAR-T cells showing greater efficacy than 4-1BB CAR-T cells. Importantly, CAR-T cells derived from IgG4-RD patients demonstrated effective *in vitro* functionality, highlighting their potential for clinical application (93).

4.15 Ulcerative colitis

Given that carcinoembryonic antigen (CEA) is overexpressed in both human colitis and colorectal cancer, CEA-specific CAR regulatory T cells (Tregs) were developed. These cells suppress the severity of colitis in the T-cell-transfer colitis model and reduce the subsequent colorectal tumor burden in the azoxymethane–dextran sodium sulfate model for colitis-associated colorectal cancer (94).

5 Clinical studies and cases of CAR-T therapy for immune mediated inflammatory diseases

The clinical cases of CAR-T therapy for IMIDs were summarized in Table 2. Generally, CAR-T cells have exhibited significantly longer persistence in clinical cases than preclinical studies (Tables 1, 2).

5.1 Lymphoma concurrent with SLE

CAR-T therapy for lymphoma has shown dual benefits, improving both lymphoma and concurrent SLE or anti-phospholipid syndrome (95, 96). Two studies have demonstrated the therapeutic potential of CD19 CAR-T cell therapy in patients with concurrent SLE and lymphoma. A 41-year-old female with a 20-year history of SLE, recently diagnosed with stage IV diffuse large B-cell lymphoma, who received BCMA-CD19 compound CAR-T cells, exhibited reductions in nuclear, cytoplasmic, and granular ANA and total immunoglobulin levels, achieving remission of both SLE and lymphoma (97). Furthermore, fifty-eight patients with concurrent B-cell non-Hodgkin lymphoma and IMIDs who received CD19 CAR-T cell therapy showed significant reductions in inflammatory markers, seronegative conversion of autoantibodies, and a decrease in the use of steroids and disease-modifying anti-rheumatic drugs (98).

5.2 Case series of SLE

A case series with follow-up of eight SLE patients has raised cautious optimism for anti-CD19 CAR-T cell therapy in SLE, particularly regarding deep B-cell depletion, disease remission, seroconversion, cytokine modulation, refractory immune thrombocytopenia, reactivity profiles, GMP-grade preparation, and safety (9, 10, 99–104). In contrast to adult SLE, anti-CD19 CAR-T cell therapy also rescued pediatric SLE patients with/without lupus nephritis (LN) (105–107). More importantly, a Phase 1 study of BCMA and CD19-directed compound CAR-T therapy demonstrated the ability to simultaneously reset the humoral and B-cell immune systems and deplete disease-causing autoantibodies derived from B cells and long-lived plasma cells in patients with SLE/LN (108). CNS involvement, including encephalitis and transverse myelitis, is a severe manifestation of SLE. Notably, a 21-year-old man with SLE and progressive transverse myelitis showed improved neurological function, muscular strength, and regression of MRI lesions after treatment with a second-generation CAR T-cell therapy (CD19 binder and 4-1BB co-stimulation), along with decreased CSF protein (109). Increasing evidence indicates that plasmacytoid dendritic cells (pDCs) play a significant role in the development of IMIDs, primarily through the overproduction of proinflammatory cytokines (110). Two Phase 2 trials have suggested that the

TABLE 2 Clinical studies and cases of CAR-T therapy for immune mediated inflammatory diseases.

First author	Year	Recognition domain	Carrier cells	Target cells	Dosage	Persistence
SLE with lymphoma						
Jacob L Schmelz (96)	2019	CD19 scFv	CAR- T	CD19+B cells		30 days
Wenli Zhang (97)	2021	CD19-BCMA	CAR- T	BCMA-CD19	5.3*10^6/kg	2 months
Jiasheng Wang (98)	2023	CD19 scFv	CAR- T	CD19+B cells		6 months
Case series of SLE						
Dimitrios Mougiakakos (99)	2021	CD19 scFv	CAR- T	CD19+B cells		
Andreas Mackensen (9)	2022	CD19 scFv	CAR- T	CD19+B cells	1.0*10^6/kg	10 days
Afroditi Boulougoura (101)	2023	CD19 scFv	CAR- T	CD19+B cells	1.0*10^6/kg	3 months
Xue He (107)	2025	CD19 scFv	CAR-T	CD19+B cells	1 × 10^5	60 days
Melanie Hagen (109)	2024	CD19 scFv and 4-1BB	CAR-T	CD19+B cells	1.0*10^6/kg	12 weeks
Daniel Nunez (100)	2023	CD19 scFv and 4-1BB	CAR-T	CD19+B cells	1 × 10^8	3 months
Tobias Krickau (105)	2024	CD19	CAR- T	CD19+B cells	1.0*10^6/kg	
Weijia Wang (108)	2024	CD3ζ, CD28 and 41BB	cCAR-T	BCMA and CD19+B cells	3.0*106/kg	3 months
Mengtao Li (102)	2024	CD19	CAR- T	CD19+B cells	5*10^3/kg	14days
Josefina Cortés Hernández (103)	2023	CD19 scFv	CAR T	CD19+B cells	12.5*10^6	28 days
J. Cortés-Hernández (104)	2024	CD19 scFv	CAR T	CD19+B cells	12.5*10^6	
C. Bracaglia (106)	2024	CD19 scFv	CAR T	CD19+B cells	1.0*10^6/kg	3 months
Lymphoma concurrent with anti-phospholipid syndrome						
Eleonora Friedberg (114)	2025	CD19 scFv	CAR T	CD19+B cells		1 year
Jacob L Schmelz (96)	2020	CD19 scFv	CAR T	CD19+B cells		30 days
Idiopathic inflammatory myopathy						
Fabian Müller (115)	2023	CD19 scFv	CAR- T	CD19+B cells	1.0*10^6/kg	
Ann-Christin Pecher (116)	2023	CD19 scFv	CAR- T	CD19+B cells	1.23*10^6/kg	
Jule Taubmann (117)	2024	CD19 scFv	CAR- T	CD19+B cells	1.0*10^6/kg	
Chuan Qin (61)	2024	CD19 scFv	CAR-T	CD19+ CD27− IgD+ naive B cells	1.0*10^6	18 months
Fabian Müller (10)	2024	CD19 scFv	CAR- T	CD19+B cells		
Rebecca Nicola (118)	2024	CD19 scFv	CAR- T	CD19+B cell	1× 106 CAR T cells/kg	8 months
Xiaobing Wang (120)	2024	CD19 scFv	CAR- T	CD19+B cells	1.0*10^6/kg	3 months
Jenell Volkov (119)	2024	CD19 scFv	CAR T	CD19+B cells	1.0*10^6/kg	4 months
Systemic sclerosis						
Christina Bergmann (121)	2023	CD19 scFv	CAR- T	CD19+B cells	1.0*10^6/kg	
Wolfgang Merkt (122)	2024	CD3ζ, CD28 and 41BB	CAR- T	CD19+B cells	5.0*10^6/kg	
Fabian Müller (10)	2024	CD19 scFv	CAR- T	CD19+B cells		
Janina Auth (124)	2025	CD19 scFv	CAR T	CD19+B cells	1.0*10^6/kg	3, 6, 9, and 12 months

(Continued)

TABLE 2 Continued

First author	Year	Recognition domain	Carrier cells	Target cells	Dosage	Persistence
Rheumatoid arthritis						
Aiden Haghiki (126)	2024	CD19 scFv	CAR- T	CD19+B cells	1×10^8	5 months
Merav Lidar (129)	2025	CD19 scFv	CAR T	CD19+B cells	1.0*10^6/kg	100 days
Aliya Masihuddin (128)	2024	CD19 scFv	CAR-T	CD19+B cells		
Fredrik N Albach (127)	2025	CD19 scFv	CAR-T	CD19+B cells		56 days
Yujing Li (130)	2025	anti-IL-6 single chain variable fragments (scFv) and anti-TNFα scFv	CAR-T	CD19/aIL-6/aTNFα		
Aiden Haghiki (126)	2024	CD19 scFv	CAR- T	CD19+B cells	1×10^8	5 months
Pemphigus vulgaris						
Felix Fischbach (132)	2024	CD19	CAR- T	CD19+B cells	1.0*10^8	
Multiple sclerosis						
Felix Fischbach (132)	2024	CD19 scFv	CAR T	CD19+B cells	1.0*10^8	3 months
Neuromyelitis optica spectrum disorders						
Chuan Qin (134)	2023	BCMA scFv	CAR- T	B-cell maturation antigen (BCMA)	1.0*10^6/kg	2 years
Myasthenia gravis						
Volkan Granit (135)	2023	plasma cells	rCAR-T	B-cell maturation antigen (BCMA)	3.5*10^6/kg	
Aiden Haghikia (136)	2023	CD19	CAR-T	B cells and autoantibodies	1.0*10^8	62 days
Dai-Shi Tian (58)	2024	CD8	CAR-T	B cells	1.0*10^6/kg	18 months
Jeremias Motte (137)	2024	CD19 scFv	CAR-T	B cells	1 × 10^8	154 days
Stiff-person syndrome						
Simon Faissner (138)	2024	CD19 scFv	CAR T	CD19+B cells	1 × 10^8	3 months
ANCA-associated vasculitis						
Ioanna Minopoulou (139)	2025	CD19 scFv	CAR T	CD19+B cells	1.0*10^6	6 weeks

elimination of pDCs is beneficial in improving both systemic and cutaneous lupus erythematosus (111, 112). A preliminary study demonstrated the feasibility of producing CD123-targeted CAR-T cells from the T cells of patients with IMIDs, as well as their *in vitro* cytotoxicity against circulating autologous pDCs (113).

5.3 Lymphoma concurrent with anti-phospholipid syndrome

The current treatment for antiphospholipid syndrome (APS) involves indefinite anticoagulation with vitamin K antagonists to prevent thrombotic recurrence, with no curative option to permanently eradicate antiphospholipid antibodies (aPL). However, a patient with relapsed or refractory diffuse large B-cell lymphoma (DLBCL) and a nearly 30-year history of APS achieved DLBCL remission, as assessed by PET-CT, and normalization of anticardiolipin antibodies at 1-year follow-up after CAR T-cell treatment (96). Another 65-year-old woman with SLE, APS (triple aPL positivity), and DLBCL achieved ongoing complete remission of DLBCL after anti-CD19 CAR T-cell treatment, along with seroconversion of lupus anticoagulant, anti-cardiolipin, and anti-beta2 glycoprotein I antibodies (114). Both cases suggest a promising role for targeted cell therapy in treating pro-thrombotic autoimmune disorders.

5.4 Idiopathic inflammatory myopathy

Several adult cases of CD19 CAR-T cell therapy in Anti-synthetase syndrome have demonstrated resolution of muscle, lung, and skin inflammation, despite prior failure of two B-cell-depleting antibodies, suggesting that the depth of B-cell depletion is significantly greater with CAR-T cell-based therapy than with

antibody-based B-cell depletion (10, 115–117). A satisfactory response to anti-CD19 CAR-T cell therapy has recently been reported in a 12-year-old caucasian boy with refractory juvenile dermatomyositis, which was resistant to multiple lines of immunosuppressive treatment, including B-cell depletion with rituximab (118).

There are no FDA-approved therapies for the immune-mediated necrotizing myopathy (IMNM) subtype of idiopathic inflammatory myopathy. The first IMNM patient in the RESET-Myositis™ phase I/II trial (NCT06154252) treated with fully human 4-1BBz anti-CD19 CAR T-cell therapy (CABA-201) showed decreased CK levels, improved muscular strength, depleted peripheral B-cells, and a reduction in autoantibodies to baseline (119). Additionally, a patient with anti-signal recognition particle IMNM, refractory to multiple available therapies, who was treated with BCMA-CAR-T cells, demonstrated a favorable safety profile, sustained reduction in pathogenic autoantibodies, and persistent clinical improvements over 18 months (57). Encouragingly, treatment with genetically engineered, healthy donor-derived CD19-targeted CAR-T cells (TyU19) alleviated severe skeletal muscle damage in a patient with refractory IMNM and reversed extensive fibrotic damage to critical organs in two patients with diffuse cutaneous systemic sclerosis (120).

5.5 Systemic sclerosis

The first promising experience with CD19-targeted CAR-T cells in diffuse cutaneous systemic sclerosis (SSc) was reported in a 60-year-old man, who exhibited rapid improvement in heart function, as assessed by 68Ga-FAPI-04 PET-CT, joint health from MRI, and skin fibrosis, alongside seroconversion of antinuclear antibody (ANA) and anti-RNA polymerase III autoantibodies, as well as stable lung function parameters (121). A 38-year-old woman with Scl70+ systemic sclerosis (SSc) and rapidly progressive non-specific interstitial pneumonia, treated with third-generation CD19 CAR-T cells, showed sustained amelioration of lung function and dramatic regression of imaging findings, including the disappearance of Fcγ-receptor-activating immune complexes (122). Four additional patients with SSc, treated with CD19 CAR-T therapy, did not exhibit disappearance of anti-Scl70 antibodies, and their EUSTAR activity index did not regress to negative levels (10). The absence of anti-Scl70 antibody seroconversion, along with reduced EUSTAR activity in all affected organs, suggests that prolonged CAR-T cell persistence may promote seroconversion, potentially leading to extended B-cell depletion, impaired vaccination responses, and an immunocompromised state (123). Therefore, the benefit-risk balance of pretreatment, CAR-T cell persistence and the duration of B-cell depletion must be carefully evaluated. Encouragingly, the same research group recently confirmed that CD19-targeting CAR T-cell therapy may prevent the progression of fibrotic and vascular organ manifestations in SSc patients (124).

5.6 Rheumatoid arthritis

Universal anti-fluorescein isothiocyanate (FITC) CAR-T cells, in combination with FITC-labelled citrullinated peptides, have been shown to specifically redirect and eliminate hybridoma cells and autoreactive B cell subsets from RA patients by specifically recognizing FITC-labelled citrullinated peptide epitopes. The cytotoxicity of these CAR-T cells was strictly dependent on the presence of the peptides in a dose-dependent manner (125). Several cases of RA with coexisting myasthenia gravis, SSc, Sjogren's syndrome, and DLBCL demonstrated that CD19-directed CAR T-cell therapy not only achieved stable clinical remission but also led to seroconversion of anticitrullinated protein antibody (ACPA), indicating the eradication of ACPA-specific plasma cells (126–129). Notably, in three patients with difficult-to-treat RA, fourth-generation CD19-targeted CAR T-cells (CD19/aIL-6/aTNFα) improved symptoms, reduced effusion, synovitis, osteitis, and tendinitis (via ultrasound and MRI), and induced seroconversion of rheumatoid factor and anti-CCP antibodies (130). This study presents a novel therapeutic approach that combines TNF and IL-6 inhibition with B-cell depletion in a single CAR T-cell platform, enhancing RA treatment efficacy while also mitigating CRS.

5.7 Pemphigus vulgaris

A first-in-human trial provided preliminary evidence of the efficacy of DSG3-CAART in patients with active anti-DSG3 mucosal PV. This therapy specifically lyses human anti-DSG3 B cells, decreases target cell burden, reduces serum and tissue-bound autoantibodies, and enhances DSG3-CAART engraftment (131).

5.8 Multiple sclerosis

Two patients with progressive MS who received CD19 CAR-T cell therapy (KYV-101) showed CAR-T cell presence and expansion in the cerebrospinal fluid, without neurotoxicity, alongside a reduction in intrathecal antibodies in one patient (132). The FDA has granted Fast Track Designation to the autologous, fully human CD19 CAR-T cell product candidate, KYV-101, for the treatment of patients with refractory progressive MS (133).

5.9 Neuromyelitis optica spectrum disorders

A first-in-human clinical study of CT103A indicated that BCMA CAR T-cell therapy resulted in improvements in disability and quality of life outcomes, a reduction in AQP-4 antibodies, and a manageable safety profile in patients with relapsed or refractory AQP4-IgG seropositive neuromyelitis NMOSD (134).

5.10 Myasthenia gravis

In contrast to the conventional DNA-based approach, autologous BCMA RNA chimeric antigen receptor T cells (BCMA rCAR-T), as exemplified in the Descartes-08 trial, resulted in clinically meaningful reductions in all measures of myasthenia gravis severity, including the induction of minimal symptom expression and the elimination of dependence on intravenous immunoglobulin infusions in adults with generalized myasthenia gravis (NCT04146051) (135). A 33-year-old woman with anti-acetylcholine receptor (anti-AChR)-positive generalized myasthenia gravis received a second-generation anti-CD19 CAR T construct (KYV-101), resulting in a 70% reduction in pathogenic anti-AChR antibodies and the maintenance of protective vaccination IgG titers, which paralleled an improvement in the patient's muscle strength and reduction in fatigue (136).

Two patients with highly relapsed and refractory myasthenia gravis—one with AChR-IgG and the other with MuSK-IgG—treated with BCMA CAR-T, resulting in B cell lineage reconstitution, exhibited favorable safety profiles and sustained clinical improvements (58). The safe administration of anti-CD19 CAR T cells in two women with concomitant myasthenia gravis and Lambert-Eaton myasthenic syndrome resulted in profound B cell depletion and normalization of acetylcholine receptor and voltage-gated calcium channel N-type autoantibody levels, which paralleled significant neurological responses (137).

5.11 Stiff-person syndrome

A 69-year-old female with a 9-year history of treatment-refractory stiff-person syndrome, characterized by deteriorating episodes of stiffness, received an infusion of autologous anti-CD19 CAR T cells (KYV-101), resulting in a reduction of GABAergic medication, as well as improvements in leg stiffness, gait, walking speed, and daily walking distance (138).

5.12 ANCA-associated vasculitis

In a 52-year-old male patient with severe, therapy-refractory proteinase-3 (PR3)- AAV, anti-CD19 CAR T-cell therapy induced both clinical remission and seroconversion of PR3-ANCA (139). Initial bone marrow biopsies, taken post-rituximab and before anti-CD19 CAR T-cell therapy, showed $CD19^{+}/CD20^{-}$ B cells, including plasma cells in bone marrow. However, a second biopsy after CAR T-cell therapy revealed CAR T cells and the absence of CD19+ B cells or $CD19^{low}$ plasma cells in bone marrow, while $CD138^{+}/CD19^{-}$ plasma cells persisted. These findings confirm limited ability of rituximab to eliminate tissue-resident B cells in primary and secondary lymphoid organs and highlight effective B-cell depletion in the bone marrow following anti-CD19 CAR T-cell therapy.

5.13 Ulcerative colitis

There are two cases of IBD-like colitis following CAR T cell therapy for lymphoma have been reported, highlighting the need for close monitoring of lower gastrointestinal symptoms in patients undergoing CAR T-cell therapy (140, 141). More importantly, additional data is required to validate the effectiveness and safety of CAR-T therapy in patients with IBD.

6 Safety of CAR-T therapy

6.1 Risk of infection

As cytopenia and hypogammaglobulinemia are potential side effects of CAR T-cell therapy, infection has become an increasingly recognized complication, arising from both host-related factors and CAR T-associated factors (142). Like hematological malignancies, patients with IMIDs undergoing CAR T-cell therapy typically have relapsed/refractory disease with a history of multiple treatments, can lead to immune exhaustion, decreased bone marrow reserve with preexisting cytopenia, and delays in post-treatment immune recovery (143). Compared to patients undergoing stem cell therapy, post-CAR T-cell immune reconstitution is less well understood. Research on infection and immune recovery after CAR T-cell therapy is essential for developing effective prophylactic and treatment strategies for these patients (144). Hypogammaglobulinemia occurs in most patients after CAR T-cell therapy, requiring enhanced immunologic monitoring to identify those at high risk for severe infections and mortality who may benefit from immunoglobulin replacement (145).

6.2 Cytokine release syndrome and neurotoxicity syndrome

Two toxic effects of CAR T-cell therapy are of particular concern: cytokine release syndrome, which is mediated by CAR T-cell activation upon target cell engagement and the release of proinflammatory mediators such as IL-6, and neurotoxicity syndrome, which involves endothelial activation and disruption of the blood–brain barrier (146). Cytokine release syndrome rates in cancer range from 42% to 93% across all therapeutic cell products (146), however, in IMIDs, the occurrence was minimal, with no high-grade or prolonged bone marrow toxicity observed (10). More importantly, the key risk factor for both toxic effects in tumors is a high target cell burden, which is not present in IMIDs.

6.3 Secondary malignancy after CAR-T therapy

Although FDA adds boxed warning of secondary cancer to CAR T-cell therapies in hematologic malignancy, there is no solid

evidence to support a higher risk of secondary cancer among IMID patients treated with CAR T than those treated with other therapies. A retrospective comparative cohort study showed a comparable safety profile of CAR T-cell therapy for cancer patients with and without pre-existing autoimmune or inflammatory disease (147). Notably, the DESCAR-T registry indicates a very low risk of T cell malignancy in patients with hematologic malignancies following CAR T-cell therapy, and integration site analysis suggests that the therapy may have contributed to the development of secondary T cell malignancy (148). Alternative factors, including prior multiple lines of treatment, immune dysfunction and clonal hematopoiesis of underlying disease, likely play a greater role than insertional mutagenesis in predisposing CAR-T-treated patients to secondary hematologic malignancies (148). Although the benefits of CAR T-cell therapy for IMID outweigh the risks, vigilant monitoring for secondary cancer remains essential.

7 Clinical trials of CAR-T for immune mediated inflammatory diseases

Currently, 23 trials are registered in the clinicaltrials.gov database to evaluate the efficacy of CAR-T therapy in IMIDs, including SLE, Sjögren's syndrome, scleroderma, pemphigus vulgaris, neuromyelitis optica, and myasthenia gravis, among others (Supplementary Table S1). Emerging clinical results from recent EULAR, ACR, EHA meeting provide preliminary human data on how targeted cell therapy programs the immune system to durably reverse autoimmunity—an outcome previously unattainable with targeted biologic agents and stem cell therapies (Supplementary Table S2).

Several prospective clinical trials are underway to assess the safety and efficacy of CAR19-T cell therapy for SLE. An open-label, single-arm, multicenter phase 1/2 study (NCT05798117) provided preliminary data from the first three sentinel patients with severe refractory SLE who received YTB323, indicating favorable safety, CAR-T cell expansion, B cell depletion, and initial efficacy (103). Another pilot trial indicated that BCMA-CD19 CAR-T therapy in 13 patients with SLE or LN could eliminate autoantibodies, reset the B cell and humoral immune systems, and provide long-term, medication-free remission with a single dose (149).

8 Future directions and challenges

Building on advances in CAR T immunoengineering and insights gained from the use of conventional CAR T cell therapies in cancer, greater efforts have been directed toward targeting multiple factors implicated in the pathogenesis of IMIDs. Although the successful application of TyU19 and KYV-201 in IMIDs encourages the further exploration of allogeneic CAR-T therapy in patients with severe and refractory IMIDs (120), the optimal timing for allogeneic CAR-T therapy in autoimmunity requires further investigation due to the potential unknown short- and long-term safety risks and toxicity associated with off-the-shelf CAR-T therapies (150–152).

All commercially available CAR-T cell therapies express second-generation CARs containing a single costimulatory domain, whereas third-generation CAR-Ts, which incorporate two costimulatory domains, have demonstrated promising clinical efficacy and remarkably low procedure-specific toxicity in acute lymphoblastic leukemia (153). The efficacy of third-generation CAR-Ts in IMIDs requires further investigation. Many patients with B cell malignancies unfortunately fail to achieve durable responses to CAR-T therapy. Further research is needed to address resistance to CAR-T therapies, even though such resistance has not been observed in patients with IMIDs to date (154). The high costs and regulatory challenges of viral vectors in CAR T therapies have led to growing interest in non-viral gene delivery as a potential alternative (155). However, low knock-in efficiency remains a challenge in non-viral CAR-T cell production, underscoring the need for advancements in non-viral vector design and clinical manufacturing (156). As different IMIDs with shared cellular biology are often treated with similar therapies, more strategically designed cross-disease and cross-discipline basket trials should be implemented to mitigate the risk of failure in early-phase CAR-T cell therapy studies in IMIDs (157).

In comparison to targeted biologics and stem cell therapies, CAR-T cell therapy offers highly individualized characteristics, allowing for the precise recognition and targeting of immune-dysregulated cells, which enhances efficacy and reduces side effects. Although the outlook for CAR-T cell therapy in the treatment of IMIDs is highly optimistic, targeted cell therapies still face numerous challenges that limit their widespread translation and commercialization, including the optimization of proliferation and persistence, enhancement of potency, and development of effective, scalable manufacturing processes (158–161). Cutting-edge basic research, driven by immunoengineering approaches, could help address these challenges and pave the way for a new class of therapeutics focused on immunomodulation rather than immunosuppression (162, 163).

9 Conclusions

Despite advancements in targeted biologic therapies for IMIDs, challenges such as issues with long-term maintenance, non-responsiveness, and adverse side effects continue to pose significant barriers. Stem cell therapy is increasingly recognized as a standard salvage treatment, while CAR-T therapy holds promising potential for achieving long-lasting remissions. A clear understanding of the differences between these therapies is crucial for accurately evaluating the current status and future prospects of CAR-T therapy. Ongoing research is critical to addressing existing limitations and improving treatment outcomes for IMIDs.

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Conflict of interest

Author HX was employed by company Antengene Corporation.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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EDITED BY
Guobao Chen,
AbbVie's Cambridge Research Center,
United States

REVIEWED BY
Nataša Radulović,
University of Belgrade, Serbia
Shiyu Xiao,
Northwestern University, United States

*CORRESPONDENCE
Zhenjie Chen
ruoshui8000@126.com
Jingwei Zhou
13910634708@163.com

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Low-dose cyclophosphamide combined with chinese herbal medicine Shuli Fenxiao formula for the treatment of intermediate-to-high risk primary membranous nephropathy

Yiran Du[1], Yuning Liu[1], Zhiyan Chen[2], Yingnan Liang[1], Xia Li[1], Ying Wang[1], Jie Lv[1], Zhongjie Liu[1], Zhen Wang[1], Weihong Chen[1], Qingqing Liu[1], Xiaowen Li[1], Zhenjie Chen[1*] and Jingwei Zhou[1*]

[1]Department of Nephrology, Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing, China, [2]Xiyuan Hospital, Department of Nephrology, China Academy of Chinese Medical Sciences, Beijing, China

Background: At present, the side effects of hormonal and immunosuppressant therapy for intermediate-to-high risk primary membranous nephropathy (PMN) are relatively large, and the remission rate is limited, so more safe and effective regimens are needed.

Methods: This study is a clinical prospective case series study. 31 patients were finally included. The intervention was cyclophosphamide (CTX) combined with "Shuli Fenxiao formula(SLFX formula)", and the patients were treated for 24 weeks. The observation nodes were baseline, 2 weeks、12weeks and 24weeks after treatment.

Results: At 12 weeks of treatment, 38.7% of patients achieved partial response. At 24 weeks of treatment, 61.3% of patients achieved partial response and 24.5% achieved complete response. All Anti-phosholipase A2 Receptor Antibody (Anti-PLA2R) seropositive patients achieved immune remission. 24-hour urine total protein quantification (24hUTP) decreased from a median of 6.1 (IQR, 4.6-8.4) g/d to 2.7 (IQR, 0.6-8.7) g/d ($P<0.001$). Serum albumin (ALB) increased from 27.2 $\pm$ 6.4 g/L to 31.9 $\pm$ 8.0 ($P<0.05$). Within 24 weeks of follow-up after discharge, no patients relapsed. During the treatment follow-up period, 6 adverse events occurred in 31 patients, 1 patient developed heart failure during the treatment period, which was not considered to be clearly associated with treatment regimen or nephrotic syndrome (NS), 3 patients were infected, and 2 patients had liver impairment.

Conclusion: The results suggest that the combination of CTX and SLFX formula dramatically decreased Anti-PLA2R titers and 24hUTP levels, increased ALB in short term. The combination was safe and had few adverse effects. It has the

potential to be used as a potential option for the clinical treatment of intermediate-to-high risk PMN patients, particularly for elderly patients with contraindications to corticosteroid use or those with refractory disease.

International Traditional Medicine Clinical Trial Registry: http://itmctr.ccebtcm.org.cn/, identifier ITMCTR2025000355.

KEYWORDS

membranous nephropathy, therapeutic, side effects, integrative medicine, Shuli Fenxiao formula

1 Introduction

Primary membranous nephropathy (PMN) is a renal-confined autoimmune disease which is the leading cause of nephrotic syndrome (NS) in adults, accounting for approximately 30 percent of cases (1). According to epidemiological statistics, the prevalence of PMN has risen by 13% in recent years, and this increase is linked to environmental pollution and declining population size (2). Proteinuria is the primary clinical symptom of PMN, and the primary pathological alterations include the formation of immune complexes between target antigens and autoantibodies on the glomerular podocyte membrane, which further thickens the basement membrane, damages the podocytes, and ultimately destroys the glomerular filtration barrier. This pathologic damage is irreversible. As the disease progresses, approximately two-thirds of patients with PMN will develop end-stage renal disease after 20 years, severely affecting the life expectancy of patients (3). Anti-PLA2R antibody titer is a critical biomarker for assessing disease activity in membranous nephropathy. With effective treatment, the antibody titer progressively declines, and immunological remission generally occurs earlier than clinical remission (4). Following the achievement of immunological remission, rigorous monitoring of proteinuria and renal function is essential to evaluate the likelihood of the patient attaining clinical remission (5).

Currently, the optimal treatment plan for patients with PMN is determined by the various risk categories. The Global Organization for Improving the Prognosis of Kidney Disease (KDIGO) (6) proposes in the clinical practice guidelines for kidney disease that patients with PMN should be risk-stratified based on renal function, proteinuria quantification and antibody titers. About one-third of individuals with low-risk PMN who receive regular testing and basic supportive care will have spontaneous remission. Hormonal or immunosuppressive medication is necessary to manage the progression of the disease in high-risk PMN patients and intermediate-risk PMN patients with a progressive increase in proteinuria. The glomerular filtration barrier will be severely damaged as a result of the ongoing high levels of proteinuria and titer antibody levels, increasing the risk of renal failure.

For intermediate-to-high risk PMN, the first-line treatment is long-term cyclophosphamide (CTX) combined with glucocorticoid (GC). The combination of CTX and GC reduces the risk of end-stage renal disease by inhibiting immunity, alleviating renal immune damage, reversing the development of renal sclerosis and fibrosis, and inducing long-term remission of kidney disease (7). Even in patients receiving low-dose CTX 1.5 mg/kg/day, CTX with GC has been clinically shown to be quite hazardous (8). The prognosis and treatment compliance of PMN patients are significantly impacted by the potential for severe adverse responses, such as liver damage, bone marrow suppression, gonadal suppression, and malignancies, that can result from long-term use of CTX in conjunction with GC.

Due to the severe adverse effects of combination between CTX and GC, current studies have proposed steroid-free hormone regimens for patients with high-risk PMN. Xing HL. et al. (9) found that the low-dose rituximab (RTX) combining with low-dose tacrolimus (TAC) regimen induced serologic remission earlier than the classical regimen of GC combining with TAC in patients with intermediate-to-high risk PMN, with no significant difference in adverse effects between the two groups. Studies have shown that PMN is ineffective with both GC and CTX alone, and the combination of CTX is 50%-60% effective (10). Compared to the combination, CTX alone lowers the risk of opportunistic bacterial, fungal, and viral infections; however, long-term use of the medication increases the risk of thrombocytopenia, gonadal suppression, and unpleasant malignancies like leukemia, hemorrhagic cystitis, and bladder cancer (11).

In our previous study, we found that the clinical use of Shuli Fenxiao formula (SLFX formula) in the treatment of intermediate-risk PMN patients could reduce proteinuria and increase serum albumin (12). In animal experiments, it was found that "SLFX formula" is likely to reduce LC3, P62, and unblock the autophagy pathway, thereby counteracting the damage of podocytes and slowing down the progression of the disease (13). Based on this, this study used "SLFX formula" to replace GC to conduct a prospective study in patients with intermediate-to-high risk PMN to explore the efficacy and side effects of "SLFX formula" combined with low-dose CTX.

2 Materials and methods

2.1 Study design

This study was a real-world prospective own before-and-after controlled study that selected PMN patients who attended outpatient clinics or wards of Dongzhimen Hospital, Beijing University of Traditional Chinese Medicine, between May 2021 and December 2023, and screened and enrolled all intermediate-to-high risk PMN patients who met the criteria. The study was approved by the Ethics Committee of Dongzhimen Hospital with the ethical approval number 2021DZMEC-030-01, and all included patients gave informed consent and signed the informed consent form.

2.2 Inclusion and exclusion criteria

Inclusion Criteria: All patients were diagnosed with biopsy-proven PMN or serum Anti-PLA2R positivity. Patients met the following criteria prior to initiating combination therapy: 1) those aged 18-80 years; 2) estimated glomerular filtration rate (eGFR) > 30 ml/min/1.73 m^2; 3) a pathologically confirmed diagnosis of membranous nephropathy (stage 1-4) or Anti-PLA2R positivity by renal puncture biopsy; 4) met the diagnostic criteria for intermediate-to-high risk PMN; reference was made to the KDIGO Practice Guidelines for Clinical Management of Glomerular Diseases, 2021, and the Up to date guidelines, evaluated on the basis of temporal trends in clinical and serologic parameters; 5) patients who had received Renin Angiotensin System Inhibitor (RASi) for more than 3 months and still had 24-hour urine total protein quantification (24hUTP) quantification > 3.5 g/d; 6) no prior treatment with hormones and other immunosuppressive agents.

Exclusion Criteria: 1) pregnant, breastfeeding, or had a plan to become pregnant in the near future; 2) needed corticosteroid immunotherapy; 3) other types of MN, such as rapidly progressive membranous nephropathy (50% decrease in eGFR within 3 months) and secondary membranous nephropathy (SMN), such as infection, autoimmune disease, drugs, tumors, heavy metals, and so on; 4) who had used or were using steroids or other immunosuppressants in the last 6 months; 5) with heart failure, abnormal liver function, abnormal coagulation function, and current infection; 6) who were allergic to the study drugs; 7) with serious mental illness or with the presence of steroids or other immunosuppressants; 8) malignant/uncontrollable high blood pressure; 9) other reasons for the current unstable renal function, such as acute renal injury caused by massive hematuria; 10) white blood cell count < 3.0×10^9/L or other blood disorders; 11) electrocardiogram showing prolonged QT interval or severe arrhythmia, or concomitant use of medications that may cause prolongation of the QT interval.

2.3 Treatment

In order to suppress the renal immune response, all patients received oral CTX 50 mg once daily for 24 weeks. The Chinese herbal medicine was administered in the form of SLFX formula, 250 ml twice daily with hot water, from the outpatient pharmacy of Dongzhimen Hospital of Beijing University of Traditional Chinese Medicine (BUTCM). The Supplementary Material details the precise composition of the Chinese herbal medicines, the dosage, and how it was adjusted based on the patient's condition.

During the medication period, blood pressure and blood lipids of the patients were kept as under control as feasible, and appropriate basic treatment was given according to the KDIGO clinical practice guidelines. This included: 1) adequate dietary protein (0.8-1.0 g/kg/day), adequate caloric intake, and a low-salt diet (<3 g/day); 2) administration of RASi to control blood pressure around 125-130/75-80 mmHg; 3) use of statins (HMG CoA reductase inhibitors) to control the patient's hyperlipidemia; 4) if serum albumin (ALB) is below 20-25g/L or there are other risk factors for thrombosis, then anticoagulation with low molecular heparin or warfarin is used.

2.4 Outcome measures

2.4.1 Primary outcome

The primary outcome was a composite of complete remission and partial remission. According to the 2021 KDIGO guidelines: 1) complete remission is defined as: 24hUTP < 0.3g/d, serum ALB > 35g/L, and normal renal function: 2) partial remission is defined as: 0.3g/d < 24hUTP < 3.5g/d or > 50% decrease from baseline levels, significant improvement in serum ALB, and stable renal function; and 3) failure to remit is defined as: failure to achieve the above criteria, with 24hUTP > 3.5g/d, < 50% decrease from baseline level, and worsening renal function.

2.4.2 Secondary outcome

Secondary outcomes included changes in 24hUTP, serum creatinine (Scr), ALB, cholesterol (CHOL), low density lipoprotein (LDL-C), and triglyceride (TG) levels from baseline to 24 weeks after the start of treatment. Changes in serum Anti-PLA2R titers and relapse rates were also assessed. Immune remission was defined as ELISA-negative serum Anti-PLA2R titers decreased by more than 50% compared to the baseline value. Relapse was defined as a 24hUTP > 3.5 g/d after achieving complete or partial remission. All relapses were reviewed to differentiate clinically insignificant fluctuations in 24hUTP values from clinical relapses.

2.4.3 Serious adverse events

Serious adverse events including events such as hospitalization or life-threatening or organ damage. Follow-up was performed during the 24-week treatment period. Patient's blood and biochemical test results were regularly reviewed to assess for events such as serious infections, hematological disorders, liver and kidney impairment.

2.5 Monitoring nodes

All patients were monitored from the start of combination therapy (initial administration of CTX and SLFX formula) until the

date of the outpatient visit at the last dose of therapy. The monitoring points were at the baseline visit, 2 weeks later, 12 weeks later, and 24 weeks later, with routine blood and liver function tests at 2 weeks to assess safety, and the following laboratory values at the 12-week and 24-week visits: routine blood, full biochemistry, and urine protein quantification.

2.6 Statistical analysis

The Case Report Form(CRF) table was designated for data collection and statistically was performed using STATA 18.0 software, with $P < 0.05$ considered statistically significant. Descriptive statistics were performed for the general information and baseline clinical indicators of the included patients, with continuous variables expressed as median and interquartile range (IQR) or mean and standard deviation, as appropriate, and categorical variables expressed as frequency and percentage. For continuous information, data satisfying normal distribution were compared within groups using t-tests. As appropriate, data that did not satisfy normal distribution were compared between groups using the nonparametric Wilcoxon test. Categorical data were compared between components using the chi-square test. If the data met the overall distribution, they were analyzed using the bivariate Pearson linear correlation test. If data did not conform to normal distribution, Spearman correlation analysis was used. Baseline parameters were compared using the Wilcoxon rank sum test or the Fisher exact test. Kaplan-Meier method was used to examine time to partial remission and time to complete remission from first enrolment. If the primary outcome was not achieved during the study period, patients were reviewed at the last follow-up date. Differences between Kaplan-Meier curves were assessed using the log-rank test and the Cox proportional risk model, and all comparisons were two-tailed.

3 Results

3.1 Baseline data information

From May 2021 to December 2023, a total of 31 patients with intermediate-to-high risk PMN in our wards and outpatient clinics were enrolled in this study. Patients with membranous nephropathy had been definitively diagnosed by renal puncture pathological biopsy or underwent a positive serum Anti-PLA2R test. The baseline Scr distribution was 67.5 (IQR, 57.5-84.1) umol/L, eGFR was 101.3 (IQR, 89.4-115.3) ml/min·1.73m^2, 24hUTP distribution was 6.1 (IQR, 4.6-8.4) g/d, serum ALB distribution was 27.2 ± 6.4 g/L, and Anti-PLA2R antibody titer distribution was 73.2 (IQR, 9.3-298.5) RU/ml. Specific baseline data information is shown in Table 1. Combined with serological data and renal puncture biopsy, 80.6% of patients were diagnosed with PLA2R-associated PMN. 19 patients underwent renal biopsy for definitive diagnosis, with the largest proportion of patients with stage I PMN, 22.6%, and the specific information is shown in Table 1.

3.2 Primary outcome

3.2.1 Effect of the combination regimen on response rates in the short term

As shown in Table 2, of the 31 patients who received CTX in combination with SLFX formula, the efficiency was 38.7% at 12 weeks and 64.5% at 24 weeks. During the study period, 19 patients achieved complete remission or partial remission. The median time to achieve remission was 4.8 (IQR, 2.0-6.0) months (Figure 1a). 8 patients achieved complete remission during the study period, and all of the patients who achieved complete remission had a 24hUTP < 0.3g/d and serum ALB > 35g/L during the treatment period.

Stratification of patients according to 24hUTP, Anti-PLA2R and renal pathology levels revealed differences in time to remission across strata. Among them, patients with lower baseline proteinuria levels had shorter time to remission (hazard ratio = 4.85, log rank $P = 0.03 < 0.05$) (Figure 1b). There was no difference in time to complete remission when stratified by anti-PLA2R status (hazard ratio = 1.92, log rank $P = 0.38$) (Supplementary Figure S1). Time to remission was shorter in patients with less severe renal pathological stage (hazard ratio = 6.75, log rank $P = 0.03 < 0.05$) (Supplementary Figure S2).

Table 3 summarized the changes in Scr, serum ALB, 24hUTP, and Anti-PLA2R antibodies in the 11 patients who failed to achieve remission during the study period, and although proteinuria remission was not achieved, all serum Anti-PLA2R-positive patients achieved immuno-serological remission after 24 weeks of treatment.

3.3 Secondary outcomes

3.3.1 Effect of the combination regimen on nephrotic syndrome parameters

Changes in nephrotic syndrome parameters in patients after 24 weeks of treatment were shown in Figures 2a–f. For nephrotic syndrome, 24-hour urine protein quantification decreased from a median of 6.1 (IQR, 4.6-8.4) g/d to 2.7 (IQR, 0.6-8.7) g/d ($P<0.001$). Scr increased from 27.2 ± 6.4 g/L to 31.9 ± 8.0 ($P=0.009<0.05$). There was a downward trend in total CHOL and LDL from baseline, and the difference was not statistically significant (P>0.05).

3.3.2 Effect of the combination regimen on renal function parameters

Changes in renal function of patients before and after treatment were shown in Figures 3a, b. Although the Scr showed a decreasing trend and the estimated glomerular filtration rate both increased after treatment compared to before, the difference between before and after treatment was not statistically significant in both cases ($P > 0.05$).

3.3.3 Effect of combined regimen on serum PLA2R antibody titers

Serum Anti-PLA2R antibody titers were shown in Figure 4, and after 24 weeks of treatment, all patients (n=20) who were seropositive for Anti-PLA2R at baseline achieved immune remission. Anti-PLA2R antibody titers before and after treatment were 73.2 (IQR, 9.3-298.5) and 0.0 (IQR, 0.0-15.5) IU/ml, respectively, which were significantly different ($P<0.001$).

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TABLE 1 Baseline clinical characteristics of participants.

Variable	Total
No. of patients	31
Age, y	50.6 ± 14.5
Female sex	10 (32.3%)
Systolic BP, mm Hg	139.7 ± 18.8
Diastolic BP, mm Hg	82.9 ± 9.0
Serum creatinine, μmol/L	67.5 [57.5,84.1]
eGFR(mL/min/1.73m^2)	101.3 [89.4,115.3]
24hUTP(g/d)	6.1 [4.6,8.4]
24hUTPcategory	
<4g/d	6 (19.4%)
4-8g/d	16 (51.6%)
>8g/d	9 (29.0%)
Nephrotic syndrome	27 (87.1%)
Albumin, g/L	27.2 ± 6.4
Uric acid, μmol/L	389.5 ± 88.0
Triglycerides, mmol/L	2.1 [1.6,4.3]
Low density lipoprotein, mmol/L	4.8 [3.1,6.6]
Total cholesterol, mmol/L	7.2 [5.8,8.6]
PLA2R-associated disease	
Yes(n, %)	25 (80.6%)
No(n, %)	4 (12.9%)
Unknown(n, %)	2 (6.5%)
Diagnosis of renal puncture biopsy	
Yes (n, %)	19 (61.3%)
No (n, %)	12 (38.7%)
Phase I	7 (22.6%)
Phase I-II	2 (6.5%)
Phase II	4(12.9%)
Phase II-III	1 (3.2%)
Atypical	1 (3.2%)
Anti-PLA2R antibody titer, RU/mL	73.2 [9.3-298.5]

Normally distributed data are represented as mean ± standard deviations, non-normally distributed data are presented as median [interquartile range], and frequency data are expressed as n (%). Nephrotic syndrome is defined as 24hUTP > 3.5 g/d, ALB < 30 g/L, and peripheral edema.

3.4 Adverse events

Serious adverse events were listed in Table 4. Five serious adverse events were identified during the combined follow-up time of 31 patients. The most common adverse events were CTX-induced infections (n=3), and hepatic impairment (n=2). None of the patients experienced deterioration of renal function and the need for renal replacement therapy during the treatment period. Also no patient developed malignancy.

TABLE 2 Response rate of participants at 3, 6 months.

Time	CR	PR	NR	Efficiency
12 w	1 (3.2%)	11 (35.5%)	19 (61.3%)	12 (38.7%)
24 w	8 (25.8%)	11 (35.5%)	12 (38.7%)	19 (61.3%)

CR, complete response after treatment; PR, partial response after treatment; NR, no response after treatment.

4 Discussion

Pathological damage of PMN is mainly manifested as the destruction of the glomerular basement membrane. In the pathological progression of PMN, immune complexes are deposited in the glomerular podocyte membrane, resulting in the destruction of the glomerular basement membrane. At this point, the patient has a small amount of proteinuria leaking, which is at low risk of PMN. With the increase of basement membrane destruction, protein leakage increases, and more serious pathological damage such as sclerosis and fibrosis occur in the kidneys, the patient is in the intermediate-to-high risk stage, with high Anti-PLA2R antibody levels and massive proteinuria. Patients in the intermediate-to-high risk groups usually have severe and irreversible pathological injuries, which have a greater impact on the development of glomerular fibrosis and are at high risk of progression to end stage kidney disease(ESKD) (14). The KDIGO guidelines recommend long-term treatment with immunosuppressants in combination with GC to prevent the irreversible damage to the glomerular basement membrane from worsening on the basis of conventional treatment.

The initial therapy for high-risk PMN has been CTX with GC. Clinical studies have shown that immunosuppressive regimens for membranous nephropathy typically have a duration of response of more than six months (15). However, during long-term treatment with CTX combined with GC, patients often have adverse reactions such as infection, liver insufficiency, cytopenias, and gastrointestinal disorders, which makes it difficult for patients to adhere to treatment. A study primarily assessing safety outcomes found that patients treated with CTX and GC had a higher incidence of adverse events in PMN compared with RTX monotherapy (16), with infection, leukopenia, and Cushing syndrome being more common. Severe infection, as a common adverse reaction of GC combined with CTX, is the main cause of disease progression and death in patients with MN. Patients with MN often have hypoproteinemia and low serum IgG levels. In addition, immunosuppressants and hormone therapy can impair the

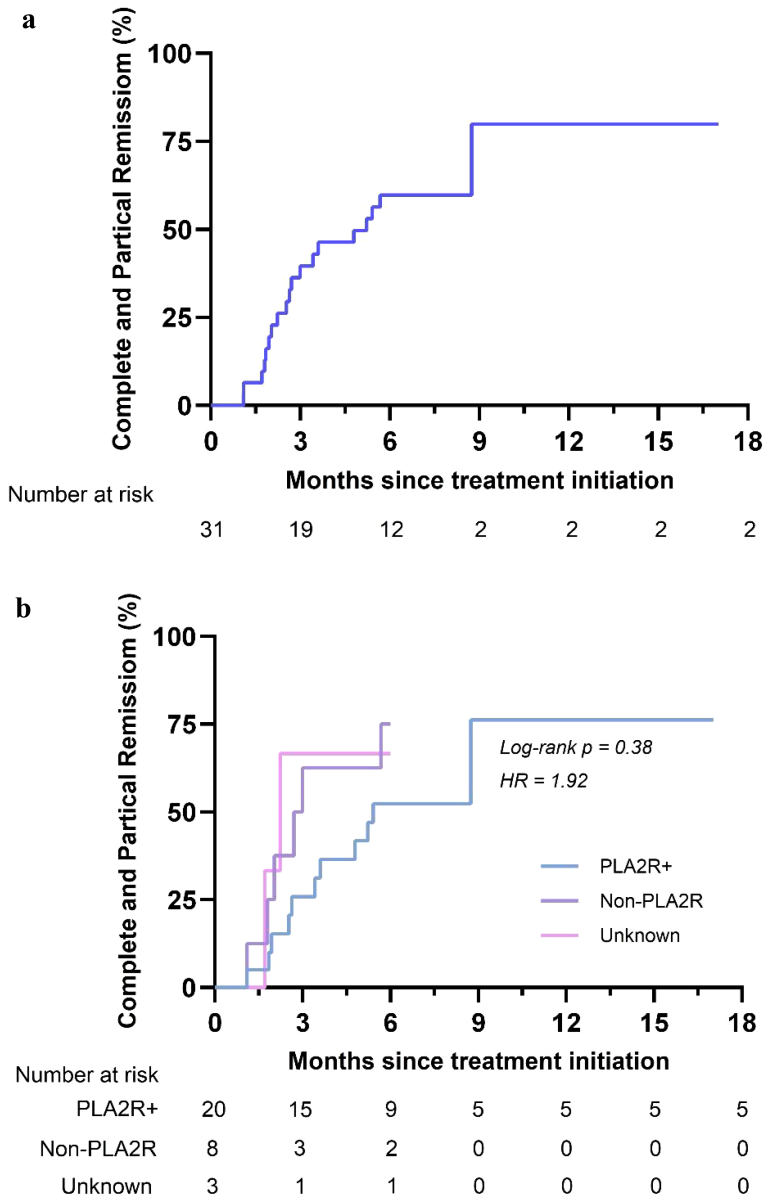

FIGURE 1
Kaplan-Meier curves for partial and complete remission. Kaplan-Meier curves for the overall group **(a)** and stratified by 24hUTP **(b)**.

patient's innate immune, cellular immunity, and humoral immunity. Together, these factors increase the risk of infection, including conditionally pathogenic bacteria, fungi, and viruses (17).

Although steroid-free therapy has been proposed to improve renal impairment in patients, CTX alone still has side effects such as liver function impairment and hematologic impairment (18–20). Patients with PMN treated with CTX have a three-fold higher incidence of malignancy compared with those who do not receive CTX (21). For example, in the STARMEN study (22), 4 of 5 serious infections (80%) occurred in the CTX-GC group, and in the RI-CYCLO study (23), 5 patients (14%) in the CTX-GC group had serious adverse events, mainly leukopenia and pneumonia. These side effects are associated with myelosuppressive and immunosuppressive mechanisms of CTX.

In previous studies, we found that SLFX formula can attenuate the toxicity of immunosuppressants by reducing inflammatory expression, inhibiting apoptosis, regulating immune response, and other multi-target and multi-pathways (24). Simultaneously, during clinical practice, it was shown that patients with PMN tended to have much lower urine protein, higher serum albumin, and less edema (12).

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TABLE 3 Patients who did not achieve clinical remission.

Patient	Sex	Age	PLA2R Association	Cr (μmol/L)		ALB (g/L)		24hUTP (g/d)		PLA2R (RU/ml)		Immunologic remission[b]	Clinical outcome
				Initial[a]	End[a]	Initial	End	Initial	End	Initial	End		
1	M	60	Y	80.5	66.9	21.2	20	6.0	8.7	419.2	253	Y	NR
2	F	78	Y	143.9	178	20.8	21.4	10.7	15.8	397.6	6.87	Y	NR
3	M	25	Y	72.9	64.6	16.2	16.9	8.8	10.1	165.9	87.2	Y	NR
4	M	37	Y	84.1	56.7	23.2	22.2	6.7	6.7	83	43	Y	NR
5	M	35	N	59.0	58.9	33.2	33.7	8.3	8.8	n/a	n/a	n/a	NR
6	M	37	N	69.0	72.0	35.4	34	3.9	3.6	131	–	–	NR
7	F	51	Y	64.6	59.5	19.8	20.4	16.2	12.0	557.7	34.4	Y	NR
8	M	74	Y	65.7	91	28.2	30.9	5.7	5.4	206.9	23.4	Y	NR
9	M	69	N	133	129	26.5	31.5	5.4	5.9	n/a	n/a	n/a	NR
10	M	64	Y	85.5	68.8	25.2	25.7	12.3	12.9	127.7	40.7	Y	NR
11	M	59	Y	78.6	88.4	23.9	23.5	12.2	8.8	63.4	–	n/a	NR

M, male; *F*, female; *Y*, yes; *N*, no; *Cr*, creatinine; *ALB*, Serum albumin; *n/a*, not applicable; *24hUTP*, 24-hour urine total protein quantification; *PLA2R*, phospholipase A2 receptor; *NR*, non-remission. a. Initial values represent values at the start of treatment; End values represent values at last follow-up. b. Immunologic remission is defined as serum Anti-PLA2R titers decreased by more than 50% compared to the baseline value.

Radix Astragali (Astragalus membranaceus polysaccharides), Bupleurum (saikosaponin), Rhizoma smilacis glabraea cocos, Scutellaria, and other active ingredients of traditional Chinese medicine have been found to be the main drugs of SLFX formula in pharmacological studies (25–27). These ingredients have detoxifying and synergistic effects on CTX, including anti-inflammatory, antioxidant, anti-fibrotic, immune system regulation, and other effects.

In this study, a prospective trial was conducted to explore the short-term clinical efficacy of SLFX formula combined with CTX in patients with intermediate-to-high risk PMN. In this study, we used the lowest dose of CTX (50 mg qd) for the same patient's treatment regimen with a cumulative CTX dose of 9 g, and we found that after 24 weeks of CTX combined with traditional Chinese medicine, the clinical remission rate of patients with PMN reached 64.5%, and the treatment time required was shorter for patients with lower urine protein levels and milder pathologic stages. The parameters of NS in all patients improved after treatment, which was manifested by a decrease in 24-hour urine protein quantification and an increase in serum ALB expression. However, there was no significant change in renal function (as measured by serum Scr and eGFR). In addition, serum Anti-PLA2R antibodies decreased during treatment, with a total of 13 patients (65%) turning negative with Anti-PLA2R antibodies after 6 months of treatment.

In order to better analyze the therapeutic effect of SLFX formula combined with CTX, we selected large RCTs in the treatment of membranous nephropathy, including the STARMEN trial (22) and the RI-CYCLO trial (23), and compared the clinical outcome criteria of CTX plus GC in the treatment of CTX in terms of clinical response, immune remission, and relapse, as shown in Table 5. We found that the short-term response rate in this study was slightly inferior to that of CTX-GC in the absence of hormones. Considered to be related to: 1) The short duration of treatment in this study and the lack of follow-up. Previous studies (28) have shown that the duration of treatment and follow-up of MN disease is 12-24 months, and the response rate may further increase with the extension of treatment and follow-up time. 2) Serum Anti-PLA2R antibody levels (29) have been shown to be an early predictor of late proteinuria remission, as well as the risk of renal outcome remission and progression to ESKD. The patients included in this study were patients with intermediate-to-high risk membranous nephropathy, with a large basal 24hUTP quantification and Anti-PLA2R antibody titers, a longer course of disease remission, and a slightly higher remission rate than expected. 3) According to previous studies (30, 31), it is difficult to achieve clinical efficacy in the treatment of single-agent CTX or GC in the treatment of membranous nephropathy, and the addition of traditional Chinese medicine on the basis of single-agent CTX in this study has improved the clinical effective rate and reduced the side effects of hormones, so it is considered that this regimen has certain efficacy for patients with intermediate-to-high membranous nephropathy. In addition, the combination of traditional Chinese medicine with CTX has improved the side effects of CTX monotherapy under the condition of certain clinical efficacy, suggesting that this regimen can be used in the treatment of PMN patients for a long time in clinical practice.

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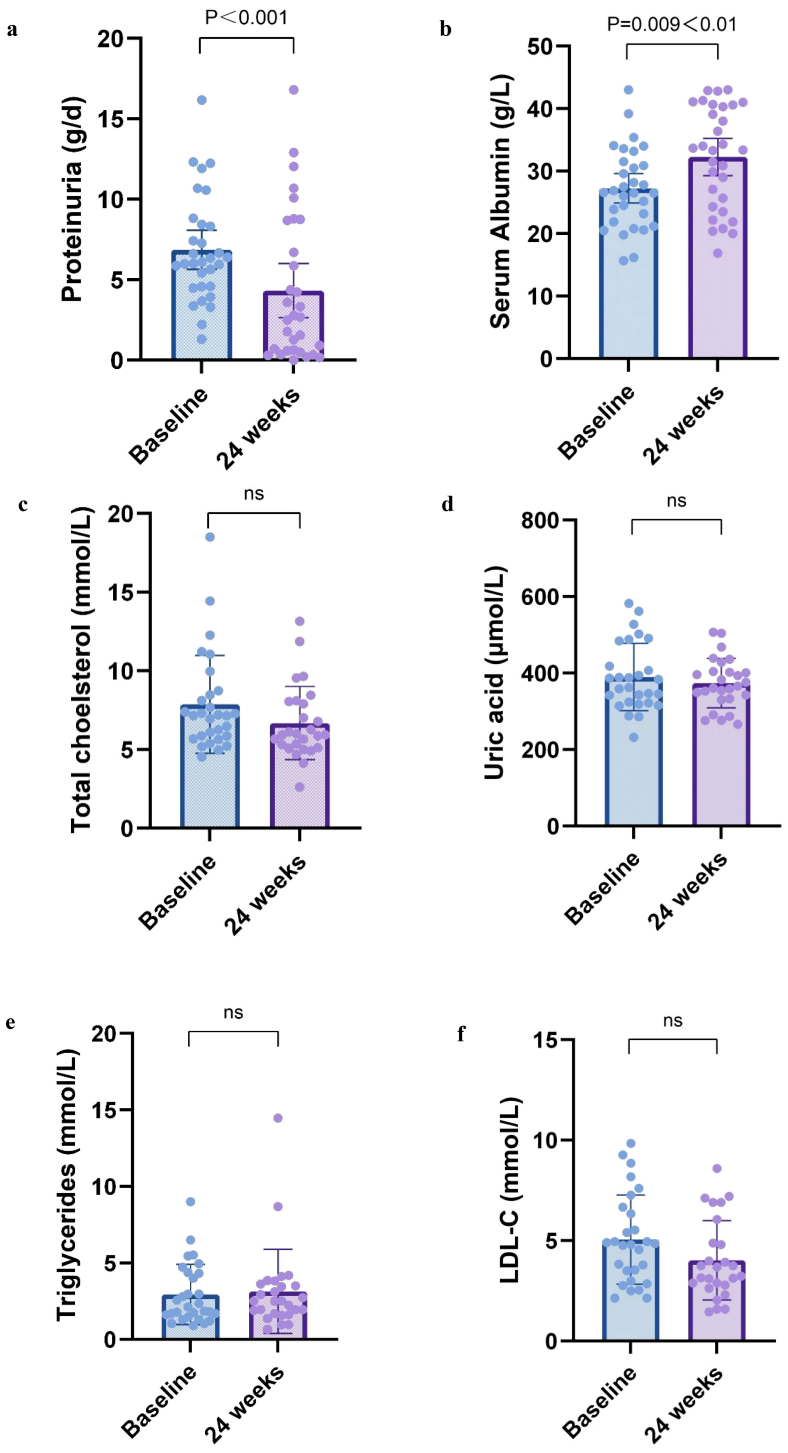

FIGURE 2
Nephrotic syndrome parameters at 6 months. Change in **(a)** proteinuria, **(b)** serum albumin, **(c)** total cholesterol, **(d)** uric acid, **(e)** triglycerides, and **(f)** ldl-c from baseline to 12 months. All box and whisker plots represent median (IQR) and the minimum-maximum range measurements. Longitudinal differences from baseline to 12 months were analyzed with the Wilcoxon signed-rank test.

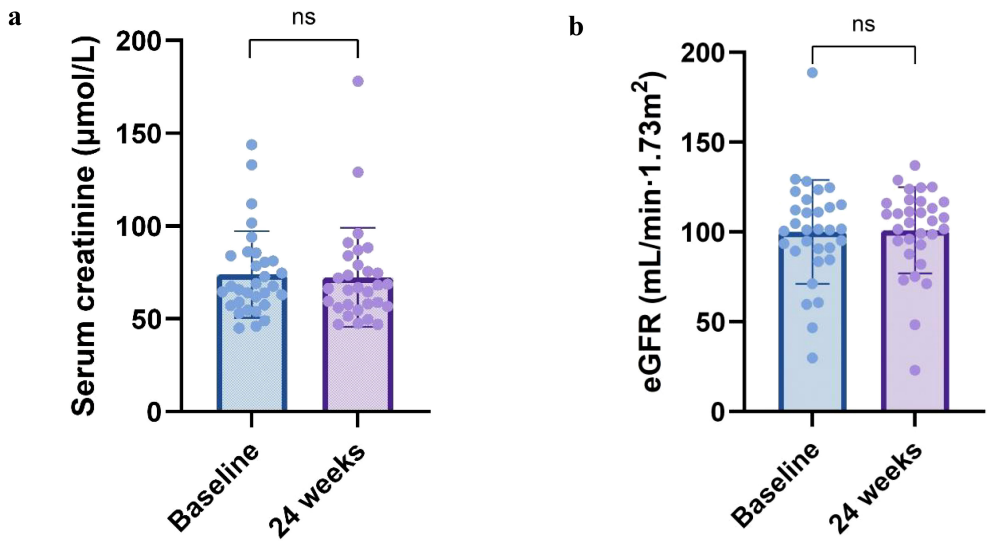

FIGURE 3
Renal function parameters at 24 weeks. Change in **(a)** serum creatinine, **(b)** eGFR from baseline to 24 weeks. All box and whisker plots represent median (IQR) and the minimum-maximum range measurements. Longitudinal differences from baseline to 12 months were analyzed with the Wilcoxon signed-rank test.

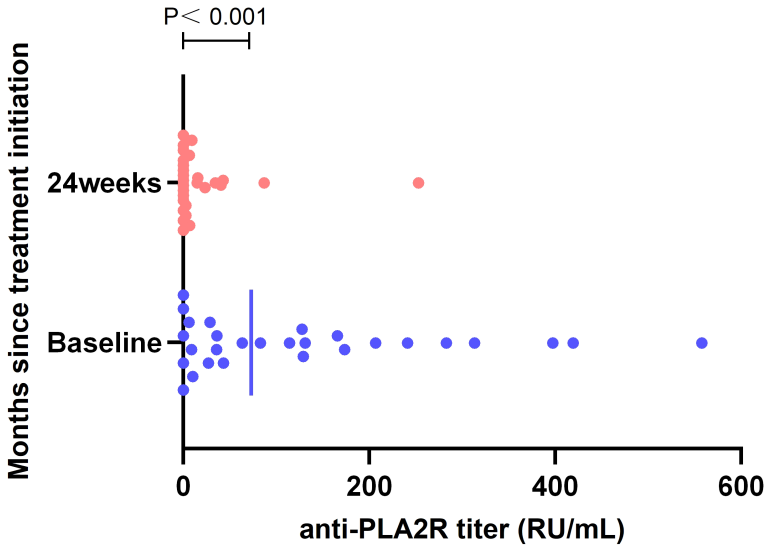

FIGURE 4
Anti-PLA2R titers with treatment. There is a significant decline in titer of anti-PLA2R antibodies over time compared to baseline. ELISA reference ranges: negative, <14 RU/mL; borderline, 14-20 RU/mL; positive, > 20.0 RU/mL. ELISA, enzyme-linked immunosorbent assay; IQR, interquartile range; PLA2R, phospholipase A2 receptor; RU, relative units.

TABLE 4 Serious adverse events.

Adverse Event	No. of Events
Lung infections	1
Heart failure	1
Urinary tract infections	2
Abnormal liver function	1
Nausea, diarrhea	1

In this study, we found that the combination of traditional Chinese medicine with CTX had a good clinical effect on patients with low baseline proteinuria and early pathological stage, while serum Anti-PLA2R antibody titers did not show a significant correlation with prognosis, which was considered to be related to the small sample size of this study. In patients who are immune remission without clinical remission, it is considered that proteinuria levels may decrease over the next 6 to 24 months. At the same time, for some patients with pre-existing renal impairment and/or mild decline in renal function at baseline, persistent

TABLE 5 Comparison of basic information and mitigation information between three studies.

Variable		This Study	STARMIN	RI-CYCLO
Regimen		SLFX formula-Cyclophosphamide	Corticosteroid-Cyclophosphamide	Corticosteroid-Cyclophosphamide
Duration		24 w	12 m	12 m
Initial PLA2R(+)		25 (31)	29 (37)	46 (71)
Initial 24hUTP		6.1 [4.6,8.4]	7.4 [4.8–11.3]	1.7 ± 0.5
Clinical response(%)	12w	38.7	51.2	/
	24w	61.3	74.4	65
Complete response(%)	12w	3.2	2.3	/
	24w	25.8	12.2	/
Immune response(%)	12w	/	/	/
	24w	100	92	/
Recurrence(%)	48w	0	/	5.4

proteinuria reflects the potential for residual structural destruction due to scarring rather than active disease.

Regarding side effects, CTX and traditional Chinese medicine work together to lower the risk of side effects including infection. During the 6-month course of treatment, there were only three infections, one of which was thought to be caused by an underlying illness. Furthermore, one patient experienced heart failure during treatment, which was thought to be related to the patient's age and prior underlying conditions rather than the treatment regimen or NS. Two patients also experienced abnormal liver function during the follow-up period, which was resolved after stopping CTX. One patient developed nausea and diarrhea, which were not excluded from intolerance to taking traditional Chinese medicine. Because the lowest dose of CTX (50 mg once a day) was used in the same patient in this study, the cumulative dose during the treatment period was 9 g, and there was no high-dose GC therapy. In contrast, the current KDIGO guideline-recognized standard cytotoxicity-based regimen (the "Ponticelli regimen") administers a cumulative dose of CTX of 15.75 g over 3 months and a cumulative dose of prednisone of 14.77 g over 3 months in 70 kg of individuals with normal renal function. This regimen is significantly lower than the dose range typically associated with gonadal toxicity, bladder toxicity, and malignancy (32).

A few other limitations of this research should be addressed. First, this is a single-arm study, so there is a lack of data from the control group, which could have provided more insight into the efficacy of this hormone-free regimen for medium-to high-risk PMN. Second, the detection rate of Anti-PLA2R antibodies was lower than expected, which may have limited the ability to fully evaluate the efficacy of this regimen.

This study suggests that, using the classic "Ponticelli regimen" from large RCTs as a reference, the efficacy and safety of CTX combined with the SLFX Formula are relatively favorable, making it a potential option for the clinical treatment of intermediate-to-high risk membranous nephropathy (MN) patients, particularly for elderly patients with contraindications to corticosteroid use or those with refractory disease. This study is an exploratory trial, and future large-scale randomized controlled trials will be conducted to further validate the efficacy of this regimen for intermediate-to-high-risk PMN. Additionally, animal experiments and cell experiments will be carried out to explore the relevant mechanisms of action of the traditional Chinese medicine SLFX Formula.

5 Conclusion

Our findings confirmed that the combination of traditional Chinese medicine SLFX Formula and low-dose CTX in the treatment of patients with intermediate-to-high risk PMN can achieve certain clinical efficacy in the short term, and reduce the adverse effects of CTX monotherapy.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethics Committee of Dongzhimen Hospital. The studies were conducted

in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

YD: Writing – original draft. YLiu: Investigation, Methodology, Resources, Writing – review & editing. ZYC: Formal analysis, Writing – review & editing. YLia: Data curation, Investigation, Writing – review & editing. XL: Data curation, Investigation, Writing – review & editing. YW: Data curation, Investigation, Writing – review & editing. JL: Data curation, Investigation, Writing – review & editing. ZL: Data curation, Investigation, Writing – review & editing. ZW: Data curation, Investigation, Writing – review & editing. WC: Data curation, Investigation, Writing – review & editing. QL: Data curation, Investigation, Writing – review & editing. XL: Data curation, Investigation, Writing – review & editing. ZJC: Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. JZ: Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing.

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EDITED BY
Feng Dong,
AbbVie, United States

REVIEWED BY
Hanyong Bae,
Sungkyunkwan University, Republic of Korea
Jiang Weng,
Sun Yat-Sen University, China

*CORRESPONDENCE
Seiya Kitamura
seiya.kitamura@einsteinmed.edu

†These authors have contributed equally to this work

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SuFEx-enabled high-throughput medicinal chemistry for developing potent tamoxifen analogs as Ebola virus entry inhibitors

Lucas Dada[1†], Emiko Nagai[1†], Sashank Agrawal[2†], Ariel S. Wirchnianski[3], Ian A. Wilson[2,4], Kartik Chandran[3] and Seiya Kitamura[1*]

[1]Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY, United States, [2]Department of Integrative Structural and Computational Biology, The Scripps Research Institute, La Jolla, CA, United States, [3]Department of Microbiology & Immunology, Albert Einstein College of Medicine, Bronx, NY, United States, [4]Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA, United States

Ebola virus (EBOV) causes severe hemorrhagic fever with a high mortality rate in humans. In acute infection, an abnormal immune response results in excessive inflammatory cytokines and uncontrolled systemic inflammation that can result in organ damage and multi-organ failure. While vaccines and monoclonal antibody therapies are available, there is an urgent need for effective small-molecule antivirals against EBOV. Here, we report on the optimization of tamoxifen, an EBOV-glycoprotein (GP) binder that inhibits viral entry, using our Sulfur-Fluoride Exchange (SuFEx) click chemistry-based high-throughput medicinal chemistry (HTMC) strategy. Using a "Direct-to-Biology" approach, we generated a focused library of 2,496 tamoxifen analogs overnight and screened them in a cell-based pseudo-EBOV infection assay. The HTMC workflow enabled the development of a potent EBOV entry inhibitor with submicromolar EC_{50} cellular antiviral activity and more than 50-fold improvement in binding affinity against EBOV-GP compared to the parent compound. Our findings underscore the use of SuFEx-enabled HTMC for rapidly generating and assessing potential therapeutic candidates against viral and immune-mediated diseases in a cell-based assay.

KEYWORDS

Ebola, small molecule antiviral drugs, drug discovery, SuFEx, direct-to-biology

1 Introduction

Ebola virus (EBOV) is an enveloped, single-stranded, negative-sense RNA virus in the family *Filoviridae* (1). EBOV infection in humans can lead to Ebola virus disease (EVD), a clinical syndrome initially characterized by nonspecific symptoms, which later progress to

severe gastrointestinal issues and hemorrhagic complications with a lethality rate as high as 90% (2–4). While other orthoebolaviruses, such as Sudan virus and Marburg virus, can also cause human disease with substantial mortality, EBOV has been responsible for the majority of recorded human outbreaks and therefore, remains to be of considerable public health concern (5). The unprecedented 2014–2016 EVD epidemic in West Africa, as well as the 2022 outbreak in the Democratic Republic of the Congo have underscored the potential of EVD to trigger severe health emergencies on a regional scale (6, 7).

EBOV causes an acute and serious viral hemorrhagic fever disease, which is often fatal if left untreated. EBOV primarily targets host macrophages leading to cell activation and systemic cytokine storm. Fatal infection is associated with an inhibited interferon response and lymphopenia. Cytokine storms are a hallmark of EVD and play a central role in its pathogenesis, marked by the induction of both pro- and anti-inflammatory responses (8, 9). Despite the high mortality rate associated with EVD, some patients survive and, in certain cases, develop chronic manifestations that may resemble inflammatory or autoimmune conditions (10). It has been demonstrated that this EBOV-induced autoimmunity is involved in post EVD syndrome (11). These features of EVD highlight the necessity for effective therapeutic approaches against EBOV. Recently, EBOV vaccine and monoclonal antibody-based therapeutics have been approved by the FDA (12, 13). However, there are currently no FDA-approved small-molecule drugs with demonstrated efficacy against filovirus infection and/or disease in humans, despite the increasing frequency with which these viruses are causing outbreaks of global concern (14). Therefore, there is a pressing and unmet need for effective therapies to prevent EBOV entry and subsequent infection.

In the pursuit of drug discovery against EVD, several high-throughput screening campaigns have been conducted that yielded hit compounds (15, 16). However, only limited medicinal chemistry optimization and *in vivo* follow-up studies were performed previously, partially due to the iterative cycle of medicinal chemistry process being time-consuming and labor-intensive. Our group has developed the first-of-its-kind high-throughput medicinal chemistry (HTMC) platform using click chemistry reactions, in particular Sulfur-Fluoride Exchange (SuFEx) reactions (17–21), to accelerate the medicinal chemistry process. The unanticipated discovery that iminosulfur oxydifluoride (difluoride, RN=S(O)F_2)-containing molecules react overnight with amines to yield products with >80% conversion in a biocompatible condition (22) allowed us to perform large-scale structure-activity relationship (SAR) studies directly from reaction mixtures. We have used this new type of click chemistry reaction to rapidly synthesize focused libraries of lead compound analogs in a miniaturized format, directly assess the products with biological assays (also known as "Direct-to-Biology (D2B)" approach) (23), and develop drug-like ligands with improved biological potency. We have previously demonstrated the utility of our SuFEx-based HTMC method to improve the potency and specificity of chemical probes against a bacterial pathogenic protease, a human leukemia-associated transcriptional coactivator, the influenza hemagglutinin stem, and molecular glues (17–21).

In this study, we applied our SuFEx-based HTMC platform to an inhibitor of the EBOV-glycoprotein (GP) for the expedited analysis of structure-activity relationships. Tamoxifen, an EBOV-GP inhibitor (24, 25), was used as a starting scaffold for diversification because of its suitability for the synthetic preparation of a SuFExable analog. With the support of an automated liquid handling robot, a focused library of 2,496 compound analogs was synthesized in a single batch and screened using a cell-based EBOV entry assay. This study provides a large-scale SAR dataset of tamoxifen analogs as EBOV entry inhibitors, which will be valuable for the further development of small molecule therapeutics against EVD. Importantly, the HTMC workflow enabled the discovery of an analog with a 50-fold improved binding affinity compared to tamoxifen. Our study showcases the successful application of the SuFEx-based HTMC platform for the accelerated structure-activity relationship study of small molecule inhibitors, ultimately providing next-generation therapeutic modalities against viral infection and autoimmune diseases.

2 Materials and methods

2.1 Cells and viruses

The Vero African green monkey kidney cells in this study were obtained from American Type Culture Collection (ATCC), Cat# CCL-81. Vero cells were cultured in Dulbecco's modified Eagle medium (DMEM, Corning, NY) containing 10% fetal bovine serum (FBS, Phoenix Scientific, St. Joseph, MO), 1% penicillin-streptomycin (Corning, NY), and GlutaMax (Thermo Fisher Scientific, Waltham, MA). Cells were maintained at 37°C in 5% CO_2. Generation and propagation of recombinant vesicular stomatitis virus (rVSV) encoding enhanced green fluorescent protein (eGFP) in the first position and replacing VSV G with the EBOV-GP (EBOV/H.sap-tc/COD/76/Yambuku-Mayinga) or Lassa virus (LASV)-GP (Josiah) were previously described (26, 27).

2.2 Computational protocol

Docking studies were carried out using Glide-SP (Schrödinger modeling suite, versions 2024-1 to 2024-3) to obtain the binding mode for tamoxifen into the proposed binding site. Standard option joint with the SP algorithm was applied for pose generation and evaluation.

Abbreviations: CC_{50}, 50% cytotoxicity concentration; Direct-to-biology, D2B; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EBOV, Ebola virus; EC_{50}, half-maximal effective concentration; eGFP, enhanced green fluorescent protein; EVD, EBOV disease; GP, glycoprotein; HTMC, high-throughput medicinal chemistry; *J*, NMR coupling constant; K_D, dissociation constant; LASV, Lassa virus; MST, microscale thermophoresis; n.d., not determined; NMR, nuclear magnetic resonance; PDB, protein databank; py, pyridine; RT, room temperature; SD, standard deviation; SI, selectivity index; SuFEx; Sulfur(VI)-Fluoride Exchange; THF, tetrahydrofuran; VLP, virus-like particle; VSV, vesicular stomatitis virus.

The X-ray structure for the Zaire EBOV-GP in complex with toremifene (PDB: 5JQ7) was used as a template for the docking.

2.3 Chemical synthesis

The details of chemical synthesis and characterization are described in Supplementary Material. The SuFEx-based library synthesis was adapted from a method described previously (17) in 384-well plate format. Briefly, to a DMSO solution of the iminosulfur oxydifluoride derivative **1** was added amine library in DMSO and sodium phosphate buffer (pH 9.0, 0.2 M) subsequently. The compounds were synthesized with difluoride concentration at 1 mM and a solvent mixture DMSO:buffer 3:1. The reaction mixtures were shaken at room temperature overnight and then used directly for activity measurement with 5000-fold dilution.

2.4 Screening and EC_{50} measurement using an EBOV-GP-pseudotyped virus

The virus was titrated to achieve an infection rate of approximately 50%. Vero cells were seeded at a density of 2.0 × 10^4 cells/well in a 384-well plate and incubated for 24 hours at 37°C in 5% CO_2. The cell culture medium was then replaced with 20 µL/well of virus culture medium (DMEM containing 2% FBS, 1% penicillin-streptomycin, and GlutaMax), and 0.2 µL/well of the compound library in DMSO was added using the Bravo Automated Liquid Handling Platform (Agilent Technologies, Santa Clara, CA). Subsequently, 20 µL/well of virus solution was added, and the cells were incubated for 16 hours at 37°C in 5% CO_2. Infected cells were stained with Hoechst (Thermo Fisher Scientific, Waltham, MA) to visualize nuclei and fixed with 4% paraformaldehyde. Infectivity of VSV pseudotype was measured by automated enumeration of $eGFP^+$ cells using a Cytation 5 reader (BioTek, Winooski, VT), as previously described (26). Quantification was done using Gen5 data analysis software (BioTek, Winooski, VT). For each plate, DMSO-treated infected and non-infected control wells were included to calculate the inhibition rate as: Inhibition rate (%) = (Infection control - Sample)/(Infection control - Non-infection control) × 100.

Dose-response assays were performed under the same experimental conditions as the library screening. Compounds were prepared in two-fold serial dilutions in DMSO and 0.2 µL/well was added to the cell culture media using the Bravo Automated Liquid Handling Platform. The EC_{50} values were calculated using GraphPad Prism 10.

2.5 Cytotoxicity measurement

Cells were seeded under the same conditions as the antiviral assay. The cell culture medium was replaced with 40 µL/well of virus infection medium without virus, and compound solutions prepared as two-fold serial dilutions were added at 0.2 µL/well using the Bravo platform. After 16 hours, Promega® CellTiter-Glo® 2.0 (Promega, Madison, WI) was added, and cell viability was assessed following the manufacturer's instruction. The CC_{50} values were calculated using GraphPad Prism 10.

2.6 Expression and purification of EBOV-GP trimer protein

The gene fragment encoding the extracellular domain of the Zaire EBOV (strain Mayinga-76) glycoprotein (UniProt ID: KB-Q05320) was synthesized as described previously (28). This gene was inserted into the mammalian expression vector pHCMV3, with an Ig Cκ leader sequence at the N-terminus to enable secretion. A foldon trimerization domain from bacteriophage T4 fibritin was added to the C-terminus to promote trimerization, along with a 6×His tag for affinity purification.

The plasmid was transfected into human Expi293S cells to produce the EBOV-GP trimers. After six days of incubation, the culture medium containing the secreted trimers was collected. The protein was purified using Ni-NTA affinity chromatography with Ni Sepharose Excel resin and dialyzed overnight into 1× PBS. The sample was concentrated and further purified via size-exclusion chromatography on a Superdex 200 HiLoad 16/600 column pre-equilibrated with 1× TBS. The protein peak corresponding to the EBOV-GP trimers was identified, collected, and concentrated to a final concentration of 2 mg/mL.

2.7 Microscale thermophoresis (MST)

Recombinant EBOV-GP protein was labeled using the Monolith Protein Labeling Kit RED-tris-NTA 2nd Generation dye (Cat #MO-L018, NanoTemper Technologies, Germany) following the manufacturer's instructions. Specifically, 125 nM EBOV-GP was incubated with 25 nM dye in 25 mM HEPES pH 7.5, 100 mM NaCl, 0.005% tween-20 in the dark at room temperature for 30 min. The sample was centrifuged for 10 min at 4°C and 14,000 g and the supernatant was transferred to a fresh tube. To determine the K_D of EBOV-GP to tamoxifen and its analogs, 125 nM labeled EBOV-GP was incubated with increasing concentrations of small molecules in the same buffer with 1% DMSO. Samples were loaded into standard glass capillaries (Monolith NT.155 Capillaries) and analyzed by MST using a Monolith NT.115 Blue/Red, LED power and IR laser power of 60%. Samples showed no aggregation according to post-run analysis using the Monolith data collection software (NanoTemper). Fraction bound and error were generated by NanoTemper software (MO.Affinity Analysis) and K_D values were determined using GraphPad Prism 10 and nonlinear fit of one-site specific binding.

2.8 Crystallization and structure determination

For crystallization of EBOV-GP trimers, the protein was concentrated to 8 mg/mL in a buffer containing 20 mM Tris (pH 8.0) and 150 mM NaCl. Crystals were grown at 20°C in a solution of 9% PEG 6000 and 100 mM sodium citrate (pH 5.2). Complex

structures were obtained by soaking EBOV-GP crystals in a 5 mM solution of the target compound for a few minutes, followed by cryoprotection with 20% glycerol and rapid plunging into liquid nitrogen for storage before data collection. Diffraction data were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) on beamline BL12-1. EBOV-GP crystals soaked with compound **4R** diffracted to a resolution of 2.59 Å. Data indexing, integration, and scaling were performed using HKL2000 (29). Structures were solved by molecular replacement (MR) with Phaser in Phenix (30) with PDB 6F6I as the MR model, and subsequent model building and refinement were conducted using Coot and Phenix.refine (31, 32). Structural quality was assessed with MolProbity (33), and further validation was performed using the PDB validation server. Data collection and refinement statistics are summarized in Supplementary Table 1.

3 Results

3.1 SuFExable tamoxifen analog design and validation of biological activity

The first step of the SuFEx-enabled HTMC workflow requires installation of a SuFExable difluoride moiety on the lead molecule (Figure 1A). This difluoride analog can be readily synthesized by reacting thionyl tetrafluoride ($O=SF_4$) gas with the lead compound functionalized with a primary amine. A structural analysis of EBOV-GP protein + ligand complex was performed to determine the modification site on the lead molecule. Due to the absence of an experimentally determined crystal structure for the complex of EBOV-GP with tamoxifen, molecular docking was used to predict the binding mode of this ligand. For this purpose, we employed a crystal structure of toremifene (i.e. an analog of tamoxifen) bound to EBOV-GP (PDB entry: 5JQ7) as a template (34). Given the chemical structural similarity between tamoxifen and toremifene, we expected that both molecules would share similar binding conformations.

The docking suggests that tamoxifen aligns closely with the binding conformation of toremifene, preserving key interactions with crucial residues within the active site (Supplementary Figure 1). The aromatic rings B and C of tamoxifen adopt a similar orientation to the corresponding rings in toremifene, forming a π-stacking between these rings and Tyr517, as well as an ionic interaction between the protonated tertiary amine and the negatively charged side chain of Glu100. Additionally, the docking analysis highlights a solvent-exposed area at the *meta* position on the aromatic ring A, making it a prime candidate site for further

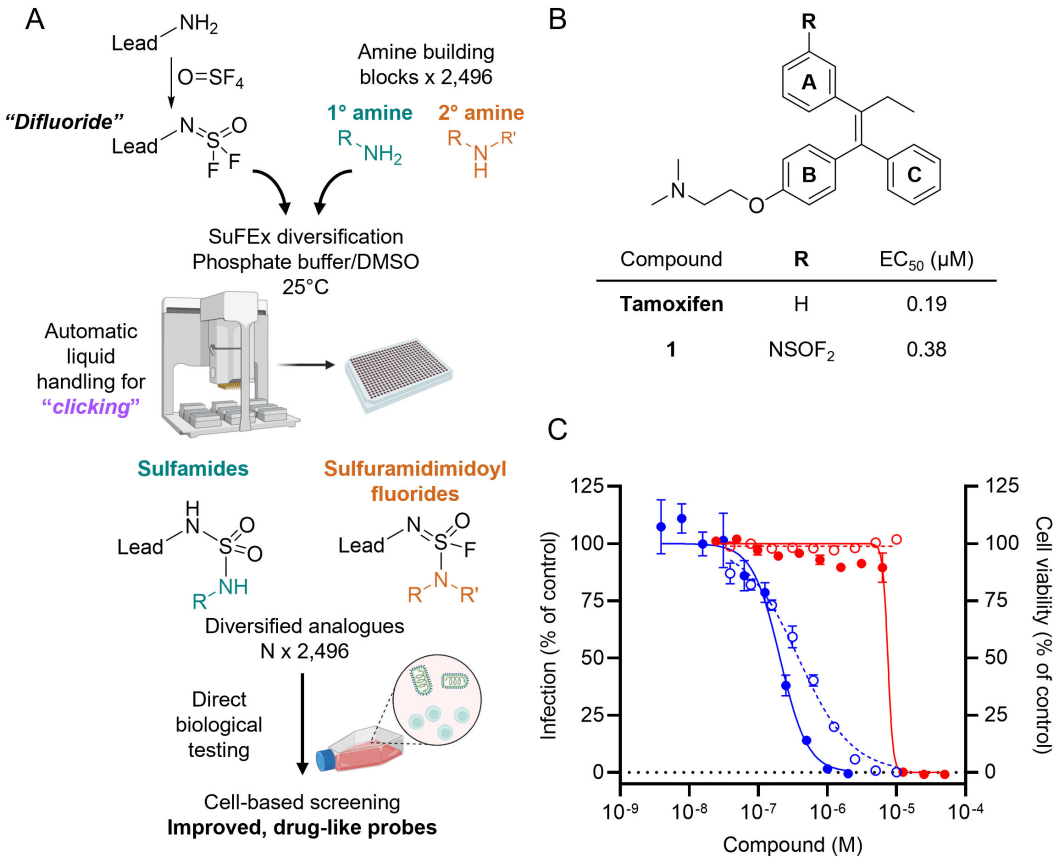

FIGURE 1
HTMC-based diversification of EBOV entry lead inhibitor tamoxifen. **(A)** Schematics of SuFEx-based HTMC workflow. **(B)** Chemical structure and EC_{50} of tamoxifen and the difluoride analog **1** against VSV-EBOV-GP (Vero cells). **(C)** Dose-response curves of VSV-EBOV-GP infection (blue) and Vero cell viability (red) for tamoxifen (●, solid lines) and compound **1** (○, dotted lines). Mean ± SD values are shown (n = 3).

functionalization. By targeting these solvent-exposed positions, we hypothesized that expanding the molecule into unoccupied regions of the binding pocket could form additional interactions with adjacent residues, thus enhancing both binding affinity and cellular anti-infective efficacy.

Guided by the predicted structure, we designed a synthetic route (Supplementary Figure 2) to introduce a difluoride group at the *meta* position of ring A of tamoxifen based on previous reports for the synthesis of analogs with the triphenylethylene core (25). The difluoride-containing analog **1** was successfully synthesized, and its chemical structure was verified by NMR and LC-MS analyses, as described in the Supplementary Material.

With analog **1** in hand, we assessed its antiviral activity and cytotoxicity, along with that of tamoxifen, using a virus-like particle (VLP) assay (26). The assay employs vesicular stomatitis virus (VSV) particles displaying EBOV-GP (VSV-EBOV-GP), in place of the native glycoprotein G, to infect Vero cells in culture. This approach obviated the need for high-security BSL4 facilities required for working with authentic filoviruses. The VSV-EBOV-GP also encodes an enhanced GFP, which allows for direct quantification of infected cells by fluorescence imaging. The use of a cell-based assay provides advantages for drug discovery, as it more accurately reflects the compounds' activity in a biologically relevant environment and accelerates the profiling of cell-active compounds. This infection model enabled us to evaluate the inhibitor's effectiveness in blocking the processing of viral GP, a crucial step for EBOV infection. Additionally, the VSV assay provided insight into whether the proposed structural modifications were tolerated by the lead compound without significantly compromising its activity.

The results showed that the lead compound tamoxifen has an EC_{50} value of 0.19 μM, while our difluoride-functionalized analog **1** exhibited an EC_{50} of 0.38 μM, with both values being within the same magnitude (Figure 1B). Importantly, compound **1** did not exhibit cytotoxicity up to 10 μM (Figure 1C). These findings indicate that the structural modifications to introduce the difluoride moiety do not critically reduce the biological activity of the lead compound, consistent with the docking predictions. The results support the effectiveness of the docking-guided design and validate the choice of the modification site on the tamoxifen aromatic ring.

3.2 SuFEx-enabled HTMC

Once an appropriate SuFExable derivative of the lead compound has been identified, the next step in the HTMC workflow (Figure 1A) is the diversification reaction with an amine-fragment library. This strategy allows the preparation of a focused library of lead compound analogs with expanded diversity containing sulfamide or sulfuramidimidoyl fluoride linkages, from primary or secondary amines respectively (Figure 2A).

A library of 2,496 primary and secondary amine fragments were individually reacted with the difluoride-containing analog in 384-well plates overnight at room temperature using a 1:3 phosphate buffer:DMSO solvent mixture (see Materials and Methods for further details). The reaction conditions employed for diversification were determined in a preliminary assessment of the difluoride reactivity with a small set of representative amines. Reagents concentration, temperature, pH, and solvent composition were evaluated to identify the optimal conditions for generating a high-quality library, allowing us to screen the compound set without additional purification. The synthesis of the library was performed using an automated liquid handling robot Agilent Bravo BenchCel, enabling efficient generation of 2,496 analogs in a single batch. An LC-MS analysis of a randomly selected wells was performed to evaluate the quality of the library. The results indicated a generally high conversion rate, validating the suitability of the library for the D2B screen in the subsequent step.

The synthesized library was screened at 200 nM using the VSV-based EBOV entry assay. The library solutions were dispensed over Vero cells in 384-well plates. Cells treated with the compound were infected with VSV-EBOV-GP, and 16 hours post-infection, their nuclei were stained and then fixed. The infection rate was assessed by measuring eGFP expression levels, normalized to the number of nuclei. This approach allowed us to screen the compound set in singlicate with an assay Z′-factor of 0.77, indicating robust assay performance. The screening results are illustrated in Figure 2B as a scatter plot of % inhibition. After validating the hits by triplicate, the most potent molecules were manually synthesized in milligram quantities for further dose-dependent biochemical and cell-based characterization. This approach allowed us to confirm the efficacy and refine the profile of the most promising candidates. The structure of the representative hits synthesized for validation is shown in Figure 2A (compounds **2**-**4**), and the dose-response inhibition profile in Figure 2C. Compound 4 was identified as one of the top hits in the primary screening, with an EC_{50} value of 241 nM. Notably, compound **4** demonstrated an almost 2-fold increase in cellular antiviral potency compared to **1**.

3.3 Structure-activity relationships and biophysical analysis

To further understand the structure-activity relationships, we synthesized a small library of analogs of the identified molecules and subjected to dose-response antiviral and cytotoxicity studies. Specifically, we focused on modifications to the oxolane ring (Table 1, Supplementary Figure 3). These modifications included altering the ring size (**5**), investigating different substitution patterns on the oxolane ring (**6**, **7**), and replacing the oxolane with an N-methyl pyrrolidine ring (**8**) as well as other N-substituted analogs with varying polarity and chain lengths (**9** – **15**). Despite exploration of these broad structural modifications, no significant improvement in activity was observed with the ring changes. Since compound **4** was identified as a racemic mixture, we synthesized and evaluated both *R* and *S* enantiomers (Table 1). Interestingly, the *S*-configured compound (**4S**) exhibited a superior EC_{50} value of 92 nM, compared to its *R*-counterpart (**4R**) of 351 nM, indicating the importance of the chirality on the improved potency and specific

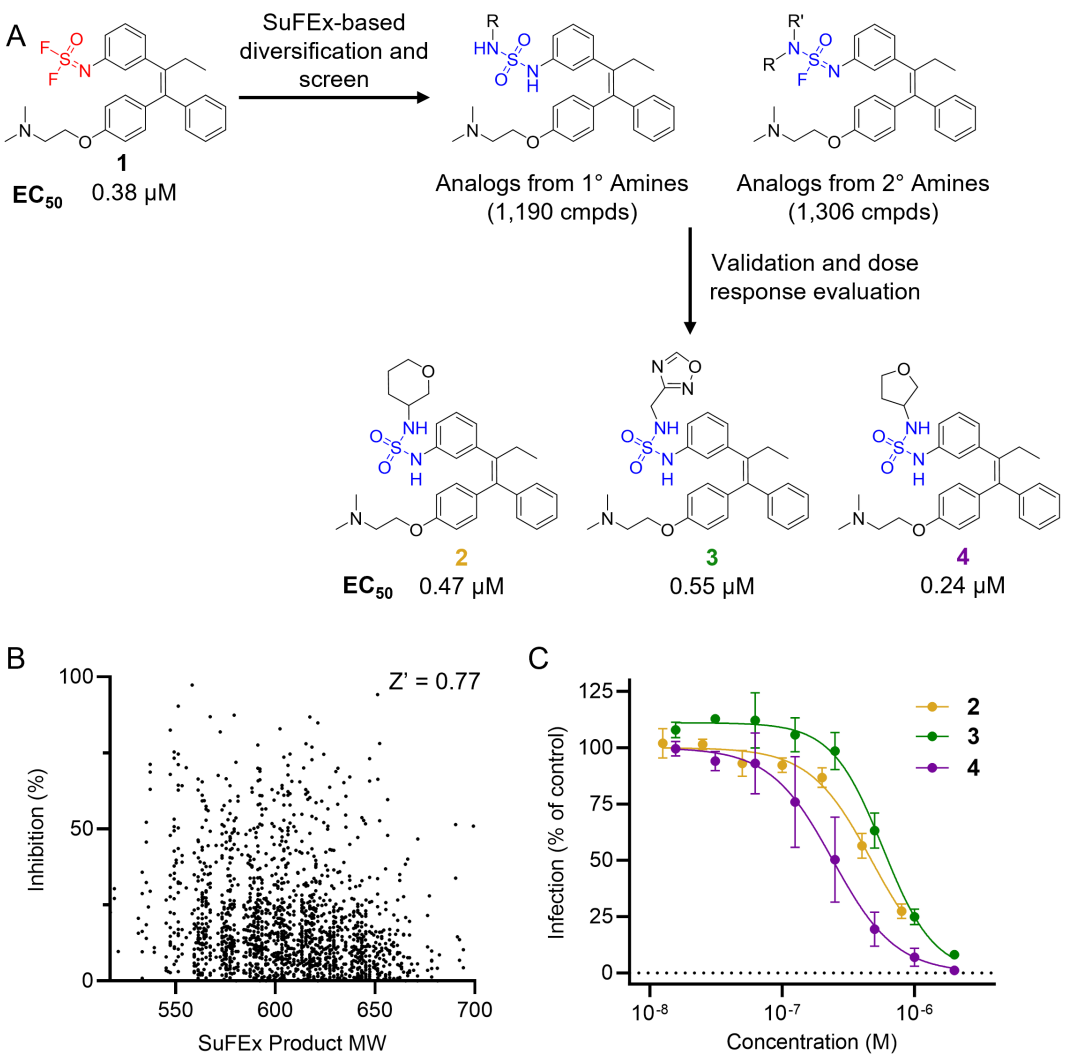

FIGURE 2
Reaction schematics and screening results of HTMC against EBOV entry. **(A)** HTMC workflow from a difluoride functionalized lead compound to the improved compounds. Chemical structures and EC_{50} (VSV-EBOV-GP, Vero cells) of representative hit molecules (compounds **2, 3**, and **4**) are shown. **(B)** Scatter plot for the tamoxifen-based HTMC library screening. Screening was performed at a small-molecule concentration of 200 nM. **(C)** Dose-response curves for representative hits identified from the HTMC library screening. Hit molecules were resynthesized in mg scale, purified, and chemically characterized, and their antiviral potency was measured against VSV-EBOV-GP using Vero cells. Mean ± SD values are shown (n = 3).

interaction with GP-protein. The cytotoxicity studies showed variable CC_{50} values among the synthesized analogs. Compounds **4S** and **4R** did not show significant cytotoxicity below 10 μM with an excellent selectivity index (SI) of 150 for **4S** (Table 1).

To confirm the direct interaction of **4R** and **4S** with EBOV-GP, the equilibrium dissociation constants (K_D) for the interaction inhibitor-protein were measured. The recombinant protein was expressed in Expi293S cells and purified using Ni-NTA affinity and size-exclusion chromatography. Then, we evaluated the interaction of both enantiomers as well as the parent tamoxifen with EBOV-GP by microscale thermophoresis (MST). As shown in Figure 3A, **4S** displayed a stronger binding affinity against EBOV-GP, with a K_D value of 0.63 μM, compared to **4R** with a K_D value of 6.4 μM. In comparison, the corresponding value for tamoxifen was 32 μM. This trend mirrors the observed differences in cellular potency, where **4S** demonstrated enhanced efficacy at low submicromolar concentrations in blocking EBOV entry, while **4R** required higher concentrations to achieve a comparable effect (Table 1). It is worth noting the 50-fold affinity enhancement in the biochemical assay between **4S** and tamoxifen, providing further validation of improved potency of the inhibitor developed through our HTMC platform.

We then determined the X-ray structure of **4R** in complex with EBOV-GP to 2.59 Å resolution (Figure 4, Supplementary Table 1). The electron density for **4R** is well defined (Figure 4B) and the compound binds within the extensive hydrophobic cavity of EBOV-GP, a known target site for other inhibitors. It adopts a similar pose to its analog, toremifene, with conserved interactions involving the three aromatic rings and the dimethylethanamine group of the parent scaffold (34). Compared to toremifene, **4R** occupies a larger portion of the available cavity, with its additional moiety engaging a hydrophobic subpocket, potentially contributing to its improved binding. Unfortunately, we

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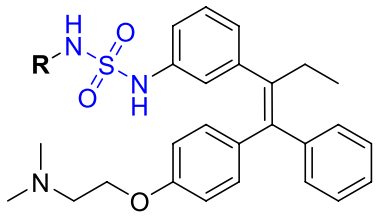
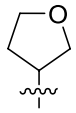
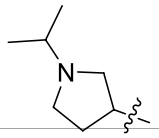
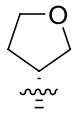
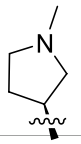
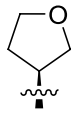
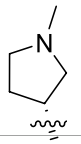
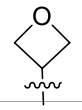
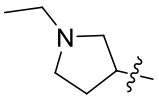
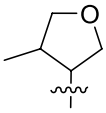
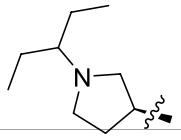
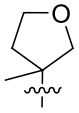
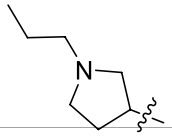
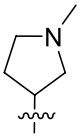
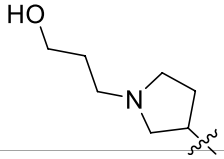
TABLE 1 Structure-activity relationships of compound 4 analogs against VSV-EBOV-GP.



Cmpd	R	EC_{50}[a] (nM)	CC_{50}[b] (μM)	SI[c]	Cmpd	R	EC_{50}[a] (nM)	CC_{50}[b] (μM)	SI[c]
4		240	n.d.	n.d.	**9**		790	3.4	4.3
4R		351	15	43	**10**		664	3.0	4.5
4S		92	14	150	**11**		575	3.6	6.3
5		156	6.8	44	**12**		841	4.0	4.8
6		201	14	70	**13**		813	5.3	6.5
7		268	8.4	31	**14**		618	3.1	5.0
8		145	2.0	14	**15**		1720	7.7	4.5

[a]EC_{50} values were measured against VSV-EBOV-GP (Vero cells).
[b]CC_{50} values were measured against Vero cells using Promega® CellTiter-Glo® 2.0. Reported EC_{50} and CC_{50} values are the average of triplicate measurements with at least two data points above and at least two below the EC_{50}.
[c]SI: Selectivity index = CC_{50}/EC_{50}. n.d. = not determined.

were unable to obtain a structure for **4S**, as it renders the crystal unstable, possibly due to conformational changes when the compound is soaked into the crystal, and leads to poor diffraction. Nevertheless, the complex structure of EBOV-GP and **4R** validates the docking model of our lead molecule and provides structural insights into the improved potency of the developed molecules.

Finally, we evaluated the viral selectivity and specificity of compound **4S** by comparing the inhibitory activity against VSV-EBOV-GP to VSV-G and VSV particles displaying LASV-GP (VSV-LASV-GP, Figure 3B). Tamoxifen and our compound **4S** showed selective inhibition of VSV-EBOV-GP but not VSV-G or VSV-LASV-GP, indicating that its antiviral activity is specific to EBOV-GP. This result supports the hypothesis that compound **4S** does not induce non-specific effects on viral entry mechanisms shared by multiple viruses or inhibit proteins involved in later stages of the EBOV infection, such as cathepsins, which are known to mediate viral entry through endosomal processing. Furthermore, the lack of activity in VSV-G and VSV-LASV-GP models reduces the likelihood that the compound's mechanism involves general endosomal disruption or interference with acidic environments. This selectivity, in concert with the chiral preference of **4S** over its *R*-isomer in binding EBOV-GP, suggests that the compound engages specific molecular interactions with EBOV-GP that are crucial for its activity.

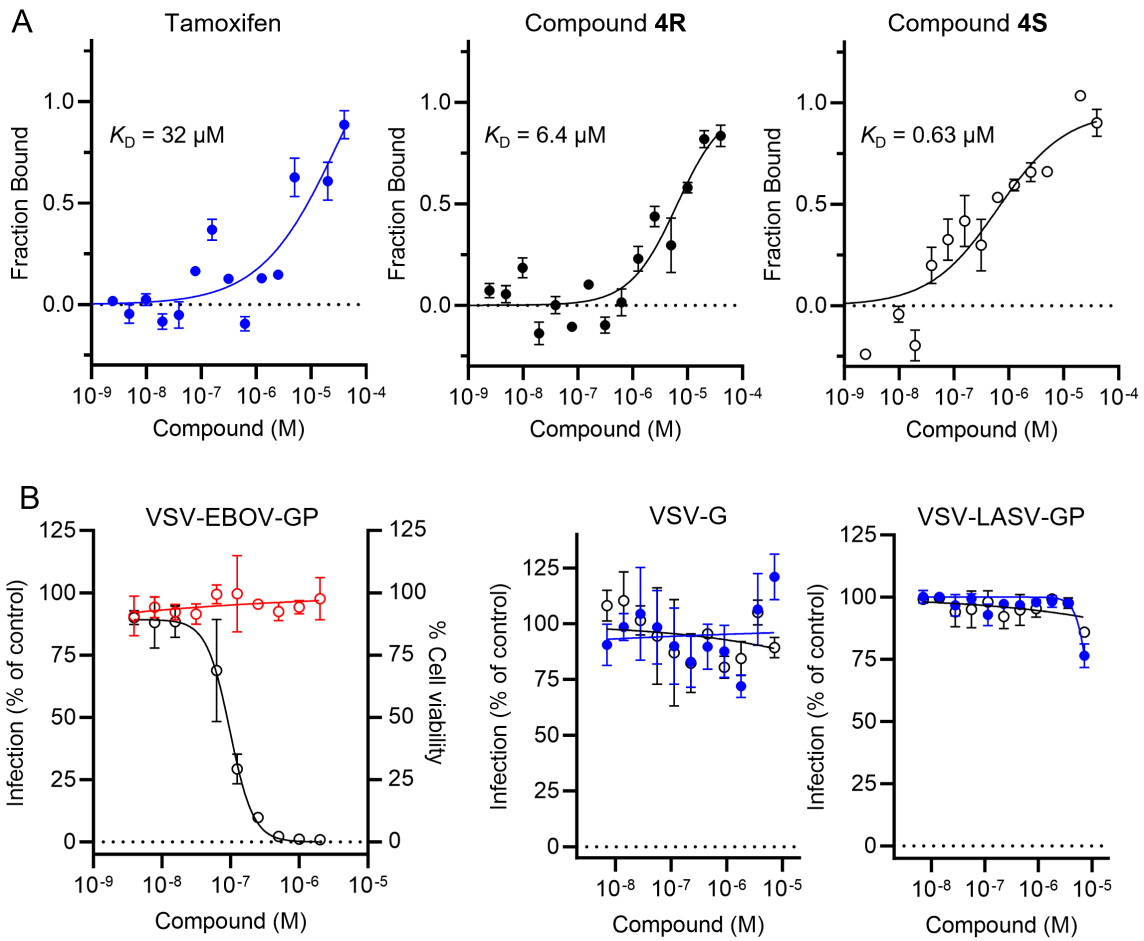


FIGURE 3
Characterization of the top tamoxifen analog **4S** identified from the HTMC campaign. **(A)** The biophysical affinity of tamoxifen, **4R** and **4S** towards EBOV-GP as measured by MST. Error bars indicate the standard deviation of three technical replicates (n = 3). **(B)** Dose-response curves of VSV-EBOV-GP infection (○) and Vero cell viability (○) for compound **4S** (left panel). Tamoxifen (●) and compound **4S** (○) were tested for their ability to inhibit VLPs displaying the VSV-G glycoprotein (middle panel) and LASV-GP (right panel). Mean ± SD values are shown (n = 3).

4 Discussion

In this study, we leveraged our unique SuFEx-based HTMC platform to generate a large-scale library of tamoxifen analogs with the aim of identifying potent inhibitors of EBOV entry. This approach enabled a rapid SAR analysis that accelerated the identification of novel EBOV inhibitors, highlighting the potential of HTMC workflow as a powerful tool for antiviral drug discovery. Our inhibitor discovery efforts were motivated by the need for effective small-molecule capable of modulating EBOV infection, especially given that current treatment options primarily include vaccines and monoclonal antibodies. While these approaches have shown promise, there remains an unmet need for potent small molecules that can be translated to clinical application. This work also demonstrated a successful application of our SuFEx-based HTMC platform for accelerated optimization of a potential antiviral compound, as we have shown previously for other biological targets (17–21).

To develop this strategy, we employed tamoxifen as a lead scaffold to design an analog with a required SuFExable hub for accelerated diversification. Although other analogs of tamoxifen, such as toremifene and clomiphene, have been extensively characterized as EBOV entry inhibitors (24), tamoxifen was the most suitable scaffold in terms of synthetic design to access to the required functionalized difluoride. The SuFEx-enabled HTMC approach applied here is especially well-suited to drug discovery for infectious diseases such as EVD, where the iterative cycle of lead optimization can be a significant bottleneck. Previous efforts on the optimization of tamoxifen analogs of these compounds have relied on traditional medicinal chemistry. Our SuFEx-based D2B platform eliminates the need for extensive purification. The biocompatible conditions employed for diversification enabled crude products to be tested directly in a cell-based assay. Furthermore, the method facilitated exploration of structural modifications that improve binding affinity and potency, as evidenced by the development of compounds with targeted changes that demonstrated improved antiviral efficacy without compromising cellular viability.

The design basis of our tamoxifen analog functionalized with a difluoride (compound **1**) stems from the binding interactions observed in the predicted complex of tamoxifen with EBOV- GP,

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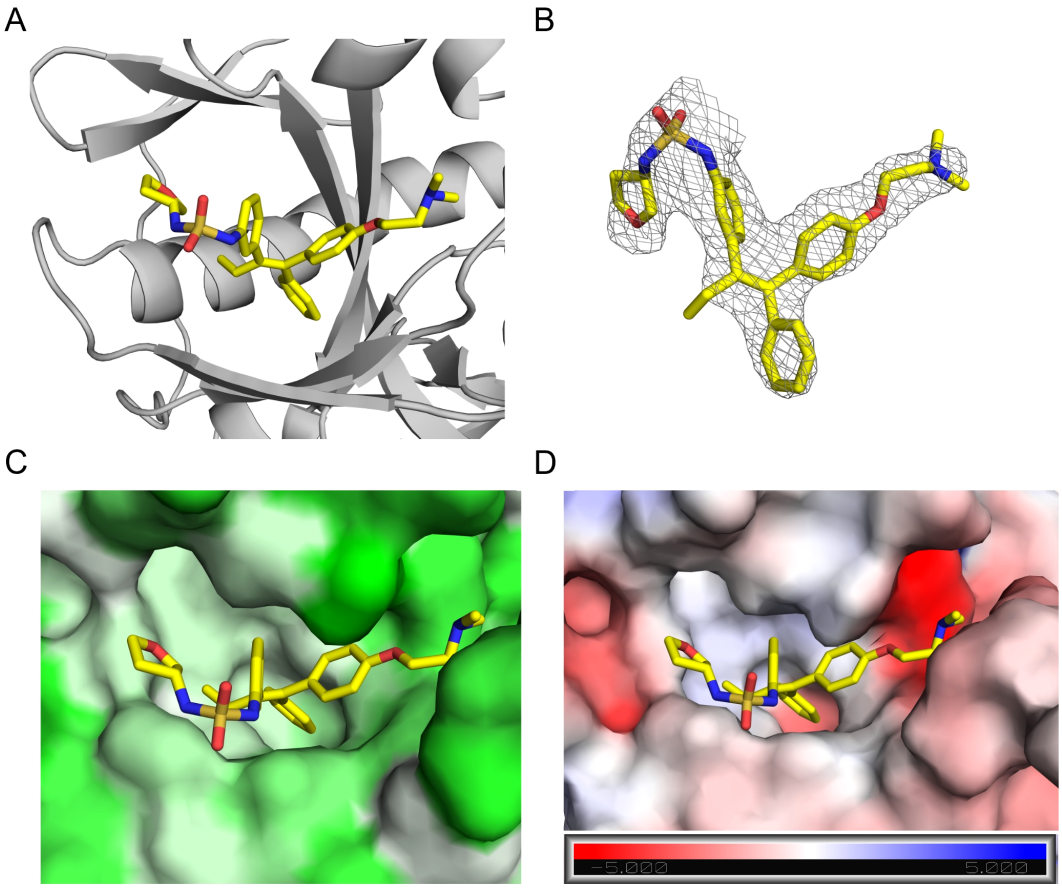

FIGURE 4
Structure of EBOV-GP in complex with compound **4R**. **(A)** Crystal structure of EBOV-GP trimer bound to **4R**. The compound is depicted as sticks, and the protein as a cartoon representation. **(B)** 2Fo-Fc electron density map contoured at 1 sigma for **4R**. **(C)** **4R** bound within the hydrophobic binding pocket of EBOV-GP. The surface is colored according to hydrophobicity, with pale green indicating the most hydrophobic regions and green the least hydrophobic. **(D)** Electrostatic surface potential of the binding pocket, colored from red (most negative) to blue (most positive), with **4R** bound. The color scale for electrostatics is shown at the bottom of the figure.

which suggested sites for functionalization to enhance binding affinity. By targeting the unoccupied pocket, we aimed to improve interaction around the binding pocket. The crystal structure of EBOV-GP with **4R** provided further validation to our docking approach for lead design.

This approach led to compound **4S** that demonstrated sub-micromolar inhibition of EBOV entry and a 50-fold improvement in binding affinity against EBOV-gp over tamoxifen as measured by MST. The stronger binding of **4S** to EBOV-GP, as reflected in its enhanced K_D, is consistent with its superior cellular potency, suggesting that improved target affinity is likely to contribute to its increased functional activity. However, it is worth noting that the improvement of cellular potency is around 2-fold compared with tamoxifen. Elucidating the apparent discrepancy between the improvement in biophysical affinity and cellular potency is the subject of further study.

Our structure-activity relationship put in evidence the chiral selectivity of the hit identified, with the analog **4R** showing less potency than its corresponding *S* enantio-counterpart, as measured by biophysical affinity as well as cellular antiviral activity. The significance of stereochemistry in **4S** exemplifies the precision achievable with this SAR-guided platform and highlights the potential for designing small molecules for enhanced interactions with viral proteins. These findings underscore the importance of chiral optimization in the development of effective EBOV entry inhibitors and validate **4S** as a particularly promising lead compound.

Unfortunately, we could not determine the crystal structure of the most active compound, **4S**. However, an analysis of the pose adopted by compound **4R** in the binding pocket of EBOV-GP revealed that the newly introduced oxolanyl ring, resulting from the SuFEx reaction, is positioned near the region where the β13-β14 loop (residues 190 to 214) is expected. This region has also been observed to be disordered in previous structures (34, 35). Structural analyses of toremifene bound to EBOV-GP have shown that this scaffold binds at the same site at the entrance of the binding pocket by expelling the DFF lid (residues 192–195) and positioning the A ring of the scaffold (Figure 1B) within this region (34). We propose that this flexible fragment could be modulating the enantioselectivity of the inhibitors identified in this work. However, additional experiments are required to verify this hypothesis.

In conclusion, this study demonstrates the effectiveness of our SuFEx-based HTMC platform in identifying promising EBOV entry inhibitors, contributing to the growing therapeutic modalities available for tackling the challenges associated with viral infections. This work paves the way for future studies to further optimize these lead compounds and assess their *in vivo* efficacy in EBOV infection. More broadly, the results of our HTMC workflow underscore the capability of this technology to accelerate the design and development of drug candidates in the field of viral infection, immunology, and autoimmune diseases.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. The data presented in the study is deposited in the RCSB PDB repository, accession number 9NNU. Further inquiries can be directed to the corresponding author.

Author contributions

LD: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. EN: Formal analysis, Investigation, Writing – review & editing, Data curation, Validation. SA: Formal analysis, Investigation, Data curation, Methodology, Resources, Writing – review & editing. AW: Investigation, Writing – review & editing, Resources, Validation. IW: Writing – review & editing, Supervision, Funding acquisition, Project administration. KC: Funding acquisition, Project administration, Supervision, Writing – review & editing, Investigation, Resources. SK: Funding acquisition, Investigation, Project administration, Resources, Writing – review & editing, Supervision, Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that Generative AI was used in the creation of this manuscript. To refine English during the revision of the manuscript. The generated sentences were further refined extensively by authors.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1533037/full#supplementary-material

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