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Immunotherapies against infectious diseases, 2nd edition

Edited by
Saba Tufail, Mohammad Asif Sherwani and Najmul Islam

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Immunotherapies against infectious diseases, 2nd edition

Topic editors

Saba Tufail — Aligarh Muslim University, India

Mohammad Asif Sherwani — University of Alabama at Birmingham, United States

Najmul Islam — Aligarh Muslim University, India

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EDITED AND REVIEWED BY
Victoria Bunik,
Lomonosov Moscow State University, Russia

*CORRESPONDENCE
Saba Tufail
✉ sabatufail12@gmail.com
Mohammad Asif Sherwani
✉ asherwani86@gmail.com

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Editorial: Immunotherapies against infectious diseases

Saba Tufail[1]*, Mohammad Asif Sherwani[2]* and Najmul Islam[3]

[1]Department of Genetics, University of Alabama at Birmingham, Birmingham, AL, United States, [2]Department of Dermatology, University of Alabama at Birmingham, Birmingham, AL, United States, [3]Department of Biochemistry, Jawaharlal Nehru Medical College (JNMC), Faculty of Medicine, Aligarh Muslim University, Aligarh, India

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Editorial on the Research Topic
Immunotherapies against infectious diseases

Immunotherapy is employed to harness the body's natural defense mechanisms to treat/manage disease (1–3). The rise of emerging and re-emerging infectious diseases, coupled with the potential cancer risks associated with oncogenic infectious agents and the limited efficacy of existing treatments (4), has attracted attention of scientific community toward immunotherapy. Advancement in immunotherapies comprising vaccines, monoclonal antibodies, cytokines, T cells, and checkpoint inhibitors, holds significant potential in addressing not only cancer but infectious diseases as well. This Research Topic aimed to explore recent advancements in utilizing immunotherapy for the treatment and management of infectious diseases. We strived to incorporate studies that assess the efficacy of various immunotherapeutic approaches, including vaccines, in preventing, treating, and managing infectious diseases.

This Research Topic elaborates the current research on immunotherapies (including vaccines) against various infectious diseases. Among around 15 different types of submissions received, we were able to collect a total of 9 articles that includes 4 Original Research articles, 2 Brief Research reports, 1 Review article, 1 Minireview, and 1 Opinion article. These articles presented diverse infectious diseases, including emerging and re-emerging diseases. We were pleased to have original research articles exhibiting studies in cell culture, mice as well as patients. This indicates that the findings of this Research Topic range from pre-clinical to clinical studies and may likely benefit and be of interest to a wider scientific community. Moreover, the studies included in the Research Topic are focused on immunotherapies against various viruses, protozoa and bacteria. In the coming paragraphs, we briefly discuss the published studies in this Research Topic.

Studies highlighting immunotherapies against viral diseases

Immunotherapeutic efforts to enhance antiviral immunity have been applied for quite some time (5–8), however, the present strategies need improvement to harness full potential of immunotherapy. Langan et al. present a novel immunotherapy strategy for against viral diseases, termed Artificial Immune Modulation Adoptive Cell Therapy (AIM ACT). This innovative approach utilizes AIM nanoparticles and is applicable to patients suffering from infections such as Epstein-Barr Virus (EBV), Human T-lymphotropic virus

1 (HTLV-1), human papillomavirus (HPV), or human immunodeficiency virus (HIV). In AIM ACT, the initial step involves collecting leukapheresis material from the patient. Subsequently, $CD8^+$ T cells are enriched using paramagnetic AIM nanoparticles, which serve as artificial antigen-presenting cells (aAPCs) to amplify $CD8^+$ T cells targeting key viral antigens. Following a 14-day expansion period, the AIM nanoparticles are removed, and the AIM ACT therapy is prepared for infusion into patients. Langan et al. present preclinical findings demonstrating the efficacy of this approach in expanding $CD8^+$ T cells targeting viral antigens associated with Epstein-Barr Virus (EBV), Human T-lymphotropic virus 1 (HTLV-1), as well as high-risk Human Papillomavirus (HPV) types 16 and 18, utilizing healthy donor cells. The final AIM ACT formulations consists of a mixture of CD3+/CD4+ T cells, predominantly targeting various antigens from EBV, HTLV-1, or HPV, with over 90% of these T cells exhibiting the memory phenotype. Furthermore, EBV-specific AIM ACT cells exhibit functional activity, showcasing antigen-specific cytotoxicity and cytokine profile.

Immunocompromised individuals are highly vulnerable to opportunistic infections and malignancies. Traditional antiviral and antifungal medications often pose significant toxicity risks, exhibit limited efficacy, and can lead to long-term resistance development. The administration of pathogen-specific Cytotoxic T-Lymphocytes (CTLs) has demonstrated minimal toxicity and efficacy in combating infections such as Cytomegalovirus, Adenovirus, Epstein-Barr virus, BK Virus, and Aspergillus. However, this therapy faces significant limitations including regulatory challenges, high cost, and the absence of public cell banks. Conversely, $CD45RA^-$ cells containing pathogen-specific memory T-cells offer a less complicated manufacturing and regulatory process, are more cost-effective, feasible, safe, and hold potential effectiveness. Sanz et al. introduce a therapeutic approach aimed at clearing viruses and particular fungi through donor lymphocyte infusions (DLIs) comprising $CD45RA^-$ cells containing pathogen-specific memory T-cells from a healthy donor. The goal is to bolster and enhance the patient's cellular immunity until their own immune system is restored. They report preliminary findings from a study involving six immunocompromised patients; four afflicted with severe infectious diseases and two with EBV lymphoproliferative disease. Each patient underwent multiple infusions of safe familial CD45RA T-cells as adoptive passive cell therapy, comprising memory T-cells specific to Cytomegalovirus, Epstein-Barr virus, BK virus, and Aspergillus. Results indicate that utilizing familial CD45RA T-cells containing pathogen-specific cytotoxic T-lymphocytes represents a viable, safe, and potentially effective approach for treating severe pathogenic infections in immunocompromised patients via third-party donor sources.

Yu et al.'s report evaluates the status and pattern of antiviral therapy among outpatient cases of herpes zoster in China. They found that throughout the study duration, there was a progressive rise in the prescription of antiviral medications. Valaciclovir and famciclovir emerged as the primary prescribed antivirals, although their usage varied significantly across different hospitals. Conversely, prescriptions for acyclovir exhibited a declining trend. Despite not being recommended, the utilization of topical antivirals continued to increase. The annual expenditure remained consistent, attributed to the diminishing daily drug costs (DDCs) of valaciclovir and famciclovir. Overall, antiviral treatments closely align with contemporary recommendations, barring the utilization of topical antivirals. The insights gained from this study hold potential for optimizing healthcare resource allocation and herpes zoster management among Chinese outpatient populations.

The other interesting report by Godinez et al. details a descriptive analysis exploring user-generated social media dialogues on Reddit pertaining to FDA-approved HIV pre-exposure prophylaxis (PrEP) treatments. Examining thousands of Reddit posts, they identified 315 posts coded for PrEP, with 105 posts (33.33%) focusing specifically on user conversations regarding the transition of PrEP prevention. Notably, users displayed a keen interest in switching to emtricitabine and tenofovir alafenamide (Descovy), particularly citing concerns such as poorer adherence or existing side effects associated with emtricitabine and tenofovir disoproxil fumarate (Truvada). The analysis unveiled major themes including discussions on the cost disparity between Descovy and Truvada, apprehensions regarding side effects, changes in insurance coverage, and conversations surrounding the donation of Truvada to other users post-transition. These findings underscore the significance of leveraging social media platforms for digital pharmacovigilance, providing insights into emerging challenges related to HIV prevention, treatment, and adherence as experienced by patients.

In the opinion article by Alape-Girón et al., the authors highlight that preclinical data, alongside findings from completed clinical trials, warrant further exploration into the potential of equine pAbs as a broad-spectrum, cost-effective, and scalable treatment for COVID-19. They advocate for the comparability to antivenoms, suggesting that these therapeutics could be manufactured under Good Manufacturing Practices (GMPs) in low- and middle-income countries equipped with the necessary technology, and made available at prices feasible for economies with limited resources.

Studies on immunotherapies against non-viral diseases

In addition to studies on anti-viral immunotherapies, this Research Topic also includes immunotherapeutic studies on human protozoa, bacterial and fungal pathogenic microbes. Phares et al. present findings on LD10, an active 18-amino acid derivative derived from a previously reported peptide (9, 10). *In vitro* experiments revealed that LD10 exhibited superior potency in disrupting PD-1 receptor signaling compared to LD01. Moreover, when administered prophylactically alongside an adenovirus-based malaria vaccine, LD10 treatment led to a greater expansion of antigen-specific CD8+ T cells secreting IFN-γ compared to LD01 treatment. Studies on dosing regimens established that a single dose of LD10 at the time of immunization with AdPyCS, a circumsporozoite (CS) protein of *Plasmodium yoelii*, was adequate to enhance the quantity of vaccine-induced antigen-specific T cells *in vivo*. Utilizing humanized mice with functional human $CD8^+$ T cells, LD10-mediated modulation of human T cell responses was demonstrated. Furthermore, it was shown that LD10 could be expressed and secreted by a recombinant MVA vector, thereby enhancing the expansion of antigen-specific $CD8^+$ T cells. These

findings collectively establish LD10 as a potent immunomodulator that enhances T cell responses and supports the delivery of peptide-based immunomodulators via a viral vector.

Tahir et al. elaborate a survey-based study conducted across various countries to assess public acceptance of vaccines. They focused on the general population of Pakistan, aiming to evaluate their knowledge, attitudes, and practices regarding the Typhoid Conjugate Vaccine (TCV) and their willingness to receive the booster dose of TCV. Their findings revealed that while the Pakistani population possessed general awareness about the benefits of vaccination and the importance of booster doses, there was a lack of understanding regarding the availability and effectiveness of TCV provided by the government, particularly in combating typhoid fever. Despite a favorable inclination toward vaccination promotion, the study highlighted the persistence of certain religious and national misconceptions, underscoring the need for targeted intervention. However, the populace exhibited willingness to comply with booster doses of TCV, indicating an understanding of the importance of completing vaccine courses for disease prevention. The study suggests collaborative efforts among healthcare authorities, media outlets, and government officials to organize seminars and campaigns at both local and national levels, leveraging electronic and print media to address misconceptions about immunization, raise awareness about diseases, their consequences, modes of transmission, and the critical role vaccines play in disease prevention and saving lives.

Cubillos-Angulo et al. provide a comprehensive review examining the rationale and obstacles to the development and implementation of host-directed therapies (HDTs) against tuberculosis. Their analysis succinctly outlines medications that have completed or are currently undergoing evaluation in ongoing clinical trials. The study underscores the potential of combined multi-drug treatment with HDT in enhancing therapy effectiveness by reducing treatment duration, improving cure rates, and minimizing residual tissue damage. Additionally, Qadri et al. delve into recent advancements in immunotherapies targeting infectious diseases. They explore various immunotherapeutic approaches and their applicability in combating a wide range of infectious diseases caused by various bacterial and fungal pathogens.

In summary, the articles within this Research Topic gather insights from diverse experts in the fields of immunotherapy and infectious diseases, addressing various challenges and proposing potential solutions, thus enriching the field with new perspectives and understandings. The articles included studies from various countries of the world and on different pathogens, which further enriches our knowledge and opens avenues for further studies.

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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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Heterologous Hyperimmune Polyclonal Antibodies Against SARS-CoV-2: A Broad Coverage, Affordable, and Scalable Potential Immunotherapy for COVID-19

Alberto Alape-Girón[1,2], Andrés Moreira-Soto[3], Mauricio Arguedas[1], Hebleen Brenes[4], Willem Buján[2,5], Eugenia Corrales-Aguilar[6], Cecilia Díaz[1,2], Ann Echeverri[5], Marietta Flores-Díaz[1], Aarón Gómez[1], Andrés Hernández[1], María Herrera[1], Guillermo León[1], Román Macaya[5], José Arturo Molina-Mora[6], Javier Mora[6], Aarthi Narayanan[7], Alfredo Sanabria[5], Andrés Sánchez[1], Laura Sánchez[1], Álvaro Segura[1], Eduardo Segura[1], Daniela Solano[1], Claudio Soto[4], Jennifer L. Stynoski[1], Mariángela Vargas[1], Mauren Villalta[1], Jan Felix Drexler[3] and José María Gutiérrez[1]*

[1] Instituto Clodomiro Picado, School of Microbiology, University of Costa Rica, San Pedro, Costa Rica, [2] School of Medicine University of Costa Rica, San Pedro, Costa Rica, [3] Institute of Virology, Charité Medical University of Berlin, Berlin, Germany, [4] Instituto Costarricense de Investigación y Enseñanza en Nutrición y Salud, Ministry of Health, Cartago, Costa Rica, [5] Caja Costarricense del Seguro Social, San Jose, Costa Rica, [6] Research Center for Tropical Diseases, School of Microbiology, University of Costa Rica, San Pedro, Costa Rica, [7] National Center for Biodefense and Infectious Diseases, College of Science, George Mason University, Fairfax, VA, United States

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Edited by:
Zhiliang Hu,
Nanjing Second Hospital, China

Reviewed by:
Thierry Burnouf,
Taipei Medical University, Taiwan
Leonardo M. R. Ferreira,
Medical University of South Carolina, United States

****Correspondence:***
Alberto Alape-Girón
alberto.alape@ucr.ac.cr

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INTRODUCTION

The emergence and dissemination of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the resulting COVID-19 pandemic triggered a global public health crisis. Although several SARS-CoV-2 vaccines have been developed, demand far exceeds supply, access to them is inequitable, and thus, populations in low- and middle-income countries are unlikely to be protected soon (1). Furthermore, there are no specific therapies available, which is a challenge for COVID-19 patient care (2). Thus, the appearance of SARS-CoV-2 variants and reports of reinfections associated with immune escape (3, 4) highlight the urgent need for effective and broad coverage COVID-19 therapeutics.

Intravenous administration of human or heterologous antibodies is a therapy successfully used in patients with viral respiratory diseases (5). Accordingly, formulations containing SARS-CoV-2 specific antibodies are an attractive therapeutic option for COVID-19 patients (6). SARS-CoV-2 specific antibodies could limit infection by direct virion neutralization and/or by targeting infected cells for elimination via complement or antibody-mediated cytotoxicity (6).

Specific SARS-CoV-2 antibody-based therapeutics include convalescent plasma (CP), monoclonal antibodies (mAbs), human polyclonal IgG formulations purified from CP or transgenic animals, and heterologous hyperimmune polyclonal antibodies (pAbs) (6). Although the window for using antibody-based therapeutics varies, clinical data show that they are mainly effective if administered early after symptoms onset (6).

CONVALESCENT PLASMA TRANSFUSION

CP transfusion contributes to viral clearance and improves patient survival when administered promptly and has been therapeutically used worldwide during the COVID-19 pandemic in hospitalized patients (6, 7). However, it is worth mentioning that in some developing countries CP transfusion may heighten the risk of transmission of blood-borne pathogens if proper donors screening and virus testing procedures are not applied (8, 9). Furthermore, uncertainty remains about the therapeutic efficacy of CP transfusion, as controlled clinical trials have provided variable results in terms of mortality and need for mechanical ventilation (7). The diversity among reported results is likely due to the heterogeneity of trial designs, anti-SARS-CoV-2 plasma titer variation, and differences in latency to product administration (7). Therefore, more studies are required to establish the optimal doses and timing for CP transfusion to COVID-19 patients.

MONOCLONAL ANTIBODIES

Over 80 mAbs have been shown to block the interaction between the SARS-CoV-2 S1 glycoprotein and its cellular receptor, thus neutralizing virus infectivity *in vitro* (10). Some of those mAbs demonstrate therapeutic efficacy to curtail viral burden and lung inflammation in animal models (10). The neutralization mechanisms of mAbs against SARS-CoV-2 *in vivo* are not fully understood, but optimal protection correlates with Fc effector functions (11).

Approximately 30 SARS-CoV-2 neutralizing mAbs are undergoing clinical trials in COVID-19 patients (10). Some were granted emergency authorization since they reduced viral load, disease severity, and hospitalization in randomized, controlled phase II clinical trials (10). However, mAbs are unaffordable for healthcare systems in many developing countries due to their high cost (> USD 1,500/vial), meaning that most infected people would not have access to them (12).

Another obstacle for COVID-19 therapy with mAbs is the emergence of viral variants harboring changes in the receptor-binding domain (RBD) of the S1 glycoprotein (13). The variants of concern (VoC) exhibit enhanced transmissibility or virulence, circulate worldwide, and include those designated as alpha, beta, epsilon, gamma, and delta, first detected in the UK, South Africa, Brazil, USA, and India, respectively (13). Therapeutic mAbs, and antibodies in the plasma of vaccinated or convalescent individuals, fail to neutralize VoC efficiently (13–17).

HUMAN AND HETEROLOGOUS HYPERIMMUNE POLYCLONAL ANTIBODY FORMULATIONS

Intravenous IgG (IVIGs) formulations purified from CP pools are a cheaper option (> USD 300/vial) than mAbs for therapy of COVID-19 patients (18, 19), which furthermore could be used prophylactically. Human IVIGs could be also purified from the plasma of genetically modified transchromosomic bovines hyperimmunized with SARS-CoV-2 antigens (20).

Preparation of IVIGs formulations from CP is feasible for some developing countries. However, it requires strict donor screening for high levels of SARS-CoV-2 neutralizing antibodies, as well as the absence of blood-borne pathogens and antibodies against human leucocyte or neutrophil antigens to limit the risks of Transfusion Related Acute Lung Injury (21). Thus, this therapy depends on rigorous blood bank systems that are often scarce in low- and middle-income countries (22). Due to these logistical hurdles and lack of infrastructure, it is difficult for most developing countries to rapidly establish large-scale manufacturing capacity to prepare IVIGs formulations against SARS-CoV-2 (23).

Results were recently reported about a single-center, single-blind, placebo-controlled phase I/II clinical trial (NCT04521309) with anti-SARS-CoV-2 IVIGs purified from CP in Pakistan with 55 hospitalized severe or critical COVID-19 patients (24). The data showed that this immunotherapy is safe, increases survival, and reduces the disease progression risk (24). Similarly, IVIGs purified from the plasma of transchromosomic bovines hyperimmunized with the SARS-CoV-2 S1 glycoprotein are well-tolerated by non-hospitalized COVID-19 patients according to a phase Ib clinical trial (NCT0446917) performed in the USA (20). This pAbs formulation will be evaluated in a phase II/III clinical trial (NCT04518410) in non-hospitalized COVID-19 patients ongoing in the USA.

Another promising therapy for COVID-19 patients is the intravenous administration of heterologous pAbs, purified from plasma of animals hyperimmunized with SARS-CoV-2 proteins (23). Such formulations of intact or fragmented hyperimmune equine/ovine pAbs are therapeutics with a proven path to regulatory approval, which have been successfully used worldwide for decades as therapies against rabies virus infection or as antivenoms to treat patients bitten or stung by venomous animals (25). Several manufacturers from developed and developing countries regularly supply formulations of heterologous pAbs as therapeutic antivenoms which comply with Good Manufacturing Practices (GMPs) and show good safety and efficacy profiles in the treatment of envenomings by animal bites and stings (26–29). The early and late adverse reactions induced by GMP-prepared formulations of heterologous pAbs are mainly mild, and if occur can be monitored and pharmacologically controlled (30, 31). Indeed, heterologous pAbs formulations are approved by the Food and Drug Administration, as therapies for envenoming by snakes, scorpions, and spiders, as well as for digoxin poisoning, botulism, and as anti-thymocyte globulin in immunosuppressive regimens (32). They are also approved by the European Medicines Agency as therapies for avian influenza and rabies virus infections, snakebite envenoming, hemolytic uremic syndrome, and colchicine poisoning.

Formulations of equine pAbs against the SARS-CoV S1 glycoprotein control coronavirus infectivity in cultured cells and animal models (33). Similarly, IgG and F(ab')2 formulations from horses immunized with MERS-CoV virus-like particles exhibit *in vivo* neutralization (33). Recently, three F(ab')2 formulations from plasma of horses hyperimmunized with recombinant

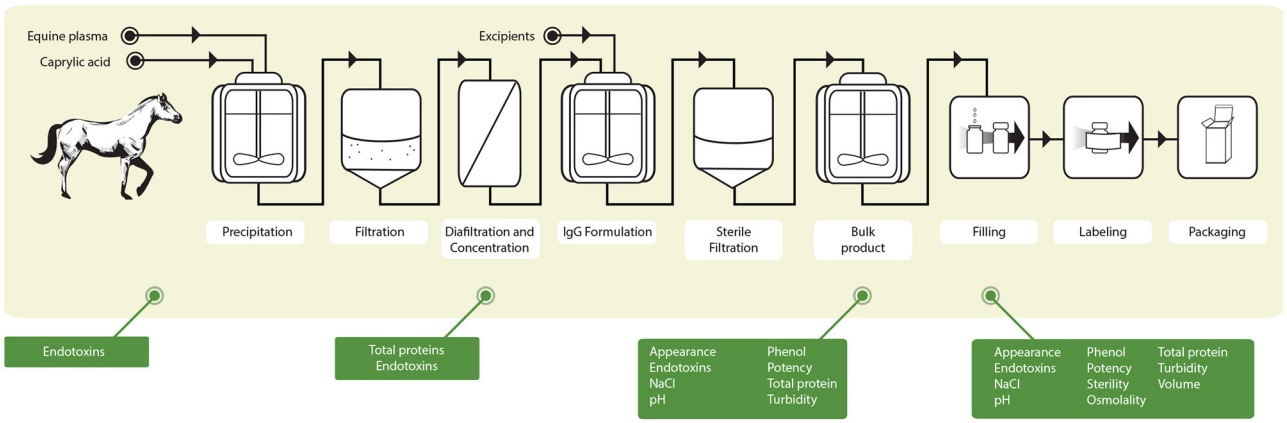

FIGURE 1 | Schematic summary of the protocol followed for the production of the anti-SARS-CoV-2 ICP at the Clodomiro Picado Institute in Costa Rica. Groups of horses were immunized with either the S1 viral protein or a mixture of S1, N and SEM viral proteins. After immunization, horses were bled, and the plasma was separated and fractionated according to the scheme shown. The green boxes depict the quality control assays carried out during the process of fractionation and of the final product. In addition, ELISA antibody titers against viral proteins and neutralization of virus infectivity in cell culture were done by plaque reduction neutralization tests to ensure viral neutralization efficacy [see Leon et al. (37) for details].

SARS-CoV-2 S1 glycoprotein or its RBD were described (34–37). These formulations are significantly more potent than COVID-19 convalescent plasma at neutralizing SARS-CoV-2 infectivity (35–37). Moreover, a multi-center, double-blind, placebo-controlled phase II/III clinical trial (NCT04494984) with RBD-specific equine F(ab')2 fragments performed in Argentina showed that this immunotherapy is well-tolerated and that clinical improvement of hospitalized severe COVID-19 patients is achieved (38). Five clinical trials are registered to test equine F(ab')2 formulations against SARS-CoV-2 that were produced by anti-venom-manufacturing laboratories in India, Argentina, Brazil (two trials), and Mexico (NCT04834908, NTC04913779, NCT04834089, NCT04573855, NCT04514302), although patient recruitment has only begun in India and Argentina.

In contrast to mAbs, equine pAbs against the SARS-CoV-2 S1 glycoprotein recognize multiple epitopes, which reduces the risk of viral escape. Long-term experience underscores that equine pAbs can be produced on a large scale and at a much lower cost (< USD 35/vial) than mAbs or IVIGs. The high neutralization potency of equine pAbs (35–37), likely results from a higher affinity than pAbs from CP because horses are exposed to optimized hyperimmunization protocols using repeated doses of viral antigens in the presence of adjuvants. Also, it is worth mentioning that equine antibodies have a low risk of viral contamination, considering that the horse plasma fractionation technologies include viral removal/inactivation steps (39).

THE COSTA RICAN EXPERIENCE

Given the urgent need for affordable COVID-19 treatments, two intact-IgG formulations were produced at the Clodomiro Picado Institute (ICP) of the University of Costa Rica from the plasma of horses hyperimmunized with either S1 (anti-S1) or a mixture of S1, N, and SEM mosaic (anti-Mix) SARS-CoV-2 recombinant proteins (37) (**Figure 1**). Pools of 25 L of hyperimmune plasma were used to prepare pilot batches of 500 vials of 10 mL, which were manufactured in 70 days, from the start of horse immunization to the final quality control tests of the products (37) (**Figure 1**). The fractionation process included two steps with robust viral inactivation/removal activity (caprylic acid precipitation and phenol addition) to ensure the viral safety of the products, as required by the WHO (21). The capacity of both formulations to neutralize virus infectivity (USA-WA1/2020 isolate) *in vitro* is 80 times higher than that of pooled human convalescent plasma (37). Additionally, the equine IgGs in these formulations activate human FcγRIIIA *in vitro*, suggesting downstream mediation of effector functions via interaction with Fc receptors in immune cells (37).

We compared the clinical safety and efficacy of the anti-S1 and anti-Mix pAbs formulations in a Bayesian pick-the-winner type phase IIa clinical trial (NCT04610502) in 27 COVID-19 patients with two or more risk factors. The single-dose immunotherapy with 550 mg of equine pAbs was well-tolerated, as adverse reactions were mild and resolved without sequelae. Most adverse reactions were cutaneous and easily controlled with a standard regimen of antihistamine drugs and steroids, as those induced by horse-derived antivenoms produced at the ICP and used in the therapy of snakebite envenomings (26, 27). Moreover, a decrease in viral load following infusion of these formulations and the good clinical recovery of most patients provide preliminary support for the clinical rationale of this COVID-19 therapy. A randomized, multi-center, double-blind, placebo-controlled, dose-finding phase IIb/III clinical trial (NCT04838821) with 173 patients is underway at hospitals of the Costa Rican Social Security Fund (Caja Costarricense del Seguro Social) to conclusively establish safety and efficacy, and to determine the optimal dose of the anti-S1 equine pAbs formulation (designated "Anti-SARS-CoV-2 ICP") to treat moderate and severe COVID-19 cases. If the ongoing trial demonstrates the efficacy and safety of this formulation, its clinical use would be therapeutic

and not prophylactic, owing to the heterologous nature of equine antibodies.

Both formulations of equine pAbs prepared at the ICP effectively inhibit the infectivity of two SARS-CoV-2 early isolates (WA1/2020 and Gisaid_EPI_ISL_406862, from USA and Germany, respectively) and five VoCs (alpha, beta, epsilon, gamma, and delta) with similar neutralizing potencies (40). The 50% Inhibitory Concentration (IC50) range in a plaque reduction neutralization assay was 0.146–1.078 μg/mL, which is much lower than the antibody concentrations presumably circulating in the patients' blood in the ongoing phase IIb/III clinical trial, given treatment doses of 12–56 mg/kg.

CONCLUDING REMARKS

The preclinical data, along with those obtained in completed clinical trials, prompt further investigations regarding the potential of equine pAbs targeting SARS-CoV-2 as a broad coverage, low-cost, and scalable treatment for COVID-19. Similar to antivenoms, those therapeutics can be readily produced under GMPs in low- and middle-income countries that have this technology in place and distributed at prices accessible to resource-constrained economies. WHO standardized guidelines are available to produce heterologous pAbs for therapeutic use in humans (21), which should allow for the worldwide production of SARS-CoV-2 specific formulations within a relatively short time. Following the protocol described in Leon et al. (37), after immunizing 20 horses with S1 protein produced in insect cells, industrial batches of 5,000 vials of 10 mL of Anti-SARS-CoV-2 ICP can be manufactured from 250 L of hyperimmune plasma pools every 2 months. Antivenom-manufacturing laboratories operating in Argentina, Australia, Bolivia, Brazil, Colombia, Costa Rica, Egypt, France, Mexico, India, Peru, South Africa, Thailand, UK, USA, and Venezuela could quickly adapt existing platforms to produce equine pAbs against SARS-CoV-2 or future emerging viral agents to cope with the global need for affordable therapeutic options during pandemic crises.

If ongoing clinical trials corroborate the safety and efficacy of equine pAbs against SARS-CoV-2, the international community should support existing antivenom manufacturing laboratories in several countries to allow for the production of these new therapeutic antibody formulations with their platforms. If this is done cooperatively and effectively, the world, and particularly low- and middle-income countries, could have a readily available therapeutic option for COVID-19 patients.

AUTHOR CONTRIBUTIONS

MF-D, AA-G, JS, AM-S, and JMG took the lead in writing the manuscript. All authors provided critical feedback, take responsibility for the overall content and integrity of the work, concur with the submission and have contributed to, read, and approved the final version.

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Conflict of Interest: Several authors of this manuscript are employees of the Instituto Clodomiro Picado at the University of Costa Rica, a public research institute with no commercial interests, where these antibody formulations were developed, and where they eventually will be manufactured for use in the Costa Rican public health system.

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EDITED BY
Saba Tufail,
Aligarh Muslim University, India

REVIEWED BY
Tibor Bakacs,
Alfred Renyi Institute of Mathematics, Hungary
Tatsuo Suzutani,
Fukushima Medical University, Japan

*CORRESPONDENCE
Lingyan Yu
lingyanyu@zju.edu.cn
Gang Han
3199022@zju.edu.cn

†These authors have contributed equally to this work

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Antiviral treatment in outpatients with herps zoster in six major areas of China, 2010–2019

Zhenwei Yu[1†], Yuhua Zhao[2†], Jiayi Jin[1], Jianping Zhu[1], Lingyan Yu[3*] and Gang Han[1*]

[1]Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China, [2]Affiliated Xiaoshan Hospital, Hangzhou Normal University, Hangzhou, China, [3]Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China

Objective: The objective of this study was to assess the status and trends of antiviral treatment in outpatients with herpes zoster in China.

Methods: Prescription data on antiviral drugs were extracted from the database of the Hospital Prescription Analysis Program of China according to the inclusion criteria. Yearly prescriptions and costs were calculated, and trends were analyzed. The trends were further stratified by age, sex, and specific drug use. The distribution of defined daily costs (DDCs) of valaciclovir and famciclovir were analyzed, and trends in the median DDCs were identified.

Results: A total of 132,911 prescriptions from 49 hospitals located in six major areas of China were included in the analysis. The yearly prescriptions containing antivirals increased from 8,819 in 2010 to 16,361 in 2019. The percentage of prescriptions for patients aged 65 years and above also increased (27.7% in 2010 to 31.0% in 2019), and the number of prescriptions for females was higher than those for males ($P < 0.001$). The average cost of antivirals per prescription decreased; thus, the yearly cost showed no increasing trend. The main prescribed antivirals were valaciclovir and famciclovir, which progressively increased in prescriptions. The use of acyclovir decreased during the study period. Prescriptions containing topical formulations, acyclovir and penciclovir, both increased. The DDCs of valaciclovir and famciclovir decreased dramatically.

Conclusion: The use of antivirals has increased over the decade, while the cost has not. Antiviral treatments adhere well to recent recommendations, except for the use of topical antivirals. The findings of this study may benefit the healthcare source allocation and management of herpes zoster in China.

KEYWORDS

acyclovir, valaciclovir, famciclovir, prescription, cost

Introduction

Herpes zoster, also known as shingles, is an infection caused by latent varicella zoster virus reactivation and is usually characterized by a prodromal period with burning pain for two–three days, followed by a vesicular eruption in the dermatomal distribution

of the infected ganglion (1). The global incidence of HZ ranges between 3–5 per 1,000 person-years (2). The incidence rate in China is similar, and it is estimated that at least 2.77 million cases occur in China annually (3, 4). It is also estimated that persons who live to 85 years of age have a 50% risk of herpes zoster in the absence of the herpes zoster vaccine (5).

Although most cases of herpes zoster are self-limited and will resolve within a few weeks, nearly all patients experience pain, with impact their normal functioning, reduces quality of life, and results in productivity losses. Herpes zoster is also associated with certain complications, the most common being post-herpetic neuralgia, a pain that persists long beyond cutaneous healing (1). Herpes zoster and its complications have been reported to cause approximately 67,000 quality-adjusted life years (QALYs) and incur costs of $2.4 billion in direct medical costs and productivity losses annually in the US (6). Economic studies in other countries and in China have also revealed that herpes zoster was associated with impaired quality of life and substantial health care resource use (7–10). Thus, attention should be paid to the management of herps zoster.

Antiviral therapy is recommended for all patients with herpes zoster, especially in patients with severe infection, old age, and immunocompromised status (1, 5, 11–13). The timely use of antiviral drugs, usually within 72 h of rash onset, can reduce viral replication, shorten the duration of symptoms, and prevent complications (1). Understanding the status of antiviral therapy for patients with herpes zoster would be helpful for improving disease management. However, little is known about this issue, especially in China. Thus, we conducted this cross-sectional study to assess the patterns and trends of antiviral therapy for patients with herpes zoster over the past decade.

Methods

Study design and ethics

This study was designed as a database-based, cross-sectional study. Ethical approval was obtained from the Ethics Committee of Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University (Reference Number 20210924-33). Informed consent was waived because of the retrospective nature of the study.

Data collection

Data on prescriptions were obtained from the Hospital Prescription Analysis Cooperative Project of China, which is widely used in pharmacoepidemiology studies (14–19). Prescriptions that met the following criteria were included for analysis: (1) Prescriptions from hospitals located in six major area of China (Beijing, Shanghai, Guangzhou, Chengdu, Hangzhou and Tianjin); (2) Prescriptions from hospitals that participated the program continuously, from 2010 to 2019; (3) Prescriptions written during 2010 and 2019; (4) Prescriptions written for adult outpatients (aged 18 years and above) with the diagnosis of herps zoster; (5) Prescriptions containing at least one antiviral drugs. The antiviral drugs were limited to acyclovir (J05AB01 and D06BB03), valacyclovir (J05AB11), famciclovir (J05AB09), penciclovir (D06BB06) and ganciclovir (J05AB06) in this study.

Analysis

The yearly prescription numbers of antiviral drugs for patients with herpes zoster were represented by the yearly eligible prescription numbers. The corresponding yearly cost was obtained by adding up the cost of all antiviral drugs in that year. It should be noted that this study did not extrapolate sampled data. Trends in yearly prescriptions and costs were analyzed and further stratified by specific drugs.

The percentages of valaciclovir (or famciclovir) prescriptions were evaluated at the hospital level. They were obtained by dividing the yearly prescriptions that contained valaciclovir (or famciclovir) by the total yearly eligible prescriptions in specific hospitals, and the yearly distribution of percentages was represented by a violin plot.

The defined daily cost (DDC), also known as the cost of a defined daily dose, was studied at the prescription level. DDCs were calculated for specific prescriptions using the following equation, and their distribution was also analyzed.

$$\text{Defined daily cost} = \frac{price\ of\ drug}{strength\ of\ price\ unit / defined\ daily\ dose} \quad (1)$$

The trends in the identified values were tested using the Mann–Kendal test, and the trends in percentages were tested using a log-linear test. The differences in the percentages of prescriptions for males and females were tested using the chi-square test. Statistical analysis was performed using R software. Statistical significance was set at $p < 0.05$.

Results

Demographic characteristics of prescriptions and overall trends

A total of 132,911 prescriptions from 49 hospitals were included in this study. The yearly prescriptions and corresponding costs are shown in Figure 1A. Yearly prescriptions increased progressively from 8,819 in 2010 to 16361 in 2019 ($P < 0.001$). The average cost per prescription

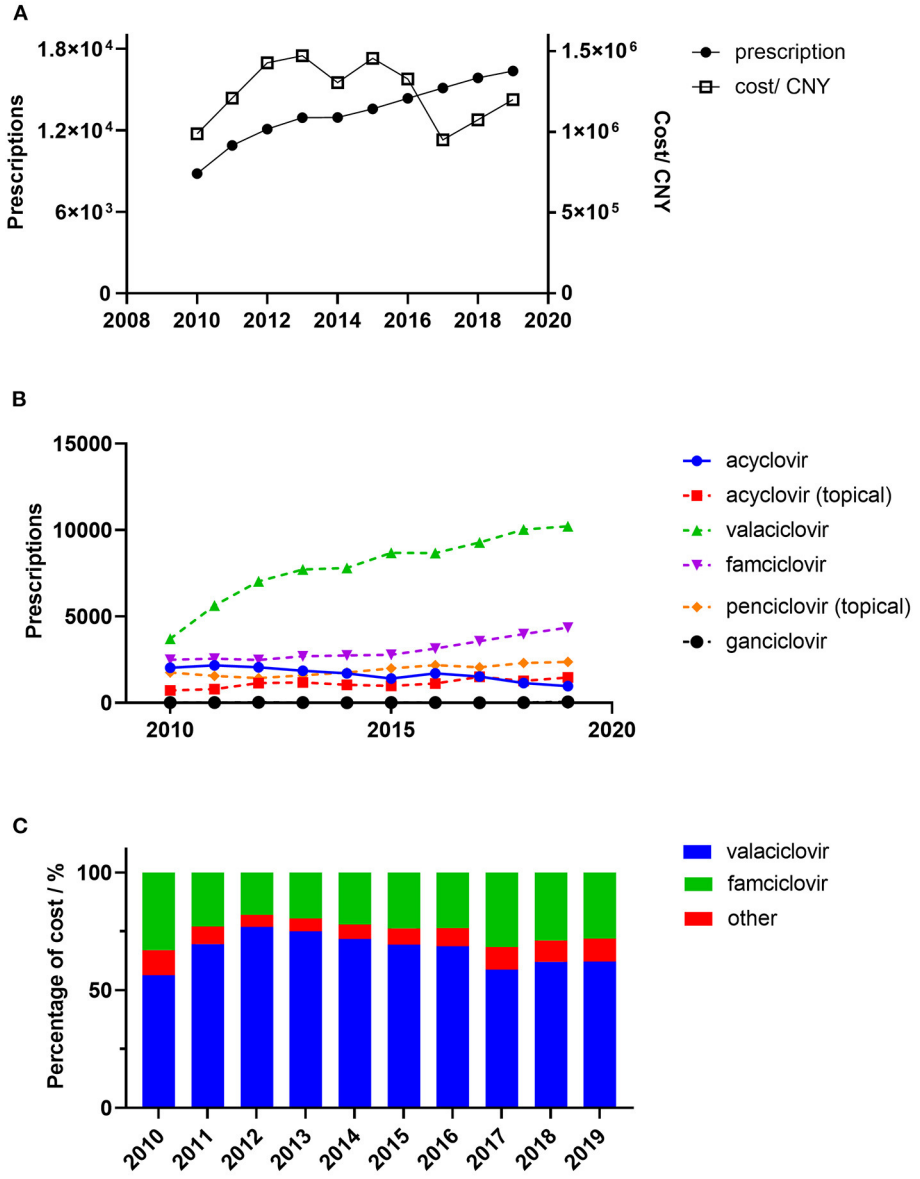

FIGURE 1
Trends in the prescription of antiviral drugs for patients with herps zoster in six major areas of China. **(A)** Overall yearly prescriptions and cost; **(B)** Yearly prescription of individual drugs; **(C)** Antiviral cost percentages. Percentage of cost means the percentage of overall yearly cost of specific drug.

is shown in Table 1, which reveals a significant decreasing trend over the study period ($P = 0.012$). Thus, the yearly cost fluctuated over the decade and showed no significant trend ($P = 0.721$). The demographic characteristics of patients with antiviral prescriptions are shown in Table 1. The percentage of prescriptions for females and males did not change over the study period ($P = 0.737$). However, the number of prescriptions for females was higher than those for males in each year (all $P < 0.05$). The majority of prescriptions were for patients aged 45–64 years, and, in this age range, the percentage of prescriptions remained constant ($P = 0.364$). However, the percentage of prescriptions for patients aged 65 years and above increased progressively ($P = 0.019$).

Trends in the prescriptions of specific antiviral drugs

The yearly prescriptions of each antiviral drug are shown in Figure 1. Valaciclovir and famciclovir were the major prescribed antiviral drugs over all of the years. Both of

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TABLE 1 Characteristics of patients with antiviral prescriptions and average cost per prescription.

		2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Age (year)	18–44	2,630 (29.8)	3,214 (29.5)	3,795 (31.4)	3,939 (30.5)	3,788 (29.3)	4,018 (29.6)	4,198 (29.3)	4,331 (28.7)	4,549 (28.7)	4,554 (27.8)
	45–64	3,745 (42.5)	4,628 (42.5)	5,067 (41.9)	5,469 (42.3)	5,640 (43.6)	5,825 (42.9)	6,230 (43.4)	6,389 (42.3)	6,629 (41.8)	6,729 (41.1)
	≥65	2,444 (27.7)	3,050 (28.0)	3,221 (26.7)	3,512 (27.2)	3,519 (27.2)	3,739 (27.5)	3,918 (27.3)	4,387 (29.0)	4,676 (29.5)	5,078 (31.0)
Sex	Male	3,992 (45.3)	4,957 (45.5)	5,647 (46.7)	6,020 (46.6)	5,882 (45.4)	6,170 (45.4)	6,564 (45.8)	6,985 (46.2)	7,188 (45.3)	7,455 (45.6)
	Female	4,827 (54.7)	5,935 (54.5)	6,436 (53.3)	6,900 (53.4)	7,065 (54.6)	7,412 (54.6)	7,782 (54.2)	8,122 (53.8)	8,666 (54.7)	8,906 (54.4)
Average cost (CNY)		112.1 ± 91.0	111 ± 91.2	118.2 ± 117.7	114 ± 144.6	100.9 ± 95.5	107.3 ± 111.6	92.6 ± 95.0	62.9 ± 87.2	67.8 ± 92.1	73.4 ± 103.9

Data were presented as prescription numbers (percentage%). Average cost was presented as mean ± standard deviation in CNY.

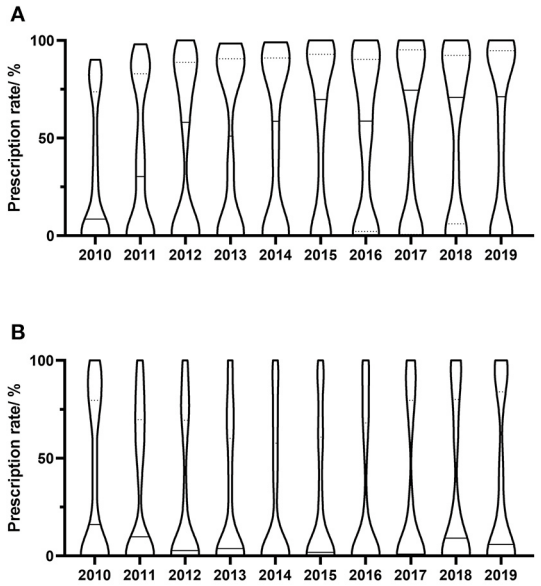

FIGURE 2
Yearly distribution of antivirals prescriptions in individual hospitals. **(A)** Valaciclovir; **(B)** Famciclovir. Prescription rate means the yearly percentage of prescriptions containing specific drug. Each column shows the distribution of prescription rate of prescriptions containing specific drug, and the width of the column represents the relative number of hospitals. The dotted lines in column represent the first quartile and the third quartile of the distribution, and the solid line in column represents the median of the distribution.

their prescriptions increased progressively during the 10 years (both $P < 0.001$), while prescriptions that contained acyclovir decreased over the study period ($P = 0.002$). The percentages of prescriptions that contained valaciclovir and famciclovir in individual hospitals were calculated, and their yearly distributions are shown in Figure 2. Our findings indicate that the treatment patterns differed greatly among hospitals. Some hospitals prescribe valaciclovir to most patients, while others prescribe valacyclovir to a very small percentage of patients.

Ganciclovir was rarely used, and its prescription was stable ($P = 0.530$). There were also two topical formulations, acyclovir and penciclovir, both of which showed increasing trends in prescription numbers over the study period ($P = 0.020$ and $P = 0.002$, respectively).

Trends in cost of specific drugs

From a cost perspective, valaciclovir and famciclovir were the major prescribed antiviral drugs (Figure 1C). However, the cost shares showed no significant trends for either drug ($P = 0.447$ and $P = 0.345$, respectively). The distribution

of valaciclovir and famciclovir DDCs in each year is shown in Figure 3. There were dramatic decreases in the DDCs of valaciclovir and famciclovir (as shown by the trend in yearly median DDC, $P < 0.001$ and $P = 0.001$, respectively). The median DDC of valaciclovir in 2019 was approximately one-tenth of that in 2010, and the median DDC of famciclovir in 2019 was approximately half of that in 2010. The percentage of the other four antiviral drugs was <10% of the total cost and did not change during the study period (Figure 1C, $P = 0.340$).

Discussion

To the best of our knowledge, this is the first study to assess the patterns and trends of antiviral therapy in patients with herpes zoster in China. The yearly prescriptions containing antiviral drugs for patients with herpes zoster have been increasing progressively. The main prescribed antiviral drugs during our study period were valacyclovir and famciclovir. The DDCs of valaciclovir and famciclovir decreased progressively over the study period, and the yearly cost was stable.

The increase in yearly antiviral prescriptions may be due to the increased incidence of herpes zoster. A temporal increase in the incidence of herpes zoster has been reported over the past several decades across several countries (2). A recent study concluded that herpes zoster incidence rates in the US population increased in all age groups from 1991 to 2016 (20). Data on trends in the incidence rate of herpes zoster in China are limited. A recent study reported that the overall incidence of herpes zoster in the population aged over 50 years in China was 6.64 per 1000 person-years (10). Another possible reason for the increased prescription of antivirals could be the increasing demand for better management of herpes zoster in China. It has been reported that only 58% of patients with herpes zoster received antivirals in the UK (21). This may be due to a lack of confidence in antivirals and delayed treatment. The population of outpatients receiving oral antivirals in the US was also near 64%, (22) which is similar to the percentage of Chinese patients receiving antivirals (10). Oral antiviral medications were found to be effective in reducing the duration of acute neuritis symptoms and the risk of complications. Our findings indicate that efforts should be made to increase the percentage of patients receiving antiviral treatment.

Age is widely acknowledged as an independent risk factor for herpes zoster (3, 10). In our study, prescriptions for patients aged <45 years only accounted for a small proportion, and the percentage of patients for this age range continues to decrease. Notably, prescriptions for patients aged over 65 years increased progressively during our study period. Routine vaccination for individuals over 60 years has shown considerable effect in terms of reducing the incidence of herpes zoster, but a recent study showed that the vaccination willingness was only 16.6% in Chinese aged 50-69 years (23, 24). Elderly patients are more likely to benefit from antiviral therapy, however, older adults are also more likely to develop drug adverse events (25). Moreover, the studied antivirals mainly undergo renal excretion and should be closely monitored in patients with reduced renal clearance, which is common in elderly patients (11, 26). The number of prescriptions for females was greater than those for males, which is in accordance with epidemiological findings of female sex being more significantly associated with herpes zoster (3, 10).

As this study focused on outpatients, and only oral and topical antivirals were analyzed. Certain novel antivirals, such as brivudine, are not usually suggested or commonly supplied in Chinese hospitals nor were they included in this study analysis. In this study, the overall use of valaciclovir was greater than that of famciclovir. Moreover, the use of these two agents differed greatly among hospitals. A literature report on antiviral drug use in the district of an eastern city of China during 2015 and 2017 found that acyclovir was the major antiviral drug (10). When looking at the situation in other countries, the use of antivirals also differed greatly. A study in the UK found that major antiviral agent was acyclovir in a period of 2000–2011 (21). However, the major antiviral prescribed to US outpatients was valaciclovir, followed by acyclovir and famciclovir (22). A survey of German primary physicians revealed that famciclovir and valaciclovir were the most commonly prescribed drugs (27). Interestingly, acyclovir was found to be the only antiviral drug prescribed to patients with herpes zoster in a New Zealand study (28).

Oral valaciclovir, famciclovir, and acyclovir are all recommended as they are proven to be superior to placebo in reducing the amount of time to complete cessation of pain (1). Valaciclovir is a prodrug of acyclovir with high bioavailability, and famciclovir is the prodrug of penciclovir. There were no differences in cutaneous and pain endpoints between famciclovir and valaciclovir (1). A recent randomized trial showed that famciclovir was not superior to acyclovir in the efficacy of herpes zoster treatment in adults (29). Furthermore, the three antivirals were found to have similar efficacy and safety profiles (1, 29). Valacyclovir and famciclovir are preferable to acyclovir because of their better pharmacokinetic profiles, easier dosing schedules, and probable higher levels of antiviral drug activity (5, 12). Given the prescription trends, it seems that physicians in China follow the guidelines well.

We found that treatment patterns differed greatly among hospitals. Some hospitals prescribe valaciclovir to most patients, while others prescribe valacyclovir to a very small percentage of patients. A similar situation was found with the use of famciclovir. The choice of valaciclovir or famciclovir may be affected by physician prescription habits, drug supply, and accessibility; however, either is reasonable, especially at the end of this study, when the DDCs of the two agents were found to be similar.

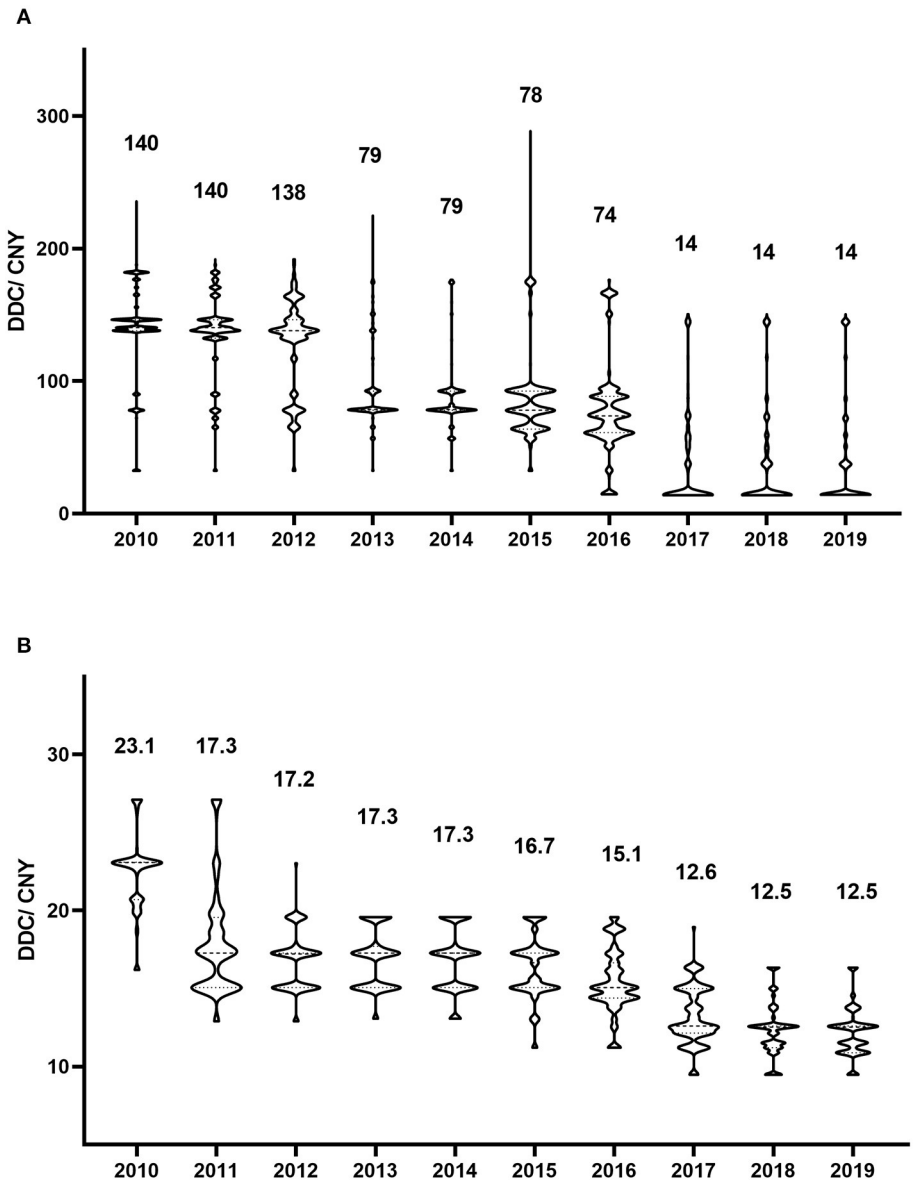

FIGURE 3
Defined daily cost of antivirals in each year. **(A)** Valaciclovir; **(B)** Famciclovir. The numbers represent the median defined daily cost for each year.

There is insufficient evidence to support the use of topical treatment for acute herpes zoster, and topical antivirals are not recommended in various expert opinions and guidelines (1, 11, 12). However, the prescription of the two topical antiviral formulations is increasing.

Cost is another important factor that influences the choice of antiviral therapy by physicians and patients. The health insurance system can also be a concern, and it is interesting to note that the overall cost of antivirals did not increase, despite the progressive increase in prescriptions, during our study period. The share in cost of valaciclovir or famciclovir did not change with their increasing use, which was due to a dramatic decrease in the DDCs of valaciclovir and famciclovir. Valaciclovir was expensive at the beginning of the study, but the median DDC in 2019 was approximately one-tenth of that in 2010. The reason for this was the implementation of the volume-based purchasing program and the national centralized drug purchasing pilot program in China (30–32). Many countries are facing the challenge of ever-increasing pharmaceutical expenditures, and it is common practice worldwide that lowering drug prices and reducing drug expenditures by volume-based drug procurement (31, 33,

34). Our results indicated that these programs reduced drug expenditure, increased the use of policy-related drugs, and may increase drug accessibility.

This study has some limitations. First, the sampling hospitals were located in six major areas of China, which may have resulted in a sampling bias. Furthermore, the severity of herpes zoster, as well as the clinical outcomes of antiviral treatment, have not been well studied. Other commonly co-used drugs for patients with herpes zoster, such as analgesics, should be evaluated in future studies.

Conclusion

We assessed the status and trends of antiviral therapy in outpatients with herpes zoster in China over a decade. During the study period, the prescription of antiviral drugs increased progressively. The main prescribed antivirals were valaciclovir and famciclovir, but the use of these two differed greatly among hospitals. Acyclovir showed a decreasing trend in prescriptions. Topical antivirals are not recommended, however, their use continues to increase. The yearly cost remained stable, due to the decreasing DDCs of valaciclovir and famciclovir. The antiviral treatments adhere well to recent recommendations, except for the use of topical antivirals. The findings of this study may benefit the healthcare source allocation and management of herpes zoster in Chinese outpatients.

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 authors.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conceptualization, project administration, supervision, validation, writing—review and editing: LY and GH. Data curation: ZY, YZ, JJ, and JZ. Formal analysis and investigation: ZY and YZ. Methodology: ZY, LY, and GH. Resources, software, visualization, and roles/writing—original draft: ZY. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.942377/full#supplementary-material

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EDITED BY
Mohammad Asif Sherwani,
University of Alabama at Birmingham, United States

REVIEWED BY
Shashank Gupta,
Division of Intramural Research, National Heart, Lung, and Blood Institute (NIH), United States
Faraz Ahmad,
University of Missouri, United States

*CORRESPONDENCE
Bruno B. Andrade
bruno.andrade@fiocruz.br

†These authors have contributed equally to this work

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Host-directed therapies in pulmonary tuberculosis: Updates on anti-inflammatory drugs

Juan M. Cubillos-Angulo[1,2,3†], Betânia M. F. Nogueira[2,3†], María B. Arriaga[1,2,3], Beatriz Barreto-Duarte[1,3,4,5], Mariana Araújo-Pereira[1,2,3], Catarina D. Fernandes[3], Caian L. Vinhaes[1,3,6], Klauss Villalva-Serra[1,2,3,4], Vanessa M. Nunes[4], João P. Miguez-Pinto[4], Eduardo P. Amaral[7] and Bruno B. Andrade[1,2,3,5,6*]

[1]Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Brazil, [2]Faculdade de Medicina, Universidade Federal da Bahia, Salvador, BA, Brazil, [3]Multinational Organization Network Sponsoring Translational and Epidemiological Research Initiative, Salvador, Brazil, [4]Curso de Medicina, Universidade Salvador, Salvador, Brazil, [5]Programa de Pós-Graduação em Clínica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [6]Bahiana School of Medicine and Public Health, Bahia Foundation for the Development of Sciences, Salvador, Brazil, [7]Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, United States

Tuberculosis (TB) is a lethal disease and remains one of the top ten causes of mortality by an infectious disease worldwide. It can also result in significant morbidity related to persistent inflammation and tissue damage. Pulmonary TB treatment depends on the prolonged use of multiple drugs ranging from 6 months for drug-susceptible TB to 6–20 months in cases of multi-drug resistant disease, with limited patient tolerance resulting from side effects. Treatment success rates remain low and thus represent a barrier to TB control. Adjunct host-directed therapy (HDT) is an emerging strategy in TB treatment that aims to target the host immune response to *Mycobacterium tuberculosis* in addition to antimycobacterial drugs. Combined multi-drug treatment with HDT could potentially result in more effective therapies by shortening treatment duration, improving cure success rates and reducing residual tissue damage. This review explores the rationale and challenges to the development and implementation of HDTs through a succinct report of the medications that have completed or are currently being evaluated in ongoing clinical trials.

KEYWORDS

host-directed therapy, *Mycobacterium*, tuberculosis, adjunct therapy, immunotherapies

Introduction

Tuberculosis (TB) is caused by infection with *Mycobacterium tuberculosis* (*Mtb*), and represents one of the most important infectious diseases worldwide (1). Until the COVID-19 pandemic, TB was the leading cause of death from a single infectious agent (2).

Throughout the past century, TB morbidity and mortality have declined significantly as a result of a number of factors including improved socioeconomic conditions, introduction of intradermal Bacilli Calmette-Guerin vaccine (BCG), particularly in children younger than 5 years old (3, 4) and most importantly with the introduction of antimycobacterial treatment (5). The use of highly effective therapy against HIV, a co-infection primarily responsible for increased TB incidence and death over the past decades, has also positively impacted TB control (4, 6). Notably, widespread access to anti-TB medications resulted in the closure of inpatient hospitals with a shift to outpatient-based treatment (5).

While worldwide efforts to curb TB incidence and mortality have been effective, the COVID-19 pandemic and subsequent limited access to health services has reversed years of progress in providing essential TB services and reducing TB disease burden (1). In addition, new challenges to control TB include continued insufficient treatment success rates, low treatment adherence and the emergence of drug resistant TB infections (7). In 2020, 132,222 individuals were diagnosed with multidrug resistant or rifampicin resistant TB (MDR/RR-TB) along with 25,681 subjects classified as extensively drug resistant TB (XDR-TB) patient, a decrease in 22% compared with 2019 (201,997) that reflects underdiagnosis of this condition (1).

Current TB treatment relies on a combination of multiple antimicrobial drugs with treatment duration ranging from 6 months for drug-susceptible TB to 6–20 months for MDR/RR-TB and even longer in cases of XDR-TB or poor clinical response (8). Globally, the TB treatment success rate is 85% for drug-susceptible TB and 57% for MDR/RR-TB (1). Outcomes are affected by several factors ranging from social determinants to the long duration and complexity of medication regimens, which directly impact patient adherence to the therapeutic protocol as well as drug toxicity (9). While changing social factors to improve treatment success is a complex, lengthy, and gradual process, development of more effective, affordable, and well-tolerated medications may shorten treatment duration and reduce collateral effects thereby improving treatment outcomes.

Two different strategies have been adopted to develop novel therapeutics: (1) traditional search for new antimycobacterial drugs, (2) host directed therapy (HDT) capable of modulating the immune response to TB as adjuvant therapy to current anti-TB treatment. The development of new anti-TB drugs is a lengthy and costly process (10), thus the study of HDT may offer an effective alternative that is readily available.

Host-directed therapy (HDT) has emerged as an attractive adjuvant treatment option using repurposed approved immune modulation therapy. It has become apparent that the determinants of TB immunopathogenesis and the mechanisms underlying successful infection control involve the following domains: inflammation (**Figures 1A–C**) (11), cellular metabolism (**Figure 1D**) (12), and the mechanisms used by *Mtb* to evade the immune system (13; **Figure 1E**). HDTs aim to modulate host factors to enhance favorable responses and dampen host detrimental responses, which contribute to tissue damage and perpetuation of mycobacterial multiplication (14). If proven to be beneficial, HDT may aid in resolving unmet needs in TB treatment, thereby resulting in improved adherence, reduction of resistant strains, shortened treatment duration and *Mtb* transmission in the community with increased cure rates and fewer chronic sequelae caused by excessive inflammatory response to TB (15).

Currently, there are many potential therapies with ongoing research at different stages in the pipeline for HDT in TB. Most are repurposed drugs in pre-clinical or clinical studies to be used as adjuvants with anti-TB therapy. This review aims to describe the rationale used in the development of HDTs, their potential and main challenges as adjuvant therapy, as well as to provide a succinct report of the medications that have completed or are evaluated in ongoing clinical trials (CT), registered in the ClinicalTrials.gov database.

Tuberculosis and the immune system

After infection with *Mtb*, the development of active disease results from both pathogen and host factors (16). Immune response against *Mtb* is a very complex and dynamic process, involving different cell types, cytokines and chemokines (17). Multiple inflammatory cells such as macrophages, monocytes, dendritic cells, neutrophils, epithelioid cells, and multinucleated giant cells, enclosed by B and T lymphocytes, accumulate at the tissue level to form a granuloma (18). Myeloid cells produce many cytokines and chemokines that are critical to recruit additional leukocytes from capillary vessels (19, 20). The interaction between differentially localized populations of intracellular *Mtb* and the cellular organelles will dictate whether *Mtb* replicates or restrict its growth through control of intracellular bacilli (21). The majority of *Mtb* exposed individuals contain primary infection with the formation of granulomas. Nevertheless, it is possible that a small proportion of bacilli survive, driving infection into a latent stage. The other potential outcome is increased hypoxic necrotic centers, rich in lipids and foamy macrophages that fail to control bacterial replication, ultimately leading to granuloma caseation (20). This process is responsible for the latter formation of cavities and destruction of alveolar cells, vessels, and bronchi, with consequent bacilli spread (19). The fate of granulomas is

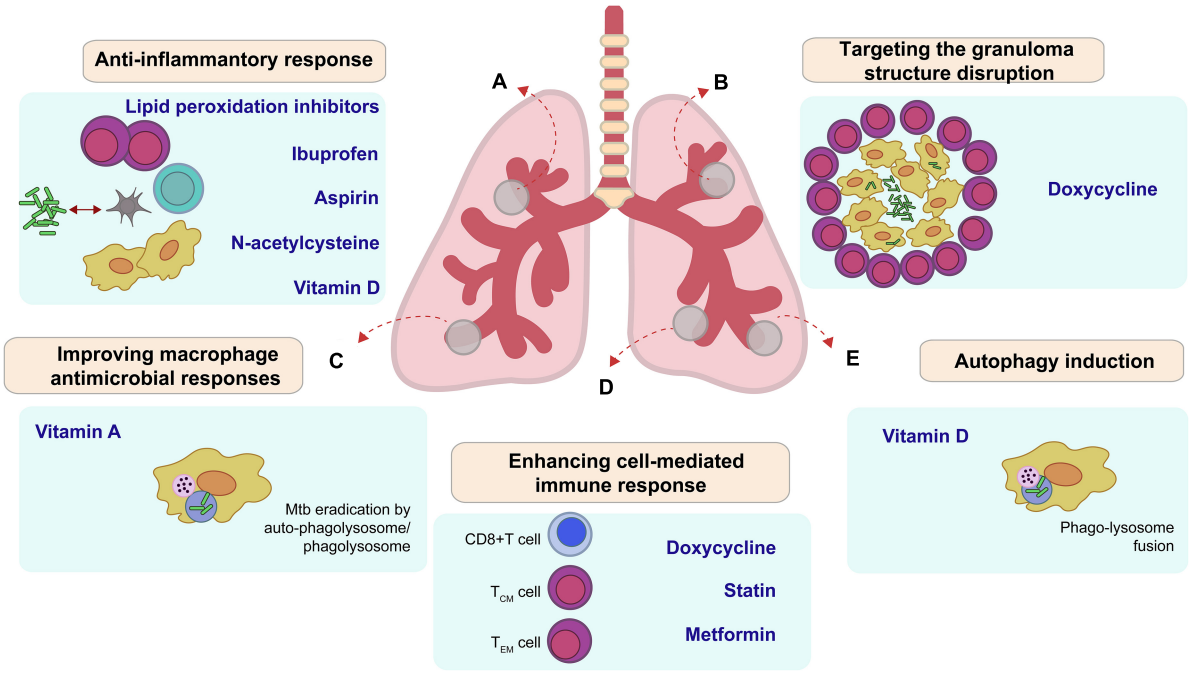

FIGURE 1
Main potential host therapeutic targets (HDT) improve outcome in *Mycobacterium tuberculosis*. **(A)** Lipid peroxidation inhibitors, Ibuprofen, Aspirin, N-acetylcysteine, and Vitamin D suppress proinflammatory responses, which decrease inflammation and tissue damage during active stage of the disease. **(B)** Doxycycline changes the integrity of granuloma and enhances drug accessibility. **(C)** Vitamin A reduces bacilli growth by apoptosis or auto-phagolysosome. **(D)** Doxycycline, Statin, and Metformin regulate cell-mediated immune responses, including antigen-specific T cell responses. **(E)** Some HDT like vitamin D induce autophagy in infected cells.

determined by a variety of host factors that involve a network of inflammatory cytokines, eicosanoids, prostaglandins, and other mediators contributing to disease exacerbation as well as tissue necrosis (19, 20). Therefore, modulating host immune response could potentially optimize TB treatment, although the ideal target for HDTs remains unclear (22).

Currently, multiple therapies that act on different host immune targets are under investigation including the following: modulation of vascular endothelial growth factor (VEGF) potentially reduces central necrosis and improves drug delivery (23); reduction of neutrophil-mediated inflammation (i.e., aspirin) to limit severe tissue damage (24); and modulation of tumor necrosis factor alpha (TNFα), transforming growth factor beta (TGF-β), and Interleukin-1 beta (IL-1β) may reduce lung damage (25).

Potential advantages of host-directed therapy in tuberculosis

Some medications studied as HDT in TB are used for other conditions and offer a wealth of clinical experience and research, such as acetylsalicylic acid or statins, to bypass the need to explore the safety and toxicity properties in prolonged use. Furthermore, most of the studied drugs are already available worldwide with accessible costs, facilitating, and accelerating their incorporation into routine practice if benefit in TB treatment is proven in robust CTs (26). The use of HDTs may avoid the undesirable adverse effects with prolonged use of repurposed antimicrobials such as oxazolidonones, carbapenems, and fluorquinolones in TB treatment (27). Among other adverse effects, long-term therapies employing broad-spectrum antibiotics will contribute to the emergence of antibiotic-resistant strains of *Mtb* as well as other opportunistic pathogens (28). Lastly, anti-inflammatory effects offered by some HDT agents may lead to potential benefits in the host by reducing tissue damage and improving long-term quality of life (14).

The challenge of finding effective host-directed therapy

In vitro studies play an essential role in the screening of potential drugs (29), while animal studies allow the understanding of immunopathology, as well the confirmation of mycobacterial infection control, as measured by the drug impact in mycobacterial load, time to sterilization of lesions, tissue damage size and overall survival (30). With the knowledge obtained through both experimental models, clinical studies

to validate these findings will be critical to address questions beyond drug efficacy (31; **Figure 2**).

Safety and toxicity

Though many drugs re-purposed as HDTs have known safety profiles, they must be evaluated in the context of individuals with TB, given that factors such as malnutrition, co-infection with HIV and inflammation tend to alter drug metabolism (32, 33).

Drug-drug interactions

The concomitant use of HDT with anti-TB therapy needs to be evaluated. For instance, mild risk of hepatotoxicity of some drugs could be potentiated when used along with anti-mycobacterial drugs. Additionally, it is important to evaluate drug-drug interactions with antiretroviral therapy, as co-infection with TB-HIV is common and of particular concern (26).

Effectiveness in different populations

People living with HIV (PWH) and those with other types of immunosuppression, children, and individuals with resistant TB may have different immune responses to TB and thus could respond differently to HDTs (34). Similarly, ethnic differences in TB immune response may also be reflected in different responses to HDTs (35). It is critical that clinical studies include ethnically diverse and clinically relevant populations.

Effectiveness in different clinical forms of tuberculosis

While most studies focus on pulmonary TB, extra-pulmonary TB has a high prevalence in some areas, particularly in countries with a high burden of TB (36) with worse outcomes when compared with pulmonary TB (37–39). For instance, having tuberculous meningitis or disseminated TB is associated with lower cure rates and higher mortality rates (37–39).

Determine the most effective protocol for host-directed therapy

There may be an ideal time for use during disease course depending on how the host immune response is modulated, and administration in the wrong time frame may be deleterious (31, 40).

Evaluation of the possibility of reducing total treatment time

Earlier sterilization and control of inflammation may result in shorter antimycobacterial treatment durations leading

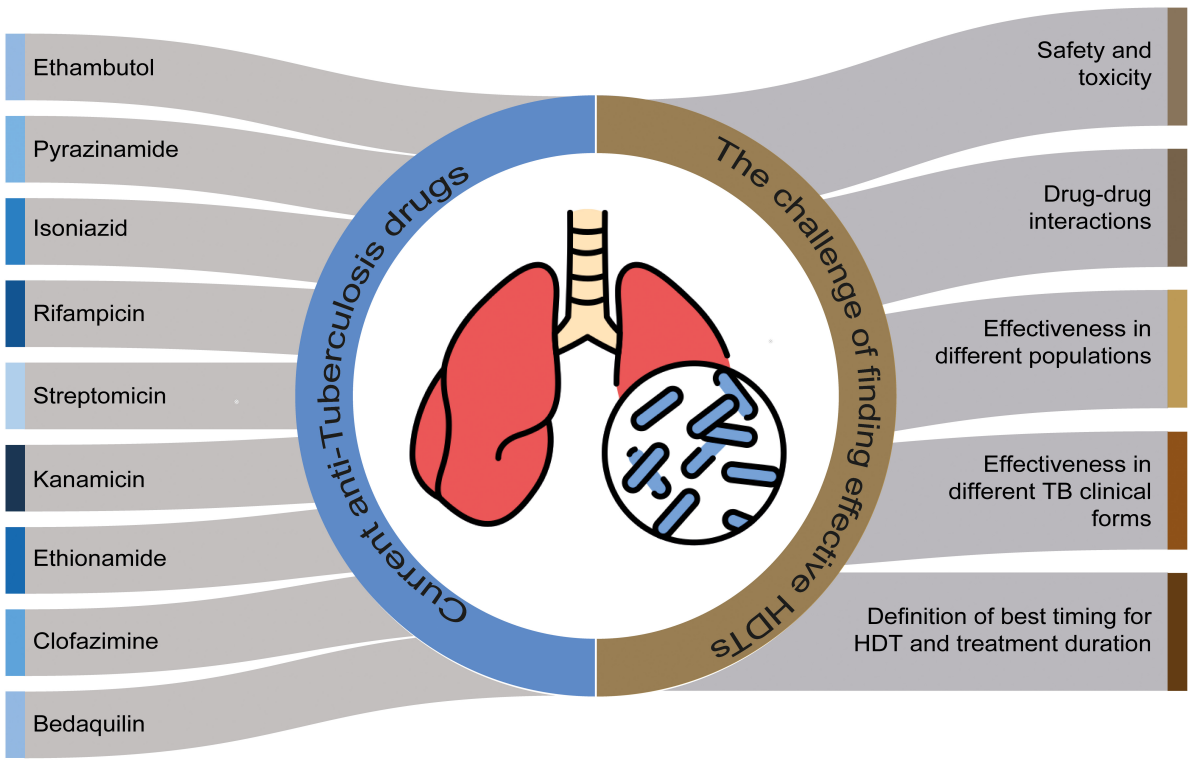

FIGURE 2
The challenge of finding effective host-directed therapy (HDTs) and the current anti-tuberculosis therapy. Perspectives for clinical studies for HDT efficacy.

to improved patient adherence and increased likelihood of cure (41).

Drugs targeting the anti-inflammatory response

Aspirin

Aspirin is a drug based on acetylsalicylic acid that performs antiplatelet (42), anti-inflammatory (43), and analgesic (44) functions and is a potential adjuvant in the treatment of TB (45, 46).

The anti-inflammatory role of aspirin has been increasingly studied for modulating neutrophil-mediated inflammatory responses. The effect of low-dose aspirin seems to enhance control of bacillary load and improve survival in the late stages of TB in C3HeB/FeJ murine model (45). A study in C3HeB/FeJ mice found that low-dose aspirin had an anti-inflammatory effect in the later stage of active TB by reducing excess, non-productive inflammation, while enhancing Th1-cell responses for the elimination of bacilli (47). In BALB/c mice, aspirin administration enhanced the effect of pyrazinamide and resulted in additional clearance of viable mycobacteria in the lungs and spleen during the initial phase of TB treatment (48). However, the combination of aspirin and Isoniazid-like treatment of murine pulmonary TB was associated with increased mycobacterial load in the spleen and lungs (48). Together, these findings highlight the urgent need for additional clinical studies to assess the impact of timing in disease course and the efficacy of concomitant aspirin use with different anti-TB drug combinations.

In addition to the anti-inflammatory effects, the antiplatelet role of aspirin may also be beneficial in TB treatment given that TB promotes a basal state of hypercoagulability that favors thromboembolic events (49) and platelets have been directly associated with pro-inflammatory status (50). A cohort study of pulmonary TB patients from Taiwan found that low doses of aspirin were associated with decreased morbidity and increased survival of patients on anti-TB regimen, without increasing the risk of bleeding (46). Similarly, a phase two CT in HIV-unexposed adults with tuberculous meningitis found that 1,000 mg of aspirin reduced 3-month mortality rates for this group of patients (51). A hypercoagulation state is present in tuberculous meningitis, leading to vascular complications (49). Using aspirin in this scenario has previously been shown to reduce the incidence of strokes and mortality at 3 months (52).

The results of these preliminary studies are underpowered and require additional robust CTs to prompt a change in current treatment guidelines. Nevertheless, they suggest that aspirin may aid TB treatment in all cases or in subgroups, in those with pulmonary TB or tuberculous meningitis. Importantly, the use of aspirin would be easily implemented given the low cost, high availability and limited side effect profile.

The ClinicalTrials.gov database lists one ongoing multi-center, phase IIB, placebo controlled, randomized CT that aims to evaluate the efficacy and safety of aspirin and ibuprofen as adjunct drugs in TB treatment, as detailed in **Table 1**.

Ibuprofen

Ibuprofen is a non-steroidal anti-inflammatory drug that inhibits both COX1 and COX2 cyclooxygenases. It is widely used and has an excellent safety profile, even in children (76). In a mouse model mimicking active TB in humans, the use of ibuprofen reduced bacillary load and affected lung area, leading to increased survival (77). These effects are mainly attributed to inhibition of the synthesis of PGE2, which inhibits phagocytosis, bacterial killing, production of nitrite (76) and T-helper 1 cytokines, and production of tumor necrosis factor α (TNF-α) (78). Another study in a murine model noted that ibuprofen enhanced the bactericidal effect of pyrazinamide during TB treatment (48). As noted above, this approach is currently under investigation in a phase II multi-center placebo-controlled trial (**Table 1**).

Antioxidants: N-acetylcysteine and lipid peroxidation inhibitors

N-acetylcysteine (NAC) is a potent antioxidant widely used as a mucolytic agent in chronic obstructive pulmonary disease (COPD) and cystic fibrosis (79). The role of NAC in TB therapy remains under study. To date, prior studies have indicated that NAC might reduce host oxidative responses (80), reduce pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α (81) and have direct antimycobacterial properties (81, 82).

A study conducted in Brazil compared oxidative stress status in plasma of patients with pulmonary TB, latent TB infection, and healthy uninfected individuals (82). Pulmonary TB patients exhibited higher levels of oxidation products and a reduction of antioxidants compared with latent TB cases or uninfected controls. Cultures were exposed to different doses of NAC and the authors found decreased oxidative stress in treated macrophages and reduced mycobacterial growth when exposed to a high concentration of NAC (82). The capacity of NAC to control *Mtb* infection was further tested *in vivo* in a mouse (C57BL/6) model, resulting in a significant reduction of mycobacterial loads in the lungs (82).

In a phase II randomized CT that evaluated the adjuvant use of NAC in hospitalized individuals with TB-HIV (RIPENACTB study) (80), NAC-treated patients exhibited a significant increase in glutathione levels and total antioxidant status along with lowered levels of lipid peroxidation, a toxic process of oxidative stress response. In this study, the adjuvant use of NAC was not unsafe (80). Similarly, another randomized CT in those with newly diagnosed pulmonary TB in India reported a significant increase in glutathione peroxidase levels in patients receiving NAC and conventional anti-TB therapy compared to placebo group (patients under TB therapy only). Patients

TABLE 1 Clinical trials investigating drugs for host directed therapy in pulmonary tuberculosis (TB).

Adjunctive HDT	Principal investigator and year (last update posted)	Study setting (s)	Trial registration	Type, dose and route of treatment (intervention)	References (PMID or clinical trials website)	Status	Next step drug
Aspirin	(53)	South Africa	NCT04575519	300 mg of Aspirin	https://clinicaltrials.gov/ct2/show/NCT04575519?term=aspirin&cond=Tuberculosis&draw=2&rank=4	Recruiting	Evaluate safety and efficacy of the adjunctive use with TB therapy
Ibuprofen	(53)	Georgia and South Africa	NCT04575519	400°mg Ibuprofen (twice daily during)	https://www.clinicaltrials.gov/ct2/show/NCT04575519?term=ibuprofen&cond=Pulmonary+Tuberculoses&draw=2&rank=1	Recruiting	Determine the impact of ibuprofen on long-term antituberculosis drugs and know the side effects in humans.
N Acetyl Cysteine	(54)	Tanzania	NCT03702738	N-acetylcysteine 1,200 mg	https://clinicaltrials.gov/ct2/show/NCT03702738?term=N+Acetyl+Cysteine&cond=Tuberculosis&draw=2&rank=1	Recruiting	Evaluate the synergize with current therapies in TB and multi-drug-resistant (MDR)-TB treatment
	(55)	Brazil	NCT03281226	N-acetylcysteine 1,200 mg (600°mg twice daily)	https://clinicaltrials.gov/ct2/show/NCT03281226?term=N+Acetyl+Cysteine&cond=Tuberculosis&draw=2&rank=3	Unknown	
Vitamin D	(56)	Indonesia	NCT05073965	1000IU Vitamin D		Completed	Need to standardize the doses and optimize the schedule of administration.
	(57)	Pakistan	NCT01130311	Vitamin D (cholecalciferol) 600,000 IU intramuscular		Completed	
	(58)	Indonesia	NCT00677339	Vitamin D3, "Calciferol Strong®" 50,000 IU (1,250 mcg, 1 tablet)		Completed	
	(59)	Pakistan	NCT02169570	600,000 IU of (I/M) Vitamin D	https://clinicaltrials.gov/ct2/show/NCT02169570?term=Vitamin+D&cond=Tuberculosis&draw=2&rank=13	Unknown	
	(60)	Mexico	NCT02464683	Vitamin D 200 IU (oral dose)	https://clinicaltrials.gov/ct2/show/NCT02464683?term=Vitamin+D&cond=Tuberculosis&draw=2&rank=2	Unknown	
	(61)	India	NCT00366470	3.3°ml (100,000 IU) dose of Vitamin D		Completed	

(Continued)

TABLE 1 (Continued)

Adjunctive HDT	Principal investigator and year (last update posted)	Study setting (s)	Trial registration	Type, dose and route of treatment (intervention)	References (PMID or clinical trials website)	Status	Next step drug
	(62)	Bangladesh,	NCT01580007	500 mg orally (5,000 IU) Vitamin D		Completed	
	(63)	Ethiopia	NCT01698476	5,000 IU of Vitamin D (cholecalciferol tablets)		Completed	
	(64)	India	NCT00507000	Vitamin D 60,000 IU	https://clinicaltrials.gov/ct2/show/NCT00507000?term=Vitamin+D&cond=Tuberculosis&draw=2&rank=5	Unknown	
	(65)	South Africa	NCT02968927	Vitamin D		Unknown	
	(66)	Tanzania	NCT00311298	Vitamin D 5 μg/200 IU		Completed	
	(67)	United Kingdom	NCT03011580	9,600 IU/day Oral Vitamin D	https://clinicaltrials.gov/ct2/show/NCT03011580?term=Vitamin+D&cond=Tuberculosis&draw=2&rank=17	Completed	
Doxycycline	(68)	Singapore	NCT02774993	Doxycycline 100 mg	https://clinicaltrials.gov/ct2/show/NCT02774993	Completed	Results from phase II may provide insights regarding safety and efficacy. New CTs to be performed, including greater sample size and different TB clinical forms besides pulmonary TB.
Vitamin A	(69)	Malawi	NCT00057434	Vitamins A 8,000 IU		Completed	Larger CTs looking at effects of Vitamin A in clinical outcomes (death/cure/relapse)
	(66)	Tanzania	NCT00311298	Vitamin A 5,000 IU		Completed	
	(70)	India	NCT00801606	Vitamin A 250 mg		Completed	
Statin	(31)	South Africa	NCT03882177	Pravastatin 40 mg, 80 mg, 100 mg and 160 mg	https://clinicaltrials.gov/ct2/show/NCT03882177?term=Statin&cond=Tuberculosis%2C+Pulmonary&draw=2&rank=2	Recruiting	Dose finding studies. Phase II CTs are ongoing. If promising results, Phase III trials.
	(71)	South Africa	NCT04147286	Atorvastatin 40°mg	https://clinicaltrials.gov/ct2/show/NCT04147286?term=Statin&cond=Tuberculosis%2C+Pulmonary&draw=2&rank=4	Recruiting	

(Continued)

TABLE 1 (Continued)

Adjunctive HDT	Principal investigator and year (last update posted)	Study setting (s)	Trial registration	Type, dose and route of treatment (intervention)	References (PMID or clinical trials website)	Status	Next step drug
	(72)	United Kingdom	NCT04721795	Atorvastatin oral 30/40° mg	https://clinicaltrials.gov/ct2/show/NCT04721795?term=Statin&cond=Tuberculosis%2C+Pulmonary&draw=2&rank=1	Recruiting	
	(73)	Philippines, Singapore, Uganda, Vietnam	NCT04504851	Rosuvastatin 10° mg	https://clinicaltrials.gov/ct2/show/NCT04504851?term=Statin&cond=Tuberculosis%2C+Pulmonary&draw=2&rank=3	Not yet recruiting	
Metformin	(74)	Thailand	NCT05215990	Metformin 500 Mg Oral	https://clinicaltrials.gov/ct2/show/NCT05215990?term=Metformin&cond=Tuberculosis%2C+Pulmonary&draw=2&rank=1	Recruiting	Phase II and dose finding studies
	(75)	South Africa	NCT04930744	Metformin hydrochloride 500 mg	https://clinicaltrials.gov/ct2/show/NCT04930744?term=Metformin&cond=Tuberculosis%2C+Pulmonary&draw=2&rank=2	Recruiting	

HDT, host-directed therapy; IU, international unit; NCT, the National Clinical Trial.

receiving NAC-based adjunctive therapy exhibited significant reduction of radiological lung infiltration, faster sputum conversion and more regulated immunological response, when compared to the group without NAC. A substantial body weight gain and improved antioxidant status was noted in the intervention group suggesting a potential promising role for NAC as adjuvant anti-TB therapy (83). Moreover, NAC may play a role in preventing hepatotoxicity of anti-TB usual therapy. An Iranian randomized CT (84) evaluated the effect of adjuvant NAC in those undergoing four drug anti-TB therapy compared with those without NAC therapy. Liver enzyme levels including aspartate aminotransferase, alanine aminotransferase and bilirubin were significantly lower following 1 and 2 weeks of NAC treatment. In this study NAC co-administration appeared to reduce the risk of hepatotoxicity commonly associated with anti-TB therapy.

N-acetylcysteine is a low-cost drug that seems to be safe for use in pulmonary TB treatment. Nevertheless, its effectiveness is still to be proven. One CT is currently in progress to clarify this question (**Table 1**).

Autophagy induction

Vitamin D

Vitamin D (VITD) participates in the reabsorption of calcium from the bone and intestine and has a fundamental role in bone constitution and remodeling (85). It also acts as an immunomodulatory hormone and influences other processes including central nervous system function and cardiovascular health (86, 87).

In the presence of *Mtb*, VITD plays an essential role in activated macrophages and monocytes in response to antigen exposure by enhancing levels of 1,25(OH) 2D in monocyte/macrophages from normal human hosts (88). The increased levels of 1,25(OH) 2D induces the expression of cathelicidin, an antimicrobial protein responsible for killing infectious agents like *Mtb* (89). Some studies have reported that VITD can down-regulate the expression of mTOR protein, thus inducing autophagy (90). Importantly, VITD deficiency has been associated with susceptibility to TB infection in a comparative cross-sectional study that identified a high prevalence of VITD deficiency among newly diagnosed TB patients and in their household contacts (91). To date, 10 completed studies and four ongoing related to VITD and pulmonary TB were identified in the ClinicalTrials.gov records (**Table 1**).

The use of VITD as adjunctive therapy has had conflicting results regarding improvement of sputum conversion. Faster sputum conversion rates were found in TB patients receiving adjunctive VITD supplementation in a randomized placebo-controlled CT in Indonesia (92) and China (93). Another CT from Bangladesh demonstrated enhanced intracellular killing

of *Mtb* in macrophages *ex vivo* in combination with increased sputum culture conversion at week 4 and at week 8 compared to the placebo group (94). This effect, however, was unable to be replicated in a number of CTs of VITD supplementation in TB disease (93, 95–97). Two CTs with MDR/RR-TB patients in Georgia (95) and Mongolia (96) identified higher sputum culture conversion with high dose of VITD use, suggesting a possible role in this subset of patients with MDR/RR TB. Another randomized controlled trial found that daily VITD administration in TB infected patients led to enhanced clinical recovery, particularly those with lower levels of VITD and an elevated TB score at enrollment (97).

Although lower levels of VITD are commonly observed in individuals with pulmonary TB, clinical and bacteriological results from randomized controlled trials of adjunctive VITD supplementation have demonstrated limited clinical benefits. A meta-analysis found that there is no evidence to support the adjuvant use of VITD. Most studies are limited by small sample sizes and include only HIV-uninfected adults with pulmonary TB (98). Additional larger studies are needed to investigate the effects of VITD and other micronutrients in specific TB treatment subgroups that have worse prognosis, immunosupression, MDR/RR-TB and individuals with diabetes.

Targeting the granuloma structure disruption

Doxycycline

Matrix metalloproteinases (MMPs) are proteolytic enzymes capable of degrading collagen and other structural proteins (99). When highly expressed, as in inflammatory conditions, they contribute to tissue damage (100). TB leads to upregulation of MMPs and an imbalance between MMPs and tissue inhibitors of metalloproteinases (TIMPs) (101, 102). This imbalance is associated with TB severity and extent of TB lesions, as well as formation of lung cavitation (103), which in turn is associated with high bacillary burden, delayed sputum culture conversion, emergence of drug resistance, and higher transmission of *Mtb* (104). MMPs also play a role in the formation of granulomas (105) and are responsible for the breakdown of the blood brain barrier, leading to poor outcomes in cases of central nervous system TB (106–108). In this context, inhibitors of MMPs may be a particularly effective HDT for TB. Doxycycline is the only FDA−approved MMP inhibitor (102). It is originally used as a bacteriostatic antibiotic of the tetracycline class. It is also an inhibitor of MMP1 and MMP9 (102) which may be responsible for reducing pulmonary cavity volume and loss of granuloma size (68). Doxycycline has been shown to inhibit mycobacterial growth in animal and *in vitro* models (109, 110). A pilot phase 2 CT (Doxy-TB) (68), comparing doxycycline plus standard TB therapy to placebo plus standard TB therapy has been concluded with results pending (**Table 1**). Doxycycline is a licensed, safe and affordable drug, with significant potential to improve TB outcomes (111). Future CTs are needed with larger sample sizes and different TB clinical forms besides pulmonary TB, such as central nervous system TB.

Improving macrophage antimicrobial responses

Vitamin A

Vitamin A deficiency is a serious and widespread public health problem (112). It is more common during infection and can increase the severity of infectious diseases and the risk of death (112).

A recently systematic review/meta-analysis found that supplementation with vitamin A associated with earlier sputum conversion, decreased abnormalities in chest radiography, and improved lung function in patients undergoing TB treatment (113). A randomized controlled trial with a 2 × 2 factorial design in Qingdao, China determined that adjunctive supplementation with vitamin A did not improve time to smear conversion in pulmonary TB patients (93). Conversely, a double-blind, placebo-controlled study in patients with newly diagnosed TB found that vitamin A supplementation was associated with earlier sputum smear conversion (114). Furthermore, a randomized placebo-controlled, double-blind, two-by-two factorial trial evaluated the use of multivitamin/mineral supplementation with vitamin A and found a significant decrease in mortality during treatment of sputum-positive TB patients co-infected with HIV (66).

Three CTs registered with ClinicalTrials.gov with the status of completed were identified for Vitamin A supplementation in TB treatment (**Table 1**). The CT NCT00057434 conducted in Malawi did not identify a survival benefit with micronutrient supplementation with vitamin A in adults with HIV and pulmonary TB (69). Another CT (NCT00311298) found that multi-vitamin/mineral supplementation (Vitamin A) and zinc decrease mortality during treatment in patients with HIV and pulmonary TB (66). The remaining CT (NCT00801606) observed that micronutrient supplementation (Vitamin A) during treatment is associated with weight gain in children with TB though did not impact the clearance of lesions by chest X-ray (115).

These studies highlight conflicting results, though significantly lack evaluation of impact on clinical relevant outcomes (cure/death/recurrence). Larger trials with clinical important outcomes are needed to better evaluate the adjuvant use of vitamin A in TB treatment.

Enhancing cell-mediated immune response

Statins

Mycobacterium tuberculosis has been shown to thrive in cholesterol rich environments, as cholesterol can improve both

survival and growth of the pathogen in a host cell. *Mtb* uses cholesterol in the macrophage membrane to bind and enter the cell (116). Subsequently, after the infection, macrophage accumulates lipid bodies forming foamy cells that utilize cholesterol as the main source of nutrition for bacteria. The lipid bodies have also been associated with *Mtb* growth restriction, drug resistance and delayed phagosome maturation due to enhanced IL-10 induction (117).

Statins are the most used cholesterol reducing drugs. They are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme reductase (HMG-CoA), with both cholesterol lowering and anti-inflammatory and immunomodulatory functions and offer the most promising candidates for adjuvant HDT against TB (15, 118). The mechanism of action of statins against TB continue to be studied though research to date has identified the following: restrict the generation of foamy cells that otherwise support *Mtb* persistence by decreasing cholesterol biosynthesis (119); promote phagosome maturation and autophagy (120); increase percentages of Natural killer T (NKT) cells within cultures and expression of co-stimulatory molecules on monocytes, with higher secretion of IL-1b and IL-12p70 (121, 122); inhibit TGF-β (123, 124). Animal studies have found that simvastatin therapy in *Mtb*-infected mice reduces dissemination of *Mtb* from the lungs (120), promotes killing of intracellular *Mtb* by macrophages and enhances the bactericidal activity of isoniazid (117) and rifampin (125). A study in mice evaluated the treatment-shortening potential of therapy with statins plus anti-TB drugs and found a reduced time required to eradicate TB infection with no increased risk of relapse (126).

Some retrospective studies in Taiwan and South Korea have evaluated the role of statin use in preventing TB finding that chronic statin users had a lower risk of developing TB, compared to non-statin users (118, 127). Though intriguing, these study findings unfortunately have not been reproduced in different populations distinct from those with COPD and diabetes and lack adjustment for important confounding factors (128–132). A meta-analysis of data from nine of these cohort studies concluded that statin use was associated with reduced incidence of active TB (133). To our knowledge no retrospective studies have evaluated the impact of statins during TB treatment on clinical outcomes.

Four ongoing CTs of statins in TB may offer further clarity (**Table 1**): StAT-TB, a phase 2B dose finding study using pravastatin (NCT03882177); ATORTUB, using atorvastatin (NCT04721795); ROSETTA, using rosuvastatin (NCT04504851), and StatinTB (NCT04147286), a proof-of-concept phase II study testing the use of atorvastatin in reducing lung inflammation after TB treatment.

Given the pre-clinical studies results and the number of ongoing CTs, statins may represent one of the most promising drugs in the pipeline for HDT. If proven beneficial in clinical studies, its use in clinical practice can be easily implemented, considering its low cost and wide availability though likely will need additional safety testing given the possibility of hepatoxicity.

Metformin

Metformin is the first-line therapy in those with type 2 diabetes (134) and is a safe and widely used medication. Results from multiple studies suggest that concurrent use of metformin for TB may be beneficial even in non-diabetic individuals (135).

How metformin acts against TB remains unclear. Pre-clinical studies have found that it facilitates phagosome-lysosome fusion and promotes expression of 5′ adenosine monophosphate-activated protein kinase (AMPK) (14), an enzyme usually activated during metabolic stress that controls energy homeostasis (136). As such, metformin may promote increased production of reactive oxygen species (ROS) and subsequent killing of intracellular *Mtb* (14).

A study performed by Singhal et al. in TB infected mice revealed that metformin reduced intracellular growth of *Mtb*, enhanced the efficacy of anti-TB drugs, and reduced lung damage and inflammation (137). This study served as a proof-of-concept demonstrating that metformin may be an option as adjunctive therapy of TB. Another study evaluating the sterilizing role of metformin found no differences in *Mtb* burden in the metformin adjuvant group versus TB treatment alone (138). Some possible reasons for the divergent results are the different model mouses and the different TB drugs used in both studies. Singhal et al. used a single anti-TB drug (isoniazid or ethambutol), whereas Duta NK et al. employed four drugs, which may have masked a possible role of metformin in sterilization. The inclusion of rifampin may also have altered the pharmacokinetics of metformin (138). Alternatively, it may be that metformin acts in an immunomodulatory role that is more clinically relevant than sterilization. Another study with mice found that metformin enhanced the effectiveness of *Mtb*-specific CD8 + T cell responses in local and systemic sites during infection, with increased decreased mortality and anti-mycobacterial properties and decreased inflammatory cytokine production such as TNF (139). Furthermore, Bohme J. et al. found that metformin enhances the immunogenicity and protective efficacy of BCG in mice (140).

Prior retrospective studies in patients with type 2 diabetes and TB suggest that metformin use was beneficial during TB treatment with reduced risk of cavitary TB (137), more rapid sputum conversion (141), lower mortality despite significantly higher glycated hemoglobin values (137, 142) and lower risk of recurrence (143) when compared to the use of other diabetes treatment regimens during TB treatment.

Considering the evidence above, the drug safety profile, wide availability, and low cost, there remains an important role for CTs to evaluate the effectiveness of metformin as TB adjunctive therapy. Currently, ClinicalTrials.gov reports two CTs investigating Metformin use in individuals with pulmonary TB that are currently recruiting participants in Thailand and South Africa (**Table 1**).

Conclusion

Novel TB interventions beyond antimicrobials are urgently needed to improve treatment effectiveness with shorter duration and without increased adverse effects. HDTs offer a promising strategy through repurposing of pre-existing drugs, that often are widely available with low cost, and therefore easily implemented if efficacy and safety are proven in robust CTs. Currently, HDT drugs are in different stages of research with primarily pre-clinical studies with conflicting conclusions. New CTs, ideally multicenter, are underway to answer questions regarding HDT drugs in TB treatment, including all populations of clinical relevance. The existent TB networks or consortia that include standardized cohorts of patients with confirmed TB represent an opportunity for harmonized CTs with heterogenous populations and homogeneous protocols. We suggest the use of these networks or consortia to expedite quality research in this field.

Author contributions

JC-A, BN, and BB-D contributed to conception and design of the study. JC-A, BN, CF, CV, KV-S, VN, JM-P, MA-P, MA, and EA also collected the information and organized the different subsections. JC-A, BN, and BA wrote the first draft of the manuscript. BA supervised the project execution. All authors contributed to manuscript revision, read, and approved the submitted version.

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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

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EDITED BY
Lourdes Planelles,
Khalifa University, United Arab Emirates

REVIEWED BY
Manuela Zonca,
PsiOxus Therapeutics, United Kingdom
Gorjana Rackov,
BioMed X GmbH, Germany

*CORRESPONDENCE
Gabriel M. Gutierrez,
gabemgutierrez@gmail.com

†These authors have contributed equally to this work

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Viral delivery of a peptide-based immunomodulator enhances T cell priming during vaccination

Timothy W. Phares[1†], Jing Huang[2,3†], Vinayaka Kotraiah[4], Mary J. Hauser[5], Arban Domi[5], Sreenivasa Oruganti[5], Cecille D. Browne[6], Peter Buontempo[7], Marc Mansour[8], James Pannucci[7], Moriya Tsuji[2,3] and Gabriel M. Gutierrez[9]*

[1]Thunder Biotech, Provo, UT, United States, [2]The Aaron Diamond AIDS Research Center, New York, NY, United States, [3]Department of Medicine, Columbia University Irving Medical Center, New York, NY, United States, [4]Leidos Life Sciences, Leidos Inc., Frederick, MD, United States, [5]GeoVax Inc., Atlanta, GA, United States, [6]Thermo Fisher Scientific, San Diego, CA, United States, [7]The MITRE Corporation, McLean, VA, United States, [8]MM Scientific Consultants Inc., Halifax, NS, Canada, [9]Hibiscus Biotechnology, LLC, Rockville, MD, United States

Modern, subunit-based vaccines have so far failed to induce significant T cell responses, contributing to ineffective vaccination against many pathogens. Importantly, while today's adjuvants are designed to trigger innate and non-specific immune responses, they fail to directly stimulate the adaptive immune compartment. Programmed cell death 1 (PD-1) partly regulates naïve-to-antigen-specific effector T cell transition and differentiation by suppressing the magnitude of activation. Indeed, we previously reported on a microbial-derived, peptide-based PD-1 checkpoint inhibitor, LD01, which showed potent T cell-stimulating activity when combined with a vaccine. Here we sought to improve the potency of LD01 by designing and testing new LD01 derivatives. Accordingly, we found that a modified version of an 18-amino acid metabolite of LD01, LD10da, improved T cell activation capability in a malaria vaccine model. Specifically, LD10da demonstrates improved antigen-specific $CD8^+$ T cell expansion when combined prophylactically with an adenovirus-based malaria vaccine. A single dose of LD10da at the time of vaccination is sufficient to increase antigen-specific $CD8^+$ T cell expansion in wild-type mice. Further, we show that LD10 can be encoded and delivered by a Modified Vaccinia Ankara viral vector and can enhance antigen-specific $CD8^+$ T cell expansion comparable to that of synthetic peptide administration. Therefore, LD10da represents a promising biologic-based immunomodulator that can be genetically encoded and delivered, along with the antigen, by viral or other nucleic acid vectors to improve the efficacy and delivery of vaccines for ineradicable and emerging infectious diseases.

KEYWORDS

vaccine, PD1, immunomodulator, $CD8^+$, viral delivery, infectious disease

Introduction

The development of effective vaccines remains key to eradicating pathogens worldwide. In order to develop successful vaccines, a potent and sustained protective immunity is needed, comprising humoral and cellular immune responses as both are essential for effectively eliminating pathogens. The inability to elicit strong, durable, and protective T cell immunity, particularly $CD8^+$ T cell responses, has posed a major obstacle for vaccines and constitutes the primary reason that vaccine development efforts fail, especially for intracellular pathogens (Seder and Hill, 2000). Malaria is a classic example of a disease for which a vaccine is challenging to develop due to lack of T cell immunity.

In an effort to overcome the limitations of current vaccines, adjuvants that enhance T cell immunity are being developed (Counoupas et al., 2017; Halbroth et al., 2018; Thakur et al., 2019). Currently, the majority of adjuvants are designed to generate innate inflammatory danger signals. While these danger signals are essential for innate activation, including antigen presentation and cytokine production, there is limited direct effect on T cells (Petrovsky, 2015; Powell et al., 2015). Thus, novel adjuvants or immunomodulators that directly enhance the expansion and durability of vaccine-induced antigen-specific T cells are needed. Further, novel adjuvants could help decrease the vaccine dosage required to elicit sufficient protective immunity, thereby reducing both toxicity and cost, the latter being a crucial consideration for vaccines intended for developing countries.

Programmed cell death 1 (PD-1) is the most well-characterized checkpoint-inhibitory receptor, and its function is to regulate the threshold, strength, and duration of T cell responses to antigen presentation (Okazaki et al., 2013). PD-1 is rapidly upregulated upon naïve T cell activation, which is required to minimize damage to the host from uncontrolled inflammation during and after infection (Ahn et al., 2018). In non-human primates, immunization with an SIV Gag adenovirus-based vaccine in combination with an anti-PD-1 monoclonal antibody (mAb) significantly elevated peak Gag-specific T cell responses (Finnefrock et al., 2009). Further, we recently showed that antagonizing the PD-1 receptor during prophylactic immunization with an adenovirus-based or radiation-attenuated sporozoite-based malaria vaccine significantly enhanced the number of antigen-specific $CD8^+$ T cells (Kotraiah et al., 2020; Phares et al., 2020). These observations suggest that PD-1 modulation may be a critical T cell-focused immunomodulator capable of enhancing T cell expansion and differentiation, resulting in increased numbers and functionality of effector and memory T cells. Similar strategies of combining PD-1 antagonists with a therapeutic vaccine have been used experimentally in cancer therapy to positive effect and are being explored clinically (Massarelli et al., 2019; Verma et al., 2019; Kaumaya et al., 2020; Ott et al., 2020; Peng et al., 2021). Importantly, adjuvant systems such as alum increase the expansion and expression of PD-1 on T cells and may limit their function and their maturation towards effector- and memory-T cell status (MacLeod et al., 2011). Therefore, combining a checkpoint modulator with a traditional adjuvant system may help promote a more balanced immune response in the host. Further, since delivery of vaccine antigens by viral vectors, including Adenoviral and Modified Vaccinia Ankara (MVA) vectors, is a proven strategy for inducing antigen-specific T cell response (Fougeroux and Holst, 2017; Vitelli et al., 2017; Coughlan et al., 2018; Rampling et al., 2018; Folegatti et al., 2020; Förster et al., 2020), combining a checkpoint antagonist with a viral vector-based vaccine may prove advantageous.

While mAb-based checkpoint inhibitors developed to treat cancer can effectively restore immune function, they do not readily lend themselves to the field of infectious disease vaccinology. Due to their long serum half-life, anti-PD-1 mAbs can trigger severe immune-related adverse events (irAEs) and precipitate autoimmune disease (Brahmer et al., 2010; Topalian et al., 2012). Thus, administering such mAbs to a healthy population as a prophylactic vaccine adjuvant poses an unacceptable safety risk. Therefore, peptide-based biologics could be a safer alternative modality to Abs, as peptides have a shorter pharmacokinetic profile and thereby reduce the likelihood of irAEs. Further, peptides offer greater formulation and delivery options and rapid synthetic manufacturing (AlDeghaither et al., 2015; Fosgerau and Hoffmann, 2015; Marqus et al., 2017; Borrelli et al., 2018). Specifically, co-delivery of an immunomodulator genetically encoded in a viral vector would greatly reduce the costs of the vaccine's formulation. Therefore, we aimed to explore a peptide-based immunomodulator that has potentially greater efficacy in a malaria vaccine model and to demonstrate its delivery by a viral vector platform.

In the current study, we report on LD10, an active, 18-amino acid derivative of our previously reported peptide (Phares et al., 2020). *In vitro*, LD10 demonstrated greater potency at impairing PD-1 receptor signaling relative to LD01. Further, when combined prophylactically with an adenovirus-based malaria vaccine, LD10 treatment resulted in greater expansion relative to LD01 treatment of antigen-specific, IFN-γ-secreting $CD8^+$ T cells. Dosing regimen studies established that a single dose of LD10 at the time of immunization with AdPyCS, a circumsporozoite (CS) protein of *Plasmodium yoelii*, was sufficient to enhance the number of vaccine-induced, antigen-specific T cells *in vivo*. Using humanized mice that mimic the human immune system (HIS) and possess functional human $CD8^+$ T cells, we demonstrate LD10-mediated modulation of human T cell responses. Moreover, we show that LD10 can be expressed and secreted by a recombinant MVA vector that enhances antigen-specific $CD8^+$ T cell expansion. Collectively, these data establish that LD10 is a potent immunomodulator that

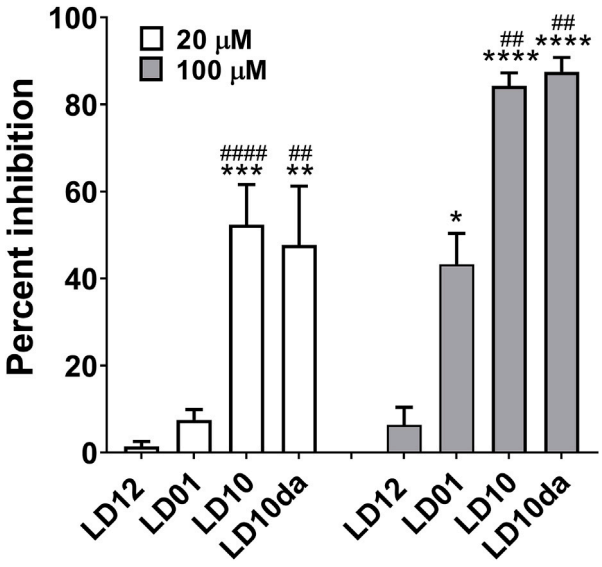

FIGURE 1
PD-1 receptor signaling is inhibited by LD10. Jurkat cells expressing PD-1 were incubated with LD12, LD01, LD10, or LD10da at 20 μM and 100 μM for 1 h. Programmed death-ligand 1 (PD-L1)-expressing cells were then added to the assay and co-cultured for 2 h. Chemiluminescence was detected, and the percent of inhibition for the peptides was calculated using the formula described in the *Materials and methods* section. Data represent 3–8 independent experiments and are expressed as the mean ± SEM percent inhibition. Significant differences between LD12 and LD01, LD10, or LD10da at 20 μM and 100 μM, respectively, were determined using a two-tailed unpaired *t*-test and denoted by * ($p < 0.05$), ** ($p < 0.001$) *** ($p < 0.0005$), and **** ($p < 0.0001$). Significant differences between LD01 and LD10 or LD10da at 20 μM and 100 μM, respectively, were determined using a two-tailed unpaired *t*-test and denoted by $^{\#\#}$ ($p < 0.01$) and $^{\#\#\#\#}$ ($p < 0.0001$).

enhances T cell responses and supports delivery of peptide-based immunomodulators *via* a viral vector.

Results

LD10 reduces programmed cell death 1 receptor signaling in a functional cell-based assay

In order to identify the pharmacophore of LD01, our previously reported 22-amino acid peptide-based PD-1 immunomodulator (Phares et al., 2020), we generated a series of LD01 variants. Analysis of these variants revealed that an 18-mer, in which the first four amino acids of LD01 are deleted, exhibited increased activity in the human PathHunter® PD-1 Signaling Bioassay (Figure 1); we named this 18-mer LD10. In addition, pharmacokinetic analysis *via* liquid chromatography tandem mass spectrometry (LC-MS/MS) of plasma from naïve mice treated intravenously (IV) with a single 200 μg dose of LD01 revealed that the intact peptide circulated for less than 5 min, with metabolites of LD01 detected up to 120 min after administration (Phares et al., 2020). A major LD01 metabolite that was identified in the mouse plasma was the 18-mer LD10 we designed and tested in Figure 1.

These observations prompted us to investigate whether LD10 represented the active metabolite of LD01 *in vivo*. The ability of LD01 and LD10 to demonstrate inhibition in the human PathHunter® PD-1 Signaling Bioassay are shown in Figure 1. In addition to LD01 and LD10, we also tested LD12, a derivative of LD01 that is mutated to severely diminish activity in the PD-1 Signaling Bioassay (Phares et al., 2020) and considered a negative control peptide. LD10da, in which the first amino acid of LD10 was modified to a D-amino acid and the C-terminus was capped with an amide group to protect against terminal degradation by serum proteases was also tested. Peptides were tested at 20 μM and 100 μM. Note, the IC_{50} of a positive control anti-PD1 antibody ran in parallel with the peptides was ~80 nM. As reported (Phares et al., 2020), incubation with 20 μM LD01 showed minimal inhibition (~8%) of PD-1 signaling (Figure 1). In contrast, treatment of cells with 20 μM LD10 or LD10da resulted in a mean inhibition of ~52% and ~48%, respectively (Figure 1). Moreover, while LD01 resulted in a mean inhibition of ~43% at 100 μM, LD10 and LD10da each yielded a ~2-fold greater reduction in PD-1 signaling, with mean inhibitions of ~84% and ~88%, respectively (Figure 1). These results indicate that LD10 is an active metabolite of LD01 and that terminal modifications in LD10da appear to have no impact on the activity in the PD-1 Signaling Bioassay. Further, the data indicate that LD10 and LD10da have greater potency relative to LD01 at impairing PD-1 receptor signaling in the current functional cell-based assay.

LD10da enhances antigen-specific $CD8^+$ T cell expansion following adenovirus-based vaccination

Recently, we demonstrated that LD01, when administered in combination with an adenovirus-based or irradiated sporozoite-based prophylactic malaria vaccine, significantly enhances antigen-specific $CD8^+$ T cell numbers (Phares et al., 2020). To assess whether LD10 increases antigen-specific $CD8^+$ T cell expansion following vaccination, we used as a model vaccine the recombinant replication-defective adenovirus serotype 5 expressing the entire *P. yoelii* circumsporozoite protein (AdPyCS) (Rodrigues et al., 1997; Phares et al., 2020). The D-amino acid substitution at the N-terminus and the addition of an amide group at the C-terminus of LD10 in LD10da had no impact on its activity *in vitro* (Figure 1). Since these modifications also have the potential to reduce degradation from serum exopeptidases, LD10da was pursued in these *in vivo* studies.

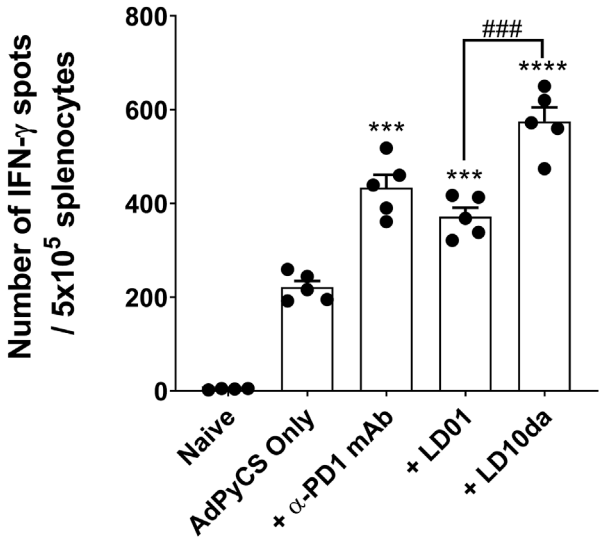

FIGURE 2
LD10da increases antigen-specific CD8⁺ T cell numbers following AdPyCS vaccination. At D12 post-AdPyCS immunization, immunogenicity was assessed by measuring the number of splenic PyCS-specific, IFN-γ-secreting CD8⁺ T cells using the ELISpot assay after stimulation with the H-2kd restricted CD8 epitope SYVPSAEQI. Data are expressed as the mean ± SEM. Data from one of two independent experiments are shown with $n = 5$ per group. Significant differences between AdPyCS alone and treated mice were determined using a two-tailed unpaired *t*-test and denoted by *** ($p < 0.0005$) and **** ($p < 0.0001$). Significant differences between LD01 and LD10da were determined using a two-tailed unpaired *t*-test and denoted by ### ($p < 0.0005$).

Firstly, mice were immunized with AdPyCS intramuscularly (IM). Subsequently, they were treated intraperitoneally (IP) with the α-PD-1 mAb, LD01 or LD10da on day 1 (D1), D3, D5, and D7 (Figure 2). The doses of α-PD-1 mAb and peptides delivered were 200 μg and 100 μg per injection per mouse, respectively. The AdPyCS only group was treated with water, the solvent used to dissolve the peptides, as a control. Splenocytes were isolated from the spleen on D12, and the relative number of PyCS-specific, IFN-γ-secreting CD8⁺ T cells was assessed using an ELISpot assay. Of note, the number of spots seen with naïve splenocytes typically does not exceed five (data not shown). As previously reported (Phares et al., 2020), α-PD-1 mAb and LD01 treatment significantly enhanced the number of PyCS-specific, IFN-γ-secreting CD8⁺ T cells by ~2 and ~1.7-fold, respectively, relative to that of mice immunized with AdPyCS alone (Figure 2). Likewise, LD10da significantly increased the number of PyCS-specific, IFN-γ-secreting CD8⁺ T cells (Figure 2), with a ~2.6-fold change compared to that of mice receiving AdPyCS immunization alone. Notably, LD10da treatment resulted in greater expansion of PyCS-specific, IFN-γ-secreting CD8⁺ T cells relative to LD01 treatment, corroborating the proposition that LD10da may have greater immuno-enhancer potency (Figure 1). Taken together, the data indicate that LD10da treatment significantly enhances the expansion of vaccine antigen-specific T cells *in vivo*.

A single dose of LD10da at the time of vaccination increases antigen-specific CD8⁺ T cell expansion

As described above, the initial dosing regimen for LD10da comprised four administrations, one each on D1, D3, D5, and D7 (Figure 2). To determine whether a single dose of LD10da is sufficient to enhance antigen-specific CD8⁺ T cell numbers following AdPyCS vaccination, mice were treated once on D1, D3, D5, or D7 (Figure 3). Of note, delivering LD10da subcutaneously (SC), relative to IP, does not significantly impact its ability to enhance antigen-specific CD8⁺ T cell expansion (Supplementary Figure S1); therefore, SC delivery was used in these studies. As shown in Figure 3A, treatment with 100 μg LD10da on D1, D3, D5, and D7 significantly increased antigen-specific CD8⁺ T cell numbers relative to mice that were administered AdPyCS alone. Similarly, antigen-specific CD8⁺ T cell expansion was significantly enhanced when LD10da treatment was limited to D1 or D3 (Figure 3A). By contrast, a single administration of LD10da on D5 or D7 following immunization had no effect on antigen-specific CD8⁺ T cell numbers (Figure 3A). Of note, the degree of elevated numbers of antigen-specific CD8⁺ T cells was comparable between SC and IM administration with a single LD10da dose on D1 post-immunization (Figure 3A).

Since a single LD10da dose on D1 following immunization yielded an equivalent increase in antigen-specific CD8⁺ T cell expansion, we next evaluated whether a single LD10da dose administered concurrently with AdPyCS vaccination (0 h) is efficacious (Figure 3B). Indeed, the numbers of antigen-specific CD8⁺ T cells were significantly increased (~3-fold) when a single LD10da injection was given at the time of vaccination compared to AdPyCS immunization alone (Figure 3B). The absence of increased CD8⁺ T cell expansion with a single administration of LD10da on D7 (Figure 3B) corroborated our previous results (Figure 3A). Taken together, the data indicate that a single SC or IM dose of LD10da at the time of AdPyCS immunization is sufficient to enhance vaccine-induced, antigen-specific T cells *in vivo*, thus indicating its potential as a vaccine adjuvant.

LD10da promotes vaccine-induced, antigen-specific CD8⁺ T cell expansion in human immune system mice

Reduced PD-1 receptor signaling by LD10da in a human functional cell-based assay suggests that the peptide modulates human T cell responses (Figure 1). To confirm this, we used

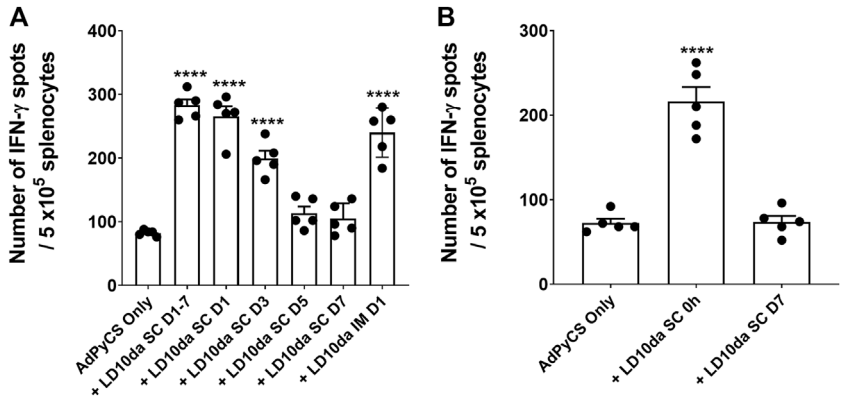

FIGURE 3
A single dose of LD10da administered concurrently with AdPyCS vaccination increases antigen-specific CD8$^+$ T cell numbers. **(A,B)** At D12 post-AdPyCS immunization, immunogenicity was assessed by measuring the number of splenic PyCS-specific, IFN-γ-secreting CD8$^+$ T cells using the ELISpot assay after stimulation with the H-2kd restricted CD8 epitope SYVPSAEQI. AdPyCS was administered IM. **(A)** A 100-μg dose of LD10da was given SC either on D1, D3, D5, and D7, or only on D1, D3, D5, or D7 post-immunization. **(B)** A 100-μg dose of LD10da was given SC on D7 or immediately (0 h) post-immunization. Data are expressed as the mean ± SEM. Data from one of two independent experiments are shown with n = 5 per group. Significant differences between AdPyCS alone and treated mice were determined using a two-tailed unpaired t-test and denoted by **** ($p < 0.0001$).

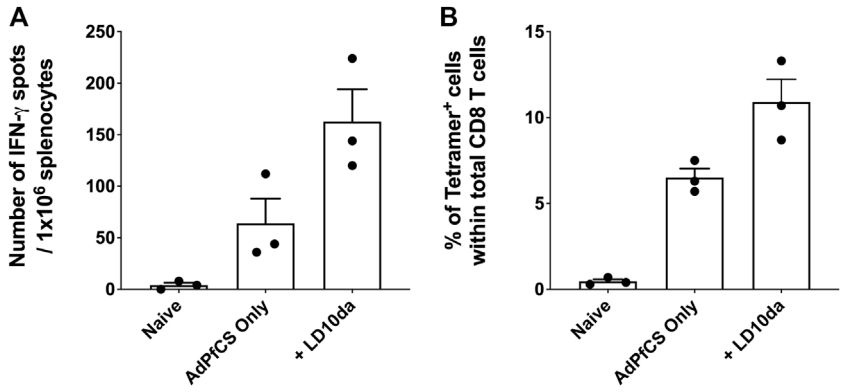

FIGURE 4
LD10da increases antigen-specific CD8$^+$ T cell expansion in AdPfCS-vaccinated HIS mice. At D12 post-AdPfCS immunization, immunogenicity was assessed by measuring the number of splenic PfCS-specific, IFN-γ-secreting CD8$^+$ T cells using the ELISpot assay after stimulation with the HLA-A2.1-restricted CD8 epitope YLNKIQNSL **(A)**, or splenocytes from mice were stained directly *ex vivo* for CD3, CD8, and YLNKIQNSL-specific tetramer and analyzed by flow cytometry **(B)**. Data are expressed as mean ± SEM. Data are from a single experiment with n = 3 per group.

humanized mice that mimic the HIS and possess functional human CD8$^+$ T cells and dendritic cells (Huang et al., 2014; Li et al., 2016; Coelho-Dos-Reis et al., 2020). As detailed in the *Materials and methods* section, HIS mice were generated by engrafting NOD/SCID/IL2Rgamma^{null} (NSG) mice with human hematopoietic stem cells (HSCs) following the transduction of genes encoding several human cytokines and human leukocyte antigen (HLA)-A2.1 by adeno-associated virus serotype 9 vectors (Huang et al., 2014). Flow cytometry analysis confirmed that 85%–95% of the peripheral blood mononuclear cells of HIS mice consist of human CD45$^+$ leukocytes, as previously published (Huang et al., 2014). The HIS mice were treated with LD10da at the time of vaccination with a recombinant replication-defective adenovirus expressing the *P. falciparum* circumsporozoite protein (AdPfCS). The dosing regimen for LD10da in the HIS mice was 20 μg on D1, D3, D5, and D7. Of note, the percentages of splenic PD-1$^+$ T cells in HIS mice increased following vaccination with AdPfCS (data not shown). As shown in Figure 4A, treatment with LD10da increased antigen-specific CD8$^+$ T cell numbers—albeit not significantly—relative to

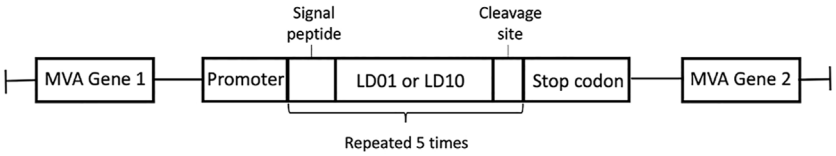

FIGURE 5
MVA vector construction. Schematic of MVA-5X.LD01 and MVA-5X.LD10 vectors illustrating the design of peptide sequences inserted into the MVA genome between two essential genes under control of an MVA-specific promoter. LD01 and LD10 sequences are preceded by a signal sequence-routing peptide for secretion and followed by a cleavage site to separate duplicated peptides. The secretion signal, peptide sequence, and cleavage site are repeated five times, and transcription is terminated with a stop codon.

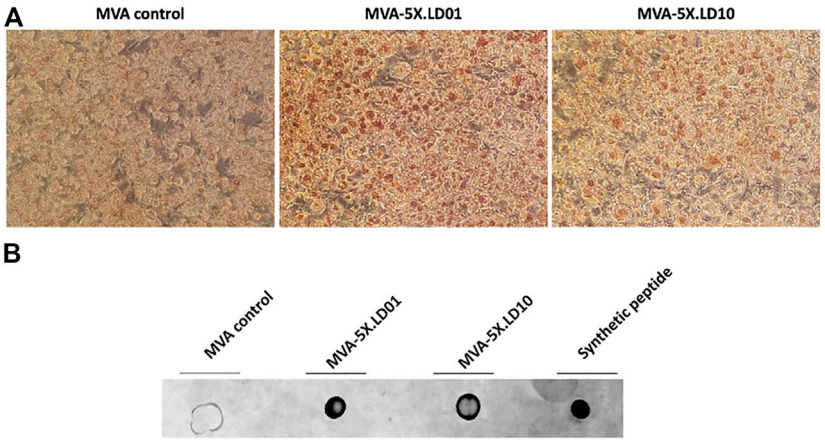

FIGURE 6
LD01 and LD10 are produced and secreted by MVA-infected cells. **(A)** DF-1 cells were infected with MVA-5X.LD01, MVA-5X.LD10 or parental MVA. Two days post-infection, cells were fixed, permeabilized, and stained with an antibody (Ab) specific to LD01 and LD10. Results show that the peptides are detected intracellularly. Representative images are shown. LD01- and LD10-positive cells are stained brown. Photomicrographs are presented at a magnification of ×20. **(B)** DF-1 cells were infected with MVA-5X.LD01, MVA-5X.LD10, or parental MVA. Two days following infection, supernatant was harvested, concentrated, and dotted onto a membrane along with chemically synthesized peptide (LD01) and probed with an Ab specific for LD01 and LD10.

AdPfCS immunization alone without treatment. Similarly, the percentages of splenic HLA-A2.1/YLNKIQNSL-tetramer-specific $CD8^+$ T cells were increased following LD10da treatment relative to AdPfCS alone (Figure 4B). This limited experiment provides supporting evidence that LD10da-mediated enhancement of human T cell responses in this *in vivo* model is consistent with its inhibitory activity in the cell-based human PD-1 assay.

Construction and characterization of an modified vaccinia ankara virus expressing peptide-based immunomodulators

Peptide-based therapeutics offer an alternative modality to mAbs and provide a shorter pharmacokinetic profile, thus reducing the likelihood of irAEs. Furthermore, peptide-based biologics can offer a greater number of formulation and delivery options, such as expression *via* a viral vector. To establish whether LD10 could be expressed by a viral vector, we constructed a recombinant MVA virus that encodes five repeats of the LD10 sequence in polycistronic format (MVA-5x.LD10) (Figure 5). In addition to the LD10 construct, a similar recombinant MVA virus expressing five repeats of the LD01 sequence was constructed (MVA-5x.LD01) (Figure 5). To facilitate peptide secretion, a signal sequence was added prior to LD01 or LD10, and a dual cleavage site was added following the sequences in order to facilitate production of the monomer LD01 or LD10 from the polycistronic design.

To determine whether the recombinant MVA vectors express LD01 or LD10, immunohistochemistry was performed on infected cells using a mAb that recognize LD01 and LD10. Of

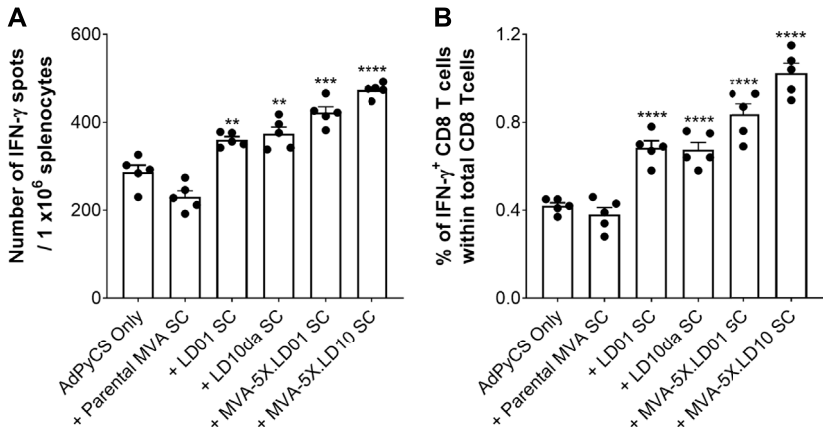

FIGURE 7
Delivery of LD01 or LD10 *via* a viral vector enhances expansion of vaccine-induced, antigen-specific CD8$^+$ T cells. **(A,B)** At D12 post-AdPyCS immunization, immunogenicity was assessed by measuring the number of splenic PyCS-specific, IFN-γ-secreting CD8$^+$ T cells using the ELISpot assay **(A)** and flow cytometry **(B)** after stimulation with the H-2kd-restricted CD8 epitope SYVPSAEQI. A 100-μg dose of LD01 or LD10da was given SC immediately following vaccination. For viral vectors, 10^8 $TCID_{50}$ of MVA-5X.LD01, MVA-5X.LD10, or parental MVA was injected SC following vaccination. Data are expressed as mean ± SEM. Data from one of two independent experiments are shown with $n = 5$ per group. Significant differences between AdPyCS alone and treated mice were determined using a two-tailed unpaired *t*-test and denoted by ** ($p < 0.001$), *** ($p < 0.0005$), and **** ($p < 0.0001$).

note, cells were fixed and permeabilized with a 50:50 solution of methanol/acetone. Cells infected with the parental MVA vector showed no specific staining (Figure 6A). However, cells infected with either MVA-5X.LD01 or MVA-5X.LD10 vectors showed positive staining (Figure 6A), indicating intracellular expression of the peptides. To confirm that LD01 or LD10 is being secreted by the recombinant MVA vector, a dot blot was performed on infected cell supernatants (see *Materials and methods*). As shown in Figure 6B, the parental MVA vector showed negligible signal. By contrast, both MVA-5X.LD01 and MVA-5X.LD10 vector samples demonstrated positive staining, arguing for secretion of LD01 and LD10. Similarly, LC-MS/MS of the cell supernatants identified LD01 and LD10 fragments (data not shown), corroborating the dot blot results. Taken together, these data strongly suggest that LD01 and LD10 are expressed and secreted by the recombinant MVA vectors.

Delivery of LD01 or LD10 by modified vaccinia ankara increases antigen-specific CD8$^+$ T cell numbers following AdPyCS vaccination

Having confirmed that LD01 and LD10 are expressed in and secreted from cells infected with peptide-encoding MVA constructs (Figure 6), we then assessed whether vaccine-induced, AdPyCS-specific CD8$^+$ T cell expansion was observed following treatment with MVA-encoding LD01 or LD10. A parental MVA vector was included as a negative control, while synthetic LD01 and LD10da served as positive controls. As shown in Figure 7, treatment with 100 μg of LD01 or LD10da directly following vaccination significantly increased antigen-specific CD8$^+$ T cell numbers relative to AdPyCS alone. Similarly, injection of 10^8 $TCID_{50}$ of MVA-5X.LD01 or MVA-5X.LD10 enhanced antigen-specific CD8$^+$ T cell expansion, which contrasted the treatment with the parental MVA vector (Figure 7). Taken together, these *in vivo* results indicate that the delivery of LD01 or LD10 *via* the MVA vector results in immunomodulatory activity that is likely due to their expression *in vivo*. As such, these results corroborate that peptide-based immunomodulators can be successfully delivered by viral vector.

Discussion

Previously, we showed that peptides are a viable and potentially favorable alternative to mAb-based checkpoint inhibitors for use in infectious disease indications. Improvements in the safety profile, manufacturing costs, and delivery options are among the key advantages of peptide-based biologics. Here we expand on the evidence in favor of peptide-based checkpoint antagonists by generating derivatives of the LD01 peptide, which led to the identification of LD10, a major metabolite whose application concurrent with a model vaccine antigen yields a greater expansion of antigen-specific T cells. We further modified the LD10 peptide by the addition of a C-terminal D-amino acid and an N-terminal amide group (LD10da) with the aim of improving stability. Indeed, we were able to detect the 18-amino acid LD10da peptide for

approximately 1 h by LC-MS/MS *via* a pharmacokinetic study in mice treated SC with a single 200 µg dose (data not shown). Therefore, enhanced durability of the LD10 derivative improves the peptide's immune-modulating properties.

Based on the increased stability of LD10da, we performed a dose-sparing evaluation of the immunomodulator by administering only a single dose at the time of vaccination. Remarkably, this single co-administered dose of LD10da was sufficient to increase antigen-specific $CD8^+$ T cell expansion in wild-type and HIS mice. Note that the number of HIS mice used per group was limited due to challenges in breeding NSG mice, availability of HLA-A*0201 matched HSCs, and a long duration (>15 weeks) following HSCs engraftment to ensure human lymphocyte expansion. Therefore, we were unable to obtain another cohort of mice to repeat the experiment. We intend to confirm the HIS mice results in future studies; therefore, the results of this experiment should be interrupted with caution. While the 2-3 fold increase in antigen-specific $CD8^+$ T cell expansion following a single dose of LD10da demonstrates a targeted biological effect, whether this improves vaccine efficacy following parasite challenge has yet to be determined and are warranted future studies.

The rationale underlying the remarkably rapid peptide activation of the T cell compartment may be the rapid upregulation of PD-1 *in vivo* upon activation of naïve CD8 T cells. This upregulation can occur 24 h after lymphocytic choriomeningitis virus (LCMV) infection and in less than 4 h after viral peptide injection (Ahn et al., 2018). Accordingly, our findings strongly suggest that modulation of PD-1 signaling does indeed occur shortly after vaccination, and LD10da likely acts on the early activated T cells. Further, it is possible that LD10da binds to T cells constitutively expressing PD-1, as is the case for regulatory CD4 T cells (Tregs) (Francisco et al., 2010). In fact, the PD-1:PD-L1 (programmed death-ligand 1) axis has been shown to play a role in regulating Treg development, expansion, and function (Cai et al., 2019; Lin et al., 2019). For example, PD-L1-deficient antigen-presenting cells only minimally converted naïve CD4 T cells to Tregs, indicating an essential role of PD-1:PD-L1 engagement for Treg induction (Francisco et al., 2009). Further, Francisco et al. (2009) reported that PD-L1 enhances and sustains FOXP3 expression and the suppressive function of Tregs. Similarly, it was shown that dendritic cells overexpressing PD-L1 when co-cultured with naïve CD4 T cells promoted Treg generation, with PD-L1 blockade resulting in reduced Treg expansion (Lin et al., 2019). Thus, it is possible that LD10da binds to the basal PD-1 on Tregs and thereby decreases expansion or inhibitory function, allowing for a greater number of vaccine-induced, antigen-specific $CD8^+$ T cells. While we have yet to assess the Treg responses following AdPyCS vaccination in the absence or presence of our peptide-based immunomodulators, we recently reported that LD01 treatment of mice infected with a lethal malaria strain resulted in survival that was associated with lower numbers of $FOXP3^+Tbet^+CD4^+$ Tregs (Phares et al., 2020). Tregs also constitutively express cytotoxic T-lymphocyte antigen 4 (CTLA4), which is critical for their suppressive abilities. In this regard, we have obtained preliminary data showing that LD10da and LD01 antagonize a novel allosteric site that is shared by the CD28 family of checkpoint receptors (data not shown), of which CTLA4 is a member. Therefore, these peptide-based immunomodulators may be modulating Treg activity by disrupting both PD-1 and CTLA4 signaling.

In addition to modifying T cell responses there is a growing body of literature demonstrating PD-1 expression on myeloid cells mediates cellular dysfunction (Huang et al., 2009; Strauss et al., 2020; Phares et al., 2021). For example, we recently showed in a cecal-ligation and puncture-induced murine polymicrobial sepsis model that LD01 treatment alleviated aspects of phagocyte immune dysfunction (Phares et al., 2021) corroborating the pathologic role of PD1 in altering microbial clearance and innate immunity in sepsis (Huang et al., 2009). Further, ablation of myeloid cell-specific PD-1 in a tumor model resulted in reduced tumor accumulation of myeloid-derived suppressor cells and increased T effector memory cell function enhancing overall antitumor protection (Strauss et al., 2020). Interestingly, myeloid cell-specific PD-1 ablation also increased cholesterol (Strauss et al., 2020), a molecule that drives myeloid cell expansion and differentiation and promotes antigen presenting function, which raises the possibility that the improved T cell function is partially due to enhanced antigen presentation in the tumor model. Similarly, in the AdPyCS vaccine model the increased antigen-specific $CD8^+$ T cell expansion observed after PD-1 blockade may be a consequence of enhanced antigen presentation by myeloid cells, something we intend to assess in future studies.

While we opted to use the AdPyCS vaccine model to rapidly test many LD01 derivatives, including LD10, we recognize that the dosing regimen of our peptides for traditional vaccines with a prime/boost(s) regimen may differ. In fact, in a therapeutic cancer vaccine model, the timing and order of vaccine and anti-PD-1 treatment have been shown to be crucial in determining optimal $CD8^+$ T cell responses and therapeutic outcomes (Verma et al., 2019). Preliminary studies that we conducted with soluble vaccine antigens in a 3-dose regimen showed that the number of antigen-specific T cells generated in mice treated with LD01 following the second and third immunization was greater than the numbers of these cells generated with treatment after each immunization (data not shown). Additionally, in a whole-cell vaccine model, we have obtained data indicating that LD10da treatment following a primary and booster vaccination reduces bacterial challenge burdens in the nasal passage and lungs to a greater degree than did administration of the LD10da only after the primary immunization (data not shown). Accordingly, dosing regimens to modulate PD-1 activity are likely dependent on an optimized

schedule of priming and boosting relative to antigen stimulation, a hypothesis that we intend to test in future studies.

Given that the more stable LD10 peptide has been proven to be a potent immunomodulator in a vaccine formulation, we aimed to establish proof of a potential delivery platform for future vaccine development efforts by encoding the peptide in the MVA platform. Following IM infection of mice with recombinant MVA-expressing green fluorescent protein (rMVA-GFP), GFP^+ cells were identified as myocytes and interdigitating cells having a macrophage or dendritic cell morphology (Altenburg et al., 2017). Further, GFP^+ cells were detected in the draining lymph nodes as well as systemically in white blood cells and splenocytes (Altenburg et al., 2017). Additionally, *in vitro* human peripheral blood mononuclear cell (PBMC) assays and *ex vivo* mouse lung explants showed that rMVA-GFP predominately infects dendritic cells (Altenburg et al., 2017). These data suggest that our peptide-based immunomodulators are expressed by MVA at the site of injection and/or within the lymphoid tissues. Of note, MVA is also detected in lymphoid organs following SC injection (Ramirez et al., 2003). Expression of LD10 or LD01 within the lymphoid tissues is ideal given that these tissues are the primary site of T cell activation, differentiation, and expansion. Moreover, our peptides are believed to be secreted locally during T cell priming because MVA preferentially targets dendritic cells (Altenburg et al., 2017) and because these professional antigen-presenting cells directly interact with or are in close proximity to T cells during immune responses. In future efforts, we plan to identify the specific cell(s) that may be expressing the peptide-based immunomodulators and to specify the duration of expression and circulating levels. These data are important for advancing our understanding of the basis for the immunogenicity of MVA-based vaccines and for informing effective vaccine designs and delivery strategies.

In addition to our reported efforts on MVA-based vaccine development, we are currently developing chimeric antigen receptor (CAR) T cells, which have been genetically engineered to express LD10. Here the secreted LD10 would not only bind to the PD-1 expressed by the CAR T cell, but also potentially engage and relieve dysfunction of endogenous T cells. We have also proceeded with the development of oncolytic viruses that express our peptide-based immunomodulators. With such viruses, which can be engineered to replicate selectively in tumor tissues, LD01 or LD10 would be produced within the infected cell and released into the tumor microenvironment following virus-induced lysis. Moreover, we are developing a dissolving microneedle patch that facilitates a painless means of delivering our peptides into the dermis, which easily accesses the lymphatic system. As microneedle patches are a viable means of circumventing the challenges associated with conventional vaccine delivery, we envision combining our peptide-based immunomodulators with vaccine antigens. In addition to the microneedle patch, we are currently testing other delivery platforms, including a solid-dose implant that is needle-free and delivers the peptides transcutaneously into the dermis. Akin to the microneedle patch, our peptide-based immunomodulators could be combined with vaccine antigens in a single implant. All of the aforementioned efforts may allow us to develop potent T cell-stimulating infectious disease vaccines that can be stably deployed and easily administered in more austere environments.

Materials and methods

Ethics statement

All animal experiments were carried out in strict accordance with the Policy on Humane Care and Use of Laboratory Animals of the United States Public Health Service. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at The Rockefeller University (Assurance No. A3081-01). Mice were euthanized with CO_2, with every effort made to minimize suffering. Human fetal liver samples were obtained *via* a non-profit partner (Advanced Bioscience Resources, Alameda, CA, United States). As no information was obtained that would identify the subjects from whom the samples were derived, Institutional Review Board approval for their use was not required, as previously described (Huang et al., 2014).

Programmed cell death 1:Programmed death-ligand 1 cell-based reporter assay

For the PathHunter® PD-1 signaling assay (cat# 93-1104C19; Eurofins DiscoverX; Fremont, CA, United States), Jurkat cells expressing PD-1 and SHP1 proteins, each fused to a fragment of DiscoverX's enzyme fragment complementation (EFC) system, were co-incubated with ligand-presenting cells. This led to PD-1 activation and SHP1 recruitment to the PD-1 receptors, bringing together the two EFC fragments and generating a chemiluminescent signal. In this assay, LD01, LD10, LD10da, and LD12 were assessed at 20 μM and 100 μM, whereas anti-PD-1 mAb controls were assessed at 10 different concentrations. In brief, PD-1-expressing Jurkat cells (20,000 cells/well) were seeded in a total volume of 50 μl into white-walled, 96-well microplates in assay buffer. Serial dilution of LD01 stock was performed to generate an ×11 sample in assay buffer, and 10 μl of the ×11 test sample was added to PD-1 cells and incubated at 37°C for 1 h U2OS cells expressing PD-L1 (50 μl, 30,000 cells/well in assay buffer) were then added to the assay. Cells in co-culture were incubated at room temperature (RT) for 2 h (PD-1 assay). The assay signal was generated using the PathHunter® Bioassay Detection Kit for both assays. Detection reagent 1 (10 μl) was added to the assay and incubated at RT for 15 min. Detection reagent 2 (40 μl) was added to the assay and incubated at RT for

1 h. Microplates were read following signal generation with an EnVision™ plate reader (PerkinElmer, Waltham, MA, United States) for chemiluminescent signal detection. LD01 activity and anti-PD-1 mAb activities were analyzed using the CBIS data analysis suite (ChemInnovation Software, Inc., San Diego, CA, United States). For antagonist mode assays, the percentage of inhibition by the peptides was calculated using the following formula: percent inhibition efficacy = 100% × [1 − (mean RLU of test sample − mean RLU of vehicle control) / (mean RLU of EC80 control − mean RLU of vehicle control)].

Mice

Female BALB/c mice 6–8 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME, United States). NOD/SCID/IL2Rgamma^{null} (NSG) mice exhibiting features of both severe combined immunodeficiency mutations and interleukin (IL)-2 receptor gamma-chain deficiency were also purchased from The Jackson Laboratory and maintained under specific pathogen-free conditions.

Generation of human immune system-CD8 mice

Recombinant AAV9 (rAAV9) vectors encoding human IL-3, IL-15, GM-CSF, and HLA-A*0201 were constructed as previously described (Huang et al., 2014). Four-week-old NSG mice were transduced with rAAV9 encoding HLA-A*0201 by perithoracic injection and with rAAV9-encoding HLA-A*0201 and AAV9-encoding human IL-3, IL-15, and GM-CSF, by IV injection, as previously described (Huang et al., 2014). Two weeks later, mice were subjected to 150-Gy total body sub-lethal irradiation for myeloablation, and several hours later, each transduced, irradiated mouse was engrafted IV with 1×10^5 HLA-A*0201$^+$-matched, CD34$^+$ human HSCs. CD34$^+$ HSCs among lymphocytes derived from HLA-A*0201$^+$ fetal liver samples were isolated using a Human CD34 Positive Selection Kit (STEMCELL Technologies, Vancouver, BC, United States) (Lepus et al., 2009). At 14 weeks post-HSC engraftment, the reconstitution status of human CD45$^+$ cells in the blood of HIS-CD8 mice was determined by flow cytometry analysis, as previously described (Huang et al., 2014).

AdPyCS and AdPfCS vaccines

A recombinant serotype 5 adenovirus that expressed *P. yoelii* circumsporozoite protein (PyCS), AdPyCS, was constructed as previously described (Rodrigues et al., 1997). A recombinant adenovirus serotype 5 (Ad5) expressing a GFP alone in its transgene, AdGFP, was previously constructed (Shiratsuchi et al., 2010). A recombinant Ad5 expressing *P. falciparum* CSP (AdPfCS) was also previously constructed as described (Altenburg et al., 2017). Briefly, a gene encoding a full-length PfCSP was codon-optimized and synthesized, and then inserted into pShuttle-CMV, which was used to make the recombinant AdPfCS. Each BALB/c mouse was immunized IM with 5×10^9 virus particles of AdPyCS, whereas each HIS-CD8 mouse was immunized IM with 1×10^{10} virus particles of AdPfCS.

ELISpot assay and flow cytometry to measure antigen-specific CD8$^+$ T cells

As previously described (Li et al., 2016), the relative numbers of splenic PyCS-specific, IFN-γ-secreting CD8$^+$ T cells of AdPyCS-immunized mice were determined by ELISpot assay using a mouse IFN-γ ELISpot Kit (Abcam, Cambridge, MA, United States) and a synthetic 9-mer peptide, SYVPSAEQI (Peptide 2.0, Chantilly, VA, United States) corresponding to the immunodominant CD8$^+$ T cell epitope within PyCS (Li et al., 2016). In brief, after the collection of splenocytes from mice 12 days after AdPyCS immunization, 5×10^5 splenocytes were placed in each well of the 96-well ELISpot plates pre-coated with IFN-γ Ab and incubated with the SYVPSAEQI peptide at 5 μg/ml for 24 h at 37°C in a CO_2 incubator. After the ELISpot plates were washed, they were incubated with biotinylated anti-mouse IFN-γ Ab for 2–3 h at RT, followed by incubation with avidin conjugated to horseradish peroxidase for 45 min at RT in the dark. Finally, the spots were developed after the addition of the ELISpot substrate (Abcam).

To identify the number of IFN-γ-secreting CD8$^+$ T cells in each well, the mean number of spots (for duplicates) counted in the wells incubated with splenocytes in the presence of the peptide was subtracted by the mean number of spots (for duplicates) counted in the wells that were incubated with splenocytes only. The percentage of antigen-specific IFN-γ$^+$ T cells among splenocytes of immunized mice were also determined by intracellular cytokine staining. Briefly, splenocytes harvested from vaccine-immunized animals were stimulated for 6 h by co-culture with 5 μg/ml SYVPSAEQI peptide at 37°C in the presence of Brefeldin A Solution (cat# 420601, BioLegend). Cells were then incubated for 15 min in the presence of TruStain FcX™ PLUS (anti-mouse CD16/32) (cat# 101319, BioLegend) before surface staining for 30 min with α-CD3ε-PE/Cy7 (cat# 100220, BioLegend), α-CD8α-PE/Cy5 (cat# 100710, BioLegend), and α-CD4-AF700 (cat# 100536, BioLegend). Permeabilization was performed using Fixation Buffer (cat# 420801, BioLegend) followed by washing with Intracellular Staining Perm Wash Buffer (cat# 421002, BioLegend) according to the manufacturer's instructions. Cells were stained intracellularly for 30 min with α-IFN-γ-APC (cat# 505810, BioLegend). Flow cytometry analyses were performed

using an LSRII (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo™ v10 (TreeStar, Ashland, OR).

Staining with HLA-A/0201 tetramer loaded with YLNKIQNSL peptide

An allophycocyanin (APC)-labeled human HLA-A*0201 tetramer loaded with the peptide YLNKIQNSL, corresponding to the PfCSP $CD8^+$ T cell epitope (Blum-Tirouvanziam et al., 1995; Bonelo et al., 2000), was provided by the NIH Tetramer Core Facility. Twelve days after immunization of HIS-CD8 mice with AdPfCS, the spleens were harvested, and splenocytes were stained with APC-labeled human HLA-A*0201 tetramer loaded with YLNKIQNSL and PE-labeled anti-human CD8 Ab (BioLegend). The percentage of HLA-A*0201-restricted, PfCSP-specific $CD8^+$ T cells among the total human $CD8^+$ T cell population was determined using a BD™ LSR II flow cytometer (Franklin Lakes, NJ), as previous performed (Li et al., 2016).

Modified vaccinia ankara construction and seed stock preparation

Two recombinant MVAs, MVA-5x.LD01 and MVA-5x.LD10, were constructed that encode five repeats of LD01 or LD10 sequences in polycistronic format. A tissue plasminogen activator signal sequence was added prior to LD01 or LD10 to route the peptides for secretion from the cell, and a dual cleavage site composed of the porcine teschovirus-1 2A sequence followed by a furin cleavage peptide were added following the LD sequences to facilitate production of monomer peptides from the polycistronic design. The starting material for recombinant virus production was parental MVA that had been harvested in 1974 before the appearance of Bovine Spongiform Encephalopathy/Transmissible Spongiform Encephalopathy (BSE/TSE) and that had been plaque-purified three times using certified reagents from sources free of BSE. A shuttle vector was used to insert the LD01 or LD10 sequences (U.S. Patent 10,799,581 and EP Patent 3512536) between two essential genes of MVA by means of homologous recombination. The chosen insertion site was previously identified as supporting high expression and insertion stability. Genetic stability of the transgene inserts was confirmed by PCR to ensure the correct size. All inserted sequences were codon-optimized for MVA. Silent mutations were introduced to interrupt homo-polymer sequences (>4G/C and >4A/T), which reduce RNA polymerase errors that possibly lead to frameshift mutations. All vaccine inserts were placed under control of the modified H5 early/late vaccinia promoter. Vectors, Research Seed Virus (RSV), and Research Stocks (RS) were prepared in a dedicated room at GeoVax, with full traceability and complete documentation of all steps using BSE/TSE-free raw materials and, therefore, can be directly used for production of cGMP Master Seed Virus (MSV).

For production of RSV for animal studies, a chicken embryo fibroblast cell line, DF-1 cells (ATCC, CRL-12203), were seeded into sterile tissue culture flasks and infected with MVA-5x.LD01 or MVA-5x.LD10 at an MOI of 0.01. Cells were recovered 3 days post-infection, disrupted by sonication, and bulk harvest material clarified by low-speed centrifugation. The clarified viral harvest was purified twice using sucrose cushion ultracentrifugation. The purified viruses were titrated by limiting dilution in DF1 cells, diluted to 1×10^8 $TCID_{50}$/ml in sterile PBS + 7% sucrose, dispensed into sterile vials, and stored at −80°C. For *in vivo* studies, mice were injected with 50 µl containing 5×10^7 $TCID_{50}$ of MVA into each hind footpad for a total dose of 1×10^8 $TCID_{50}$ MVA per mouse.

Production of anti-LD01/LD10 mAb

KLH-conjugated LD01 peptide formulated in Sigma Adjuvant System® (cat# S6322) was used to immunize SJL/J mice IM. Following two similar IM boosts at 2-week intervals, the mice were culled, and spleens and lymph nodes were collected. Splenocytes and lymphocytes were isolated and fused to HL-1 mouse myeloma cells and cultured for 13 days. On D13, colonies were manually selected and transferred to selection media. Culture supernatants were screened for specificity by ELISA using plate-coated BSA-conjugated peptides. Supernatants were screened against BSA-conjugated LD01 and LD10 peptides. Two clones (3F11 and 7G10) were selected based on their high binding affinity to both peptides and on the high concentration of supernatant Ab. Monoclonal cultures of these two clones were expanded and the supernatants were used to purify the Abs. Cell suspensions, containing at least 8.0×10^7 cells in two T-75 flasks, were aseptically transferred to 2 ml × 50 ml centrifuge tubes and centrifuged at 1,000 rpm for 5 min. The resulting cell pellet was resuspended in 25 ml of HyClone™ HYQSFMMAB media + 5% FBS and slowly added to a 250-ml bag containing 225 ml of HyClone™ HYQSFMMAB media + 5% FBS. The bag was placed in an incubator set at 5% CO_2, 37°C for 10–14 days. After 10–14 days of growth, the contents of the 250 ml bag were transferred to a 250 ml centrifuge bottle, and 10 ml of Neutralization Buffer (1 M Tris, 1.5 M NaCl, pH 8.5) was added to it; it was then centrifuged at 8,600 rpm for 10 min using a Sorvall GSA rotor. The supernatant was filtered using a 0.45-µm bottle top filter.

A 5-ml protein A column connected to a FPLC Purification System was washed with 25 ml of ultra-pure water followed by 25 ml of 50 mM TRIS containing 250 mM NaCl (pH 8.0). The filtered supernatant was loaded onto the column at a flow rate of 7 ml/min. The column was further washed with 15 ml of 50 mM

TRIS, 250 mM NaCl, pH 8.0. Elution fractions were collected in 15-ml tubes containing 800 µl of Neutralization Buffer (1 M Tris, 1.5 M NaCl, pH 7.4). The Ab was eluted with 20 ml of 50 mM glycine (pH 3.0) and dialyzed against 1–2 L of 1× PBS (pH 7.4; depending on the volume of purified Ab) on a stirrer at 4°C overnight. The dialyzed Ab was sterile-filtered and aliquoted for storage.

Dot blot assay

DF-1 cells were infected at a multiplicity of infection of 0.5 with parental MVA, MVA-5X.LD01 or MVA-5X.LD10 and 48 h later supernatant was collected. In order to concentrate secreted peptide, supernatant was passed through Pierce C-18 tips (cat# 87782, Thermo Fisher Scientific). Twenty microliters from each sample and 125 ng of synthetic LD01 peptide were spotted onto a PVDF membrane, allowed to dry at room temperature, then blocked with Intercept blocking buffer (cat# 927-70001, LI-COR Biosciences) for 30 min at room temperature. The membrane was incubated overnight at 4°C in primary Ab (Leidos, clone: 7G10) diluted in blocking buffer at 1:1,000. Three washes with PBST (PBS with 0.05% Tween-20) were performed, and the membrane was probed for 1 h with anti-mouse-680RD (1:10,000; cat# A-21058; Invitrogen). The membrane was then washed again and imaged using Odyssey imager.

Immunocytochemistry assay

DF-1 cells were infected at a multiplicity of infection of 0.5 with parental MVA, MVA-5X.LD01 or MVA-5X.LD10 for 48 h, subsequently cells were fixed in 1:1 methanol:acetone and washed with water. Cells were then probed with a mouse anti-LD01/LD10 Ab (Leidos, clone: 3F11) at room temperature for 1 h. Three washes with water were performed and the cells were stained for 1 h with anti-mouse-IgG-HRP (1:1,000; VWR, cat# 10150-400). The cells were then washed again and developed with AEP substrate kit (cat#ab64252, Abcam). Images of stained cells were captured at ×20 magnification using light microscopy.

Data analysis

Statistical analyses were performed using Prism (GraphPad Software, Inc., La Jolla, CA, United States). The two-tailed unpaired *t*-test was used to determine differences between groups. Data are expressed as mean ± SEM and $p < 0.05$ was considered statistically significant.

Data availability statement

The full complement of data accumulated for these studies is available upon request.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) at The Rockefeller University (Assurance No. A3081-01).

Author contributions

TP and JH contributed equally. GG, VK, TP, JH, MH, AD, SO, and MT contributed to the conceptualization and study design. Data organization and formal analyses were performed by GG, VK, TP, PB, JH, MH, AD, SO, and MT. Methodology was designed and experiments were performed by MH, AD, SO, JH, and MT. Supervision was contributed by GG, VK, TP, MM, and JP. The writing of the original draft was completed by TP, JH, MT, MH, and CB. Manuscript review and editing were performed by JP, GG, PB, and MM.

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Conflict of interest

TP was employed by Thunder Biotech. VK was employed by Leidos Inc. MH, AD, and SO were employed by GeoVax Inc. CB was employed by Thermo Fisher Scientific. PB and JP were employed by The MITRE Corporation. MM was employed by MM Scientific Consultants Inc. GG was employed by Hibiscus BioVentures, LLC.

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/fphar.2022.1029636/full#supplementary-material

SUPPLEMENTARY FIGURE S1
IP and SC administration of LD10da demonstrate similar increases in antigen-specific $CD8^+$ T cell numbers following AdPyCS vaccination. At D12 post-AdPyCS immunization, immunogenicity was assessed by measuring the number of splenic PyCS-specific, IFN-γ-secreting $CD8^+$ T cells using the ELISpot assay after stimulation with the H-2kd restricted CD8 epitope SYVPSAEQI. A 100-μg dose of LD10da was given either IP or SC on D1, D3, D5, and D7 post-immunization. Data are expressed as the mean ± SEM fold increase in the number of IFN-γ spots with the levels from AdPyCS alone taken as one. Data from one of two independent experiments are shown with $n = 5$ per group.

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EDITED BY
Mohammad Asif Sherwani,
The University of Alabama at Birmingham, United States

REVIEWED BY
C. David Pauza,
Viriom Inc., United States
Mengying Zhang,
Biogen Idec, United States

*CORRESPONDENCE
Jerome Zeldis
✉ jzeldis@neximmune.com

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AIM^{TM} platform: A new immunotherapy approach for viral diseases

David Langan, Ruipeng Wang, Keshanti Tidwell, Selome Mitiku, Alison Farrell, Catrina Johnson, Adam Parks, Lauren Suarez, Shweta Jain, Sojung Kim, Kristi Jones, Mathias Oelke and Jerome Zeldis*

NexImmune Inc., Gaithersburg, MD, United States

In addition to complications of acute diseases, chronic viral infections are linked to both malignancies and autoimmune disorders. Lack of adequate treatment options for Epstein-Barr virus (EBV), Human T-lymphotropic virus type 1 (HTLV-1), and human papillomavirus (HPV) remains. The NexImmune Artificial Immune Modulation (AIM) nanoparticle platform can be used to direct T cell responses by mimicking the dendritic cell function. In one application, AIM nanoparticles are used ex vivo to enrich and expand (E+E) rare populations of multi-antigen-specific CD8^{+} T cells for use of these cells as an AIM adoptive cell therapy. This study has demonstrated using E+E CD8^{+} T cells, the functional relevance of targeting EBV, HTLV-1, and HPV. Expanded T cells consist primarily of effector memory, central memory, and self-renewing stem-like memory T cells directed at selected viral antigen peptides presented by the AIM nanoparticle. T cells expanded against either EBV- or HPV-antigens were highly polyfunctional and displayed substantial in vitro cytotoxic activity against cell lines expressing the respective antigens. Our initial work was in the context of exploring T cells expanded from healthy donors and restricted to human leukocyte antigen (HLA)-A*02:01 serotype. AIM Adoptive Cell Therapies (ACT) are also being developed for other HLA class I serotypes. AIM adoptive cell therapies of autologous or allogeneic T cells specific to antigens associated with acute myeloid leukemia and multiple myeloma are currently in the clinic. The utility and flexibility of the AIM nanoparticle platform will be expanded as we advance the second application, an AIM injectable off-the-shelf nanoparticle, which targets multiple antigen-specific T cell populations to either activate, tolerize, or destroy these targeted CD8^{+} T cells directly in vivo, leaving non-target cells alone. The AIM injectable platform offers the potential to develop new multi-antigen specific therapies for treating infectious diseases, cancer, and autoimmune diseases.

KEYWORDS

T cell, ACT, AIM, aAPC, NexImmune, viral, immunotherapy, nanoparticle

Introduction

Many viral infections, if not cleared during the acute stages, may become chronic. Chronic infections are often associated with intermittent recrudescence, autoimmune complications and/or malignancies (1–5). Both the innate and acquired immune systems are involved. Antigen-presenting cells (APCs), especially dendritic cells (DCs), can modulate both types of immunity by displaying antigen derived peptides and cytokine release to direct antigen-specific T cell and other immune cells. In this report, we will describe our work utilizing AIM nanoparticles as artificial APCs (aAPCs), or synthetic DCs, designed to present antigenic peptides in the context of class I human leukocyte antigen (HLA) and simultaneously deliver a second co-stimulation signal to expand antigen-specific T cells for adoptive cell therapies (ACT). In a second application, the AIM nanoparticle is the therapeutic. Injectable nanoparticles with signal 1 (peptide loaded class I HLA) and signal 2 (co-stimulatory anti-CD28) proteins activate and expand viral antigen-specific $CD8^+$ T cells without affecting non-antigen-specific cells. Both the aAPC and AIM injectable approaches simultaneously engage antigen-specific T cells through their peptide loaded HLA molecule and a co-stimulatory second signal that can up or down regulate the function of the engaged T cell. As described below, these approaches offer potential treatments of a broad range of viral diseases.

Adoptive cell therapies has shown increasing promise for treating Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) infections, specifically in immune compromised patients after allogeneic stem cell transplantation (2, 6, 7). These patients may benefit from virus-specific ACT used either prophylactically or therapeutically (2). Additionally, both preclinical models and clinical trials using ACT have supported its utility to treat virally driven malignancies associated with human papillomavirus (HPV) and EBV (2–4). EBV targeted ACT might further elucidate links between EBV infection and multiple sclerosis (5). Multiple challenges need to be addressed for ACTs to expand on early successes. These challenges include addressing disease escape mechanisms, increasing T cell persistence, antigenic heterogeneity including emergence of neo-antigens, reducing on-target off-tissue adverse events, reducing manufacturing cost, and product inconsistency (8, 9).

With the Artificial Immune Modulation (AIM) ACT system, NexImmune may overcome many of these challenges to develop cellular therapies for patients with virally driven diseases (**Figure 1**). Incorporation of magnetic based systems to generate infectious disease specific ACT have shown promise (6, 7), for reasons including the reduction in the manufacturing time compared to other clinically tested methods (10, 11). The AIM Enrichment and Expansion (E+E) system is a reproducible, closed manufacturing process that consistently produces T cells for ACT comprised of non-genetically engineered multiple antigen specific effector and long-lived memory T cells from autologous or allogeneic donor leukopaks (12). Two AIM ACT clinical trials are currently ongoing: one for relapsed/refractory multiple myeloma (MM) [NCT04505813] and another for acute myeloid leukemia (AML) [NCT04284228]. An additional trial for HPV-related Head and Neck cancer will be initiated.

Here we provide an overview of the AIM E+E system used to manufacture T cells for ACT, and report preclinical results from using this system to expand $CD8^+$ T cells directed against viral antigens of Epstein-Barr virus (EBV), Human T-lymphotropic virus type 1 (HTLV-1), or high-risk human papillomavirus (HPV) types 16 and 18 from HLA-A*02:01 healthy donor cells. Each of the final AIM ACT products consists of a mixture of $CD3^+/CD4^-$ T cells that are mostly specific for multiple antigens from EBV, HTLV-1 or HPV with greater than 90% of these T cells being of the T cell memory phenotype [*i.e.*, effector memory (Tem), central memory (Tcm), or stem cell-like memory (Tscm)]. In addition, EBV-specific AIM ACT cells demonstrate functional activity in terms of antigen-specific cytotoxic killing and cytokine profile.

Materials and methods

Nanoparticles, reagents, and cell lines

Briefly, nanoparticles were manufactured by direct conjugation of humanized HLA-Ig dimer and anti-CD28 antibody to MACS Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously (13). Antibodies used for flow cytometry were purchased from Miltenyi Biotec and are described in **Supplementary Table 1**. Cell lines were originally purchased from American Type Culture Collection (ATCC, Manassas, VA). To generate red fluorescence protein (mKate2) expressing cell lines for Incucyte studies, cells were transduced with Incucyte NucLight Red Lentivirus (EF1a, Puromycin) and selected under 2 μg/ml puromycin (Sartorius, Goettingen, Germany). The amino acid sequence of HLA-A*02:01 and beta-2-Microglobulin (ß2M) (GenBank AYV97550.1 and AAA51811.1) were codon optimized and cloned downstream of separate EF1α promoters in a PiggyBac cDNA cloning vector (PB533A-2, System Biosciences, Palo Alto, CA). The expression vector was co-transfected into HeLa cells along with a PiggyBac transposase helper plasmid from System Biosciences (PB210PA-1) to mediate transposition into the host genome for stable cellular expression of membrane-bound HLA-A*02:01 and ß2M. Cells were passaged in 1 mg/ml G418 for selection of stable expression from the cell lines (**Supplementary Figure 1**). CellEvent Caspase-3/7 Green Detection Reagent, CellTrace Far Red, and Sytox Blue Dead Cell Stain were purchased from Thermo Fisher Scientific.

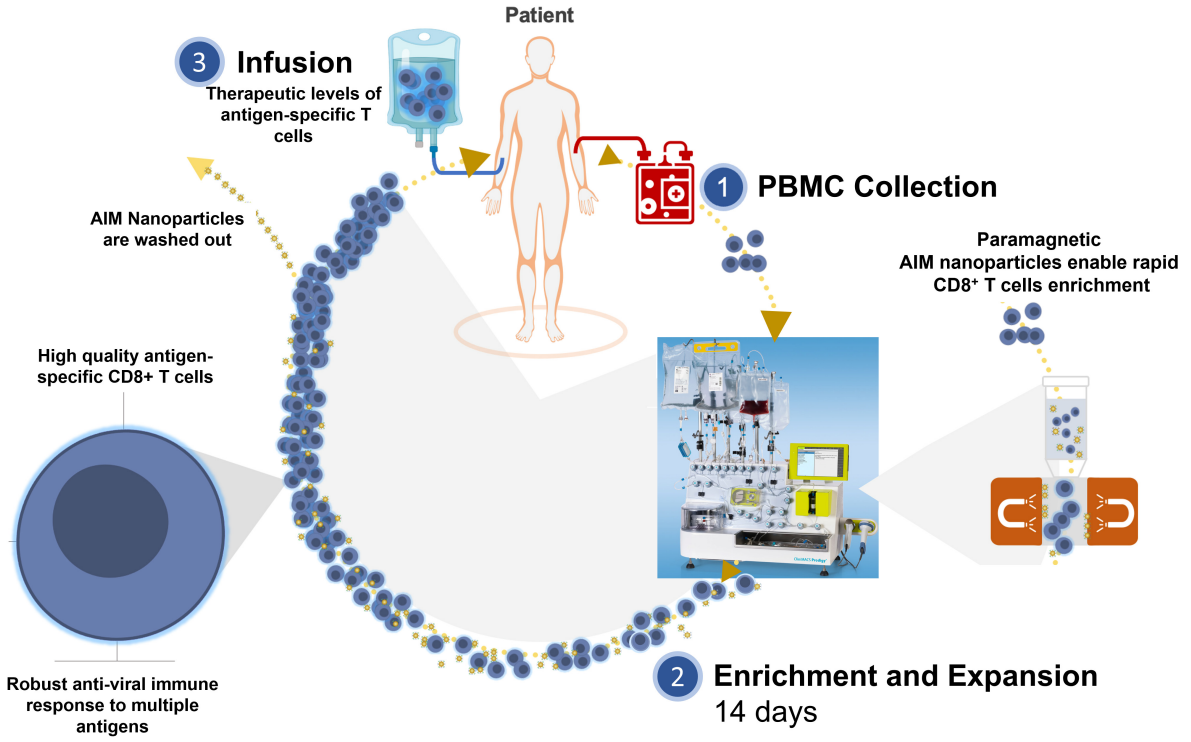


FIGURE 1
Artificial Immune Modulation Adoptive Cell Therapy (AIM ACT). (1) Leukapheresis material is collected from a patient with infection (*e.g.*, EBV, HTLV-1, HPV, and HIV). (2) The $CD8^+$ T cells are enriched for using paramagnetic AIM nanoparticles that function as artificial APCs (aAPCs) to expand $CD8^+$ T cells specific for immunodominant viral antigens. (3) After 14 days expansion, AIM nanoparticles are washed out and the AIM ACT is ready to be infused into patients.

Enrichment and expansion of T cells

Leukapheresis mononuclear cells from HLA-A*02:01 healthy donors were obtained from commercial vendors. Donors were not tested for EBV, HTLV-1, or HPV. Cells were processed according to the NexImmune AIM platform to produce an ACT, as previously described (14). As previously described, on day 0, after $CD4^+$ T cell depletion, $CD8^+$ T cells were enriched on a CliniMacs Prodigy (Miltenyi Biotec) using AIM nanoparticles loaded with the peptides listed in Table 1 (12). The enriched cells were seeded into a G-Rex culture system (Wilson-Wolf Manufacturing) and expanded for 14 days. The E+E cells were collected on Day 14 to freeze and store in liquid nitrogen until further testing.

Cell counting and flow cytometry analysis

Donor cells were counted with the NucleoCounter NC-200 (Chemometec, Lillerod, Denmark). Dead cells were stained with Zombie Aqua for 10 min in the dark. Surface staining of cells was done at 4°C for 10 min with anti-human antibodies in PBS supplemented with 2% FBS and 0.01% sodium azide (FACS Buffer). Cells were then washed once with FACS buffer before analyzing. For the intracellular cytokine assay, cells were stained with anti-CD107a overnight during the period of activation. Then cells were washed once with PBS before staining with Zombie Aqua (BioLegend, San Diego, CA) and washed again with FACS buffer before surface staining as described above. Next cells were fixed and permeabilized with 1X eBiosciences Fixation/Permeabilization solution. Intracellular staining was performed with antibodies in 1X eBioscience Permeabilization Buffer. Cells were then washed twice with FACS Buffer and analyzed. For the flow-based killing assay, nuclear RFP expressed endogenously was used to detect tumor cells and CellTrace Far Red was used to detect PBMCs. Cells were analyzed on a MacsQuant10 or MacsQuant16 flow cytometer (Miltenyi Biotec). Data was analyzed with FlowLogic software (Inivai Technologies, Mentone, Australia).

Culturing tumor cell lines and E+E cells

Cell lines were cultured in RPMI supplemented with 10% fetal bovine serum (16140-071), 1 mM sodium pyruvate (11360070), 1X MEM non-essential amino acids (11140-035),

TABLE 1 HLA-A2 restricted peptides from viral driven diseases tested in the AIM ACT system.

Virus	Name	Protein	Sequence	Diseases
HTLV-1	Tax LLF	Tax	LLFGYPVYV (20)	Adult T-cell leukemia/Lymphoma (ATL), HTLV-1-associated myelopathy
HTLV-1	Tax SFH	Tax	SFHSLHLLF (19)	
HTLV-1	GPP ALL	Pol	ALLGEIQWV (55)	
HTLV-1	GPP SLI	Pol	SLISHGLPV (55)	
HTLV-1	GPP FMQ	Gag	FMQTIRLAV (55)	
EBV	LMP2 CLG	LMP2	CLGGLLTMV (56)	Infectious mononucleosis, Multiple sclerosis, chronic active EBV (CAEBV), lymphoproliferative disease, certain malignancies including lymphomas and carcinomas
EBV	LMP2 FLY	LMP2	FLYALALLL (57)	
EBV	BMLF1	BMLF1	GLCTLVAML (58)	
EBV	BRLF1	BRLF1	YVLDHLIVV (59)	
EBV	EBNA3	EBNA3	LLDFVRFMGV (60)	
EBV	LMP1	LMP1	YLQQNWWTL (61)	
HPV-16	HPV2	E7	YMLDLQPETT (62)	Squamous cell carcinomas (*e.g.*, head and neck, cervical, anal, penile), warts
HPV-16	HPV21	E6	TIHDIILEC (63)	
HPV-18	HPV38	E6	LFVVYRDSI	
HPV-18	HPV42	E7	FQQLFLNTL (64)	
N/A	SVN1	Human /BIRC5	QMFFCFKEL (65)	

1X 2-Mercapthoethanol (21985-023), and 1X MEM Vitamin (11120052). These Gibco cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA). Human PBMCs or E+E cells were thawed from cryopreservation the day before *in vitro* assay testing and allowed to rest overnight in TexMACS medium (Miltenyi Biotec) with cytokine supplements (14). The following day, tumor cells, PBMCs, or E+E cells were prepared in single suspension with fresh media before performing specific assays as described below.

Intracellular cytokine staining (ICS) assay

E+E cells were prepared in fresh TexMACS the day after thawing and $1.0x10^5$ cells/well were seeded into a 96-well round-bottom plate (Corning, Corning, NY). Either peptide loaded nanoparticles or phorbol 12-myristate 13-acetate/Ionomycin in Invitrogen's Cell Stimulation Cocktail (plus protein transport inhibitors, 500X) (Thermo Fisher Scientific) were used to stimulate cells. Cells alone with Invitrogen's eBioscience Protein Transport Inhibitor Cocktail (Thermo Fisher Scientific) were used as the unstimulated control and when stimulating cells with peptide loaded nanoparticles. Cells were fixed, permeabilized, and stained before analyzing as described above.

In vitro incucyte-based killing assay

Incucyte compatible tumor cell lines expressing red fluorescence protein (mKate2) were generated by transducing cells with Incucyte NucLight Red Lentivirus (EF1a, Puromycin) according to manufactures recommendation (Sartorius). Tumor cells were seeded at $5.0*10^3$ cells per well in a clear 96-well flat-bottom plates (Corning) the evening prior to starting a killing assay. The following day E+E cells were added to wells to achieve the desired effector to target ratio. For assessment of antigen-specific killing by E+E cells, A375 tumor cells were pulsed with an EBV peptide or irrelevant peptide (10 μg/ml) 2-4 h prior to adding the E+E cells. For negative controls no E+E cells were added. Phase-contrast and fluorescent images were taken every 4 h using the Incucyte S3 (Sartorius). IncuCyte S3 2019A software was used to analyze images. From each image RFP- puncta corresponding to tumor cell nuclei were enumerated and reported as relative cell number normalized to the starting cell number.

Flow cytometry caspase-3/7-based killing assay

Autologous donor PBMCs were washed with non-supplemented RPMI and stained at 37°C for 20 min with CellTrace Far Red (0.1 μM). After staining, cells were washed twice with supplemented RPMI. Tumor cells expressing nuclear RFP were dissociated into single cell suspension using Accutase solution (STEMCELL Technologies) and washed with supplemented RPMI. Donor PBMCs or tumor cells were then added to 96-well round-bottom plates (Corning) at $5.0*10^4$. E+E cells were then added to wells to achieve the indicated E:T ratio. Controls received no E+E cells. CellEvent Caspase-3/7 Green was added to each well (250 nM final). After culturing at 37°C for 4-7 h, SYTOX Blue dead cell stain was added (0.5 μM final) and plates were incubated in the dark for 5 min before flow cytometry analysis.

Calculations and statistical analysis

The percentage of antigen-specific killing in the incucyte killing assays was calculated as follows:

$$\%\ Antigen\text{-}specific\ killing = \frac{(Mean\ \%\ tumor\ cell\ number\ in\ controls - \%\ tumor\ cell\ number\ with\ peptide\ pulsed)}{(Mean\ \%\ tumor\ cell\ number\ in\ controls)}$$

The percentage of caspase 3/7 positive tumor or autologous PBMC cells was calculated as follows:

$$\%\ Caspase\ 3/7\ positive = \frac{(\%\ positive\ with\ E+E\ cells - Mean\ \%\ positive\ in\ controls)}{(100\% - Mean\ \%\ positive\ in\ controls)}$$

Statistical difference between groups was tested by either student T-test or ratio paired T test as indicated using GraphPad Prism 9 software. Significant difference is reported where p-values are ≤ 0.5.

Results

HTLV-1 and EBV antigen specificity of AIM E+E T cells

The list of HLA-A2 restricted EBV, HTLV-1, and HPV peptides used in the AIM E+E manufacturing system are listed in **Table 1**.

Using the AIM E+E system with EBV peptides, the final expanded T cells included significant numbers of antigen-specific $CD8^+$ T cells to both lytic (BMLF1 and BRLF1) and latent (LMP1, LMP2, and EBNA3) EBV antigens. Six EBV peptides from the following proteins LMP2, BMLF1, BRLF1, EBNA3, and LMP1 were used to generate AIM ACT from two healthy donors. The estimated frequency of the E+E $CD8^+$ T cells to each EBV peptide ranged from 0.25% to 36.77% (**Figure 2A**). The total frequency of all cells to the six EBV peptides was 95.45% for EBV Donor 1 and 69.23% for EBV Donor 2. Among the E+E cells from both donors, the frequencies to LMP2 FLY (36.77% and 19.0%), BMLF1 (18.29% and 15.02%), and BRLF1 (22.6% and 15.95%) were the highest, whereas the frequency to LMP2 CLG (2.56% and 0.25%) was relatively low.

Using the AIM E+E system with HTLV-1 peptides, the summed total frequency of E+E $CD8^+$ T cells to all five HTLV-1 peptides was 46.69% for Donor 1 and 17.64% for Donor 2. The frequency to each antigen ranged from 0 to 34.06% (**Figure 2B**). The frequencies to Tax LLF (33.06% and 9.62%), GPP FMQ (8.55 and 6.56%), and Tax SFH (3.3% and 1.45%) were the highest among expanded cells from both donors, whereas $CD8^+$ T cells showed a relatively low frequency to GPP ALL (0.34% and 0.0%) and GPP SLI (0.44 and 0.01%).

Memory phenotype of AIM E+E T cells

For a T cell ACT to be potent and durable, it will ideally consist of a heterogeneous population of memory T cell phenotypes that can combine cytotoxic capacity with long-term persistence and immunologic memory (12). We define Memory T cells as those with the phenotype of either Tem ($CD62L^-CD45RA^-$), Tcm ($CD62L^+CD45RA^-$), or Tscm ($CD62L^+CD45RA^+CD95^+$).

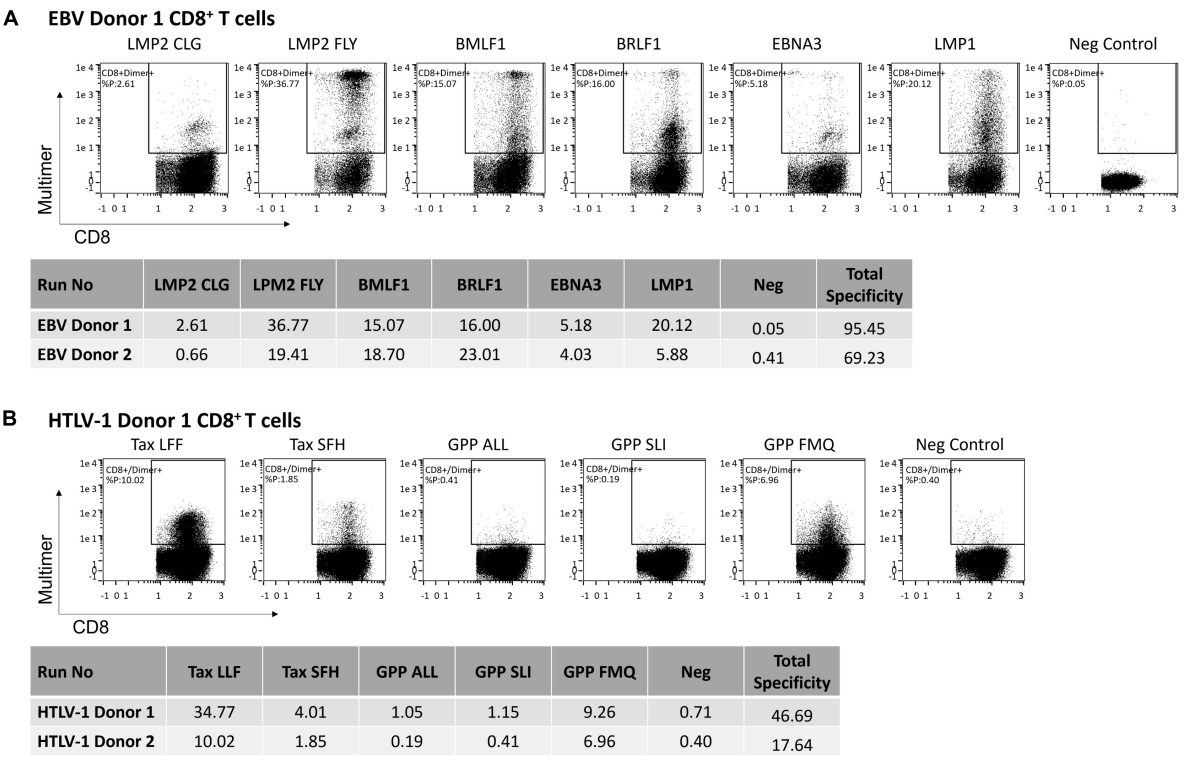
The AIM platform consistently expanded clinically relevant numbers of T cells containing greater than 90% Tscm, Tcm, and Tem cells in 14-days. **Figure 3** displays the memory phenotypes of T cells post-enrichment from leukopaks from two healthy donors and again on day 14 after expansion using EBV peptide loaded nanoparticles. The Tem compartment was the largest subset (58-59%) among the T cells immediately post-enrichment. After expansion (Day 14), the Tem subset of total cells was reduced to 30-44% with a shift in phenotype favoring the Tcm subtype from 12-13% to 47-66% of total cells. Tcm cells are the primary reservoir of proliferating T cells and are responsible for immunologic memory. Of the expanded total T cells, expression of CD95 by some $CD62L^+CD45RA^+$ T cells showed that 4-5% were Tscm. Both naïve T cells (Tn) and terminally differentiated effector memory T cells expressing CD45RA (Temra) cells constitute less than 5% of the total T cell population.

Polyfunctional activity of EBV specific AIM E+E T cells

EBV-specific AIM ACT cells from two healthy donors were generated using AIM nanoparticles loaded with the 6 different EBV antigen-peptides described below and in **Table 1**. Assessment by intracellular cytokine staining (ICS) showed that most of the T cells responded to a peptide by expressing Type1 cytokines, IFNγ and TNFα, and by displaying surface CD107a (**Figure 4**). Although there was T cell specificity to LMP1 (5.47 and 20.07%), upon stimulation with LMP1 loaded nanoparticles very few cells expressed cytokines. The total percentage of $CD8^+$ T cells that responded to these EBV peptides ranged from 80 to 100%.

Antigen specific cytotoxicity of AIM E+E cells

Adoptive cell therapies that targets multiple viral antigens may help to overcome issues that arise from circulating T



Run No	LMP2 CLG	LPM2 FLY	BMLF1	BRLF1	EBNA3	LMP1	Neg	Total Specificity
EBV Donor 1	2.61	36.77	15.07	16.00	5.18	20.12	0.05	95.45
EBV Donor 2	0.66	19.41	18.70	23.01	4.03	5.88	0.41	69.23

Run No	Tax LLF	Tax SFH	GPP ALL	GPP SLI	GPP FMQ	Neg	Total Specificity
HTLV-1 Donor 1	34.77	4.01	1.05	1.15	9.26	0.71	46.69
HTLV-1 Donor 2	10.02	1.85	0.19	0.41	6.96	0.40	17.64

FIGURE 2
The frequency of antigen-specific CD8$^+$ T cells among cells enriched and expanded to target HLA-A2 restricted viral peptides. **(A)** EBV peptides (6 total) or **(B)** HTLV-1 peptides (5 total) were used to generate E+E cells from two healthy donors. Reported is the frequency of CD8$^+$ T cells that bind peptide-loaded HLA-A2-Ig dimer. Also shown is the total frequency of CD8$^+$ T cells to each of the EBV or HTLV-1 peptides. Total frequency is normalized using an irrelevant peptide, the negative control.

cell heterogeneity between donors and antigenic heterogeneity of infected cells. This heterogeneous immune response is illustrated by our observations made from leukopaks from two health donors. EBV-specific expanded T cells were cocultured with HLA-A2 positive target cells that were pulsed with one of the six EBV peptides used for enrichment and expansion. The expanded T cells from Donor 1 had antigen-specific cytotoxic activity directed to all six peptides but the cells from Donor 2 were directed to only 4 peptides. The greatest cytotoxic activity was directed against LMP2 FLY, BLMF1, and BRLF1 (Figure 5). This corresponds to the same peptides for which the highest frequencies (Figure 2) and polyfunctional activities (Figure 4) were observed.

NEXI-003 is an ACT therapy targeting HPV-related cancers. The HPV-16 and HPV-18 strains are targeted using peptides from the immunodominant E6 and E7 antigens of these strains and by adding a fifth peptide from the tumor-associated antigen Survivin. In Table 2 and Figure 6, the numerical, phenotypic, and functional characterization of the preclinical runs (n = 4) for NEXI-003 are summarized. In Supplementary Figures 2, 3, the gating strategy used to determine the frequencies of these cell subsets are shown with representative FACS plots. These expanded cells consisted of highly pure populations of CD3$^+$/CD4$^-$ T cells, greater than 90% (96.82% ± 2.89%). Cells were 69% ± 13% CD8$^+$ T cells and these CD8$^+$ T cells showed multi-antigen specificity to these HPV-related cancer antigens with a total frequency ranging from 17.66% to 56.34%.

Preclinical validation of NEXI-003: An AIM ACT to treat HPV-related cancer

The mean total memory phenotype of the T cells was 95% (Figure 6B) which is consistent with what has been observed with the EBV-antigen expanded AIM ACT (Figure 3), as well as other AIM ACT against HTLV-1 and various tumor-associated antigens (Data not shown). An average of 71% of T cells produce 3-4 functional markers (*i.e.*, CD107a, IL-2, IFNγ, and TNFα) upon non-specific stimulation with PMA/ionomycin (Figure 6C). The AIM ACT cells consistently showed cytotoxic activity directed at both HPV-16 and HPV-18 positive HLA-A2 expressing tumor cells lines (Figures 6D, E). *In vitro* testing of the HPV-related cancer specific T cells determined that no significant cytotoxic activity was directed at autologous PBMCs (Figure 6F). In Figure 6G, a representative killing assay with

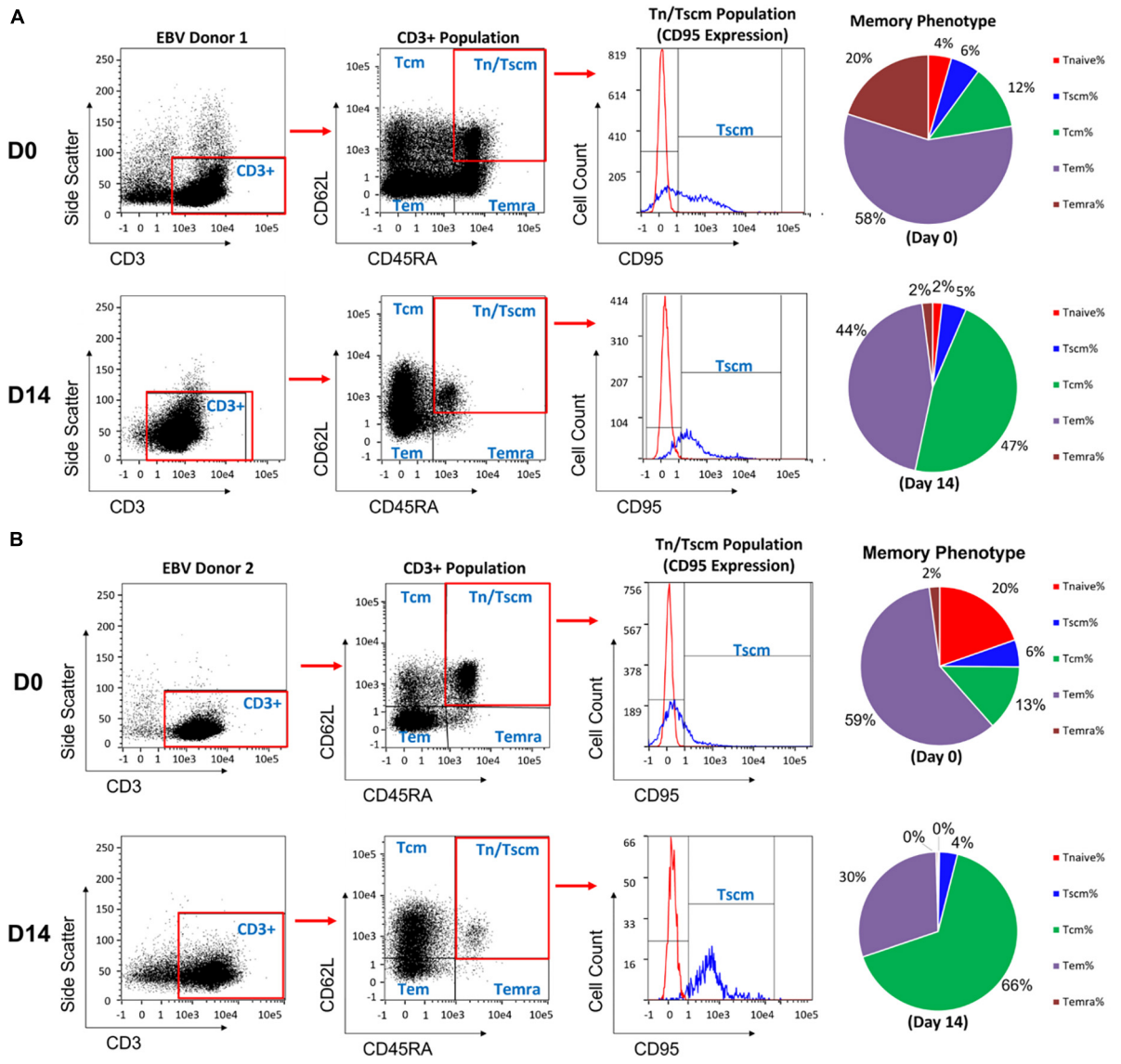


FIGURE 3
Phenotype of the T cells on day 0, before expansion, and on day 14 after enrichment and expansion for EBV antigen specific T cells using the AIM ACT system. **(A,B)** Results are shown from two healthy donors. After completion of expansion, T cells from each run were taken for phenotyping memory cell populations by staining for CD62L vs CD45RA. The naïve and stem cell-like memory cells were further distinguished by CD95 expression. The pie charts show the distribution of memory T cell subsets at day 0 and day 14 of the process. Central memory T cells (Tcm), effector memory T cells (Tem), effector memory T cells re-expressing CD45RA (Temra), stem cell-like memory T cells (Tscm), naïve T cell (Tn).

HPV Donor 2 expanded cells shows sustained cytotoxic activity *in vitro* over 72 h.

Discussion

Central to the AIM ACT application and used to manufacture ACT products for virally driven diseases are paramagnetic nanoparticles that are designed to display HLA-Ig dimer molecules loaded with disease relevant peptides (signal 1) as well as co-stimulatory anti-CD28 antibody (signal 2). Antigen peptide loaded AIM nanoparticles selectively enrich, activate, and expand populations of antigen-specific $CD8^+$ T cells from the naïve and memory repertoire (15). AIM nanoparticles were previously shown to elicit T cell responses to a dominant peptide from CMV, pp65 (NLVPMVATV) (16). Ongoing experiments to evaluate AIM nanoparticles loaded with different viral peptides are focused on EBV, HTLV-1, and HPV. The intention is to expand this work to other viruses including HIV.

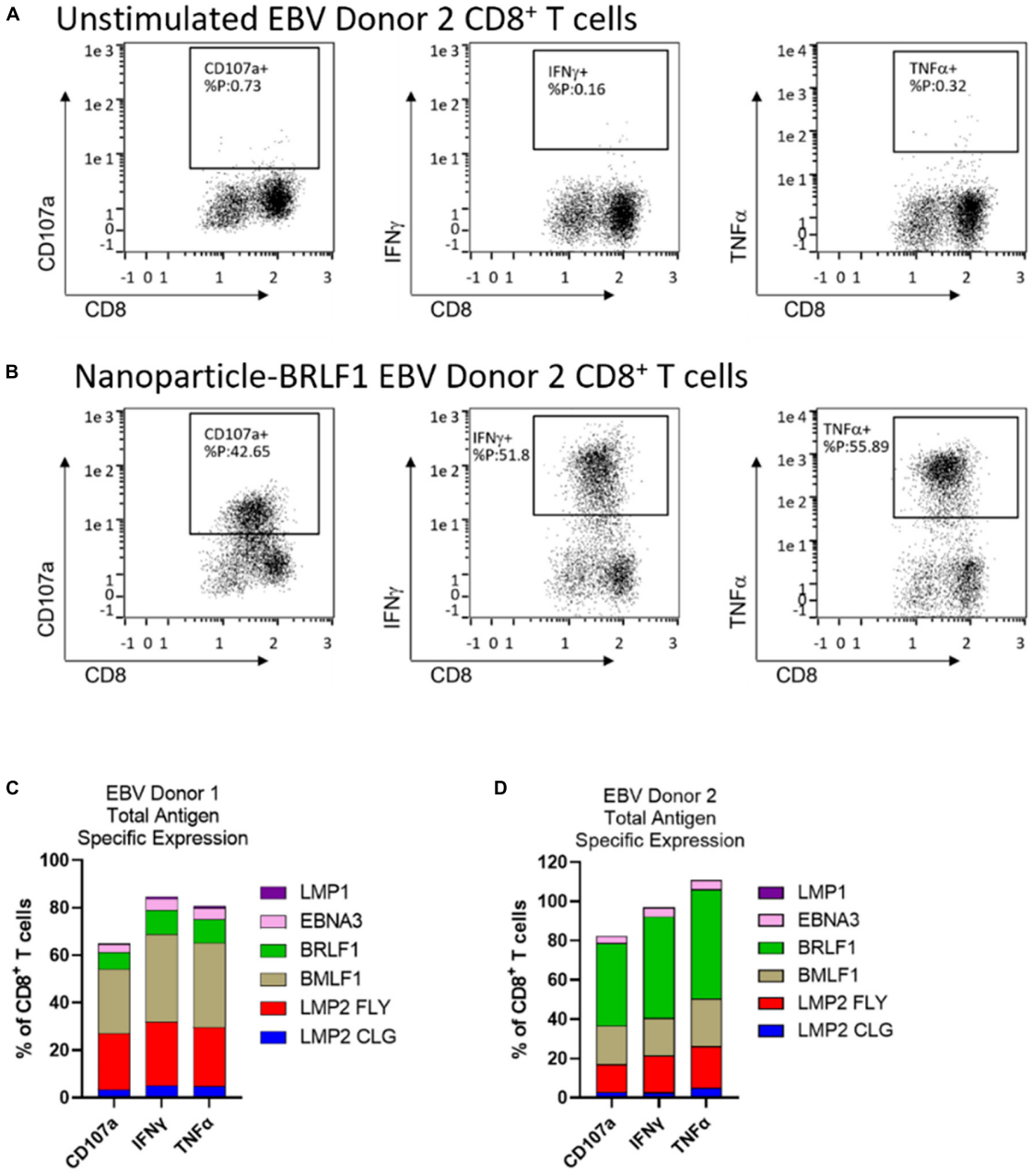


FIGURE 4
AIM ACT cells selected on 6 EBV peptides produce a polyfunctional response upon antigen-specific restimulation. Intracellular cytokine staining was run on EBV antigen-specific E+E cells from 2 healthy donors. Cells were left unstimulated or were restimulated with nanoparticles loaded with one of the six EBV peptides. Representative plots show the expression of functional markers by $CD8^+$ T cells from EBV Donor 2 when **(A)** left unstimulated or when **(B)** stimulated with BRLF1 loaded nanoparticles. The cumulative percentage of $CD8^+$ T cells from **(C)** EBV Donor 1 and **(D)** EBV Donor 2 that express each functional marker in response to stimulation with peptide loaded nanoparticles. Conditions were run in duplicate wells.

Most adults have been infected with EBV at some time during their life. Often adolescent and adult infections result in mononucleosis. $CD8^+$ T cell responses to both lytic and latent antigens occur, but at a higher frequency for some lytic antigens (17, 18). Our results are consistent with previous findings that show the peptides we tested are immunodominant antigens in donors that are likely to have been previously infected with EBV. Multiple experiments are ongoing to characterize and compare the final T cells derived from EBV infected patients with or without EBV-related disorders. HTLV-1 viral infections are associated with adult T cell leukemia/lymphoma (ATL) and a progressive neurological disorder known as

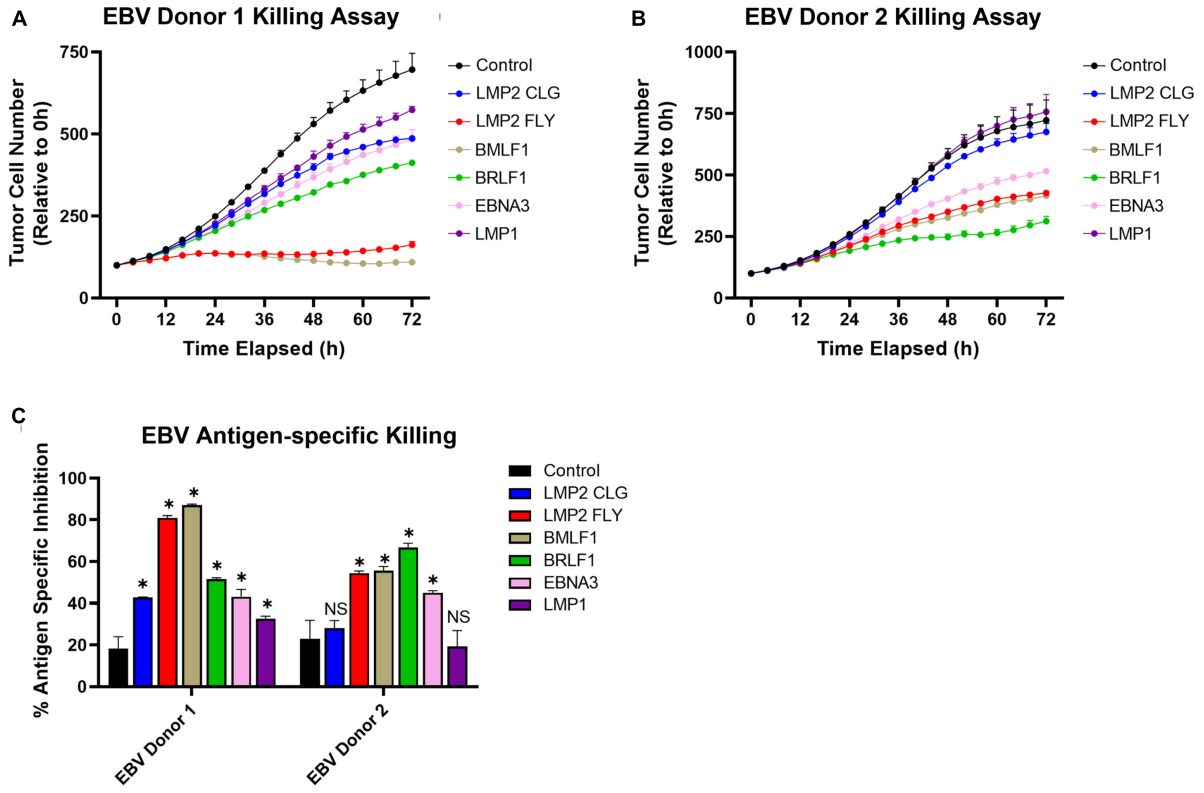


FIGURE 5
Multi-antigen specific killing by EBV specific E+E cells from 2 healthy donors. A375 target cells were pulsed with peptides (10 ug/ml); one of six peptides used to enrich and expand T cells, a control HLA-A2-restricted peptide, or no peptide. Then T cells were added to coculture for 72 h at an effector to target ratio of 10:1. Target cells were enumerated by fluorescent microscopy to identify RFP expressing live A375 cells. **(A,B)** Reported is the relative number of tumor cells compared just after adding E+E cells (0 h). **(C)** Reported is the percentage reduction in tumor cell number at 72 h compared to when no effector cells were added. Statistical difference was determined by student T test comparing the relative tumor cell number at 72 h for each condition with when the control peptide was pulsed ($p > 0.05$ = NS, $^*p \leq 0.05$). Conditions were run in duplicate wells.

TABLE 2 With the AIM platform a highly pure population of T cells is consistently generated for NEXI-003.

Donor	Viability	$CD3^+$ %	$CD8^+$ T cell %	γδ T cell %	$CD4^-$ T cell %
Donor 1	94.6	96.63	83.86	5.43	95.36
Donor 2	88.6	96.15	60.13	32.89	95.53
Donor 3	88.1	98.48	76.69	15.04	96.29
Donor 4	89.7	99.48	56.34	39.92	99.33

* $CD3^+$ %, $CD8^+$ T cell %, and $CD4^-$ T cell % from identity staining panel.
* γδ T cells % taken from TCR staining panel.

HTLV-1 associated myelopathy or tropical spastic paraparesis (HAM/TSP). Developing an AIM ACT against the HTLV-1 virally infected cells is showing promise. The antigen peptide targets we used have been described and show that peptides from the Tax 1 protein are immunodominant and predominant to those of Gag and polymerase (Pol) (19, 20).

The AIM E+E clinical system employed in these experiments is a two-step closed process that uses the CliniMacs Prodigy (Miltenyi) for the enrichment of antigen-specific $CD8^+$ T cells and the GREX culture system for expansion of the specific $CD8^+$ T cells over a 14 day period (Figure 1). Since the AIM nanoparticles present only HLA class I restricted peptides and $CD4^+$ T cells have been found to expand non-specifically under these culture conditions eventually outgrowing the antigen-specific $CD8^+$ T cells of interest, $CD4^+$ T cells are initially removed. This also removes potential regulatory T cells from the culture and therefore from the final product, which could impact the expansion of the antigen-specific T cells as well as the functionality of the ACT. In murine models, regulatory T cells have been shown to restrict $CD8^+$ T cell memory and inhibit tumorigenic immunity elicited by $CD8^+$ and $CD3^+/CD4^-CD8^-$ T cells (21, 22). AIM ACT consist of ≥ 90% $CD3^+/CD8^+$ and $CD3^+/CD4^-CD8^-$ T cells, the latter being primarily gd T cells. Several recent reviews highlight the increasing interest in using gd T cells for cancer treatment and more recently for viral infections (23–26). In our AIM ACT trials we have confirmed that E+E cells are well tolerated with no induced graft-vs-host disease (GVHD) or other grade 3-4

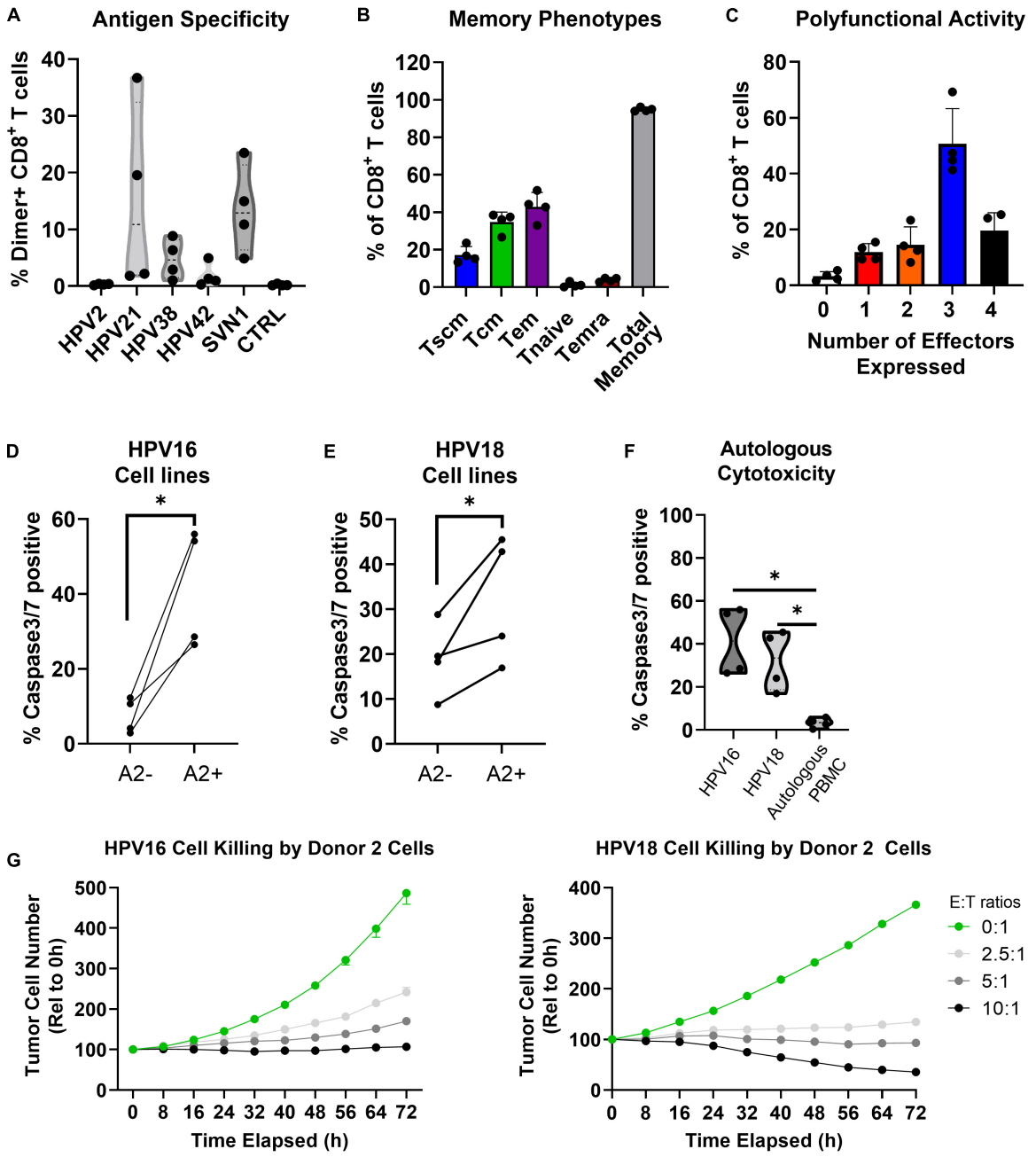


FIGURE 6
In the preclinical validation of NEXI003, the AIM platform consistently generated high quality cytotoxic T cells for HPV-associated cancer treatment. Results are reported from four independent runs for the validation of NEXI-003 (n = 4). **(A)** The percentage of peptide specific (Dimer+) cells among CD8$^+$ T cells (Mean ± StdDev). **(B)** The memory phenotype of CD8$^+$ T cells. **(C)** The percentage of CD8$^+$ T cells that express 0-4 total functional markers (*i.e.*, CD107a, IL-2, IFNγ, and TNFα) upon stimulation with PMA/Ionomycin (Mean ± StdDev). **(D–F)** The percentage of live target cells that were caspase-3/7 positive after coculture with E+E cells at a 10:1 E:T ratio. Target cells that were HLA-A2 positive (*i.e.*, CaSki or HeLa-A2) are directly compared to those that were HLA-A2 negative (*i.e.*, SiHa or HeLa) or were autologous PBMCs. Statistical difference tested by ratio paired T test (*$p \leq 0.05$). **(G)** HPV-16$^+$ or HPV-18$^+$ HLA-A2 expressing cell lines (*i.e.*, CaSki or HeLa-A2, respectively) were cultured for 72 h alone or with E+E cells from Donor 2 at E:T ratios of 2.5:1-10:1. Reported is the tumor cell number relative to the 0 h (Mean ± SEM; n = 3). Effector cell to target cell ratio (E:T ratio).

adverse events while observing clinical activity and persistence of our infused antigen-specific CD8$^+$ T cells (27). Different methods of generating ACT for viral diseases have been applied in the clinic with some clinical success (2–4, 6, 7). In a small clinical trial with 10 HSCT patients, monocyte-derived DC expanded allogeneic donor-derived T cells specific to 4 viruses

were given a median of 63 days post-transplant. No reactivation of EBV, adenovirus, or varicella zoster virus was observed and 6 of 10 patients showed CMV reactivation (28). Only 1 patient required additional antiviral therapy after T cell infusion and no patient died during the 12 months of follow-up (28). Patients displayed anti-viral immunity against all 4 viruses after infusion. Also, grade II-IV acute GVHD occurred in 3 of the patients following T cell infusion. This supports the use of ACT for such disease treatment and prevention.

While some *ex vivo* T cell expansion protocols produce large numbers of T cells, in many cases these cells include more Temra cells with short-lived clinical potency. It is believed that terminally differentiated Temra are short-lived and die soon after transfer and antigen exposure. Temra cells are a significant proportion of the DC mediated T cell expanded products and may not be able to control malignancies on a long-term basis due to lack of persistence (29–31). This was also shown with an ACT for viral infections. CMV-seropositive HSCT patients infused with less differentiated CMV-specific memory T cells were conferred prolonged protection from CMV-reactivation compared to patients that received more terminally differentiated memory cells (32). The less differentiated T cell compartments (*i.e.*, Tscm and Tcm) are capable of self-renewal and replenish the Tem compartment cells. This is critical for immunological memory and the persistence of ACT T cells after infusion (32, 33). These less differentiated subsets of T cells associated with long-term survival are among the T cell populations that are enriched and expanded using the AIM platform. In murine and non-human primate models, $CD8^+$ T cells with a less differentiated memory phenotype (similar to human Tscm) engraft more efficiently and persist longer *in vivo* (33–36). Ichikawa et al. compared cell expansion systems using anti-CD3/CD28 Dynabeads, autologous DCs, or AIM nanoparticles to expand MART-1 antigen-specific $CD8^+$ T cells from melanoma patients (14). MART1-specific $CD8^+$ T cells demonstrated higher expansion using DCs and AIM nanoparticles vs. Dynabeads, approximately 1,000-fold from pre-enriched numbers. Expansion conditions with Dynabeads may not have been optimal but may also perform better when antigen-specific expansion is unnecessary such as with genetically modified cell therapies, like CAR T cell systems (37). The percentage of MART-1 specific expanded cells on day 14 was 10-times greater with AIM nanoparticles than either DCs or Dynabeads. Not only did the AIM nanoparticle provide the highest antigen-specific cell numbers, but these cells also had a greater percentage of self-renewing Tscm, longer telomers, and less terminally differentiated T cells than DC expanded cells from the same donors. Noteworthy, the resulting phenotype is the same whether the starting population comes from a healthy donor or a donor with cancer (14). Similarly, the EBV-antigen specific cells that are enriched at D0 are likely to have a predominance in memory phenotypes, as compared to antigen-specific T cells for other AIM ACT indications, because greater than 90% of the general population has been exposed to EBV. These results suggest that previous exposure to a virus may not detract from the ability to generate AIM ACT with memory T cells, including Tscm that are functional and capable of eliminating infected cells.

In addition to therapeutic safety and persistence, a potent anti-viral response is important to clinical success. The anti-viral or anti-cancer activity of $CD8^+$ T cells is mediated by a polyfunctional Type1 cytokine response (*e.g.*, IL-2, IFNγ, and TNFα) (38, 39). Autologous IFNγ signaling, for example, increases $CD8^+$ T cell numbers and cytotoxic activity (40, 41). In a separate study comparing aAPCs and monocyte-derived DC expanded viral antigen-specific T cells, aAPC expanded T cells had a greater percentage of polyfunctional cells expressing Type1 cytokines (42). The T cell polyfunctional response appeared to be regulated, in part, by the initial method of expansion. Monocyte-derived DC expanded T cells showed signs of senescence or exhaustion after multiple rounds of stimulation whereas cells initially activated with aAPC sustained their polyfunctional activity. To control latent virus reemergence, a sustained polyfunctional anti-viral activity is important for preventing disease recrudescence. The total frequency of $CD8^+$ T cells with a Type1 cytokine response to these EBV peptides was equivalent to or greater than the total frequency of EBV peptide-specific cells estimated by multimer staining. The observation from testing EBV Donor 2 E+E cells suggests that some antigen-specific cells may not be detected by peptide loaded multimer staining and that the frequency of cells to some of these peptides may be higher than reported in **Figure 2**. LMP1 specific T cells response to LMP1 loaded nanoparticles was low. Recognition of peptide: HLA by $CD8^+$ T cells can occur without inducing a strong functional response and activation during expansion can lead to exhaustion. The identification of clinically relevant $CD8^+$ T cells targeting LMP1 as well as ACT specific to LMP1 has proven difficult (17, 18). LMP1 remains a promising clinical target because of its expression alongside LMP2, for which we observed strong functional responses from T cells, in tumors without EBV lytic proteins expressed, such as type II latency tumors. In patients with EBV-associated tumors that received an ACT of LMP1/2-specific CD8+ T cell, 28 of 29 patients receiving this ACT as an adjuvant therapy remained in remission at a median of 3.1 years after CTL infusion and of 21 patients with relapsed or resistant disease at the time of CTL infusion, 11 had complete responses (43). Further characterization of the T cell responses to LMP1 and LMP2 by ELISPOT analysis showed that many patients had fewer T cells respond to LMP1 than LMP2 and that the response to LMP1 often waned relatively quickly after infusion.

Two AIM ACT clinical trials are ongoing, and the clinical trial for HPV-related Head and Neck cancer will initiate by year's end. Chronic infection from oncogenic viruses is estimated to contribute to 10% of cancers globally (44, 45). Of the oncogenic viruses, HPV accounts for 4.6-5.2% of cancer,

with HPV-16 and HPV-18 being the most prevalent strains associated with cancers of the cervical, vulva, vagina, penis, anus, and oropharynx. Although vaccines are highly effective at preventing disease, HPV-related cancers have low cure rates and relapsed patients have a poor prognosis (44, 46). The E6 and E7 oncogenic proteins of HPV are ubiquitously expressed in tumor cells and as such are ideal targets for ACT. In current therapeutic vaccination and immunotherapeutic strategies E6 and E7 are the primary target antigens (47, 48). A study looking at predictive markers of cervical cancer concluded Survivin was strongly correlated with HPV cervical cancer (49). The overall cytotoxic activity of E+E cells directed at HPV-positive HLA-A2 expressing cell lines did not appear to be correlated to the γδ T cell percentages. Unlike for AIM ACT, γδ T cell expansion protocols often incorporate phosphoantigens recognized by the gammadelta T cell receptor (TCR) and IL-15 from the common gamma-chain receptor family (50, 51). Most of the $CD3^+/CD4^-CD8^-$ T cells were γδ T cells which have been shown by others to be clinically safe (23, 24), though potential contribution to efficacy is unknown. In clinical trials with AIM ACT for oncological diseases the γδ T cell percentages can vary, and no adverse impact of γδ cells were observed. Also, corroborating the safety of the γδ T cell in ACT are the results showing that high γδ T cell percentages did not contribute to significant autologous PBMC killing (**Figure 6F**).

The AIM E+E manufacturing system incorporates off-the-shelf nanoparticle technology capable of producing clinically relevant numbers of EBV, HTLV-1, and HPV multi-antigen-specific $CD8^+$ T cells from HLA-A*02:01 healthy donor leukopaks. The consistent quantity and quality of final T cells produced are sufficient to support dose escalation Phase I trials for safety and efficacy (cell number data not shown). Multi-antigen-specific $CD8^+$ T cells may be of benefit for treating viral infections and their complications including autoimmune diseases and cancers. Work to enrich and expand viral specific $CD8^+$ T cells from infected patients is proceeding.

Exploiting the AIM platform E+E system also enables high-throughput screening and selection of relevant peptides for other viral diseases. Four HPV peptides for NEXI-003 were selected from the initial list of 44 HLA-A2 restricted peptides in under 2 months (52). Each peptide was tested using the AIM E+E screening system and evaluated based on the ability to enrich and expand HPV-specific $CD8^+$ T cells that can recognize and kill HPV-antigen expressing target cells. These findings for EBV, HTLV-1, and HPV and the previous findings described highlight the potential for using the AIM platform to develop immunotherapies for other viral infections. The flexibility offered by the AIM platform enables substitution of peptides with other viral protein targets and neo-antigens. As a function of the platforms flexibility there is the ability for an individualized immunotherapy that is adapted to a patient's specific antigen heterogeneity, including a shift in antigen expression over time because of immune pressure.

The AIM platform allows for two types of therapies using AIM nanoparticles: adoptive cell therapy (ACT) and direct injectable therapy (INJ) (**Supplementary Figure 4**). Due to their long-lived and self-renewing phenotype, once infused into patients these cells (*i.e.*, NEXI-001 and NEXI-002) were found to expand, persist, and traffic to the site of disease. T cell receptor-sequencing (TCR-Seq) with a lower limit of detection of ∼1 in 1×10^5 cells was performed on the T cell products and patient samples before and after lymphodepletion and ACT. The results confirmed that the T cell product contained many dominant T cell clones that were not detected in the patients' blood at baseline but rapidly expended and persisted after ACT. In addition, multimer staining of the T cell product and patient blood samples after ACT confirmed the presence and expansion of the multimer positive T cells in vivo. While the AIM ACT system expands T cells for infusion *ex vivo*, the AIM INJ system is being developed for direct injection into the patient. Vaccination with peptide pulsed DCs can reconstitute a patient's anti-viral immunity and is a promising method to treat viral infections (53, 54). The AIM INJ is an off-the-shelf therapy that is designed to directly engage the T cells while bypassing host DC's that may be compromised in the face of disease. The INJ nanoparticle delivers simultaneous signaling that enables precise engagement, activation, and expansion of effector $CD8^+$ T cells to kill infected cells and eliminate acute disease, while providing a memory response for sustained clinical protection. We are currently creating new AIM nanoparticles that incorporate other HLA class I subtypes including *A01, *A03, *A11, *A24, and *B7, allowing for the targeting of a broader patient population. In summary, AIM technology represents a new scalable and cost-effective therapeutic platform that has the potential to cure viral diseases.

Data availability statement

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

Author contributions

DL wrote the first draft of the manuscript. RW, LS, SJ, SK, KJ, MO, and JZ contributed to the project conception. DL, RW, KT, SM, AF, and CJ performed the data acquisition. All authors contributed to data analysis, manuscript revision, read, and approved the submitted version.

Conflict of interest

DL, RW, KT, SM, AF, CJ, AP, LS, SJ, SK, KJ, MO, and JZ were employees of NexImmune, Inc.

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Supplementary material

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Saba Tufail,
Aligarh Muslim University, India

REVIEWED BY
Jasim Khan,
University of Alabama at Birmingham, United States
Luca Castagna,
Azienda Ospedaliera Ospedali Riuniti Villa Sofia Cervello, Italy
Sabiha Khatoon,
Texas Tech University Health Sciences Center, United States

*CORRESPONDENCE
Antonio Pérez-Martínez
✉ aperezmartinez@salud.madrid.org

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Familial CD45RA$^-$ T cells to treat severe refractory infections in immunocompromised patients

Karima Al-Akioui Sanz[1], Carlos Echecopar Parente[2], Cristina Ferreras[1], Marta Menéndez Ribes[1], Alfonso Navarro[1], Carmen Mestre[1], Laura Clares[1], José Luis Vicario[3], Antonio Balas[3], Raquel De Paz[4], Eduardo López Granados[5], Elena Sánchez Zapardiel[5], Carlos Jiménez[6], María López-Oliva[6], Esther Ramos[7], Francisco Hernández-Oliveros[8] and Antonio Pérez-Martínez[1,2,9]*

[1]Hospital La Paz Institute for Health Research, Madrid, Spain, [2]Department of Pediatric Hemato-Oncology, La Paz University Hospital, Madrid, Spain, [3]Histocompatibility Unit, Transfusion Center of Madrid, Madrid, Spain, [4]Cell Therapy Unit, Department of Hematology, La Paz University Hospital, Madrid, Spain, [5]Immunology Unit, La Paz University Hospital, Madrid, Spain, [6]Department of Nephrology, La Paz University Hospital, Madrid, Spain, [7]Intestinal Rehabilitation Unit, Pediatric Gastroenterology and Nutrition Unit, La Paz University Hospital, Madrid, Spain, [8]Department of Pediatric Surgery, La Paz University Hospital, Madrid, Spain, [9]Department of Pediatrics, Faculty of Medicine, Universidad Autónoma de Madrid, Madrid, Spain

Background: Immunocompromised patients are susceptible to high-risk opportunistic infections and malignant diseases. Most antiviral and antifungal drugs are quite toxic, relatively ineffective, and induce resistance in the long term. The transfer of pathogen-specific Cytotoxic T-Lymphocytes has shown a minimal toxicity profile and effectiveness in treating Cytomegalovirus, Adenovirus, Epstein - Barr virus, BK Virus and *Aspergillus* infections, but this therapy have the main limitations of regulatory issues, high cost, and absence of public cell banks. However, CD45RA$^-$ cells containing pathogen-specific memory T-cells involve a less complex manufacturing and regulatory process and are cheaper, feasible, safe, and potentially effective.

Methods: We present preliminary data from six immunocompromised patients: four who had severe infectious diseases and two who had EBV lymphoproliferative disease. All of them underwent multiple safe familial CD45RA$^-$ T-cell infusions as adoptive passive cell therapy, containing Cytomegalovirus, Epstein - Barr virus, BK virus, and *Aspergillus*-specific memory T-cells. We also present the method for selecting the best donors for CD45RA$^-$ cells in each case and the procedure to isolate and store these cells.

Results: The infusions were safe, there was no case of graft-versus host disease, and they showed a clear clinical benefit. The patients treated for BK virus nephritis, Cytomegalovirus encephalitis, Cytomegalovirus reactivation, and disseminated invasive aspergillosis experienced pathogen clearance, complete resolution of symptoms in 4-6 weeks and a lymphocyte increase in 3 of 4 cases after 3–4 months. Donor T cell transient microchimerism was detected in one patient. The two patients treated for EBV lymphoproliferative disease underwent chemotherapy and several infusions of CD45RA$^-$ memory T-cells containing EBV cytotoxic lymphocytes. Donor T-cell microchimerism was observed in both patients. The viremia cleared in one of the patients, and in the other, despite the viremia not clearing, hepatic

lymphoproliferative disease remained stable and was ultimately cured with EBV-specific Cytotoxic T-Lymphocytes.

Conclusion: The use of familial CD45RA$^-$ T-cells containing specific Cytotoxic T-lymphocytes is a feasible, safe and potential effective approach for treating severe pathogen infections in immunocompromised patients through a third party donor. Furthermore, this approach might be of universal use with fewer institutional and regulatory barriers.

KEYWORDS

CD45RA$^-$ T cells, adoptive cell therapy (ACT), BK virus (BKV), Epstein-Barr virus (EBV), Cytomegalovirus (HCMV), *Aspergillus*, mDLI

1. Introduction

Advances in supportive care continue to improve the outcomes of immunocompromised patients. However, despite the improvements in achieving early diagnosis, the use of prophylactic therapies and the establishment of early treatment, infectious diseases remain a significant cause of morbidity and mortality in patients with immuno-deficiencies. These immunocompromised patients are also susceptible to Epstein-Barr virus (EBV) infections or reactivations, responsible in many cases for the post-transplantation lymphoproliferative disease (PTLD) (1). These patients are more susceptible to infection due to lack of pathogen-specific T-cell immunity, which is essential for resolving several infectious diseases, especially those caused by viruses (2, 3).

Drug therapies involve costly agents, are associated with side effects and do not act on the main cause of the severity, latency and recurrence of the infection, which is the absence or evasion of specific cellular immunity (4). Cell therapies present a new paradigm in treating both infectious and tumor diseases. Between approved T-cell therapies we find Donor Lymphocyte Infusions (DLI) of Cytotoxic T-Lymphocytes (CTLs) to reestablish the immune system after a transplant or to treat Severe Combined Immunodeficiencies (5–9). The infusion of specific CTLs has also been shown effective and safe in treating refractory infections in immunocompromised patients, especially those infections caused by Human Cytomegalovirus (HCMV), Adenovirus (AdV), EBV and BK Virus (BKV) (4, 10, 11). For patients whose donor lacks virus-specific cellular immune memory, the use of third party CTLs has been shown to have a safe clinical effect and has been associated with long-term viral control (10, 12). For these patients, a higher Human Leukocyte Antigens (HLA)-match has been associated with better systemic survival of the transferred cells (13). However, the generation of CTLs is a costly process in both time and resources, requiring highly skilled manufacturing technologists and the regulation issues can be struggling (10, 14, 15).

A simpler and cheaper alternative to the use of CTLs is the mDLI (memory donor lymphocyte infusion) of CD45RA$^-$ T cells. The CD45RA$^+$ fraction is supposed to cause lethal graft-versus-host disease (GvHD), but the mDLI of CD45RA$^+$ depleted cells has proven to be safe and effective, given that these cells are less alloreactive. That is because of their lower proportion of naïve-T cells and greater proportion of memory T-cells (5, 7, 15–18). CD45RA$^-$ cells from a familiar donor or a third-party donor, which contains pathogen-specific memory T-cells, can be isolated from the peripheral blood of a convalescent donor and infused directly into the patient (6–8, 17, 19, 20).

Here we present a treatment focused on clearing virus and specific fungus by mDLIs of CD45RA$^-$ cells containing pathogen-specific memory T-cells from a healthy donor. The objective is providing and improving the cellular immunity of the patient until he recovers its own one. This procedure might be effective in any T-cell mediated infection, above all when immunocompromised patients present sustained lymphopenia. However, we have tested it only with viruses HCMV, EBV, BKV, and AdV, and the fungus *Aspergillus*, which usually act as opportunistic or latent pathogens that cause problems in immunocompromised patients, and that are becoming increasingly resistant to anti-viral or anti-fungal drugs. With emergent viruses such as SARS-CoV-2, this procedure is being tested in a clinical trial with promising results (20), so we decided to extend this procedure to other infectious diseases. We also present the methodology to detect, isolate and infuse those CD45RA$^-$ cells containing pathogen-specific memory T-cells in a novel cell therapy, with major potential for use in all hospitals in the near future.

2. Materials and methods

2.1. Patients characteristics and diseases

We conducted in the University Hospital La Paz a study with immunosuppressed patients (five children and one adult) who suffered with refractory and severe infectious disease or lymphoproliferative disease associated with EBV that did not respond to classical management. **Table 1** lists the patients' characteristics. In all cases this therapy was applied when antivirals or antifungals ineffective and/or organ toxicity. A general scheme of the whole procedure is represented in **Figure 1**.

2.2. Donor selection, cell processing, and detection of specific memory T-cells

The donors were family members in all cases, and the selection was based on HLA genotype compatibility with the patients after hematopoietic stem cell transplantation (HSCT) (it should be at least

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TABLE 1 Patient characteristics and pathogen-associated disease.

Patient	Sex	Age (years)	Primary disease	Immuno-suppression therapy	Lympho-cytes at time of infection	Infectious disease	Viral copies or GMN at diagnosis (copies/μl)	Standard therapy and duration	Viral copies or GMN after standard therapy (copies/μl)
1	F	37	Kidney transplantation	Prednisone Everolimus Tacrolimus	670/μl	BKV nephritis	5.6×10^5	IS minimization, leflunomide and IGs (4 months)	7.5×10^3
2	M	19	Chronic granulomatous disease and MUD HSCT	Methylprednisolone (0.5 mg/kg/d) Cyclosporin	50/μl	HCMV encephalitis	3.69×10^7 in LCR, 4.97×10^3 in serum	Foscarnet (7 days), ganciclovir and specific HCMV IGs (parenteral and intrathecal) (2 months)	2.3×10^3
3	M	7	Multivisceral transplantation	Methylprednisolone (2 mg/kg/d) Sirolimus	1.310/μl	HCMV systemic infection	1.14×10^3	Ganciclovir (2 months), Foscarnet (1 month) and weekly IGs (2.5 months)	< 1,000
4	F	15	CTLA4 haploinsufficiency and MUD HSCT	Methylprednisolone 0.4 mg/kg/day Abatacept	870/μl	Lung disseminated invasive aspergillosis	2.99	Micafungin. Surgical resection (x2). Voriconazole and amphotericin B (2 years).	0.52
5	F	12	Multivisceral transplantation	Methylprednisolone 0.3 mg/kg/day Tacrolimus Everolimus	230/μl	Liver EBV PTLD	1.2×10^5	IS minimization, Rituximab and chemotherapy (2,5 years)	1.07×10^5
6	F	9	Primary Immunodefficiency	None	340/μl	EBV DLBCL	4.09×10^5	Rituximab and chemotherapy (8 months)	0

BKV, BK virus; HCMV, cytomegalovirus; DLBCL, diffuse large B-cell lymphoma; EBV, Epstein Barr virus; HSCT, hematopoietic stem cell transplantation; MUD, match unrelated donor; PTLD, post-transplant lymphoproliferative disease; GMN, galactomannan; IG, immunoglobulin; IS, immunosuppression.

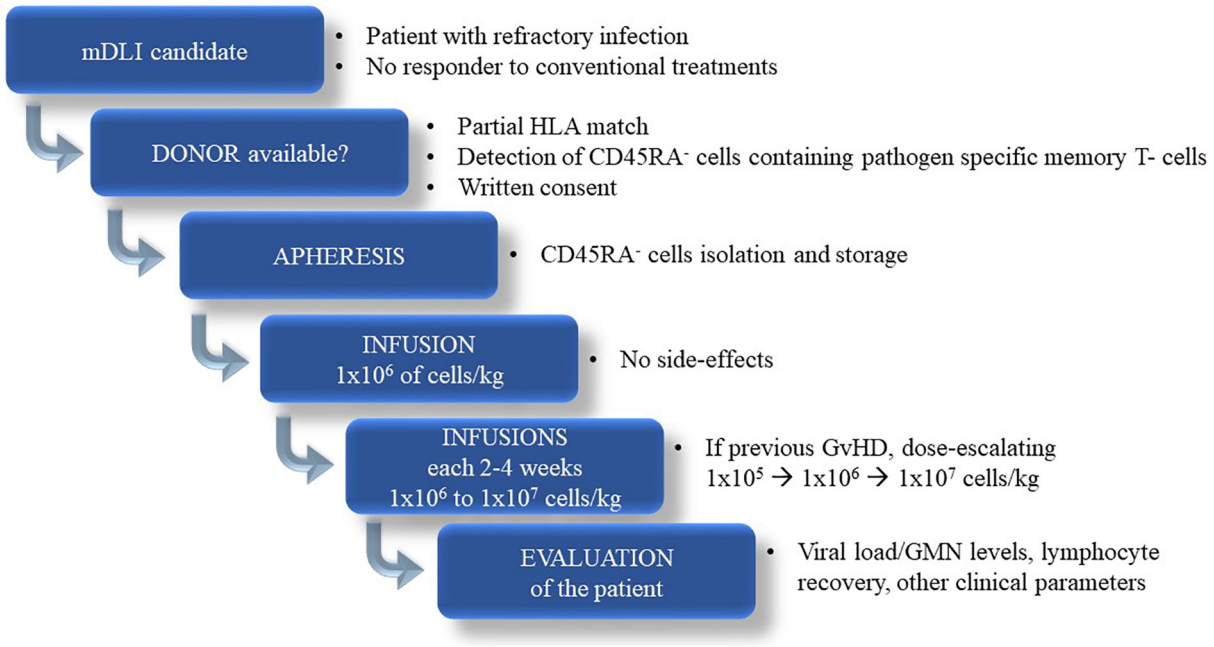

FIGURE 1
Scheme describing the general procedure followed in our center to treat immunocomprimised patients with infectious diseases.

partial match) and the presence of specific memory T-cells against the pathogen related to the patients' disease. These data is included in Table 2. After providing their informed consent, HLA typing of the eligible donors was performed at the Community of Madrid Transfusion Center (Madrid, Spain) on two independent samples by sequence-specific oligonucleotide and next-generation sequencing.

The assay to check the presence of specific memory T-cells against a particular pathogen was performed as previously described by our group with certain modifications (19). Briefly, peripheral blood mononuclear cells (PBMCs) samples were collected from potential healthy donors and isolated using density gradient centrifugation (Ficoll-Paque; GE Healthcare, Chicago, IL, USA). Cells were resting overnight (O/N) in TexMACS medium (Miltenyi Biotec, Germany) supplemented with 10% AB serum (Sigma-Aldrich, Saint Louis, MO, USA) and 1% penicillin/streptomycin (Sigma-Aldrich). Approximately 1 million cells were then stimulated with specific peptides at a final concentration of 0.6 nmol/ml. Peptides are small fragments measuring 8–12 amino acids in length that cover specific epitopes of the virus. In this case the peptides used were PepTivator® CMV pp65 (UniProt ID: P06725, Miltenyi Biotec) for HCMV; EBV-PT consensus premium grade human (Miltenyi Biotec) for EBV; BKV small T (ST) antigen (UniProt ID: P15000), BKV LT antigen (UniProtKB Acc. no. P14999), BKV VP1 capsid protein (UniProt ID: P14996) and BKV VP2 protein (UniProt ID: P14997) for BKV (Miltenyi Biotec); and PepTivator® AdV Select for AdV, a peptide pool of different proteins coming from virus serotypes 2 and 5 (Miltenyi Biotec). For *Aspergillus fumigatus* we used the MACS GMP *Aspergillus fumigatus* Lysate 4 mg, (Miltenyi Biotec), with a final concentration of 1 μg/ml.

To detect specific memory T- cells against BKV, AdV, or *Aspergillus*, cells were rested O/N at 37°C in supplemented medium After 5 h stimulation with pooled peptivators or the *Aspergillus* lysate, depending on each case, interferon-gamma (IFN-γ) was caught and labeled (Human IFN-γ Secretion Assay-Detection Kit, Miltenyi Biotec). Basal IFN-γ production by PBMCs was included as a background control in the absence of stimulation. To consider a sample positive it had to have 0.1% of IFN-γ^+ cells (0.01% for EBV) out of the total cells with a minimum of 150,000 events analyzed. In addition, the sample had to contain at least twice the number of IFN-γ^+ cells than in the negative control, as well as positive control based on plate-bound cells stimulated with mouse anti-human CD3 and co-stimulated with purified CD28/CD49d. After incubation, cells where stained using the following fluorochrome-conjugated anti-human surface antibodies: CD45RA FITC, CD27 APC, CD3 VioGreen, CD4 PECy7, CD8 APC Cy7, L/D 7AAD, and IFN-γ PE. Cell acquisition was then performed using a Navios cytometer (Beckman Coulter), acquiring a mean of 150,000 cells. To detect specific memory T-cells against CMV and EBV, same process was performed, but incubation was for 6 h and adding Brefeldin A (Sigma-Aldrich), and IFN-γ production capacity was analyzed by intracellular flow cytometry staining. The antibodies used were the following: surface antibodies 7-AAD PC5.5, CD45 KO, CD8 APC-AF700, and CD4 APC-AF750 (Beckman Coulter Inc.), and intracellular marker IFN-γ- PacificBlue (Beckman Coulter). The cell adquisition was made by DxFlex cytometer (Beckman Coulter). The analysis was performed using FlowJo 10.7.1 software (FlowJo LLC) in all cases.

2.3. Donor inclusion and CD45RA T-cell depletion

After confirmation of HLA compatibility and a positive memory T-cells response against the pathogen of interest, the donors' clinical history was reviewed and a physical examination was done. Non-mobilized apheresis was then performed at the Bone Marrow Transplantation and Cell Therapy Unit of University Hospital La Paz (Madrid, Spain) using a CliniMACS Plus cell separation system (Miltenyi Biotec).

2.4. CD45RA$^-$ T cells infusion

Obtained allogenic CD45RA$^-$ T cells were frozen as previously described (20) and infused into the patients weekly and then monthly depending on the cases and the patients' state. Table 3 includes number of cell infusions and cells dose administrated in each case.

In cases of leukemia, established doses of 1×10^6 escalating to 1×10^7 cells/kg are infused. After transplantation, the doses established for CTLs infusions are very variable, from 1×10^3 to 1×10^9 cells/kg (21). However, beyond the transplantation setting, there are no established doses for these procedures, but there are a number of examples that have proven to be safe (20). In this study, when the patients were at risk of GvHD, the minimum dose infused was 1×10^5 cells/kg of weight; however, the normal dose was usually 1×10^6 cells/kg. In all cases, subsequent doses were decided based on the clinical evaluation, polymerase chain reaction (PCR) levels and immunologic follow-up.

2.5. Lymphocyte reconstitution, viral load and microchimerism

Lymphocytes levels were measured by a routine hemogram, and viral load was defined by PCR. Microchimerism analysis was performed in the Community of Madrid Transfusion Center

TABLE 2 Donor characteristics.

Patient	Familiar donor, specificity	IFN-γ^+ T cells (%)	HLA-matching
1	Brother (Match)	0.4	A*24 B*44 B*45 C*02 C*16 DRB1*01 DRB1*10 DQB1*05
2	Brother (Mismatch related donor)	1.61	A*03, DRB1*07 DQB1*02
3	Mother (Haploidentical)	0.62	A*68 B*51 C*15 DRB1*08 DQB1*04
4	Father (Haploidentical)	0.28	A*11 C*07 B*49 DRB1*13 DQB1*06
5	Father (Haploidentical)	0.17	DRB1*07 DRB1*13 DRB1*02 DRB1*06
6	Mother (Haploidentical)	0.3	A*24 C*04 B*35 DRB1*11 DQB1*03 DPB1*04

TABLE 3 Infusion characteristics, response and outcomes after therapy with familial CD45RA$^-$ T cells containing pathogen-specific memory T cells (BAL, Broncho-alveolar lavage; GMN, galactomannan).

Patient	Number of infusions	Dose of infusions (CD45RA$^-$ T cells/kg)	Lymphocyte count before infusion (x10^3/μl)	Best lymphocyte count after infusion (x10^3/μl)	Viral copies or GMN before infusion (copies/μl)	Best response after infusion (copies/μl)	Best T cell donor chimerism (%)	Concomitant therapy during infusions	Duration of concomitant therapy during infusion	Outcome
1	14	10×10^7/kg	0.53	1.12	7.5×10^3	<1,000	0.5-0.25	IS withdrawn. IGs	2 months	Alive with stabilization of renal function
2	12	5.01×10^6/kg	0.38	1.44	2.3×10^3	0	Not performed	Ganciclovir/ Valganciclovir then replaced by Letermovir. Specific HCMV IGs (parenteral and intrathecal).	Ganciclovir/ Valganciclovir (6 months). Letermovir. Specific HCMV IGs (6 months).	Alive with encephalitis sequelae
3	6	5.4×10^5/kg	0.9	1.46	<1,000	0	Not detected	Letermovir and weekly IGs	4 months	Died of septic shock (not associated with therapy)
4	11	1.2×10^5/kg 5×10^5/kg 1×10^6/kg	0.89	1.03	0.52	Serum and BAL negative GMN	Not detected	Posaconazole	1 year	Alive and Lansky score 100%
5	10	1.53×10^7/kg	0.02	0.54	1.07×10^5	<3,500	1	Chemotherapy. IS withdrawn. IGs	1 month	Alive in CR after EBV CTLs
6	6	1×10^7/kg	0.04	0.18	0	0	1, <1	Rituximab. Third line chemotherapy. IGs every 2 weeks	1 month	Alive in CR after MUD HSCT

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(Madrid, Spain) as previously described (20). For each donor-recipient pair, DNA was isolated using the QIAamp Blood Kit (Qiagen, Germany). The microchimerism analysis was monitored weekly for 3-4 consecutive weeks, and performed based on detecting insertion/deletion polymorphism (INDELs) by qPCR technology (sensitivity 0.01–0.05%). To this end, commercial reagents for screening of informative alleles (Mentype DIPscreen, Biotype, Dresden, Germany) and quantitative chimerism analysis (Mentype DIPquant qPCR, Biotype) were employed. The percentage of donor alleles was calculated based on the DDCt qPCR method using Chimerism Monitor 2.1 software (Biotype), with b-globin as the reference gene.

3. Results

3.1. Detection of pathogen-specific CD45RA$^-$ memory T-cells

Potential donors for adoptive cell therapy were tested to check their response against different pathogens, namely HCMV, AdV, EBV, BKV, and *Aspergillus*. The presence of CD45RA$^-$ memory T cells responsive to all pathogens was detected in all donors (**Figure 2**). The donors therefore underwent apheresis and subsequent infusions to patients were performed. However, the infection was resolving in patient with AdV; thus, cells were stored for future use. This detection process is effective and could be an accepted and extended diagnostic procedure in the near future.

3.2. Adoptive cell therapy with CD45RA$^-$ cells containing pathogen-specific memory T-cells to treat infectious diseases

Four of the six patients included in this study had severe refractory infections that where not responding to classical therapies. An adoptive cell therapy with CD45RA$^-$ T cells containing pathogen-specific memory T cells therefore appeared as the best treatment option. The four cases are described below. **Table 3** presents a summary of the response and treatment outcomes with familial CD45RA$^-$ T cells.

3.2.1. BKV nephritis (patient 1)

The first patient was a 37-year-old woman who underwent a kidney transplantation due to chronic kidney failure secondary to focal and segmental glomerulonephritis. The patient was prescribed rejection prophylaxis (prednisone, tacrolimus and everolimus), and as a result she developed lymphopenia, with lymphocytes counts of 670/μl. Approximately 9 months after transplantation, and despite changing the medication, the patient showed a progressive worsening of the renal function and a rise of both creatinine levels and plasmatic BKV copies. The patient was therefore diagnosed with BKV nephritis.

Given the situation, and after an ineffective immunosuppression-therapy reduction, Leflunomide plus intravenous immunoglobulins (IGs) were suggested. For 4 months the patient was not improving, so cell therapy in combination with standard therapy was suggested. One of the patients' brother had identical HLA and 0.4% of CD3$^+$ cells that were IFN-γ^+ (**Table 2** and **Figure 2**). Thus, weekly infusions of 1×10^7 cells/kg of CD45RA$^-$ T donors' cells started. A week after first infusion, T-cell donor chimerism was 0.5% and after the second infusion it was 0.25%. Then it was undetectable. A month later, the patient showed stable kidney function and viral replication dropped to the minimum. After 2 months, lymphocyte counts increased (**Figure 3**); therefore IGs were suspended and infusions were performed every 2 weeks for 4 months and then monthly until the present. The patient is currently undergoing monthly infusions, and there are no signs of worsening renal function while the viral replication continued under control, with rising lymphocytes levels. The infusion of CD45RA$^-$ T-cells showed no side effects, and there was no GvHD. The total duration of the treatment remains uncertain.

3.2.2. HCMV encephalitis (patient 2)

Patient 2, a 19-year-old man with chronic granulomatous disease who underwent HSCT from a haploidentical match unrelated donor, was receiving DLIs every 2 weeks to promote immune reconstitution in combination with GvHD prophylaxis (methylprednisolone and cyclosporine). Two months after HSCT patient suffer a HCMV reactivation, treated with valganciclovir, and 2 months later, the patient developed an encephalitis caused by HCMV with high viral copies in blood and cerebrospinal fluid (CSF). At this point the immune reconstitution was incomplete and his total lymphocyte count was 50/μl.

To treat HCMV encephalitis correctly, and since valganciclovir was not effective, patient's sensitivity to antivirals was tested and he was sensitive to foscarnet, ganciclovir and cidofovir. Then, patient received foscarnet and ganciclovir first, suspended because of their high toxicity, followed by IGs weekly. After 2 weeks ganciclovir was reintroduced, without side-effects this time, but without any improve of the patient's state. After 2 months with a progressive worsening and with elevated viral copies in blood and CSF, adoptive cell therapy with CD45RA$^-$ T-cells was indicated. The patient's brother shared three HLA alleles and a total of 1.61% of CD3$^+$ cells that were IFN-γ^+ against HCMV (**Table 2** and **Figure 2**). Infusions of 5×10^6 cells/kg of CD45RA$^-$ cells were combined with ganciclovir and IGs. Only 2 weeks after infusion the patient showed progressive neurological improvement of his symptoms and a decrease of HCMV viral copies in CSF. Blood viral copies were undetectable 1 month after the infusion and CSF viral copies were undetectable 2 months later. The number of lymphocytes progressively increased and stood at 1,000/μl seven months later (**Figure 3**). Microchimerism was not performed in this case.

To date, the patient has been clinically stable without new neurologic symptoms or reactivation, but dealing with important sequelae due to HCMV ventricle-encephalitis. He maintains treatment with letermovir and monthly infusions of CD45RA$^-$ T cells to accelerate immune reconstitution. The CD45RA$^-$ T-cells infusions were safe and effective in this patient, showing no side effects or GvHD and with a decrease of viral load and an increase in lymphocyte number.

3.2.3. HCMV reactivation (patient 3)

The next patient was a 7-year-old boy with Trichohepatoenteric Syndrome, who underwent multivisceral transplantation with spleen preservation at 6 years of age. The patient developed GvHD with skin and eye involvement 10 months post-transplantation due to the intestinal graft resident lymphocytes. He therefore started on methylprednisolone as immunosuppression therapy, and

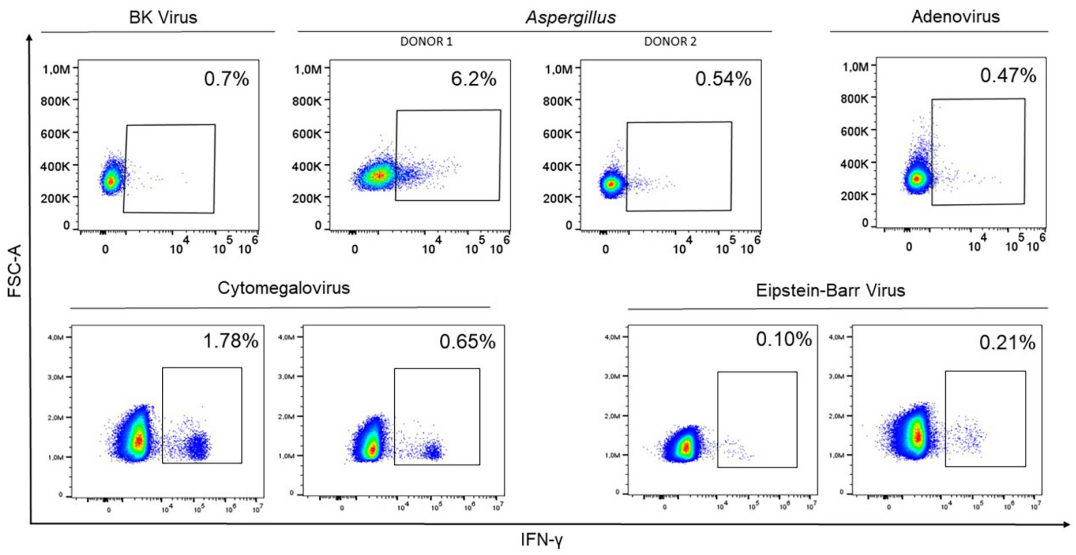

FIGURE 2
Representation of interferon-gamma (IFN-γ) expression by flow cytometry in 8 potential donors within the CD45RA$^-$ subpopulation after co-culturing peripheral blood mononuclear cells with the mixture of 4 BKV peptides (ST, LT, VP1, VP2), *Aspergillus fumigatus* Lysate at a final concentration of 1 μg/ml for *Aspergillus*; PepTivator® AdV Select for Adenovirus; PepTivator® CMV pp65 for HCMV; and EBV-PT consensus premium grade human for EBV. In all cases, the cell response was sufficient for use in cell therapy. Donor apheresis was therefore performed, and CD45RA$^-$ T cells were isolated and infused.

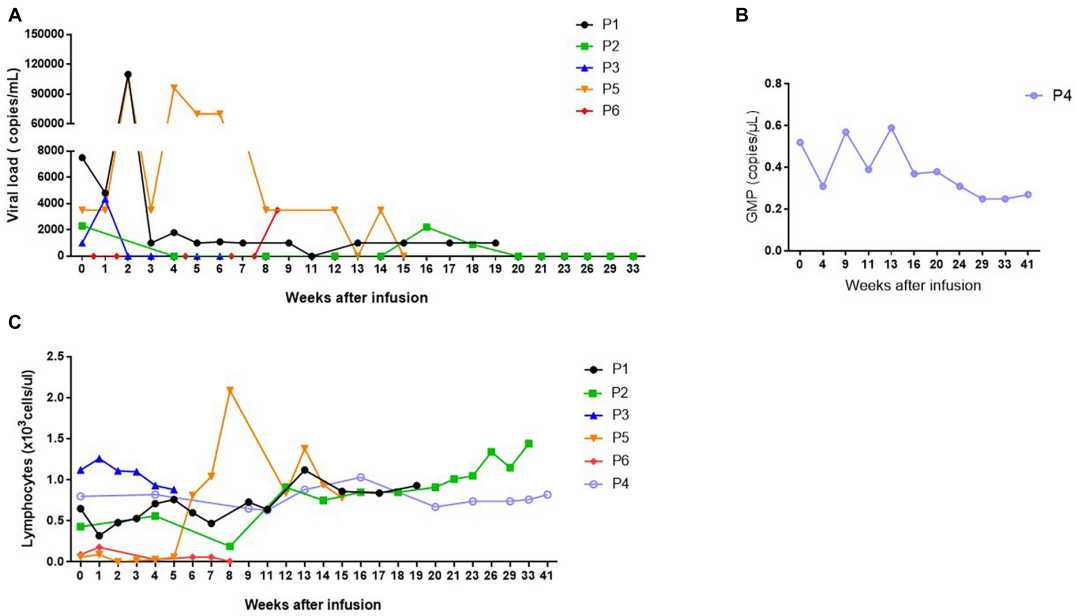

FIGURE 3
Graphical representation of **(A)** viral load (copies/ml), **(B)** galactomannan (GMP) (copies/μl) and **(C)** lymphocyte (×10^3 cells/μl) progression while the infusions were performed. As expected, the viral load and GMP decrease with adoptive cell therapy with CD45RA$^-$ T-cell infusions containing pathogen-specific memory T cells, while lymphocyte count increase.

rejection and HCMV prophylaxis (sirolimus and valganciclovir respectively). However, virus reactivation was detected and he started on treatment with ganciclovir and intravenous IGs, reduced immunosuppression dosage and, as the patient's GvHD was also worsening, he initiated treatment with ruxolitinib, resulting in a decreasing lymphocyte count.

After one month, and despite changing medication several times (foscarnet for one month and then ganciclovir for another month), new rise in the viral copies was detected. Patient's mother was HLA-haploidentical and has 0.62% of CD3$^+$ cells that were IFN-γ$^+$ (Table 2 and Figure 2). Thus, the patient started weekly infusions of donor's CD45RA$^-$ T cells at a dose of 5 × 10^5 cells/kg. After two infusions, the viral copies were undetectable and remained so throughout the treatment (Figure 3), although microchimerism was not detected.

A total of 6 infusions were administered, with no side effects and controlling HCMV infection. However, lymphocyte count did not improve and GvHD was worsening despite an immunosuppression

increase. The infusions were therefore suspended in case they were contributing to the worsening of GvHD, even if this was not suspected since we were not able to detect even microchimerism from the mother (all the chimerism detected was from the intestinal graft donor). Nevertheless, his clinical condition continued progressively worsening until he finally died of septic shock.

3.2.4. Lung disseminated invasive aspergillosis (patient 4)

The last patient was a 15-year-old girl with severe combined immunodeficiency, specifically a cytotoxic T-lymphocyte antigen 4 (CTLA-4) haplo-insufficiency. She underwent HSCT from an identical match unrelated donor and, despite being under fungal prophylaxis with micafungin, a high level of galactomannan (GMN) was detected 8 months after the HSCT. She initiated treatment with voriconazole and 4 months later, it was changed by amphotericin B because of an azole resistance. Infection was in that way controlled.

However, as an HSCT complication, the patient developed steroid-refractory bronchiolitis obliterans syndrome 7 months later, which required immunosuppression therapy with abatacept. As a result, GMN levels rose again. In a PET-CT scan invasive aspergillosis with some lung aspergillomas were detected. The treatment, consisting in surgery and azoles, controlled the infection. Same events happened one year later, however, this time GMN levels remained high, despite the new aspergilloma-removing surgery and the treatment with voriconazole and with amphotericin B. A schematic representation of the evolution of the patient is represented in **Supplementary Figure 1**. At this point, the HSCT donor was studied and was found immunologically competent against *Aspergillus* (Donor 1, **Figure 2**), but was not available for apheresis. Therefore, patient's father, who was HLA-haploidentical and has 0.28% of $CD3^+$ cells that were IFN-γ^+ was chosen as a donor (Donor 2, **Figure 2**). Patient get monthly infusions of 1×10^5 cells/kg $CD45RA^-$ T-cells combined with posaconazole. Two months later there was a significant reduction in GMN levels but with a few rebounds (**Figure 3**), so the dose was increased to 5×10^5/kg and the infusions performed every 2 weeks.

Although in this case, we saw no significant changes in lymphocyte counts (**Figure 3**) and no microchimerism was found, GMN levels remained negative 4 months after the therapy, so the infusions were changed to monthly with a higher dose of 1×10^6/kg. The patient maintains this therapy and is currently clinically stable with no new rebounds in GMN levels more than one year and a half after cell therapy start. No side effects were found associated with the cell infusions, there were no signs of GvHD and we observed an improvement in the lymphocyte recovery.

3.3. Adoptive cell therapy with $CD45RA^-$ T cells containing EBV-specific memory T cells against EBV associated lymphoproliferative syndromes

Two of the six patients involved in our study experienced PTLD associated to EBV and were not responding to standard treatments. The patients therefore required cellular therapy to control the viral reactivation. The two cases are described below. **Table 3** provides a summary of the response and treatment outcomes with familial $CD45RA^-$ T-cells.

3.3.1. PTLD after a multivisceral transplantation (patient 5)

This patient is a 12-year-old girl with intestinal failure secondary to postsurgical superior mesenteric vein thrombosis, who underwent multivisceral transplantation. She developed acute skin GvHD three months after the transplantation and was treated with high doses of corticosteroids, ruxolitinib and photopheresis for a month. After that, the patient get rejection prophylaxis; consequently, she developed severe lymphopenia, with a total lymphocyte count of 230/μl. At that moment, a blood reactivation of EBV was detected, so immunosuppression was minimized and rituximab was initiated. After a while, the patient developed a monomorphic PTLD, a diffuse large B-cell lymphoma (DLBCL). A PET-CT showed supra-diaphragmatic (cervical, axillary, and pleural effusion) and infra-diaphragmatic (liver injury and doubtful renal) involvement (stage III according to Murphy's classification).

Immunosuppression therapy was minimized, rituximab was maintained and the patient started chemotherapy following Inter-B-NHL ritux 2010 protocol in the high-risk group B. She received one COP cycle and two R-COPADM. There was an initial good response of the DLBCL to chemotherapy, but viral copies of EBV remained elevated in serum despite rituximab, and the patients' lymphocytes count was still very low.

In this context, patient's father was studied as potential donor. He presented a 0.17% of $CD3^+$ IFN-γ^+ cells and he was HLA-haploidentical, so the patient initiated infusions of 1.53×10^7 cells/kg of $CD45RA^-$ T-cells twice a week. A 1% of T-cell microchimerism was detected after the first two infusions and it get undetectable after that. EBV copies initially decreased, but did not go negative, and the patient still has lymphopenia (**Figure 3**). No side effects or GvHD were observed after infusions.

After 3 months, the patient had a lymphoma in progression in spite of first-line chemotherapy, and an EBV reactivation without significant response to rituximab and familial $CD45RA^-$ T-cells. For this reason, chemotherapy and $CD45RA^-$ T- cells infusions were suspended, and infusions of specific CTLs against EBV twice a week started. After two infusions of 5.7×10^7 cells/ml of specific CTLs, the number of viral copies decreased significantly, lymphocyte counts improved considerably and the patient showed good clinical response. After 6 infusions, EBV copies in blood were undetectable, the patient presented normal lymphocyte counts and a partial response of PTLD was finally achieved. The treatment with specific CTLs was then interrupted. To date, 14 months after the end of treatment, the patient is still clinically stable without signs of disease progression. No side effects related to the use of $CD45RA^-$ T-cells or CTLs were observed.

3.3.2. Diffuse large B-cell lymphoma and EBV infection in a patient with primary immunodeficiency (patient 6)

The last patient was a 9-year-old girl with a diagnosis of unaffiliated primary congenital immunodeficiency. She had a history of primary EBV infection when she was 5 years old, and since then she maintained persistently positive viral copies in serum.

At 8 years of age, the patient was diagnosed with a DLBCL (stage III according to Murphy's classification). She started standard therapy with rituximab and first line chemotherapy (prednisone and cyclophosphamide). Three months later she started second line therapy following Inter-B-NHL protocol as high-risk group C1,

receiving 2 COPADM cycles and one CYVE. Finally, 2 months later she received a third line of treatment with a first R-ICE cycle as rescue therapy. After rescue therapy, EBV copies became negative, but infusions of CD45RA$^-$ T-cells were performed in order to prevent future reactivations. Her mother was HLA-haploidentical and presented 0.3% of CD3$^+$ IFN-γ^+ T-cells (**Table 2** and **Figure 2**). First, weekly infusions of 1×10^7 CD45RA$^-$ cells/kg were performed, without any side effects or GvHD. No microchimerism was detected. A second R-ICE was administered during the infusions, and no significant changes in the total lymphocyte count was observed, probably due to concomitant treatment with chemotherapy. The patient received a total of 6 infusions with no EBV reactivations, which allow HSCT. More than one year after the HSCT, the patient is still at complete remission, with no new EBV reactivation.

4. Discussion

Here we present a treatment focused on the ability to clear various viruses (HCMV, AdV, BKV, EBV), and the fungi *Aspergillus* by mDLI of CD45RA$^-$ cells containing pathogen-specific memory T-cells of a familial donor. We conducted a study with six patients, children and adults who were immunosuppressed and who experienced either an infection or reactivation of different viruses or EBV-associated lymphoproliferative disease. Given that these patients did not respond to classical treatments, this therapy provides a new, safe and effective curative procedure. These mDLIs were used as well in a preventive way to avoid reactivations, as in patient 6. We present as well the methodology to detect, isolate and infuse CD45RA$^-$ T cells in this novel cell therapy.

Cell reconstitution therapies are routinely performed to recover the immune system after HSCT (5–7, 22), a process that is essential for patient survival. Cells from the HSCT donor are usually employed to perform this recovery, but if the donor does not have memory cells against a specific pathogen, the patient is at risk of infection or reactivation of a latent virus. Thereby, cell therapy with CD45RA$^-$ T cells appears to be the best option for avoiding and controlling infections after HSCT. What is more, in immunocompromised patients who have the same disease and progress with lymphopenia or lack of memory cells against a specific virus, CD45RA$^-$ T cells are a life drug alternative to conventional treatments. In many cases, there are more effective or are the only option when there is a lack of appropriate drugs. Indeed, more and more resistances to anti-viral or anti-fungal drugs urge to develop new solutions and treatments for not only extreme but also common infections in healthy donors without comorbidities or previous pathologies (23, 24).

Adoptive cell therapy with CD45RA$^-$ T cells is as well an alternative to CTLs, usually used to treat infections in immunocompromised patients. Some examples employed to treat the infections described in this article are the clinical trial by Muftuoglu et al., for treating multifocal leukoencephalopathy caused by the John Cunningham Virus (genetically similar to BKV) (10); or the use of CTLs proposed by Bao et al., to treat HCMV infections in stem cell transplantation patients (25). However, CTLs need to be isolated, characterized and expanded *in vitro*, a costly and lengthy process that our therapy is not subject to. The therapy that we propose is not considered an advanced cell therapy and therefore does not require substantial cell modification, making the process easier and cheaper, without losing effectiveness.

One essential factor to consider in our therapy is the presence of a repertoire of memory lymphocytes with proven antiviral activity in the donor's CD45RA$^-$ subpopulation. To confirm that activity we used the IFN-γ production in presence of small peptides or a lysate that simulate the contact with a real pathogen. Previous studies by our laboratory reported the presence of SARS-CoV-2-specific T-cell subpopulations within CD45RA$^-$ memory T cells in the blood of convalescent donors that infused might clear virally infected cells and confer T-cell immunity for subsequent reinfections, representing an off-the-shelf living drug (19, 20). In this study, we are applying the same technology to treat other infectious diseases, with visible results.

The other essential factor to take in account when selecting the best donor is the HLA compatibility, which must be at least partial to permit TCR/HLA pathogen recognition. CD45RA$^-$ T cells are mainly CD4$^+$ cells, with a less proportion of CD8$^+$ cells. In the case of HCMV infections, it is known that deficiencies in the response of class I HLA-restricted CD8$^+$ cytotoxic T lymphocytes are important in the pathogenesis of the disease in immunocompromised recipients of allogeneic transplants (26). However, in the two HCMV patients that were infused in this study, a clearance of the virus was achieved. Therefore, we can obtain a good response despite the less proportion of CD8$^+$ cells in our product. Moving on, EBV presentation is mediated by HLA-class II alleles before the latent phase of the infection; CD4$^+$ T cells recognize particles presented by MHC-class II in the surface of infected B lymphocytes and eliminate them. In later stages of the infection, HLA class I alleles are the ones that induce a strong specific cytotoxic CD8 + T cell response (27). Therefore, CD45RA$^-$ T cells might be more effective in early stages of the infection or when there is not an active infection. On the contrary, CTLs infusions would be more effective when the infection is spread, as we observed in our study patients. Indeed, patient 5 needed CTLs infusions to contain the infection, while in patient 6, who was receiving the infusions in a prophylactic manner, the CD45RA$^-$ T-cells were effective avoiding viral load increase. It is true that CTLs are more effective, but they are also more alloreactive, what increases the risk of having GvHD or tissue inflammation. On the other side, BKV immunity is associated with a multifunctional population of CD4 + T cells with both T-helper and T-cytotoxic properties (28). That might be the reason why CD45RA$^-$ T cells infusion in patient 1 was completely effective. Finally, immunology of *Aspergillus* is complex and not completely defined, but T regulatory cells (which were contained in CD45RA$^-$ T-cells infused to the patient 4) might have a crucial role in the responses against this pathogen (29, 30).

In this study we performed weekly and monthly mDLI of CD45RA$^-$ T cells to patients who had HCMV, BKV, EBV, and *Aspergillus* infections that progressed with lymphopenia and who did not respond to standard therapies. We observed improvement in the general symptoms for a number of the patients, as well as increased lymphocyte counts and reduced viral and GMP loads, sometimes to zero. When performed, microchimerism between donors and patients cells was observed after the first and second infusion, and then it was undetectable, coinciding with the reduction of viral loads and the increase of lymphocyte counts. Therefore, when autologous lymphocytes get recovered, donor's lymphocytes are reduced to minimum and are not detected. None of the patients experienced adverse effects due to infusions or GvHD reactions, and the infusions did not interfere with the treatments the patients were undergoing

for other diseases. Thereby, this therapy can be combined with other procedures like HSCT, CTLs infusions, and different antiviral and antifungal drugs.

However, this study has some limitations. A larger patient cohort would be useful for studying specific reactions and characteristics of the infections. Obtaining more information and follow up of the patients would be of use; for example, more data on microchimerism, or donor's CD45RA$^-$ T cells function and interactions in the patient is essential for future research. Also a study of how immune lymphocyte reconstitution develops and a phenotyping of the different immune system cells subsets would be very interesting for future approaches Finally, as we propose, the possibility of generating a biobank of living drugs accessible to all patients will be useful for providing this treatment to those who might need it.

In conclusion, after this study we can affirm that the use of familial multiple CD45RA$^-$ T cells containing specific memory T-cells of a third-party donor is a feasible, safe and potential effective approach for treating severe pathogenic diseases in immunocompromised patients non-responders to antiviral treatments or with side effects due to their toxicity. This therapy provides and improves the cellular immunity of the patient until he recovers its own one and protects from opportunistic infections. Furthermore, this approach might be of universal use with fewer institutional and regulatory barriers, since Good Manufacturing Practices are not required.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this manuscript.

Author contributions

AP-M: conceptualization and resources. KAS and AP-M: methodology. KAS, CE, and AP-M: research and writing of the original draft. AP-M and CF: supervision. All authors: writing, reviewing, and 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1083215/full#supplementary-material

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EDITED BY
Saba Tufail,
Aligarh Muslim University, India

REVIEWED BY
Hemant Khuntia,
Siksha O Anusandhan University, India
Carlos Mier-Aguilar,
University of Alabama at Birmingham,
United States

*CORRESPONDENCE
Muhammad Sohaib Asghar
✉ sohaib_asghar123@yahoo.com

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Knowledge, attitudes, and practices of the general population of Pakistan regarding typhoid conjugate vaccine: findings of a cross-sectional study

Muhammad Junaid Tahir[1], Musharaf Zaman[2], Junaid Saffi[1], Muhammad Sohaib Asghar[3]*, Waleed Tariq[1], Faizan Ahmed[1], Rabia Islam[4], Usman Shakeel Farooqui[5], Irfan Ullah[6], Muhammad Saqlain[7], Kaleem Ullah[8] and Ali Ahmed[9]

[1]Department of Medicine, Lahore General Hospital, Lahore, Pakistan, [2]Department of Anesthesia, Pakistan Red Crescent Teaching Hospital, Lahore, Pakistan, [3]Department of Internal Medicine, Dow University of Health Sciences-Ojha Campus, Karachi, Pakistan, [4]Department of Medicine, Punjab Medical College, Faisalabad, Pakistan, [5]Department of Internal Medicine, Dow Medical College, Karachi, Pakistan, [6]Department of Medicine, Kabir Medical College, Gandhara University, Peshawar, Pakistan, [7]Department of Pharmacy, Quaid-I-Azam University, Islamabad, Pakistan, [8]Department of Hepatobiliary Surgery and Transplant, Pir Abdul Qadir Shah Jeelani Institute of Medical Sciences, Gambat, Pakistan, [9]Department of Pharmacy, School of Pharmacy, Monash University, Subang Jaya, Selangor, Malaysia

Typhoid fever, a common enteric disease in Pakistan, caused by *Salmonella typhi*, is becoming an extended drug-resistant organism and is preventable through the typhoid conjugate vaccine (TCV). Public adherence to preventive measures is influenced by knowledge and attitude toward the vaccine. This study investigates the knowledge, attitudes, and practices of the general population of Pakistan toward TCV. The differences in mean scores and factors associated with typhoid conjugate vaccine knowledge, attitudes, and practices were investigated. A total of 918 responses were received with a mean age of 25.9±9.6, 51% were women, and 59.6% had graduation-level education. The majority of them responded that vaccines prevent illness (85.3%) and decrease mortality and disability (92.6%), and typhoid could be prevented by vaccination (86.7%). In total, 77.7 and 80.8% considered TCV safe and effective, respectively. Of 389 participants with children, 53.47% had vaccinated children, according to the extended program on immunization (EPI). Higher family income has a higher odds ratio (OR) for willingness toward booster dose of TCV [crude odds ratio (COR)=4.920, *p*−value <0.01; adjusted odds ratio (aOR)=2.853, *value of p* <0.001], and negative attitude regarding the protective effect of TCV has less willingness toward the booster dose with statistical significance (COR=0.388, *value of p*=0.017; aOR=0.198, *value of p*=0.011). The general population of Pakistan had a good level of knowledge about the benefits of TCV, and attitude and practices are in favor of the usage of TCV. However, a few religious misconceptions are prevalent in public requiring the efforts to overcome them to promote the usage of vaccines to prevent the disease and antibiotic resistance.

KEYWORDS

typhoid fever, enteric fever, extended drug resistance, willingness, booster dose

Introduction

Typhoid fever is a systemic infection characterized by fever, vomiting, and diarrhea caused by *Salmonella typhi* (*S. typhi*) (1, 2). The disease is transmitted by consuming fecal-contaminated water and food (3). Annually, typhoid affects 11 to 20 million people with 128,000 to 161,000 deaths worldwide.[4] Underprivileged communities and children are at higher risk for typhoid fever (4). Children younger than 5 years of age had the highest rates of morbidity and mortality (12.6% of cases and 17% of deaths), followed by children aged 5 to 9 years (56% of cases and 59% of deaths). Typhoid fever is also a common disease in Pakistan, affecting 451.7 people per 100,000 each year (5). The improvements in sanitary infrastructure significantly control enteric fever in developed countries. The misusage of antibiotics in lower-middle-income countries (LMICs) for the treatment of *S. typhi* is growing antibiotic resistance, raising danger to global public health (1, 6). The mortality rate due to *S. typhi* had also increased, attributing to antibiotic resistance. *S. typhi* strains resistant to antibiotics such as chloramphenicol, ampicillin, trimethoprim–sulfamethoxazole, fluoroquinolones, and third-generation cephalosporin are labeled as extended drug-resistant (XDR) (7).

Pakistan had one of the highest rates of typhoid cases in South Asia. The rising level of antibiotic resistance in the country is raising global fear. The dire state of the sewage and water system along with low vaccination rates, non-compliance to treatment, and overcrowding are the major elements contributing to the spread of XDR typhoid within Pakistan (8). Furthermore, in 2016, XDR typhoid outbreak occurred in Sindh, Pakistan (9). The prevalence of positive cultures of *S. paratyphi* A raised from 0.2% in 2017 to 0.3% in 2018, then up to 0.9% in 2019 and increasing the prevalence of XDR typhoid fever across the country, indicating spread outside Sindh (9). Without active and effective measures, the preexisting and increased burden of XDR typhoid is expected to worsen, given the strain coronavirus disease 2019 (COVID-19) is putting on the healthcare system (10, 11). The prevalence of diseases, disabilities, and deaths has been significantly reduced because of vaccination (12).

Vaccines prevent 4–5 million people from dying from fatal diseases each year (13). Numerous success stories against polio, tetanus, influenza, hepatitis B, diphtheria, pertussis, measles, mumps, and rubella (MMR) had been made possible by effective vaccination. Typhoid vaccination had been proven effective for the prevention and control of typhoid. Pakistan is the first country to introduce typhoid conjugate vaccine (TCV) in the extended program on immunization (EPI) and had vaccinated over 30 million children in the country with an effectiveness of 95% against protection from typhoid (14). The first single dose of TCV provides prolonged immunity and is effective and safe for children between 6 months and 2 years of age (15).

The vaccine coverage gap does, however, exist between nations and even within a nation (16). The World Health Organization (WHO) identified vaccine reluctance as one of the threats to global health (17). Vaccine hesitation had been caused by several factors, including erroneous safety worries about immunization as a result of measles, diphtheria, and pertussis outbreaks (12). Influential religious and political figures and false religious beliefs greatly influenced vaccine refusal (18, 19). Personal or philosophical convictions, such as the idea that a healthy lifestyle will prevent sickness or that active immunity obtained after natural infection is superior to that acquired by vaccination, are other factors contributing to vaccination refusal (20) (Figures 1–3).

The vaccination rates of Pakistan's population are below the required levels worldwide (21). The vaccination rates for Bacillus Calmette–Guérin (BCG), polio combined with DPT (Diptheria, Pertussis, and Tetanus), and measles are each stated to be at 80, 65, and 67%, respectively (21). This is a result of logistical challenges, inadequately qualified medical staff, inadequate parental education and awareness, and political, religious, and commercial influences on the promotion of vaccine products (21–23). Education and vaccines are the most powerful tools, proven to be valuable, against preventable diseases (24, 25). Previously, in Pakistan, the literature had been published regarding awareness and attitudes toward TCV involving a specific and localized population (26). We are conducting this study among the general population of Pakistan to assess the knowledge, attitude, and practices regarding TCV and willingness toward the booster dose of TCV.

Materials and methods

Study design and study setting

A cross-sectional, web-based study was conducted among the general population of Pakistan, involving participants from all provinces, from 15 October 2021 to 30 November 2021.

Ethical considerations

Participation in this study was completely voluntary. Before beginning the survey, each participant provided informed consent and was given the option to withdraw from the study at any time before submitting their response. The anonymity of the participants was assured by not collecting any personal information such as name, email, or any other contact information. The review committee of Pir Abdul Qadir Shah Jeelani Institute of Medical Sciences, Gambat, Pakistan, with reference number: IRB/22/13, approved ethical approval for the study. Furthermore, the STROBE checklist is followed to report this study.

Sample size

The population of Pakistan is 227.3 million with a median age of 23.2 years and 51.5 and 48.5% being men and women, respectively, with the literacy rate of 62.3% (27, 28). The online Rao soft calculator $[n = {}^{Nx}I\ ((N-1)E^2 + x)]$ was used to calculate the sample size of the study (29). A minimum sample size of 846 was calculated with a 98% confidence interval, 50% proportion of the population, and 4% margin of error. A total of 918 responses were included with a 96% response rate.

Data collection

A Google® form was used to collect the responses through a convenient sampling method. The questionnaire link was circulated

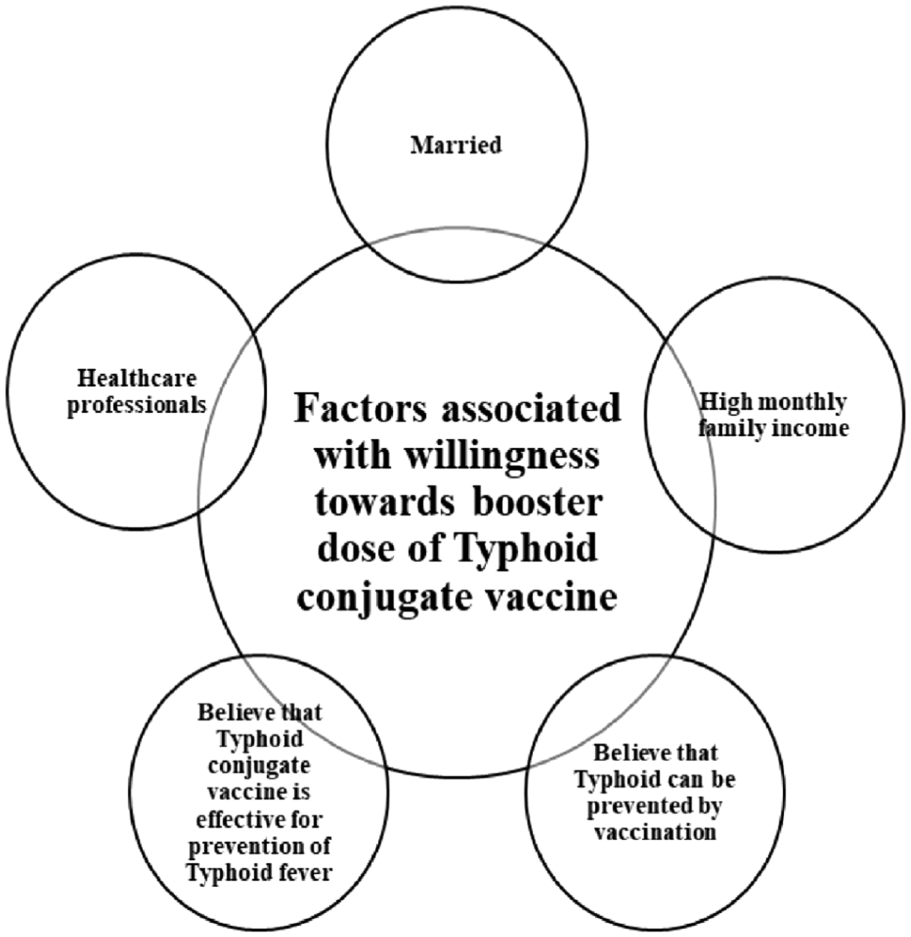

FIGURE 1
Factors associated with willingness toward booster dose of typhoid conjugate vaccine.

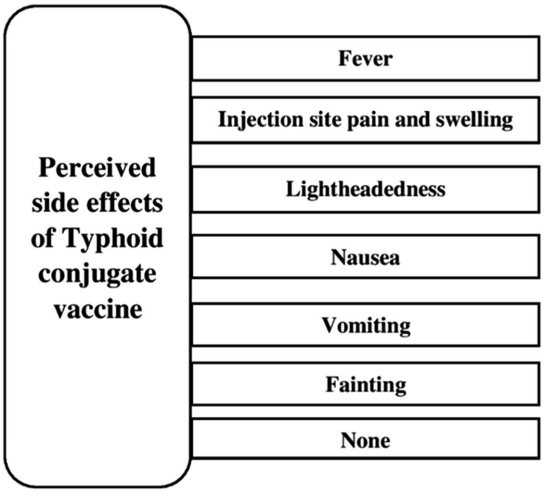

FIGURE 2
Perceived side effects of typhoid conjugate vaccine.

among friends and contacts in multiple social media groups such as WhatsApp and Facebook. Individuals were unable to understand the language of the survey, and non-Pakistani nationals were excluded from the study.

Questionnaire designing

The questionnaire was designed after a substantial literature review (12, 16, 24, 30, 31). It was validated by medical education and public health experts. To assess the relevancy and simplicity of the questionnaire, a pilot study was conducted with 40 individuals from the target population. The questionnaire was comprised of demographic information and questions about knowledge, attitude, and practices regarding TCV. The sociodemographic characteristics include age, gender, education, occupation, residence, marital and employment status, monthly family income, and the number of household individuals. The knowledge section was comprised of action, benefits, availability, cost, source of information, side effects of TCV, and awareness about the TCV vaccination program conducted by the Government of Pakistan. The attitude assessed participants' beliefs about safety, efficacy, recommendation, and need for TCV for children. Furthermore, attitudes regarding religious beliefs toward vaccines were also inquired. Regarding practices of TCV, vaccination of own child, to comply with the

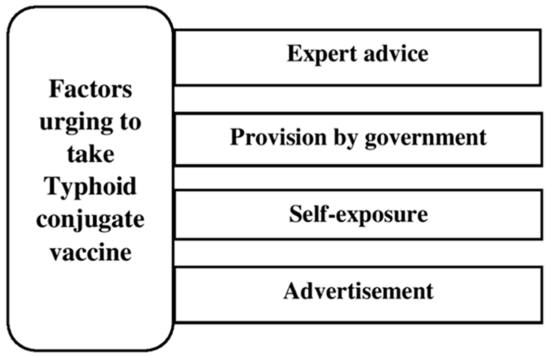

FIGURE 3
Factors urging to take typhoid conjugate vaccine.

booster dose, and family restrictions regarding vaccine were inquired.

Statistical analysis

The statistical package for social sciences (SPSS) version 21 (IBM) was used for data analysis. Mean and standard deviation were used to present the numerical variables, and for categorical data, frequencies and percentages were used. Depending upon the nature of the variables, inferential analysis was applied. Univariate and multivariate logistic regression analyses of sociodemographic, knowledge, and attitude with willingness for a booster dose of TCV were conducted, and a *value of p* of <0.05 was considered significant.

Results

Sample characteristics

A total of 918 responses were received with a response rate of 96%. The mean age and number of family members were 25.9 ± 9.6 and 5.9 ± 3.7, respectively. Regarding sociodemographic information, 51% (468) were women, 25.7% (236) were married, and 39.5% (363) were employed. Among the majority of the people, 82.7% (759) belonged to urban areas, 59.6% (547) had graduation-level education, and 60.2% (533) had a monthly family income of more than 50,000 PKR (Table 1).

TABLE 1 General characteristics and demographics of the study participants (*n*=918).

Variables	Characteristics	Frequency/ percentage
Age (in years)	Mean (SD)	25.9 (9.6)
Gender	Female	468 (51.0%)
	Male	450 (49.0%)
Marital status	Unmarried	682 (74.3%)
	Married	236 (25.7%)
Residence	Rural	159 (17.3%)
	Urban	759 (82.7%)
Monthly family income (PKR)	< 25,000	162 (17.6%)
	25,000–50,000	203 (22.1%)
	> 50,000	553 (60.2%)
Level of education	No formal education	25 (2.7%)
	Primary up to Matric	68 (7.4%)
	Secondary/Intermediate	191 (20.8%)
	Graduate	547 (59.6%)
	Post-graduation	87 (9.5%)
Number of household members	Mean (SD)	5.9 (3.7)
Employment status	Unemployed	555 (60.5%)
	Employed	363 (39.5%)
Occupation	Healthcare workers	253 (27.6%)
	Others/No occupation	665 (72.4%)

PKR, Pakistani Rupee; SD, standard deviation.

Assessment of knowledge in the participants

The majority of the people (783) responded that vaccine is used to prevent illness (85.3%), 834 (90.8%) reported that healthy child needs vaccination, 850 (92.6%) reported that vaccine lowers disability and mortality, and 796 (86.7%) reported that typhoid could be prevented by vaccination. In total, 54.2% (498) were aware of TCV, and 52.1% (478) of TCV vaccination programs conducted by the Government of Pakistan. Mass media [35.1% (322)] and healthcare workers [16.2% (149)] were major sources of information. Regarding side effects, fever [462 (50.3%)] and injection site pain and swelling [453 (49.3%)] were considered the most common reactions to TCV (Table 2).

Assessment of the attitude of participants

In total, 77.7% (713) and 80.8% (742) considered TCV safe and effective, respectively, and 91.8% (843) were in favor of vaccination programs designed by healthcare authorities. In total, 92.3% (847) would advise TCV to family and friends, 617 (67.2%) would strongly recommend others to vaccinate their children, and 53.3% (489) followed expert advice for TCV vaccination. Only 21.6% (198) had fear about TCV, and 13.1% (120) considered it a conspiracy theory against Pakistan; however, 623 (67.9%) believed that it may contain contents forbidden by Islamic law (Table 3).

Assessment of practices of participants

Only 389 (42%) participants had children, and 208 (22.7%) had vaccinated their children according to EPI including TCV. In total, 763 (83.1%) would comply with the booster dose, and 829 (90.3%) had family restrictions regarding vaccination. The majority would consult with the doctor for side effects of vaccination [533 (58.1%)], the missed dose of TCV [842 (91.7%)], and the next vaccination [845 (92.0%)] (Table 4).

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TABLE 2 Knowledge about typhoid as a disease and vaccination of the study participants (n=918).

Variables	Characteristics	Frequency (%)
What do you think vaccination do?	I do not know	45 (4.9%)
	It's just an injection	22 (2.4%)
	It's used to cure disease	68 (7.4%)
	Prevent illness	783 (85.3%)
Do you know that even healthy child needs	No	84 (9.2%)
vaccination?	Yes	834 (90.8%)
Do you know vaccination decreases the rates of mortality and disabilities?	No	68 (7.4%)
	Yes	850 (92.6%)
Do you know typhoid could be prevented by	No	122 (13.3%)
vaccination?	Yes	796 (86.7%)
Do you know about Typhoid Conjugate	No	420 (45.8%)
Vaccine?	Yes	498 (54.2%)
Do you know that the government of Pakistan had started typhoid vaccination in different districts of Pakistan?	No	440 (47.9%)
	Yes	478 (52.1%)
How did you come to know about typhoid vaccination?	Doctors and Nurses	149 (16.2%)
	Friends And Relatives	139 (15.1%)
	Mass Media (TV/Newspaper)	322 (35.1%)
	Personal knowledge	126 (13.7%)
	I do not know	182 (19.8%)
Do you know which age group children should get typhoid vaccination?	0–5 years	335 (36.5%)
	5–13 years	246 (26.8%)
	More than 13 years	98 (10.7%)
	I do not know	239 (26.0%)
What do you know about booster dose?	Given to cover missed dose	71 (7.7%)
	Gives more protection	667 (72.7%)
	I do not know	180 (19.6%)
Is typhoid vaccine available free in Pakistan or patient bear its price?	Patient bear its price	208 (22.7%)
	Typhoid vaccine available free in Pakistan	498 (54.2%)
	I do not know	212 (23.1%)
What side effects does TCV have?	Fever	462 (50.3%)
	Lightheadedness	187 (20.4%)
	Nausea	173 (18.8%)
	Vomiting	102 (11.1%)
	Fainting	41 (4.5%)
	Pain and swelling at injection site	453 (49.3%)
	None	35 (3.8%)
	I do not know	478 (52.1%)

TV, Television; TCV, Typhoid Conjugate Vaccine.

Regression analysis of factors associated with willingness for a booster dose of typhoid vaccine

Being married has a significant association with willingness for a booster dose of TCV [crude odds ratio (COR) = 0.285, *value of* p <0.001; adjusted odds ratio (aOR) = 0.562, *value of* p =0.038]. Participants with a monthly family income of more than 50,000 PKR have higher OR than those with lower income (COR = 4.920, *value of* p <0.01; aOR = 2.853, *value of* p < 0.001). Based on univariate and multivariate analyses, a negative attitude regarding the protective effect of TCV has less willingness toward the booster dose with statistical significance (COR = 0.388, *value of* p = 0.017; aOR = 0.198, *value of* p = 0.011) (Table 5).

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TABLE 3 Attitude of the study participants toward typhoid vaccination (*n*=918).

Variables	Characteristics	Frequency (%)
Are you in favor of obligatory vaccination	No	75 (8.2%)
programs designed by the health authorities?	Yes	843 (91.8%)
Will you advice your relatives and family	No	71 (7.7%)
members to immunize their children?	Yes	847 (92.3%)
Do you believe that typhoid conjugate vaccine is safe?	No	36 (3.9%)
	Yes	713 (77.7%)
	I do not know	169 (18.4%)
Do you believe that typhoid conjugate vaccine is effective for prevention of typhoid fever?	No	34 (3.7%)
	Yes	742 (80.8%)
	I do not know	142 (15.5%)
Will you recommend vaccine to other children?	Discourage taking vaccine	31 (3.4%)
	Only if they ask	142 (15.5%)
	Strongly recommend	617 (67.2%)
	No comments	128 (13.9%)
Will you vaccinate the child if he/she is already sick?	No	536 (58.4%)
	Yes	382 (41.6%)
Do you have any fears about this vaccination?	No	720 (78.4%)
	Yes	198 (21.6%)
What encourages you to get the child vaccinated with Typhoid Conjugate Vaccine?	Advertisements	28 (3.1%)
	Expert Advice	489 (53.3%)
	Government Provides Vaccination	274 (29.8%)
	Self-experience	98 (10.7%)
	Will not vaccinate my child	29 (3.2%)
Would you support it if it is added in Govt. vaccination program?	No	62 (6.8%)
	Yes	856 (93.2%)
Do you think it is an international conspiracy against Pakistani children?	No	798 (86.9%)
	Yes	120 (13.1%)
Do you think it may contain contents forbidden by Islamic law?	No	199 (21.7%)
	Yes	623 (67.9%)
	I do not know	96 (10.5%)

Discussion

Typhoid conjugate vaccine (TCV) had been proven effective in reducing the burden of *S. typhi*-induced morbidity and mortality, especially in regions with XDR *S. typhi*. The routine TCV vaccination prevents antimicrobial resistance and misusage of antibiotics. This study assessed the knowledge, attitude, and practices of the general population of Pakistan regarding TCV. In total, 85.3% of our study population responded that vaccines prevent illnesses consistent with the study by Sankar et al. that 91.86% responded as vaccines provide prevention against illnesses (30). Only 54.2% of participants were aware of TCV being lower than the study by Hanif et al. as 75% of the sample population knew about TCV in Sindh, Pakistan (21). It can be attributed to the possibility that Hanif et al. had conducted the study in the vicinity of the vaccination center having the probability of raising consciousness and awareness about the vaccines.

Mass media (35.1%) and doctors and nurses (16.1%) followed by friends and relatives are the major sources of information for TCV while Debnath et al. had reported that healthcare workers (39.2%), mass media (25.6%), and relatives (12.8%) are the sources of knowledge for vaccines and vaccine-preventable diseases (31). Humans are now living in the era of mass and social media, and people depend on technological services even for basic daily needs making it the major source of information (23, 32). Social media campaigns had been proven effective in promoting awareness about health information such as their appeal, broad coverage, rapid provision, and cost-effectiveness (33).

The majority of the participants have a positive attitude toward TCV, as it was considered safe and effective by 77.7 and 80.8%, respectively. Memon et al. depicted that 75.7 and 87% believed that TCV is safe and effective, respectively (26). However, participants responded that TCV may contain contents forbidden by Islamic

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TABLE 4 Practice of study participants toward typhoid vaccination (*n*=918).

Variables	Characteristics	Frequency
Have you given your children the obligatory vaccines including typhoid vaccine?	No	69 (7.5%)
	Yes	208 (22.7%)
	Given vaccines other than typhoid	112 (12.2%)
	I have no children	529 (57.6%)
Was the vaccine from Government Campaign or taken privately?	Government Campaign	262 (28.5%)
	Privately	87 (9.5%)
	Not vaccinated	34 (3.7%)
	Not applicable	535 (58.3%)
Would you comply with a booster dose?	No	155 (16.9%)
	Yes	763 (83.1%)
Do you have family restrictions regarding this vaccine?	No	89 (9.7%)
	Yes	829 (90.3%)
What side effects you think it has?*	Fever	462 (50.3%)
	Lightheadedness	187 (20.4%)
	Nausea	173 (18.8%)
	Vomiting	102 (11.1%)
	Fainting	41 (4.5%)
	Pain and swelling at injection site	453 (49.3%)
	None	35 (3.8%)
	I do not know	478 (52.1%)
What would you do if the child experience any of the side effect following vaccination?	Believe that vaccination is working	115 (12.5%)
	Consult a doctor	533 (58.1%)
	Give fever medication at home	197 (21.5%)
	Stop giving the vaccination	26 (2.8%)
	I do not know	187 (20.4%)
Who would you contact if the child missed the dose?	Doctor	842 (91.7%)
	Nurse	16 (1.7%)
	Pharmacist	21 (2.3%)
	Family or friends	14 (1.5%)
	Handle myself	25 (2.7%)
Who will you contact for next vaccination?	Doctor	845 (92.0%)
	Nurse	23 (2.5%)
	Pharmacist	28 (3.1%)
	Family or friends	22 (2.4%)

*This question had multiple options to choose with.

law (67.9%), have fear about the vaccine (21.6%), and is an international conspiracy against Pakistan (13.1%). Memon et al. reported that TCV is an international conspiracy against Pakistani children (73.5%), harmful (24.3%), and may have harmful contents (11.4%), and 92% would accept the vaccine if their issues and concerns were answered (26). Previously, religious leaders had spread the idea that polio vaccination will cause vaccine-induced infertility. Similarly, for the COVID-19 vaccine, people believed that it will make the population sterile, or the person getting vaccinated will die in 2 years (18). Similarly, the assumptions had been made against the other vaccines as TCV being comprised of harmful contents and would have hazardous effects on human health in the long run.

The advice from an expert (53.3%) and the provision of vaccination by the government (29.8%) would encourage the participants to vaccinate the children with TCV. Tahir et al. reported that the compulsion of vaccination by the government (48.06%) and the recommendation by the doctor (39.37%) are the reasons leading to vaccination against COVID-19 (12).

Healthcare workers are the most significant and trustworthy source of knowledge to the public for disease prevention and treatment, especially immunization, educating about the benefits of

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TABLE 5 Regression analysis of factors associated with willingness for booster dose of typhoid vaccine.

Variables	Univariate	*p*-value	Multivariate	*p*-value
Gender				
Male	1.000	-	1.000	–
Female	1.652 (1.163–2.346)	0.005*	1.132 (0.735–1.744)	0.573
Age groups				
≤20 years ($n = 199$)	3.658 (1.738–7.702)	0.001*	0.875 (0.307–2.497)	0.803
21–30 years ($n = 574$)	5.236 (2.624–10.448)	<0.001*	1.520 (0.606–3.815)	0.372
31–50 years ($n = 107$)	1.434 (0.672–3.063)	0.351	0.968 (0.402–2.331)	0.942
>50 years ($n = 38$)	1.000	-	1.000	-
Marital status				
Unmarried	1.000	-	1.000	-
Married	0.285 (0.199–0.409)	<0.001*	0.562 (0.326–0.969)	0.038*
Residence				
Urban	1.000	-	1.000	-
Rural	0.255 (0.173–0.376)	<0.001*	0.673 (0.403–1.124)	0.130
Monthly family income (PKR)				
< 25,000	1.000	-	1.000	-
25,000–50,000	1.090 (0.694–1.710)	0.709	0.826 (0.496–1.374)	0.461
> 50,000	4.920 (3.138–7.714)	<0.001*	2.853 (1.655–4.916)	<0.001*
Number of household members				
1–2 ($n = 56$)	2.036 (0.874–4.740)	0.099	2.133 (0.850–5.354)	0.107
3–4 ($n = 195$)	1.890 (1.138–3.140)	0.014*	1.414 (0.795–2.516)	0.239
5–6 ($n = 414$)	1.561 (1.050–2.321)	0.028*	1.066 (0.675–1.684)	0.784
>6 ($n = 253$)	1.000	-	1.000	-
Level of Education				
No formal education	0.250 (0.099–0.634)	0.003*	0.692 (0.221–2.165)	0.526
Primary up to Matric	0.514 (0.257–1.028)	0.060	1.382 (0.618–3.093)	0.431
Secondary/Intermediate	2.214 (1.154–4.247)	0.017*	2.013 (0.942–4.305)	0.071
Graduate	2.168 (1.249–3.763)	0.006*	1.370 (0.729–2.576)	0.328
Post-graduation	1.000	-	1.000	-
Employment status				
Employed	0.589 (0.416–0.833)	0.003*	1.024 (0.634–1.653)	0.923
Unemployed	1.000	-	1.000	-
Occupation				
Healthcare workers	3.635 (2.147–6.155)	<0.001*	2.339 (1.314–4.163)	0.004*
Others/No occupation	1.000	-	1.000	-
Do you know typhoid could be prevented by vaccination?				
No	0.146 (0.097–0.222)	<0.001*	0.337 (0.196–0.580)	<0.001*
Yes	1.000	-	1.000	-
Do you know that Government of Pakistan have started typhoid vaccine drive?				
No	0.505 (0.354–0.719)	<0.001*	0.667 (0.421–1.056)	0.084
Yes	1.000	-	1.000	-
Do you believe that typhoid conjugate vaccine is safe?				
No	0.790 (0.384–1.627)	0.523	1.595 (0.756–3.366)	0.267
Yes	6.707 (4.518–9.957)	<0.001*	2.078 (0.571–7.566)	0.220

(Continued)

TABLE 5 (Continued)

Variables	Univariate	*p*-value	Multivariate	*p*-value
I do not know	1.000	-	1.000	-
Do you believe that typhoid conjugate vaccine is effective for prevention of typhoid fever?				
No	0.388 (0.178–0.845)	0.017*	0.198 (0.057–0.688)	0.011*
Yes	6.417 (4.254–9.680)	<0.001*	1.379 (0.686–2.771)	0.367
I do not know	1.000	-	1.000	-
Do you have any fears about this vaccination?				
No	2.965 (2.045–4.299)	<0.001*	1.117 (0.672–1.856)	0.670
Yes	1.000	-	1.000	-
Do you think it is an international conspiracy against Pakistani children?				
No	1.000	-	1.000	-
Yes	0.389 (0.252–0.601)	<0.001*	0.979 (0.496–1.933)	0.952
Do you think it may contain contents forbidden by Islamic law?				
No	6.642 (4.437–9.943)	<0.001*	1.988 (1.060–3.728)	0.032*
Yes	1.469 (0.869–2.484)	0.151	0.968 (0.427–2.196)	0.939
I do not know	1.000	-	1.000	-

PKR, Pakistani Rupee. Significant *p*-values are denoted by *. The dependent variable compliance with a booster dose of typhoid vaccine has been responded to as 'Yes'.

vaccination, and myths prevalent about the vaccine in society. Therefore, good communication between HCWs and the general population would promote vaccination and encourage them to vaccinate themselves and others in society (26). Majority, would consult a doctor for the missed dose of vaccine (58.1 %) and for side effects of vaccine (91.7%), and for the next vaccination (92.0%) corresponding with Sankar et al. as 70.2 and 29% would contact a doctor and nurse, respectively, for a missed dose of vaccine (30).

The higher income, education level, and less number of household members had more willingness for a booster dose of TCV. The higher income level contributes more to the affordability of medical treatments and utilizes available health facilities for healthy living and protection against diseases (34). Similarly, a higher education level raises awareness and knowledge regarding healthcare and reduces false beliefs resulting in a positive attitude toward the prevention and treatment of diseases and a willingness for vaccination. Older age, higher level of the perceived threat from the diseases, and positive attitude toward the vaccine are other factors contributing to the willingness for vaccination (35). The sociodemographic groups with low levels of education and income had lower willingness toward vaccination suggesting the modification in strategies to overcome the social inequalities to provide equal opportunities to vaccinate (36).

Limitations of the study

This study, however, would have some limitations. First, convenient sampling was utilized for which there would be chances of selection bias. It was an online survey, and people with the facility to the Internet had only participated in the study. Second, this study would have recall bias. Third, compared to Pakistan's overall population, our sample size was more educated, wealthier, and more urban. Finally, despite the fact that the replies were anonymous because the study was self-reported, participants may have felt under pressure to adopt the favorable attitudes and perceptions that were socially acceptable. This study calls for additional research focused on the population residing in rural areas and with low education levels.

Conclusion

The general population of Pakistan had knowledge about the benefits of vaccination and the role of booster doses but was found to be lacking regarding the provision of TCV by the government in the country and its effectiveness against typhoid fever. The attitude and practices were reported to be in favor of promoting vaccination, but some false religious and national beliefs are also prevalent demanding the need to be addressed. People will also comply with the booster dose of TCV, indicating the awareness of the general population about the importance of completing a treatment course of vaccine for disease prevention. Furthermore, healthcare authorities, media, and government professionals should collaborate to conduct seminars and campaigns on domestic and national levels and should utilize electronic and print media to make an effort to address negative views about immunization and raise awareness about the disease, its adversities, mode of transmission, and the role of vaccines in the prevention of diseases and saving human lives.

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 human participants were reviewed and approved by Pir Abdul Qadir Shah Jeelani Institute of Medical Sciences, Gambat. The patients/participants provided their written informed consent to participate in this study.

Author contributions

IU, MT, and MS conceived the idea. MZ, JS, and UF collected the data. MA analyzed and interpreted the data. MZ, MT, JS, WT, FA, RI, UF, and AA performed write-up of the manuscript. AA, MA, MT, WT, KU, and MS reviewed the manuscript for intellectual content critically. All authors approved the final version of the manuscript.

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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
Mohammad Asif Sherwani,
Independent Researcher, Birmingham, AL, United States

REVIEWED BY
Suhail Muzaffar,
University of Alabama at Birmingham, United States
Artur Queiroz,
Northwestern Medicine, United States

*CORRESPONDENCE
Timothy Ken Mackey
✉ tkmackey@ucsd.edu

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Analysis of online user discussions on Reddit associated with the transition of use between HIV PrEP therapy

Hector Godinez[1], Qing Xu[1,2], Tiana J. McMann[2,3], Jiawei Li[1] and Timothy Ken Mackey[1,2,3]*

[1]S-3 Research, San Diego, CA, United States, [2]Global Health Policy and Data Institute, San Diego, CA, United States, [3]Global Health Program, Department of Anthropology, University of California, San Diego, San Diego, CA, United States

In 2019, the U.S. Food and Drug Administration (FDA) approved emtricitabine and tenofovir alafenamide (Descovy) as another option for HIV pre-exposure prophylaxis (PrEP) prevention for high-risk adults and adolescents. With the introduction of this new PrEP, millions of current users on emtricitabine and tenofovir disoproxil fumarate (Truvada), another PrEP medication currently used to prevent HIV transmission, have options of whether to continue their current treatment regime or transition to new treatment options. The objective of this study was to conduct a descriptive analysis to characterize user-generated social media conversations on Reddit associated with FDA-approved PrEP prevention treatment options. Key themes identified were associated with perceptions, knowledge, and attitudes associated with the transition of use of different PrEP medications. Data were collected retrospectively and prospectively from the Reddit platform for posts with keywords filtered for HIV, PrEP, and FDA-approved PrEP prevention treatment from October 2020 to December 2020. We chose the Reddit platform based on prior studies that have identified PrEP user conversations and insights on access challenges for specific AIDS communities, such as gays and men who have sex with men (MSM). Reddit posts were then manually annotated using an inductive content coding approach for key themes regarding the transition of use and other emergent themes from user-generated content. Formal coding of text data was conducted with refined codes, and sub-codes created. A total of 3,120 posts were analyzed from Reddit resulting in 315 posts that were coded for PrEP and 105 posts (33.33%) specific to user discussions regarding the transition of PrEP prevention. Overall, users expressed interest in drug switching to Descovy, particularly in the context of poorer adherence or concerns about existing side effects associated with Truvada. Other major themes included discussions about the cost of Descovy, apprehension about side effects in comparison to Truvada, insurance coverage changes, and discussions about the donation of Truvada to other users after transitioning. Among these discussions, topics related to sexual minorities, including MSM, reported concerns when considering a switch in their HIV prevention regime. Understanding the changing public perception associated with the introduction of new HIV prevention is important in the context of market access, patient safety, pharmacovigilance, and health equity, particularly among high-risk populations such as MSM. Results support the use of social media from a digital pharmacovigilance perspective to better understand emerging HIV prevention, treatment, and adherence challenges experienced by patients.

KEYWORDS

PrEP, HIV/AIDS, drug switching, pharmacovigilance, infoveillance, infodemiology, minority health, social media

1. Introduction

In 2020, ∼37.7 million people were living with human immunodeficiency virus (HIV) globally, and as of 2021, over 28.2 million people were accessing anti-retroviral therapy (ART), representing an increase of more than 20 million people accessing treatment since 2010 (1). This is in large part due to a large reduction in anti-retroviral therapy (ART) prices as well as increases in the availability of treatment options approved for HIV (2, 3). In addition to ARTs, pre-exposure prophylaxis (PrEP) is a highly effective medication, that when taken as prescribed, is highly effective for preventing HIV infection, including for those at risk through sex or injection drug use (4). Importantly, certain sexual and gender minorities (SGMs), including men who have sex with men (MSM) and transgender women, have been disproportionately impacted by HIV infection and may have different attitudes toward PrEP therapy that can impact uptake and adherence (5, 6). Understanding the willingness to use PrEP therapy among these at-risk SGM populations, including in the context of drug transition and switching behavior, is critical to reducing HIV incidence.

In 2012, emtricitabine and tenofovir disoproxil fumarate (brand name Truvada) was approved by the U.S. Food and Drug Administration (FDA) for use in HIV-negative individuals to reduce the likelihood of contracting HIV, in addition to its use to treat active infections (7, 8). Since Truvada's approval as the first drug for HIV prevention using pre-exposure prophylaxis (PrEP), other drugs have also received approval for a PrEP indication in an oral formulation. In 2019, the FDA approved emtricitabine and tenofovir alafenamide (brand name Descovy) for PrEP in at-risk adults and adolescents (9). Truvada is a tenofovir disoproxil fumarate (TDF), and Descovy is a tenofovir alafenamide (TAF).

Although the chemical composition of these therapies is different, studies have found both formulations to be non-inferior to each other (10). Descovy's approval came with reports that its safety profile might be more favorable, with early evidence of fewer side effects compared to other PrEP options. This factor was considered important, as clinicians cited concerns about side effects when prescribing PrEP (11). However, evidence to date does not support that one drug has better safety and efficacy over the other, with a recent meta-analysis finding a difference in viral suppression and bone and renal side effects only when using a boosting agent with TAF (12). Apart from these considerations, cost and access issues are an ongoing concern as these drugs lacked generic treatment options at certain periods of time (e.g., the FDA approved a generic version of Truvada in June 2017) (9, 13). Hence, the potential benefits of increased competition and patent expiry have also been discussed and anticipated (13). Additionally, medical mistrust among SGMs and other minority populations regarding the effectiveness of PrEP may also represent a critical barrier to uptake and adherence (14–19).

Abbreviations: HIV, Human immunodeficiency virus; PrEP, Pre-exposure prophylaxis; FDA, The U.S. Food and Drug Administration; MSM, Men have sex with men; ART, Anti-retroviral therapy; TDF, Tenofovir disoproxil fumarate; TAF, Tenofovir alafenamide; AIDS, Acquired immunodeficiency syndrome; LGBTQ, Lesbian, gay, bisexual, transgender, queer; PEP, Post-exposure prophylaxis; SGM, Sexual and gender minority.

Despite active conversation about the similarities, differences, and possible benefits vs. risks of these different FDA-approved PrEP therapy regimes among the HIV/AIDS community, few studies have specifically examined perceptions, attitudes, and behaviors associated with the transition of use for PrEP, though several studies have been published assessing barriers and facilitators to PrEP therapy as discussed on social media (20–23). Furthermore, few studies have examined social media to characterize how willingness to use PrEP may be impacted by perceptions and attitudes about drug transitions, particularly among SGMs as a specific population of interest. This study adds to this growing body of literature on social media and PrEP by conducting a descriptive analysis identifying and analyzing Reddit posts specific to users reporting their lived experiences with transitions of use attitudes for PrEP prevention therapy, while also examining whether these conversations included users who discussed issues related to or specifically self-identified as racial, ethnic, or sexual/gender-identification minorities (e.g., LGBTQ).

2. Method

2.1. Data collection

We used the programming language Python™ to develop a custom programming script to collect publicly available posts from Reddit. Posts were collected from Reddit over a 60-day study period (13 October 2020–11 December 2020) and allowed us to collect both retrospective data from posts that occurred prior to the date of collection and prospective data, posts collected starting on the date of collection. This data collection method and time period were chosen as it was deemed sufficient to collect both past and recent conversations regarding PrEP. Specifically, data were collected by retrieving posts from search results and sub-Reddits filtered for common PrEP and HIV-associated keywords including "HIV," "PrEP," "Truvada," and "Descovy." These keywords led to additional associated keywords and hashtags identified in Reddit conversations relating to HIV prevention and PrEP that were used for additional data collection. We chose the Reddit platform based on prior studies that have identified PrEP user conversations and insights on access challenges for specific AIDS communities, such as gay and MSM.

2.2. Data analysis

To identify, characterize, and elucidate conversation associated with the two approved PrEP medications, Truvada and Descovy, we manually annotated all collected posts. First, we identified all signal posts. Posts were deemed as "signal" if they were (a) user-generated (i.e., not posted by organizations or media outlets); and (b) conversation discussing topics relevant to Truvada and Descovy accessibility, use and or transition of use, effectiveness, insurance coverage, and associated barriers. Advertisements of the drug, public announcements, and posts not related to Truvada and Descovy were specifically excluded from analysis as the focus of this study was to identify and characterize user-generated posts regarding behavior associated with PrEP, and with a specific

focus on drug transitions, not drug promotion or HIV health education and promotion. To classify the content of Reddit posts, a general inductive approach was utilized to code the textual data for descriptive analysis (24). All identified signal posts were reviewed by the first author (HG) and notes were taken on general themes of posts. Formal coding of text data was conducted with refined codes, and sub-codes were created. A final coded dataset was reviewed by the second author (QX), and differences in code definitions and applications were reconciled by the first and second authors. Inductively derived codes (see Table 1) were then included in the analysis if they included content discussing the two FDA-approved PrEP drugs "Truvada" and "Descovy." A final coded dataset was reviewed by the first, second, and last authors (TKM) to assess whether any differences in code definitions and application occurred with any differences reconciled by consensus on the correct classification. The first and second authors achieved high inter-coder reliability for signal coding (kappa = 95.32). Additionally, the text and metadata (e.g., gay label and rainbow emoji) of Reddit posts associated with PrEP content that was relevant to the themes generated for this study were also coded to determine whether they included comments or discussions related to SGM issues, or whether users specifically self-identified as gay, lesbian, or bisexual (sexual minority status) or whether they self-reported their expression of their gender identity as different from their sex assigned at birth (gender minority status) (25). A visual summary of the study methodology used is shown in Figure 1.

3. Results

3.1. Overview

We collected a total of 2,349 unique Reddit posts over the study period. Descriptive analysis and manual annotation of all collected posts identified 85 (4.47% or the entire dataset) signal posts. Posts that did not meet our inclusion criteria for this study but were nevertheless relevant to PrEP facilitators and barriers included posts discussing differences between post-exposure prophylaxis (PEP) and PrEP therapy, confusion regarding PrEP therapy, experiences regarding healthcare providers' lack of PrEP knowledge and training, and comments believing that Descovy advertisements only targeted certain minority populations were excluded. Based on the publicly available text in Reddit posts, the largest representation from a minority group were for members associated with the sexual minority community (95.29%, $n = 81$), specifically gay and MSM. A summary of parent and sub-code book results and corresponding de-identified examples are reported in Table 1.

3.2. Data analysis

According to our data analysis, all signal posts were categorized into two main parent-level topics: (i) pre-transition: posts regarding users who currently take Truvada, users recognizing a new PrEP medication has been approved, and users sharing or seeking knowledge regarding the differences between Truvada and Descovy and (ii) post-transition: sharing the experience of switching from Truvada to Descovy, or asking questions associated with the transition experience. In the context of PrEP drug transition, all discussions detected concerned a pathway of transition or switching from Truvada (pre-transition) to Descovy (post-transition).

Among all signal posts, 62 posts (72.94% of total signal posts) were classified in the pre-transition parent topic (please see Figure 2 for the visual depiction of themes). We observed four main themes among these posts: conversations sharing or seeking knowledge on how to switch from Truvada to Descovy (A-3) that had the highest number and percentage of posts of all topics ($n = 24$, 22.86%); followed by topic (A-1) associated with users sharing or seeking knowledge on potential side effects from Descovy and also discussing reasons why this influenced their decision of not to engage in drug transition ($n = 15$, 17.65%); topic (A-4) included posts in which users expressed interest in switching to Descovy because of the purported side effects experienced from Truvada ($n = 12$, 28.23%); and topic (A-2) focused on sharing or seeking insurance coverage information associated with drug switching to a new PrEP treatment and how this impacted their decision to transition or not to transition to another PrEP treatment ($n = 11$,12.94%).

Among posts classified in post-transition parent topics ($n = 23$, 27.06%), we also observed four main themes. A major theme (B-3) that emerged was the discussion of side effect management after switching to Descovy ($n = 11$, 12.94%). Another topic (B-2) included discussions regarding mixing Truvada and Descovy medications (i.e., primarily focused on questions by users regarding how they should use (co-use) remaining Truvada medication after switching to and initiating Descovy) ($n = 6$, 7.07%). Topic (B-4) contained posts regarding users seeking information on how they can donate their remaining Truvada to other people who might benefit from prevention treatment access ($n = 4$, 4.71%). A final sub-topic (B-1) included general discussion of the experience of switching medications and why users switched to Descovy.

4. Discussion

Our study conducted a descriptive analysis of over 2,000 Reddit posts and found that just under 5% included specific discussions about transitions of use between Truvada and Descovy, with the vast majority of these posts originating from user-generated comments or posts related to SGM issues or from users self-identifying as members of the LGBTQ community, specifically gay and MSM populations. Key insights generated from these discussions include knowledge seeking and information sharing by users to help navigate decisions about transitioning to new PrEP therapy (pre-transition) and concerns that arose after switching from Truvada to Descovy (post-transition).

Our results add to insights generated by prior studies that have examined social media for clues regarding barriers and facilitators associated with PrEP, including specifically among gay and MSM populations. For example, a 2018 systematic review examined both peer-review studies and online posts to identify barriers and facilitators to PrEP among MSM, detecting six overarching categories that included online sources acting as facilitators to PrEP uptake and concerns about perceived side

TABLE 1 Inductive code list and identified sub-codes.

Topic level	Code number	Description	Examples	Total
Pre-Switch Level (72.38% of all signals)	A-1	Sharing or seeking knowledge regarding potential side effects from Descovy and also discussing reasons why this influenced their decision not to engage in drug transition	"So I got prescribed descovy [sic] today. I haven't taken it yet. Before I do, I just wanted to get everyone's thoughts and opinions on it. Should I expect any real side effects [sic] the first few days? I know it is and I feel stupid for asking but it's completely safe right? Thanks" "I just started PrEP on Truvada like LAST WEEK [sic] and now Descovy is FDA approved. I pinged my doc if I should switch when my current supply of Truvada is gone and she responded by prescribing Descovy. I'm waiting for her to answer the question of [sic] 'should I switch now?'. I feel like the lower side effects are great but it's also [sic] 'bleeding edge' for PrEP so I'm not sure how I feel about that. So guys who have been on PrEP longer than I have: Are you switching to Descovy?"	15 (17.65%)
	A-2	Sharing or seeking insurance coverage information associated with drug switching to a new PrEP treatment and why this impacted their decision to transition or not to transition to another PrEP treatment	"I want to get on PrEP before I start having condomless sex with my fwb specifically Descovy since it has less [sic] side effects. However, my insurance company . . . told me that I will have a co-pay [sic] around $200 for only a 30-day prescription! What should I do?"	11 (12.94%)
	A-3	Asking for or sharing knowledge on the process of switching medication	"What are your experiences with truvada and descovy [sic]? Did you tolerate one better? How did you start the conversation with your doctor? I'd appreciate any advice you can give me."	24 (22.86%)
	A-4	Interest in switching to Descovy after having side effects from Truvada	"Last night I topped (i almost never have sex) and a guy and the condom broke. I didn't notice until it was over so I don't know how long it was broken for (but the whole thing only lasted like 7 min?). . . I went to the doctor to ask about PEP today. She didn't really seem to know what it was (she was a physician's assistant) and went and called the doctor and came back basically saying sure we'll give it to you. I didn't feel really assured by that. I went and got the prescriptions [truvada tivicay (sic)] and took them for the first day so far I feel okay. But I am really afraid of the potential side effects I keep reading about online. Especially the severe shit like liver/kidney failure. Should I be taking this given what my situation is?"	12 (28.23%)
Total				62 (72.94%)
Post Switch Level (21.90% of all signals)	B-1	Sharing experience and reasons for switching from Truvada to Descovy	"I had bad long lasting [sic] side effects with Truvada for PreP so I thought I'd make this post to help others with the same experience with Truvada. With Truvada, I had diarrhea and stomach cramping always 30 min after ingestion. Doctors told me to continue the medication as it would get better with time. Went on for months and it was the same thing. A week ago, I switched over to Descovy, and it's been fantastic compared to Truvada. Only minimal diarrhea for the first 3 days and then nothing. Its [sic] been a week now and I'm not experiencing any side effects. Doctor [sic] informed me it was most likely due to the fact that the tenofovir in Truvada is absorbed by your body in lesser doses than the tenofovir in Descovy is which is absorbed [sic] a higher quantity. Whether this is true or not I have no idea. I just know that I'm experiencing no side effects with Descovy besides the[sic] mild diarrhea the first few days. Hope this helps!"	2 (2.35%)
	B-2	Asking for advice on mix using Truvada and Descovy	"I've been trying to take both but pure for men says to take it 2 h after or before taking any other medication. What is your advice and does pure for men lessen the protection and benefits of descovy [sic]"	6 (7.06%)
	B-3	Asking for advice on managing side effects	"Just started taking descovy [sic] about 2 weeks ago and have been experiencing the worst gas of my life. Has anyone had a similar experience? Also, any advice on how to manage the side effects would be appreciated." "Seems like I am having the same side effects with my Descovy as I did with Truvada (sucks because I switched because of this). Side effects for me are stomach problems and not being able to put on weight (for me being as thin as I am this is not necessarily a good thing before someone says you should be happy with this side effect)."	11 (12.94%)
	B-4	Asking for advice on donating leftover Truvada	"My gay GP just switched me from Truvada to Descovy not because I've had any side effects but just because my plan would cover it and if Descovy has a lower chance of side effects why not? When he switched me, I told him I'd just refilled my Truvada and he said I'd probably have to wait until the Truvada ran out to go onto the Descovy. OH NO... He sent in the prescription for Descovy and it was ready at my pharmacy the next morning. So now I have a sealed original packaging completely untouched 30-day bottle of Truvada that I will not [sic] ever use. Last I heard these retail for ~$1,800. So, I don't want to just flush it. I live in Los Angeles so I'm sure there's someone in need who could use it! Is there some way I can donate it? Is that even legal? If so, how would I find a place that would take it? If not... any ideas of how I might be able to get it into the hands of someone in need? It is criminally expensive medication so I don't just want to throw it away. All advice welcome PM me if you're more comfortable."	4 (4.71%)
Total				23 (27.06%)

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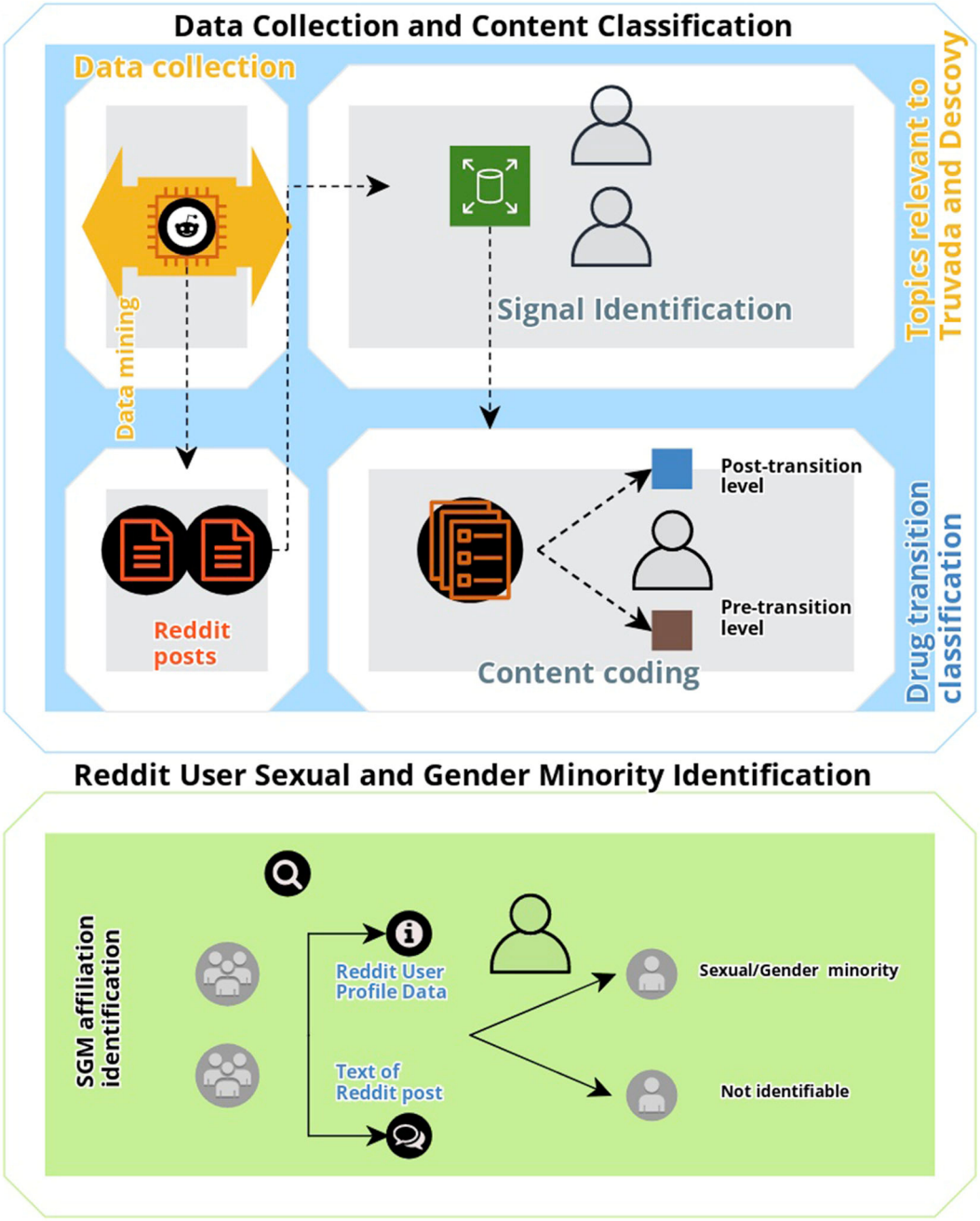

FIGURE 1
Overview of study methodology.

effects (20). A 2021 study specifically examined characteristics of PrEP-related content on Instagram for Truvada, including discussion by users about side effects (21). A recent 2022 study analyzed user perceptions and attitudes toward PrEP across multiple social media platforms (Twitter, YouTube, Instagram, Tumblr, and Reddit) and identified specific barriers to PrEP access including among minority online users (22). Finally, another 2022 study specifically examined conversations from Reddit users on PrEP from 2014 to 2019 and identified discussions about PrEP initiation and side effects as well as other themes (23).

Adding to these prior studies that examined social media for HIV and PrEP topics, this study was able to identify specific lived experiences regarding transitions of use for PrEP therapy, a topic subject to possible social stigma as it relates to HIV status, sexual orientation, and HIV-related risk behavior. However, due to Reddit's relative anonymity as an online platform and the ability to up and downvote content, it appears to be a platform

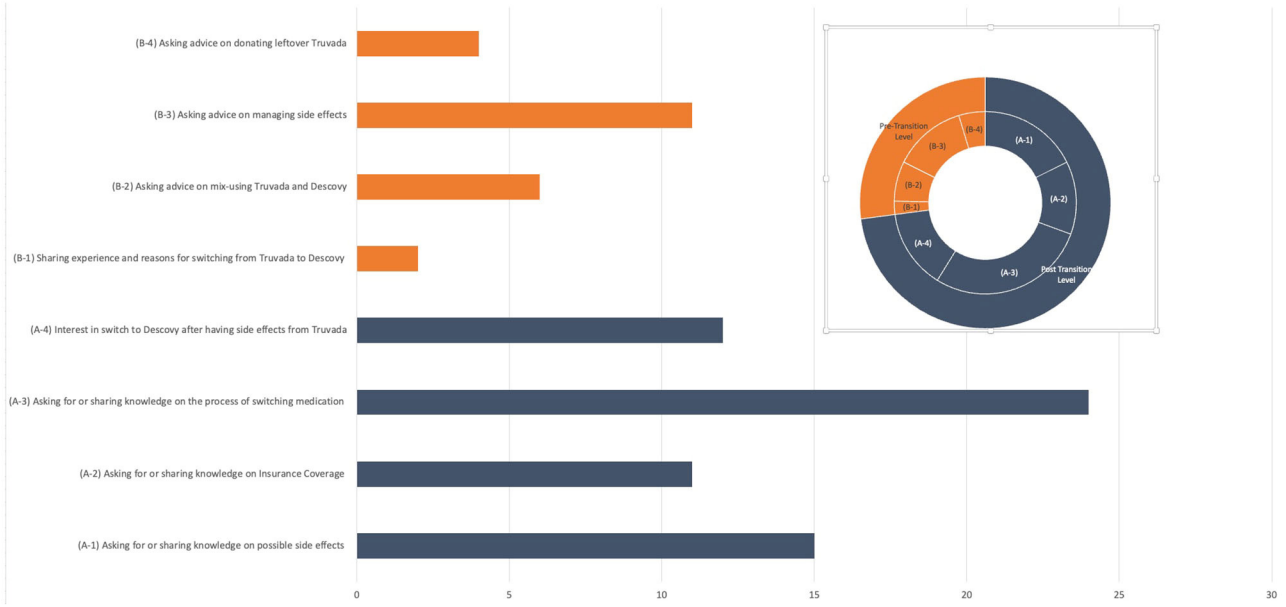

FIGURE 2
Content breakdown into sub-topics.

where users may be more willing to discuss stigmatizing topics (26). Specifically, we observed that Reddit users actively shared their lived experiences with PrEP to aid in online peer-to-peer decision-making about the benefits and risks of PrEP therapy, including in the context of facilitating the transition of use or initiation of new HIV therapy. Importantly, users who may lack sufficient information to make informed decisions about PrEP therapy options may utilize these online communities as primary health information-seeking sources, even before or in the absence of consulting with a healthcare professional.

Furthermore, discussion of the impact of perceived side effects of different PrEP medications was prominent in the drug transition of use decisions, along with the discussion of access (e.g., insurance coverage) and cost. An additional consideration not specifically detected in this study but likely influencing the transition of use among users was the financial incentives associated with switching PrEP therapy. Descovy's regulatory approval for PrEP therapy occurred in 2019, just over 1 year prior to the generic availability of Truvada. At its introduction to the market, Descovy was offered with large commercial discounts which resulted in it launching at a 12% lower average net price than Truvada (27). These financial incentives led to changes in PrEP use. Specifically, within the first 9 months of it entering the market, nearly 30% of patients switched from Truvada to Descovy (28). In addition to the initial lower costs, patients may also prefer transitioning from Truvada to Descovy due to early reports that Descovy was less toxic and had fewer side effects, despite these claims since being refuted (10, 29). New themes that emerged from this study also included questions about the co-use of PrEP therapy and ways to donate unused medicines to other patients, unique in the context of examining behaviors associated with the transition of use for HIV/AIDS drugs.

Importantly, these findings provide insights that are important to post-market surveillance and pharmacovigilance efforts attempting to better understand perceptions of uptake, risk, and safety for PrEP among a community of predominantly gay and MSM users at higher risk for HIV. However, this study may not provide an extensive list of barriers and facilitators to accessing and adhering to PrEP therapy, as many people who contemplate HIV prevention or treatment may not openly discuss the unique complications they experience due to the stigmatization associated with this topic whether online or offline. Results can help inform how patients and specific gay and MSM populations in English-speaking countries experience and react to perceived adverse events and side effects and how it may impact the HIV care continuum of initiating, adhering to, and potentially transitioning between PrEP in preventing transmission (30). Importantly, the HIV burden remains high among African, Asian, and other non-English speaking countries. As such, most PrEP users were reported as residing in Africa in 2020 (31).

Hence, future studies should adopt methodologies used in this study for non-English language social media posts to better understand the unique barriers and facilitators in regions representing the largest HIV burden and greatest PrEP use, while also examining topics not explored in this study, such as the targeting of PrEP product advertising or outreach toward specific SGM populations. Future studies should also further validate results generated from social media with other sources of patient experiences (e.g., real-world data, prescribing and reimbursement information, and electronic health records) and integrate these novel data sources into a more proactive system of interdisciplinary pharmacovigilance to ensure better uptake and adherence to PrEP. In particular, the use of digital mixed methods that combine social listening approaches with in-depth qualitative data (e.g.,

focus groups) and robust quantitative data (e.g., surveys) designed specifically for at-risk SGM populations can better triangulate insights into PrEP barriers that can inform the development of future interventions and patient outreach.

5. Limitations

This study is primarily exploratory and descriptive in nature and has certain limitations. First, we only collected data from Reddit and limited our analysis to keywords and terms in the English language. Hence, the findings are not generalizable to all social media users who discuss transitioning between PrEP therapy, including those in other countries with higher HIV burdens but who are underrepresented in the population of Reddit users. Additionally, this study may represent an underestimate of people's experience with PrEP therapy and may not provide an extensive list of barriers to treatment due to the social stigma associated with HIV and discussing HIV-related care, including via online communities. Our collected and analyzed data sample size was also relatively small, which could lead to sampling bias. Hence, future studies should expand data collection and analysis approaches to different phrases and keywords associated with an individual's HIV-related risk behavior to obtain a more representative corpus of social media conversations and increase the sample size of data that can be analyzed. In addition, Reddit is a social media platform. There is a potential bias in using social media data including population biases, behavioral biases, content biases, and linking biases. Future studies should consider triangulating results from social listening approaches used in this study with other data sources (e.g., focus groups and survey instruments). Future studies should also expand data collection to additional platforms, languages, and specific keywords associated with HIV exposure and risk behavior to generate a more representative corpus of PrEP-related social media conversations.

Data availability statement

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

Ethics statement

All information collected during this study was available in the public domain, did not include any deleted posts, and the study did not involve any interaction with users. Any user indefinable information was removed from study results and any racial and/or ethnic identifiers were presented as aggregated results to ensure anonymity. This study has been approved by WCG Institutional Review Board's (WCGIRB's). WCG IRB is registered with the Office for Human Research Protections (OHRP) and FDA as IRB00000533.

Author contributions

HG, QX, TJM, JL, and TKM jointly conceived the study, drafted the study, conducted data collection and analysis, and wrote and agreed to the final version of this manuscript.

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Conflict of interest

HG, QX, TJM, JL, and TKM were employees of the startup company S-3 Research LLC.

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