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Induction of immune tolerance: Addressing unmet medical need in immune mediated diseases and immune responses to biologics

Edited by
Amy Rosenberg, David Markusic, Sophie Tourdot and Christoph Königs

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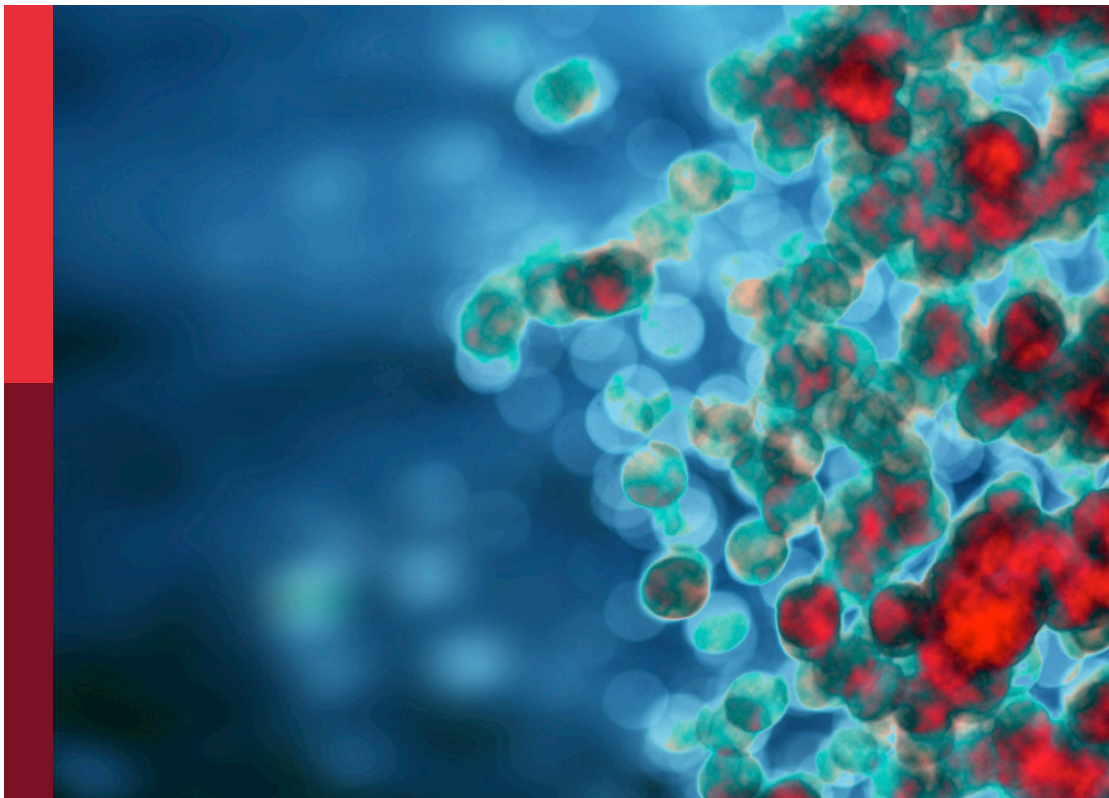

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Induction of immune tolerance: Addressing unmet medical need in immune mediated diseases and immune responses to biologics

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Editorial: Induction of immune tolerance: addressing unmet medical need in immune mediated diseases and immune responses to biologics

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Editorial on the Research Topic

Induction of immune tolerance: addressing unmet medical need in immune mediated diseases and immune responses to biologics

This Research Topic focuses on 1) strategies for immune tolerance induction in immune mediated diseases and 2) prevention and mitigation strategies for immunogenicity of biological therapeutics.

Immune tolerance approaches in immune mediated diseases

The critical overarching strategy for immune tolerance induction in the settings of autoimmunity, transplantation and allergy includes 1) interrupting effector mechanisms 2) restraining innate activation and 3) boosting regulation (Figure 1). Moreover, emphasis is placed on safety considerations, the role of epitope spread, and the need for mechanistic studies for informing future studies (Huffaker et al.).

A key concern for inducing antigen specific tolerance using a single agent in the context of pre-existing autoimmunity, allergy, or anti-drug antibodies (ADA) is the possibility of boosting extant responses and worsening disease, realized in a study employing an altered peptide ligand in MS (1). This outcome stressed the importance of developing combinatorial approaches to minimize this possibility. This is illustrated in the multifactorial treatment approaches for allergy and autoimmunity. In allergy, antigen specific desensitization treatments target both IL-4 signaling with Dupilumab, an anti-IL-4R mAb (interrupt effector mechanisms), combined with sublingual grass desensitization (boost regulation); and in the CATNIP trial in which Tezepelumab, an anti-TSLP mAb (interrupt effector mechanisms) is co-administered with Cat Allergen Extract (boost

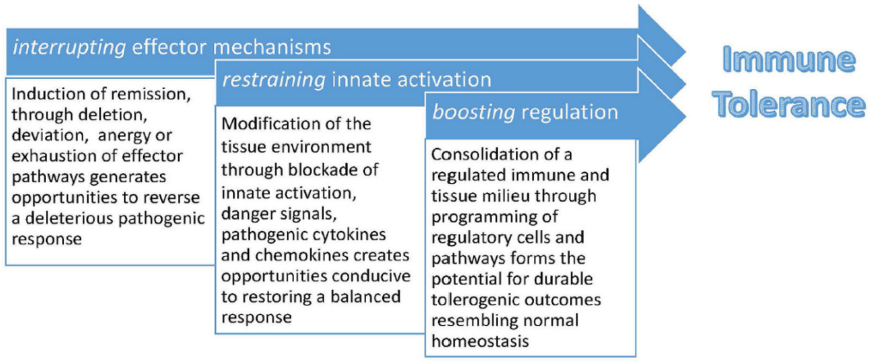

FIGURE 1
Strategy for immune tolerance induction as elucidated by the Immune Tolerance Network.

regulation). In Type 1 diabetes (T1D) initial focus was on interrupting effector mechanisms by depleting or modulating Teff cells with Teplizumab (a CD3mAb) (2), or Alefacept (LFA3-Ig) (3). While both induced marked CD8+ T cell exhaustion and prolonged the development of clinical T1D from precursor stages, neither agent changed the level of Tregs, and the patients ultimately developed T1D, further illustrating the need to "boost regulation" in this context. For tolerance to organ transplantation, the need to more effectively delete donor reactive T cells and boost Tregs or Tr1 cells to the relevant alloantigens is paramount as studies promoting tolerance *via* autologous hematopoietic stem cell transplant fail in the majority of patients. Approaches anticipated or in ongoing studies include administration of "tolerance promoting" cell therapies (the ITN TEACH trial), or infusion of alloantigen specific Tregs (ITN LITMUS Trial), and especially promising, engineered Tregs that either express target alloantigens or CARs or TCRs reactive to alloantigens (4).

In this Research Topic, Docampo et al. highlight the need for multimodal approaches in the treatment of relapsing-remitting multiple sclerosis (RRMS). GWAS studies indicate that the vast majority of SNPs associated with MS risk pertain to immune function related genes, especially HLA haplotype (HLA-DR15). Environmental risk factors include Epstein Barr virus (EBV) infection, whose key role has recently been elucidated (5). Brain homing CD4+T cells are the lynchpin in driving proinflammatory B cell and CD8+ cytolytic Teff function, and, activated by MBP, PLP and MOG derived peptides, have a clear functional hierarchy Th1>Th17. Approved therapeutics for RRMS mainly focus on disrupting effector mechanisms and Docampo et al. argues for incorporation of tolerance boosting regimens into existing treatment protocols as evaluated in studies of EAE.

The key principles elucidated by the ITN including safety, the role of epitope spread in disease progression (and tolerance), and the importance of mechanistic studies to define the critical parameters mediating or highly correlating with tolerance induction are discussed by Schurgers and Wraith. Antigen Processing Independent T cell Epitopes (Apitopes) are CD4+ T-cell epitopes that bind directly to MHC II in the conformation recognized by cognate T cells. Moreover, Apitopes bind selectively to "steady state" tolerogenic dendritic cells, the basis for which requires further elucidation.

Tolerance in T1D is explored in several novel approaches in this Research Topic. Sun et al. examined the potential for the regulatory adjuvant, kynurenin, produced by indoleamine 2, 3 dioxygenase (IDO), to increase Tregs in the setting of an autoantigen GAD65 vaccine. With previous studies of GAD65-Alum (Diamyd®) reportedly indicating safety, the potential to increase the efficacy of the immunomodulatory vaccine by the addition of kynurenin was investigated. Meanwhile, Zhang et al. modified peptide residues in contact with the TCR and found that substitution of a single amino acid by its D isoform at a TCR contact residue reduced autoreactive CD8 T cell function, T cell organ infiltration, and inflammatory responses in the pancreas. Maulloo et al. investigated induction of antigen specific tolerance through liver targeting of Antigen-N-acetylglucosamine glycopolymer conjugates showing that, as with IV delivery, Treg generation induced robust tolerance when the glycopolymer was administered SC. And finally, Al-Mrahleh et al. used IFNg and TNFa treated Wharton's Jelly- Derived Mesenchymal Stromal Cells to induce tolerogenic dendritic cells and Tregs in PBMC from T1D patients, upregulating IL-10 and TGF-b, and downregulating IL-17 and IFN-g, but not IL-6. A major limitation of these studies was that they were limited to *in vitro* assays and require further validation under *in vivo* inflammatory conditions.

Focus on tolerance to therapeutic proteins

In "Driving CARs to BARs: The winding road to specific Regulatory T cells for Tolerance," Scott provides an outstanding historical review of the development of Treg therapies as well as a critique of individual approaches: TCR vs CAR T regs vs B cell Antigen Receptor (BAR) Tregs (6). BAR Tregs elude issues regarding the HLA-matching restrictions for TCR Treg donor-recipient combinations and conformational epitope matching of CAR T scFV variable domains by expressing on Treg the target antigen to which antigen specific B cells bind, inducing B cell anergy or elimination. Thus, BAR Tregs are appropriate for antibody-mediated disease.

In studies by Lagassé et al. endogenous NK cells were found critical to FVIII tolerance mediated by a FVIII Fc-fusion protein (rFVIIIFc), through its binding to FVIII-specific memory B cells *via* the FVIII moiety and to FcγRIIIA/CD16 on NK cells, which killed FVIII-specific memory B cells *via* ADCC.

ADA to highly effective TNF mAbs in treatment of inflammatory bowel disease (Crohn's and Ulcerative Colitis) abrogate efficacy in up to 65% of patients, causing devastating adverse events including hospitalization and surgery. As Shakhnovich et al (7) make clear, aside from medication adherence, Therapeutic Drug Monitoring (TDM) is the single, most critical step for both preventing and overcoming immunogenicity in clinical practice. However, it remains to be determined whether immune tolerance to TNF mAbs is induced as well as the underlying mechanisms. Of important note, the authors allege that TDM also has the potential to "reverse immunogenicity" with TDM-based dose adjustments.

The remaining manuscripts in this Research Topic focus on a broad variety of topics related to the monitoring, risk assessment, and treatment of unwanted immune responses to different therapeutic modalities.

De Groot et al. describe a personalized immunogenicity risk assessment (PIMA) tool based on the patients' mutation and HLA haplotype in the context of Pompe Disease treated with Enzyme Replacement Therapy (ERT). PIMA tools have the potential to better identify patients at risk for ADA formation for other lysosomal storage diseases treated with ERT, thus identifying those who would benefit from prophylactic immune tolerance induction.

Vultaggio et al. discuss approaches to predict, identify the mechanism, and prevent or mitigate ADA mediated hypersensitivity reactions (HSR) directed against biologics, including a broad range of protein-based therapies.

Gross et al. delved into major immunogenicity issues pertaining to Adeno-associated viruses (AAV)-based gene therapies including antibodies to AAV capsid determinants, as well as severe adverse events stemming from complement activation. High priority approaches to address immunogenicity include modification of AAV capsid antigens, drug regimens to limit development of ADA, and strategies to transiently eliminate anti-capsid antibodies.

Balcerek et al. with UCSF investigators reported that release criteria for Treg batches in clinical studies of autoinflammatory disease and organ transplant were met in 88% of 7 phase I trials, indicating a consistent manufacturing process produces cellular products meeting standards for critical product quality attributes. The main factor correlating with level of ex-vivo expansion of Tregs was the starting number of Tregs.

Nabhan, M et al. focused on the mechanisms that underlie the interaction of mAb aggregates with antigen presenting cells, emphasizing that antibody aggregates act as danger signals recognized by innate immune cells, to trigger innate and adaptive immune responses (8).

Shah et al. focused on microneedle delivery of therapeutics or vaccines to facilitate optimal immune enhancing or immunomodulatory strategies, as well as eliminating obstacles to current storage and delivery methods.

Sakowska et al. provided a review of the immune pathways involved in cancer immune evasion and, reciprocally, development of autoimmune disease in which similar molecular players work in opposite directions.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

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Publisher's note

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ORIGINAL RESEARCH
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Immune Tolerance-Adjusted Personalized Immunogenicity Prediction for Pompe Disease

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Infantile-onset Pompe disease (IOPD) is a glycogen storage disease caused by a deficiency of acid alpha-glucosidase (GAA). Treatment with recombinant human GAA (rhGAA, alglucosidase alfa) enzyme replacement therapy (ERT) significantly improves clinical outcomes; however, many IOPD children treated with rhGAA develop anti-drug antibodies (ADA) that render the therapy ineffective. Antibodies to rhGAA are driven by T cell responses to sequences in rhGAA that differ from the individuals' native *GAA* (nGAA). The goal of this study was to develop a tool for personalized immunogenicity risk assessment (PIMA) that quantifies T cell epitopes that differ between nGAA and rhGAA using information about an individual's native GAA gene and their HLA DR haplotype, and to use this information to predict the risk of developing ADA. Four versions of PIMA have been developed. They use EpiMatrix, a computational tool for T cell epitope identification, combined with an HLA-restricted epitope-specific scoring feature (iTEM), to assess ADA risk. One version of PIMA also integrates JanusMatrix, a Treg epitope prediction tool to identify putative immunomodulatory (regulatory) T cell epitopes in self-proteins. Using the JanusMatrix-adjusted version of PIMA in a logistic regression model with data from 48 cross-reactive immunological material (CRIM)-positive IOPD subjects, those with scores greater than 10 were 4-fold more likely to develop ADA ($p<0.03$) than those that had scores less than 10. We also confirmed the hypothesis that some GAA epitopes are immunomodulatory. Twenty-one epitopes were tested, of which four were determined to have an immunomodulatory effect on T effector response *in vitro*. The implementation of PIMA V3J on a secure-access website would allow clinicians to input the individual HLA DR haplotype of their IOPD patient and the GAA pathogenic variants associated with each GAA allele to calculate the patient's relative risk of developing ADA, enhancing clinical decision-making prior to initiating treatment with ERT. A better understanding of immunogenicity risk will allow the implementation of targeted immunomodulatory approaches in ERT-naïve settings, especially in CRIM-positive patients, which may in turn improve the overall clinical outcomes by minimizing the development of ADA.

The PIMA approach may also be useful for other types of enzyme or factor replacement therapies.

Keywords: Pompe Disease (glycogen storage disease type II), enzyme replacement therapy (ERT), immune tolerance induction (ITI), anti-drug antibodies (ADA), acid alpha-glucosidase (GAA), cross-reactive immunological material (CRIM), Tregitope, personalized immunogenicity assessment (PIMA)

INTRODUCTION

Infantile-onset Pompe Disease (IOPD) is a fatal autosomal recessive glycogen storage disorder caused by a deficiency of the enzyme acid alpha-glucosidase (GAA), which breaks down lysosomal glycogen. The deficiency of lysosomal GAA leads to the accumulation of glycogen and damage to skeletal, cardiac and smooth muscles (1). Children who are born with IOPD present with hypotonia and hypertrophic cardiomyopathy within the first days to weeks of life, and lethal cardiorespiratory failure occurs if treatment is not initiated within the first 6 months (2). The introduction of enzyme replacement therapy (ERT) with recombinant human acid alpha-glucosidase (rhGAA) has vastly improved IOPD patient survival and quality of life. However, children who have IOPD and are treated with rhGAA can develop IgG anti-drug antibodies (ADA) to ERT. The development of high and sustained antibody titers (HSAT) results in reduced efficacy of the replacement therapy, and clinical decline (3).

The development of ADA to rhGAA is influenced by the presence or absence of endogenous GAA, defined as cross-reactive immunologic material (CRIM). Individuals who are CRIM-negative have a complete absence of GAA and are at the highest risk of ADA, whereas those who are CRIM-positive may be more immune tolerant to ERT, due to prior exposure to endogenous GAA (4). However, one-third of CRIM-positive IOPD children still develop high and sustained or intermediate ADA titers, putting them at risk for clinical decline similar to CRIM-negative individuals (5). All CRIM-negative patients develop HSAT. An Immune Tolerance Induction (ITI) protocol using a short course of Rituximab, Methotrexate, and IVIG has been successful for the treatment and prevention of ADA, and is the standard of care for all CRIM-negative IOPD children (6–8). As only one-third of CRIM-positive IOPD develop ADA, and it is difficult to predict exactly which CRIM-positive children are at high risk, the cost-benefit profile of ITI treatment with rituximab, methotrexate, and IVIG is not favorable, for these individuals. Improved methods for differentiating high-risk from low-risk CRIM-positive subjects and correctly identifying those that should be treated with ITI *versus* those who can be carefully watched instead, are needed.

We previously established a tool for personalized immunogenicity risk assessment (now called PIMA) that quantifies T cell epitopes that differ between nGAA and rhGAA (9) using information about an individual subject's GAA gene and HLA DR haplotype. Here we improved on the previous version of the PIMA method by taking into consideration potential tolerizing epitopes in GAA. First, we re-evaluated the original method using information from 48 CRIM-positive IOPD subjects, whose HLA and GAA genotype data were available, and we then tested progressive improvements in three new versions of the PIMA tool (V2, V3, and V3J) that weighted selected factors, such as conservation of T cell epitopes with proteins in the human genome beyond conservation with GAA (**Figure 1**). We then asked which of four versions of PIMA would best align with clinical outcomes as measured by ADA titers.

Of the new PIMA prediction models, the final version (V3J), which included more precise definition of HLA DR alleles for each subject and a correction for T cell epitopes in nGAA that may be inducing tolerance to the recombinant replacement enzyme (rhGAA), performed better than the intermediate versions. This version of PIMA integrates information generated by JanusMatrix, a computational tool that identifies T cell epitopes that have extensive conservation in the human genome (at their TCR face), which may be epitopes that activate circulating regulatory T cells (10).

We also investigated the potential for selected epitopes identified by JanusMatrix to induce regulatory T cell responses *in vitro*. Several of the putative Treg epitopes identified in GAA significantly suppress effector memory T cell response in a standardized Treg bystander assay. This important discovery of potential regulatory T cell epitopes in nGAA may improve the assessment of immunogenicity risk for IOPD and for a range of enzyme replacement therapies.

And finally, a first-generation PIMA website (Pompe-PIMA) has been developed for use in clinical decision making. Once the clinician inputs the patient-specific nGAA sequence(s) and HLA DRB1 alleles, an ADA risk estimate that is based on V3J can be calculated. After further validation and regulatory approval, the website may be used by clinicians to assess the relative risk associated with ERT therapy for their individual CRIM-positive IOPD patient.

Abbreviations: Tregitope, Regulatory T Cell epitope; Tregs, Regulatory T Cells; MHC, Major Histocompatibility Complex also referred to Human Leukocyte Antigen (HLA) complex in humans; TCR, T Cell Receptor; IOPD, Infantile Onset Pompe Disease; LOPD, Late-onset Pompe Disease; ERT, Enzyme Replacement Therapy; ITI, Immune Tolerance Induction; ADA, Anti-Therapeutic Antibodies; GAA, acid alpha-glucosidase; rhGAA, recombinant human GAA; nGAA, native or endogenous-expressed GAA of patient; CRIM, cross-reactive immunological material; GSD, glycogen storage disease; HSAT, High and Sustained Antibody Titers SIT, Sustained Intermediate Titers.

MATERIALS AND METHODS

Enrollment of IOPD Cohorts

Recruitment

Children with a confirmed diagnosis of IOPD were enrolled in Duke University Medical Centers. IOPD was defined as the

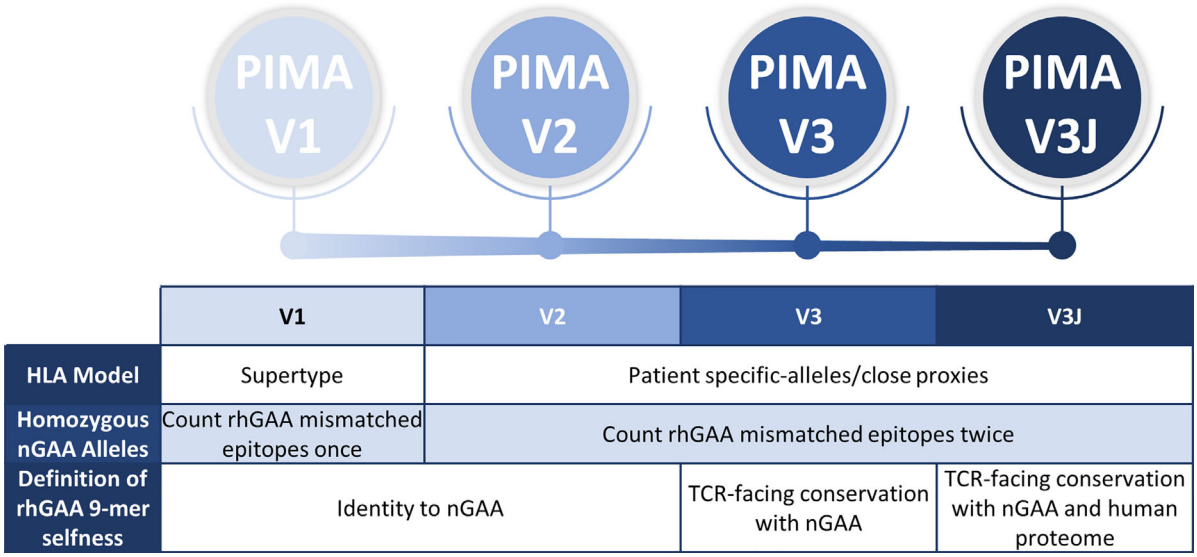

FIGURE 1 | Overview and evolution of the PIMA scoring algorithms used to calculate the ADA risk assessment score. V1 was previously published. V2-V3 are intermediate steps to developing V3J as described here. V2 added subject-specific HLA DR epitope prediction, V3 added conservation with nGAA at the TCR face of epitopes, and V3J examine the potential for certain nGAA epitopes to be tolerogenic by comparing the sequence to other human genome epitopes. The most accurate for this cohort was V3J, which adjusted the prediction for T cell epitopes that are cross-conserved with other self-epitopes (not confined to nGAA). In contrast to V1, where epitopes mismatched between rhGAA and nGAA of individuals with homozygous nGAA mutations were counted once, V2-V3J included mismatched epitopes twice in the calculations, once for each allele. We then identified specific T cell epitopes in GAA that generated tolerance *in vitro*. Individual immune tolerance to nGAA sequences may diminish the risk of ERT-related ADA.

presence of hypertrophic cardiomyopathy in the first year of life. Parents of subjects were provided with a written consent approved by the Institutional Review Board (IRB) [(Pro00001562; Determination of Cross-Reactive Immunological Material (CRIM) Status and Longitudinal Follow-up of Individuals with Pompe disease; LDN6709 Site 206; ClinicalTrials.gov NCT01665326]. Subjects were selected for the present study based on the following inclusion criteria: 1) a confirmed diagnosis of Infantile-onset Pompe disease (IOPD), 2) CRIM-positive status determined as described previously (4), 3) received ERT with rhGAA, 4) had a skin/blood sample available for HLA haplotyping, 5) did not receive immune tolerance induction, and 6) availability of at least 6 months of follow-up data. Clinical data including CRIM status, *GAA* variants, GAA enzyme activity, age at ERT initiation, and longitudinal anti-rhGAA IgG antibody titers were extracted from medical records (**Supplementary Table S1**).

CRIM Status, HLA Typing and *GAA* Sequencing

CRIM status was assessed by Western blot reactivity to a pool of monoclonal and polyclonal anti-GAA antibodies capable of recognizing both native and recombinant GAA (11, 12) from subject's fibroblast cultures and/or PBMC (4, 13). Study subject HLA DR haplotypes were determined by PCR, using a sequence-specific oligonucleotide probe (SSP) typing test (One Lambda, Inc.). Mutations in the nGAA gene were determined by PCR amplification followed by Sanger DNA sequencing. The methodology employed here was developed by the Duke University Health System Clinical Molecular Diagnostic Laboratory (4).

ADA Titers and Classification of Subjects

ADA titers were determined by Sanofi Genzyme using enzyme-linked immunosorbent assays (ELISA) and confirmed using radioimmunoprecipitation as described previously (6). Subjects whose ADA titers repeatedly exceed 51,200 after more than 6-months on rhGAA were classified as high and sustained antibody titers (HSAT) (5, 11). Subjects whose ADA titers fell between 12,800 and 51,200 within the first year of ERT were classified as sustained intermediate titers (SIT). Based on ADA titers, subjects were stratified into two groups; 1) High ADA, subjects who developed ADA titers in HSAT or SIT range (≥12,800) and 2) Low ADA, subjects who maintained ADA titers of ≤6,400.

Immunoinformatic Assessment of GAA: The Evolution of PIMA and Selection of T Cell Epitopes

The PIMA approach to assessing an individual patient's HLA-specific risk for immunogenicity has been described previously (9). Each patient's nGAA sequence, as well as the reference sequence for rhGAA, is parsed into overlapping 9-mer frames by an epitope prediction tool called EpiMatrix (14, 15) and each frame is evaluated with respect to the specific Class II HLA DR alleles expressed by the patient. The EpiMatrix algorithm is based on coefficient matrices representing all 20 natural amino acids and nine peptide binding pockets for each HLA allele, so that the coefficients for each amino

acid in a novel peptide can be summed and normalized to generate a Z-score indicative of binding likelihood. The coefficients are derived based on empirical binding data, and Z-scores above 1.64 (the top 5% of a distribution of random peptides) are considered significant hits, likely to bind. Each 9-mer frame is assigned a normalized z-score using EpiMatrix; this z-score is used in the calculations.

EpiMatrix focuses on HLA DR, as it is usually expressed at the highest levels on antigen presenting cells (16, 17) and has been associated with therapeutic protein immunogenicity; no predictions were performed on HLA DP or DQ. Those 9-mer peptides predicted to bind to HLA DR, found in the rhGAA sequence, but absent in at least one of the patient's nGAA alleles, are considered potential inflammatory (T effector) epitopes and included in the calculation of the PIMA score. Henceforward, this approach will be referred to as PIMA V1. **Figure 2** illustrates how new epitopes introduced from the rhGAA therapeutic could potentially drive an ADA response in IOPD subjects. (**Figures 2A, B**). Notably, PIMA does model the impact of stop codons, where every section of rhGAA following the stop codon is considered potentially foreign to the patient, and of frame-shift mutations, where the out-of-frame translations are compared to rhGAA (and to the remainder of the human proteome in V3J) to assess the foreignness of the therapeutic protein. PIMA is not yet able to model the impact of splice-site variants due to the heterogeneous nature of gene products from these mutations.

In this study, three additional candidate scoring approaches were evaluated: PIMA V2, V3 and V3J. For PIMA V1, predictive models for the following supertype alleles were available: HLA DRB1*0101, *0301, *0401, *0701, *0801, *1101, *1301 and *1501.

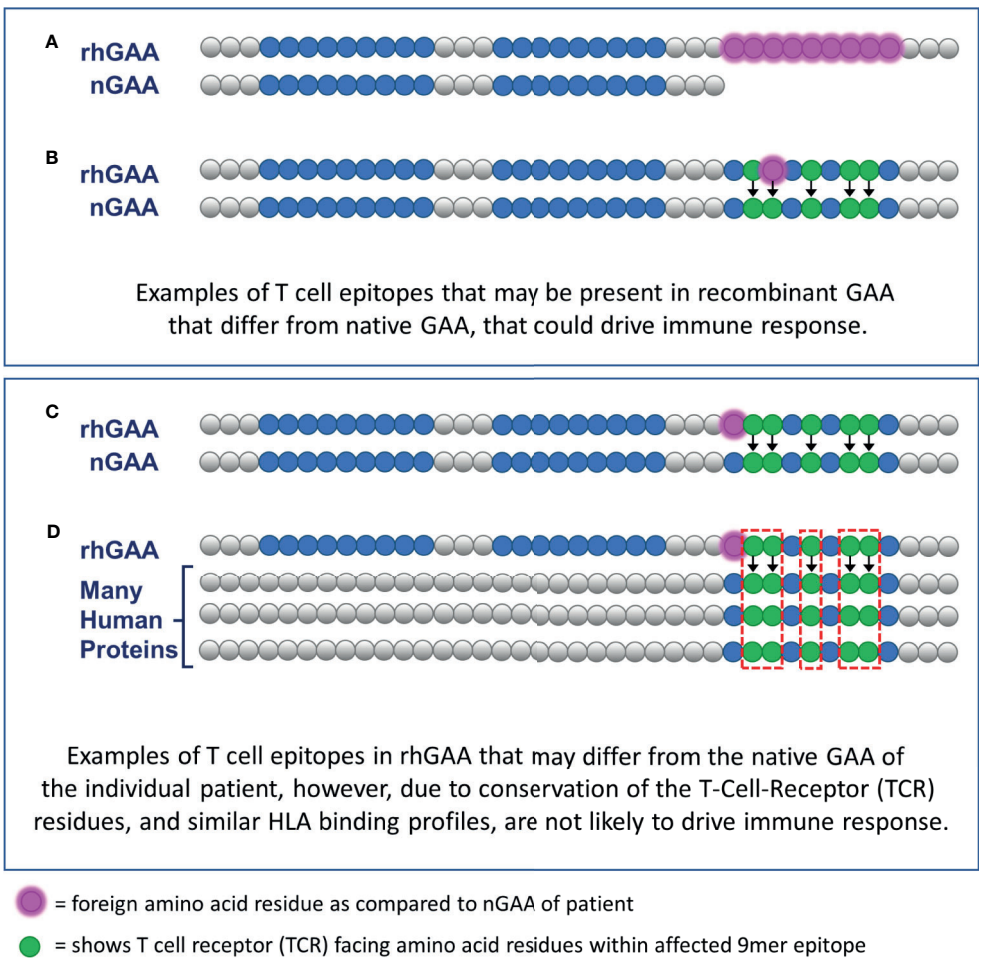

FIGURE 2 | Epitope differences in therapeutic GAA from endogenously expressed native GAA predicted to drive ADA or be tolerized. CRIM-positive IOPD subjects who express residual nGAA may be tolerant to epitopes conserved, for their HLA, with the rhGAA replacement protein. **(A)** T cell epitopes contained within the rhGAA may be recognized as "foreign" if they are within the truncated or mutated portions of the patient-specific nGAA. **(B)** T cell epitopes within the rhGAA that contain T cell receptor (TCR)-facing residues that are different from those found in nGAA may be sufficient to generate a different T cell phenotype response. **(C)** T cell epitopes within the rhGAA that contains different MHC-facing residues but the same TCR-facing residues as epitopes found in nGAA are predicted to be tolerated by the immune system (this hypothesis was included in PIMA V3). **(D)** The presence of a T cell epitope in the rhGAA sequence with TCR-facing residues highly cross-conserved with several self-human proteins may not appear as foreign and would also be tolerated by the immune system. (This hypothesis was included in PIMA V3J).

Since the publication of V1, many new HLA DR allele epitope prediction models have been developed for use in EpiMatrix. PIMA V2 evaluated whether expanding the set of HLA DR alleles used for T cell epitope prediction improved the performance of the tool. In V2, IOPD subject-specific subtypes were used instead of the eight supertypes (e.g. HLA DRB1 subtype 0403 instead of DRB1 supertype 0401). If a prediction model for a patient's HLA DR allele is not available in our EpiMatrix tool, a close proxy was selected based on HLA DR binding pocket similarity (by examining the preferred side chains) and that was used for T cell epitope prediction. The new, more precise subject-specific subtypes were also incorporated into the subsequent V3 and V3J analyses.

The next step was to refine the selection of T effector epitopes for the third PIMA model (V3). In previous models, we considered all epitopes that were not identical in sequence to nGAA to be potential T cell epitopes. We re-assessed these potential T effector epitopes in V3. Instead of automatically counting non-identical sequences as T cell epitopes, we considered only whether the epitopes were different from nGAA at the T cell receptor face. If so, they counted as T effector epitopes, but if they were conserved at the TCR face (even if their HLA DRB1 HLA-face was different, and if it was still predicted to bind to the same HLA DRB1), we considered the epitopes to be 'null' or not T effector epitopes (**Figure 2C**). To perform this analysis, we used the JanusMatrix tool (10). In retrospective and prospective studies we have determined that TCR conserved epitopes may be tolerated, deleted during the thymic selection process, or actively regulatory (18–20). For PIMA V3, GAA-like epitopes were excluded from the calculation of the PIMA score.

In some subjects, mutations are caused by frameshifts. For each frameshift and non-sense mutations, the anticipated expressed GAA protein product is compared to the therapeutic rhGAA sequence. The sequence of the rhGAA which does not align with the truncated protein product is considered to be mismatched and is scored as a foreign protein as follows: The mismatched sequence is parsed into 9-mer frames and evaluated for potential HLA binding hits to the patient's specific HLA DRB1 haplotype. For PIMA V1 and PIMA V2, the HLA-epitope hit values are then added up to calculate to overall PIMA score. In the case of V3 and V3J, the predicted binding epitopes are further evaluated for T cell receptor facing residues and cross-conservation to the human proteome (V3J).

Beyond finding epitopes that may be tolerated, JanusMatrix can be used to identify putative regulatory T cell epitopes if the TCR face is extensively conserved with other epitopes from the human genome (10, 21, 22). Therefore we searched for putative Treg epitopes in GAA using the study subject HLA DR alleles, and then identified potential Treg epitopes, specific to each subject, for PIMA V3J. This version of PIMA discounts additional epitopes defined by JanusMatrix that may or may not be conserved in nGAA but are conserved (at their TCR face) within *other* human proteins (**Figure 2D**). We used the UniProt Reviewed Human Proteome as the database for comparison (23, 24). T cell epitopes that had high JanusMatrix scores, indicating high conservation to other human proteins, were not included in the calculation of the PIMA score. Some of these 'regulatory' epitope sequences were produced as peptides and were also evaluated *in vitro*.

Selection and *In Vitro* Validation of Putative Regulatory T Cell Epitopes in GAA

The first step in the search for Treg epitopes was to use EpiMatrix to analyze the sequence of recombinant GAA replacement enzyme. This analysis considered the complete GAA sequence and the globally representative set of HLA DR supertype alleles (25). Several categories of putative T cell epitopes were identified based on their EpiMatrix cluster score and their ability to bind across multiple HLA DRB1 alleles. Next, to determine cross-conservation with the human proteome, each of the clusters was screened using JanusMatrix. In general, JanusMatrix Human Homology Scores above two are considered significant, indicating an elevated level of conservation between the TCR-facing features of the input peptide and the TCR-facing features of the proteins resident within the human genome. Those epitopes with higher conservation scores were considered to be putative Treg or regulatory T cell epitopes. **Supplemental Table S2** describes the peptides that were tested, including their predicted binding affinity (using EpiMatrix) and their corresponding JanusMatrix score. T effector epitopes that were used as controls are also shown in this table.

Twenty-one GAA-derived putative regulatory T cell epitopes were identified and synthesized for *in vitro* evaluation and validation studies. Twelve were promiscuous epitopes that were predicted to bind across multiple HLA DRB1 alleles (and therefore relevant to a wide range of haplotypes), while nine putative GAA regulatory T cell epitopes were more HLA DR restricted by the HLA DRB1 of the individual IOPD subjects included in the study cohort. T cell assays were performed using a diverse panel of healthy donor PBMCs (as subject-specific PBMCs were limited for *in vitro* studies). The positive (regulatory T cell epitope) control for this assay was a Treg epitope similar to the previously identified Tregitopes (26), FV621. This control peptide is a Factor V peptide that modulates CD4+ memory T cell responses and induces bystander suppression of T effector immune response *in vitro* in a standardized Tetanus Toxin Bystander Suppression Assay (TTBSA) (27).

HLA Binding Assays

The major histocompatibility complex proteins (MHC, also known as HLA in humans) play a critical role in the development of an effective immune response or in activating both effector and regulatory T cells to induce, or diminish immune responses, respectively. The twenty-one putative regulatory T cell epitopes from GAA were tested for *in vitro* binding to HLA DRB1*0101, *0301, *0401, *0701, *1101, *1301 and *1501 alleles. The HLA DRB1 alleles selected for the HLA binding assays represent families of Class II HLA DRB1 alleles that share similar binding peptide side-chain preferences for their binding pockets (25).

The HLA binding assay used at EpiVax was originally described by Steere et al. (28), has been standardized for in-house validation of *in silico* binding predictions. This binding assay has been described in detail in previous publications (28–30). A seven-point binding assay (0.01, 0.1, 0.3, 3.0, 10.0, 30.0 and 100.0 μM) is performed for each test peptide, in triplicate. The HLA binding information is used to calculate the IC_{50}, or the concentration at which the peptide inhibits 50% of the labeled control peptide's specific binding.

Human Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs from healthy donors were isolated from leukocyte reduction filters purchased from the Rhode Island Blood Center (RIBC) in Providence, RI. High-resolution HLA Class II DRB1 haplotyping of donor PBMCs was performed at the Transplant Immunology Laboratory at Hartford Hospital in Hartford, CT. Donors' age and sex are provided however race, ethnicity, and medical history are not available due to the anonymous nature of the blood donation process.

All assays were performed in RPMI complete medium: RPMI-1640 + GlutaMax (Life Technologies) containing 10mM HEPES buffer (Life Technologies), 2mM L-glutamine (Life Technologies), 50μg/ml Gentamicin (Life Technologies), 10% Human AB serum (Sigma), MEM Non-essential amino acids (Gibco) and 55μM β-Mercaptoethanol (Gibco).

Tetanus Toxoid Bystander Suppression Assay (TTBSA)

The TTBSA measures the inhibitory capacity of potential regulatory peptides on the recall response of human CD4 T cells to the tetanus toxoid (TT) antigen was adapted for validation of Treg epitopes and previously described (27, 31). TT vaccination is a routine, nearly universal immunization, resulting in memory T cell responses that persist for many years (32). Therefore PBMCs are considered to be a reliable source for *in vitro* assays that require TT-specific memory T cells.

Briefly, PBMCs were labeled with CFSE cell proliferation dye (eBioscience) and rested overnight at 37°C, 5% CO_2. The following day cells were stimulated with Tetanus toxoid (TT) (Astarte Biologics, cat no. 1002) at 0.5 μg/ml alone and in combination with the putative regulatory peptides or control peptide at 8, 16 or 24 μg/ml, then incubated for 6 days and analyzed by flow cytometry on day 7. CD4+ T cell proliferation, T effector activation and the ratio of regulatory to effector T cells were measured.

Statistical Analysis

Association between predictors of ADA response and outcome were evaluated by Chi-square test, or Fisher's Exact test in the case of small sample sizes, using GraphPad online tools (GraphPad Software). Prediction metrics (sensitivity, specificity, positive predictive value, negative predictive value, odds ratio) were evaluated using Microsoft Excel (2016) (33). Statistical analysis was performed using Prism software (GraphPad version 8.3). The Student's *t*-test (unless otherwise indicated, unpaired, two-tailed) was used to compare the significance of differences between TT stimulated cells to Tregitope treated cells or the indicated experimental groups. Differences were considered significant when $p < 0.05$ (*), very significant when $p < 0.01$ (**), highly significant when $p < 0.0002$ (***), and extremely significant when $p < 0.0001$ (****).

RESULTS

GAA PIMA Scores as a Predictor of ADA Status

HLA and GAA genotype data were collected from 62 individuals with IOPD in an international cohort of IOPD subjects enrolled in the Duke University IRB-approved study. These individuals include 19 CRIM-positive participants analyzed as part of our published (V1) pilot study (9). Fourteen of the 62 subjects were excluded from this analysis due the presence of splice site mutations resulting in indeterminate protein products (6) that is characteristic of late-onset Pompe disease (8). At this phase of the PIMA development splice-site variants have not been integrated into the analysis due to the heterogeneous nature of gene products from these splice-site mutations. Among the remaining 48 IOPD subjects, 19 (40%) developed high ADA titers and 29 (60%) developed low ADA titers.

Using a score threshold of +10, PIMA V1 correctly predicted ADA status for 27 (56%) of 48 subjects, the intermediate versions V2 and V3 correctly predicted for 54% of the subjects and PIMA V3J correctly predicted for 64% of the subjects (**Figure 3**). For

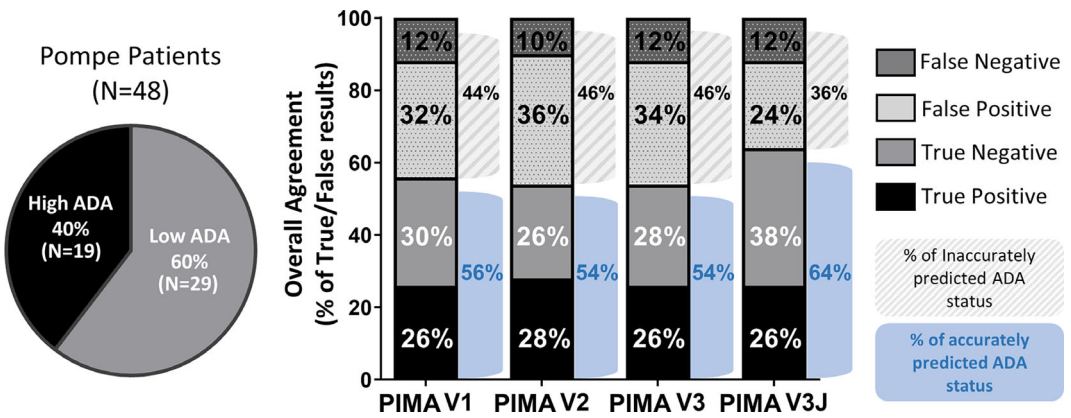

FIGURE 3 | Overall agreement of the four PIMA scoring algorithms as predictors of ADA status. Among the 48 IOPD subjects evaluated, 19 (40%) developed high ADA titers; this includes subjects with high and sustained antibody titers (HSAT) as well as sustained intermediate titers (SIT). The remaining 29 subjects (60%) developed low ADA titers. PIMA V1 (previously published) accurately predicted the ADA status for 56% of the IOPD subjects. Versions V2 and V3 accurately predicted 54% of subjects, thus further adjustment was explored. Adjusting for potential Treg epitopes with V3J improved accuracy to 64% of the subjects.

each of the analyses, correct predictions included both True Positive (predicted to and did develop high ADA) and True Negative (predicted not to and did not develop high ADA) predictions. False Negative predictions (subjects who developed high ADA contrary to predictions of low ADA) represented the smallest set among all predictions at 10-12% (5-6 out of 48 subjects).

Regression Model Improves Prediction Outcome

The four PIMA scoring algorithms were further evaluated as predictors of high ADA development in IOPD using univariate and multivariable logistic regression analysis. Odds ratios (OR) and 95% confidence intervals (95% CI) for the development of high ADA titers were calculated. Two-sided P-values <0.05 were employed to assess significance. In the univariate logistic regression analysis, IOPD subjects with PIMA scores above 10 had increased odds of developing high ADA titers compared to subjects with scores below 10.

As shown in **Table 1**, PIMA V3J reported the highest OR of 4.12 (95% CI 1.24-15.01), which was statistically significant. The regression model was slightly improved by incorporating the age of the subject at initiation of ERT as a covariable, as both PIMA score >10 and age were significantly associated with the high ADA outcome. After adjusting for age at ERT initiation, PIMA V3J scores of greater than 10 were associated with the highest adjusted OR of 4.40 (95% CI 1.21-18.21), also statistically significant (**Table 1**). For the combined risk model, age at ERT initiation was divided into two categories using the mean (19 weeks) as a cutoff (>19 and <19 weeks).

Considered together, subjects with PIMA V3J scores >10 and initiation of ERT after 19 weeks of age were 8.23 times more likely to develop high ADA than all other subjects (**Table 2**). Viewing the data from a clinical importance perspective (using PIMA to identify subjects at low risk), subjects with PIMA V3J scores <10 and ERT initiation prior to 19 weeks were 12.7 times more likely to have low ADA compared to all other subjects. If validated in future studies, the V3J PIMA score may be clinically useful for identifying IOPD subjects who may not need to be treated with ITI. We have also compared the area under the ROC curve (AUC) among univariate and multivariable logistic regression models which confirm the improved accuracy of V3J over V1 and intermediate versions V2 and V3 (**Supplementary Figure S1**). A multivariable regression using both PIMA and age as independent variables to predict high ADA titer status indicated that regression coefficients for both factors were significant, and the joint threshold model (PIMA >10 and age@ERT >19 wks) had the lowest p-value and highest AUC of all the models we explored.

Selection of GAA-Tregulatory Peptides and HLA Binding

To investigate the hypothesis that there may be tolerogenic peptides in GAA, we used JanusMatrix to identify putative tolerogenic epitopes. Epitopes that were predicted to bind promiscuously across multiple human class II HLA-DRB1 molecules were selected for testing *in vitro*, as were other epitopes that were more restricted by HLA DRB1 class. To confirm HLA binding before testing *in vitro* bystander T cell assay, we performed 7-point HLA DR binding assays. See **Supplementary Table S1** for list of all peptides tested in the *in vitro*. Within twenty-one tested peptides, we found that the 12 GAA peptides showed moderate to strong binding to the panel of multiple HLA alleles, whereas others were somewhat more restricted in the breadth of binding to the full range of HLA DR alleles (data not shown). **Figure 4** summarizes the HLA-binding results for the putative Treg epitopes in GAA evaluated in TTBSA.

Immunomodulatory Effect of GAA-Derived Peptides on the Tetanus Toxoid Mediated Recall Response of CD4 T Cell Proliferation

To determine the magnitude of immune tolerance induced by the 21 pre-selected putative Treg epitopes and 2 IOPD patient-specific Teff epitopes (**Supplementary Table S2**), we performed a Tetanus Toxoid Bystander Suppression Assay (TTBSA) for Treg epitopes and measured their capacity to inhibit the proliferative response to TT in PBMCs derived from a panel of six healthy donors and selected 7 peptides with potential inhibitory capacity compared to validated positive control FV621 (data not shown). These 7 peptides were re-evaluated in an additional panel of 5 healthy donors for the inhibition of TT-induced memory response (**Figure 5**). Four of the 21 peptides (hGAA-1a, hGAA-1b, hGAA-6, hGAA-11) significantly inhibited memory CD4+ T cell proliferation across

TABLE 1 | PIMA V3J and age at ERT initiation are significant predictors of high ADA in univariate and multivariable logistic regression analyses.

↓version	UNIVARIATE LOGISTIC REGRESSION		MULTIVARIABLE LOGISTIC REGRESSION (age in Weeks at ERT as a covariable)			
	PIMA OR (95% CI)	p-val	PIMA OR (95% CI)	p-val	Age OR (95% CI)	p-val
PIMA V1	2.32 (0.71-8.21)	0.1728	3.45 (0.93-15.04)	0.0770	1.07 (1.019-1.133)	0.0105*
PIMA V2	2.27 (0.67-8.58)	0.1997	3.40 (0.87-16.23)	0.0945	1.07 (1.019-1.132)	0.0110*
PIMA V3	2.02 (0.62-7.14)	0.2544	2.74 (0.75-11.54)	0.1418	1.07 (1.017-1.127)	0.0123*
PIMA V3J	4.12 (1.24-15.01)	0.0246*	4.40 (1.21-18.21)	0.0296*	1.06 (1.012-1.122)	0.0214*

Odds of high ADA were 2.32-4.12 times greater for subjects with PIMA V1-V3J scores >10 according to univariate logistic regression models (left). The odds ratio for the PIMA V3J model was statistically significant (p=0.0246). Multivariable logistic regression models incorporating both PIMA V1-V3J and age in weeks at initiation of ERT (right) indicate that increasing age consistently conferred 6-7% increased odds of high ADA per week after adjusting for PIMA score. In the PIMA V3J model, both the PIMA score and age variables were statistically significant (p=0.0296 and p=0.0214, respectively). Notably, the inverse interpretation is also true. For example, in the V3J univariate model, subjects with PIMA scores <10 were 4.12 times more likely to maintain low ADA. In the V3J multivariable model accounting for both age and PIMA score, patients with scores <10 were 4.4 times more likely to maintain low ADA, while each week of increasing age conferred 6-7% lower odds of maintaining low ADA.

**Significant, two-sided p-value < 0.05.*

TABLE 2 | Combined logistic regression model for high and low ADA risk.

OUTCOME	Predictor	Univariate Logistic Regression	
		OR (95% CI)	p-val
HIGH ADA	**PIMA V3J>10 & AGE @ ERT >19 WKS**	8.23 (2.28-34.31)	0.00206**
LOW ADA	**PIMA V3J<10 & AGE @ ERT <19 WKS**	12.7 (2.15-244.25)	0.0202*

*Combined logistic regression models for ADA risk indicate that IOPD subjects with PIMA V3J scores >10 and ERT initiation after age 19 weeks were 8.23 times more likely to develop high ADA than all other subjects, while subjects with PIMA V3J scores <10 and ERT initiation prior to 19 weeks were 12.7 times more likely to maintain low ADA compared to all other subjects. *Significant, two-sided p-value < 0.05, **Highly significant, two-sided p-value < 0.01.*

Name	HLA DRB1 (IC50)						
	*0101	*0301	*0401	*0701	*1101	*1301	*1501
hGAA-1a	0.25	4.83	3.01	<1	2.97	4.19	<1
hGAA-1b	<1	9.48	29.65	<1	4.90	112.25	80.45
hGAA-2	<1	<1	4.83	2.23	0.24	4.19	0.83
hGAA-6	1.20	<1	1.19	<1	1.13	1.17	0.76
hGAA-11	3.30	1.79	1.45	1.34	<1	9.45	1.69
hGAA-12	non binder	non binder	182.26	non binder	119.85	21.81	72.26
hGAA-13	3.19	<1	3.33	<1	7.36	7.54	2.00

Strong Binders (IC50 value of <10 μM)
Moderate Binders (IC50 value between 10 and 50 μM)
Weak Binders (IC50 value between 50 and 100 μM)
Non-binders (IC50 >100 μM or no concentration-dependent inhibition)

HLA Binding Assessments	n = 49
True Positive	34
False Positive	3
False Negative	9
True Negative	3

FIGURE 4 | GAA-peptides bind to HLA DR1 as predicted. Selected GAA peptides were evaluated for HLA DRB1 binding *in vitro* and IC_{50} values were calculated. hGAA-1a, hGAA-1b, hGAA-2, hGAA-6, hGAA-11 and hGAA-13 FV621 peptides bound with the multiple alleles tested (DRB1*0101, *0301, *0401, *0701, *1101, *1301 and *1501) whereas hGAA-12 was predicted to be more HLA-restricted and consequently had limited binding to HLA. A seven-point competition assay using a validated control peptide was performed; color coding reflects binding affinity IC_{50} was determined by interpolation. Using a standard Z-score threshold of 1.64 (top 5%), overall positive predictive value for EpiMatrix predictions was 92%, with sensitivity of 79%. False negatives are not uncommon when testing peptides containing significant predictions for several alleles (EpiBars), as many contain "near-miss" Z-scores in the top 10% of predicted peptides. Note that peptide GAA-12 and 13 were not selected for promiscuity: they were designed for individualized testing in a specific patient for which both the mutation and the HLA DRB1 allele restriction concurred.

all the donors tested. We have also tested 2 Teff epitopes in TTBSA in healthy donor PBMCs and found that they were not inhibitory (**Supplementary Figure S2**).

As shown for one representative donor in **Figure 5A**, Tetanus Toxoid stimulation usually expanded CD4+ T cell proliferation by ten to twenty-five-fold in a CFSE dilution assay. The addition of one of the 21 GAA-peptides (hGAA peptide 6) significantly suppressed proliferation of CD4+ T cells to TT in a dose-dependent manner (75%-90%). As shown in **Figure 5B**, the FV621 T reg epitope positive control also significantly inhibits TT-induced memory CD4+ T cell proliferation. **Figure 5B** shows the effect of selected GAA peptides on the inhibition of memory CD4+ T cells. GAA peptides 1a, 1b, 6 and 11 significantly inhibited TT-induced CD4 T cell proliferation in a dose-dependent manner (observed in TTBSA for 11 donors), while none of the other peptides had the same effect.

GAA-Derived Peptides Increased the Ratio of Tregs to Teff Cells

To further characterize the inhibitory capacity of down selected GAA derived peptides on the CD4+ T effector cell populations and investigate its impact on Tregs, we evaluated the effect of these peptides on cell surface markers in PMBC obtained from five healthy donors with diverse HLA DRB1 haplotypes. CFSE labeled PBMCs from the donors were stimulated with Tetanus Toxoid (TT) in the presence or absence of GAA-derived peptides or FV621 (as a positive control peptide) for 6 days and the proliferation of T effector and T regulatory cells was assessed. Tregs were identified by the expression of $CD127^{low}$, $CD25^{hi}$ and $FoxP3^{hi}$ (FoxP3 is a transcription factor and major regulator of Treg development but is also transiently expressed in activated T effector cells) (34) while CD4+ T effector cells were identified as $CD25^{hi}FoxP3^{int}$ in the CD4+ gated population. Data from a single representative donor in **Figure 6A** shows an expansion of $CD4+CD25^{hi}FoxP3^{int}$ T effector cells in the presence of TT alone, while co-treatment of the cultures with increasing concentrations of GAA-derived peptides significantly reduced the percentage of activated CD4+ T effector cells. In parallel, we observed a dose-dependent increase in the percentage of Tregs in cultures treated with GAA-derived peptides (**Figure 6B**).

We hypothesize that the increased frequency of Tregs that is observed when PBMC cultures of activated TT-specific effector T

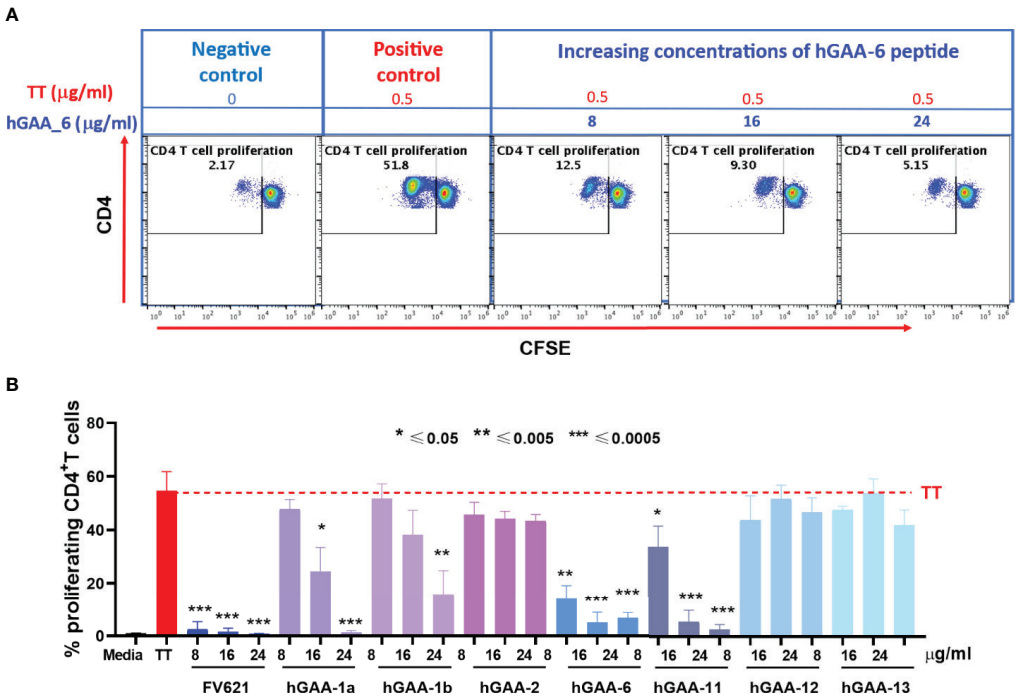

FIGURE 5 | GAA-derived peptides inhibit memory CD4 T cell response to Tetanus Toxoid (TT) in healthy donors. **(A)** Representative flow cytometry dot plots show CD4 memory T cell proliferative response to TT and dose-dependent inhibition by hGAA-6 peptide. **(B)** Inhibition of CD4+T cell recall response by GAA-peptides in TTBSA. PBMCs from healthy donors were stimulated with 0.5 µg/ml of TT with or without FV621 or GAA-peptides and analyzed at six days post-stimulation by flow cytometry for inhibition of CD4+ T cell proliferation. Data are the representative donor from 5 donors in the experiments. Significant suppressive capacity of CD4+ T cell proliferation was observed for 4 putative Treg peptides in GAA confirming their regulatory potential across all donors tested. P values * ≤ 0.05, ** ≤ 0.005 and *** ≤ 0.0005 represents statistical significance between peptide stimulation *vs* TT using a two-tailed t test. GAA peptides 1a, 1b, 6 and 11 significantly suppressed the expansion of TT-memory T cells in this *in vitro* assay.

cells are treated with increasing concentrations of GAA-peptides may be due to (1) conversion of T effectors to adaptive Tregs; (2) elimination of T effectors from the mixed population of cells due to killing by Granzyme B (35); or (3) proliferation of natural Tregs. Previous studies in D011.10 mice showed that treatment with other Treg epitopes *in vivo* converted (OVA) antigen-specific T effector cells to regulatory T cells (36). The potential for conversion of T effectors to adaptive Tregs is supported by additional published studies which show that Tregitope treatment of human PBMCs *in vitro* converted tetramer-stained Birch Pollen specific T effector cells to adaptive Tregs (26).

The ratio of activated T regulatory cells to T effector cells may be a determining factor in the maintenance of tolerance and in the potential for tolerance to ERT as well as for treatment of allergic and autoimmune diseases (37, 38). Here we have found that GAA-derived peptide treatment in the presence of TT also shifts the balance of T effector cells and T regulatory cells by increasing the ratio of Treg to Teff cells, also in a dose-dependent manner (**Figure 6C**).

DISCUSSION

Among the 48 IOPD subjects evaluated in this study, 19 (40%) developed high ADA titers, which included patients who exhibited sustained ADA titers of ≥12,800. Using the existing personalized immunogenicity risk assessment (PIMA) Version 1 (V1) (9), we found that ADA status was predicted accurately for 56% of subjects in the cohort. Intermediate versions 2 and 3 which used more specific HLA DR allele models (patient specific rather than supertype) and assessed for TCR conservation with GAA, respectively, were not significantly better than Version 1. Notably, in multivariable regression models, both age at initiation of ERT and PIMA score were significant indicators of the likelihood of developing high ADA to rhGAA. We believe that the significant effect size for age at ERT initiation underscores the importance of early assessment, while the significant finding for PIMA score supports the potential benefits of delaying ITI treatment for those individuals at lower risk of ADA. Across versions, 5-6 subjects developed high ADA despite having PIMA scores below 10. The specific characteristics of these subjects will be considered as the PIMA scoring algorithm is refined in future versions.

An additional version (PIMA V3J) adjusted for potential regulatory T cell epitopes using JanusMatrix was also tested. This improved version that integrated putatively tolerated and tolerogenic epitopes predicted ADA status accurately for 64% of subjects. To explore the interesting hypothesis that these epitopes might down-modulate T effector responses to ERT we used a validated *in vitro* T cell assay (The Tetanus Toxoid Bystander

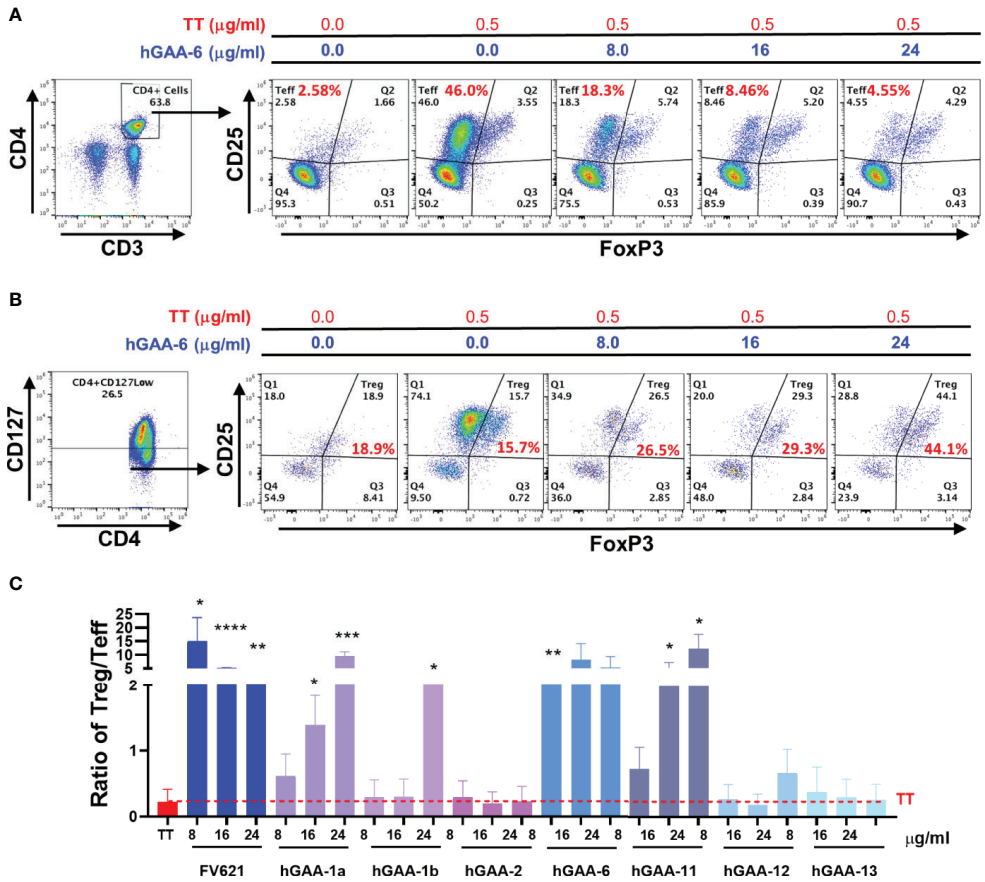

FIGURE 6 | GAA-peptides modulate T regulatory to T effector cell ratio. Healthy donor PBMCs were stimulated with Tetanus Toxoid (TT) with or without GAA-selected peptides for 7 days and analyzed by flow cytometry. **(A)** Representative flow cytometry dot plots show the effect of hGAA-6 peptide on the inhibition of T effector ($CD4+CD25^{hi}FoxP3^{int}$) cells for a single donor. **(B)** The effect of hGAA-6 peptide on regulatory T cells ($CD4+CD127^{low}CD25^{hi}FoxP3^{hi}$) in the representative donor is shown. **(C)** Representative histogram indicates the effect of selected GAA peptides on the Treg to Teff ratio in an individual donor. GAA peptides 1a, 1b, 6 and 11 significantly increased the Treg : Teff ratio similar to the FV621 Tregitope control. P values * ≤ 0.05, ** ≤ 0.005, *** ≤ 0.0005 and **** ≤ 0.00005 represents statistical significance between peptide stimulation at a given concentration *vs* TT using a two-tailed t test.

Suppression Assay (TTBSA) (27) to examine their potential regulatory effects, using blood from naïve donors. We confirmed that four of the 21 GAA epitopes tested *in vitro* appear to be significantly immunomodulatory. These four peptides inhibited the expansion of Tetanus Toxoid-specific memory CD4+ T cells in a standardized Treg assay (the TTBSA), similar to other well-defined Treg epitope peptides. Others have reported an absence of immune responses to some of the epitopes that have been confirmed to be regulatory here (39). The reasons for which 17 of the 21 peptides were not tolerogenic in the same assay is unknown, although epitope processing (lack of proper processing and presentation on the cell surface) may play a role.

In subjects who have circulating Tregs that recognize these epitopes in GAA, treatment with the drug may activate antigen-specific T regulatory cells, contributing to the induction of tolerance to the GAA therapy while limiting the development of ADA. In future studies, we intend to assess the effect of these peptides in the TTBSA with blood samples from IOPD subjects, while also testing additional GAA T effector and GAA Treg epitopes using naïve donor and IOPD subjects' T cells. In keeping with the hypothesis, tolerance to GAA has been observed in late-onset Pompe Disease (LOPD (40).

The discovery of Treg epitopes in self proteins has implications for protein therapeutics and may help explain why some subjects unexpectedly develop tolerance to ERT or blood factor therapy. For example, we have identified a Treg epitope in Factor V that may induce tolerance to Factor VIII in certain hemophilia A subjects (27). The potential for human proteins to have internal Treg epitopes may transform the prediction of ADA development for Pompe patients and has important implications for other protein-based replacement therapies (41). The putative GAA Treg epitopes are similar to Treg epitopes first discovered in IgG (Tregitopes, T regulatory epitopes) (26) in 2008. These Tregitope sequences were synthesized as peptides and encoded in viral vectors (AAV) and shown to suppress inflammatory responses to co-administered antigens (Ag) (such as diabetes antigens, AAV capsid, MOG protein, OVA, and other antigens) *in vitro* and *in*

vivo (36, 42–44). Co-delivery of Tregitopes in conjunction with target Ag appears to be critical to the induction of antigen-specific tolerance (45); antigen-specific tolerance may also be operational in the setting of enzyme replacement, providing the IOPD individual has circulating Tregs that recognize these GAA Treg epitopes with tolerance to rhGAA.

In the context of GAA, we hypothesize that exogenous rhGAA may be able to induce tolerance in CRIM-positive children due to the engagement of pre-existing GAA-specific regulatory T cells. Circulating Tregs may be found in subjects who have been exposed to nGAA that contains these GAA sequences. Other subjects (such as CRIM-negative subjects, or other subjects who have key mutations or truncations in the region of the GAA Treg epitope), may not have Tregs that respond to these sequences. Tolerance to GAA may be 'personalized' since it is both native GAA-sequence-specific and HLA DR-haplotype dependent. Therefore, a personalized immunogenicity risk assessment such as the PIMA V3J tool may be the most accurate means of assessing the risk of an immune response to replacement rhGAA.

The means by which the GAA-specific immunomodulatory T cells modulate immune responses deserves further study. Their effect may be due to i) production of immunosuppressive cytokines, e.g., TGFβ, IL-10 and IL-35, ii) upregulation of effector T cell-specific transcription factors important for the expression of CXCR3 and survival of Tregs (46)(e.g., T-bet), iii) competing with effector T cells for the growth factor IL-2 by sustained expression of the IL-2Rα subunit, CD25, iv) inducing cytolysis of T effector cells by producing perforin and granzyme and v) modulating dendritic cell maturation and function, all of which are known mechanisms of action for Tregs (47). As blood samples are relatively limited in pediatric subjects, and many CRIM-positive subjects are now treated pre-emptively with immune-suppressive therapies, validation of the putative Treg epitopes in GAA will require a concerted effort and close collaboration with IOPD families and clinicians.

ADA also develop in IOPD individuals who have splice site mutations that result in indeterminate protein products (6). Fourteen of the 62 subjects were excluded due to presentation with splice site mutations which may not directly change the nGAA sequence (8). As the presence or absence of residual GAA is difficult to assess in these subjects, we have yet to resolve how to accurately predict the tolerance induced by putative Treg epitopes in these subjects. An *in vitro* test (TTBSA) could be developed using their peripheral blood cells that could guide their ADA risk assessment.

No comparison to publicly available tools was made because the type of analysis performed by JanusMatrix is not available elsewhere. These tools have been compared to on-line tools in other settings such as for cancer, please see reference (48). Several additional studies have demonstrated the utility of EpiMatrix and JanusMatrix for identifying HLA DR restricted T effector and putative T regulatory epitopes in human proteins (10, 20). Similar analyses performed by other groups have suggested that 'self-like' epitopes may be tolerated or tolerogenic (49).

Further validation of these models in prospective studies will be necessary before the models are implemented in clinical settings. The best predictive model (V3J) has been incorporated into a web-based "**P**ersonalized **IM**munogenicity **A**ssessment" tool (PIMA) (**Figure 7**) to facilitate additional research. This website is

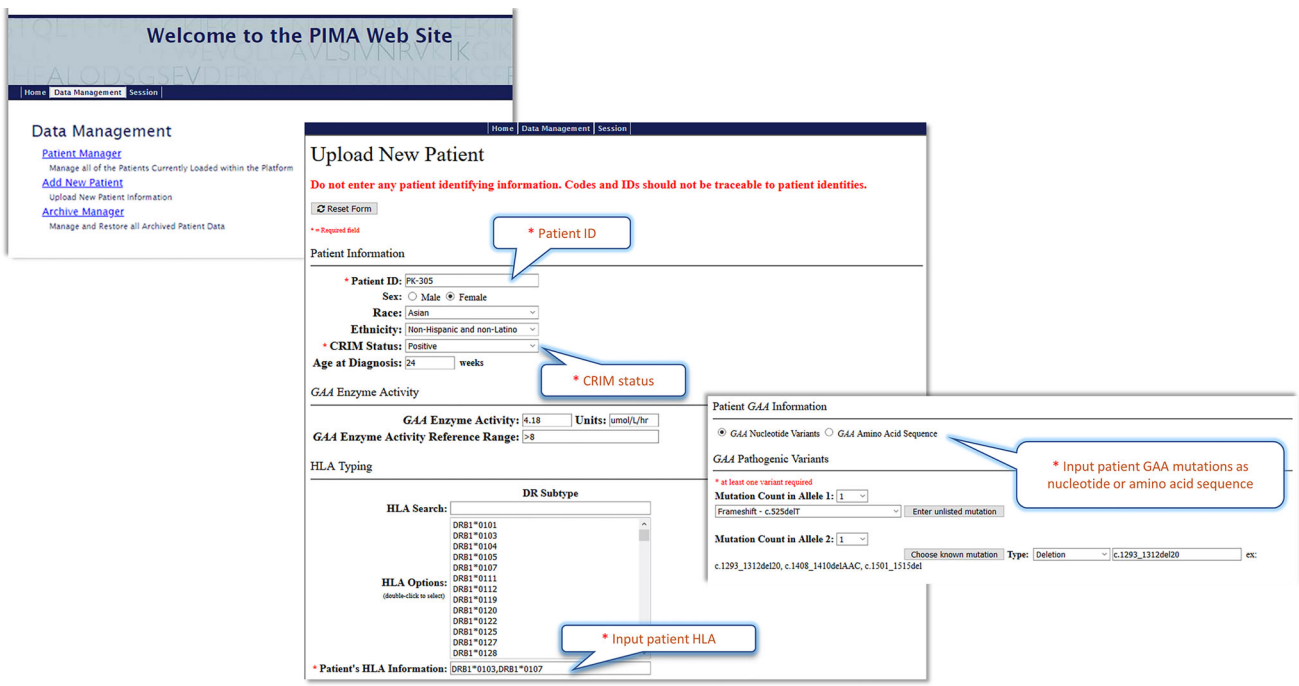

FIGURE 7 | The Pompe PIMA user interface prototype. The upper screenshot shows the Pompe PIMA homepage with data management options. The lower two screenshots from the Upload New Patient page feature the required (*) input fields to generate the IOPD patient's individualized ADA risk assessment score.

available for use in pre-clinical research studies. Further validation studies may enable clinicians to input IOPD HLA-type and GAA mutations and generate PIMA scores to predict ADA for their IOPD patients. We anticipate that personalizing treatment using the PIMA tool may assist clinicians in their efforts to improve clinical outcomes for Pompe disease children.

DATA AVAILABILITY STATEMENT

All relevant data generated for this study are included in the article/**Supplementary Material**. The raw data supporting the conclusions of this manuscript will be made available by the authors to any qualified researcher upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Duke Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

PSK, ADG, MA, and WDM conceptualized and designed the study, experiments and *in silico* tool development. Experiments, *in silico* implementation of algorithms were performed by MA, WDM, FET, SK, SL, JSY and SM. IOPD patient cohort data was collected by AKD, CL, and PSK. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.636731/full#supplementary-material

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frontiers in Immunology

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Factor VIII-Fc Activates Natural Killer Cells *via* Fc-Mediated Interactions With CD16

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The most challenging complication associated with Factor VIII (FVIII) replacement therapy is the development of neutralizing anti-drug antibodies, or inhibitors, which occur in 23-35% of severe (FVIII level <1%) hemophilia A (HA) patients and are a serious hindrance to effective management of HA. Consequently, strategies that can either prevent anti-FVIII inhibitors from developing or "tolerize" individuals who develop such antibodies represent a clinically important unmet need. One intervention for patients with high-titer inhibitors is immune tolerance induction (ITI) therapy. Although ITI therapy is the only clinically proven strategy to eradicate anti-FVIII inhibitors, mechanisms of inhibitor reduction remain unknown. Factor VIII Fc-fusion (rFVIIIFc) is an enhanced half-life antihemophilic factor used in replacement therapy for HA. Fc-fusion is a successful protein bio-engineering platform technology. In addition to enhancement of plasma half-life *via* neonatal Fc receptor (FcRn) binding, other Fc-mediated interactions, including engagement with Fc gamma receptors (FcγR), may have immunological consequences. Several case reports and retrospective analyses suggest that rFVIIIFc offers superior outcomes with respect to ITI compared to other FVIII products. Previously we and others demonstrated rFVIIIFc interactions with activating FcγRIIIA/CD16. Here, we investigated if rFVIIIFc activates natural killer (NK) cells *via* CD16. We demonstrated rFVIIIFc signaling *via* CD16 independent of Von Willebrand Factor (VWF):FVIII complex formation. We established that rFVIIIFc potently activated NK cells in a CD16-dependent fashion resulting in IFNγ secretion and cytolytic perforin and granzyme B release. We also demonstrated an association between rFVIIIFc-mediated NK cell IFNγ secretion levels and the high-affinity (158V) CD16 genotype. Furthermore, we show that rFVIIIFc-activated $CD16^+$ NK cells were able to lyse a B-cell clone (BO2C11) bearing an anti-FVIII B-cell receptor in an antibody-dependent cellular cytotoxicity (ADCC) assay. These *in vitro* findings provide an underlying molecular mechanism that may help explain clinical case reports and retrospective studies suggesting rFVIIIFc may be more effective in tolerizing HA patients with anti-FVIII inhibitors compared to FVIII not linked to Fc. Our *in vitro* findings suggest a potential use of Fc-fusion proteins acting *via* NK cells to target antigen-specific B-cells,

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in the management of unwanted immune responses directed against immunogenic self-antigens or therapeutic protein products.

Keywords: Fc-fusion, immunogenicity, Fc gamma receptors, natural killer cells, antibody-dependent cellular cytotoxicity

INTRODUCTION

Hemophilia A (HA) is a genetic disorder caused by a deficiency in functional Factor VIII (FVIII) levels. FVIII replacement therapy is used to treat HA. Several novel bioengineered FVIII therapeutic protein products have been approved in the last decade. One of these is a recombinant FVIII Fc-fusion protein (rFVIIIFc) designed to enhance the plasma half-life of FVIII. The most challenging complication associated with FVIII replacement therapy is the development of neutralizing anti-drug antibodies, or inhibitors, which occur in 23-35% of severe (FVIII level <1%) HA patients and are a serious hindrance to the effective management of hemophilia A (1, 2). Consequently, strategies that can either prevent anti-FVIII antibodies from developing or "tolerize" individuals who develop such antibodies represent a clinically important unmet need. The clinical intervention for patients with high-titers of inhibitors is immune tolerance induction (ITI); i.e. high-dose FVIII infused over several months to tolerize the immune system. Depending on the protocol, studies have shown that ITI is successful in 50% to 88% of patients (3, 4). The success rate for "rescue ITI" (use of an alternative product for those who have failed an initial ITI regimen) is much lower. Several retrospective studies and case reports (5–7) have shown that rFVIIIFc, (i) has a lower median time to tolerization and (ii) is often successful in rescue ITI. However, these data sets are small and not conclusive. Although ITI therapy is the only clinically proven strategy to eradicate anti-FVIII inhibitors, mechanisms of inhibitor reduction remain unknown.

Therapeutic Fc-fusion protein products have been used to successfully treat many diseases (8–10). An Fc-fusion protein used as a drug consists of an immunoglobin Fc-domain linked to a bioactive protein or peptide which provides the pharmacological effect. The Fc-domain affects the biophysical and biochemical properties of the active moiety, making it a better therapeutic (11). A common impetus for developing an Fc-fusion protein is to increase the plasma half-life *via* interaction of the Fc with the neonatal Fc-receptor (FcRn). Following endocytosis, Fc-fusion proteins bind to FcRn located within acidified endosomes where they are recycled instead of continuing down a catabolic pathway (12). Additionally, the larger size of the fusion-protein can lead to slower renal clearance (13). Finally, the Fc-domain allows affinity purification using Protein A or Protein G, simplifying the manufacturing process and providing cost savings (14). Due to these advantages, 15 Fc-fusion products have been approved by the U.S. FDA and many more are in the pipeline.

Besides recycling of the protein mediated by Fc-FcRn, the IgG1 Fc-domain also engages with several canonical and non-canonical Fc-receptors (FcRs) (15, 16). These FcRs play important roles in many immunological responses (16, 17). The FcR relevant to this study is Fc gamma receptor IIIA (FcγRIIIA/CD16), an activating FcγR, with low to medium affinity for IgG1 Fc; primarily expressed by monocytes, macrophages and natural killer (NK) cells (18). The binding of the IgG1 Fc-domain to CD16 on NK cells combines the specificity of antibodies to the potent effector function of NK cells through a phenomenon called ADCC (19). Several clinically used monoclonal antibodies (mAbs) (e.g. trastuzumab and rituximab) specifically destroy cancer cells by exploiting ADCC (20, 21). These therapeutic mAbs bind to molecules expressed on the target cells with their antigen binding (Fab) regions and to NK cells *via* the Fc-FcγRIIIA interaction. When fused to the IgG1 Fc-domain a protein antigen that binds to a cell surface receptor on a specific cell can, in principle, target and lyse that cell *via* ADCC. An example is a protein antigen that specifically binds to the B-cell receptors (BCR) on a B-cell while the Fc moiety engages with CD16 on NK cells. Such targeted destruction of memory B-cells could provide a tool for controlling anti-drug antibody immune responses to therapeutic proteins.

We previously reported the unexpected observation that monomeric rFVIIIFc bound and signaled *via* CD16 using BW5147:hCD16ζ reporter cells (22). Thus, we hypothesized that specific killing of memory B-cells expressing anti-FVIII BCR by $CD16^+$ NK cells and mediated by rFVIIIFc could prove a mechanistic explanation for the recent clinical findings vis-à-vis ITI. In this study, we investigated the hypothesis that rFVIIIFc mediates selective destruction of memory B-cells that express anti-FVIII antibodies. We demonstrate that rFVIIIFc engages with CD16, activates $CD16^+$ NK cells, and induces NK cell-mediated cellular cytotoxicity of an anti-FVIII B-cell clone from an inhibitor-positive hemophilia A patient.

MATERIALS AND METHODS

Materials

rFVIIIFc and rFIXFc were acquired from Biogen through a Material Transfer Agreement. Biogen provided rFVIIIFc protein (1.02 mg/mL; 4.6 μM). The rFVIIIFc protein was adjusted to the final working concentration using assay buffer and was used to achieve hyper-physiological FVIII levels in *in vitro* assays. rFVIIIFc drug product (DP) [ELOCTATE, 2270 IU vial] and rFVIII [XYNTHA, 3120 IU Solofuse syringe] were purchased from ASD Healthcare. Anti-CD20 [rituximab] and anti-CD20 [obinutuzumab] were acquired from the NIH Pharmacy. NISTmAb (RM 8671), Humanized IgG1κ Monoclonal Antibody was purchased from the National Institute of Standards and Technology. The anti-CD20 monoclonal antibodies, rFIXFc, rFVIIIFc DP and rFVIII were reconstituted with manufacturer-provided diluent per

manufacturer's instructions. The reconstituted drug products [rituximab, 10 mg/mL (69 μM); obinutuzumab, 25 mg/mL (167.5 μM); rFIXFc, 1 mg/mL (10.2 μM); rFVIIIFc DP, 100.9 μg/mL (458.6 nM); rFVIII, 101.3 μg/mL (595.9 nM)] were then adjusted to the final working concentration using assay buffer. All therapeutic proteins were frozen at -80°C as small aliquots and thawed on ice prior to use. Blocking $F(ab')_2$ fragments [anti-human CD64 (FcγRI) Clone 10.1; anti-human CD32 (FcγRII) Clone 7.3; anti-human CD16 (FcγRIII) Clone 3G8] were purchased from Ancell.

NK-92 (ATCC #CRL-2407) and PTA-6967 (ATCC #PTA-6967) were maintained in Alpha Minimum Essential medium without ribonucleosides and deoxyribonucleosides but with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate (Gibco #12561-056) and supplemented with 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 12.5% horse serum (ATCC #30-2040), 12.5% fetal calf serum (Gibco #10438-034), and 100 U/mL recombinant IL-2 (R&D Systems #202-IL), herein referred to as NK cell media.

BO2C11, a human lymphoblastoid cell line was generated from an inhibitor positive hemophilia A patient using the Epstein-Barr virus (23). Raji (ATCC #CCL-86) and BO2C11 were maintained in RPMI 1640 media supplemented with GlutaMAX (Gibco #35050-061) and 10% fetal calf serum (Gibco #10438-034).

ADCC Reporter Bioassay

The ADCC Reporter Bioassay (Promega #G7015) is a surrogate assay for monitoring cellular cytotoxicity by NK cells mediated by Fc engagement with CD16. In this assay the effector cells, Jurkat cells, are engineered to express CD16 (with the V158 polymorphism) on their surface and contain a reporter gene, nuclear factor of activated T cell response element (NFAT-RE) upstream of luciferase (24). In some experiments, (i) target (Raji) cells were not included or (ii) the manufacturer provided RPMI 1640 media was replaced with AIM-V serum free media ± 2 nM VWF (Haemotologic Technologies #HCVWF-0191) (VWF concentration equivalent to ~5% serum) or (iii) the manufacturer provided low IgG fetal calf serum was replaced with normal human serum (Assaypro #UD203011) or with VWF-deficient human serum (Assaypro #D203011).

NK Stimulation Assay

In 96-well V-bottom cell culture plates, NK-92 or PTA-6967 cells (100,000/well) were incubated overnight at 37°C, 5% CO_2 in NK cell media containing therapeutic protein products, polyclonal human IgG, or PMA (50 ng/mL) and ionomycin (1 μg/mL). For blocking studies, NK cells were pre-bound with FcγR-specific $F(ab')_2$ fragments (10 μg/mL) prior to the addition of rFVIIIFc. Following incubation, NK cells were pelleted, and cell culture supernatants were tested for human IFNγ (BD #555142), granzyme B (Invitrogen #BMS2027), and perforin (Invitrogen #BMS2306) levels by sandwich ELISA.

NK Cell Isolation

Cryopreserved human PBMCs were purchased from Cellular Technology Limited. Human NK cells were isolated from PBMCs using Miltenyi NK Cell Isolation Kit (Miltenyi #130-092-657) with MS Columns (Miltenyi #130-042-201), and a MiniMACS Separator (Miltenyi #130-042-102). Stimulation of primary human PBMC and NK cell fractions were performed as described above.

NK Cell Degranulation Assay

In 12-well cell culture plates, PTA-6967 cells (1,000,000/well) were incubated for 6 hours at 37°C, 5% CO_2 in NK cell media containing 5 μL anti-CD107a-APC (BD #560664) as well as PMA (50 ng/mL) and ionomycin (1 μg/mL), or rFVIIIFc (250 nM). After one hour, monensin (0.67 μL/mL) and Brefeldin A (1 μL/mL) were added to block endocytic trafficking. Cell samples were harvested, washed and stained for viability (Fixable Viability Dye eFluor506; eBioscience #65-2860) and surface markers (CD3-FITC (BD #561806), CD16-PE-Cy5 (BD #555408), CD56-PE-Cy5 (BD #557747)). Samples were analyzed using a BD LSRII cytometer and FlowJo version 10 software.

FVIII-Specific B-Cell Killing Assay (Modified ADCC)

In a modified ADCC assay, target BO2C11 B-cells were prepared in assay buffer (RPMI-1640 media with 5% fetal calf serum) and added (20,000 cells/well) to a V-bottom 96 well plate. rFVIIIFc was prepared to appropriate concentrations in assay buffer and added to target B-cells. Effector NK cells ($CD16^-$ NK-92 or $CD16^+$ PTA-6967) were prepared in assay buffer and added (100,000 cells/well) to the target cells and rFVIIIFc. Target cells lysed with 1% Triton X-100 served as maximal-lysis (positive) controls. Wells containing no rFVIIIFc served as spontaneous-lysis (negative) controls. The 96-well culture plates were incubated 4 hours at 37°C, 5% CO_2. Following incubation, the cells were pelleted, and the cell culture supernatants were transferred to another 96-well plate for further analysis. Lactate dehydrogenase (LDH) release was measured using CytoTox 96 Non-Radioreactive Cytotoxicity Assay according to manufacturer's instructions (Promega #G1782).

RESULTS

rFVIIIFc Signals *via* CD16

Previously we had shown that monomeric rFVIIIFc and recombinant Factor IX Fc-fusion protein (rFIXFc) bound and signaled *via* CD16 using BW5147:hCD16ζ reporter cells (22). To determine whether these findings translated into activation of $CD16^+$ NK cell function, we employed the ADCC Reporter Bioassay system (24). We tested rFVIIIFc, rFIXFc and anti-CD20 mAb (positive-control) for stimulation of CD16-mediated signaling. The luciferase signal generated by CD16-activated effector cells provides a surrogate endpoint for NK cell mediated-cellular cytotoxic responses (**Figure 1A**). When co-incubated with effector and target cells (Raji B-cells expressing the surface antigen CD20), rFVIIIFc stimulated $CD16^+$ effector cells to produce a luciferase signal to a similar degree as an assay-specific positive control anti-CD20 mAb. However, rFIXFc did not elicit a CD16-mediated signaling response (**Figure 1B**), despite having the

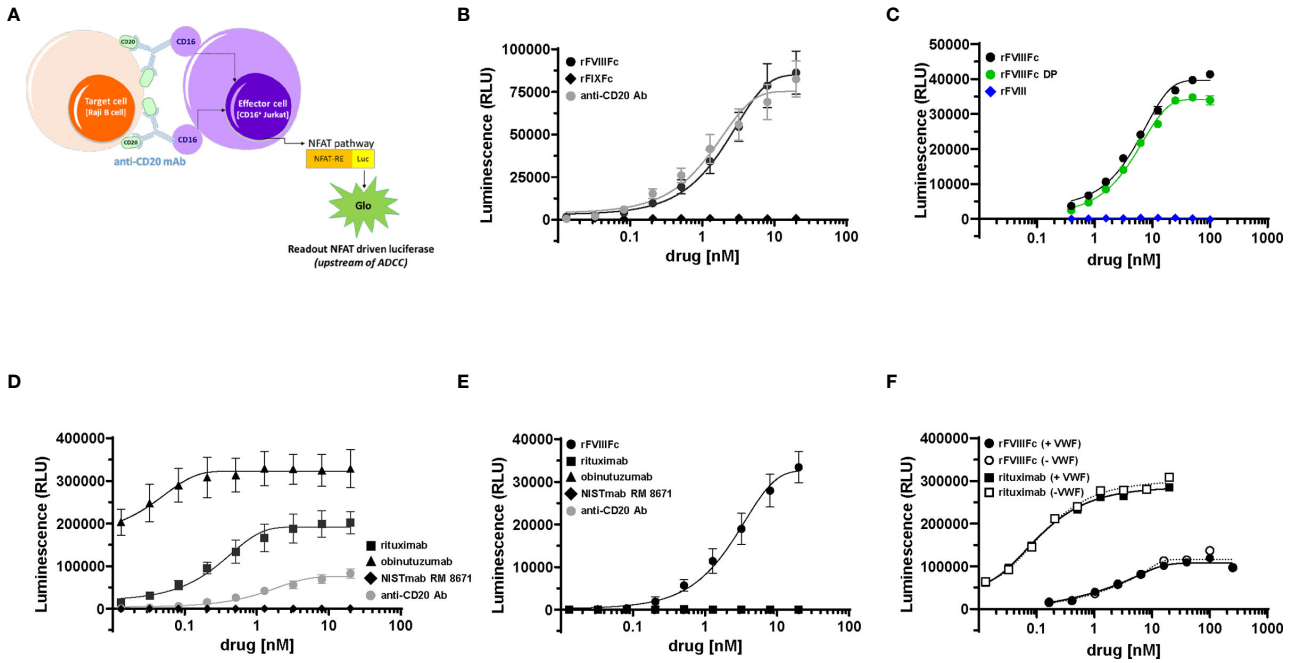

FIGURE 1 | rFVIIIFc signals *via* CD16. **(A)** An illustration depicting the ADCC Reporter Bioassay used to measure CD16-mediated responses to human IgG1 Fc-containing therapeutic protein products or control antibodies. **(B)** ADCC Bioassay Target Cells (1.25 x 10^4 $CD20^+$ Raji B-cells) were incubated with a dilution series (20 nM – 13.1 pM) of therapeutic Fc-fusion proteins (rFVIIIFc, black circles; rFIXFc, black diamonds) or anti-CD20 assay control antibody (grey circles) and then incubated with ADCC Bioassay Effector Cells (7.5 x 10^4) for 6 hours at 37°C. Following incubation, Bio-Glo Luciferase Assay Reagent was added, and luminescence was determined using a Perkin Elmer Victor X3 2030 plate reader. Plotted as mean relative luminescence units (RLU) ± standard error of mean (SEM) (n = 2-3) from two independent experiments. **(C)** ADCC Reporter Bioassay conducted as described above with dilution series (100 nM – 391 pM) of rFVIIIFc protein (black circles), rFVIIIFc DP (green circles), or rFVIII (blue diamonds); plotted as mean ± SEM (n = 2-4) from a representative of two experiments. **(D)** ADCC Reporter Bioassay conducted as described above with monoclonal antibodies as controls (rituximab, black squares; obinutuzumab, black triangles; NISTmAb RM 8671, black diamonds; anti-CD20 assay control antibody, grey circles); plotted as mean ± SEM (n=2-3) from two independent experiments. **(E)** ADCC Reporter Bioassay conducted as described above, except in the absence of ADCC Bioassay Target Cells (rFVIIIFc, black circles; rituximab, black squares; obinutuzumab, black triangles; NISTmAb RM 8671, black diamonds; anti-CD20 assay control antibody, grey circles); plotted as mean ± SEM (n = 2) from a representative of two experiments. **(F)** ADCC Reporter Bioassay experiment conducted as described above, except assay buffer constituents RPMI 1640 Medium and 4% Low IgG fetal calf serum were replaced with AIM-V serum free medium + 2 nM VWF (solid lines/closed symbols) or AIM-V serum free medium (dashed lines/open symbols) (rFVIIIFc, circles; rituximab, squares); plotted as data points (n = 1) from a representative of two experiments.

identical Fc primary amino acid sequence, hinge region, protein expression system, and manufacturer as rFVIIIFc. We observed no difference in CD16 signaling by rFVIIIFc protein and rFVIIIFc DP samples in the ADCC Reporter Bioassay (**Figure 1C**). A beta-domain deleted rFVIII therapeutic did not elicit a response suggesting rFVIIIFc's IgG1 Fc domain is responsible for the observed CD16 signaling, not the rFVIII moiety. Two B-cell targeting (anti-CD20) therapeutic mAbs (rituximab and obinutuzumab) were used as additional positive controls (**Figure 1D**), as their mechanisms of action involves ADCC (25, 26). A non-B-cell targeting mAb (NISTmAb RM 8671) was used as a negative control and exhibited no luciferase response from the $CD16^+$ effector cells (**Figure 1D**).

Since FcγRIIIA/CD16 is a low-affinity receptor for human immunoglobulin G1 (IgG1), Fc-multimerization and receptor crosslinking is a prerequisite for signaling *via* CD16 (27). For IgG1 Fc-containing proteins, protein aggregates (Fc-multimerization) could trigger FcγR signaling. Thus, we assessed the aggregate level of the rFVIIIFc samples used in our studies by non-reducing SDS-PAGE (data not shown) and size exclusion chromatography, which revealed no evidence of rFVIIIFc aggregates (**Supplementary Figure 1**). In the case of anti-CD20 mAbs, Fab engagement with CD20 on the target cell surface allows Fc multimerization, CD16 receptor crosslinking, and effector cell activation (28). Consistent with this postulate, none of the three anti-CD20 mAbs tested elicited a luciferase signaling response in the absence of $CD20^+$ Raji cells (**Figure 1E**). However, we observed rFVIIIFc stimulation of $CD16^+$ effector cells in the absence of Raji target cells (**Figure 1E**) suggesting that rFVIIIFc-CD16 signaling does not require target cell-binding and/or multimerization.

We hypothesized that rFVIIIFc elicits CD16 signaling because, under physiological conditions, FVIII molecules are associated with circulating VWF multimers (29). Thus, the VWF present in fetal bovine serum (FBS) used in the assay buffer could provide a scaffold for Fc multimerization on the NK cell and subsequent CD16 signaling. To test this hypothesis, we performed the ADCC assay with rFVIIIFc in the presence and absence of human VWF. When tested in AIM-V serum free medium, rFVIIIFc continued to stimulate luciferase activity in the $CD16^+$ effector cells. We observed no difference in the

rFVIIIFc-CD16 signaling response when serum-free medium was spiked with VWF (2 nM; equivalent to ~5% final serum concentration) (**Figure 1F**); as expected, the absence of VWF had no effect on rituximab-mediated CD16 signaling (**Figure 1F**). Furthermore, we also observed no effect of rFVIIIFc-mediated CD16 signaling when the ADCC Reporter Bioassay was performed using RPMI 1640 medium containing VWF-deficient human serum as compared to normal human serum (**Supplementary Figure 2**). These findings suggest that rFVIIIFc-mediated CD16 signaling is independent of VWF : FVIIIFc multimerization. Unlike rFVIIIFc, rFIXFc and rituximab in the absence of B-cell targets fail to activate the $CD16^+$ effector cells, suggesting that the FVIII component of rFVIIIFc may play a role in enhancing the Fc-FcγRIIIA interaction.

rFVIIIFc Activates and Induces Degranulation and IFNγ Secretion by $CD16^+$ NK Cells

Although we demonstrate engagement of rFVIIIFc with CD16, and signaling *via* a reporter-gene, this does not necessarily mean NK cells are fully activated leading to granzyme and perforin release and cytokine (IFNγ) secretion. To determine whether rFVIIIFc fully activated NK cells *via* CD16 we used two NK cell lines; NK-92 (which is $CD16^-$) and PTA-6967 (which is $CD16^+$). The $CD16^+$ NK cell line was stimulated by rFVIIIFc to secrete IFNγ, but not by any other Fc-fusion protein or antibody tested (**Figure 2A**). Even upon plate-immobilization of the Fc-containing proteins, mimicking aggregation or immune complex formation to enhance CD16 crosslinking, rFIXFc failed to stimulate IFNγ secretion by $CD16^+$ NK cells (**Supplementary Figure 3**). This is consistent with a lack of rFIXFc activity in the ADCC Reporter Bioassay (**Figure 1B**). Importantly, none of the therapeutic proteins tested (including rFVIIIFc) stimulated IFNγ secretion from the $CD16^-$ NK cell line (**Figure 2A**, open squares). These results confirmed rFVIIIFc can activate NK cells in a CD16-dependent fashion. IFNγ secretion mediated by rFVIIIFc is dose dependent with an EC_{50} of 6.4 nM (**Figure 2B**). Consistent with results shown in **Figure 1C**, we observed no difference between rFVIIIFc protein and rFVIIIFc DP in terms of inducing IFNγ secretion from $CD16^+$ NK cells, while rFVIII (without the Fc moiety) did not elicit a response (**Figure 2C**). The specificity of the rFVIIIFc : CD16 interaction was further demonstrated by pre-incubation of NK cells with αCD16 $F(ab')_2$ that completely blocked rFVIIIFc-induced IFNγ secretion by NK cells (**Figure 2D**). Pre-incubation of the NK cells with αCD64 $F(ab')_2$ or αCD32 $F(ab')_2$ fragments did not block their activation by rFVIIIFc (**Figure 2D**). This is consistent with our findings using the $CD16^-$ NK cell line (**Figure 2A**). We confirmed the surface FcγR expression profiles of NK-92 and PTA-6967 NK cells by flow cytometry (**Supplementary Figure 4**). Moreover, as observed in the ADCC Reporter Bioassay (**Figure 1F** and **Supplementary Figure 2**), the induction of $CD16^+$ NK cell IFNγ secretion was not dependent on VWF (**Supplementary Figure 5**).

Elevated surface expression of Lysosomal Associated Membrane Protein 1 (LAMP-1; CD107a) is an indirect measure of NK cell activation and the release of cytolytic granules (15). Flow cytometric analysis of NK cells (PTA-6967) showed that, compared to untreated cells, there was an almost 8-fold increase in the percentage of cells with an elevated level of LAMP1 following a 6-hour treatment with rFVIIIFc (**Figure 2E**). Treatment with PMA and ionomycin, as a positive control for CD16-independent stimulation showed an approximately 17-fold increase in the percentage of cells with elevated LAMP-1 (**Figure 2E**).

Upon activation NK cells secrete cytokines and release granules containing cytolytic proteins (30). IFNγ secretion occurs as early as three hours and continues to increase for 24 hours following rFVIIIFc stimulation (**Figure 2F**). Release of the cytolytic granule proteins granzyme B (**Figure 2G**) and perforin (**Figure 2H**), followed different kinetics compared to the IFNγ response. As a CD16-independent positive control for NK cell activation, we used PMA and ionomycin and observed IFNγ secretion as well as granzyme B and perforin release from $CD16^+$ NK cells (**Supplementary Figure 6**).

These findings (**Figures 1** and **2**) indicate that monomeric rFVIIIFc signals *via* CD16 and results in NK cell activation.

rFVIIIFc Induces IFNγ Secretion From NK Cells Isolated From Human PBMCs

In addition to NK cell lines, we assessed the effect of rFVIIIFc stimulation on primary human NK cells isolated from peripheral blood mononuclear cells (PBMCs) collected from 15 healthy donors. We observed significantly ($p < 0.0001$) elevated IFNγ secretion from human PBMCs as well as isolated NK cells ($CD3^-$ $CD56^+$) confirmed for surface CD16 expression (**Supplementary Figure 7**) and PMA/ionomycin responsiveness (**Supplementary Figure 8**) following incubation with rFVIIIFc (**Figures 3A, B**). The FCGR3A gene single nucleotide polymorphism (SNP) (rs396991) results in two FcγIIIRA allotypes, FcγIIIRA 158V and FcγIIIRA 158F (31). FcγIIIRA 158V has been demonstrated to bind IgG1 with higher affinity than FcγIIIRA 158F (31) and increased clinical responses to monoclonal antibodies with ADCC-mediated mechanisms of action (32). A subset analysis showed that elevation in secreted IFNγ by NK cells following treatment with rFVIIIFc was significant ($p < 0.05$) with cells from donors possessing at least one high affinity CD16 158V allele, and not significant with cells from donors with the low affinity 158F/F variant (**Figure 3C**).

rFVIIIFc Induces BO2C11 (FVIII-Specific B-Cell) Lysis Through Interactions With $CD16^+$ NK Cells

We tested the hypothesis that rFVIIIFc-mediated stimulation of NK cells to become effector cells (shown above) can lead to the killing of FVIII-specific B-cells. In such a mechanism the Fc-moiety would engage with CD16 on NK cells while the rFVIII would bind to anti-FVIII BCRs. rFVIIIFc-mediated cell killing of BO2C11, a B-cell clone specific for the FVIII-C2 domain (IgG4 kappa) (23), was dependent on $CD16^+$ NK cells (**Figure 4**). The rFVIIIFc-mediated BO2C11 cytotoxicity was dose-dependent with a peak cytolytic response observed at 31.25 nM rFVIIIFc (**Figure 4**). However, at higher rFVIIIFc concentrations, we observed a "hook effect" which has been reported in some

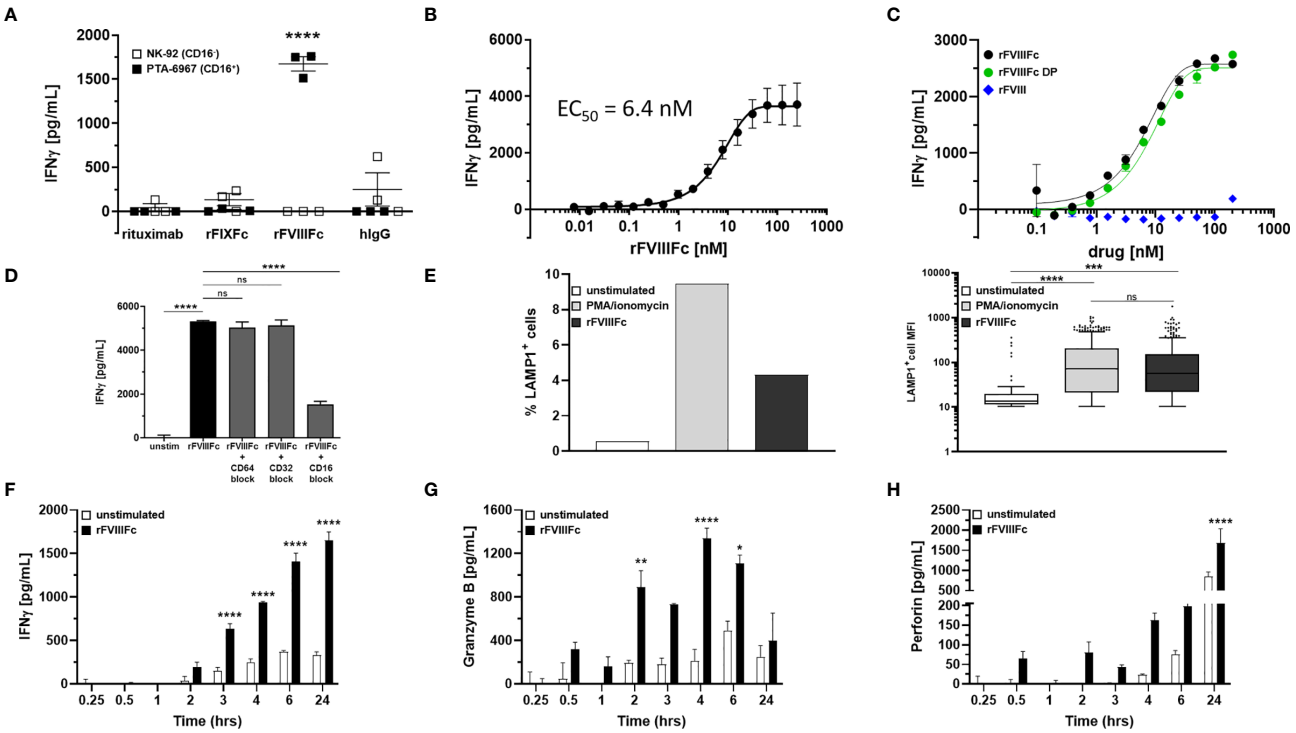

FIGURE 2 | rFVIIIFc activates and induces degranulation by CD16$^+$ NK cells. **(A)** IFNγ secretion by CD16$^-$ NK cells (NK-92; open squares) or CD16$^+$ NK cells (PTA-6967; filled squares) measured by ELISA following overnight incubation with 67 nM human IgG1 Fc-containing proteins [rituximab; rFIXFc; rFVIIIFc; human polyclonal IgG]; plotted as mean ± SEM (n=3) from a representative of two experiments. Tukey's multiple comparisons tests (two-tailed) were performed for each protein between stimulated CD16$^-$ NK cells (open squares) and stimulated CD16$^+$ NK cells (filled squares). ****p < 0.0001. **(B)** Secreted IFNγ response of CD16$^+$ NK cells (PTA-6967) following overnight stimulation with different concentrations of rFVIIIFc (250 nM to 7.6 pM); plotted as mean ± SEM (n = 6) from 2 independent experiments. **(C)** Secreted IFNγ response of CD16$^+$ NK cells (PTA-6967) as above with rFVIIIFc protein (black circle), rFVIIIFc DP (green circle), or rFVIII (blue diamond) (200 nM to 98 pM); plotted as mean ± SEM (n = 2) from a representative of two experiments. **(D)** IFNγ secretion by CD16$^+$ NK cells (PTA-6967) stimulated with rFVIIIFc (250 nM). For FcγR blockade, CD16$^+$ NK cells were pre-incubated with FcγR-specific F(ab')$_2$ fragments prior to stimulation with rFVIIIFc; plotted as mean ± SEM (n = 4). Tukey's multiple comparisons tests (two-tailed) were performed between (i) unstimulated controls and rFVIIIFc stimulated and (ii) rFVIIIFc stimulated ± FcγR blockade. ****p < 0.0001. **(E)** Left, bar graph depicting surface CD107a (LAMP1) levels (% LAMP1$^+$ cells), an indirect measure of degranulation, measured on NK cells (PTA-6967) from a single flow cytometry experiment following a 6-hour stimulation with PMA/ionomycin (50 ng/mL PMA, 1 μg/mL ionomycin) or rFVIIIFc (250 nM); right, Tukey box-and-whiskers plots depicting LAMP1$^+$ cell mean fluorescence intensity (MFI). One-way ANOVA and Tukey's multiple comparisons test performed, ***p = 0.0004, ****p < 0.0001. **(F–H)** Measurement of secreted IFNγ **(F)**, granzyme B **(G)**, and perforin **(H)** by ELISA over time from rFVIIIFc-stimulated (67 nM) CD16$^+$ NK cells (PTA-6967) plotted as mean ± SEM (n = 3). Tukey's multiple comparisons tests (two-tailed) were performed at each timepoint between unstimulated cells (open bars) and rFVIIIFc stimulated cells (filled bars). *p < 0.05, **p < 0.01, ****p < 0.0001. ns, not significant, p > 0.05.

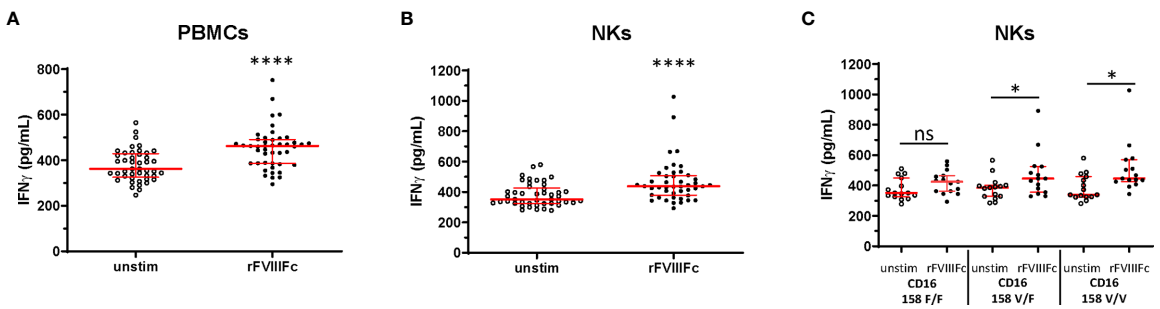

FIGURE 3 | rFVIIIFc induces IFNγ secretion from peripheral blood mononuclear cells and NKs isolated from healthy human donors. IFNγ secretion from PBMCs **(A)** or resting NK cells **(B)** isolated from 15 healthy human donors each measured in triplicate by ELISA following overnight stimulation with rFVIIIFc (250 nM). **(C)** IFNγ secretion from resting NK cells with distinct CD16 V/F 158 genotypes (CD16 158 F/F, V/F, V/V; n = 5, each measured in triplicate). **(A–C)** Median ± inter-quartile range (IQR) indicated in red. Wilcoxon signed rank tests (two-tailed) were performed for comparisons between unstimulated and rFVIIIFc-stimulated cells. *p < 0.05, ****p < 0.0001. ns, not significant, p > 0.05.

ternary complexes (33, 34) (**Figure 4**). Furthermore, we observed BO2C11-mediated enhancement of rFVIIIFc-induced IFNγ secretion by the PTA-6967 cell line (**Supplementary Figure 9**). These results indicate that rFVIIIFc can mediate killing of anti-FVIII producing B-cells.

DISCUSSION

The extended half-life rFVIIIFc product was designed and developed with the primary goal of increasing the plasma half-life (35, 36). The rFVIIIFc was approved in 2014, and substantial clinical experience with this therapeutic provided in case reports and retrospective analyses (5–7) suggest that rFVIIIFc offers superior outcomes with respect to ITI compared to other FVIII products (37). Currently, several prospective clinical studies including the verITI-8 Study (ClinicalTrials.gov # NCT03093480), the ReITIrate Study (ClinicalTrials.gov # NCT03103542), and the Hemophilia Inhibitor Eradication Trial (ClinicalTrials.gov # NCT04303572) are evaluating the efficacy of rFVIIIFc in ITI regimens. Here, we have investigated the hypothesis that rFVIIIFc could engage with CD16 and drive NK cell-mediated killing of memory B-cells expressing an anti-FVIII BCR. We propose a testable hypothesis that may explain superior outcomes with rFVIIIFc in ITI; *viz.* rFVIIIFc could engage with $CD16^+$ NK cells and mediate lysis of anti-FVIII memory B-cells. Here we provide *in vitro* evidence in support of this hypothesis.

Immune tolerance has wide applications in circumventing immune responses to proteins that are used therapeutically (38, 39) as well as in auto-immune diseases (40). However, many currently used tolerogenic approaches result in broad immune suppression, thus putting the patient at risk of infection. In the case of FVIII ITI regimens, the Malmö protocol (41), employs high-dose FVIII product (200 IU/kg daily) administered concomitantly with an immunosuppressive agent [i.e. rituximab; cycloheximide; intravenous immunoglobulin (IVIg)]. There are reports of enhanced FVIII inhibitor eradication following a FVIII ITI regimen in combination with rituximab-mediated B-cell depletion (42, 43). Notably, rituximab does not target antibody-secreting plasma cells as they do not express the CD20 surface antigen, nevertheless depletion of the $CD20^+$ memory B-cell pool may be sufficient to eradicate FVIII inhibitors and induce tolerance. However, this approach does not specifically target anti-FVIII B-cells as rituximab also depletes non-FVIII specific B-cells. A highly selective tolerogenic approach that depletes antigen-specific B-cells would avoid undesirable broad-spectrum immunosuppression.

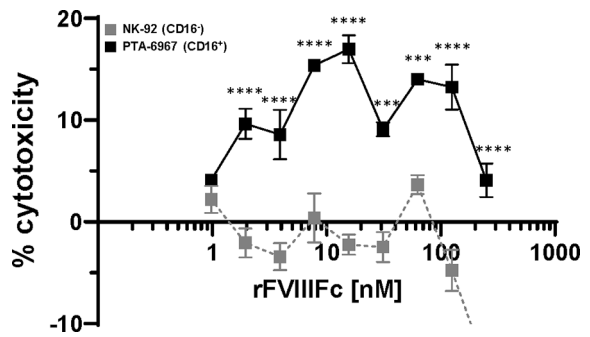

FIGURE 4 | rFVIIIFc induces BO2C11 (FVIII-specific B-cell) lysis through interactions with $CD16^+$ NK cells. Lactate dehydrogenase (LDH) release from target BO2C11 B-cells following 4 hour incubation at 37°C following incubation with rFVIIIFc (250 nM – 977 pM) and $CD16^+$ NK cells [PTA-6967, black squares] or $CD16^-$ NK cells [NK-92, grey squares] using a 5:1 effector to target ratio (100,000:20,000 cells per well); plotted as mean % cytotoxicity ± SEM (n = 4) from a representative of two experiments. % cytotoxicity was calculated as: % cytotoxicity = [(experimental well – spontaneous lysis control)/(Triton X-100 maximal lysis control – spontaneous lysis control)] * 100. Tukey's multiple comparisons tests (two-tailed) were performed at each rFVIIIFc concentration comparing responses between $CD16^-$ (NK-92) and $CD16^+$ (PTA-6967) NK cells. ***$p < 0.001$, ****$p < 0.0001$.

The Fc-domains of antibodies engage with CD16 on the surface of NK cells while the Fab domains provide exquisite selectivity for cellular cytotoxicity by binding to a specific molecule on the target cells (44). Natural non-engineered IgG1 Fc monomers bind to CD16 with low affinity (~ 1 μM) (45). Thus, binding to CD16 at levels that can result in a meaningful biological response requires that the Fc molecules form multimers or complexes thereby elevating the local concentration and resulting in receptor crosslinking (19). For instance, in our ADCC Reporter Bioassay results (**Figure 1**), binding of the anti-CD20 antibodies (e.g. rituximab and obinutuzumab) to target Raji cells is a prerequisite for engagement with $CD16^+$ effector cells (**Figure 1E**). In contrast, we observed an exception to this general rule; *viz.* the engagement of rFVIIIFc with $CD16^+$ reporter cells was independent of multimerization on the surface of a target cell (**Figures 1E** and **5A**). This is consistent with our previous finding that rFVIIIFc exhibits non-canonical FcγR and complement C1q binding and signaling properties, even in a monomeric form (22). The rFVIIIFc-mediated signals *via* CD16 in these assays could suggest a response driven by protein aggregation or the formation of immune complexes. However, non-reducing SDS-PAGE (data not shown) and size exclusion chromatography analyses of rFVIIIFc used in our studies revealed no evidence of rFVIIIFc aggregates (**Supplementary Figure 1**). Our results provided strong evidence that (unlike other Fc-fusion proteins and mAbs tested) monomeric rFVIIIFc can bind CD16 and stimulate NK cells with relatively high potency (EC_{50} 6.4 nM). This finding is consistent with a previous study demonstrating rFVIIIFc engaged CD16 on the surface of monocyte-derived macrophages (46).

A plausible explanation for the surprising rFVIIIFc signaling in the absence of the prerequisite multimerization is an alternate mechanism for complex formation. Human FVIII has very high affinity for human VWF (0.2 nM) (47), consequently VWF acts as a chaperone for FVIII increasing its circulating half-life from ~2.5 to 12 hours (47, 48). A single FVIII molecule (C2 domain) can bind to the D'D3 domains of a VWF monomer (48). VWF circulates as large multimers which could provide a scaffold for sufficient rFVIIIFc multimerization to foster engagement with CD16. To test the hypothesis that VWF:rFVIIIFc interactions contributed to

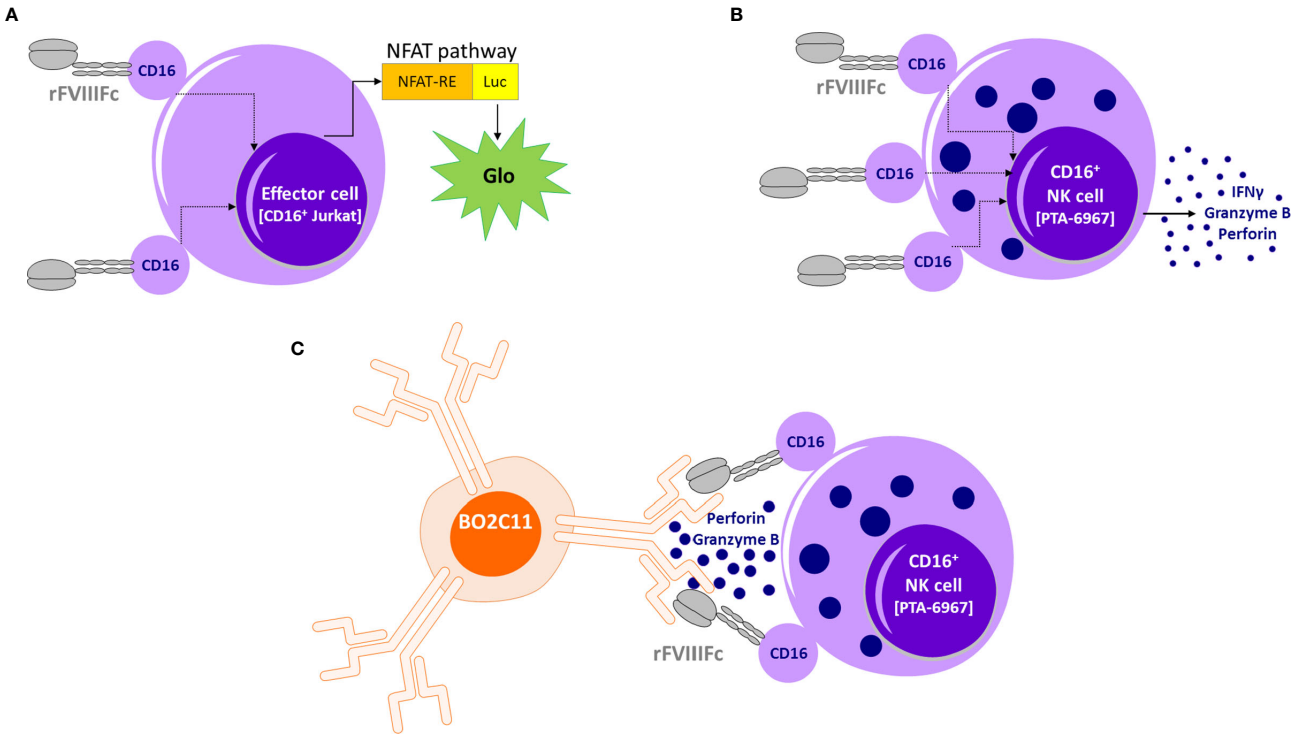

FIGURE 5 | Proposed model of rFVIIIFc : CD16 interactions. **(A)** An illustration depicting interactions between rFVIIIFc and CD16$^+$ ADCC Reporter Bioassay effector cells resulting in luciferase activity. **(B)** An illustration depicting interactions between rFVIIIFc and CD16$^+$ NK cells leading to IFNγ, granzyme B, and perforin release. **(C)** An illustration depicting interactions between rFVIIIFc and CD16 and a putative mechanism by which rFVIIIFc can target anti-FVIII memory B-cells (BO2C11).

rFVIIIFc-mediated signaling *via* CD16 we performed the ADCC Reporter Bioassay and *in vitro* NK cell stimulation in the absence and presence of human VWF. We demonstrated rFVIIIFc-mediated CD16 signaling was not abrogated in the absence of VWF (**Figure 1F** and **Supplementary Figures 2, 5**). This suggests that the FVIII component of rFVIIIFc, unlike the FIX of rFIXFc and the Fab of rituximab, enables Fc : CD16 interactions that lead to NK cell activation and effector function.

To determine whether rFVIIIFc : CD16 interactions are sufficient to selectively kill anti-FVIII memory B-cells we used BO2C11 cells. BO2C11 is a human IgG4 kappa B-cell clone with anti-FVIII C2 domain specificity obtained from a hemophilia A subject with high-level inhibitors [for details see (23, 49)]. Using BO2C11 as the target cell in an ADCC assay we demonstrated BO2C11 killing mediated by rFVIIIFc in the presence of CD16$^+$ NK cells. Understanding the mechanistic details of rFVIIIFc : CD16 interactions and under what conditions this engagement can be exploited for the killing of FVIII-specific memory B-cells lays the groundwork for the design of future Fc-fusion proteins for use as antigen-specific B-cell targeting agents in immune tolerance. Several groups have initiated efforts to design Fc-fusion proteins as antigen-specific B-cell depletion agents (50–52).

We acknowledge that rFVIIIFc can also engage with other FcγRs on a variety of immune cell types, which may complicate the extrapolation of our findings to mechanisms operating *in vivo*, in a patient. Previous *in vitro* studies have assessed rFVIIIFc's role on the activation state of macrophages (46), dendritic cells (53) and B-cells (54). We (22) and others (54) have shown the ability of rFVIIIFc to signal *via* the inhibitory FcγRIIB. Additionally, Fc-mediated interactions with FcγRs resulting in antibody-dependent cellular phagocytosis (ADCP) may also be involved in the *in vivo* setting. However, it is possible that several rFVIIIFc : FcγR-mediated interactions may collectively contribute towards the reduced immunogenicity and induced tolerance observed in hemophilia A mice (55). Therefore, *in vivo* hemophilia A mouse model or clinical studies will be needed to test which FcγR$^+$ immune cell populations are critical during high-dose rFVIIIFc ITI regimens for inducing tolerance.

The schematic in **Figure 5** provides a putative mechanism by which rFVIIIFc can target anti-FVIII memory B-cells. Our *in vitro* results, and the proposed mechanism could provide a testable hypothesis that may explain reported clinical results wherein ITI using rFVIIIFc was successful in some patients who have failed tolerizing regimens using FVIII products that were not conjugated with Fc (7). As discussed above, the concept of targeting NK cells specifically to memory B-cells that produce undesirable antibodies (to therapeutics and self-antigens) does not find easy clinical applicability due to the low IgG1 Fc : CD16 affinity. However, the fortuitous CD16 engagement by monomeric rFVIIIFc has allowed us to demonstrate that, if the Fc-moiety can be made to engage with CD16 at high affinity, then the strategy of selectively depleting BCR$^+$ memory B-cells producing antibodies to a specific

antigen is feasible *in vitro*. The biophysics and structural biology of Fc : CD16 binding are quite well understood (56). Thus protein- and glyco-engineering approaches to enhance Fc : CD16 affinities are plausible (57, 58). For instance, the obinutuzumab Fc region was glyco-engineered for higher affinity for CD16 and more potent ADCC activity (25). Future studies with Fc-engineering hold considerable promise in developing a platform-technology for selectively targeting B-cells with undesired BCRs. This approach may be extended to other target cells, such as cancer cells, using Fc-fusion proteins so that the fusion protein is designed to bind a receptor on the target tumor.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

HL, ZS, and BG designed the research. MJ provided critical reagents and critically reviewed the manuscript. HL, LH, and WJ performed the research. HL, LH, WJ, ZS, and BG analyzed the data. HL, ZS, and BG wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.692157/full#supplementary-material

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The Effect of Immunosuppressive Adjuvant Kynurenine on Type 1 Diabetes Vaccine

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Inducing antigen-specific tolerance is a promising treatment for preventing or reversing Type 1 diabetes (T1D). In contrast to a vaccine that induces immune responses against pathogens, a tolerogenic vaccine can suppress immunity against antigens causing diseases by administrating a mixture of self-antigens with an adjuvant that decreases the strength of antigen-specific response. Kynurenine (Kyn) is an endogenous substance that can inhibit the natural killer cell and T cell proliferation and promote the differentiation of naïve T cells into regulatory T cells (T_{regs}). In this study, we evaluated the efficacy of Kyn as a novel suppressive adjuvant. Kyn was co-immunized with GAD65 phage vaccine to induce T_{reg} cells and tolerogenic responses for the prevention of T1D in NOD mouse model. Mice were subcutaneously immunized two times with 10^{11} Pfu (100μL,10^{12} Pfu/ml) GAD65 phage vaccine doses mixed with 200 μg of Kyn. Serum antibodies and cytokines were detected by ELISA and electrochemiluminescence, respectively. Flow cytometry assay was used to analyze DC and Treg. MTS was used for the analysis of spleen lymphocyte proliferation. RNA sequencing was used to investigate mRNA and miRNA expression profiles in spleen lymphocytes. Compared to GAD65 phage vaccine alone, co-immunization of Kyn and GAD65 phage vaccine resulted in the prevention of hyperglycemia in 60% of mice for at least one month. Further, Kyn enhances GAD65-specific Th2-mediated immune responses; regulates the Th1/Th2 imbalance and increases the secretion of Th2 cytokines and the number of $CD4^+CD25^+Foxp3^+$T cells; suppresses DC maturation and GAD65-specific T lymphocyte proliferation. Moreover, we integrated Kyn related miRNA and mRNA expression profiles obtained from the spleen lymphocyte RNA-sequencing which was stimulated by Kyn *in vitro*. These data provide an important basis for understanding the mechanisms underlying Kyn as an immunosuppressive adjuvant which regulated the immune response. These findings suggest that Kyn can serve as an effective suppressive adjuvant candidate for Type 1 diabetes vaccines.

Keywords: immunosuppressive, adjuvant, kynurenine, vaccine, Type 1 diabetes vaccine

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HIGHLIGHTS

- Co-immunization of Kyn and GAD65 phage vaccine resulted in prevention of hyperglycemia in 60% of mice for at least one month.
- Co-immunization of Kyn and GAD65 phage vaccine enhances GAD65-specific Th2-mediated immune responses; regulates the Th1/Th2 imbalance and increases the secretion of Th2 cytokines and the number of $CD4^+CD25^+Foxp3^+$ T cells; suppresses DC maturation and GAD65-specific T lymphocyte proliferation.
- RNA sequencing provides an important basis for understanding the mechanisms underlying Kyn as an immunosuppressive adjuvant which regulated the immune response.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease attributed to the immune-mediated progressive destruction of β cells in the pancreatic islets, which results in hyperglycemia. Autoantibodies against insulin, including 65 kDa glutamic acid decarboxylase (GAD65), insulinoma-associated protein 2 (IA-2), and zinc transporter 8 (ZnT8), are proteins associated with secretory β-cell granules. It can be used as biomarkers of T1D-associated autoimmunity. These proteins can be identified months to years before the onset of T1D and served as developing risk markers (1, 2).

Infiltration of islet antigen-specific T cells, activation of pro-inflammatory antigen-presenting cells, and loss of $Foxp3^+$ regulatory T cells (Tregs) are three of the most T1D characteristic immunopathological processes (3).

Nearly 20 million people suffer from T1D worldwide. Although life-long insulin treatment can alleviate symptoms and delay organ damage, it does not reverse the antigen-specific T cell responses toward β cells. Therefore, a novel treatment strategy is required to improve therapeutic effects. Some scholars (4) believe that β cell autoantigens presented in non-inflammatory contexts can regulate auto-reactive T cells and generate β cell protection. Recovering antigen-specific tolerance or down-regulating the immune response to non-harmful antigens is a promising way to treat T1D.

GAD65 is a major autoantigen in T1D. T-cell reactivity and autoantibodies against GAD65 are early markers of this autoimmune disease process. GAD antibodies have been found in nearly 70–80% of T1D patients at the time of diagnosis (5). Preclinical studies have demonstrated that the administration of the isoform GAD65 in non-obese diabetic (NOD) mouse model can prevent autoimmune destruction of pancreatic β-cells. In nearly 80% of NOD mice, long-term normoglycemia was restored by repeated administration of GAD65-alum. Moreover, the injection of aluminum salts of GAD65 (GAD65-Alum) in mice has been shown to reduce GAD-specific Th1 Teff cells (6, 7). Also, GAD65-Alum (Diamyd®) has been recently tested in phase II and III clinical trials (8–11), showing drug safety. In addition, Diamyd® appeared to be superior to placebo in preserving residual β-cell function at 12–15 months. Moreover, an expansion of $IL\text{-}10^+CD4^+$ T cells was observed, indicating the regulatory compartment's induction (12). Despite these vaccines' success in mouse models, these vaccines could not induce $CD4^+Foxp3^+$ Tregs that can balance between beneficial and harmful effects of inflammation.

Regulatory T (Treg) cell is believed to have a key role in preventing autoimmunity. Animal studies have illustrated that $CD4^+Foxp3^+$ Tregs can induce tolerance by suppressing the functions of Th1 cells and DCs or by releasing inhibitory cytokines such as TGFβ or IL-10 (13), and form the primary mechanism of peripheral tolerance (14). The most promising immunotherapy for autoimmune disease treatment in humans is autologous Treg cell therapy (15). Some studies (16) have shown that dexamethasone and rapamycin (rapa) could significantly increase cell numbers and function $CD4^+CD25^+$ Treg cells in animal models. However, these immunosuppressive drugs have side effects, such as induction of infection and tumor formation.

Nowadays, many kinds of autoantigen-specific T1D trials involving oral and nasal insulin or recombinant human GAD65 formulated with alum have been continuously applied (17). However, no suitable immunosuppressive drug or immunosuppressive adjuvant can be used in combination with it. Therefore, finding new immunosuppressive drugs or immunosuppressive adjuvants is a key factor for the success of this treatment strategy.

Kynurenine (Kyn) is a tryptophan metabolite produced through tryptophan-2, 3-dioxygenase (TDO) degradation in the liver under physiological conditions (18), and through the indoleamine 2, 3 dioxygenase (IDO) in the extrahepatic tissues including blood and lymph tissue during infection, inflammation, or oxidative stress (19, 20). It has been demonstrated that Kyn activates the cytosolic aryl hydrocarbon receptor (AHR) in a ligand-receptor manner (21), and endogenously regulates systemic inflammation and tolerance. Additionally, AHR has been implicated in various immune functions, including reduce the activity of natural killer (NK) cells (22), inhibit the NK cell and T cell proliferation (23), and promote the differentiation of naïve T cells into Treg instead of into Th17 cells through preventing dendritic cell maturation (24), which supports the role of AHR as an important player in determining the T cell differentiation (25). Because the Kyn-AHR axis has an effect on the proliferation of Tregs, it has been considered a potential therapeutic target for the treatment of autoimmune disorder. In our previous studies, we found that Kyn can serve as an effective suppressive adjuvant for vaccines. Otherwise, Kyn is an endogenous substance that is safer than exogenous substances when considered as a vaccine adjuvant.

Abbreviations: T1D, Type 1 diabetes; Kyn, kynurenine; GAD65, 65 kDa glutamic acid decarboxylase; Tregs, regulatory T cells; NOD, non-obese diabetic; AHR, aryl hydrocarbon receptor.

In this study, Kyn was co-immunized with GAD65 phage vaccine to induce Treg cells and tolerogenic responses for prevention of T1D in the NOD mouse model. We provided direct evidence that Kyn, as a novel suppressive adjuvant, promotes Foxp3$^+$ Treg induction, suppresses dendritic cell maturation and GAD65-specific T cell proliferation, and significantly increases IL-10, IL-4 and TGF-β1, decreases of IFN-γ and IL-2 in the NOD mouse model. We also analyzed the molecular information provided by transcriptome sequencing of mRNA and miRNA in an *in vitro* Kyn assay, providing a new understanding of the underlying immune response mechanism and a new idea for the development of suppressive adjuvants.

MATERIALS AND METHODS

GAD65 Phage Vaccine Preparation

The recombinant GAD65 phage vaccine expressing the 190–320 amino acid sequence of huGAD65 (GenBank: M81882.1) was constructed in the T7 phage display system by our laboratory. The huGAD65 gene shares 95% amino-acid identity and 98% conservation with mGAD65 (26), respectively. Briefly, we inoculated 50 µl 10^{11} pfu/ml GAD65 phage into 5 ml fresh *Escherichia coli strain* BLT5403 with OD600 = 0.6–0.8, cultured at 37°C and 150 rpm for 3 h. The cultures were diluted 50-fold into 1,000 ml of *Escherichia coli strain* BLT5403 with OD600 = 0.6–0.8, cultured at 37°C and 150 rpm for 3–6 h. Bacteria were collected by centrifugation (30 min at 5,000 rpm.) The supernatant was mixed in 1:5 volume solution containing 20% polyethylene glycol 8000 (PEG-8000) and 2.5 M NaCl, then kept at 4°C overnight to precipitate the phage particles. After that, the precipitate was collected by centrifugation (30 min at 10,000 rpm.), dissolved in 4 ml of phosphate-buffered saline (PBS), and centrifuged at 10,000 rpm for 30 min. The phage particles were then purified by sequential centrifugation of the PBS phage suspension at 10,000 rpm for 30 min. The precipitate was then dissolved in 1 ml of 50 mM Tris-HCl buffer, pH 7.5. Then, an ultrafiltration membrane with a molecular weight of 100 kD was used to remove endotoxin. After ultrafiltration, the content of endotoxin in phage preparation was less than 100 EU/ml. Then the phage concentration was adjusted to 10^{12} PFU/ml, and the formaldehyde was added to phage particles at a volume ratio of 1:4,000 to inactivate the phage to obtain the GAD65 phage vaccine.

Animal Immunization

Female NOD mice of 4–6 weeks of age were purchased from GemPharmatech Co, Ltd (Nanjing, China). All the animals were housed in a light- and temperature-controlled environment. All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of the Institute of Medical Biology, Chinese Academy institutional animal care and conducted according to the AAALAC and the IACUC guidelines.

The mice were randomly divided into four groups (12 animals per group). They were subcutaneously immunized (subcutaneous injection at different sites on the back of mice) with phage vaccine alone or co-immunized with Kyn (Sigma, K8625) in 100 µl final volume listed in **Table 1**. The animal immunization schedule and detection program are shown in **Figure 1**. Briefly, mice were sacrificed, and splenocytes were harvested at different time points. Blood samples were collected, and sera were stored at −20°C until tested.

Antibodies and Synthetic Peptide

Fluorescently labeled anti-mouse monoclonal antibodies including anti-mouse CD4-FITC (RM4-5), anti-mouse CD25-APC (PC61.5), anti-mouse/Rat-Foxp3 PE (FJK-16s), anti-CD11c-PE (N418), anti-CD80-APC (16-10A1), anti-IL10-FITC (JESS-16E3), and isotype controls were purchased from eBioscience (San Diego, CA, USA). GAD65 specific peptide acid sequences were TYEIAPVFVLLEYVT, EYVTLKKMREIIGWPGGSGD, KKGAAALGIGTDSVI, ALGIGTDSVILIKCDERGK.

TABLE 1 | Animal groups and dosage.

Groups	Dose	Adjuvant	Injection Times	No.
Control	100 µl (10^{12}Pfu/ml)		2	12
Control + KYN	100 µl (10^{12}Pfu/ml)	200 µg KYN	2	12
GAD65	100 µl (10^{12}Pfu/ml)		2	12
GAD65 + KYN	100 µl (10^{12}Pfu/ml)	200 µg KYN	2	12

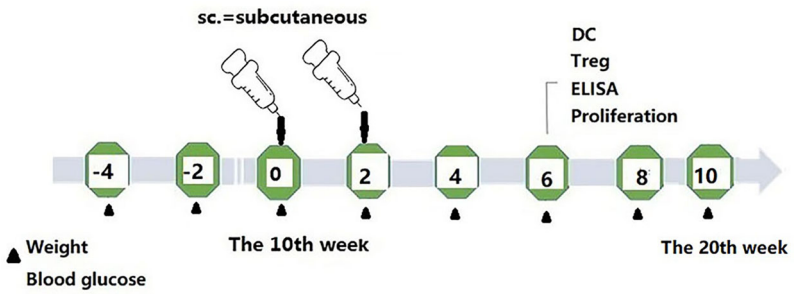

FIGURE 1 | Schematic Map of immunization schedule.

Determination of Serum Antibodies and Cytokines

Determination of Serum Antibodies by ELISA

The 96-well flat-bottom plates were coated with purified recombinant GAD65 protein (purified by our lab) at a concentration of 0.1μg/ml in coating buffer at 4°C overnight. Plates were washed five times with washing buffer and blocked with blocking solution at 37°C for 1 h. After that, mice sera were serially diluted two-fold in blocking solution (starting at 1:100–1:3,200), and 100μl was added to each well. After incubation for 1 h, the plates were washed five times and incubated with 1:2,000 diluted HRP-labeled goat anti-mouse IgG1 and IgG2a antibodies (Invitrogen, USA) at 37°C for 1 h. After the final wash, 100μl of ABTS HRP substrate was added per well and plates were incubated for 5 min; the reaction was stopped by adding 25 μl of ABTS peroxidase stop solution. The optical density (OD) of the plate was measured at 450 nm by ELISA plate reader (UVP, California, USA). The ELISA test reagents were from the ELISA Kit Anti-Mouse ABTS® System (KPL Protein Detector™).

Determination of Serum Antibodies and Cytokines by Electrochemiluminescence

The concentrations of the IL-2, IL-4, IL-10, IFN-$\gamma$and TgF-β_1 in the serum samples were examined using the MSD ECLIA according to the manufacturer's instructions (MSD, Rockville, MD, USA). For quality control, a standard curve was prepared. The highest point and background point were selected for the standard point to confirm that the sample concentration was within the range of the standard curve. In the preliminary experiment, the sample was diluted two and eight times; eight times had undetectable factors, which indicated that dilution of at least two times was recommended. Three repeated tests were performed on each sample.

Spleen Lymphocyte Proliferation Analysis

Mice splenocytes were harvested after 4 weeks of the last treatment. Splenocytes were recovered as a single cell suspension in RPMI 1640 medium (Hyclone). Erythrocytes were removed from splenic suspensions by lysis in ACK Lysis Buffer (Gibco™) for 2 min at 37°C. After that, the 1,640 complete culture medium containing 10% fetal bovine serum was added, and the cells were inoculated into 96-well plates at the concentration of 1×10^5/ml. GAD65 specific peptides were added as antigen stimulation (final concentration 5 μg/ml); PMA (positive control, final concentration 5 μg/ml) was added as positive antigen control. After 48 h of stimulation, the 20 μl MTS (Promega) was added to each well at the last 4 h of incubation. The wavelength of 490 nm was used to measure light absorption value of each well by ELISA plate reader (UVP, California, USA).

Flow Cytometry Assay

Analysis of DC

Mice splenocytes were harvested after 4 weeks of the last treatment. Mice spleen lymphocytes were harvested and washed two times with PBS containing 2% BSA. Cells were then adjusted to the concentration of 1×10^6/ml and incubated for 48 h at 37°C and 5% CO_2 with 100 μl GAD65 peptides pool containing 5 μg/ml of each individual peptide as described previously. Cells were processed as described previously and stained with anti-CD11c-PE (N418) and anti-CD80-APC (16-10A1). After permeabilization, samples were stained with intracellular anti-IL10-FITC (JESS-16E3) fluorescent-labeled monoclonal antibody. After washing two times, samples were re-suspended with PBS and immediately analyzed on CytoFLEX S Flow Cytometer (BECKMAN COULTER, USA). The flow data was processed using FlowJo10.4 software.

Analysis of Treg

Mice spleen lymphocytes were harvested as previously described, and then adjusted to the concentration of 1×10^6/ml and incubated for 48 h at 37°C and 5% CO_2 with 100 μl GAD65 peptide pool containing 5 μg/ml of each individual peptide as described previously. After that, cells were stained with anti-mouse CD4-FITC (RM4-5), anti-mouse CD25-APC (PC61.5) antibodies. Then, the cells were fixed with Fix/Perm buffer (eBioscience, America), incubated in permeabilization buffer (eBioscience, America), and stained with anti-mouse/Rat-Foxp3 PE (FJK-16s) antibody. After washing two times, samples were re-suspended with PBS and immediately analyzed on CytoFLEX S Flow Cytometer (BECKMAN COULTER, USA) as previously described.

RNA Sequencing

We also provided insight into the mechanisms of Kyn and its influence on the immune response to immune cells. Mouse splenocytes were performed to assess the immune suppressive properties of kynurenine and to determine that the adjuvants used were indeed biologically active. Balb/C mice spleen lymphocytes (three mice in each group) were isolated as described above, after that they were stimulated for 12 h by Kyn (40 μg/ml), which performed at least three independent experiments. Splenocyte samples were collected after treatment, after which the total mRNA and microRNA libraries were prepared and sequenced. Total RNA was extracted using the RNAfast200 kit (Fastagen Biotech, Hefei, China) according to the manufacturer's protocol. The quality control of the isolated RNA (concentration, RIN, 28S/18S, and size) was performed with Agilent 4200 Bioanalyzer (Agilent Technologies, Santa Clara, USA). The strand-specific RNA-seq libraries were prepared using a NEBNext® Ultra™ I RNA Library Prep Kit for Illumina (NEB, MA, USA) following the manufacturer's instructions. The libraries were assessed on the Agilent Bioanalyzer 4200 system and sequenced on the Illumina Xten platform. RNA sequencing and reads alignment were performed by GMINIX Biotechnology Corporation (Shanghai, China). Reads were aligned to mouse genome version mm9. MicroRNA sequencing and RNA sequencing data are deposited at the Gene Expression Omnibus (GEO; accession number: GSE164304; GSE165737). Differentially expressed miRNAs/mRNAs were selected based on the following criteria: |log2 fold change| > = 1.2 and P-value <0.05.

Validation of Differentially Expressed miRNA and mRNA

The expression of the selected miRNAs was detected by the stem-loop qRT-PCR method. Total RNA was extracted to obtain cDNA by reverse transcription-PCR using GoScript Reverse Transcription System (Promega, USA) according to the manufacturer's protocol. The qPCR assay was performed in CFX96 Touch Real-Time PCR Detection System (BioRad, Berkeley, USA). The cycling parameters of qPCR reaction were as follows: 95°C for 5 min, then 95°C 10 s, and 60°C 30 s for 40 cycles (miRNAs); 95°C for 5 min, then 95°C 10 s, and 50°C 30 s for 35 cycles (mRNAs), followed by a melting curve to record the specific PCR product. In the qPCR experiment, the relative expressions were calculated using the 2–ΔΔCt method with GAPDH as an endogenous control for mRNA and U6 as an internal control for miRNA. Each reaction was conducted in triplicate. The corresponding primers were shown in **Supplementary Table 1**.

Statistical Analyses

Data are presented as means ± standard deviations (SDs), and statistical analyses were performed with professional statistical computer SPSS software. A p value of <0.05 was considered statistically significant.

RESULTS

The Effect of Kyn on Suppressed Hyperglycemia and Diabetes

To study the potential immunosuppressive adjuvant effects of Kyn, we immunized mice with the GAD65 phage vaccine premixed with the Kyn adjuvant. The immunization and sample collection schedule is shown in **Figure 1**. Blood glucose levels and body weight of NOD mice were detected at 6, 8, 10, 12, 14, 16, 18, and 20 weeks. Our results showed that (**Figures 2A, B**) subcutaneous administration of GAD65 vaccine, or GAD65 vaccine + Kyn, prevented the development of hyperglycemia in 50% (3/6) and 67% (4/6) of NOD mice at least one-month (from the 14th week to 18th week), respectively. Clinical diabetes was defined by hyperglycemia (blood glucose levels >10.3 mmol/l) in fasted animals (27); the protective effect began after the 14th week of initial hyperglycemia in our study. In contrast, no significant

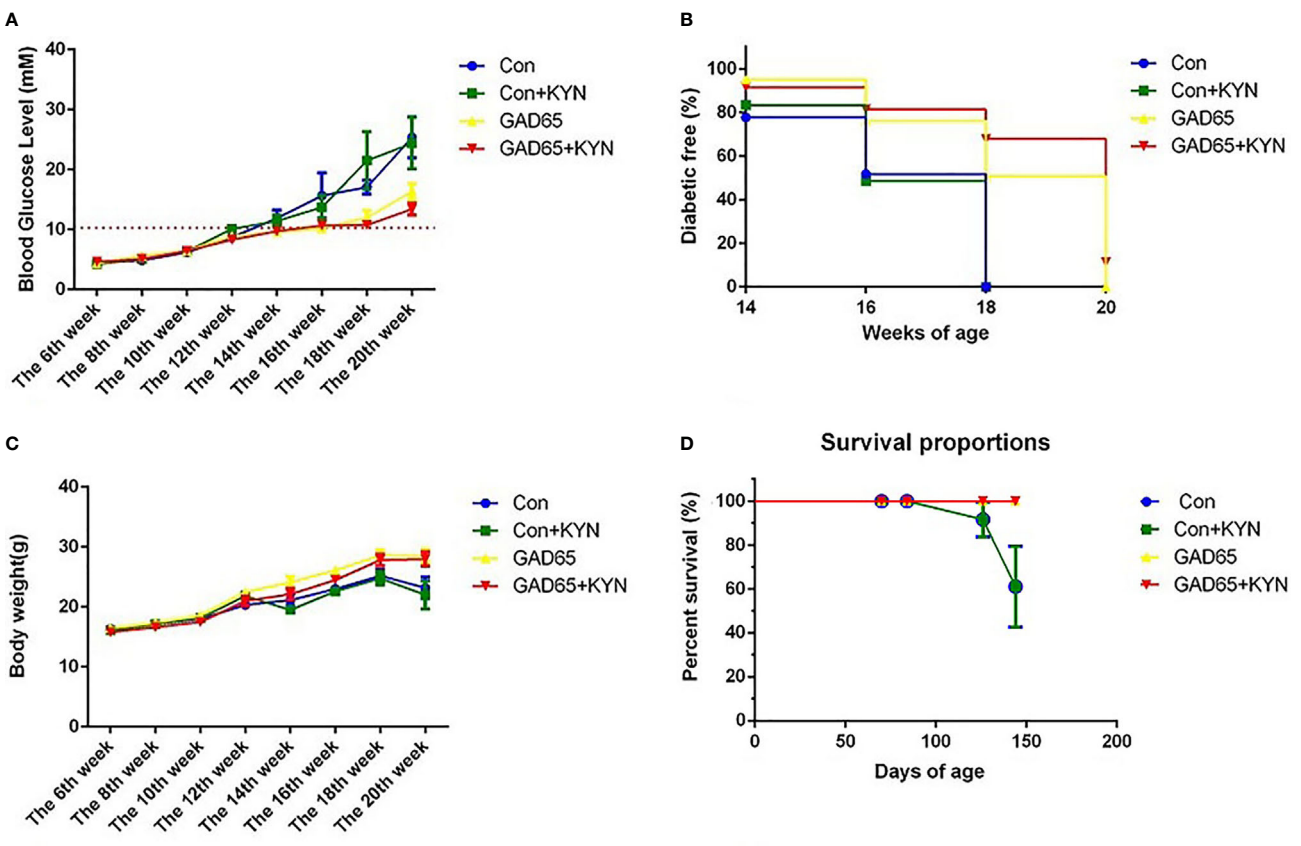

FIGURE 2 | NOD mice were immunized with phage vaccine with or without KYN on weeks 10 and 12. **(A, C)** Blood glucose/body weight was measured on weeks 6, 8, 10, 12, 14, 16, 18, and 20. The body weight of mice grew when treated with GAD65 phage vaccine with KYN. **(B)** Mice immunization of GAD65 phage vaccine with KYN were significantly remission from hyperglycemia in 60% at least one month. **(D)** The survival curves.

remission of hyperglycemia was observed in the control group and control + Kyn group. Weight loss is a significant symptom of T1D, and we found no changes in weight loss in GAD65 and GAD65 + Kyn groups. During the 144 days of observation period (**Figures 2C, D**), mice in control and control + Kyn groups had a mortality rate of 33.3% (2/6), while no death was observed in GAD65 and GAD65 + Kyn groups (mice were in good condition, weighing between 25 and 30 g). Thus, these results indicated that co-immunization of Kyn and GAD65 phage vaccine could significantly temporarily reverse diabetes in NOD mouse model.

The Effect of Kyn Shifted the Th1/Th2 Balance Toward Th2

Since the proportion of Th1/Th2 cells is not balanced and the immune response is skewed to Th1 in diabetic NOD mice and diabetic patients, it is important to examine whether Kyn can decrease GAD65-specific inflammatory profiles of T cells. Comparing the antibody responses generated by GAD65 phage immunizations with or without Kyn adjuvant, the IgG1 and IgG2a isotypes produced by both immunization ways were detected. DNA injection into the muscle can produce a Th1 type immune response, and mainly IgG2a antibodies in mouse models (28). Compared to the group immunized with GAD65 phage alone, a significantly enhanced level of IgG1 was obtained in the group immunized with GAD65 phage plus Kyn as an adjuvant. IgG1 anti-GAD65 antibodies resulted in ratios of IgG1 to IgG2a >1 that was indicative of Th2 polarization (**Figure 3**).

To further determine whether the immunosuppressive effect of Kyn on diabetes was mediated by Th2 cells, electrochemiluminescence assay was performed to examine the effects of Kyn on the generation of Th1 and Th2 cytokines. We detected the production of IFN-γ, IL2, IL-4, IL-10 cytokines associated with Th1 and Th2 responses in the serum of immunized NOD mice. Our results revealed that IL-4 and IL-10 (Th2 cytokines) were significantly up-regulated (**Figure 3**), while production of IL-2 and IFN-γ (Th1 cytokines) was significantly down-regulated in Kyn co-immunized mice with GAD65 phage vaccine compared with other groups, which is consistent with IgG isotype data. These results indicated that Kyn could enhance the GAD65 vaccination by shifting the Th1/Th2 balance toward Th2. We also found that the Tgfβ1 was increased when immunized with the adjuvant Kyn + GAD65 vaccine compared with other groups in NOD mice.

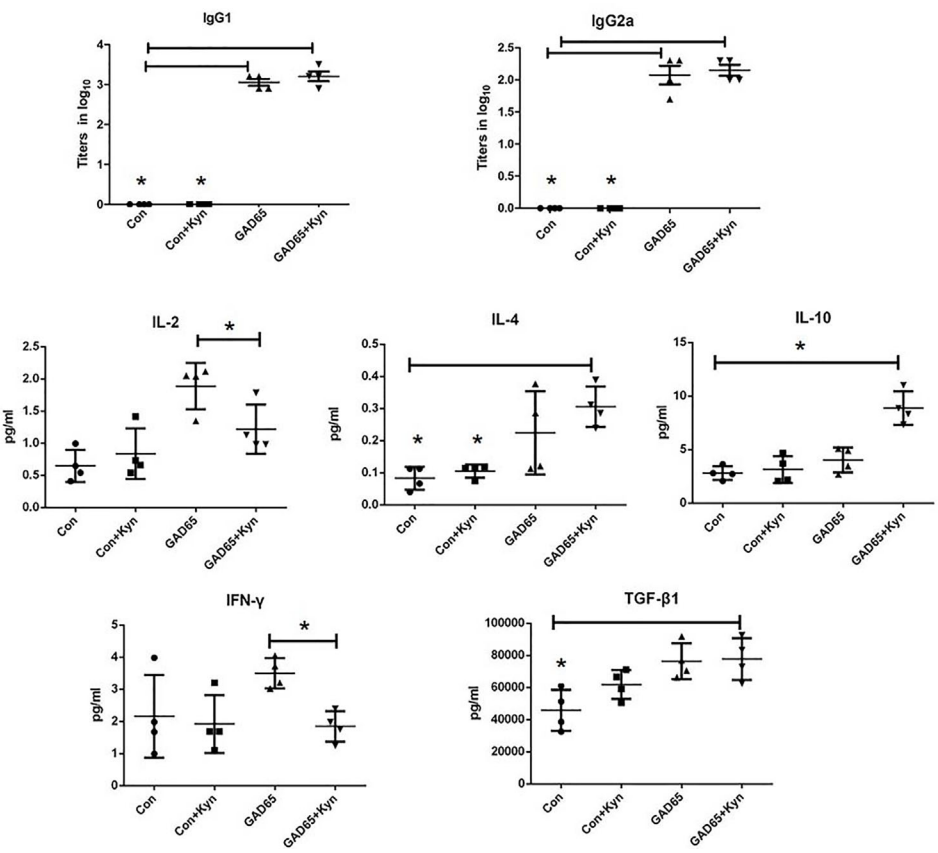

FIGURE 3 | Sera samples were collected to detect cytokines with MSD electochemiluminescence. GAD65 phage vaccine co-immunized with KYN generated significantly lower secretion of IL-2, IFN-γ than the GAD65 phage group did, and higher secretion of IL-10, IL-4, TGF-β1, than any other groups did (*P < 0.05).

GAD65 Phage Vaccine + Kyn Decreases GAD65-Specific T Cell Proliferation

In the case of T1D, auto-reactive T cells attack islet cells, most of which are insulin-specific cytotoxic $CD8^+$ T cells. To explore the potential underlying mechanisms of GAD65 phage vaccine + Kyn on diabetes, we measured the autoantigen-specific T cell activation and proliferation *in vivo* using T cell proliferation assay in response to GAD65. As shown in **Figure 4**, the proliferative response to GAD65 was significantly lower ($P < 0.05$) in spleen lymphocytes isolated from GAD65 phage vaccine + Kyn immunized mice than those from the GAD65 phage vaccine immunized mice. The proliferation was specific to GAD65 peptide since challenge with the control BSA peptide *in vitro* had no effect ($P < 0.05$). Our data demonstrated that GAD65 phage vaccine + Kyn vaccination could inhibit the proliferation of GAD65 auto-reactive T cells *in vivo*.

Kyn Can Suppress Dendritic Cell Maturation

DC promotes immunity and mediates T cell tolerance by direct elimination, Treg induction, or co-adjustment. Immune tolerance can be induced by adoptive transfer of immature or semi-mature DCs, or by self-antigen-presenting DCs under steady-state conditions (29). Twenty-eight days after the last immunization, single cells of the spleen lymphocyte were collected, stained with anti-CD11c-FITC, anti-CD80-APC, and anti-IL10-PE, and analyzed by fluorescence-activated cell sorting (FACS) to detect the mature state of DCs. As shown in **Figures 5A, B**, the proportion of mature DC cells ($CD11c^+CD80^+$) in the group immunized by Kyn + GAD65 was significantly lower than that in the group immunized by GAD65 alone, indicating that Kyn can inhibit the maturation of DC cells when stimulated by GAD65 peptides ($P < 0.05$).

We also detected the changes in IL-10 expression in DC cells (**Figures 5C, D**). The highest percentage of IL-10 expression in DC cells immunized by Kyn + GAD65 phage vaccine was 14.2%, which was significantly higher compared to other groups, indicating that Kyn could enhance the ability of DCs to express IL-10. At the same time, we also observed that Con + Kyn or GAD65 phage vaccine group could induce more secretion of IL10 in immature DC cells compared with the control group ($P < 0.05$). This result confirmed that GAD65, as an autoimmune antigen of T1D, can induce certain immune tolerance, and Kyn could enhance this tolerance effect.

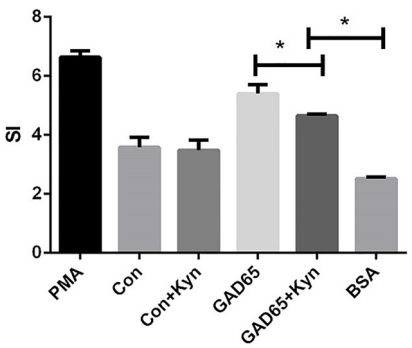

FIGURE 4 | Effects of suppressive adjuvant Kyn on the GAD65 specific T cell proliferation. The proliferative response to GAD65 was significantly lower in spleen lymphocytes isolated from GAD65 phage vaccine + Kyn immunized mice than those from the GAD65 phage vaccine immunized mice (*$P < 0.05$).

GAD65 Phage Vaccine + Kyn Increases $CD4^+CD25^+$ Treg Cells in the NOD Mouse Model

Treg is a subset of $CD4^+$ T cells which are characterized by the expression of transcription factor Foxp3 and helps to keep inflammation under control and lower the autoimmune disease risk in healthy individuals (30). They have been proved to maintain their regulatory functions for a long-term even in the absence of antigens that induced their generation and are stable and transferable (31), thereby permitting the expected induction of these cells to suppress unwanted immunity (13). In this study, we evaluated the effect of the Kyn co-immunized with GAD65 phage vaccine on the regulation of Treg cells in spleen lymphocytes. Spleen lymphocytes were isolated and re-stimulated in culture with the GAD65 peptides. The cells were then stained with anti-CD4-FITC, anti-CD25-APC, anti-FoxP3-PE and analyzed by fluorescence-activated cell sorting (FACS). As shown in **Figure 6**, with the administration of Kyn, the Kyn + GAD65 group produced a higher proportion of $CD4^+CD25^+Foxp3^+$ T cells in $CD4^+$ T cell populations compared with the GAD65 group ($P < 0.05$). A similar result was observed in the GAD65 group; the proportion of $CD4^+CD25^+Foxp3^+$ T cells in $CD4^+$ T cell populations was significantly improved compared with control or control + Kyn group, respectively ($P < 0.05$). These results indicated that GAD65, as an autoimmune antigen of T1D, could increase the proportions of $CD4^+CD25^+Foxp3^+$ T cells, and immunosuppressive adjuvant Kyn could specifically increase this enhanced effect, which may be associated with the suppression of T1D progression in the spleen lymphocytes.

Gene Expression Profile of Spleen Lymphocytes Stimulated by Kyn *In Vitro*

Analysis of miRNA Expression Profiles

RNA-sequencing technology uses ultra sequencing technologies to determine vaccine adjuvant transcriptomic profiles *in vivo* and *in vitro* (32–34). Modigliani Y et al., confirmed that miRNA expression has an important role in vaccination with aluminum adjuvant (32). In our study, 91 distinctly expressed miRNAs were identified between the Kyn stimulated group and the NC group (>1.2-fold change and $p < 0.05$ as screening criteria). Among them, 46 (50.5%) miRNAs were up-regulated, and 45 (49.5%) were down-regulated (**Supplementary Figure 2A**). The detailed information of differentially expressed miRNAs can be seen in **Supplementary Table 2**. Additionally, hierarchical clustering analysis showed the expression profile of 91 differentially expressed miRNAs in different treatment groups. The results indicated that these miRNAs were divided into two major

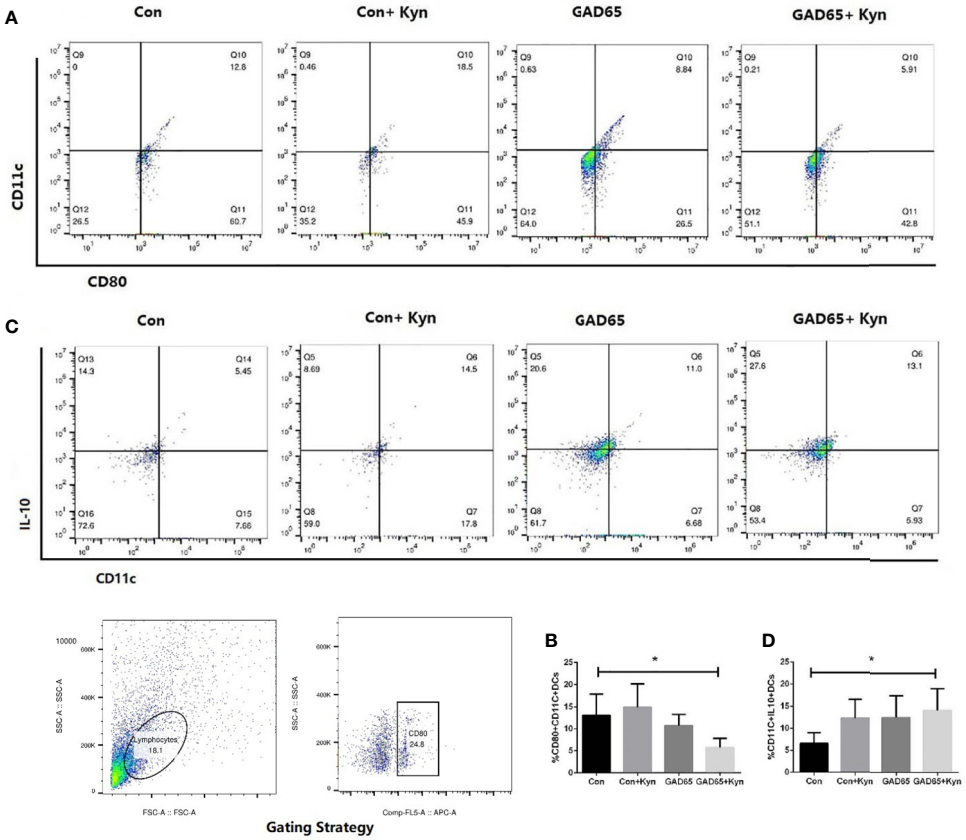

FIGURE 5 | Kyn suppressed mouse dendritic cell maturation. At the 16th week, mice spleen lymphocytes were stained and immediately analyzed on Flow Cytometer. Compared with GAD65 phage vaccine immunization alone, significantly lower percentages of $CD80^{+}CD11c^{+}$ DC cells were observed in GAD65 phage vaccine co-immunized with KYN, which suggested that KYN may suppress dendritic cell maturation **(A, B)**. At the same time, immatured-dendritic cells in the co-immunized group secreted more IL10 than the control group did **(C, D)** (*P < 0.05).

clusters, one representing the negative controls and one representing Kyn stimulated group. The data illustrated that these common expression patterns of miRNAs were significantly different from NC groups (**Supplementary Figure 2B**). Validation of expression levels for randomly selected miRNAs was analyzed using qRT-PCR (**Supplementary Figure 1A**); the results were consistent with the expression trends shown in the miRNA sequencing results for the selected miRNAs.

Analysis of mRNA Expression Profiles

Transcriptome sequencing was used to analyze the mRNA expression profiles of spleen lymphocytes stimulated by Kyn (>1.2-fold change and p<0.05 as screening criteria). After 12 h of stimulation, differentially expressed mRNAs matching these criteria were depicted in volcano maps (**Supplementary Figure 2C**). A total of 1,436 differentially expressed genes were significantly different between the Kyn stimulated group and the NC group (**Supplementary Table 3**). Among these differentially expressed mRNAs, 713(49.7%) were up-regulated and 723 (50.3%) were down-regulated. Additionally, cluster analysis indicated that the expression of differentially expressed mRNAs was significantly different between the Kyn-stimulated group and the NC group (**Supplementary Figure 2D**). Validation of these trends was performed on randomly selected mRNAs and was demonstrated using qRT-PCR (**Supplementary Figure 1B**).

miRNA Target Prediction and Integration of miRNA and mRNA Expression Profiles in Kyn-Stimulated Spleen Lymphocytes

Functional Analysis of Common Differentially Expressed miRNAs

To further study the role of the identified differentially expressed miRNAs, we first predicted the miRNA target genes *via* targetscan (http://www.targetscan.org/, mirnada (http://www.microrna.org/ and miRWalk (http://129.206.7.150/) databases. A total of 1,073 target mRNAs were predicted from the intersection of the three databases. These target mRNAs, which were predicted by the bioinformatics software and confirmed by transcriptome sequencing, were identified as the preliminarily putative target genes. Then, the preliminarily putative target genes that present a

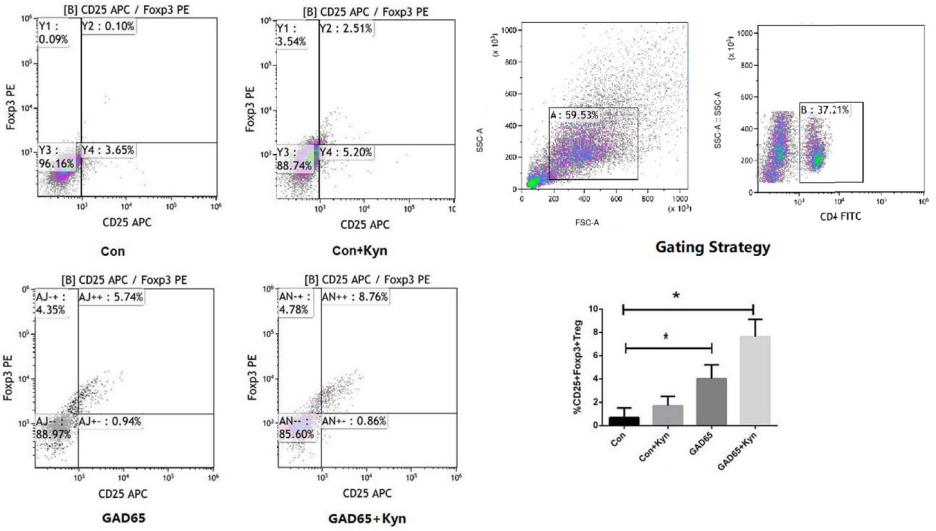

FIGURE 6 | Kyn induced $CD4^+CD25^+Foxp3^+$ Treg cells. At the 16th week, mice spleen lymphocytes were stained and immediately analyzed on Flow Cytometer. Notably, Kyn still strongly enhanced the percentages of $CD4^+CD25^+Foxp3^+$ Treg cells when co-immunized with GAD65 phage vaccine (*$P < 0.05$).

negative regulatory relationship between miRNA-target mRNAs were included. Finally, the differentially expressed target mRNAs that passed through these screen processes were subject to analysis using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). All Gene Ontology can be seen in **Supplementary Table 4**, the significant top 25 GO terms were described in **Figure 7**. Up gene GO analysis (**Figure 7A**) results showed that the five most-enriched terms are translation, cytoplasmic translation, negative regulation of transcription by RNA polymerase II, negative regulation of transcription, DNA-templated and positive regulation of transcription by RNA polymerase II. Down gene GO analysis (**Figure 7B**) results showed that the five most-enriched terms are regulation of transcription by RNA polymerase II, immune system process, defense response to virus, innate immune response and cellular response to interferon-beta. These data could provide evidence that miRNA potentially up-regulated GO is mostly involved in gene transcription, but down-regulated GO is mostly involved in immune response.

KEGG analysis of these target mRNAs illustrated that the putative target genes of the selected common miRNAs were enriched into 186 KEGG signaling pathways. Of these signaling pathways, 91 KEGG signaling pathways were up-regulated, and 95 were down-regulated. The top 25 signal pathways with the most enriched genes were depicted by bubbles (**Figure 8**). Among those signaling pathways **(Supplementary Table 5)**, all involved in the modulation of Kyn stimulated signal pathways, including metabolic pathways, FoxO signaling pathway, cytokine–cytokine receptor interaction, Toll-like receptor signaling pathway, NF-kappa B signaling pathway, TNF signaling pathway, MAPK signaling pathway, IL-17 signaling pathway, were enriched in both up and down pathways. Th17 cell differentiation, Th1 and Th2 cell differentiation, PI3K–Akt signaling pathways were significantly enriched in down pathways. In general, these pathways mostly focused on metabolic regulation and immune regulation. These 11 pathways were selected, and the miRNA–mRNA–pathway regulatory network was generated based on the previously selected miRNA-target mRNAs pairs (**Figure 9**). There were 39 differentially expressed miRNAs including 25 up-regulated miRNAs and 14 down-regulated miRNAs, and 58 differentially expressed mRNAs included 17 up-regulated mRNAs and 41 down-regulated mRNAs which were involved in these 11 pathways. In the miRNA–mRNA-pathway-net, we observed that some genes have an important regulatory effect in these pathways that up-regulated gene Gadd45a, Il12b, IL10 and down-regulated gene Igf1, Il1r1, PIK3R1 which were located in the center nodes (**Supplementary Figure 3**).

Functional Analysis of Specific Differentially Expressed miRNAs

After KEGG signaling pathway enrichment analysis, the miRNA–mRNA regulatory network map was generated in **Figure 10**. Bioinformatics analysis demonstrated that some specific miRNAs play a significant role in regulating the lymphocyte function when stimulated by Kyn. Among the up-regulated miRNAs with more predicted targets, mmu-miR6916-5p had 40 predicted targets, followed by mmu-miR674-5p and mmu-miR34a-5p with 33 and 22 predicted targets, respectively. There were few predictive targets for down-regulated miRNAs, although mmu-miR155-3p had 34 predictive targets. Consequently, our data could provide evidence that there is a close relationship between miRNA changes, host gene expression, and suppressive adjuvant effects.

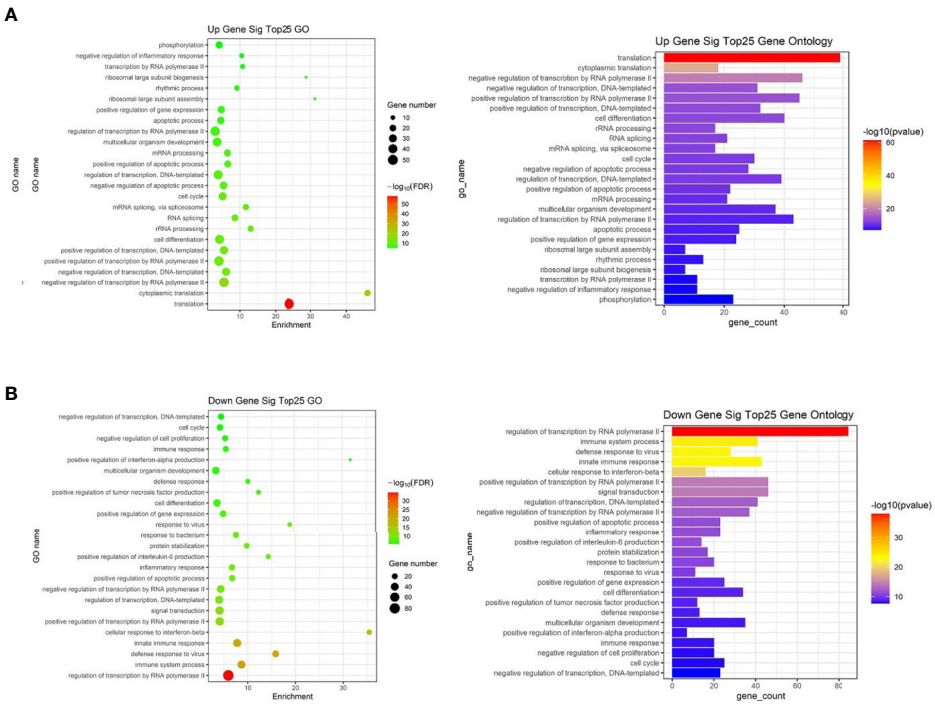

FIGURE 7 | The top 25 significantly enriched GO terms of the overlapping target genes of differentially expressed miRNAs in spleen lymphocytes of Balb/C mice (Kyn stimulated *vs* negative control). **(A)** Up gene Go analysis. **(B)** Down gene Go analysis.

DISCUSSION

Although animal model studies have demonstrated that GAD65 vaccination can be used to prevent autoimmune diabetes (35–39), the preventive efficacy needs to be further improved. Successful clinical treatment may be hindered by the uncertainty of major auto-antigens, an insufficient dosage of antigen and lack of an ideal adjuvant or low Treg induction to confer long time tolerance effects (40). In order to address these issues, we designed an immunosuppressant-kynurenine (Kyn), as adjuvant co-immunized with a phage-displayed vaccine containing the 190–320 amino acid sequences of GAD65 to induce Treg cells and tolerogenic responses and to prevent diabetes I disease in the NOD mouse model.

Our study indicated that subcutaneous administration of GAD65 phage vaccine + Kyn could prevent the development of hyperglycemia in 60% (4/6) of NOD mice for at least one month, which was highly effective for the suppression of T1D in NOD mice. Weight loss is a significant symptom of T1D. In our study, no significant changes in weight loss were observed in the GAD65 phage vaccine and GAD65 phage vaccine + Kyn groups. In addition, no death was observed in these two groups, and mice were in good condition (weighing between 25 and 30 g). This greater efficacy may be due to the combination of GAD65 antigen with the immunosuppressant-kynurenine, thus providing effective immunotherapy against T1D.

In T1D, GAD65 has been identified as a primary auto-antigen in the pancreas. Some studies (41–46) indicated that the administration of recombinant GAD65 protein or peptide in NOD mice can induce immune tolerance against pancreatic β cells and thus prevents or delays the development of insulitis and diabetes. Based on human clinical trials and animal studies (47), the proposed mechanism includes induction and proliferation of GAD65-specific Tregs, which down-regulate antigen-specific auto-reactive T cells and prevent them from attacking the pancreatic β-cells. Therefore, inducing the proliferation of antigen-specific regulatory T cells may be one of the most effective ways to prevent or treat autoimmune diseases.

The use of Tregs as a method to treat inflammation and autoimmune diseases has been proposed and well-received by scientists (30). Many clinical trials have proved that the use of vaccines to treat autoimmune diseases cannot effectively induce the production of antigen-specific Treg cells, thus inhibiting the role of pathological T cells. Consequently, it is necessary to add inhibitory adjuvants in the process of vaccine use. Our previous studies have shown that Kyn may be used as a suppressive adjuvant to reduce the immunogenicity of HAV, a TD antigen, *in vivo*, and LPS, a TI antigen, *in vitro* (48). As an endogenous ligand of AHR, Kyn can activate the AHR signaling pathway and may control immunity and autoimmunity while providing us with a new opportunity for autoimmune disease therapeutic. In this study, GAD65-specific $CD4^+CD25^+FoxP3^+$Treg cells were significantly increased in the GAD65 vaccine + Kyn group compared with the vaccine single immunization group.

We also discovered that the proportion of mature DC cells ($CD11c^+CD80^+$) was significantly lower in mice co-immunized

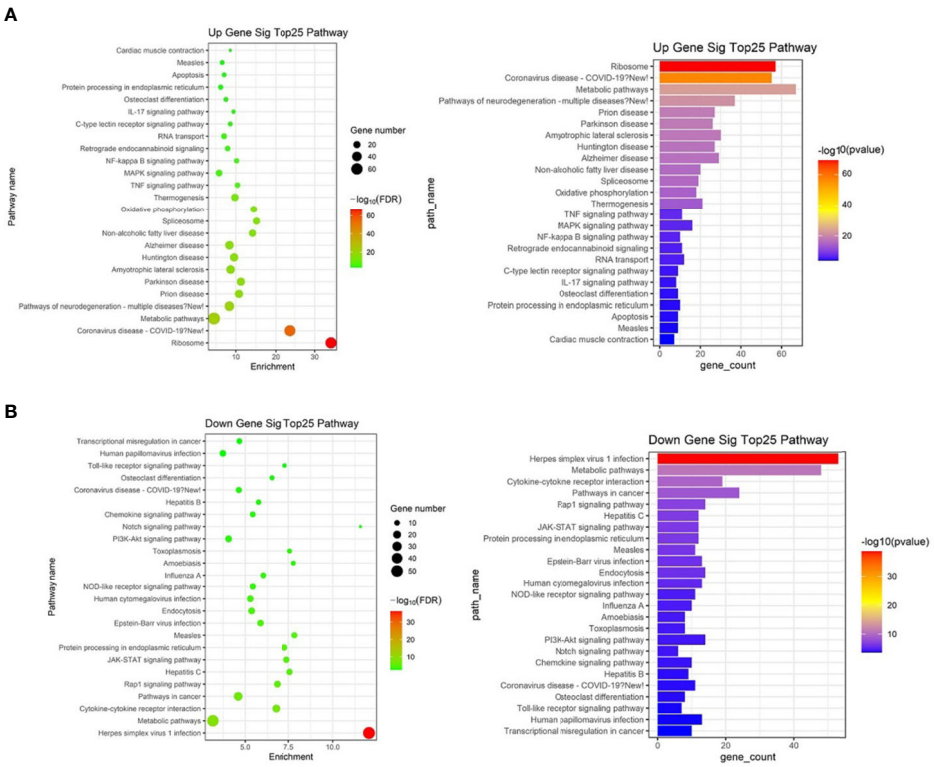

FIGURE 8 | The top 25 significantly enriched KEGG-pathway analyses of overlapped target genes of differentially expressed miRNAs in spleen lymphocytes of Balb/C mice (Kyn stimulated *vs* negative control). **(A)** Up gene KEGG analysis. **(B)** Down gene KEGG analysis.

with Kyn, indicating that Kyn as an adjuvant could inhibit the maturation of DC cells. The highest proportion of IL-10 expression in DC cells in the GAD65 co-immunization Kyn group was 14.2%, which was significantly higher than that in other groups, indicating that Kyn could enhance the ability of DC cells to express IL-10. The mature state of DC cells stimulated by antigen directly affected the production of the immune response. Under normal physiological conditions, the fully matured DCs secrete the pro-inflammatory cytokines, such as IL-1β, TNF, IL-12, and IL-6 (49). However, DCs can also perform the opposite function by making T cells tolerant against the autoantigen-directed immune response, which is necessary to reduce the autoimmune reactions. The immunogenic and tolerogenic functions of DCs depend on the balance between activating and inhibitory signals during DC maturation (50).

In T1D patients and NOD animal models, the skewed Th1/Th2 balance which leads to autoimmune destruction of the β-cells in the pancreas is a progressive phenomenon, resulting in the continual loss of these cells (51, 52). Our results showed higher IgG1 than IgG2a in both GAD65 vaccine and GAD65 vaccine + Kyn adjuvant groups, indicating that the vaccine treatment could induce Th2 type immune response in NOD mice, mainly regulating humoral immunity. In our study, protection from diabetes by Kyn + GAD65 phage immunization was also associated with a significant reduction of T cell proliferation and of IFN-γ secretion by T cells responding to GAD65. Additionally, an increase in the release of IL-10 and TGFβ1 was detected in Kyn + GAD65 phage vaccinated mice, indicating that Kyn + GAD65 phage immunization shifted the diabetogenic Th1 response to the regulatory Th2 response. IL-10 is a strong anti-inflammatory cytokine, which plays an important role in inhibiting Th1 cells by inhibiting the production of proinflammatory cytokines such as IFN-γ, IL-1 and TNFα. TGF-β, which induces immune tolerance and counteracts the immunostimulatory effects of checkpoint inhibitors, has an immunosuppressive effect (53). The secretion of IL10 and TGF-β and the production of Treg cells complement each other, which is the key factor for the feasible treatment of autoimmune diseases. T1D is related to the immune imbalance caused by excessive activation of Th1 cells and inhibition of Th2 cells. Therefore, whether T1D can be cured or not depends on whether the damaged Th1/Th2 balance can be effectively restored by immune regulation.

Our data suggested that Kyn can be used as an immunosuppressive adjuvant in autoimmune disease. Understanding how lymphocytes interact with adjuvants is crucial to understanding the mechanisms of this adjuvant and will be critical in the rational design of future vaccines against many diseases. In the present study, RNA and miRNA sequencing was performed in mouse spleen lymphocytes stimulated by Kyn. Compared with the negative control group, 91 common differentially expressed miRNAs and 1,436 common

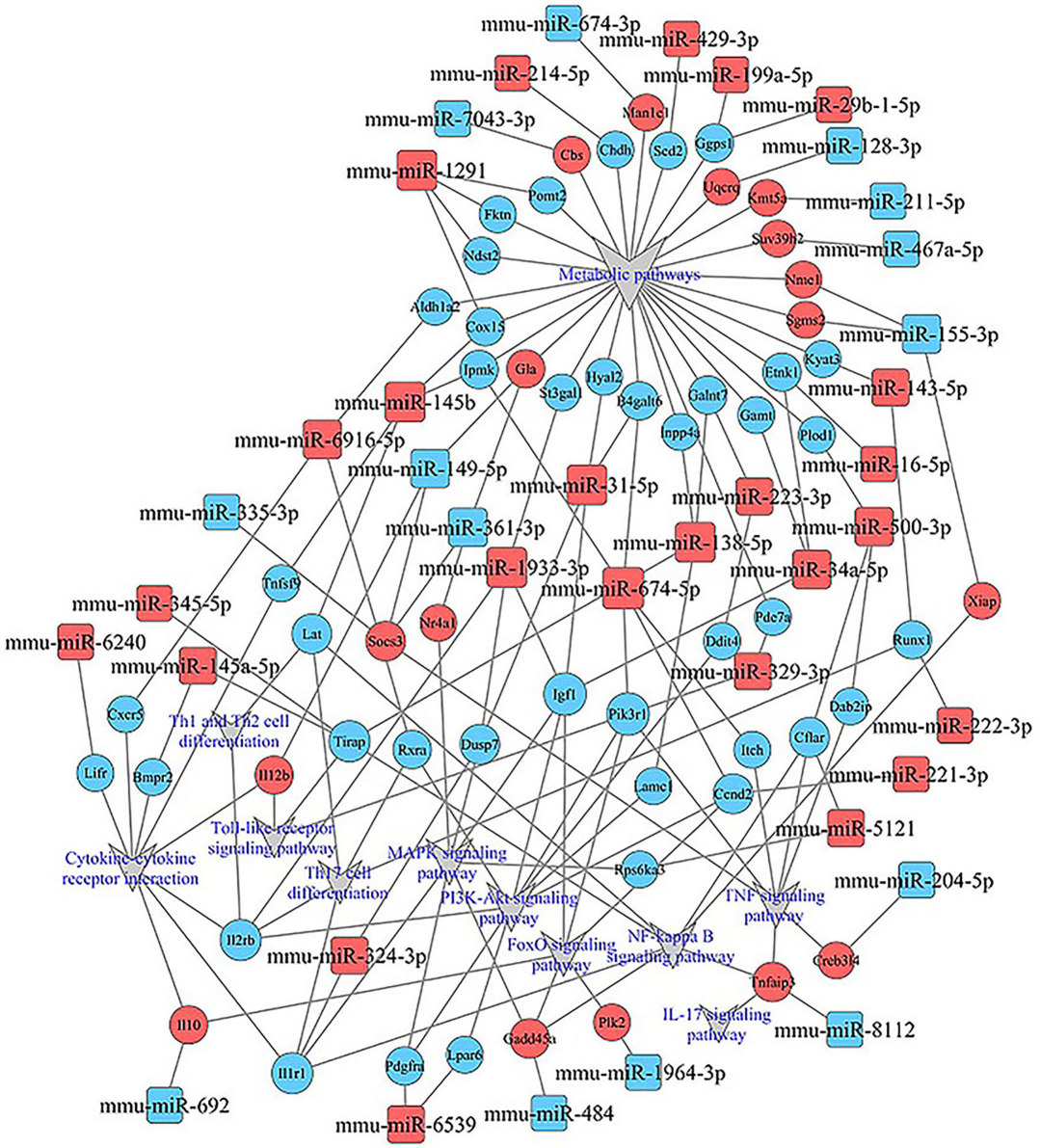

FIGURE 9 | miRNA–mRNA-pathway-net. Squares indicate identified miRNAs, while circles represent the corresponding target genes. Blue indicates down-regulated miRNAs or mRNAs while red indicates up-regulated miRNAs or mRNAs. The relationship between miRNAs and genes is shown connected by gray lines. Eleven significantly enriched pathways associated with splenocytes stimulated by Kyn were depicted using the gray triangles. More details about immune related miRNA–mRNA-pathway are shown in **Supplementary Figure 3**.

differentially expressed mRNAs were found in the Kyn stimulated group. The results of the GO analysis revealed that the up-regulated genes were mainly enriched in gene expression processes, such as mRNA processing, RNA splicing and translation. The down-regulated genes were primarily involved in immune-related biological processes, such as positive regulation of tumor necrosis factor production, interferon-alpha production, and IL-6 production. According to the KEGG-pathway analysis, both up-regulated and down-regulated genes play a crucial role in metabolic pathways and pathways related to immune regulatory function. This is in agreement with other studies in which a relationship between metabolic state and the differentiation status of innate and lymphoid cells (54).

Numerous studies (55) have demonstrated that AhR plays an important role in several normal physiological processes, including development of the vasculature, construction of the central nervous system, differentiation of blood cell subsets, and

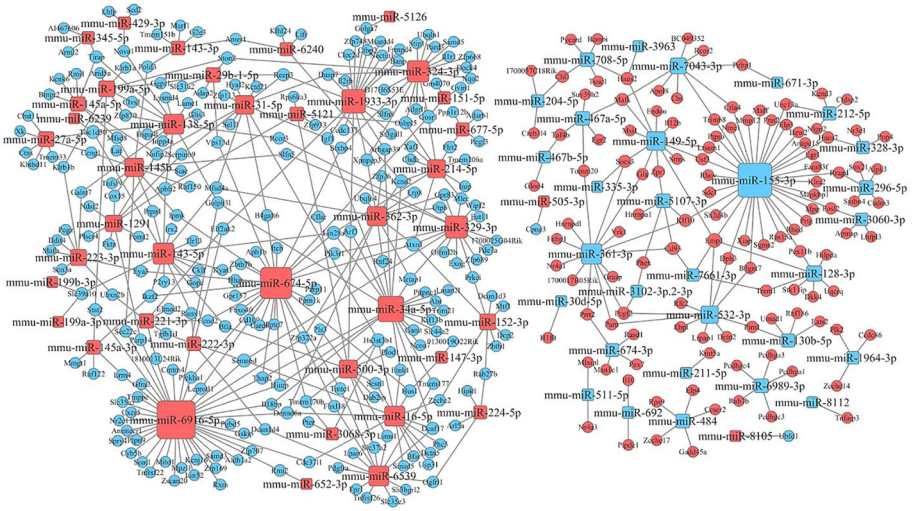

FIGURE 10 | miRNA–mRNA interaction network. The size of the point represents the regulatory capacity of a given miRNA. Squares indicate identified miRNAs, while circles represent the corresponding target genes. Blue indicates down-regulated miRNAs or mRNAs while red indicates up-regulated miRNAs or mRNAs. The relationship between miRNAs and genes is shown connected by gray lines.

the maintenance function of hepatocytes, adipocytes, and epithelial cells. Beyond that, the AhR plays a crucial role in the control of the adaptive immune response. It controls the differentiation and activity of specific T-cell subsets and influences adaptive immune responses by affecting both T cells and antigen presenting cells (APCs). Kyn is the first metabolite of tryptophan. As an endogenous substance, it activates the AHR in a ligand-receptor manner. In our study we found that Kyn can work as immunosuppressive adjuvant and mainly has a negative regulatory role in the immune related signaling pathway, offering plausible molecular mechanisms that may control immunity and autoimmunity. These findings provide us with new opportunities for targeted, therapeutic modulation of the immune response. Comparing the most significant up- or down-regulated genes, we observed that most of the genes are related to MAPK, PI3K-Akt, FoxO, and NF-kappa B signaling pathway (Gadd45a, Il12b, IL10, Igf1, Il1r1, PIK3R1). In these genes, PIK3R1 is located at the central node of miRNA–mRNA-pathway-net which indicated that it had a strong regulatory effect in the process of lymphocytes stimulated by Kyn. The immune system is highly sensitive to manipulation of PI3K signaling pathway. Some researchers (56) have observed that just a two-fold change in PI3K signal activity through Akt is sufficient to regulate lymphocyte homeostasis and induce autoimmunity in mice.

MicroRNAs (miRNAs) are increasingly being identified as key factors in the immune system regulating immune responses. Although miRNA regulation of each target results in only minor changes in gene expression, these small changes can be amplified by miRNA–mRNA-net to affect the cell behavior. These changes can be easily observed in the immune system, where miRNAs modulate many cells' ultimate fate by developing mature lymphocytes (57–59). Among the differentially expressed miRNAs, mmu-miR-329-3p (up-regulated) and mmu-miR-3066-3p (down-regulated) were most variable after stimulation with Kyn. The integrated analysis of miRNA and mRNA expression revealed that one miRNA targeting several mRNAs, mmu-miR6916-5p, mmu-miR674-5p, mmu-miR34a-5p and mmu-miR155-3p were shown to have more targets. There are no reports on the function of mmu-miR6916-5p, mmu-miR674-5p, and mmu-miR-3066-3p. MicroRNA-329-3p (miR-329-3p) has been studied in many types of human cancer (60). MiR-155 is one of the most studied miRNAs for its multiple roles in the control of the innate and adaptive immune processes. Several studies (61) demonstrated that miR-155 controls differentiation of $CD4^+$ T cells into the T helper cell subsets (Th1, Th2 and Th17) (62–64) and that it affects the development of Tregs (65, 66). MiR-155 also regulates $CD8^+$ T cells (67, 68) and is vital for normal B cell differentiation and antibody production (63, 64, 69). MiR-155 over-expression can enhance the anti-viral, as well as anti-tumor $CD8^+$ T cell responses *in vivo* (61). On the other hand, reducing the expression of miR-155 may cause downstream cascades and increase the tendency to generate Th2 cells which secrete type 2 (IL-4, IL-5 and IL-10) cytokines (70). Therefore, fully understanding vaccine factors that influence immune response has important implications. It helps direct and rationally design new and more efficacious vaccines or adjuvants with better immunogenicity and safety profiles (32).

To sum up, our data demonstrated that kynurenine, as an immunosuppressive adjuvant, can successfully help the phage vaccine to induce immune tolerance in NOD mice, thus reducing the symptoms of diabetes. As a physiological substance *in vivo*, kynurenine has superior safety as an adjuvant than other exogenous substances in theory. We believe that kynurenine may be used as a novel immunosuppressive adjuvant in autoimmune disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, GSE164304 https://www.ncbi.nlm.nih.gov/, GSE165737.

ETHICS STATEMENT

The animal study was reviewed and approved by Institute of Medical Biology, Chinese Academy institutional animal care and conducted.

AUTHOR CONTRIBUTIONS

JS and YH designed the experiments. JS, JDS, JL, MW, SJ, XW, and ZL performed the experiments. JS and JDS analyzed data. JS wrote the manuscript. YL, CM, and NH provided important analysis tools. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.681328/full#supplementary-material

Supplementary Figure 1 | qRT-PCR results. **(A)** MircoRNAs **(B)** mRNAs.

Supplementary Figure 2 | Analysis of microRNA/mRNA expression profiles in spleen lymphocytes of Balb/C mice stimulated with Kyn *in vitro* compared with the negative control group (NC). **(A, C)** Volcano plots show the differentially expressed miRNAs/mRNAs. **(B, D)** Hierarchical clustering presents the correlation of different samples.

Supplementary Figure 3 | Immune related miRNA–mRNA-pathway. Global analysis of immune related miRNA and mRNA which were enriched in both up and down pathways.

Supplementary Table 1 | The primers sequence used for q-PCR.

Supplementary Table 2 | Differentially expressed miRNA result.

Supplementary Table 3 | Differentially expressed mRNA result.

Supplementary Table 4 | Gene Ontology analysis for the target gene of differentially expressed miRNA.

Supplementary Table 5 | KEGG pathway analysis for the target gene of differentially expressed miRNA.

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A Single L/D-Substitution at Q4 of the mInsA$_{2-10}$ Epitope Prevents Type 1 Diabetes in Humanized NOD Mice

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Autoreactive CD8$^+$ T cells play an indispensable key role in the destruction of pancreatic islet β-cells and the initiation of type 1 diabetes (T1D). Insulin is an essential β-cell autoantigen in T1D. An HLA-A*0201-restricted epitope of insulin A chain (mInsA$_{2-10}$) is an immunodominant ligand for autoreactive CD8$^+$ T cells in NOD.*β2m^{null}.HHD* mice. Altered peptide ligands (APLs) carrying amino acid substitutions at T cell receptor (TCR) contact positions within an epitope are potential to modulate autoimmune responses *via* triggering altered TCR signaling. Here, we used a molecular simulation strategy to guide the generation of APL candidates by substitution of L-amino acids with D-amino acids at potential TCR contact residues (positions 4 and 6) of mInsA$_{2-10}$, named mInsA$_{2-10}$DQ4 and mInsA$_{2-10}$DC6, respectively. We found that administration of mInsA$_{2-10}$DQ4, but not DC6, significantly suppressed the development of T1D in NOD.*β2m^{null}.HHD* mice. Mechanistically, treatment with mInsA$_{2-10}$DQ4 not only notably eliminated mInsA$_{2-10}$ autoreactive CD8$^+$ T cell responses but also prevented the infiltration of CD4$^+$ T and CD8$^+$ T cells, as well as the inflammatory responses in the pancreas of NOD.*β2m^{null}.HHD* mice. This study provides a new strategy for the development of APL vaccines for T1D prevention.

Keywords: type 1 diabetes, altered peptide ligand, D-amino acid substitution, mInsA$_{2-10}$, NOD.*β2m^{null}.HHD* mice

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INTRODUCTION

Type 1 diabetes (T1D) is a spontaneous organ-specific autoimmune disease characterized by T cell-mediated elimination of insulin-producing pancreatic islet β-cells. Given the close association between major histocompatibility complex (MHC) class II molecules and type 1 diabetes has been early found (1), β-cell autoreactive CD4$^+$ T cells were the most intensively studied in both humans and NOD mice (2). However, the importance of β-cell autoantigen-specific CD8$^+$ T cells in the pathogenesis of type 1 diabetes has been heightened by multiple studies in NOD mice (3–5). Either CD8α or β2-microglobulin-deficient NOD mice do not develop diabetes (6). Similarly, diabetes does not occur in NOD mice depleted of CD8$^+$ T cells by antibody treatment (7). Human leukocyte

antigen-A*0201 (HLA-A*0201), one of the most commonly expressed MHC class I allele in Caucasians and Asians (50%), has been also indicated to contribute to the susceptibility to T1D (8). NOD.$\beta2m^{null}$.*HHD* mice, carrying human HLA-A*0201 but no murine MHC class I molecules, show significantly accelerated T1D onset (8). Thus, induction of β-cell autoreactive $CD8^+$ T-cell tolerance has been considered as a promising approach for the prevention of T1D (9).

Insulin is a primary β-cell autoantigen that initiates spontaneous T1D in both NOD mice and human (10), so induction of insulin-autoreactive T-cell tolerance can lead to prevention of T1D (11). Multiple $CD8^+$ T cell epitopes of insulin such as A chain 2-10 ($mInsA_{2\text{-}10}$) and B chain 5-14 ($mInsB_{5\text{-}14}$), which are identified to be HLA-A*0201-restricted and immunodominant in NOD.$\beta2m^{null}$.*HHD* mice with high potential T1D relevance, represent important candidates of $CD8^+$ T cell targets in human T1D patients.

Altered peptide ligands (APLs) carrying amino acid substitutions at T cell receptor (TCR) or MHC contact positions can trigger altered TCR signaling events and are suggested as useful tools to modulate autoimmune responses. For examples, administration of APLs of a known immunodominant $CD8^+$ T cell epitope with partial agonist activity (12) or nanoparticles coated with APL-MHCs complexes (13) has been shown to effectively induce autoreactive $CD8^+$ T cell tolerance and prevent T1D in NOD mice. We recently found that repeated treatment of APL of $mInsB_{5\text{–}14}$ with histidine to phenylalanine substitution at the potential TCR contact site (p6) prevents T1D *via* selectively expanding a tiny population of $CD8^+CD25^+Foxp3^+$ regulatory T cells in humanized NOD mice (14).

The introduction of D-amino acids into the sequence of peptides is widely used to improve the stability and prevent peptides from proteolytic degradation (15). On the other hand, scanning with D-amino acid has been previously exploited for molecular analysis of MHC binding or/and TCR interacting residues within a T cell epitope, since the corresponding single D-amino acid substitution minimizes the influence of charges of each original residue as well as the size and molecular weight of the native peptide (16–18). Although these limited earlier studies have shown that T cell priming capacity of a T cell epitope can be altered by introducing D-amino acid at selected residues, whether APLs derived from D-amino acid substitution in TCR contact residues of native peptide are suitable for prevention of autoimmune diseases has been rarely considered. Here, we designed two APL candidates by *in silico*-assisted substitution of L-amino acids with D-amino acids at positions 4 and 6 of $mInsA_{2\text{-}10}$, respectively, which are in close contact with the TCR and potentially important for the recognition and response of specific T cells. We found that these two APLs, $mInsA_{2\text{-}10}$DQ4 and DC6, significantly inhibited the native $mInsA_{2\text{-}10}$ peptide-induced proliferation of splenocytes from NOD.$\beta2m^{null}$.*HHD* mice *in vitro*. However, *in vivo* administration of $mInsA_{2\text{-}10}$DQ4 but not DC6 notably reduced the insulitis and effectively delayed the development of T1D in NOD.$\beta2m^{null}$.*HHD* mice. Mechanistically, systemic treatment with $mInsA_{2\text{-}10}$DQ4 blinded autoreactive $CD8^+$ T cell responses toward to $mInsA_{2\text{-}10}$ and reduced the infiltration of both autoreactive $CD4^+$ T and $CD8^+$ T cells into the pancreas in NOD.$\beta2m^{null}$.*HHD* mice.

MATERIALS AND METHODS

Mice

NOD.$\beta2m^{null}$.*HHD* mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were bred and maintained in specific pathogen-free facilities and handled according to "Principles of Laboratory Animal Care and Use in Research" (Ministry of Health, Beijing, China). All experimental protocols were approved by the Animal Ethics Committee of the Army Medical University (Third Military Medical University).

Blood Glucose Monitoring

Blood glucose was monitored using a glucometer (OneTouch Ultral; LifeScan, Milpitas, CA, USA) at weekly intervals, beginning at 10 weeks of age. Diabetes was defined as two consecutive blood glucose values above 11.1 mM.

Epitope Modification and Molecular Dynamics Simulation

We constructed the HLA-A*0201/$mInsA_{2\text{-}10}$(IVDQCCTSI) complex based on the crystal structure (PDB ID: 3MRE) by mutating of epitope, which was used as template to build other complexes by replacing the L-amino acid of $mInsA_{2\text{-}10}$ with the corresponding D-amino acid at the assigned position (listed in **Table 1**). Subsequently, the epitopes complexed to HLA-A*0201 were put into a cubic box with 0.15 M NaCl solution and kept the ensemble neutral, then 120 ns (100 ns for HLA-A*0201/epitope) molecular dynamics simulation was performed with ff14SB force filed by AMBER 16 (19, 20). The root means square deviation (RMSD) of backbone (C_α, C, N, O of main chain) between sampling and initial conformation of HLA-A*0201 and epitopes was used to monitor the states of MD simulation. The ensemble reached the stable state while the RMSD was fluctuating at any

TABLE 1 | The distance (P1–P9), average hydrogen bonds, and binding energy of epitopes.

Epitope	Sequence	Distance (Å, P1-P9)	HBonds (average)	Binding Energy (kcal/mol)
				MHC/Epitope
$mInsA_{2\text{-}10}$	IVDQCCTSI	23.52	12	–4986.95
$mInsA_{2\text{-}10}$DQ4	IVD(D-Gln)CCTSI	23.43	6	–4974.66
$mInsA_{2\text{-}10}$DC6	IVDQC(D-Cys)TSI	19.81	6	–4616.46

number for a long time (more than 20 ns), then the MD simulation was ended. The binding free energy between epitopes (included $mInsA_{2-10}$) and HLA-A*0201 was calculated with molecular mechanics/Poisson Boltzmann surface area (MM/PBSA) method for the stable conformations extracted from last 20 ns trajectory, which were used to evaluate their potential affinity (21). Sequentially, we averaged the number of hydrogen bonds between epitopes and HLA-A*0201 and the solvent accessible surface area (SASA) of epitope with the LCPO model for stable conformations (22).

Peptides and Mice Treatment

Synthetic peptides $mInsA_{2-10}$ (IVDQCCTSI), $OVA_{257-264}$ (SIINFEKL), HIV $pol_{476-484}$ (ILKEPVHGV), $mInsA_{2-10}$DC6 (IVDQC(D-Cys)TSI), and $mInsA_{2-10}$DQ4 (IVD(D-Gln)CCTSI) were synthesized with purity >95% at Chinese Peptide Company (Hangzhou, China). Cohorts of 4-week-old female NOD.$\beta2m^{null}$.*HHD* mice were intraperitoneally injected with 100 μg (1 μg/μl) in PBS, and this procedure was repeated every week until the sixth injection.

Histology

Pancreatic tissues from 12-week-old non-diabetic female NOD.$\beta2m^{null}$.*HHD* mice immunized with different peptides (10 mice per group) were fixed in 10% neutral-buffered formalin, and the paraffin-embedded samples were stained with hematoxylin and eosin (H&E). A minimum of 10 islets from each mouse were microscopically observed by two different observers, and insulitis scoring was performed according to the following criteria: 0, no infiltration; 1, peri-insulitis; 2, insulitis with <50% islet area infiltrated; 3, insulitis with >50% islet area infiltrated.

HLA-A*0201 Binding Assay

T2 cells (1×10^6 cells/ml) were incubated with each peptide (50 μg/ml) in serum-free RPMI 1640 medium supplemented with 3 μg/ml β-2-microglobulin (Sigma-Aldrich, St. Louis, MO, USA) for 16 h at 37°C. Then cells were washed and stained with anti-HLA-A2 mAb BB7.2 (purified in-house from the hybridoma obtained from ATCC), followed by incubation with FITC-conjugated goat anti-mouse IgG (Beyotime, Jiangsu, China), and analyzed using an FACSAria™ instrument (BD Bioscience, Franklin Lakes, NJ, USA).

Mouse IFN-γ ELISPOT Assays

ELISPOT plates were precoated with anti-mouse mAb (MabTech, Stockholm, Sweden) overnight at 4°C, and blocked with RPMI 1640 plus 10% FBS (HyClone Corp., Logan, UT, USA). $CD8^+$ T cells were purified from splenocytes of 12-week-old non-diabetic female NOD.$\beta2m^{null}$.*HHD* mice treated with or without different peptides using the EasySep mouse $CD8^+$ T cell isolation kit (StemCell Technologies, Vancouver, Canada). Purified $CD8^+$ T cells (purity>90%, 2×10^5 cells/well) were incubated with each peptide (50 μg/ml)-pulsed T2 cells (1×10^4 cells/well) for 24 h at 37°C. After incubation, cells were removed and plates were processed according to the IFN-γ ELISPOT kit (MabTech) manufacturer's instructions. Spots were counted using a spot reader system (Saizhi, Beijing, China).

Proliferation Assay

Splenocytes (1×10^6 cells/ml) freshly isolated from 12-week-old non-diabetic female NOD.$\beta2m^{null}$.*HHD* mice were co-cultured with 10 μg/ml indicated peptides and 10 U/ml recombinant murine interleukin 2 (rmIL-2; Peprotech, Rocky Hill, NJ, USA), followed by twice weekly rmIL-2. After incubation at 37°C for 72 h, [^{3}H] thymidine (1 μCi/well) was added for an additional 16 h of culture, and uptake of [^{3}H] thymidine was determined using a liquid scintillation counter (Beckman Coulter, Brea, CA, USA).

Real-Time RT-PCR

Pancreatic biopsy samples (n=6) from indicated peptide-treated NOD.$\beta2m^{null}$.*HHD* mice at 12 weeks of age were lysed in Trizol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted according to the manufacturer's instruction. About 500 ng of total RNA was used for reverse transcription using a PrimeScript® RT reagent Kit (TaKaRa, Shiga, Japan) in a volume of 10 μl, and products were detected using a SYBR® Premix Ex Tap™ Kit (TaKaRa). Data were collected and quantitatively analyzed on an M×3000 P Real-Time PCR System (Stratagene, Austin, TX, USA). Values were normalized using β-actin as an endogenous internal standard. The sequences used were as follows: mouse IL-6, Sense 5'-TAGTCCTTCC TACCCCAATTTCC-3', Anti-sense 5'-TTGGTCCTTAGCC ACTCCTTC-3'; mouse IL-1β, Sense 5'-GCAACTGTT CCTGAACTCAACT-3', Anti-sense 5'-ATCTTTTGGGGTCCG TCAACT-3'; mouse TNF-α, Sense 5'-CACGCTCTTCTGTC TACTGAAC-3', Anti-sense 5'-ATCTGAGTGTGAGGGTCT GG-3'; mouse IFN-γ, Sense 5'-TCAAGTGGCATAGATGTGG AAG-3', Anti-sense 5'-CGCTTATGTTGTTGCTGATGG-3'; mouse IL-17, Sense 5'- ATCTGTGTCTCTGATGCTGTTG-3', Anti-sense 5'- AACGGTTGAGGTAGTCTGAGG-3'; mouse β-actin, Sense 5'-GAGACCTTCAACACCCCAGC-3', Anti-sense 5'-ATGTCACGCACGATTTCCC-3'.

Isolation of Pancreas-Infiltration Cells

Mice were euthanized and systemically perfused by injection of physiological saline into the left heart ventricle. After removal of all visible pancreatic lymph nodes, the pancreases were cut into tiny pieces and then digested in HBSS containing 1 mg/ml collagenase IV and 1.25 μg/ml DNase (Sigma), by shaking (200 rpm) at 37°C for 15 min. Single-cell suspensions were collected after diluting the enzyme with ice-cold HBSS containing 2% FCS. Aggregates were further digested with 0.5 mg/ml collagenase IV and 1 μg/ml DNase for 10 min and 0.25 mg/ml collagenase IV and 1 μg/ml DNase for 6 min. Single-cell suspensions were washed three times, and pancreas-infiltration cells were isolated by percoll density-gradient centrifugation according to the manufacturer's instructions, and then resuspended in medium.

Flow Cytometry

Single-cell suspensions were made from spleens and pancreas-infiltrating immune cells of 12-week-old non-diabetic female NOD.$\beta2m^{null}$.*HHD* mice immunized with different peptides and resuspended in RPMI 1640 medium. Fluorochrome-conjugated

antibodies specific for surface markers used in this study were anti-CD3-FITC (145-2C11), anti-CD4-PE (GK1.5), and anti-CD8-PerCP-Cy5.5 (53-6.7) (eBiosciences, San Diego, CA, USA). Events were collected on the BD Acurri C6 flow cytometer and analyzed with FlowJo software.

Statistical Analyses

Paired t-test was used to compare autoreactive $CD8^+$ T cell responses to control peptide or $mInsA_{2\text{-}10}$ of an individual within a certain treatment group. A non-parametric ANOVA (Kruskal-Wallis test) followed by a Dunn's test was performed to analyze differences among the groups, and a log-rank test was used to assess the cumulative incidence of diabetes. Other statistical analyses were conducted by the two-tailed Student's t-test. $P<0.05$ were considered statistically significant.

RESULTS

The Treatment of Native $mInsA_{2\text{-}10}$ Epitope Fails to Prevent the Development of T1D in NOD.$\beta2m^{null}$.*HDD* Mice

We firstly verified the immunodominance of HLA-A*0201-restricted native $mInsA_{2\text{-}10}$ peptide in 12-week-old non-diabetic female NOD.$\beta2m^{null}$.*HHD* mice. As expected, IFN-γ ELISPOT analysis revealed that potent $CD8^+$ T-cell responses against $mInsA_{2\text{-}10}$ were indeed present in 12-week-old non-diabetic female NOD.$\beta2m^{null}$.*HHD* mice (**Figures 1A, B**). So we questioned whether systemic administration of the native $mInsA_{2\text{-}10}$ peptide could protect from T1D in NOD.$\beta2m^{null}$.*HHD* mice. However, $mInsA_{2\text{-}10}$ peptide showed no protective activity (**Figure 1C**), and this encouraged us to explore the APLs of $mInsA_{2\text{-}10}$ peptide with antidiabetic activity.

In Silico Rational Design of APLs of $mInsA_{2\text{-}10}$ Epitope With a Single D-amino Acid Substitution

To define the potential TCR contact residues in $mInsA_{2\text{-}10}$, we constructed the HLA-A*0201/$mInsA_{2\text{-}10}$(IVDQCCTSI) complex based on the crystal structure (PDB ID: 3MRE) by *in silico* replacing of epitope. **Figure 2A** showed amino acid residues at positions 4 and 6 were bulged out of the binding groove and potentially important for the interaction with TCR. Therefore, we generated two APL candidates *via in silico*-assisted replacing L-amino acid of $mInsA_{2\text{-}10}$ at positions 4 and 6 by the corresponding D-isomer, respectively. Then, 120 ns MD simulation for the complex of HLA-A*0201/each peptide was

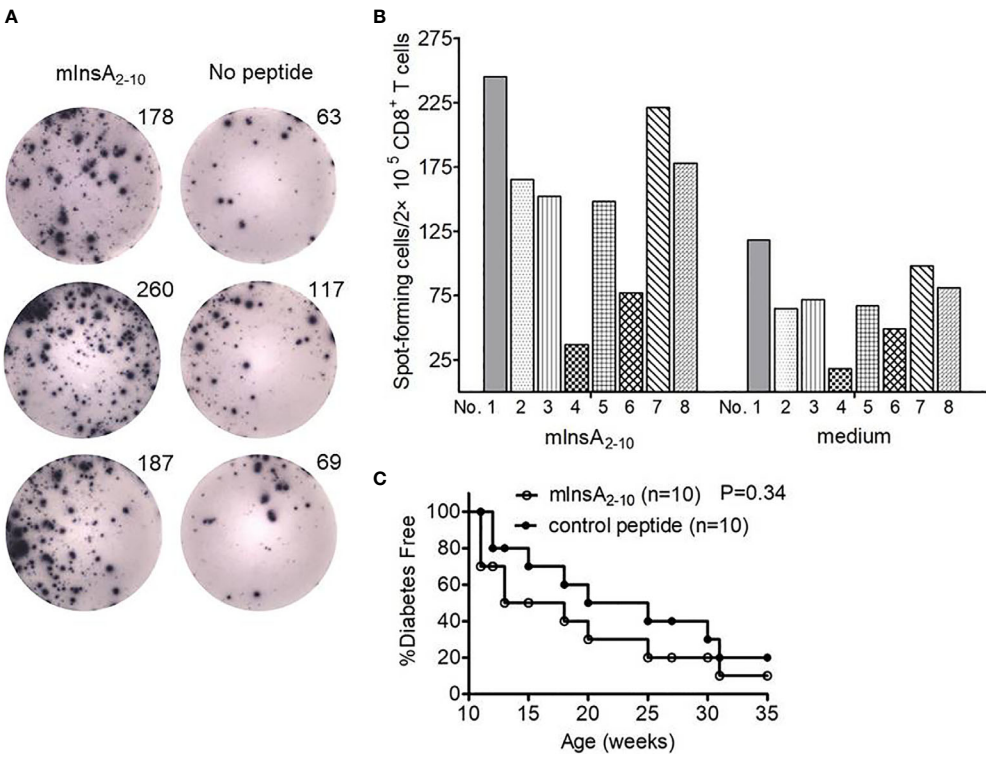

FIGURE 1 | The $mInsA_{2\text{-}10}$ epitope treatment could not prevent the development of T1D in NOD.$\beta2m^{null}$.*HHD* mice. **(A)** Representative IFN-γ ELISPOT assay demonstrated splenic $CD8^+$ T cell responses to T2 cell-loaded $mInsA_{2\text{-}10}$ (10 μg/ml), or no peptide in 12-week-old non-diabetic female NOD.$\beta2m^{null}$.*HHD* mice. **(B)** The average number of peptide-specific IFN-γ-positive spots per 2×10^5 splenic $CD8^+$ T cells in triplicate cultures was calculated for each indicated peptides (10 μg/ml) or no peptide from eight separate experiments. SDs are not shown to avoid excessive visual clutter. **(C)** Percentage of female NOD.$\beta2m^{null}$.*HHD* mice developing diabetes after 100 μg $mInsA_{2\text{-}10}$ i.p. weekly injections from 4 to 9 weeks of age (white circles, n = 10) *vs.* mice that were treated with $OVA_{257\text{-}264}$ (black circles, n = 10).

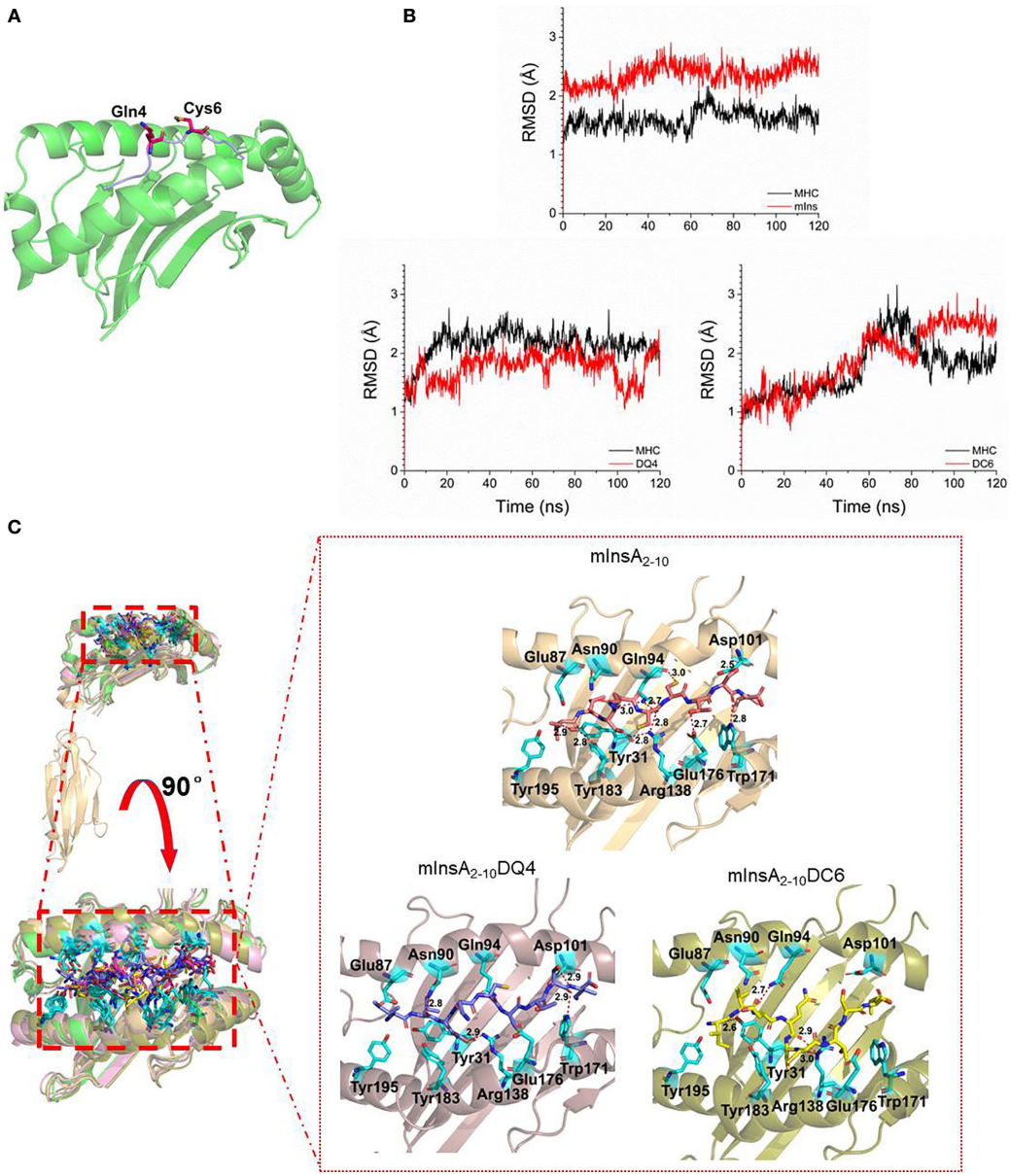

FIGURE 2 | Design of a potential antagonist peptide for $mInsA_{2\text{-}10}$. **(A)** The modeled structure of the HLA-A*0201/$mInsA_{2\text{-}10}$ complex showed that residues Gln 4 and Cys 6 bulged out of the binding groove, which were the potential TCR contact residues. **(B)** The RMSD of simulation of complex HLA-A*0201/epitope, the stable conformation for these three complexes were achieved through 120 ns MD simulation, and the RMSD fluctuated near 2Å. **(C)** The binding mode between HLA-A*0201 and epitopes, these epitopes bound to HLA-A*0201 through stronger hydrogen bonds, but $mInsA_{2\text{-}10}$ had the most hydrogen bonds.

performed, and the average structures (200 conformations) for the last 20 ns MD simulation were used to analyze their binding mode. The distance (C_α) between amino acids at positions 1 and 9, RMSD, binding energy, and the number of hydrogen bonds between each peptide and HLA-A*0201 (**Table 1**), as well as the solvent accessible surface area (**Table 2**) and the conformation comparison between APL candidates and $mInsA_{2\text{-}10}$, were analyzed. The RMSD plot showed that both HLA-A*0201 and $mInsA_{2\text{-}10}$ remained stable after 80 ns MD simulation, with the fluctuation around 1.8 Å. While HLA-A*0201 molecule complexed with APL DQ4 and DC6 reached stable after 90 ns (**Figures 2B, C**) shows $mInsA_{2\text{-}10}$ may stably bind to HLA-A*0201 through strong hydrophilic interaction. Notably, the predicted binding free energy of $mInsA_{2\text{-}10}$DQ4 to HLA-A*0201 was very similar to that of $mInsA_{2\text{-}10}$ to HLA-A*0201. Whereas, the predicted binding free energy between DC6 and HLA-A*0201

TABLE 2 | The solvent accessible surface area (SASA) of residues of epitopes.

Epitope	P1	P2	P3	P4	P5	P6	P7	P8	P9
$mInsA_{2-10}$	−51.63	−1.04	2.33	122.81	40.35	49.28	55.88	63.21	29.50
$mInsA_{2-10}$DQ4	−23.04	11.34	16.96	135.49	64.11	53.20	42.59	62.30	59.90
$mInsA_{2-10}$DC6	−48.99	2.16	87.05	56.82	−1.53	61.45	6.53	82.70	80.73

P_i is position i.

was increased (**Table 1**), indicating that the binding strength between DC6 and HLA-A* 0201 might be weakened. Thus, these results suggested that the substitution of D amino acid at position 4 rather than position 6 might have minor effect on the binding ability of peptide to HLA-A*0201 molecule.

In Vitro Analysis of the T-Cell Stimulating Potency of the Selected APL Candidates of $mInsA_{2-10}$

The binding affinity of the native peptide $mInsA_{2-10}$ and the two APLs for HLA-A*0201 molecule was evaluated *in vitro* using a T2-cell-peptide binding test. Both $mInsA_{2-10}$ and $mInsA_{2-10}$ DQ4 showed a strong HLA-A*0201-binding affinity similar to the positive control $HIVpol_{476-484}$. Whereas $mInsA_{2-10}$DC6 showed a relatively weaker binding affinity to HLA-A*0201 (**Figures 3A, B**). These results confirmed that the substitution of D-amino acid at position 4 rather than position 6 did not change the binding ability of APL to HLA-A*0201. To assess whether $mInsA_{2-10}$DQ4 and DC6 displayed any stimulating or inhibiting activity toward $mInsA_{2-10}$-reactive T cell population, the proliferation of splenocytes from 12-week-old non-diabetic female NOD.$\beta 2m^{null}$.*HHD* mice was tested upon the stimulation with $mInsA_{2-10}$ alone or plus each APL. When compared to the stimulation with $mInsA_{2-10}$ alone, the proliferation of splenocytes decreased significantly upon stimulation with $mInsA_{2-10}$ plus either $mInsA_{2-10}$ DQ4 or DC6 (**Figure 3C**). Thus, these preliminary data indicated that both $mInsA_{2-10}$DQ4 and DC6 displayed inhibitory effects on $mInsA_{2-10}$-stimulated lymphocyte proliferation *in vitro*.

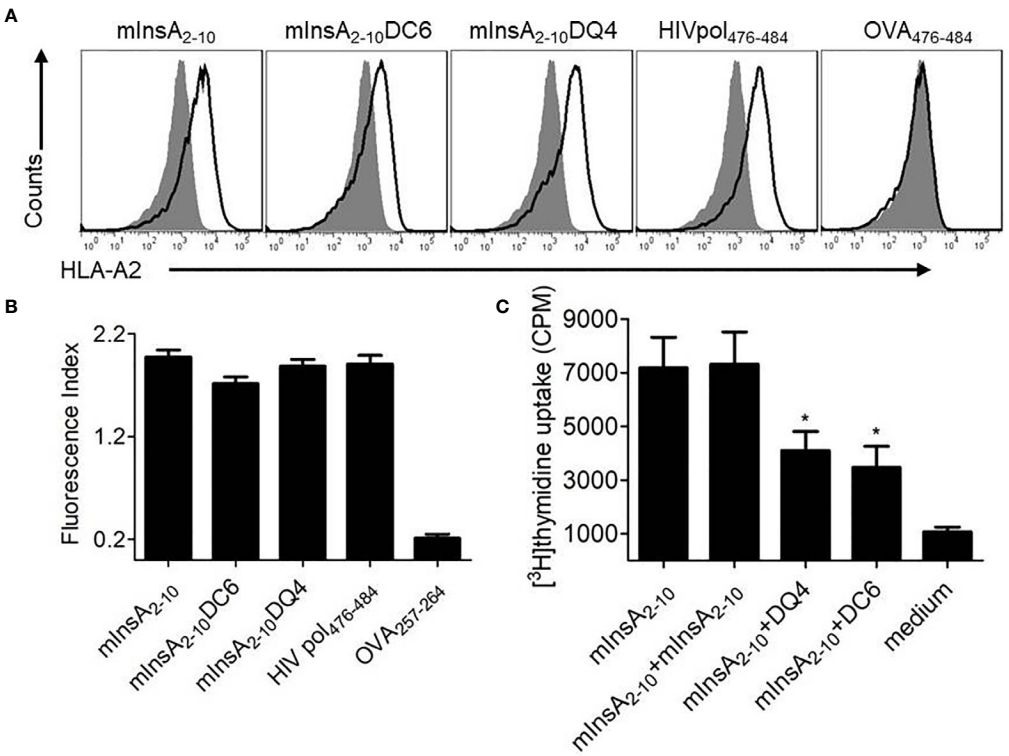

FIGURE 3 | Selection of a promising antagonist peptide for $mInsA_{2-10}$. **(A)** T2 cells were incubated with or without the indicated peptides (50 μg/ml), and the stabilization of surface HLA-A2 molecules was detected by flow cytometry. The HLA-A*0201-binding peptide HIV $Pol_{476-484}$ was used as a positive control. The H-$2K^b$-binding peptide $OVA_{257-264}$ was used as a negative control. Filled histograms, no peptide; open histograms, plus peptide. **(B)** The binding affinity was presented as the fluorescent index (FI) that was calculated as follows: FI = (mean fluorescence intensity with the given peptide – mean fluorescence intensity without peptide)/(mean fluorescence intensity without peptide). Bars represent the mean ± SEM of three independent experiments. **(C)** Splenocytes were treated with 10 μg/ml $mInsA_{2-10}$ plus 10 μg/ml indicated peptides for 72 h in 96-well plates (2×10^5 per well) at 37°C. The cell proliferation was measured by [^{3}H] thymidine incorporation. Bars represent the mean ± SEM of seven independent experiments. *P < 0.05.

The Treatment of mInsA$_{2\text{-}10}$DQ4 Reduces Insulitis and Prevents the Development of T1D in NOD.$\beta 2m^{null}$.*HHD* Mice

To investigate whether the two APLs had antidiabetic activity *in vivo*, female NOD.$\beta 2m^{null}$.*HHD* mice were injected intraperitoneally with soluble peptide mInsA$_{2\text{-}10}$, mInsA$_{2\text{-}10}$DC6, mInsA$_{2\text{-}10}$DQ4, or control peptide (OVA$_{257\text{-}264}$) in PBS, respectively, starting at 4 weeks of age. Consistently, native mInsA$_{2\text{-}10}$ peptide treatment had no protective effect on T1D. Interestingly, administration of mInsA$_{2\text{-}10}$DQ4, but not DC6, significantly suppressed the development of T1D compared with the control group (p=0.0086) (**Figure 4A**). Histopathological analysis of mInsA$_{2\text{-}10}$DQ4-treated female NOD.$\beta 2m^{null}$.*HHD* mice at 12 weeks showed less insulitis, compared to age-matched non-diabetic NOD.$\beta 2m^{null}$.*HHD* mice of mInsA$_{2\text{-}10}$, mInsA$_{2\text{-}10}$DC6, or control peptide treatment (**Figures 4B, C**). These results indicated that the treatment of mInsA$_{2\text{-}10}$DQ4 reduces insulitis and prevents the development of T1D in NOD.$\beta 2m^{null}$.*HHD* mice.

The Treatment of mInsA$_{2\text{-}10}$DQ4 Results in Loss of mInsA$_{2\text{-}10}$ Autoreactive CD8$^+$ T Cell Responses in NOD.$\beta 2m^{null}$.*HHD* Mice

To further determine whether mInsA$_{2\text{-}10}$DQ4 treatment could induce mInsA$_{2\text{-}10}$-specific CD8$^+$ T cell tolerance *in vivo*, we analyzed mInsA$_{2\text{-}10}$-pulsed T2 cells stimulated IFN-γ spots forming by purified splenic CD8$^+$ T cells from NOD.$\beta 2m^{null}$.*HHD* mice treated with mInsA$_{2\text{-}10}$DQ4, mInsA$_{2\text{-}10}$, or control peptide. As shown in **Figures 5A** ,**B**, when compared with control peptide-loaded T2 cells, the stimulation of T2 cells pulsed with mInsA$_{2\text{-}10}$ significantly increased IFN-γ spot formation of splenic CD8$^+$ T cells in both control peptide and mInsA$_{2\text{-}10}$-treated NOD.$\beta 2m^{null}$.*HHD* mice. In contrast, T2 cells pulsed with mInsA$_{2\text{-}10}$ failed to increase IFN-γ spot formation of splenic CD8$^+$ T cells in mInsA$_{2\text{-}10}$DQ4-treated NOD.$\beta 2m^{null}$.*HHD* mice. These results together indicated that treatment with mInsA$_{2\text{-}10}$DQ4 blinded peripheral mInsA$_{2\text{-}10}$-autoreactive CD8$^+$ T cell responses in NOD.$\beta 2m^{null}$.*HHD* mice.

The Treatment of mInsA$_{2\text{-}10}$DQ4 Reduces the Infiltration of CD4$^+$ T and CD8$^+$ T Cells and Inflammatory Responses in the Pancreas of NOD.$\beta 2m^{null}$.*HHD* Mice

Since insulin has been considered as an only essential autoantigen to initiate spontaneous T1D and the elimination of insulin-reactive T cells can block epitopes expansion and subsequent destruction of β cells by other autoreactive T cells in NOD mice, we questioned whether the treatment of mInsA$_{2\text{-}10}$DQ4 prevented the infiltration of CD4$^+$ and CD8$^+$ T cells and inflammatory responses in the pancreas of *NOD.*$\beta 2m^{null}$.*HHD* mice. Strikingly, both the frequency and

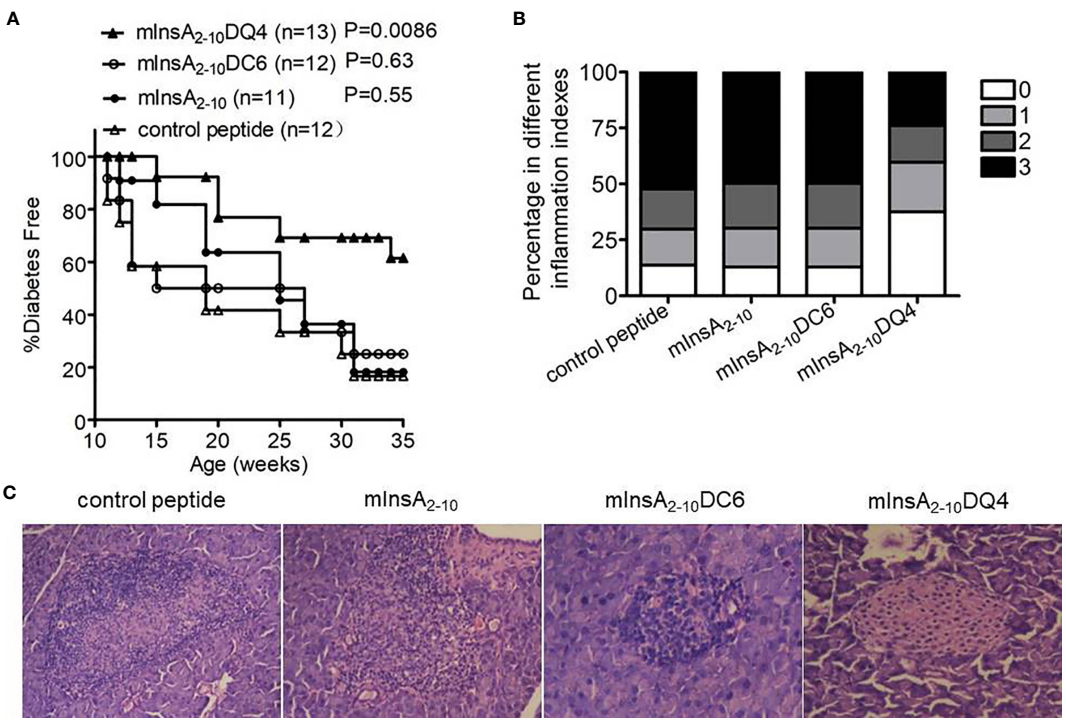

FIGURE 4 | The treatment with mInsA$_{2\text{-}10}$DQ4 prevented diabetes development in NOD.$\beta 2m^{null}$.*HHD* mice. NOD.$\beta 2m^{null}$.*HHD* female mice were injected intraperitoneally (once weekly) at 4–9 weeks of age with 100 μg of peptide mInsA$_{2\text{-}10}$, mInsA$_{2\text{-}10}$DQ4, mInsA$_{2\text{-}10}$DC6, or OVA$_{257\text{-}264}$. **(A)** Mice were monitored for diabetes development. **(B)** Histopathological evaluation of pancreatic sections from indicated peptide-treated NOD.$\beta 2m^{null}$.*HHD* mice at 12 weeks of age. **(C)** Representative hematoxylin and eosin–stained paraffin-embedded pancreas sections (200 × magnification) are shown.

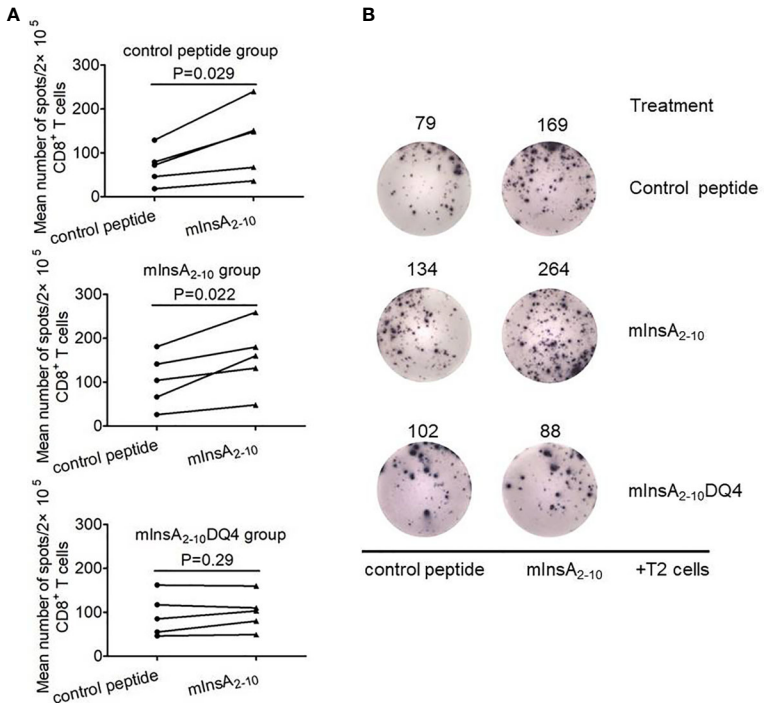

FIGURE 5 | mInsA$_{2\text{-}10}$DQ4 treatment resulted in loss of mInsA$_{2\text{-}10}$ autoreactive CD8$^+$ T cell responses. **(A)** Statistical analysis of the average number of control peptide or mInsA$_{2\text{-}10}$-specific IFN-γ-positive spots per 2×10^5 splenic CD8$^+$ T cells in triplicate cultures with a treatment group (n = 5) are shown. Significance was determined by a paired *t*-test. **(B)** Representative IFN-γ ELISPOT assay demonstrated splenic CD8$^+$ T cell responses to T2 cell loaded with mInsA$_{2\text{-}10}$ in each peptide-treated group of non-diabetic NOD.*β2m^{null}.HHD* mice at 12 weeks of age.

absolute number of pancreas-infiltrating CD4$^+$ T and CD8$^+$ T cells were markedly reduced in mInsA$_{2\text{-}10}$DQ4-treated NOD.*β2m^{null}.HHD* mice (CD4$^+$ T cells, 18.5 ± 1.7%, 12,757.4 ± 2,556.6; CD8$^+$ T cells, 1.6 ± 0.3%, 1,172.2 ± 206.1) compared with those detected in mInsA$_{2\text{-}10}$-treated (CD4$^+$ T cells, 29.3 ± 4.0%, 24,215.2 ± 2,994.9; CD8$^+$ T cells, 3.3 ± 0.4%, 2,005.8 ± 326.6) or control peptide-treated (CD4$^+$ T cells, 29.2 ± 5.7%, 23,309.2 ± 2,171.7; CD8$^+$ T cells, 3.2 ± 0.5%, 2,015.2 ± 314.6) NOD.*β2m^{null}.HHD* mice (**Figures 6A, B**). As expected, the mRNA levels of the proinflammatory cytokines IL-6, IL-1β, TNF-α, IFN-γ, and IL-17 significantly decreased in the pancreas of mInsA$_{2\text{-}10}$DQ4-treated NOD.*β2m^{null}.HHD* mice, when compared with NOD.*β2m^{null}.HHD* mice that received mInsA$_{2\text{-}10}$ or control peptide injection (**Figure 6C**). Thus, these data suggested that the treatment of mInsA$_{2\text{-}10}$DQ4 reduces the aggregation of autoreactive CD4$^+$ T and CD8$^+$ T cells and inflammatory responses in the pancreas of NOD.*β2m^{null}.HHD* mice.

DISCUSSION

T1D in both NOD mice and humans is an organ-specific autoimmune disease resulting from selective damage of pancreatic β cells by autoreactive T lymphocytes. Autoreactive CD8$^+$ T cells, which play an indispensable key role in initiation and progression of T1D, are recommended as ideal targets for the prevention of T1D. In this study, we generated an APL, mInsA$_{2\text{-}10}$DQ4, derived from D-amino acid substitution at a potential TCR contact site of an HLA-A*0201-restricted immunodominant insulin epitope. Treatment with mInsA$_{2\text{-}10}$DQ4 significantly suppressed the development of T1D in NOD.*β2m^{null}.HHD* mice *via* inducing mInsA$_{2\text{-}10}$ autoreactive CD8$^+$ T cell tolerance and preventing the subsequent infiltration of CD4$^+$ T and CD8$^+$ T cells as well as inflammatory responses in the pancreas in NOD.*β2m^{null}.HHD* mice.

It has been reported that the modification of peptides with D-amino acids instead of natural L-amino acids can not only preserve the feasible recognition properties but also significantly improve the stability and half-life of peptides (23, 24). Most of the available literatures reported that the replacement of all L-amino acid residue in T cell epitope by their D-enantiomers results in normal or reversed (retro) amide linkage (25, 26). A recent study reported that a retro-inverso-D-amino acid-based insulin B-chain (9–23) peptide blocked the presentation of native InsB$_{9\text{-}23}$ by HLA-DQ8 to autoreactive T-cells, suggesting that D-amino acid peptides may be an innovative treatment for T1D (27). Whereas, relatively few studies have been reported on a single amino acid substitution by the respective D-enantiomer in T cell epitope. For example, the individual substitutions by the corresponding D-amino acid at most positions within an I-E^d-restricted 13-mer snake toxin epitope greatly diminished its T-cell stimulating activity (17). A modified H-2D^d-restricted epitope of HIV-1 IIIB envelope

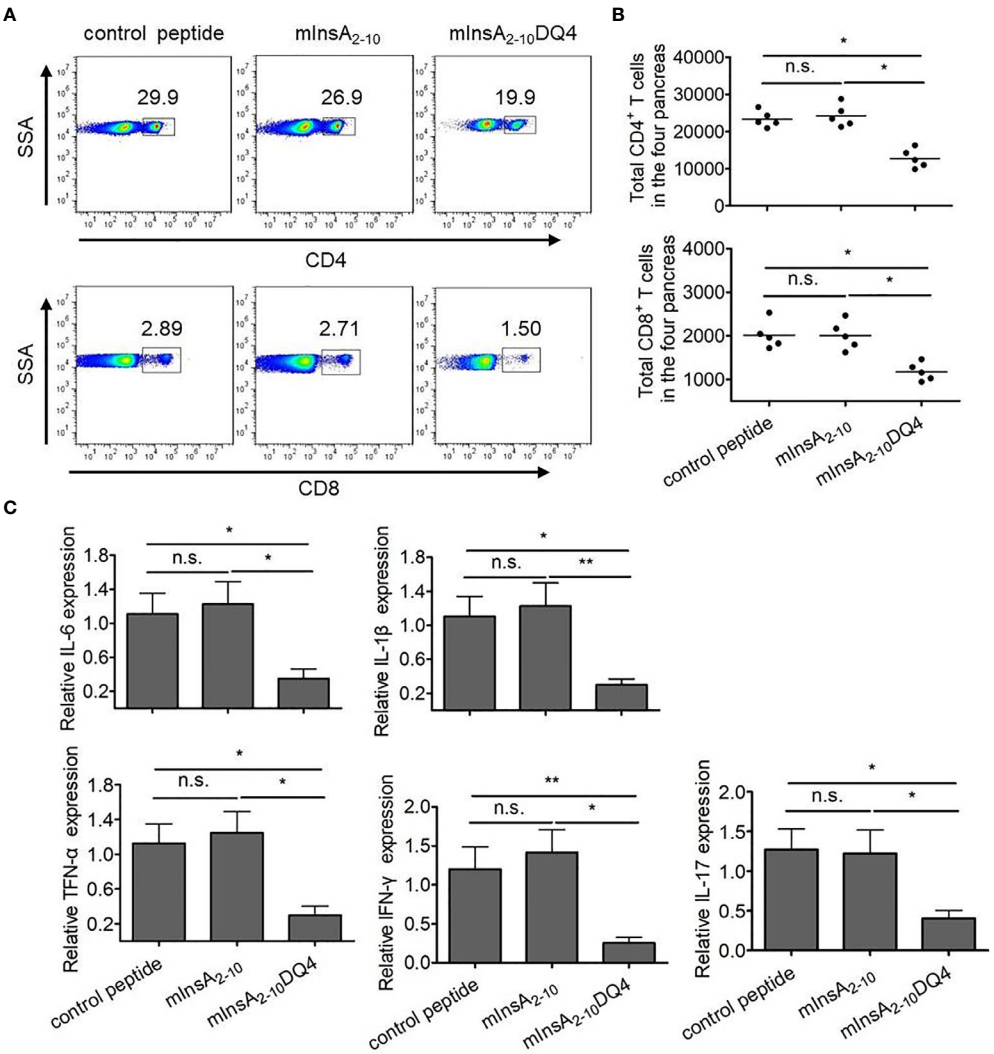

FIGURE 6 | Treatment with mInsA$_{2\text{-}10}$DQ4 notably reduced frequencies and absolute numbers of pancreas-infiltrating CD4$^+$ T and CD8$^+$ T cells in NOD.*β2m^{null}.HHD* mice. **(A)** Representative fluorescence-activated cell sorting (FACS) histogram plots for pancreas-infiltrating CD4$^+$ T and CD8$^+$ T cells from four mice. **(B)** The results for absolute numbers of these pancreas-infiltrating CD4$^+$ T and CD8$^+$ T cells are expressed as the mean ± SD. Each symbol in **(B)** represents a sample of pooled pancreas-infiltrating CD4$^+$ T and CD8$^+$ T cells from four mice. **(C)** Total RNA was extracted from the pancreas in each peptide-treated group of non-diabetic mice (n = 6). The mRNA expression levels of these cytokines were quantified by real-time RT-PCR. The data are presented as fold-change compared to the mRNA levels expressed in pancreas from control peptide-treated mice. *P < 0.05 and **P < 0.01. n.s. indicates no significance.

glycoprotein P18-I10 (RGPGRAFVTI) with a single amino acid substitution by the respective D-enantiomer at positions 324F, 325V, 326T, or 327I markedly reduced the cytotoxic activity of P18-I10-specific murine CD8$^+$ T lymphocytes (18).

Subtle changes at the TCR contact residues can dramatically alter the downstream signaling events, leading to T-cell anergy, apoptosis, or high activation (28). Since amino acid residues at positions 4 and 6 of mInsA$_{2\text{-}10}$ were predicted to be in close contact with the TCR, we generated two APL candidates *via in silico*-assisted single D-amino acid substitution at the two positions, respectively. Our *in silico* analysis predicted that single D-amino acid substitution at position 4 rather than position 6 might have minor influence on the binding affinity of epitope to HLA-A*0201. These simulation results were supported by the peptide binding assays, which showed that DQ4 had a similar HLA-A*0201-binding affinity with that of native peptide, whereas DC6 displayed a relatively weaker binding ability to HLA-A*0201. Although both two APLs showed antagonistic effects against natural peptide-stimulated T cell proliferation *in vitro*, only mInsA$_{2\text{-}10}$DQ4, but not DC6, exhibited *in vivo* antidiabetic effects in NOD.*β2m^{null}.HHD* mice. A plausible explanation is that APL DC6 has a reduced binding ability to HLA-A*0201 relative to its parent peptide, and it is difficult for DC6 to competitively bind to HLA-A*0201 *in vivo* and therefore cannot be an effective antagonist. Whereas, mInsA$_{2\text{-}10}$DQ4 not only has a similar HLA-A*0201-binding affinity with mInsA$_{2\text{-}10}$ but also has a

theoretically stronger stability *in vivo* than $mInsA_{2-10}$. Therefore, DQ4 could effectively antagonizes the natural peptide *in vivo*. The metabolic dynamics and stability of DQ4 need further study.

We did not further study the mechanisms by which DQ4 induced $mInsA_{2-10}$-autoreactive T-cell tolerance in humanized NOD mice. APLs with amino acid substitutions at TCR contact positions are suggested as useful tools to modulate T cell responses induced by the native peptide. For example, APLs of insulin B_{15-23} (LYLVCGERG) G6H and R8L, which were generated by one natural amino acid substitution at position 6 and position 8 (TCR contact sites), respectively, showed the antagonist activity of the highly pathogenic insulin B_{15-23}-reactive $CD8^+$ T cell clone G9C8 in cytotoxicity and IFN-γ production assays (29). Another study indicated that a superagonist APL with an amino acid substitution at position 6 TCR contact site proved more effective than the native peptide in blocking autoimmune diabetes by a decreased accumulation of pathogenic $CD8^+$ T cells in the pancreas (30). We hypothesized that DQ4 with an increased stability and modified TCR contact site could provide an altered antigen stimulation signal to the native $mInsA_{2-10}$-autoreactive T cells, leading to induce their anergy or apoptosis, or change their functional state *in vivo*. However, the specific molecular and cellular mechanisms need to be further studied.

At least two studies have reported the presence of specific T cell responses to human proinsulin (PPI) $_{90-99}$ or $InsA_{1-10}$ (GIVEQCCTSI) in T1D patients (31, 32). Thus, human $InsA_{2-10}$ is likely to be an immunogenic target for diabetic patients. The sequence of $mInsA_{2-10}$ (IVDQCCTSI) is highly consistent with that of $hInsA_{2-10}$ (IVEQCCTSI) with only one amino acid difference at position 3, suggesting the possibility of cross-reactivity between the two peptides in T1D patients, but further studies are needed. We also did not detect whether $mInsA_{2-10}$ DQ4 can antagonize T cell responses towards $hInsA_{2-10}$ in PBMC of T1D patients. Therefore, further studies are needed to determine whether DQ4 has potential clinical application value.

As we know, prediction of T1D onset is possible, and prevention is now a goal in T1D. T1D vaccines based on islet autoantigens are considered to be one of such strategies to prevent or delay the occurrence of T1D by modulating autoimmune responses towards pancreatic islet antigens and prevent further destruction of pancreatic β-cells in T1D-susceptible individuals or preclinical individuals (33). Interestingly, our findings confirmed again that insulin is the key autoantigen for T1D initiation, since the administration of DQ4 not only eliminated peripheral $mInsA_{2-10}$-autoreactive T cells response but also reduced the subsequent infiltration of $CD4^+$ T and $CD8^+$ T cells as well as inflammatory responses in the pancreas in NOD.$\beta 2m^{null}$.*HHD* mice. Consistent with this, administration of chemically fixed splenic antigen-presenting cells coupled with intact insulin or the dominant insulin epitopes, but not epitopes of other autoantigens, protected 4–6-week-old NOD mice from development of T1D, which also indicated insulin is the key initiating autoantigen (34). Therefore, induction of insulin-autoreactive T cell immune tolerance in prediabetic mice may reduce the autoimmune insulitis and damage of islets, and prevent the subsequent epitope spreading and the activation and recruitment of other autoreactive T cells. In this sense, T1D vaccine targeting insulin, such as APLs of insulin epitopes, has potential value in the prevention of T1D when applied to the high-risk individuals whose islet autoimmunity has not yet occurred.

In conclusion, this present study describes that an APL designed by *in silico*-assisted single D-amino acid substitution at the potential TCR contact site displayed a protective effect against T1D in humanized NOD mice. This study provides a new strategy for the development of APL vaccines for T1D prevention.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of the Army Medical University (Third Military Medical University).

AUTHOR CONTRIBUTIONS

MZ and YW performed main experiments and data analysis and drafted the manuscript. XL, GM, XC, and LNW performed experiments and/or analyzed data. ZL revised the manuscript critically for important intellectual content. LW carried out the project design, guided the research process, supervised and managed it, completed the manuscript, and provided research funds. All authors contributed to the article and approved the submitted version.

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frontiers in Immunology

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Driving CARs to BARs: The Winding Road to Specific Regulatory T Cells for Tolerance

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Chimeric antigen receptor (CAR) transduced T cells have significantly improved cancer immunotherapy. Similarly, engineering regulatory T cells (Treg) with specific receptors to endow specificity and increase efficacy of Tregs holds great promise for therapy of a variety of adverse immune responses. In this review, we focus on our approaches using retroviral transduction of specific T-cell receptors, single chain variable fragments (scFv) or antigen in models of monogenic diseases, autoimmunity and allergy. The advantages of each of these for different targets diseases are discussed as well as their potential for clinical translation.

Keywords: chimeric antigen receptor, tolerance, autoimmunity, hemophilia, allergy, EAE (experimental autoimmune encephalitis), regulatory T cell (Treg)

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INTRODUCTION

Nearly five decades ago, Gershon and colleagues at Yale proposed that immune responses can be controlled by a subset of T cells called "suppressor cells" that downregulate other lymphoid cells (1). At that time, immunologists lacked the reagents to fully characterize these suppressors and their mode of action other than cell mixing experiments. Ironically, the latter approach still remains a *sine qua non* to demonstrate their efficacy. Armed with flow cytometry and molecular biology approaches, including the discovery of FOXP3 and its association with human immunodeficiency and autoimmune diseases (2–5), suppressor cells were replaced by a well-defined entity, "regulatory T cells" (Tregs) (2, 6). Similarly, these cells have the ability to suppress a variety of immune responses *in vitro* and *in vivo*.

Clinical trials with expanded Tregs were initiated over a decade ago in transplantation and autoimmunity, as summarized in Romano et al. (7). While these cells have been used safely in multiple clinical trials, they are polyclonal and the frequency of specific Tregs is very low. Expanded Tregs express a broad repertoire of specificities, and have the potential to be non-specifically immunosuppressive (8). To overcome the latter issue and the rare frequency of specific T cells, our lab has focused the use of *specifically engineered* Tregs to suppress adverse immune responses in monogenic diseases, autoimmunity and allergy. Our studies are based on the seminal studies of Eshhar and colleagues, who first demonstrated expression of specific receptors in T cells (9). These pioneering studies have been successfully applied using single chain antibody fragments (scFv) in cancer immunotherapy worldwide (10, 11). A major example is the use of anti-CD20 engineered human T cells in the successful treatment of leukemia (10). The first successfully use of specific Tregs used expanded FoxP3-expressing transgenic T cells in an autoimmune model of multiple

sclerosis (12). Since that time, multiple laboratories have made significant contributions by engineering specificity into murine and/or human Tregs (13–25). The purpose of this manuscript is to highlight our approaches in the context of this rapidly developing field focusing on targeting specific adverse responses.

Specificity in our lab has been achieved by engineering Tregs to express receptors that can recognize the targets of adverse immune responses. Thus, we have applied this protocol in autoimmunity, hemophilia A and allergy. To achieve this goal, we have used retroviral transduction of cloned T-cell receptors (TCR's), scFv's or antigen domains in thymic-derived human natural regulatory T cells (see **Figure 1**). In this review, we describe the basic principles and progress in each of these efforts by ourselves in three disease models to achieve the ultimate goal of modulating adverse human diseases (26–31). We also summarize the advantages and disadvantages of these approaches below and in **Table 1**. We have used retroviral transduction of Tregs with CD3 and CD28 signaling domains as the basic version; efforts to modify the signaling process has been reviewed by others (16, 32).

CHOOSING TARGETS, CHOOSING RECEPTORS, ESTABLISHING COLLABORATION

Typical pharmacologic therapies for adverse immune responses are broadly immunosuppressive. An issue to achieve any specific tolerogenic therapy is the choice of targets. Indeed, many adverse responses have multiple targets, with no unique or specific antigen being attacked. Moreover, any targeted antigen may have a variety of T-cell and B-cell epitopes. Thus, the challenge in rendering Treg cell-based therapy specific depends on multiple factors: knowing the target antigen and having the appropriate receptors. This is easier in monogenic diseases wherein the target antigen is known as patients lacking this protein often produce antibodies to the missing antigen when treated therapeutically. Patients are not tolerant to the missing protein and treat the therapeutic protein as a foreign antigen. Such is the case in hemophilia A (HA), an X-linked disease with a frequency of 1 in 5000 males in which patients have mutations in the *F8* gene encoding pro-coagulant Factor VIII (FVIII). Approximately 30% of HA patients develop high-titer neutralizing antibodies against therapeutic FVIII following repeated infusions of this needed protein that inhibit the function of this life saving therapy (33, 34). Most of these inhibitors block FVIII activity by binding to two immunodominant domains, called C2 and A2, which are important for FVIII's pro-coagulant activity.

In 2012, Yongchan Kim joined my lab after a successful post-doctoral fellowship with Ethan Shevach, a Treg expert at NIH. At the same time, Kathleen Pratt joined the faculty in our department. Dr. Pratt had cloned several T-cell lines from HA patients (35, 36), and one of these T cell clones, called 17195, recognized an HLA-restricted peptide in the C2 domain of FVIII, residues 2194-2210 (35, 37). With a determination of the TCR V regions, Yongchan then inserted them into a retroviral vector and used the vector to transduce FACS-purified human Tregs (see ref. (27) for detailed methods). Purified Tregs were $CD25^{high}$ and $CD127^{low}$ and typically expressed FOXP3 and Helios transcription factors. Although less than 20% of the initially transduced Tregs expressed the 17195 T-cell receptor (TCR), this population expanded upon stimulation with the FVIII 2194-2210 peptide on HLA DR1 antigen-presenting cells (26). This stimulation also led to an increased expression in FOXP3 and Helios transcription factor, markers that confirmed the expansion of Tregs in culture. Importantly, these expanded 17195-expressing Tregs suppressed the proliferation and

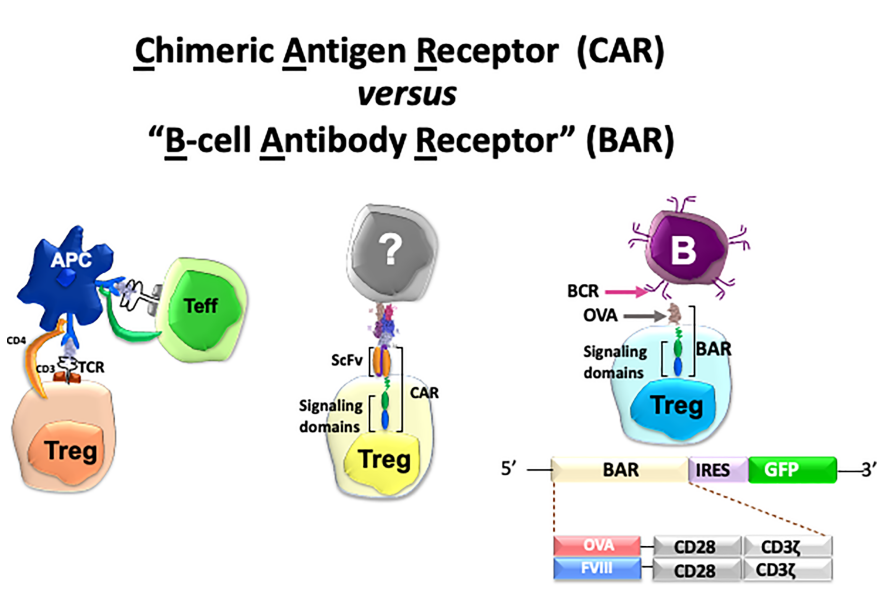

FIGURE 1 | Cartoon of three types of specific Tregs and potential targets used in our lab. See **Table 1** for details.

TABLE 1 | Advantages and disadvantages of engineered Treg approaches.

Gene modified Treg	Specificity (Target antigens)	Disease model	Cellular targets	Advantages	Disadvantages
T-cell receptor (TCR)	MHC-restricted epitopes (Factor VIII; Myelin basic protein)	Hemophilia (FVIII knockout) mice (26) Multiple sclerosis (EAE) (27)	Antigen-presenting cells	Suppression of CD4 effector proliferation and cytokine production; Suppression of antibody formation; Bystander effect for suppression; Not affected by circulating antibody	HLA-restricted; Need to clone different TCRs; may be affinity dependent
Single change Fv chimeric receptor (CAR)	Conformational epitopes (FVIII; MOG/MBP)	Hemophilia (FVIII knockout) mice (28) Multiple sclerosis (EAE) (18)	Cell surface membrane antigens	Suppression of CD4 effector proliferation and cytokine production; Suppression of antibody formation; Bystander effect for suppression; Not affected by circulating antibody	Need to recognize intact conformational epitope/domain
B-cell antigen receptor (BAR)	Antigen-specific B cell Fcε receptor on Mast cells	Hemophilia (FVIII knockout) mice (29, 30) Allergy (OVA; Peanut) (31)	B-cell receptor; IgE in Fcε receptor	Suppression of antibody formation; Bystander effect for suppression of antibody formation;?Not affected by circulating antibody	Unknown bystander effect for suppression of allergy

cytokine production by FVIII-specific T effector cells even more effectively than polyclonal Tregs *in vitro*. Moreover, these expanded human 17195-expressing Tregs also suppressed the secondary antibody response of *murine* HLA-DR1 transgenic spleen cells stimulated with FVIII *in vitro*. Overall, human 17195-expressing Tregs were able to effectively suppress the antibody response to FVIII *in vivo*, even across a xenogeneic barrier, although they were rejected within two to three weeks (26). Notably, while human 17195-expressing Tregs were specific for a single peptide in the C2 domain of FVIII, they suppressed antibody formation against the multiple epitopes in the entire FVIII protein. This suggested that suppression, while "specific" for a single FVIII peptide, may have bystander effects on the response to other epitopes in FVIII locally, presumably at the level of the antigen-presenting cells.

TCR engineered Tregs are MHC-restricted, which limits their utility to HLA-matched donor recipient combinations and limited repertoires. To generate antigen-specific Tregs that were not MHC-restricted, Jeong-Heon Yoon collaborated with Christoph Königs and Anja (ne' Naumann) Schmidt in Frankfurt. Anja had isolated several single chain (sc)Fv's that recognized FVIII domains (38) and cloned them into our standard retroviral vector for expression in Tregs. The human Tregs transduced with retroviral vector encoding scFv recognized a conformational epitope in the A2 region of FVIII (38). When these scFv-expressing human Tregs were mixed with spleen cells from mice immunized with FVIII, they suppressed the secondary response *in vitro*, and blocked antibody formation *in vivo* in hemophilic mice. Compared to TCR-engineered Tregs, the scFv-transduced Tregs suppressed the anti-FVIII immune response to the same degree at certain ratios (28). It is worth noting, then, that engineered Tregs specific for the MHC-restricted peptide in the C2 domain of FVIII and Tregs engineered to express the scFv directed at the A2 domain of FVIII both suppressed the immune response to other domains in FVIII. Thus suggesting that both types of antigen-specific Tregs exerted bystander suppression to other epitopes in the same target protein locally, but they did not non-specifically suppress the response to an unrelated antigen, *e.g.*, immunization with TNP hapten-conjugated red blood cells (28). Application of scFv-transduced Tregs in a transplant model was elegantly demonstrated in the Levings' lab (15), thus demonstrating further application of engineered Tregs for tolerance.

TREG EFFECTS IN AUTOIMMUNITY

T-cell responses against self-proteins can result in a spectrum of autoimmune diseases. Unlike monogenic diseases in which the target antigens are known, multiple potential antigens and epitopes may be recognized in autoimmunity. For instance, several antigens have been identified in Type 1 diabetes including islet antigens such as glutamic acid decarboxylase or insulin. In the central nervous system (CNS), myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) can be targeted in multiple sclerosis (MS). Based on our success and the bystander effect in hemophilia with an engineered TCR recognizing FVIII, we hypothesized that transduction of an MBP-specific TCR into expanded human Tregs might be an effective therapy in MS. In collaboration with Kai Wucherpfennig at Harvard, we engineered human Tregs to express the Ob2F3 TCR V regions that targeted an immunodominant MBP peptide, p85-99 (39). Ob2F3-expressing Tregs from either healthy adults or MS patients could suppress MBP-specific T effector cells, but were also able to suppress T cells with other specificities after Tregs had been activated through the TCR (27). Surprisingly, these Ob2F3-expressing Tregs which were MBP-specific ameliorated EAE in MOG-immunized DR15 transgenic mice (22). This suggested that bystander suppression *in vivo* might be associated with soluble factors, enhanced by cell contact between Tregs and effectors (27). These results indicated that engineered MBP-specific Tregs were able to suppress autoimmune pathology in EAE.

An important question was whether Tregs engineered with scFv's will work in autoimmunity. To answer this question, Anja Schmidt provided two single chain Fv's reactive with MBP and MOG, and we showed that these cells recognized murine CNS tissue. Alessandra Pohl in my lab then cloned these into constructs in our retroviral vector for Treg transduction. Results showed that a mixture of human Tregs expressing

these CNS "specific" scFv's were able to suppress EAE *in vivo* similar to the TCR-expressing Tregs (18); however, direct comparison of the TCR- *versus* scFv-transduced Tregs has not yet been performed. Nevertheless, these important results indicate that engineered CNS targeting CAR-Tregs have the potential to be used as a cellular therapy for MS patients.

POSSIBLE MECHANISMS OF SUPPRESSION BY ENGINEERED TREGS

Using an ingenious version of a transwell system in which neighboring microtiter well liquid contents were connected *via* an opening above the cell layer (**Figure 2**), Kim showed that suppression of effector T cells in one well only occurred when the neighboring well contained *both* Tregs and effector T cells. Thus, contact between Tregs and effector cells in one well led to suppression of effector T cells not in direct contact in a neighboring microtiter well. Based on the observation that IL-2 signaling *via* interaction with CD25 can lead to STAT5 phosphorylation, we were able to show in kinetic studies that phospho-STAT5 decreased in effector CD4 T cells at the same time it was increasing in engineered Tregs (27). These data led us to propose that, similar to classical Tregs, engineered Tregs were able to capture IL-2 produced by effector T cells upon antigen presentation in the local milieu. Overall, these results indicate that the induction of engineered Treg growth led to the activation of Tregs and release of inhibitory cytokines which mediated the bystander suppressive effect observed *in vivo*.

BAR TREGS TO TARGET B CELLS IN ADVERSE IMMUNE RESPONSES

Our results suggested that specific Tregs may block adverse immune responses at the level of the antigen-presenting cells. While we knew that adverse inhibitory antibody responses that can occur in monogenic diseases were highly T-helper cell dependent, the culprits were alloreactive B cells. We reasoned that generating specific T cells expressing antigen should be recognized by (or recognize) B cells *via* their immunoglobulin receptors. Moreover, *cytotoxic* CAR CD8 T cells could kill specific B cells recognizing the expressed antigen as shown in pemphigus by the Payne group at Penn (20). Kalpana Parvathaneni in my lab, engineered human and murine cytotoxic T cells with FVIII antigen to target the B-cell antibody receptor (BAR) and showed that these BAR-engineered T cells were capable of killing FVIII-reactive B-cell hybridomas *in vitro* and *in vivo* (40). In addition, adoptive transfer of FVIII A2- and C2-BAR CD8 T cells significantly reduced the anti-FVIII antibody formation in hemophilic mice. These data suggest that BAR-engineered T cells are a promising approach for future prophylactic treatment for patients with severe hemophilia A who are at high risk of developing inhibitors.

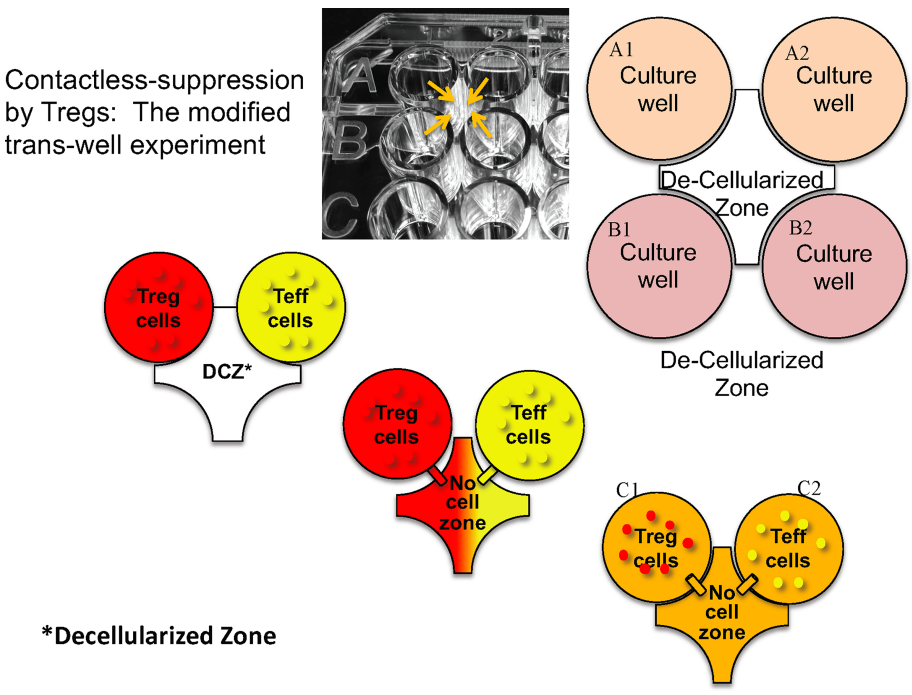

FIGURE 2 | Design of microtiter plate to test whether direct cell contact is necessary for the bystander effect. Tregs and T effectors (Teff) and placed in the same or contiguous wells with a hole created above the bottom of the wells (arrows) so that fluid but no cells can migrate. "Decellularized" zone refers to the space between the four wells into which soluble products can diffuse to neighboring wells.

Aihong (Allan) Zhang in the lab also demonstrated human BAR Tregs, which express only the C2 domain of FVIII, suppressed the secondary response of spleen cells from FVIII-immunized mice *in vitro*, thus confirming the bystander effect mediated by engineered Tregs (29). Moreover, FVIII-BAR Tregs also suppressed the primary response *in vivo*, and reduced priming to FVIII as evidenced by lower secondary antibody titers upon boosting. It is important to note that FVIII antigen domains on the surface of Tregs (or CD8 T cells) might be blocked by circulating antibody to immunodominant domains in immunized mice, thus competing with B cell targeting. However, this did not appear to be a major problem at least with low titered antibodies, as was shown in collaboration with Shiva Venkatesha (19), as well as by the Payne group in their model (20). Moreover, crosslinking the expressed domains on human T cells may even lead to expansion of these T cells.

CAN BAR TREGS WORK IN ALLERGY MODELS?

Based on the success of BAR Tregs in hemophilia, we sought to test them in an allergy model, the IgE response to ovalbumin (OVA). We hypothesized that expression of OVA in Tregs would target the OVA-specific B cells as was done for FVIII-specific B cells in hemophilia. Maha Abdeladhim immunized mice with OVA in alum, eliciting both IgE and IgG responses, and treated OVA-primed mice with OVA-BAR Tregs. Forty-eight hours later, mice were challenged with a high dose of OVA intravenously to induce anaphylaxis as measured by an acute drop in body temperature of 2-6°C. Results showed that OVA-BAR Tregs, human or mouse, blunted this temperature drop, an effect that persisted for at least one month (31). Surprisingly, no significant drop in IgE titer in primed and Treg-treated mice was observed within two weeks. However, we predict that pretreatment with BAR Tregs would prevent sensitization as it does in the hemophilia model.

We then investigated whether OVA-BAR Treg treatment targeted mast cells loaded with IgE (see **Figure 3**, right side). To this end, Maha passively sensitized *naive* Balb/c mice with anti-OVA IgE and then injected mice with OVA-BAR Tregs. To our surprise and pleasure, these OVA-BAR Tregs were very effective at blocking passive anaphylaxis, thus implicating mast cells as an important target in this system (31). Notably, BAR Tregs alone did not cause detectable release of allergic mediators despite expressing the target antigen on their surface. While the mechanism for BAR Tregs blocking anaphylaxis but not eliciting a reaction *per se* is unknown, we currently attribute this to stoichiometric differences in antigen doses. Because mast cells in patients are loaded with IgE of diverse specificities, we are currently investigating whether specific BAR Treg activity can "desensitize" to other antigens for which the mast cells are sensitized, using a peanut allergy model.

SUMMARY AND CONCLUSIONS

The studies in my lab and others (13, 15, 16) have demonstrated that Tregs can be made specific to treat a variety of adverse immune responses. The choice of which specific receptor to employ will depend on the targeted disease. For example, TCR-engineered Tregs, while highly specific, are limited by MHC diversity and by the knowledge of the variable regions of the receptor. On the other hand, scFv (CAR)-Tregs require identification of a conformational epitope in a disease. The BAR Tregs are a choice in antibody-mediated diseases as they provide a clear target, the Ig receptors recognizing antigen. Our approaches have focused on the extracellular receptor, whereas other laboratories have attempted to increase Treg signaling, with variable success (16, 41). We hope our data will be followed up by multiple efforts to increase efficacy, as well as with clinical trials to prove their efficacy and safety in the future as depicted in **Figure 4**. Efforts to create "off the shelf" generic

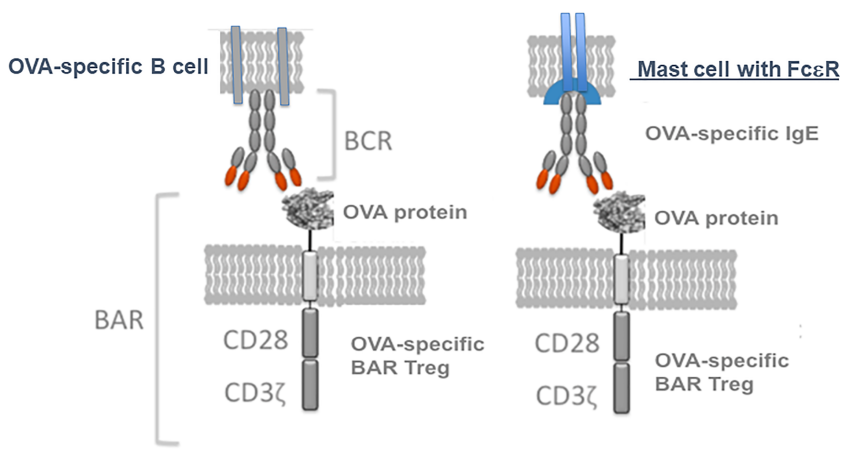

FIGURE 3 | Model for BAR Tregs interaction not only with the immunoglobulin receptor on B cells, but also Ig(E) bound *via* FcεR on mast cells. Tregs expressing an antigen like ovalbumin (OVA) can interact with either specific B cell IgM (left, the BCR) or IgE bound to mast cells (right, in sensitized individuals).

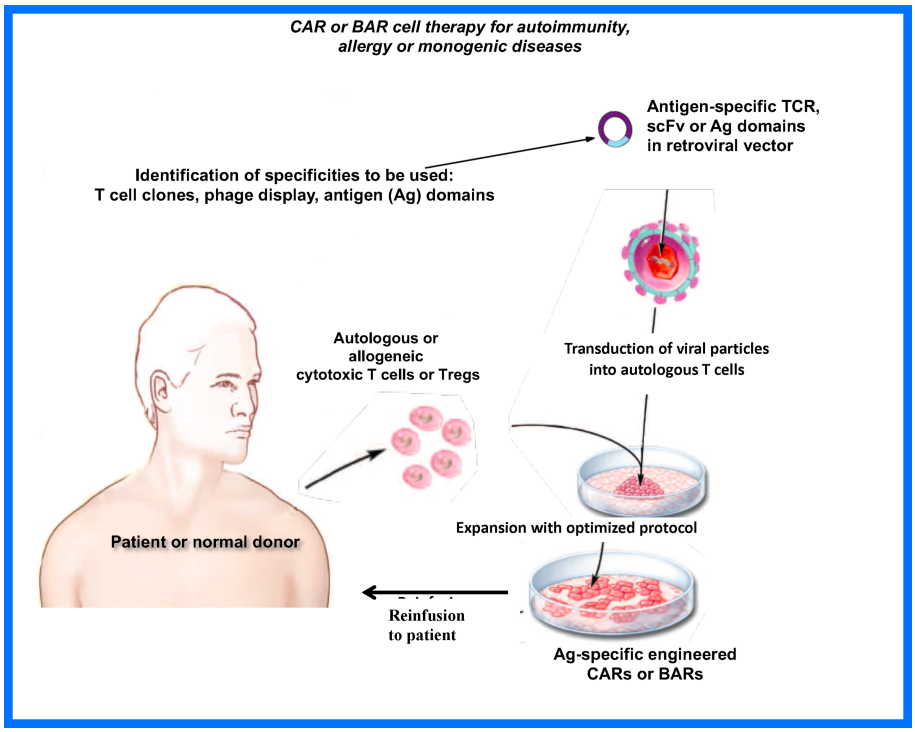

FIGURE 4 | Proposed engineered Treg therapy in patients.

Tregs using CRISPR engineering should also prove fruitful. The future is bright and we hope that our approaches provide the framework for Treg treatments for adverse immune responses.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Induction of Tolerance to Therapeutic Proteins With Antigen-Processing Independent T Cell Epitopes: Controlling Immune Responses to Biologics

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The immune response to exogenous proteins can overcome the therapeutic benefits of immunotherapies and hamper the treatment of protein replacement therapies. One clear example of this is haemophilia A resulting from deleterious mutations in the FVIII gene. Replacement with serum derived or recombinant FVIII protein can cause anti-drug antibodies in 20-50% of individuals treated. The resulting inhibitor antibodies override the benefit of treatment and, at best, make life unpredictable for those treated. The only way to overcome the inhibitor issue is to reinstate immunological tolerance to the administered protein. Here we compare the various approaches that have been tested and focus on the use of antigen-processing independent T cell epitopes (apitopes) for tolerance induction. Apitopes are readily designed from any protein whether this is derived from a clotting factor, enzyme replacement therapy, gene therapy or therapeutic antibody.

Keywords: Immunological tolerance, haemophilia A, Tr1 cell, Treg cell, immunotherapy, hypersensitivity, synthetic peptide, T cell epitope

INTRODUCTION

The last 30 years has seen an upsurge in the use of drugs produced using biological systems rather than chemical synthesis (biologics) for treatment of cancer and inflammatory diseases. This is such that in 2019, biologics, largely monoclonal antibodies and fusion proteins, constituted 73% of major drug sales. This reflects the high specificity of such agents for their disease targets. The disadvantage of large exogenous proteins as therapeutics, however, is their immunogenicity. While the incidence of immunogenicity is reduced by 'humanisation' of monoclonal antibodies, anti-drug antibodies frequently arise following repeated administration. For example, the ABIRISK consortium found antibodies to the most popular anti-TNF agent (Humira) increased in frequency with time reaching ~50% at 18 months (1). A similar issue affects people with haemophilia A or B, conditions caused by deficiency in or production of defective clotting factors VIII or IX respectively (2). A recent study showed that recombinant FVIII injections, designed to provide effective levels of clotting factor, induce anti-FVIII antibodies ('inhibitors') at a higher frequency than factor purified from plasma (3).

The cumulative incidence of inhibitors was 26.8% with plasma derived FVIII and 44.5% with the more frequently used recombinant FVIII.

FVIII inhibitors arise in people with altered FVIII genes who fail to produce a fully functional protein. In unaffected individuals, immunological tolerance to FVIII is generated by both central and peripheral tolerance mechanisms. The lack of complete protein in haemophiliacs results in ineffective tolerance and leads to susceptibility to inhibitor formation. FVIII-specific immune activation occurs through antigen-presenting cells (APC) that internalize the FVIII protein, process and present antigenic peptides (epitopes) on major histocompatibility complex class II (MHCII) molecules to $CD4^+$ T-cells in the presence of co-stimulatory signals (4). Activation of naïve B and $CD4^+$ T helper cells, including follicular helper T cells, is subsequently amplified by B-T collaboration resulting in affinity maturation and switching from IgM to IgG isotypes. FVIII inhibitors are $CD4^+$ T-cell-dependent in both murine haemophilia models (5–7) and HA patients (8) and are of high affinity i.e. B cells producing them have undergone affinity maturation. Recent studies have shown that FVIII-specific antibodies in patients with inhibitors have up to a 100-fold higher apparent affinity compared to antibodies in patients without inhibitors or healthy individuals (9). The inference from these observations is that it should be possible to control affinity maturation and inhibitor formation by induction of tolerance in FVIII-specific $CD4^+$ T cells.

INDUCING TOLERANCE TO FVIII

Approaches to induce tolerance to FVIII were reviewed recently by Lacroix-Demazes and colleagues (10). Repeated high doses of FVIII through immune tolerance induction (ITI) dampens the immune response in many but not all recipients; however, it is burdensome on patients and so expensive (11) that it is unavailable in many countries. There is a critical need for more effective and less expensive approaches to tolerance induction.

Various broad acting immunosuppressive approaches have been tested. These include co-administration of FVIII with rapamycin (12), co-stimulatory pathway modulators (13) and agents to selectively deplete B cell subsets (14). More directly antigen-specific approaches have been tested to improve the efficacy of ITI. For example, coupling FVIII to the Fc portion of immunoglobulin targets the protein to B cells *via* inhibitory Fc receptors. Both pre-clinical and early clinical studies show that this reduces inhibitor formation (15). A promising approach is to modify $Foxp3^+$ regulatory T (Treg) cells with T cell receptors specific for FVIII epitopes or a single chain Fv specific for FVIII protein (16, 17). $Foxp3^+$ Treg cells were shown to mediate linked suppression whereby Treg cells specific for a single epitope would suppress the immune response to other epitopes within the protein. This approach would involve complex modification of the patient's own Treg cells; furthermore, it is not clear how frequently Treg infusions would be required and, therefore, how practical this would be in the clinic.

An alternative approach is to administer antigens in a form that selectively induces Treg cells *in vivo*. For example, it has long been known that mucosal delivery of antigen can induce suppression through various mechanisms of tolerance including apoptosis, anergy and Treg induction (18). Herzog and colleagues have shown that FVIII fused to the B-subunit of cholera toxin B and encapsulated in plant cells can control inhibitor formation when given repeatedly by oral administration to mice (19). Oral tolerance correlated with an increase of immunoregulatory cytokines, IL-10 and TGF-β, with upregulation of the Foxp3 gene in antigen-specific cells.

Various nanoparticle-based approaches for suppression of inhibitors have been tested. Encapsulation of FVIII in phosphatidyl serine enriched liposomes targets the protein to tolerogenic APC capable of inducing Treg cells. Treatment of mice led to suppression of inhibitor antibody formation associated with an increase in $Foxp3^+$ Treg cells (20). As reviewed elsewhere (21), both macrophage and dendritic cell populations can have tolerogenic properties *in vivo*. Consequently, antigen-specific tolerance can be induced by administration of antigens linked to antibodies against receptors on steady state dendritic cells thus proving their tolerogenic potential (22). Similarly, nanoparticles containing immunosuppressive drugs such as rapamycin can be used to suppress the immune response to co-administered FVIII (23). The rapamycin study is especially interesting because the treatment was successful when rapamycin nanoparticles were injected alongside treatment with FVIII, the drug and antigen did not need to be in the same particle. This implies that the nanoparticles can promote systemic immune modulation resulting in the maintenance of tolerance to FVIII.

IMMUNOTHERAPY IN THE FACE OF A PANDEMIC

The world is currently facing the challenge of COVID-19. Not since the 1918 HINI Influenza pandemic has the human race faced such a threat from an infectious agent. This has brought immunotherapy and the use of non-specific immune modulating drugs into sharp focus. Many patients with cancer or chronic inflammatory conditions are experiencing long periods of isolation as they wait for the rest of the population to benefit from vaccination and the pandemic to recede. Clearly, we must find more selective ways to treat such conditions so that the immune system remains intact both to combat such infections and respond effectively to vaccination. Many of the approaches mentioned above fall short of an effective immunotherapy. Any approach depleting B or T cells or non-specifically modulating the function of APC will leave the individual immune compromised. We must strive for antigen-specific approaches that induce tolerance to therapeutic proteins but do not compromise the rest of the immune system. Here we will review our experience with apitopes. Apitopes are antigen processing independent T cell epitopes that selectively induce tolerance among $CD4^+$ T cells.

MECHANISM OF ACTION OF APITOPES

Apitopes are CD4 T-cell epitopes designed to bind directly to their MHC II restriction element in the correct conformation for recognition by their cognate T cell (24). These peptides are identical to the native protein but may be modified to improve solubility by addition of hydrophilic amino acids at N- and C-termini. As reviewed elsewhere (21), apitopes differ from other forms of peptide therapy since they do not depend on combination with cells or nanoparticles or chemical modification for their function. Our path towards developing apitopes for treatment of autoimmune and allergic conditions began with an investigation of mucosal tolerance (25). This showed that while oral administration of protein was unreliable as a means of inducing tolerance, high doses of peptide could be effective. However, it became clear that the oral route was relatively ineffective when compared with the intranasal route for peptide antigens as a result of the degradative nature of the gut (26). Later work revealed that subcutaneous delivery of soluble peptides was far more effective than either of the two mucosal routes (27). Initial studies showed that some known T cell epitopes were tolerogenic while others were not (28). Our work subsequently revealed that tolerogenic T cell epitopes bind directly to MHC II in a conformation that mimics the naturally processed epitope whereas non-tolerogenic epitopes either bind to MHC molecules in a cryptic conformation (29) or were insufficiently soluble to induce tolerance (30). Tolerogenic CD4 T cell epitopes are, therefore, designed as apitopes that bind directly to MHC class II molecules in the correct conformation and are sufficiently soluble to induce tolerance. We recently revealed that soluble apitopes selectively bind to steady state dendritic cells and not B cells or monocytes in lymphoid organs (24). Steady state dendritic cells are tolerogenic because they express low levels of costimulatory molecules and, importantly, selectively bind apitopes because they have peptide receptive MHC II at the cell surface (31).

Presentation of peptide antigens on steady state dendritic cells (**Figure 1**) increases the proportion of Foxp3$^+$ T cells *in vivo* but the major impact of treatment with apitopes is to convert potentially pathogenic T cells into regulatory Tr1 cells (27, 34, 35). These Tr1 cells are anergic, IL-10 producing cells that are Foxp3$^-$ but express a similar tolerance associated gene signature to the IL-10 producing cells that control immune pathology in chronic infection (36). In particular, they express the IL-10 promoting transcription factors c-Maf and NFIL3 and upregulate inhibitory receptors CTLA-4, LAG-3, TIM-3 and TIGIT (27). Recently, through analysis of T-cell receptor signalling, epigenetic modification and gene expression, we have shown that the induction of Tr1 cells involves suppression of both signalling to and chromatin priming of immune response genes. At the same time, chromatin priming of those genes associated with the Tr1 cell tolerance signature was promoted making these genes sensitive to levels of signalling below the threshold needed to activate immune response genes (32). This study explains how repeated encounter of antigen, in the form of apitopes presented by steady state dendritic cells, prevents the differentiation of pathogenic/effector cells but leads to dominant tolerance through generation of a regulatory Tr1 population.

DEVELOPMENT OF APITOPES FOR TREATMENT OF AUTOIMMUNE DISEASES

Apitopes can be used to suppress immune pathology in response to biologics, allergies and autoimmune diseases. The most

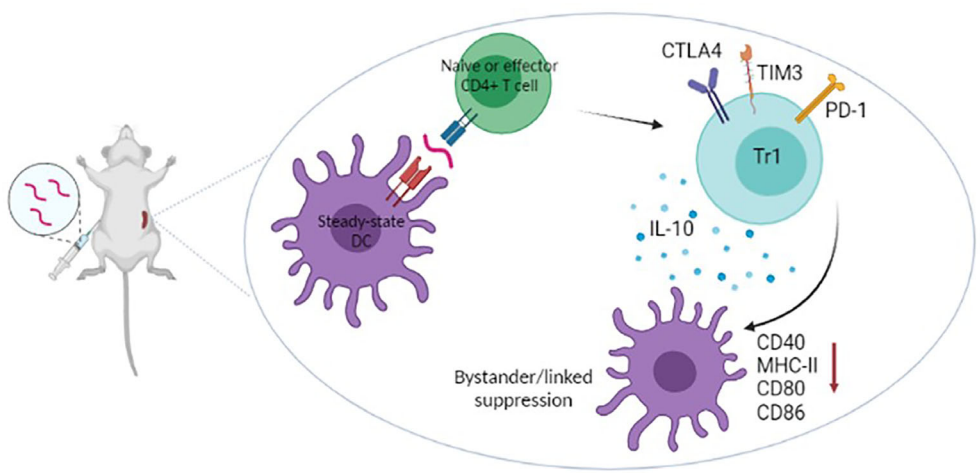

FIGURE 1 | Recent work has shown that apitopes injected subcutaneously migrate rapidly to (<5mins) and bind MHC II on steady-state DC in lymphoid organs (24). Naïve cells undergo abortive activation then, on repeated dosing, develop anergy and upregulate genes characteristic of Tr1 cells (IL-10 and inhibitory receptors) (27, 32). Tr1 cells specific for epitope A within an antigen cause downregulation of the antigen presenting machinery of antigen presenting cells (33) hence blocking their ability to present epitopes B,C,D etc from the same or associated antigens. Apitope induced Tr1 cells mediate both linked and bystander suppression.

advanced clinical programmes are for autoimmune diseases including multiple sclerosis (MS) and Graves' disease (GD). Our experience with these programmes informs the use of apitopes for aberrant responses to biologics.

Graves' disease is caused by the generation of autoantibodies specific for the thyroid stimulating hormone receptor (TSHR). These antibodies cause chronic activation of the receptor leading to excessive secretion of thyroid hormones and hyperthyroid disease. Apitope designed 2 peptides (5DK and 9B) from TSHR that were pan-DR binding, highly soluble and induced tolerance to the TSHR in a relevant HLA-DR3 transgenic mouse model (37). Apitope designs pan-DR epitopes primarily because Graves' is associated with different MHC haplotypes; therefore, while most Caucasian Graves' patients are HLA-DR3$^+$, the link to a specific HLA-DR is less clear in Asian populations. It is important to design peptides with the capacity to bind to and be recognised by people with a broad range of HLA-DR types. We have shown, for example, that peptide 5DK will suppress the response to TSHR in both HLA-DR3 and HLA-DR4 transgenic mice.

Based on our analysis of the mechanism of action of model apitope peptides, we have designed an 'in patient' dose escalation protocol that optimises the generation of Treg cells (27, 32). Patients are given either a subcutaneous or intradermal dose of peptide increasing from 25 to 800 μg of the peptide cocktail every 2 weeks. In the Graves' phase 1 study this resulted in a reduction in anti-TSHR antibody levels that correlated with a return of thyroid hormone levels to the normal range in 7 of 10 patients treated (38).

It is believed that MS is driven by the immune response to a range of different myelin antigens including myelin basic protein (MBP), proteolipid protein and myelin oligodendrocyte protein. We identified 4 dominant epitopes from MBP that could be designed as apitopes. The cocktail of 4 MBP apitopes (ATX-MS-1467) induced tolerance and promoted IL-10 secreting T cells in HLA-DR2 transgenic mice (39, 40). Two phase 1 trials showed that intradermal ATX-MS-1467 was safe with evidence of efficacy in patients with secondary progressive (39) and relapsing MS (41). A phase 2 study in relapsing MS revealed that ATX-MS-1467 not only suppressed CNS inflammation but significantly improved cognition in treated patients (41). The implication from these early trials in Graves' disease and MS is that selected T cell epitopes from within one autoantigen can control both the response to the whole autoantigen (linked suppression) and the response to other antigens within the same tissue (bystander suppression). Linked and bystander suppression mediated by antigen-specific immunotherapy with peptide epitopes has been described previously in experimental animal models (28, 35, 42).

DESIGN OF APITOPES FROM FVIII

The nature of the immune response to FVIII was reviewed recently by Varthaman and Lacroix-Desmazes (43). They emphasise the key role of CD4$^+$ T cells in supporting generation of inhibitor antibodies and summarise previous work on defining the immunodominant T cell epitopes driving the response. Although some previous studies have indicated a link with the HLA-DR type of people developing inhibitors (44) this has not been substantiated. For this reason, our approach has been to identify pan-DR binding epitopes expected to function in a wide range of the population. The combined use of various MHC-binding algorithms identified a set of 12 peptides that were then used to screen responses among human peripheral blood mononuclear cells and T cells from FVIII-immune HLA-DR transgenic mice (45). Two dominant T cell epitopes were identified spanning amino acids 545-559 and 1788-1802. Importantly, an independent analysis by Steinitz and co-workers, using a screen of overlapping peptides from the whole FVIII protein, identified the same peptides among 3 immunodominant peptides arising in HLA-DR transgenic mice immunised with Baxter's recombinant FVIII (46). In a separate study, van Haren undertook an elegant experiment whereby they allowed dendritic cells from a range of donors to process FVIII protein (47). They then eluted peptides from HLA-DR and identified the peptides by mass spectrometry. The dominant epitope identified by this alternative approach contained the FRNQASRPY sequence found within our 1788-1802 epitope. Importantly, this peptide was found in dendritic cells from a heterogeneous group of donors with a broad range of HLA-DR types hence confirming its pan-DR binding characteristics.

Peptides 545-559 and 1788-1802 were optimised for solubility by addition of lysine residues at the N- and C-termini to create apitopes P1 and P17 respectively. A combination of P1 and 17 (ATX-F8-117) was injected subcutaneously in saline in HLA-DR transgenic mice. This resulted in suppression of the immune response to FVIII as evidenced by inhibition of the T cell proliferative response to FVIII *in vitro* with cells from mice immunised with FVIII in Freund's adjuvant. Furthermore, mice treated with either peptide alone showed a suppressed immune response to FVIII. HLA-DR transgenic mice injected with 1 μg FVIII in saline solution weekly develop anti-FVIII antibodies after the 4th subcutaneous injection (46). HLA-DR transgenic mice were treated with ATX-F8-117 using a dose escalation protocol prior to repeated challenge with FVIII in saline. This significantly inhibited the generation of both total anti-FVIII and inhibitor antibodies (45). Furthermore, we have shown that treatment with ATX-F8-117 suppresses new inhibitor formation in mice previously immunised with FVIII. Importantly, FVIII inhibitor levels fell away from the time at which the apitope treatment was started. This promising result shows that dominant T cell epitopes from FVIII can be used to both prevent and reverse inhibitor formation in HLA-transgenic mice. ATX-F8-117 warrants investigation in phase 1 trials of immunotherapy in people with inhibitors.

DISCUSSION

The central role of CD4 T cells in orchestrating the immune response in allergy, autoimmune disease and the antigen-drug antibody response to biologics is clear. For example, haemophiliacs

TABLE 1 | Properties of antigen processing independent T cell epitopes (apitopes) designed for antigen-specific immunotherapy.

Apitope Property	Comment	References
Preferred route of administration	Intradermal/subcutaneous > intranasal >> oral	(25–27)
MHC II binding	Apitopes must bind MHC II in a conformation that mimics the naturally processed antigenic epitope	(29)
***In vivo* interaction with antigen presenting cells**	Apitopes bind selectively to steady-state dendritic cells rather than B cells or monocytes	(24)
Solubility	Apitopes must be soluble such that they rapidly migrate in the fluid phase to steady-state dendritic cells in lymphoid organs following subcutaneous administration	(24)
Induction of regulatory T cells in mice	Repeated administration of the model apitope Ac1-9[4Y] induces both $Foxp3^+$ Treg and $Foxp3^-$ Tr1 cells (Tr1 >> $Foxp3^+$ Treg)	(27, 33–35, 53)
Clinical development (Graves' disease)	Phase 1 trial of two peptides from the thyroid stimulating hormone receptor in patients with mild to moderate hyperthyroid disease showed a treatment related response in 7 of 10 individuals	(37, 38)
Clinical development (Multiple Sclerosis)	Phase 1 trials in secondary progressive and relapsing MS (RMS) using a cocktail of four peptides from myelin basic protein revealed no unexpected safety signals and evidence of efficacy (significant reduction of enhancing lesions) in RMS patients. Phase 2 demonstrated a similar reduction in lesion volume with improvement in cognition in RMS patients	(39, 41)
Clinical development (Inhibitor formation in haemophilia A)	Two pan-DR binding peptides from factor VIII were optimised for solubility and shown to either prevent the generation of or suppress levels of inhibitors in HLA-DR transgenic mice, previously immunised with FVIII, when the apitopes were injected subcutaneously	(45)

with a history of inhibitors who became HIV positive failed to make an anamnestic response to subsequent FVIII infusions (48). Furthermore, inhibition of CD4 T cell priming by blockade of costimulatory pathways prevented the generation of inhibitors in a mouse model of haemophilia A (49). Reinstating immunological tolerance to specific antigens through antigen-specific immunotherapy is designed to suppress the immune pathology associated with hypersensitivity conditions while leaving the immune system of an individual capable of protecting against infectious diseases, of responding to vaccination or immune surveillance of cancers. As discussed above, there is an urgent need to move away from the use of immune debilitating drugs for hypersensitivity conditions especially when faced with the threat of emerging infections and global pandemics.

Various approaches to antigen-specific immunotherapy are under development. These include the use of nanoparticles designed to target antigens to the liver or to tolerogenic APC in lymphoid organs (21). A recent advance described the use of an RNA vaccine to deliver a peptide in a tolerogenic form to treat a mouse model of MS (50). This approach would have clear advantages if it were possible to deliver an intact protein in a tolerogenic fashion.

As it stands, the most straightforward approach to antigen-specific immunotherapy is the design of tolerogenic peptide epitopes. Our work on this topic was first described in 1989 when we realised that a high affinity analogue of a T cell epitope could be used to switch off the immune response *in vivo* (51). Subsequent work showed that high affinity analogues in adjuvant caused activation induced cell death among responsive cells (52) and led to investigation of peptide delivery without adjuvants. Over the past two decades we have defined the rules governing successful tolerance induction with T cell epitopes (**Table 1**). Key features are that the peptides need to be designed as antigen processing independent epitopes (apitopes) (29), need to be highly soluble so as to reach steady state/tolerogenic dendritic cells in lymphoid organs (24), can be made more potent by optimising affinity for MHC (54) and are capable of both linked and bystander suppression. The ability of a single T cell epitope from a large, complex protein such as FVIII to suppress the response to the whole protein has been demonstrated in our recent study (45). This important observation emphasises the impact of linked suppression for both prevention of and suppression of ongoing immune responses.

The fact that simple apitopes designed from antigens, including self-antigens such as TSHR (37, 38) and exogenous antigens such as FVIII (45), can control pathogenic antibody responses to these proteins provides evidence that they can be readily designed for any antibody-mediated complication where the antigen is known. This includes other autoimmune conditions such as myasthenia gravis, allergic conditions and the aberrant immune response to biologics or enzyme replacement therapies. Our work has shown that it is now possible to control hypersensitivity conditions through antigen-specific immunotherapy thereby avoiding the use of non-specific immunosuppressive drugs.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Approaches to Establishing Tolerance in Immune Mediated Diseases

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The development of rational approaches to restore immune tolerance requires an iterative approach that builds on past success and utilizes new mechanistic insights into immune-mediated pathologies. This article will review concepts that have evolved from the clinical trial experience of the Immune Tolerance Network, with an emphasis on lessons learned from the innovative mechanistic studies conducted for these trials and new strategies under development for induction of tolerance.

Keywords: immune tolerance, allergy, transplantation, autoimmunity, clinical trial, immunomodulation, immunosuppression

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INTRODUCTION

The mission of the Immune Tolerance Network (ITN) is to advance the development of immune tolerance strategies in autoimmunity, allergy, and transplantation by conducting high-quality clinical trials with emerging therapeutic agents. Integrated mechanism-based research is a critical component of these trials that provide new insights into the success or failure of the intervention, as well as further understanding of disease pathogenesis. These results in turn provide the building blocks for further clinical trials and the design of new treatment strategies and incremental advancement towards the tolerance goal.

Central to the ITN's mission is the concept of immunologic tolerance. While specific clinical definitions of tolerance vary across the immune mediated diseases, they all center on differentiating permanent or prolonged improvements in disease that represent a significant clinical benefit over the expected natural history of disease or the standard of care. Conceptual parallels are illustrated in **Figure 1**. In the transplant setting, tolerance is defined as graft acceptance without the need for a continuous immunosuppressive regimen, or a greatly reduced regimen. In allergic disease, tolerance may be defined as prolonged unresponsiveness to antigen challenge or exposure after withdrawal of allergen immunotherapy. For autoimmune diseases, tolerance is reflected in reduced need for disease-modifying therapy or prolonged improvement of disease manifestations, such as retention of residual insulin secretion in type 1 diabetes.

However, at a molecular level, immunologic tolerance represents prolonged or permanent modulation of aberrant immune responses towards a homeostatic state. Immune self-tolerance normally occurs in T and B lymphocytes by central and peripheral mechanisms, reviewed previously (1). Central tolerance involves elimination of lymphocytes with high affinity receptors for self-components, a process that takes place in T lymphocytes and B lymphocytes during cellular maturation in the thymus and bone marrow, respectively. Self-reactive lymphocytes that are not

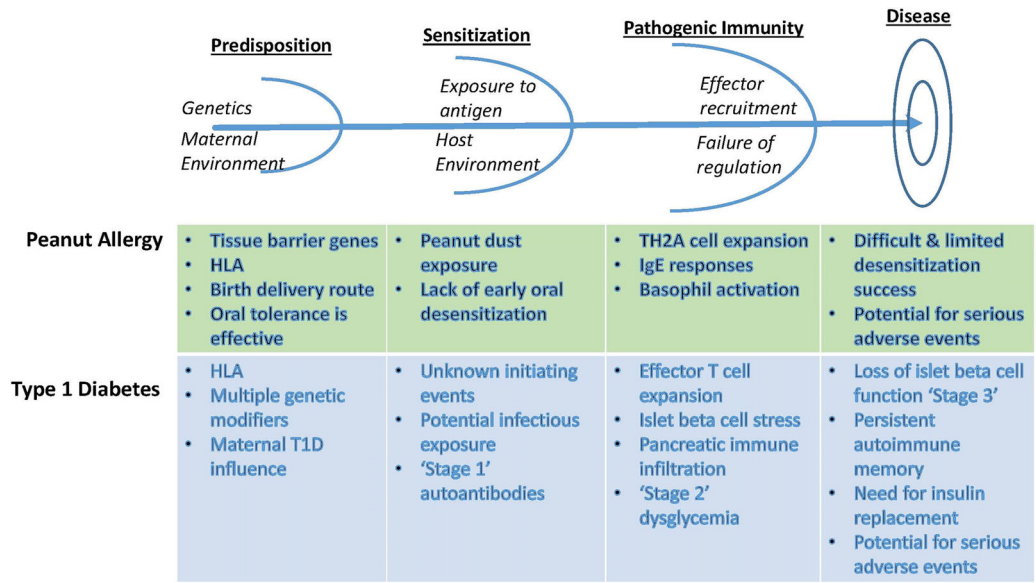

FIGURE 1 | Conceptual parallels between the approach to autoimmunity and allergy tolerance trials, using examples from T1D and from peanut allergy. Identification of early at-risk children allows for prevention strategies that rely on antigen exposure in the context of immune deviation or anergy. After initial antigen sensitization, however, additional measures are required to blunt effector responses to inhibit immune amplification events. Failing this, determinant spreading elicits robust immunity that recruits additional effector pathways and conditions inflammatory innate tissue responses, now requiring combinations of targeted therapeutics to 'reset' the immunological threshold and enable an opportunity to reestablish homeostatic balance between regulatory and effector pathways. Recognition of these differences requires appropriate staging and monitoring in order to select therapeutic options with tolerogenic potential.

eliminated centrally during development exit into the periphery where they are restrained by a variety of tolerance mechanisms, included induction of anergy, cellular exhaustion, and suppression by regulatory T cells, B cells, innate immune cells, and inhibitory cytokines.

In disease conditions, immunologic tolerance can in theory be re-established through a variety of mechanisms which may expand or augment regulatory cells (e.g. Tr1, Treg, Breg) and suppress effector responses (e.g. effector cell depletion, co-stimulation blockade, anti-cytokine therapy) that in tandem act to restore immune homeostasis and disease quiescence. In this review, we provide an overview of ITN successes, challenges, and new strategies to achieve immune tolerance in the fields of allergy, autoimmune disease, and solid organ transplantation. **Table 1** summarizes the ITN trials mentioned in this review. Summaries of all ITN trials are available at www.immunetolerance.org.

BARRIERS TO TOLERANCE

Immunological memory is a hallmark of successful immune responses, and is essential for pathogen surveillance and extinction. It is also a formidable barrier to successful immune tolerance induction. The challenge of reversing pathogenic immune responses in individuals with autoimmune disease and allergy requires not only directed therapy against immune effector cells, but also prevention of recurrent memory responses when therapy is discontinued. This concept also plays a role in transplantation, both through initial heterologous memory and subsequently in dealing with the robust alloimmune response. Innate immune activation, memory, and self-perpetuating inflammatory cascades must be restrained, allowing tissue repair to occur. Therefore, a major focus of immune tolerance strategies is to retain and expand regulatory immune mechanisms, thereby exploiting homeostatic pathways that are intrinsic to a healthy balance of immune effector and regulatory compartments.

This combination of *interrupting* effector mechanisms, *restraining* innate activation, and *boosting* regulation is the central dogma of successful immune tolerance therapy, illustrated in **Figure 2**. Therapies that achieve only one of these goals without the others, as discussed in the examples below, achieve suboptimal or transient clinical benefit. And because immunological memory is very resilient, early intervention – at the time of transplant or early in the immune process prior to the onset of clinical disease in allergy or autoimmunity – can be attempted whenever at-risk individuals can be identified.

Underlying these efforts to rebalance immune effector and regulatory responses is the recognition that drastic immune suppression is accompanied by significant safety concerns, notably infectious and neoplastic risk, as well as life-long drug administration. Consequently, the targets of immune tolerance therapy need to be selective, addressing the ITN's '*interrupting/restraining/boosting*' therapeutic dogma without creating an unacceptable level of danger. Selective targets within the adaptive immune system may involve specific subsets of T or B cells, or even antigen-specific receptors and signaling pathways;

TABLE 1 | Summary of ITN Clinical Trials Included in this Review.

Disease Indication	Trial Name	Intervention	Status	References	ClinicalTrials.gov Identifier
Allergy					
At risk for peanut allergy	LEAP LEAP-On	Peanut	Complete	(2–7)	NCT00329784 NCT01366846
Peanut allergic	IMPACT	Peanut	Complete	(8)	NCT01867671
At risk for atopy	ACTIVATE	Vaginal microbiome seeding	Ongoing		NCT03567707
Grass allergic	GRADUATE	Timothy grass + Dupilumab	Ongoing		NCT04502966
Grass allergic	GRASS	Timothy grass	Complete	(9)	NCT01335139
Cat allergic	CATNIP	Cat allergen + Tezepelumab	Complete		NCT02237196
Transplantation					
Kidney transplant	Mixed Chimerism	Immunosuppression withdrawal following donor hematopoietic stem cell transplant	Complete	(10)	NCT00063817, NCT00801632
Kidney transplant	TEACH	Immunosuppression withdrawal following donor mesenchymal stromal cells	Ongoing		NCT03504241
Liver transplant	LITTMUS	Immunosuppression withdrawal following donor specific Tregs	Ongoing		NCT03577431, NCT03654040
HLA sensitization awaiting kidney transplant	ADAPT ATTAIN	Carfilzomib plus belatacept Daratumumab plus belatacept	Planned Planned		NCT05017545 NCT04827979
Autoimmunity					
Treatment-resistant multiple sclerosis	HALT-MS BEAT-MS	Autologous hematopoietic stem cell transplant Autologous hematopoietic stem cell transplant	Complete Ongoing	(11–13)	NCT00288626 NCT04047628
ANCA associated vasculitis	RAVE	Rituximab	Complete	(14)	NCT00104299
Lupus nephritis	CALIBRATE	Rituximab + Belimumab	Complete	(15)	NCT02260934
Primary membranous nephropathy	REBOOT	Rituximab + Belimumab	Ongoing		NCT03949855
Antiphospholipid syndrome	DARE-APS	Daratumumab	Planned		Pending
New onset type 1 diabetes	AbATE	Teplizumab	Complete	(16–18)	NCT00129259
New onset type 1 diabetes	T1DAL	Alefacept	Complete	(19–21)	NCT00965458
New onset type 1 diabetes	START	Antithymocyte globulin	Complete	(22)	NCT00515099
Lupus nephritis	ACCESS	Abatacept	Complete	(23)	NCT00774852
Multiple sclerosis	ACCLAIM	Abatacept	Complete	(24, 25)	NCT01116427
Psoriasis vulgaris	PAUSE	Ustekinumab + Abatacept	Complete	(26)	NCT01999868

Summaries of all ITN trials are available at www.immunetolerance.org.

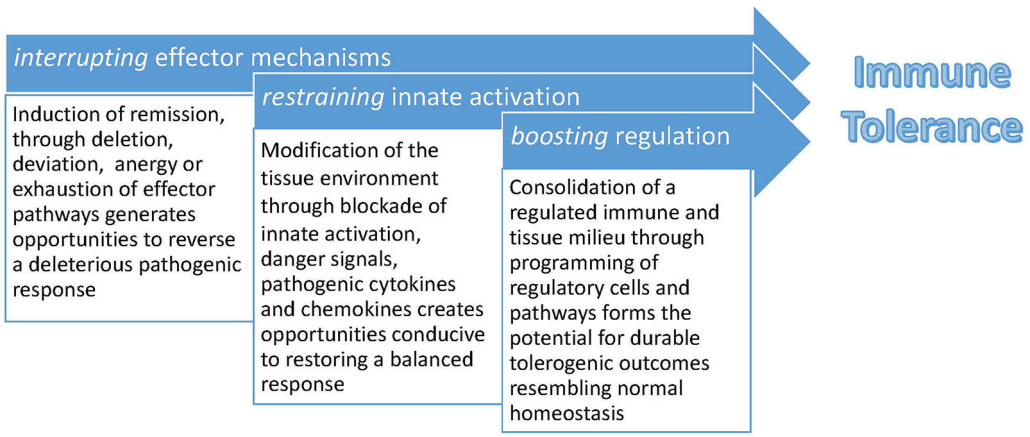

FIGURE 2 | A conceptual framework for induction and maintenance of immune tolerance. Tolerogenic therapies that focus on boosting regulatory immune responses face daunting hurdles in the form of established immunological memory and robust redundant effector activation pathways, involving both adaptive and innate immunity. Creating a host environment conducive to regulation is augmented by first inducing clinical remission and decreasing tissue inflammation. This concept creates a practical platform for combination therapies in which short-term immunosuppressive or immunomodulatory interventions are first employed, followed by emphasis on restoration of immune regulation.

and selectivity in a broader sense may target a limited set of cytokine, chemokine, or activation pathways without global immunosuppression. Rapid development over the last two decades of novel immune therapeutic agents that meet these types of selectivity requirements has enabled the design of clinical trials with aspirations for achieving immune tolerance. Although transplantation, allergy, and autoimmune diseases have many distinct characteristics, conducting such trials within the ITN provides an opportunity to study immune mechanisms and concepts across clinical disciplines while evaluating safety, therapeutic durability, and homeostatic immune reconstitution.

ALLERGY

Desensitization strategies have been a mainstay of allergy therapy for many years, for many types of environmental allergens, including hymenoptera venom and aeroallergens such as pollens, dust mites, and animal dander. On this background of clinical experience showing that allergen specificity of an allergic response that can often be modulated by controlled antigen exposure, the ITN has designed several clinical trials intended to achieve durable, long-lasting allergen desensitization, i.e. tolerance.

IgE-mediated peanut allergy typically develops in the first two years of life and for the majority affected, the disease persists into adulthood. Sensitization to peanut may involve cutaneous and enteral pathways that lead to production of TH2 cytokines and antigen-specific IgE. The Learning Early about Peanut Allergy (LEAP) trial demonstrated that early oral exposure to peanut prevented the development of peanut allergy in atopic infants including those who had already developed sensitization to peanut (2). This protection proved to be durable through the LEAP-On trial, which assessed peanut allergy status after one year of avoidance of peanut following completion of the oral peanut intervention (3). Nearly complete protection in the LEAP cohort of infants with family histories of peanut allergy and eczema, who were at high risk for development of peanut allergy, led to the publication of new public health guidelines for safely introducing peanut protein into the early childhood diet (4). This protection was highly allergen specific, as consumption of peanut did not prevent the development of other food allergies (5).

While it is possible that antigen specific prevention may be applicable for many food allergens, there are practical limitations to introducing multiple allergens in young infants, including the ability of young infants to comply with ingestion of multiple different foods in sufficient quantity to prevent allergic disease (27). Prevention strategies are needed that are personalized based on individual risk of developing a specific allergy. For example, subsequent whole genome sequencing from the LEAP trial revealed a novel association for peanut allergy with a single nucleotide variant in the mucosa-associated lymphoid tissue lymphoma translocation (*MALT1*) gene (6). *MALT1* encodes a paracaspase that acts in response to antigen binding to the T-cell or B-cell receptor leading to NFκB activation (28). The association of *MALT1* with peanut allergy was found to be independent of atopic dermatitis and egg allergy, suggesting that carrier status predisposed to a unique risk for peanut allergy specifically, and correlated with the progressive acquisition of IgE antibodies to multiple allergenic peanut protein components (6). This immunological relationship was further explored by analyzing the development of peanut specific IgE to a broad repertoire of linear epitopes after the second year of life (7). Peanut specific IgE in infants sensitized to peanut and consuming it, however, recognized conformational epitopes without expansion of peanut specific IgE reactivity to linear epitopes. These findings suggest an interaction between genetics, age of peanut exposure, and likelihood of tolerance, opening possibilities for personalized prevention and intervention strategies based on distinct phenotypic and genotypic risk factors.

Observations in another ITN study, the Oral Immunotherapy for Induction of Tolerance and Desensitization in Peanut-Allergic Children (IMPACT) trial, further emphasize this point. IMPACT was a randomized double-blind placebo controlled trial of peanut oral immunotherapy (OIT) in peanut allergic children ages 12-48 months. Successful desensitization at the end of OIT and persistent desensitization after 6 months of withholding OIT was associated with lower baseline peanut specific IgE, particularly in children below the age of 3 years (8). While tolerance was achieved in ~70% of the younger children with low initial IgE, overall the success rate was much lower, indicating an opportunity may exist early in disease during which the host immune response may be more receptive to development of tolerance. Together, LEAP and IMPACT demonstrate that the atopic march, the paradigm for the progression from atopic dermatitis to allergen-specific disease, can be halted or even reversed early in its course for at least a single allergen-specific disease.

These studies illustrate the concept that the younger immune system, not surprisingly, is more amenable to tolerization using antigen desensitization. They also suggest opportunities to attempt tolerization therapy in older, more established allergy, using agents to encourage an immune response that resembles the immature phenotype. Towards that end, two novel strategies are currently under ITN development, one using microbiome immune modulation and another using anti-cytokine agents in combination with desensitization. Microbiome modification is conceptually a way of providing an innate adjuvant to encourage tolerization, and potentially provide a complementary approach to antigen-specific preventative therapy by inhibiting the development of atopic diseases. Differences in environmental exposure and associated alterations of the microbiome (e.g., gut, nasopharyngeal, airway epithelium) have been implicated in the development of asthma and allergic disease (29). In the LEAP trial, colonization with *S. aureus* amongst young participants was associated with increased IgE production, persistence of egg allergy, and inhibition of oral tolerance to peanut (30). In the Copenhagen Prospective Studies on Asthma in Childhood 2010 mother-child cohort, the risk of asthma at 6 years [OR 2.45 (95% CI 1.32 to 4.55), P = 0.004] and allergic sensitization at 18 months [OR 1.68 (95% CI 1.01 to 2.79), P = 0.046] was higher in infants delivered by Cesarean section compared to vaginally-delivered infants. Since the increased risk of asthma was

identified in infants born by Cesarean section whose gut microbiome was less mature at 1 year of age (i.e., retained a Cesarean section microbiome signature), it is possible the infant gut microbiome may be a contributing factor to this predisposition for the development of asthma (31). In addition, in neonates who have a high risk for developing asthma, the gut microbiome produces metabolites, which in murine studies have been shown to increase pulmonary inflammation and decrease regulatory T (Treg) cell abundance in the lung (32). Taken together, these studies suggest that altering the newborn microbiome could favorably impact the risk of allergic and atopic disease. To address this hypothesis, the Vaginal Microbiome Exposure and Immune Responses in C-section Infants (ACTIVATE) trial is studying the impact of vaginal microbiome seeding on the development of allergic disease in infants delivered by Cesarean section (NCT03567707). Longitudinal samples of the gut, skin, nasal, and oral microbiomes will be collected over the child's first 3 years of life to assess for compositional factors as well as changes to the microbiome (e.g., maturation) associated with developing sensitization to food and aeroallergens.

In older individuals, with established allergic responses and mature immunological memory, a strategy to alter the allergenic immune program associated with TH2 T cell immunobiology is an alternative approach. To this end, the ITN GRADUATE trial, which is currently underway (NCT04502966), combines dupilumab (anti-IL4R) and sublingual grass desensitization to induce durable tolerance. This study builds on the previous ITN GRASS trial comparing desensitization to grass pollen between the sublingual and subcutaneous routes (9). Another cytokine strategy, using tezepelumab (anti-TSLP) to alter TH2 developmental programming, has been studied recently in the ITN CATNIP trial as a potential adjunct to desensitization in established allergic patients (NCT02237196). With recent advances in understanding TH2 diathesis, agents such as anti-OX40 (33), or other strategies targeting allergenic T effector cell subsets, known as TH2A cells (34), may provide additional therapeutic opportunities.

TRANSPLANTATION

Hematopoietic chimerism has been utilized to induce tolerance of kidney allografts in animals and humans, based on the foundational discovery that dizygotic cattle twins exhibit stable red cell chimerism and mutually accept skin grafts (35, 36). A proof of concept for chimerism as a robust mechanism of transplant tolerance was provided by recipients of allogeneic hematopoietic stem cell transplant for leukemia who did not require maintenance immunosuppression after kidney transplant from the same donor 3–11 years later (37). The ITN clinical experience, while successful at achieving transient mixed chimerism (2-3 weeks) in haplotype-matched patients, was only able to induce durable immunosuppression free graft survival in a small number of study participants (10). Similar loss of chimerism and subsequent rejection has been seen by others (38, 39), likely indicating a need for improved peripheral deletion of donor-reactive T cell clones and induction of regulatory mechanisms. Use of a donor hematopoietic stem cell product featuring CD8+/TCR- "tolerance-promoting facilitating cells" (40), various regulatory T cell strategies (41–44), or a donor mesenchymal stem cell infusion (the ITN TEACH trial, NCT03504241) are alternatives currently being evaluated for potential for inducing transplant tolerance using therapeutic cell transfer. The ITN LITTMUS trials (NCT03577431, NCT03654040) are currently testing whether infusion of alloantigen-specific regulatory T cells, generated from liver transplant recipient cells collected within the first post-transplant year, can facilitate withdrawal of immunosuppressive anti-rejection medications.

In transplantation, the importance of controlling immunological memory cannot be overemphasized. A significant proportion of transplant candidates have accumulated pre-formed antibodies to HLA antigens as a result of prior sensitizing events such as blood transfusion, pregnancy, or a previous transplant. These patients represent a growing challenge for the transplant community as their sensitized immune system makes it more difficult to find compatible potential donors. The achievement of "HLA desensitization" may be therefore considered a waypost on the road to B-cell tolerance. Trials of B cell depletion using rituximab and obinituzumab, although attractive in theory, were found to be unsuccessful in practice, likely reflecting the inability of these agents to eliminate long-lived plasma cells, as well as to sufficiently diminish memory B cells that are destined to develop into anti-HLA antibody-secreting cells (45, 46). Interestingly, the strategy of targeting antibody-secreting plasma cells alone, using agents highly effective against multiple myeloma, has also been found to be insufficient in producing a durable response (47). Elegant studies in allosensitized non-human primate (NHP) models have now shown that ensuing compensation by expanding germinal centers (GC) following plasma cell depletion underlies the rapid repopulation of plasma cells and consequent rebound in HLA antibody (48). Costimulation *via* the CD28 and CD40 pathways plays a critical role in GC interactions between Tfh cells and B cells (49, 50), and costimulation blockade in NHP has been shown to collapse GC and abrogate the humoral rebound seen after plasma cell depletion (51). Based on these observations, a "dual-targeting" strategy of HLA desensitization consisting of 1) plasma cell depletion (using proteasome inhibitors or anti-CD38 monoclonal antibody) and 2) suppression of the upstream humoral response by costimulation blockade is now being tested in two parallel ITN trials (ADAPT, NCT05017545, and ATTAIN, NCT04827979).

AUTOIMMUNITY

Rational strategies for tolerance induction in autoimmune diseases rely on alignment of pathogenic mechanisms and potential therapeutic targets. In some cases, selective targeting of effector T or B cell compartments may be an attractive way to induce remission, by creating a tissue environment less resistant to subsequent homeostatic regulation; in other cases, heterogeneity among patients with similar diseases may require more personalized strategies to optimize the likelihood of matching the appropriate therapy with an individual subject.

Numerous ITN trials, in diseases such as multiple sclerosis, systemic lupus erythematosus (SLE), anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, type 1 diabetes, and psoriasis, have documented robust changes in immune cell compartments in association with T or B cell targeting, but clinical responses are often transient. Therefore, different ITN trials have been designed to evaluate agents for selective immune depletion, immune modulation, and immune regulation, with combinations of those agents when feasible to assess synergistic mechanisms of action. A few examples that illustrate key concepts are discussed below.

Although over a dozen disease-modifying agents have been approved by the FDA for the treatment of multiple sclerosis (MS), some patients with this disease are resistant or refractory to treatment and continue to relapse and accumulate disability. In treatment-resistant relapsing MS, an immune "reset" can be accomplished with immunoablation followed by autologous hematopoietic stem cell transplant (AHSCT). In this procedure, the pathogenic immune repertoire is eliminated with interruption of autoimmune destruction of myelin in the central nervous system. Clinical trials, including the HALT-MS trial conducted by the ITN, demonstrated high efficacy in aggressive treatment-resistant relapsing MS (11, 12, 52–54). There is evidence that AHSCT alters the immune system upon reconstitution *via* thymic reactivation (55), rebalancing of regulatory and effector immune components (56–59), and T cell repertoire diversification (13). In the HALT-MS trial, dominant CD4+ T cell clones were largely replaced by a new repertoire following transplant, while CD8+ T cell clones re-emerged and better clinical outcomes post-transplant were associated with a more diverse CD8+ T cell repertoire (13). To confirm this result, the BEAT-MS multicenter trial is in progress comparing the efficacy and cost-effectiveness of AHSCT to high efficacy disease modifying agents in treatment-resistant relapsing MS (NCT04047628), with the aim of developing high quality evidence supporting consensus recommendations for utilizing AHSCT to treat resistant forms of relapsing MS (60).

Depletion of specific effector cell populations without the global changes induced by AHSCT are also effective, although with inconsistency in the durability of response. In ANCA-associated vasculitis, ANCA do not appear to cause disease by forming immune complexes or directly binding to tissues. Instead, these autoantibodies are suspected to bind neutrophils, resulting in hyperactivation of neutrophils and formation of neutrophil extracellular traps, which in turn results in vascular damage. The Rituximab for ANCA-associated Vasculitis (RAVE) trial treated patients with severe active granulomatosis with polyangiitis and microscopic polyangiitis with rituximab (anti-CD20), with the goal of depleting antibody-secreting B cells and promoting sustained disease control (14). In this study, rituximab therapy was shown to be non-inferior to cyclophosphamide, the previous standard of care for induction of disease remission. Following induction of clinical remission with rituximab therapy, the majority of patients nevertheless relapsed, indicating that immune tolerance was not achieved despite the initial clinical response. A similarly transient benefit of rituximab therapy was seen in the TrialNet study of type 1 diabetes (61, 62); interestingly, T cell transcripts indicating activation following B cell depletion correlated with reduced preservation of residual insulin secretion, suggesting a need for targeting multiple effector arms of the immune response (63).

Despite an abundance of evidence supporting a critical role for B cells in the pathogenesis of SLE, rituximab-induced B cell depletion has failed in randomized, controlled trials to show clinical efficacy in both non-renal SLE and lupus nephritis (64, 65). One possible explanation for the early failure of rituximab therapy for SLE in clinical trials may be that levels of B-cell activating factor (BAFF) increase following B cell depletion (66). In BAFF-transgenic mice, elevated levels of BAFF rescue autoreactive B-cells and prevent them from becoming anergic (67). To address the hypothesis that inhibition of BAFF following B cell depletion might improve disease control in this setting, the ITN CALIBRATE trial was undertaken to explore in lupus nephritis the efficacy of adding the anti-BAFF agent belimumab to a B cell depletion regimen combining rituximab and cyclophosphamide. Although the percentage of autoreactive naïve B cells were decreased in the group who received belimumab, the addition of a BAFF blocker did not differentiate the clinical outcomes in lupus nephritis (15). A related rituximab and belimumab combination strategy is also being studied in primary membranous nephropathy, where anti-PLA2R autoantibody production is being assessed in the ITN REBOOT trial. In this disease, in contrast to SLE, rituximab therapy has clinical efficacy for some patients. Belimumab will be initiated 4 weeks prior to rituximab therapy based on the observation that circulating memory B cells (CD19+/CD20+/CD27+) increase in numbers after belimumab therapy (68). Thus, initiating belimumab therapy prior to the intervention with rituximab use has been hypothesized to increase the proportion of memory B cells that can be depleted by this CD20-depleting antibody.

Similar to the rationale for transplantation studies noted above, plasma cell targeting is an attractive option for some antibody-mediated autoimmune diseases. Elimination of plasma cells could be beneficial for the treatment of antibody mediated diseases such as antiphospholipid syndrome (APS), a systemic autoimmune disease characterized by thrombotic and obstetric manifestations in individuals with potentially pathogenic antiphospholipid antibodies (69). Standard treatment of APS is lifelong anticoagulation, which is not always effective. Patients with APS often have persistence of antiphospholipid antibodies and are therefore at risk for future thrombosis, shining light on the antibody-secreting cell as a plausible target for effective therapy. A candidate drug for targeting the antibody-secreting plasma cells in APS and other autoimmune conditions is daratumumab, a cytolytic monoclonal antibody developed for the treatment of multiple myeloma. Daratumumab binds to CD38 expressed on plasma cells and is cytolytic for these cells. Small case studies of patients with APS, SLE, and autoimmune hematologic conditions report that treatment with daratumumab reduces autoantibody production in concert with improved clinical manifestations of disease (70–72), providing the rationale for the ITN study of

daratumumab therapy in APS with the goal of eliminating antiphospholipid antibodies.

In parallel with these depletional studies that specifically target the pathologic humoral response, other ITN trials have focused on inducing remission and tolerance in autoimmunity through T cell depletion and modulation. The ITN trials of teplizumab (anti-CD3), alefacept (LFA3-Ig), and anti-thymocyte globulin (ATG) in type 1 diabetes (T1D) are particularly informative. Teplizumab is a monoclonal anti-CD3 antibody, investigated by the ITN for over a decade in several clinical trials. In the ITN AbATE study, treatment with teplizumab delayed the loss of residual insulin secretion in patients with type 1 diabetes in a subset of subjects, with some maintaining insulin secretory function for several years (16). Teplizumab does not deplete T cells; rather, it acts as a partial agonist to induce a series of functional and phenotypic changes. For example, whole blood transcriptomic analysis in AbATE identified *EOMES*, a transcription factor and marker of T-cell exhaustion, as a correlate of changes in insulin secretion (16–18). Moreover, CD8 effector memory T cells were found to have greater expression of EOMES mRNA and higher expression levels of other exhaustion markers, namely KLRG1 and TIGIT, by flow cytometry. These KLRG1+ TIGIT+ CD8 T-cells showed impaired expression of cell cycle genes when activated, suggesting impaired proliferative capacity, and an effector T-cell exhaustion profile that correlated with improved outcomes. These studies indicate a novel mechanistic basis for tolerance, namely induction of lymphocyte exhaustion by a T cell agonist therapy. The TrialNet TN10 trial subsequently extended these findings to a population of high-risk individuals who had anti-islet cell antibodies, a marker of susceptibility to T1D, but who were not yet hyperglycemic. In this study, teplizumab induced a similar T cell exhaustion profile and resulted in an overall delay in the development of T1D by a median of 24 months (73).

The costimulatory CD2 cell surface molecule is most prominently expressed on CD4 T effector memory (Tem) cells and naïve CD8 (19). CD4 T regulatory cells (Treg) express CD2 at lower levels than any other T-cell subset. Targeting this pathway to affect the antigen-specific effector memory response preferentially over the regulatory T-cell compartment was the basis for the ITN T1DAL trial. T1DAL was a clinical trial carried out in children and adolescents with new-onset type 1 diabetes and investigated the clinical efficacy of alefacept, an LFA-3 fusion protein that blocks the costimulatory LFA-3/CD2 interaction (19–21). In this study, 30% of treated subjects retained or improved their insulin secretory function over 2 years, and another 40% showed only modest loss of islet function. Similar to treatment with teplizumab, alefacept treatment was associated with increases in the prevalence of CD8 effector memory cells expressing both KLRG1 and TIGIT; as seen in AbATE, these exhausted CD8 cells correlated with beneficial clinical response. Notably, however, in T1DAL they appeared in the blood approximately 9 months after therapy, following an earlier induction of PD1 on a CD4 Tem population. Both teplizumab and alefacept treated participants demonstrated these changes in effector populations concurrent with relative preservation of regulatory T cells, providing a proof-of-concept for the sequential combination of an induction therapy followed by consolidation of the regulatory response. This conclusion was reinforced by the findings in the ITN START trial (22) which utilized anti-thymocyte globulin in a similar study of patients with early T1D. In START, no therapeutic benefit was observed, and analysis of lymphocyte subsets indicated depletion of multiple cell lineages, notably including both regulatory and effector T cell populations. Retention or boosting of regulatory responses is therefore a major objective of current ITN therapeutic strategies, forming one of the key components in clinical trial designs.

Failure to retain regulatory T cell function may explain the disappointing outcomes in several trials using CD28 costimulatory blockade as a therapeutic tolerance strategy. Abatacept (CTLA4-Ig) is a fusion protein consisting of the extracellular domain of the CTLA4 ligand for CD80/86 coupled to a modified Fc portion of human immunoglobulin G (IgG). It acts by preventing CD80/CD86 on APCs from binding to CD28 on T cells, thereby inhibiting T cell activation and function, and serving as an effective treatment option in adult rheumatoid arthritis and juvenile idiopathic arthritis. ITN trials with abatacept in multiple sclerosis, lupus nephritis, and psoriasis, however, did not demonstrate clinical benefit for induction or maintenance of tolerance (23, 24, 26). Analysis of peripheral blood cells from the participants in the multiple sclerosis trial demonstrated that the relative proportions of activated CD4+ T follicular helper cells and regulatory T cells were both decreased in participants receiving abatacept compared with those receiving placebo (25). Similar changes following abatacept treatment, including loss of regulatory T cells, have been observed by others in a variety of other diseases (74–76). While transient immunomodulatory effects in effector cells from CD28 blockade occur, these require continued drug administration, and therefore the undesirable inhibition of regulatory T cells that also utilize CD28-dependent pathways appears to preclude effective use of abatacept as a tolerance therapy where drug discontinuation is desired.

DISCUSSION

Successful treatment interventions in transplantation, allergy, and autoimmune disease rarely allow for discontinuation of therapy and result in sustained and selective immune tolerance. However, they do occur, and in the context of clinical trials, they provide an opportunity to identify specific therapeutic targets, or combinations of targets, capable of restoring homeostatic immunity. Notably, minimization or extended drug holidays from immunosuppressive therapy also provide clinical benefit, even without achieving the ideal goal of permanent immune tolerance. Ongoing maintenance intervention may be required, for example a small dose of oral peanut (or other allergen) to maintain a non-allergic state in an individual who had previously undergone successful desensitization to that allergen.

Looking across multiple clinical trials and different clinical disciplines in ITN studies, shared patterns emerge that emphasize the need for a combination of *interrupting* effector mechanisms, *restraining* innate activation, and *boosting*

regulation. These objectives can be met through targeting of different cells and pathways in different diseases, whether B cell, plasma cell, T effector, T regulatory, cytokine or other non-lymphocytic effector mechanisms. Early intervention, and mechanism-based stratification of individuals for optimal targeting of particular immune pathways, are likely to improve success rates. Combining antigen exposure in allergic disease and transplantation with selective immunomodulation, and using initial induction strategies in autoimmunity followed by regulatory enhancement, are promising conduits for improving clinical outcomes. In the examples summarized in this article, we have learned that early intervention in the form of antigen introduction can be successful in preventing and treating peanut allergy. In autoimmune disease, immunomodulatory agents targeting T cells and inducing exhaustion pathways can delay clinical disease onset and improve the effector/regulatory T cell balance in T1D, while targeting B cells improves outcomes in ANCA-associated vasculitis. For other autoimmune diseases resistant to B cell depletion or costimulatory blockade, targeting alternative costimulatory pathways and antibody-producing plasma cells in tandem, for example, and combination strategies interrupting key hubs in the adaptive and innate immune response may be required to restore immune tolerance and tissue homeostasis. In solid organ transplantation, success has been partially achieved with an immune reset *via* hematopoietic chimerism, but other strategies including cell-based therapies and molecularly targeted agents are under investigation. Eliminating unwanted immune responses in allergy, autoimmunity, and transplantation and restoring a healthy balance of regulatory and effector immune elements is a formidable goal. Nevertheless, improved understanding of the complexities of the immune system provide opportunities that continue to provide a foundation for future tolerance-inducing strategies and success.

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All authors contributed to the article and approved the submitted version.

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Lymph Node-Targeted Synthetically Glycosylated Antigen Leads to Antigen-Specific Immunological Tolerance

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Inverse vaccines that tolerogenically target antigens to antigen-presenting cells (APCs) offer promise in prevention of immunity to allergens and protein drugs and treatment of autoimmunity. We have previously shown that targeting hepatic APCs through intravenous injection of synthetically glycosylated antigen leads to effective induction of antigen-specific immunological tolerance. Here, we demonstrate that targeting these glycoconjugates to lymph node (LN) APCs under homeostatic conditions leads to local and increased accumulation in the LNs compared to unmodified antigen and induces a tolerogenic state both locally and systemically. Subcutaneous administration directs the polymeric glycoconjugate to the draining LN, where the glycoconjugated antigen generates robust antigen-specific $CD4^+$ and $CD8^+$ T cell tolerance and hypo-responsiveness to antigenic challenge *via* a number of mechanisms, including clonal deletion, anergy of activated T cells, and expansion of regulatory T cells. Lag-3 up-regulation on $CD4^+$ and $CD8^+$ T cells represents an essential mechanism of suppression. Additionally, presentation of antigen released from the glycoconjugate to naïve T cells is mediated mainly by LN-resident $CD8^+$ and $CD11b^+$ dendritic cells. Thus, here we demonstrate that antigen targeting *via* synthetic glycosylation to impart affinity for APC scavenger receptors generates tolerance when LN dendritic cells are the cellular target.

Keywords: lymph node, subcutaneous, tolerance, glycopolymer, regulatory T cell, dendritic cell, lymphatics, co-inhibition

INTRODUCTION

Current treatments for autoimmune and inflammatory diseases are non-curative and rely on broad nonspecific immunosuppression, risking a number off-target effects, complications, and opportunistic infections that limit the long-term use of these strategies. As the underlying mechanisms of immune suppression and the identities of the disease-causing autoantigens and allergens are being increasingly unraveled, antigen-specific therapies are being put through the clinical developmental pipeline to a

greater extent (1, 2). Several strategies to induce a more directed antigen-specific immune response are under investigation (3). Subcutaneously-administered free antigens have been explored, including in clinical trials, in the context of celiac disease, diabetes and multiple sclerosis (4–6). Delivery and formulation approaches have also been explored to direct antigen to APCs for preferential uptake without activation and subsequent tolerogenic education of naïve T cells (7–11). Successful strategies have included coating PLGA microparticles with DEC-205$^+$ DC-targeting antibodies or P-D2 integrin-targeting peptides (8) or encapsulating PLGA nanoparticles with antigen (such as MOG peptide) and immunosuppressive agents such as IL-10 (9) or rapamycin (10) to promote tolerogenic DC maturation. However, these approaches are still limited in efficacy due to immunogenicity of the vehicle or ADAs that can result from repeated dosing.

Antigen glycosylation has been leveraged as an immune-modulatory tool in the context of both vaccination and tolerance (12). Since glycan binding to carbohydrate-binding receptors is a low-affinity event, multivalency of glycosylation has been shown to be beneficial in the optimal engagement of these receptors (13). Covalent attachment of carbohydrate structures from pathogens or cancer cells to immunogenic proteins has been explored to improve the efficacy of activating or tolerogenic vaccines (14). Moreover, antigens modified with glycosylation repeats have been used to target a number of lectin receptors such as the asialoglycoprotein receptor (15), DC-SIGN (16), MARCO receptor (17), and LSECtin (18).

We have shown in prior work that antigens decorated with synthetic glycopolymers of N-acetyl glucosamine (p(GluNAc)) or N-acetyl galactosamine, after intravenous (i.v.) injection, promiscuously target various subsets of hepatic APCs, resulting in antigen-specific tolerance (19, 20). Here, we investigate this approach to target draining lymph node (dLN)-resident APCs, seeking to understand whether tolerance can be induced *via* peripheral subcutaneous (s.c.) administration and to elucidate the mechanisms involved. We show that antigen-p(GluNAc) is retained to a higher extent in the dLNs, improving uptake by APCs and promoting antigen presentation so as to generate a pool of long-lived anergic antigen-specific CD4$^+$ and CD8$^+$ T cells in addition to regulatory T (Treg) cells that attenuate effector T cell responses and maintain tolerance in the face of an inflammatory antigenic challenge. We also explore differences in immunological mechanisms between tolerization *via* the LN, accessed *via* s.c. administration, and liver, *via* i.v. administration, with synthetically glycosylated antigen. Thus, we present a subcutaneously-administered biocompatible inverse vaccine platform that is promising for blunting the response to antigens, such as primary autoantigens, allergens, or protein drugs, opening the approach of glycoconjugate inverse vaccination to a new APC subset with a convenient route of administration.

MATERIALS AND METHODS

Study Design

The objective of this study was to target synthetically glycosylated antigen to LN APCs to induce antigen-specific immunological tolerance, and investigate the molecular mechanisms of tolerance. We delivered p(GluNAc)-conjugated antigen to dLNs *via* s.c. administration, and characterized the antigen distribution, retention and uptake landscape, as well as downstream effects on the antigen-specific T cell response. We furthermore elucidated the contribution of specific APC subsets, T cell regulatory populations, and co-stimulatory signaling axes to the maintenance of tolerance. Flow cytometry and fluorescence microscopy were the primary analytical techniques used, and the OTI and OTII TCR-transgenic system was the main model studied. The number of experimental replicated are indicated in figure legends.

Mice

Mice were maintained in a pathogen-free facility at the University of Chicago. All experiments and procedures in this study were performed with the approval of the Institutional Animal Care and Use Committee at the University of Chicago. Female C57BL/6N mice, aged 7-12 weeks, were purchased from Charles Rivers (strain code: 027). OTI (JAX code: 003831) and OTII (JAX code: 004194) were crossed to CD45.1$^+$ mice (JAX code: 002014) to yield congenically labeled OTI and OTII mice. Batf3$^{-/-}$ mice (also on a C57BL/6 background) were originally a donation from Justin P. Kline's laboratory at the University of Chicago, and subsequently, bred in house.

OVA-p(GluNAc) Synthesis and Characterization

Detailed synthesis and characterization methods can be found in (19). Briefly, p(GluNAc) was synthesized using a reversible addition-fragmentation chain transfer (RAFT) polymerization using an azide-modified RAFT agent, a biologically inert comonomer (N-(2-hydroxypropyl) methacrylamide, HPMA) and the glycosylated methacrylamide N-acetyl glucosamine monomer. We use a copper-free click-based reaction in aqueous solvent at room temperature to conjugate the polymers to antigens to preserve the antigen's tertiary structure and function. To this end, the OVA (Invivogen, vac-pova) is modified at terminal amines with an amine-reactive heterobifunctional bicyclononyne-decorated linker. Upon conjugation, this linker forms a reduction-sensitive chemical bond that is stable in serum but is cleaved when the conjugate encounters the reductive environment of the endosome inside the antigen presenting cell. The polymer ranges in size from 30-60 kDa, and can be visualized on a non-reducing SDS-page gel after conjugation to antigen. Conjugated OVA-p(GluNAc) was separated from unconjugated OVA by size exclusion in PBS buffer and the concentration of conjugated OVA was quantified by boiling the conjugate in reducing Laemmli buffer and running it on a reducing SDS-page gel alongside unmodified OVA samples of known concentrations. Finally, OVA-p(GluNAc) was tested for the presence of endotoxin before being used in tolerance experiments. For the synthesis of the fluorescent OVA_{647}-p(GluNAc) conjugate, Alexa Fluor™ 647 NHS ester (Thermo Fisher Scientific A20006) was first conjugated to OVA before the click linker step.

S.c. Tolerization

Unless otherwise specified, mice were injected s.c. in all four hocks at a dose of 5 μg of OVA antigen and volume of 20 μL per hock, under isoflurane anesthesia.

Whole-Organ Fluorescence Imaging of LNs

15 h after s.c. hock injections, whole cardiac perfusion was performed with PBS (pH= 7.4) under isoflurane inhalation anesthesia, after which the liver and draining axillary and popliteal LNs were isolated. The organs were cleaned by removing extra fatty tissue and washed in PBS to remove blood that could contribute to auto-fluorescence. They were imaged on the *In Vivo* Imaging System (IVIS, PerkinElmer) using an excitation wavelength of 630 nm and an emission wavelength of 650 nm. For the time-dependent antigen retention study, mice were sacrificed without perfusion at timepoints of 1 h, 6 h, 15 h, 24 h, 48 h and 72 h post-injection and draining popliteal LNs were isolated and imaged using the same procedure described above.

LN APC Biodistribution

24 h after s.c. hock injection, mice were sacrificed and draining LNs were isolated. The LN capsule was gently poked with 25 G needles. They were digested at 37°C, first with 1 mg/mL Collagenase IV and 40 μg/mL DNAse1 for 30 min, followed by 3.3 mg/mL Collagenase D and 40 μg/mL DNAse1 in 300 μL of DMEM (Gibco 11966025) supplemented with 1.2 mM $CaCl_2$ for 15 min with magnetic stirring. The LNs were gently pipetted 100 times using an electronic pipette. An equal volume of ice-cold 10 mM EDTA in PBS supplemented with 1% FBS was added to the digestion mixes to quench the enzymatic reaction for a final concentration of 5 mM EDTA, followed by pipetting for another 100 times. The cell suspensions were filtered through a 70 μM filter to generate a single cell suspension which was stained for flow cytometry. Antibodies against the following markers were used: CD45 – APC-Cy7 (BioLegend, 103116), CD31 – BV421 (BioLegend, 102423), GP38 – PE-Cy7 (eBioscience 25-5381-82), CD21/35 – FITC (BioLegend, 123407), B220 – BUV496 (BD Biosciences 564662), CD3e – BUV395 (BD Biosciences 563565), CD11c – PE (BioLegend 117308), CD11b – BV785 (BioLegend 101243), CD8 – BUV737 (BD Biosciences 612759), CD103 – PE (BD Biosciences, 561043), CD169 – BV421/BV605 (BioLegend 142421/142413), MerTK – PerCP-eF710 (eBioscience 46-5751-82), CX3CR1 – PE (BioLegend 149005), F4/80 – BUV395 (BD Biosciences 565614), and MHCII – FITC/PerCP-Cy5.5 (BioLegend 107605/BD Biosciences 612759).

Whole Mount Confocal Imaging of LN

Popliteal lymph nodes were fixed in Zinc (pH= 6.5) at 4°C for 24 h. The LNs were washed with tris buffered saline (TBS) and permeabilized with filtered tris buffered saline (TBS) 1% Triton X-100 5% DMSO (pH= 7.4) for 12 h at RT to degrade intracellular fat that could interfere with the staining. The LNs were washed and gently digested with a mixture of Collagenase IV (1 mg/mL), DNAse1 (40 μg/ml) and Collagenase D (3.3 mg/mL) enzymes in 0.5% casein in TBS supplemented with 5 mM $CaCl_2$ for 45 min at room temperature. LNs were incubated with unlabeled or biotinylated primary antibodies at 1 μg/mL in 0.5% casein in TBS overnight at 4°C. 10 μg of DNAse1 was added to the primary antibody mix as an added precaution. After thoroughly washing with 0.1% Tween TBS, followed by TBS, the LNs were gently dried and stained with secondary labeled or streptavidin conjugated $F(ab)_2$ at 3.75 μg/mL in 0.5% casein in TBS overnight at 4°C. The LNs were thoroughly washed, dried, and dehydrated by sequentially washing in 70%, 95% and finally 100% ethanol. LNs were gently compressed on a microscopy slide, mounted with 25 mg/mL of propylgalate in a 2:1 solution of benzyl benzoate in benzyl alcohol (BABB). The cover slip was placed on the LN and edges were sealed using silicone glue. The mounted LNs were imaged using an Olympus confocal microscope equipped with CellSense software. Images were acquired using four lasers (488 nm, 594 nm, 647 nm and 750 nm excitation wavelengths) and a confocal stack, and analyzed using Imaris 9.1.2 software.

Adoptive Transfer of OTI $CD8^+$ and OTII $CD4^+$ T Cells

$CD8^+$ T cells were isolated from the spleen and s.c. LNs (axillary, brachial, inguinal, popliteal, cervical) of OTI mice using the EasySep $CD8^+$ isolation kit (Stemcell 19853). Similarly, $CD4^+$ T cells were isolated from the spleen and s.c. LNs of OTII mice using the EasySep $CD4^+$ isolation kit (Stemcell 19852). Spleens were first mashed into a single cell suspension and lysed with ACK lysis buffer (Gibco A1049201). LNs were digested with 1 mg/mL Ca^{2+} supplemented Collagenase D (Roche 11088866001) for 45 min at 37°C and gently mashed into a single cell suspension. Suspensions from the LNs and spleen were pooled and subjected to magnetic cell isolation using the kits. The OTI and OTII cells were labeled with 1 μM CFSE (eBioScience 65-0850-84) for 6 min at RT, washed with sterile PBS buffer, quantified and resuspended in saline buffer for injection. $5x10^5$ - $1x10^6$ cells of each OTI and OTII cells were injected into mice *via* i.v. tail vein injection.

Challenge Following Adoptive Transfer and Tolerization

Mice received an inflammatory s.c. challenge of 20 ug EndoFit OVA (InvivoGen vac-pova) and 50 ng LPS (Sigma) total in all four hocks under isoflurane anesthesia. Mice were sacrificed under CO_2 inhalation 5 days following challenge.

Preparation of Cell Suspensions for Flow Cytometry Analysis

Draining s.c. LNs (axillary and popliteal) and the spleen were isolated from mice. Spleens were first mashed into a single cell suspension with plain DMEM media (Gibco 11966025), filtered through 70 μM cell strainers, and lysed with ACK lysis buffer (Gibco A1049201). LNs were digested with 1 mg/mL Ca^{2+} supplemented Collagenase D (Roche 11088866001) for 45 min at 37°C and gently mashed into a single cell suspension, also with DMEM and through 70 μM cell strainers. The cells were

resuspended in IMDM media (Gibco 12440053), supplemented with 10% FBS and 1% Penicillin-Streptomycin (Gibco 15140122), and counted using a LUNA automated fluorescent cell counter (Logos biosystems). Cells were seeded at a count of $1x10^6$ - $3x10^6$ per well in 96-well round-bottom plates for subsequent antibody staining for flow cytometry. Antibodies against the following markers were used: CD3 – BUV395 (BD Biosciences 563565), CD8-BUV737 (BD Biosciences 612759), CD4 – BUV496 (BD Biosciences 612952), Foxp3 – FITC (BD Biosciences 560403), CD25 – BV605 (BioLegend 120235), ST2 – BV421 (BD Biosciences 566309), Lag3 – PerCP-Cy5.5 (BD Biosciences 564673), CTLA4 – PE-Cy7 (eBioScience 17-1522-82), IFNγ – APC (BioLegend 505810), TNFα – BV605 (BioLegend 506329), IL-2 – FITC (BioLegend 503806), IL-10 – APC-Cy7 (BioLegend 505036), PD-1 – BV711 (BioLegend 135231), Tim3 – PE (BD Biosciences 566346).

Ex Vivo Antigen-Specific Restimulation

LN and spleen single-cell suspensions were seeded at a count of $1x10^6$ - $3x10^6$ per well in non-tissue culture treated round-bottom 96-well plates (Celltreat 229590), and stimulated *ex vivo* at 37°C for 2 h with either $OVA_{257-264}$ peptide (Genscript) at a final concentration of 1 μg/mL, or $OVA_{323-339}$ peptide (Genscript) at 2 μg/mL, followed by Brefeldin A at a final concentration of 5 μg/mL for another 4 h. The cells were then washed with PBS before proceeding with cytokine antibody staining for flow cytometry. For long-term restimulations, grade V OVA (Sigma A5503) was added to cells at a final concentration of 100 μg/mL for 4 days. The culture supernatant was collected and frozen for subsequent cytokine ELISA (ThermoFisher Scientific 88-7314-77) and LegendPlex™ (BioLegend 741044) assays.

In Vivo Blockade of Co-Stimulatory Molecules

Mice were administered *via* i.p. injection 250 μg of either αLag-3 (BioXCell BE0174, clone C9B7W), αPD-1 (BioXCell BE0146, clone RMP1-14) or αCTLA-4 (BioXCell BE0164, clone 9D9) on days 1, 3, 5, 7, 9 and 11 for a total of 6 injections.

In Vivo Macrophage Depletion

For the depletion study in **Figure S5A**, mice were treated s.c. in all four hocks with 250 μg of αCSF1R or an isotype IgG2a control once only on day 0 and sacrificed on day 7 to evaluate macrophage depletion. For the experiment described in **Figure 5A**, mice were treated s.c. in all four hocks with 250 μg of αCSF1R (BioXCell BE0213, clone AFS98) or an isotype IgG2a control (BioXCell BE0089, clone 2A3) on days 0, 3, 6 and 9.

Ex Vivo DC Sorting and Priming

Pooled s.c. LNs (axillary, brachial, inguinal, popliteal, cervical) were isolated from wild-type mice, and digested into a single-cell suspension as described in the "LN APC biodistribution" section above. All reagents were kept sterile and all procedures were handled in a biosafety hood when possible. The cell suspension was washed with MACS buffer and the following biotinylated antibodies were added at a final concentration of 2 μg/mL to deplete specific cell populations: αCD3 (T cells), αCD19 (B cells), αB220 (B cells), αGr-1 (neutrophils), and αNK1.1 (NK cells). The cells were washed with MACS buffer, resuspended with Dynabeads Biotin Binder (Invitrogen 11047), and placed in a magnet. The depleted LN suspension was carefully pipetted out of the tube and washed with MACS buffer before proceeding with antibody staining for FACS. The following antibodies were added at these specified dilutions in FACS buffer: Streptavidin – APC-Cy7 (1:400, BD Biosciences 47-4317-82), CD64 – PE-Cy7 (1:100, BioLegend 139314), F4/80 – PE-Cy7 (1:100, BioLegend, 123114), CD11c – APC (1:200, BioLegend 117310), MHCII – PacBlue (1:800, BioLegend, 107620), CD8α – PerCP-Cy5.5 (1:200, BioLegend, 100734), CD103 – PE (1:100, BD Biosciences, 561043), and CD11b – BV510 (1:400, BioLegend 101263). The cells were washed before staining with near-IR Live-Dead dye in PBS, and resuspended in MACS buffer for sorting. The cells were then sorted into four populations: $CD8^+$ resident ($CD11c^+MHCII^{int}CD8^+CD11b^-$, denoted as $CD8^+$ rDC1), $CD103^+$ migratory ($CD11c^+MHCII^{high}CD103^+CD11b^-$, denoted as $CD103^+$ mDC1), $CD11b^+$ resident ($CD11c^+MHCII^{int}CD8^-CD11b^+$, denoted as $CD11b^+$ rDC2) and $CD11b^+$ migratory ($CD11c^+MHCII^{high}CD103^-CD11b^+$, denoted as $CD11b^+$ mDC2) (see **Figure S5D** for the gating strategy). The sorted cells were collected in sterile RPMI media supplemented with 10% FBS, 1% Penicillin-Streptomycin, 0.1% Gentamicin and 50 μM β-mercaptoethanol. The DC populations were counted and plated at a number of 2.7×10^4 per well in triplicates in a 96-well round bottom plate. Each population was then stimulated in a 1:1 ratio with CFSE-labeled OTI and OTII cells in the presence of 2 μM of unmodified OVA or OVA-p (GluNAc) at 37 ^{0}C. Three days later, the cells were harvested, and the OTI and OTII cells were analyzed for proliferation and activation ($CD44^+$).

Ex Vivo Culture of Primary LN-LECs and Priming

LNs (axillary, brachial, popliteal, inguinal, cervical) were isolated from female WT C57BL/6 mice into plain RPMI medium. They were gently poked with 29G1/2 needles and transferred into digestion media made up of 0.25 mg/mL Liberase DH and 200 Kunitz/mL DNAse1 in RPMI media for a total of 1 h at 37°C. Every 10-15 min, the LNs were poked and the digest was pipetted up and down. At the end of 45 min, a single cells suspension is obtained, and filtered through a 70 μM cell strainer into a 50 mL conical. This was spun down, resuspended in 10 mL of αMEM media containing 1% P/S and 10% FBS and seeded into a T75 tissue culture flask at 37°C. The T75 flask was coated with a mixture of 10 μg/mL collagen I and 10 μg/mL human plasma fibronectin in 1x PBS for 30 min at 37°C prior to transferring the cells. The cells were washed with 1x PBS 24 h and 72 h post-isolation and 10 mL of fresh complete αMEM media was replaced. At day 5 post-isolation, the adhered lymph node stromal cells (~85% LECs) were detached from the surface of the T75 flask by first washing with 1x PBS and adding accutase for ~7 min at RT.

The detached cells were transferred into a 50 mL conical, spun down and resuspended in complete αMEM media. These were counted and plated at a density of 2.7×10^4 per well in a flat-bottom 96-well plate. The LECs were stimulated in a 1:1 ratio with CFSE-labeled OTI and OTII cells in the presence of 2 μM of unmodified OVA or OVA-p(GluNAc) at 37 ^{0}C. Three days later, the cells were harvested, and the OTI and OTII cells were analyzed for proliferation and activation ($CD44^+$).

Statistical Analysis

Statistically significant differences between experimental groups were determined using Prism software (version 6.07, GraphPad). All n values and statistical analyses are stated specifically in the figure legends for all experiments. For most experiments, a one-way or two-way ANOVA, followed by Tukey's or Dunnett's post-hoc test was used. Comparisons were significant if $p < 0.05$. For two-group comparisons, unpaired Student's T test was used.

RESULTS

Our previous work describes the synthesis of antigen-glycopolymer conjugates composed of synthetic polymers synthesized from N-acetylglucosamine-decorated monomers conjugated to protein or peptide antigens *via* a self-immolative linker that cleaves in response to intracellular stimuli (19) (**Figure 1A**). When injected i.v., our antigen-N-acetylglucosamine glycopolymer (p(GluNAc)) conjugates accumulate in the liver and are taken up by hepatic APCs. Upon delivery to hepatic APCs, our self-immolative linker is cleaved from the antigen, which releases the conjugated antigen in its unmodified form to allow efficient antigen processing and presentation by hepatic APCs (19). Here, we seek to understand the nature and extent to which LN APC populations can induce antigen T cell non-responsiveness and regulation when collecting the glyco-antigen under homeostatic conditions.

Antigen-p(GluNAc) Conjugate Injected Subcutaneously Accumulates in the Draining Lymph Nodes Where it Targets Various Subsets of Antigen Presenting Cells

We first determined whether s.c. injected glyco-polymerized antigen, in this case ovalbumin (OVA)-p(GluNAc), is specifically retained in the draining LNs (dLN), which we expected due to its optimal size and molecular weight (~100 kDa) for lymphatic uptake (21). Indeed, we were able to detect OVA-p(GluNAc) in the draining axillary and popliteal LNs (dLNs), using whole-organ fluorescence imaging, only when injected s.c. in the hocks but not after an i.v. injection (**Figures 1B, C**). Conversely, OVA-p(GluNAc) was only detected in the liver when injected i.v. but not s.c. (**Figures 1B, C**). We also verified that no antigen remained at the site of immunization 72 h after injection (**Figure S1A**). This demonstrates the versatility and unique trafficking profile of our synthetically glycosylated antigen platform depending on the injection route.

Following s.c. injection of an equivalent antigen dose and visualization of the fluorescence in the dLNs at different timepoints after injection, we found that OVA-p(GluNAc) localizes to the dLNs to a higher extent than unconjugated OVA (**Figure 1D**). The higher accumulation of OVA-p(GluNAc) is expressed in two ways: as absolute protein content calculated from a dose-radiant efficiency standard curve, and as a percent of the initially injected antigen dose per hock of 5 μg (**Figure 1E**). We detected a 17-fold difference in antigen accumulated (maximum at time = 8 h), and a 10-fold difference in the area under the curve, in the favor of OVA-p(GluNAc) (**Figure 1E**). Increased antigen retention in the first few days of immunization is especially important under unadjuvanted conditions where a higher antigen dose and availability need to trump transient TCR-pMHC interactions for fruitful T cell stimulation to occur (22, 23).

After confirming that antigen-p(GluNAc) accumulated in the dLN, we verified whether antigen was taken up by APCs in the LN microenvironment. We conducted a biodistribution experiment in which we assessed the types of APCs that took up antigen-p(GluNAc) and the extent to which they did 15 h after s.c. injection. OVA-p(GluNAc) was taken up by different APC types, reported as % OVA^+ within each APC subset (**Figure 1F**) or mean fluorescence intensity (MFI) of OVA^+ cells (**Figure 1G**). These APCs included various subsets of macrophages, dendritic cells and lymphatic endothelial cells (LECs) that efficiently take up antigen due to their strategic location within the LN, their phagocytic ability and expression of scavenger receptors (24–28). Using multi-parameter flow cytometry, we elucidated the contribution of specific APC subsets that took up antigen after administration of OVA-p(GluNAc). Among these were APCs found at and surveilling the subcapsular sinus such as LECs (26), $CD169^+$ subcapsular sinus macrophages (27) and $CD11b^+$ resident DC2s (24) as well as APCs that are more deeply located in the medullary or cortical regions of the LN, such as the $CD169^+$ medullary macrophages (29), cross-presenting resident $CD8^+$ DC1s (30) and T cell zone $CX3CR1^+Mertk^+$ macrophages (28). These results confirm that antigen-p(GluNAc) can traffic and be taken up by APCs located at different locations within the LN for subsequent processing and presentation (**Figures 1F, G**).

To obtain a visual confirmation for our flow cytometry results, we isolated popliteal LNs from mice that had been injected s.c. in the hind hocks with fluorescently-labeled OVA_{647}-p(GluNAc) and imaged whole mounts on a confocal microscope. We stained APCs using a combination of CD11c and CD11b for non-cross presenting DC2s (**Figure 1H**), or CD11c and CD8 for resident cross-presenting DCs (**Figure 1I**), or CD169 for subcapsular sinus and medullary macrophages (**Figure 1J**). We also stained for the basement membrane and lymphatics using antibodies to collagen IV and Lyve1, respectively (**Figures 1H, J**). OVA-p(GluNAc) was found to promiscuously co-localize with all the APC subsets imaged and mentioned above, consistent with our flow cytometry results and indicating that the mechanism of action is not preferential targeting of specific APC subsets but increased antigen uptake by LN APCs in general.

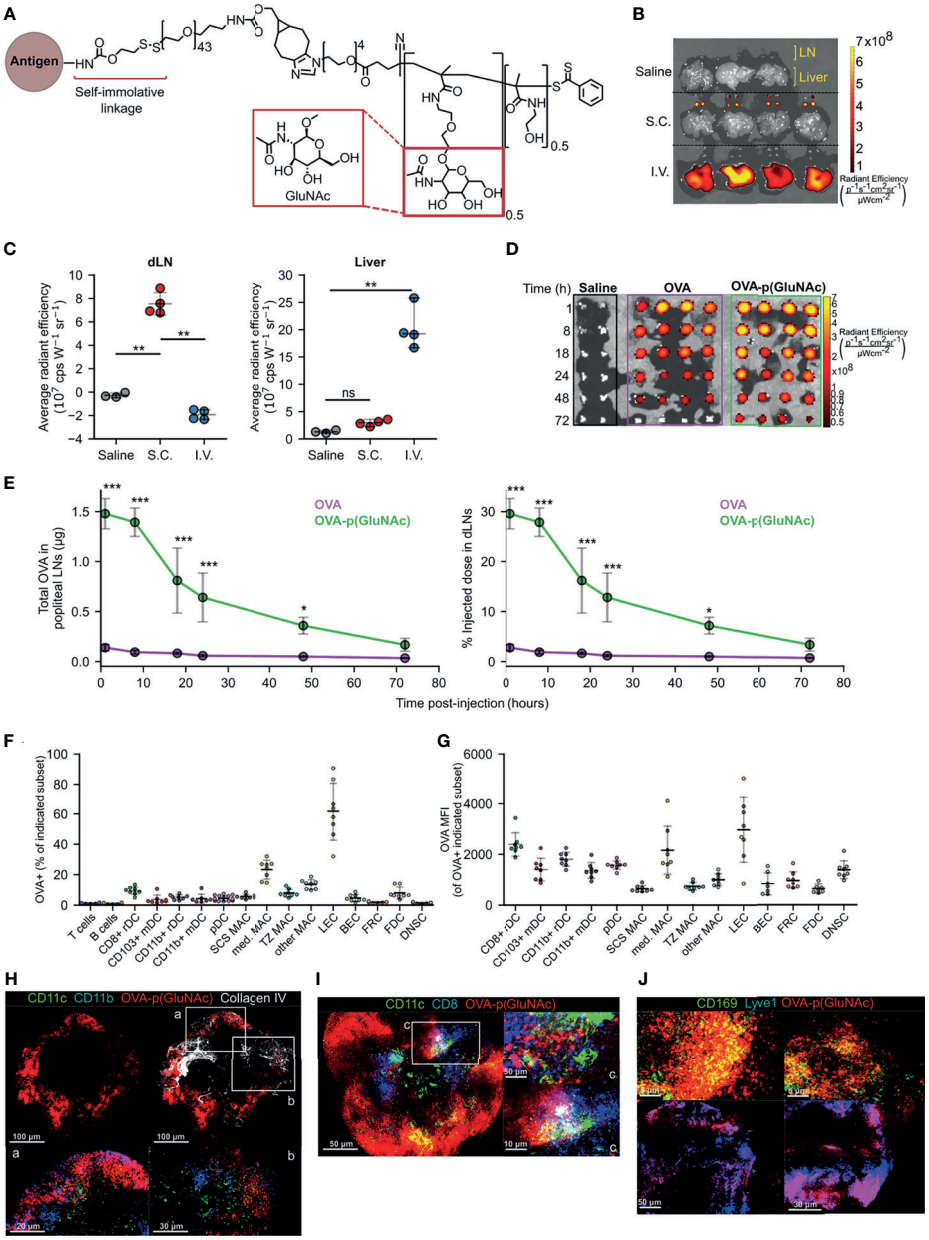

FIGURE 1 | **(A)** Structure of p(GluNAc) conjugated to an antigen lysyl side chain amine *via* a self-immolative linker. **(B, C)** Accumulation of OVA_{647} or OVA_{647}-p(GluNAc) in the dLNs and livers 15 h after s.c. or i.v. injection. **(B)** Representative NIR images of dLNs and liver. **(C)** Average NIR intensities of dLNs and livers. **(D, E)** Mice were injected with saline or 5 µg of OVA_{647} or OVA_{647}-p(GluNAc) s.c. and the draining popliteal LNs were isolated and imaged at various timepoints between 1-72 h post-injection n (dLN)= 2 for saline and 4 for OVA_{647} or OVA_{647}-p(GluNAc) at each timepoint. **(D)** Representative NIR images of dLNs. **(E)** Quantification of OVA accumulation in dLNs, expressed as µg (left) or % of initial injected dose (right). **(F, G)** Flow cytometry analysis of LN cells that took up OVA_{647}-p(GluNAc) 15 h after s.c. injection of 20 µg of OVA_{647}-p(GluNAc). DC, dendritic cell; pDC, plasmacytoid dendritic cell; MAC, macrophage; SCS, subcapsular sinus; med, medullary; TZ MAC, T cell zone macrophage; LEC, lymphatic endothelial cell ($CD45^-CD31^+gp38^+$); BEC, blood endothelial cell ($CD45^-CD31^+gp38^-$); FRC, fibroblastic reticular cell ($CD45^-CD31^-gp38^+CD21/35^-$); FDC, follicular dendritic cell ($CD45^-CD31^-gp38^+CD21/35^+$); DNSC, double negative stromal cell ($CD45^-CD31^-gp38^-$). **(F)** Percent of each cell subset that is OVA^+. **(G)** OVA_{647} MFI of each OVA^+ cell subset. **(H–J)** Representative whole mount confocal images of immunostained dLNs after s.c. injection of OVA_{647}-p(GluNAc) (red). **(H)** LNs stained versus CD11c (green), CD11b (blue) and Collagen IV (white), 8 h post-injection (p.i.). $CD11c^+CD11b^+$ DCs are shown in aqua and co-localized OVA-p(GluNAc) is shown in magenta. Scale bar ranges from 20-100 µm and is indicated in each panel. **(I)** LNs stained versus CD11c (green) and CD8 (blue) 18 h p.i. $CD11c^+CD8^+$ double positive DCs are shown in aqua and co-localized OVA-p(GluNAc) is shown in magenta. Scale bar ranges from 5-50 µm and is indicated in each panel. **(J)** LNs stained versus CD169 (green, top) and Lyve1 (blue, bottom). Co-localized OVA-p(GluNAc) with $CD169^+$ macrophages is shown in yellow (top), and OVA-p(GluNAc) co-localized with the lymphatics is shown in magenta (bottom). Scale bar ranges from 5-50 µm and is indicated in each panel. Data represent mean ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ns is not significant by one-way ANOVA using Tukey's *post hoc* test in **(C)**, and two-way ANOVA using Sidak's *post hoc* test in **(E)**.

Antigen-p(GluNAc) Leads to Antigen-Specific CD4$^+$ and CD8$^+$ T Cell Tolerance, Induction of Regulatory Subsets and Hypo-Responsiveness Upon Antigenic Challenge

We used the OTI/II OVA-reactive transgenic T cell receptor (TCR) model to assess the impact of s.c. administration of OVA-p(GluNAc) on the immune response. We adoptively transferred naïve CD45.1$^+$ OTI CD8$^+$ and OTII CD4$^+$ T cells into mice one day before they were injected s.c. with OVA-p(GluNAc) or saline as unimmunized control, and compared that to i.v. administered OVA-p(GluNAc), as we have published (19). We first determined the optimal dose at which OVA-p(GluNAc) was tolerogenic through the s.c. route. We also compared the effect of immunizing mice one vs. two times with the same molecule. We challenged mice with OVA and LPS 9 days following injection (for mice that received one dose) or 9 days following the second dose (for mice that received two doses) and assessed the OVA-specific immune response 5 days after challenge (**Figure 2A**). We observed a strong dose-dependent response in inhibiting OVA-specific CD8$^+$ T cell proliferation in the dLNs (**Figure 2B**). In mice that received one dose, significantly fewer OTI cells were recovered from the challenge site dLNs of mice that received a mid (5 μg) or higher (20 μg) dose, but not a low (1 μg) dose. The same dose-dependent reduction in OTI was observed in mice that received two doses, with lowest OTI recovery in the mice that received the highest dose (2 x 20 μg), indicating that clonal deletion was more effective with a higher dose of antigen (**Figure 2B**). These results were consistent with OTI numbers recovered from the spleen, showing that even though T cell education takes place locally in the s.c. dLNs, a systemic tolerogenic response is generated (**Figure S2A**). Furthermore, it was necessary to increase the s.c. dose in order to attain the tolerogenic behavior observed with one dose of an i.v. injection (19), suggesting that a higher threshold to suppression exists in the LN and peripheral lymphatics compared to the liver and also that antigen dose is an important modulating factor (31, 32).

We then focused on the OTI and OTII cell phenotypes in experiments performed at the optimized dose of 20 μg s.c. and in the prime-boost regimen that generated the most effective OTI antigen non-responsiveness, and we assessed the tolerogenic responses induced by OVA-p(GluNAc) compared to unconjugated OVA. In this context, tolerance induction is characterized by an abrogated T-cell response to antigenic challenge and an enrichment of antigen-specific Treg cells. Five days post-challenge on day 22, s.c. prophylactic tolerization with OVA or OVA-p(GluNAc) both resulted in a significant reduction in OTI CD8$^+$ T cell proliferation in the dLNs compared to untreated saline controls (**Figure 2C**). This result was comparable to that obtained in the spleen (**Figure S2B**). Even though OVA-p(GluNAc) did not lead to a significantly lower OTI recovery compared to unmodified OVA, it induced a number of tolerogenic signatures distinct from OVA-educated T cells. OTI cells primed with OVA-p(GluNAc) expressed significantly higher levels of co-inhibitory receptors, including PD-1 and Lag-3, compared with OVA (**Figures 2D, E**). OTI cells from the OVA-p(GluNAc) group also highly expressed Tim-3, another co-inhibitory marker of exhaustion (**Figure 2F**). OVA-p(GluNAc) also induced a sizeable subset of OTI cells that co-express PD-1 and Tim-3 (**Figure S2C**), known to mark terminally exhausted cells in the context of tumors and chronic viral infections (33). Additionally, OTI cells from both the OVA and OVA-p(GluNAc) groups had significantly down-regulated the surface expression of their TCR, indicative of a self-inhibitory and anergic response (**Figure 2G**). Upon restimulation of OTI cells isolated from OVA-p(GluNAc)-treated mice with their cognate peptide $OVA_{257-264}$ peptide *ex vivo*, a similar fraction produced the pro-inflammatory cytokine IFNγ (**Figure S2D**) but to a significantly lower extent illustrated by a 2-fold reduction in IFNγ MFI of the secretors (**Figure 2H**). OTI cells from the OVA-p(GluNAc) group secreted significantly higher levels of IL-10, an immunosuppressive cytokine known to play important roles in the induction and maintenance of tolerance (**Figure 2I**) (34).

We observed similar tolerogenic effects exerted in the OTII CD4$^+$ T cell compartment. Upon antigenic challenge, fewer OTII cells were recovered from the dLNs in both the OVA and OVA-p(GluNAc) treated mice compared to untreated saline controls (**Figure 2J**). The OVA-p(GluNAc) treatment induced significantly higher CD4$^+$ antigen-specific Foxp3$^+$CD25$^+$ Tregs (**Figure 2K**), as well as Foxp3$^+$ST2$^+$ Tregs (**Figure 2L**), both with major roles in tolerance (35). The OTII cells tolerized with OVA-p(GluNAc) more highly expressed co-inhibitory molecules such as Lag-3 (**Figure 2M**) and CTLA-4 (**Figure 2N**) and also down-regulated their TCR (**Figure 2O**). Next, we evaluated the effector function of the OTII cells upon *ex vivo* antigen reencounter, and detected cytokines either (1) produced by cells isolated from dLNs and spleen using flow cytometry after a 6-hour culture with their cognate $OVA_{323-339}$ peptide, or (2) secreted into the culture supernatant using the LegendPlex assay after a 3-day culture with full OVA protein. LN cells from OVA-p(GluNAc)-treated mice had significantly reduced IL-13 production into the supernatant, suggesting that this treatment can also be useful in suppressing Th2-mediated reactions such as allergies (**Figure 2P**). There were also lower levels of Th17 cytokines, IL-17 and IL-22 secreted (**Figures S2E, F**). Furthermore, OTII cells from both the LN and spleen produced markedly lower levels of Th1 pro-inflammatory cytokines such as IFNγ, IL-2 and TNFα, indicating an ablation of their effector response (**Figure 2Q**). OVA-p(GluNAc) suppressed the presence of polyfunctional CD4$^+$ T cells, measured by their ability to produce more than one cytokine, pointing to a dysfunctional state. The OTII cells also produced higher IL-10 levels (**Figure 2R**). Thus, we demonstrated that s.c. treatment with p(GluNAc) conjugated antigen generates antigen-specific tolerance, characterized by deletion, upregulation of surface co-inhibitory molecules, induction of both CD25$^+$ (IL-2 receptor) and ST2$^+$ (IL-33 receptor) Tregs, and an abrogation of broad-spectrum effector cytokines upon antigenic challenge.

LN-Targeted Antigen-p(GluNAc) Conjugate Induces Tolerogenic Memory *via* CD8$^+$ Regulatory Subsets That Can Suppress Adoptively Transferred Effector CD4+ T Cells

We sought to further evaluate the mechanisms of action of LN-targeted OVA-p(GluNAc) by assessing suppressive populations

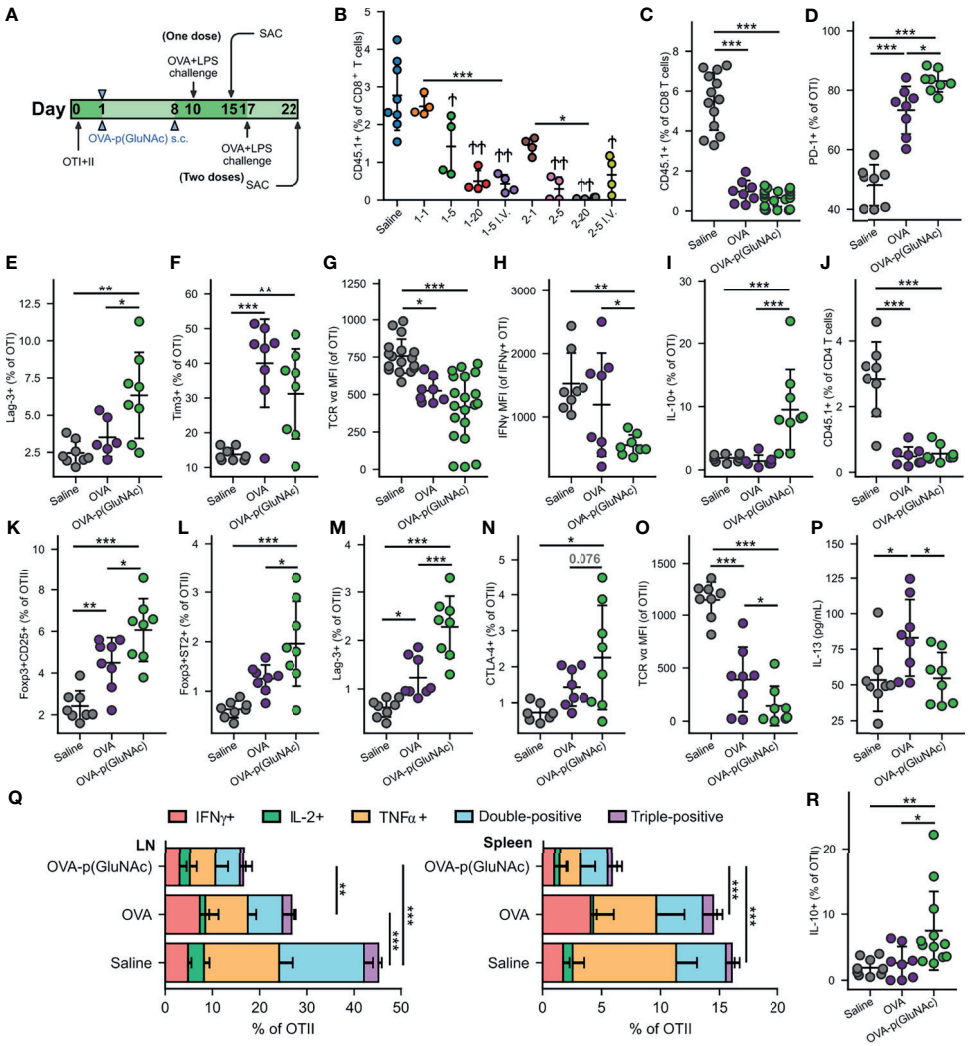

FIGURE 2 | **(A)** Experimental timeline of the dose-efficacy study, n=4. CD45.2$^+$ mice that had received an adoptive transfer of both OTI (CD45.1$^+$CD3$^+$CD8$^+$) and OTII (CD45.1$^+$CD3$^+$CD4$^+$) T cells *via* i.v. injection, were treated with saline or a low, mid or high dose of OVA-p(GluNAc) s.c. in all four hocks or i.v. in the tail vein (as benchmark) either once on day 1 or twice on days 1 and 8. 9 days following the last dose, on day 10 (for the groups that received one dose) or on day 17 (for the groups that received two doses), all mice were administered an OVA+LPS challenge s.c., and 5 days later, the dLNs and spleen were examined for an OVA-specific response. Stars above horizontal bars represent p values with respect to the i.v. groups (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001) and † indicate p values with respect to the saline group (†p ≤ 0.05, ††p ≤ 0.01). **(B)** OTI CD8$^+$ T cells recovered from dLNs at time of sacrifice. Plot legends are as follows: 1-1 (1 μg s.c., once), 15 (5 μg s.c., once), 1-20 (20 μg s.c., once), 1-5 i.v. (5 μg i.v., once), 2-1 (1 μg s.c., twice), 2-5 (5 μg s.c., twice), 2-20 (20 μg s.c., twice) and 2-5 i.v. (5 μg i.v., twice). **(C–R)** Data are representative of three pooled experiments performed at the optimal high 20 μg dose of OVA as unconjugated OVA or OVA-p(GluNAc) injected twice on days 1 and 8, followed by an OVA+LPS challenge on day 17 and sacrifice on day 22, n=8-20. **(C)** OTI CD8$^+$ T cells recovered from dLNs. **(D)** PD-1$^+$ OTI CD8$^+$ T cells in dLNs. **(E)** Lag-3$^+$ OTI CD8$^+$ T cells in spleen. **(F)** Tim-3$^+$ OTI CD8$^+$ T cells in spleen. **(G)** MFI of the TCR on OTI CD8$^+$ T cells in dLNs. **(H)** IFNγ MFI of IFNγ secreting OTI cells after a 6-h *ex vivo* restimulation with OVA$_{257-264}$ peptide. **(I)** IL-10 producing OTI CD8$^+$ T cells after a 6-h *ex vivo* restimulation with OVA$_{257-264}$ peptide. **(K)** OTII CD4$^+$ T cells recovered from dLNs. **(L)** Foxp3$^+$CD25$^+$ OTII CD4$^+$ Tregs induced in dLNs. **(M)** Foxp3$^+$ST2$^+$ OTII CD4$^+$ Tregs induced in dLNs. **(N)** Lag3$^+$ OTII CD4$^+$ T cells in dLNs. **(O)** CTLA-4$^+$ OTII CD4$^+$ T cells in dLNs. **(P)** MFI of the TCR on OTII CD4$^+$ T cells in dLNs. **(P)** IL-13 levels in the supernatant of LN cells restimulated with 100 μg/mL OVA protein for 4 days, measured by LegendPlex assay. **(Q)** OTII CD4$^+$ T cells from the dLNs (left) or spleen (right) that secreted IFNγ, IL-2, TNFα, or a combination of two or all three cytokines after a 6-h *ex vivo* restimulation with OVA$_{323-339}$ peptide. **(R)** IL-10 producing OTII CD4$^+$ T cells after a 6-h *ex vivo* restimulation with OVA$_{323-339}$ peptide. Data represent mean ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 by one-way ANOVA using Tukey's *post hoc* test.

induced in the long-term at steady-state (without an antigenic challenge), especially in the antigen-specific CD8$^+$ T cell compartment. We treated mice s.c. with either unconjugated OVA or OVA-p(GluNAc), and evaluated the OTI phenotype in the dLNs and spleen one month following the booster injection (**Figure 3A**). At day 38, we observed a significantly lower recovery of OTI cells from the dLNs of OVA-p(GluNAc)-treated mice, indicating that the activated antigen-specific CD8$^+$ T cells were deleted, resulting in a smaller pool of circulating cells (**Figure 3B**). We confirmed that OVA-p

(GluNAc) treatment leads to a substantial initial proliferation of OTI cells, measured by CFSE dilution of circulating OTI in blood 3 days post-injection (**Figure S3A**), establishing that the deletion observed with s.c. OVA-p(GluNAc) was not due to incomplete priming by LN APCs but rather abortive proliferation, similar to the mechanism observed with liver-targeted OVA-p(GluNAc) (19). Thus, p(GluNAc) conjugation enhanced clonal deletion as its tolerogenic mechanism, a phenomenon observed not only in adoptively transferred T cells but also in endogenous autoimmune disease models (36).

We next investigated whether circulating antigen-specific regulatory memory was preferentially induced in $CD8^+$ T cells educated by p(GluNAc) conjugated antigen, compared to free antigen, in the dLNs. Surviving OTI cells educated by OVA-p(GluNAc) exhibited a central memory phenotype characterized by high expression of CD44, CD62L and Ly6C in the dLNs (**Figure 3C**, left). OTI cells from the spleen also shared this phenotype, further validating that local antigen education in the dLNs is able to generate a circulating central memory T cell pool poised for immune suppression (**Figure 3C**, right) (37, 38). Not only did OVA-p(GluNAc) lead to more central memory $CD8^+$ T cells overall but the proportion of central memory cells ($CD44^+CD62L^+$) compared with effector memory cells ($CD44^+CD62L^-$) was significantly higher (**Figure 3D**).

In contrast to what we observed after challenge, OTI cells in the OVA-p(GluNAc) group had a lower PD-1 expression at steady-state (**Figure 3E**), possibly because of the absence of chronic inflammatory stimuli and feedback networks that are usually needed to maintain high PD-1 expression and an exhausted state (39). Contrarily to PD-1, Lag-3 was expressed at high levels on OTI cells (**Figure 3F**), indicating that other mechanisms exist to maintain its expression even in the absence of residual antigen or chronic inflammation, which might be through interaction with scavenger receptor LSECtin (Clec4g) expressed on LECs (40).

Importantly, we noticed a significant induction in $CD8^+$ T cells that were $Foxp3^+$, both in the dLNs and spleen of mice that had been treated with OVA-p(GluNAc) (**Figure 3G**). Along with antigen-specific $Foxp3^+$ $CD25^+$ and $Foxp3^+$ $ST2^+$ $CD4^+$ Tregs, these could also be the source of the heightened IL-10 levels secreted upon antigenic challenge (**Figure 2I**). Foxp3-expressing $CD8^+$ Tregs have been reported to be important suppressive players in autoimmune disease such as type 1 diabetes and especially in the context of transplantation where donor cells continue to express MHCI for long time periods following the graft (41). The anergic T cells were rescued in their ability to produce IFNγ by the addition of exogenous IL-2 in the restimulation culture supernatant (**Figure 3H**) (42). Since this was an ELISA measurement, it was not possible to point out the identities of the T cells that were most responsible for this reversal in effector function, but it is most likely due to both $CD4^+$ and $CD8^+$ T cells. A similar restoration or increase in cytokine production was observed when cells from the saline and OVA groups were also restimulated in the presence of additional IL-2 (**Figure S3B**). Nonetheless, tolerance induced by s.c. antigen-p(GluNAc) is long-lasting, as evidenced by the resistance to antigenic challenge three months following the tolerization dose (**Figure S3C**).

We next asked whether the antigen-specific $Foxp3^+$ $CD8^+$ T cells induced by OVA-p(GluNAc) were capable of suppressing antigen-specific effector $CD4^+$ T cells. We set up three groups to answer this question. Group #1 received a first adoptive transfer of OTI cells, followed by the OVA-p(GluNAc) tolerizing treatment and antigenic challenge but not the second OTII adoptive transfer (positive control for tolerance). Group #2 did not receive a first adoptive transfer of OTI cells but received the OVA-p(GluNAc) treatment, followed by a second adoptive transfer of OTII cells and challenge (negative control for tolerance). Experimental group #3 received both the first and second adoptive transfers, including OVA-p(GluNAc) treatments and the antigenic challenge. The purpose of the challenge following the second adoptive transfer was to activate the naïve $CD4^+$ T cells into an effector phenotype. Moreover, we chose to wait an additional 10 days prior to sacrificing the mice to give the OTI $CD8^+$ Tregs enough time to encounter the effector OTII cells in the face of a potentially overwhelming LPS-induced inflammatory environment (**Figure 3I**). We found that OTII cells from group #3 were significantly suppressed compared to OTII cells from group #2 at day 41. OTII cells from the dLNs and spleen of group #3 were recovered in smaller numbers (**Figure 3J**), more highly expressed Lag-3 (**Figure 3K**), and were impaired in their ability to produce IL-2 and TNFα cytokines upon restimulation with their cognate peptide (**Figures 3L, M**). This shows that antigen-specific $CD8^+$ Tregs induced with p(GluNAc)-conjugated antigen are long-lived and contribute to suppression of antigen-specific effector $CD4^+$ T cells.

Inhibition of LAG-3 Signaling Completely Reverses $CD4^+$ and $CD8^+$ T Cell Tolerance Induced by LN-Targeted OVA-p(GluNAc)

We have demonstrated that OTI $CD8^+$ and OTII $CD4^+$ T cells engage the co-inhibitory module by up-regulating several surface immunosuppressive molecules, including PD-1 and Lag-3. We thus sought to investigate the role of these co-inhibitory signaling pathways in the tolerogenic mechanism of action of LN-targeted antigen-p(GluNAc) glycoconjugates. We set up a tolerance experiment as described above, but where we administered, during the OVA-p(GluNAc) priming window, i.p. injections of 250 μg blocking antibody against either Lag-3, PD-1 or CTLA-4, or no antibody for a total of 6 injections (**Figure 4A**). We challenged the mice with OVA and LPS 6 days following the last dose of blocking antibody and assessed the impact on the OTI and OTII T cell response 5 days after challenge. The antigen-specific $CD8^+$ T cell deletional tolerance established with OVA-p(GluNAc) was completely ablated to the non-tolerized saline levels when Lag-3, PD-1 or CTLA-4 was blocked in both the dLNs and spleen, though a slightly larger effect was observed with Lag-3 neutralization (**Figure 4B**). Antigen non-responsiveness was also reversed in the OTII compartment but not to levels seen in the saline-treated mice, except with αLag-3

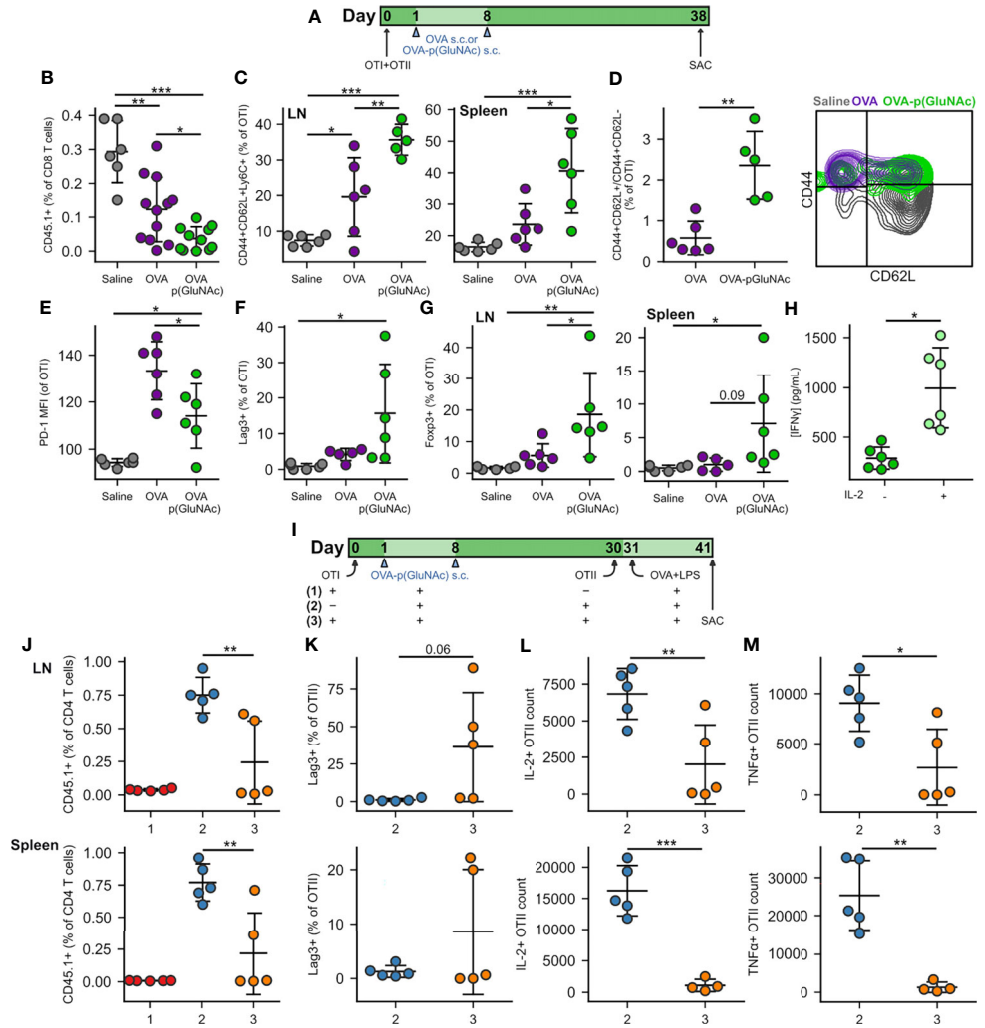

FIGURE 3 | **(A)** CD45.2$^+$ mice that had received an adoptive transfer of 1x10^6 of both OTI (CD45.1$^+$CD3$^+$CD8$^+$) and OTII (CD45.1$^+$CD3$^+$CD4$^+$) T cells *via* i.v. injection, were treated s.c. in all four hocks on days 1 and 8 with saline, or 20 μg of OVA or OVA-p(GluNAc) (5 μg per hock). On day 38, all mice were sacrificed and the dLNs and spleen were analyzed for OTI and OTII T cell phenotype. **(B)** OTI CD8$^+$ T cells recovered from dLNs. **(C)** Central memory OTI CD8$^+$ T cells in dLNs (left) and spleen (right). **(D)** Ratio of central memory to effector memory OTI CD8$^+$ T cells (left) and representative flow cytometry contour plot of the memory subsets (right) induced in the spleen. **(E)** PD-1 MFI on OTI CD8$^+$ T cells in dLNs. **(F)** Lag-3$^+$ OTI CD8$^+$ T cells in dLNs. **(G)** Foxp3$^+$ OTI CD8$^+$ T cells in dLNs (left) and spleen (right). **(H)** Splenocytes from the OVA-p(GluNAc) group were restimulated with 100 μg/mL OVA in culture media alone or supplemented with 200 Units/mL (~12 ng/mL) exogenous IL-2, and IFNγ levels were measured in the supernatant 3 days later by ELISA. **(I)** CD45.2$^+$ mice received a first adoptive transfer of 1x10^6 OTI CD8$^+$ T cells (groups 1,3) or no cells (group 2), followed by two s.c. OVA-p(GluNAc) treatments on days 1 and 8 for all groups. On day 30, mice from groups 2 and 3 received a second adoptive transfer of 5x10^5 OTII CD4$^+$ T cells. All mice were administered an OVA+LPS challenge on day 31, and 10 days later, the dLNs and spleen were examined for the OTII CD4$^+$ T cell response. **(J)** OTII CD4$^+$ T cells recovered from dLNs (top) and spleen (bottom). **(K)** Lag-3$^+$ OTII CD4$^+$ T cells in dLNs (top) and spleen (bottom). **(L)** Numbers of IL-2 producing OTII cells in dLNs (top) or spleen (bottom) after a 6-h *ex vivo* restimulation with OVA$_{323-339}$ peptide. **(M)** Numbers of TNFα secreting OTII CD4$^+$ T cells in dLNs (top) or spleen (bottom) after a 6-h *ex vivo* restimulation with OVA$_{323-339}$ peptide. Data are pooled from two independent experiments (n= 5-12), and represent the mean ± SD.
*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 by one-way ANOVA using Tukey's *post hoc* test in **(B, C, E–G, J)**, and unpaired Student's T test in **(D, H, K–M)**.

and αCTLA-4 in the spleen (**Figure 4C**). Antigen-specific CD4$^+$ Treg induction was also abrogated and diminished back to saline levels, especially with Lag-3 neutralization (**Figure 4D**). Upon restimulation with OVA$_{257-264}$ peptide, IFNγ production by OTI CD8$^+$ T cells was completely restored with αLag-3 but only partially with αPD-1 and αCTLA-4 (**Figure 4E**). Similar trends were observed in IFNγ and TNFα production in the OTII CD4$^+$ T cell compartment, albeit not to equivalent levels as with OTI (**Figures 4F–H**). OTII CD4$^+$ T cell cytokine impairment was not rescued in the spleen, indicating that there is more of a CD4$^+$ T cell local effect in the dLNs (**Figures S4A, B**). Therefore, these inhibitory signaling pathways investigated are important axes of T cell tolerance induced by LN-targeted antigen-p (GluNAc) glycoconjugates.

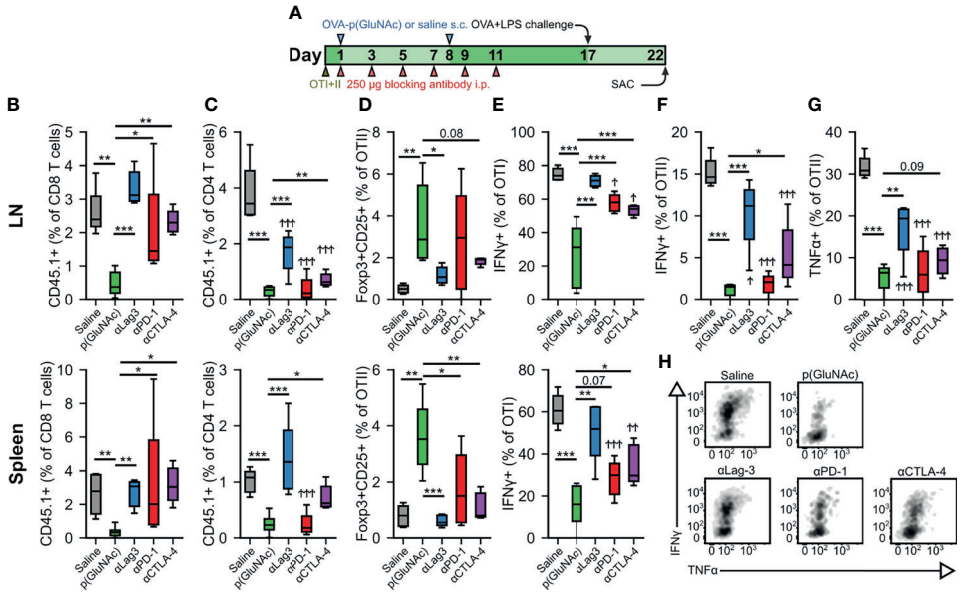

FIGURE 4 | **(A–F)** CD45.2$^+$ mice that had received an adoptive transfer of 1x10^6 of both OTI (CD45.1$^+$CD3$^+$CD8$^+$) and OTII (CD45.1$^+$CD3$^+$CD4$^+$) T cells *via* i.v. injection, were treated on days 1 and 8 with saline, or 20 µg of OVA-p(GluNAc) s.c. in all four hocks (5 µg per hock). On days 1, 3, 5, 7, 9 and 11, mice were also treated with 250 µg of either αLag-3, αPD-1 or αCTLA-4. On day 17, mice were given a s.c. OVA+LPS challenge, and were sacrificed 5 days later to evaluate the OTI and OTII T cell phenotype in the dLNs and spleen. **(B)** OTI CD8$^+$ T cells recovered from dLNs (top) and spleen (bottom). **(C)** OTII CD4$^+$ T cells recovered from dLNs (top) and spleen (bottom). **(D)** Antigen-specific OTII CD4$^+$ Tregs in dLNs (top) and spleen (bottom). **(E)** IFNγ secreting OTI CD8$^+$ T cells after a 6-h *ex vivo* restimulation with $OVA_{257-264}$ peptide from dLNs (top) and spleen (bottom). **(F)** IFNγ producing OTII CD4$^+$ T cells after a 6-h *ex vivo* restimulation with $OVA_{323-339}$ peptide. **(G)** TNFα secreting OTII CD4$^+$ T cells after a 6-h *ex vivo* restimulation with $OVA_{323-339}$ peptide. **(H)** Representative flow cytometry plots depicting IFNγ$^+$ and TNFα$^+$ OTI CD8$^+$ T cells from dLNs after a 6-h *ex vivo* restimulation with $OVA_{257-264}$ peptide. Data are pooled from two independent experiments (n= 5-10), and box-and-whisker plots represent the median, first and third quartiles. Statistical differences w.r.t saline were determined by one-way ANOVA using Dunnett's *post hoc* test, and one-way ANOVA using Tukey's *post hoc* test w.r.t OVA-p(GluNAc). Stars above horizontal bars represent p values with respect to the OVA-p(GluNAc) group (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001) and † indicate p values with respect to the saline group (†p ≤ 0.05, ††p ≤ 0.01, †††p ≤ 0.001).

OVA-p(GluNAc) Presentation to CD4$^+$ T Cells and Cross-Presentation to CD8$^+$ T Cells Is Mediated by Dendritic Cells

The biodistribution experiment described in **Figure 1** showed that several professional and semi-professional APCs were responsible for antigen-p(GluNAc) uptake in the dLNs, but their contribution to antigen presentation to naïve CD4$^+$ and CD8$^+$ T cells in the dLNs remained to be elucidated. To tease out the contribution of specific APC subsets to antigen presentation, we evaluated the proliferation of OTI and OTII cells 3 days post-s.c. immunization in transgenic mice that lacked the APC subsets of interest or in wild type C57BL/6 mice where those APC subsets were depleted using monoclonal antibodies. We first focused our attention on macrophages, which we showed are major uptakers (**Figures 1F, G**). We compared the initial proliferative response of OTI and OTII cells in wild type mice that received 250 µg of anti-CFS1R depleting antibody or isotype control s.c. on days 0, 3, 6 and 9. These mice received an adoptive transfer of CSFE-labeled OTI and OTII cells on day 7 and 20 µg OVA-p(GluNAc) s.c. on day 8. They were also administered daily i.p. injections of FTY-720 inhibitor to trap the T cells in the LNs in order to maximize exposure of the T cells to peptide-bearing MHC expressing APCs (**Figure 5A**). A problem with antibody depletion such as with anti-CSF1R is the systemic dissemination associated with i.v. or i.p. injections of the antibody (43). In order to limit macrophage depletion to the dLNs, we administered the antibody s.c. in the hocks in the same way that we immunized the animals. We found that, compared with clodronate depletion, this local antibody injection depleted macrophage populations of interest, namely CD169$^+$ SCS and medullary macrophages as well as more deeply located TZMs, in LNs only but left splenic macrophages intact (**Figure S5A**). We observed extensive but similar OTI and OTII proliferation in both the αCSF1R-treated and isotype-treated mice, indicating that LN macrophages are dispensable to the priming of CD4$^+$ and CD8$^+$ T cells in response to s.c. administered antigen-p (GluNAc) (**Figure 5B**).

To determine the contribution of another major uptaker, cross-presenting DCs, to s.c. OVA-p(GluNAc) immunization, we used Batf3$^{-/-}$ mice that lack cross-presenting CD8$^+$ DCs (44). We verified that there were minimal residual DCs in the LNs of these mice due to compensatory Batf1 expression (**Figure S5B**). We followed the same schedule as described above (**Figure 5A**). OTI cells proliferated significantly less in the Batf3$^{-/-}$ mice compared to wild type mice, showing that these DCs play an important role in the cross-presentation of OVA-p(GluNAc);

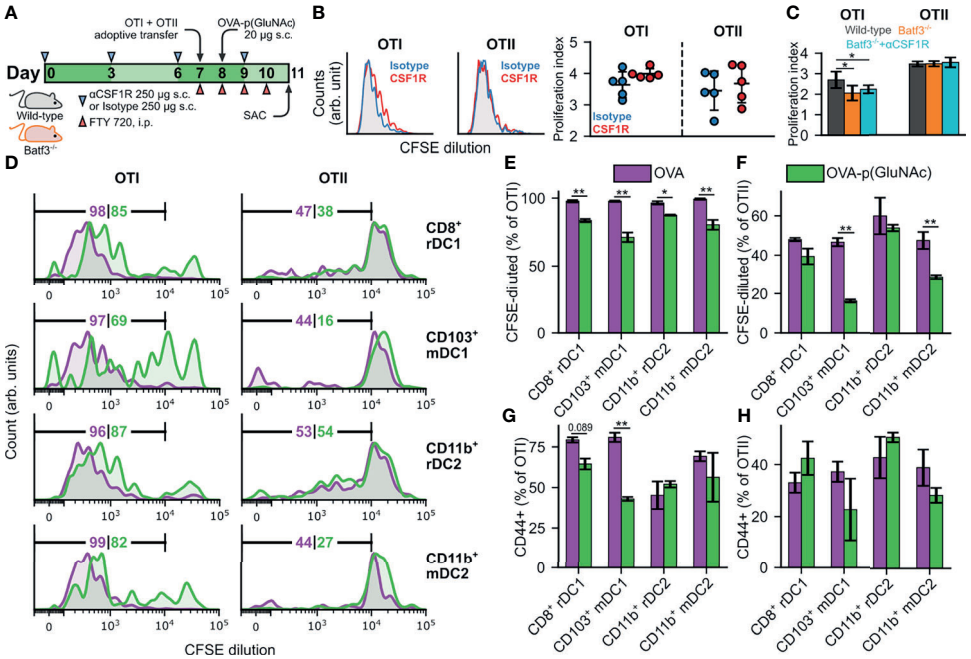

FIGURE 5 | **(A)** CD45.2$^+$ mice of wild-type (WT) or Batf3$^{-/-}$ genotype were treated s.c. in all four hocks with 250 µg of αCSF1R or an isotype IgG2a control on days 0, 3, 6 and 9. On day 7, mice received an adoptive transfer of CFSE-labeled OTI CD8$^+$ T and OTII CD4$^+$ T cells *via* i.v. injection, followed by a s.c. administration of 20 µg OVA-p(GluNAc) on day 8, and daily i.p. injections of FTY 720 inhibitor starting on day 7. On day 11, mice were sacrificed and the dLNs and spleen were examined for OTI and OTII proliferation. **(B)** (Left) Representative flow cytometry histograms of the CFSE dilution undergone by OTI CD8$^+$ T (left) and OTII CD4$^+$ T (right) cells in the dLNs of WT mice in the isotype control (blue) and αCSF1R (red) conditions. (Right) Quantitative analysis of the OTI and OTII T cell proliferation index in dLNs of WT mice treated as described above. **(C)** Quantitative analysis of the OTI and OTII T cell proliferation index in dLNs of Batf3$^{-/-}$ mice and WT mice treated as described above. **(D–H)** DCs were FACS sorted from s.c. LNs (axillary, brachial, inguinal, popliteal, cervical) of WT mice into four populations: CD8$^+$ resident (CD8$^+$ rDC1), CD103$^+$ migratory (CD103$^+$ mDC1), CD11b$^+$ resident (CD11b$^+$ rDC2) and CD11b$^+$ migratory (CD11b$^+$ mDC2), and stimulated *in vitro* in a 1:1 ratio with CFSE-labeled OTI CD8$^+$ T and OTII CD4$^+$ T cells in the presence of 2 µM of OVA or OVA-p(GluNAc). 3 days later, the OTI and OTII T cells were analyzed for proliferation and activation (CD44$^+$). **(D)** Representative flow cytometry histograms of the CFSE dilution (numbers indicate percent proliferated) undergone by OTI CD8$^+$ T (left) and OTII CD4$^+$ T (right) cells induced by each DC subset in the OVA (purple) and OVA-p(GluNAc) (green) groups. **(E)** Quantitative analysis of the OTI CD8$^+$ T proliferation. **(F)** Quantitative analysis of the OTII CD4$^+$ T proliferation. **(G)** CD44$^+$ OTI CD8$^+$ T cells. **(H)** CD44$^+$ OTII CD4$^+$ T cells. The graphs show means ± SD, n = 5. *p ≤ 0.05, **p ≤ 0.01 by unpaired Student's T test in B, one-way ANOVA using Tukey's *post hoc* test in C, and two-way ANOVA using Sidak's *post hoc* test in **(E–H)**.

OTII cells were unaffected, as anticipated (**Figure 5C**). To confirm that macrophages were not involved, we further depleted these subsets through s.c. αCSF1R antibody injections in Batf3$^{-/-}$ mice according to the above-described schedule (**Figure 5A**) and observed no further change in proliferation of OTI cells (**Figure 5C**). Even though we identified that cross-presenting DCs were important, they are evidently not the only APC involved, since we obtained non-negligible residual OTI proliferation in the Batf3$^{-/-}$ mice (**Figure 5C**).

Because we still saw substantial OTI proliferation in Batf3$^{-/-}$ mice, we sorted DC subsets from WT LNs and assessed their ability to present GluNAc-delivered OVA to T cells *in vitro* in order to identify the important DC players. We isolated the subcutaneous LNs (axillary, brachial, inguinal, popliteal, cervical) from wild-type mice and sorted the LN digests into four populations: CD8$^+$ resident (CD11c$^+$MHCII^{int}CD8$^+$CD11b$^-$, denoted as CD8$^+$ rDC1), CD103$^+$ migratory (CD11c$^+$MHCII^{high}CD103$^+$CD11b$^-$, denoted as CD103$^+$ mDC1), CD11b$^+$ resident (CD11c$^+$ MHCII^{int}CD8$^-$ CD11b$^+$, denoted as CD11b$^+$ rDC2) and CD11b$^+$ migratory (CD11c$^+$MHCII^{high}CD103$^-$CD11b$^+$, denoted as CD11b$^+$ mDC2). We then stimulated each population *in vitro* in a 1:1 ratio with CFSE-labeled OTI and OTII cells in the presence of 2 µM of unmodified OVA or OVA-p(GluNAc). 3 days later, the OTI and OTII cells were analyzed for proliferation and activation (antigen experience), measured by dilution of the CFSE dye and CD44 expression, respectively. We made four main observations: (1) OVA-p(GluNAc) presentation elicited mainly a CD8$^+$ T cell response (i.e. proliferation and activation), (2) presentation was not limited to cross-presenting DC1s, but DC2s were also important, (3) LN-resident subsets were more important than migratory populations for both DC1s and DC2s and, (4) OVA-p (GluNAc) generally resulted in a lower OTI and OTII proliferation and activation compared to unmodified OVA, indicative of an early tolerogenic skewing of T cell fate (**Figures 5D–H**). We also assessed the ability of LECs (the other major uptaker) to present OVA-p(GluNAc), and, while they did, they did so to a lower extent compared to DCs (**Figure S5C**). Thus, we established that

DCs, alongside being good uptakers, are also the main LN APC involved in presenting s.c. administered antigen-p(GluNAc) to naïve $CD4^+$ and $CD8^+$ T cells.

DISCUSSION

Our previous work has demonstrated that synthetically glycosylated antigen may be useful as an inverse vaccine platform for inducing antigen-specific tolerance (19). The versatility and mild conditions of the antigen conjugation chemistry to our glycopolymer ensure that the strategy can be universally applied to any antigen that contains a native or engineered primary amine. A synthetically-glycosylated inverse vaccine has now entered phase I clinical trials for inducing tolerance in the context of celiac disease (ClinicalTrials.gov Identifier: NCT04248855). Although our previous work investigated targeting hepatic APCs (19), in this study we investigate tolerance induction mediated by targeting LN-resident APCs accessed through s.c. injection.

LNs are the site of tightly orchestrated responses that can be guided toward immunity or tolerance, depending on context (45). Similar to the liver, antigen dose and frequency, formulation and co-formulation with modulatory signals, as well as specific APC players determine the immunological response (46). Here, we focus on understanding the mechanisms of action of synthetically glycosylated inverse vaccines on LN-resident APCs, and to what extent dose and dose frequency may need to be adapted to achieve tolerance. Delivering the antigen conjugated to a glycopolymer may be beneficial for lymphatic absorption and channeling to LN-resident APCs and then for uptake *via* binding to their scavenger receptors to promote tolerance.

When delivered s.c., antigen conjugated to p(GluNAc), in this case OVA-p(GluNAc), rapidly drains and accumulates in the dLNs, to a significantly higher extent than unmodified OVA, which is consistent with particle filtration dynamics in the dLNs (**Figure 1E**). Glyco-polymerization alters the physicochemical properties of the antigen in important ways: the molecular weight is increased by 30-70 kDa, resulting in a net neutrally charged, branched polymeric particle. These nanoparticles drain into the lymphatics and accumulate in the dLNs, whereas smaller particles may be rapidly filtered through floor lymphatic endothelial cells and into systemic circulation *via* high endothelial venules, and larger microparticles may be preferentially captured by migratory APCs at the site of injection for subsequent trafficking to the dLNs (21, 47). However, given that OVA alone resulted in similar tolerogenic outcomes as OVA-p(GluNAc) in some instances, such as in total numbers of OTI and OTII recovered upon challenge, it is possible that OVA is taken up and processed by APCs more rapidly than OVA-p(GluNAc), resulting in negligible signal at the measured timepoints (**Figure 1E**). Thus, conjugation to p(GluNAc) may not significantly lengthen the residence time in the LNs but may only delay enzymatic cleavage in the endosome. This mechanistic distinction may be further explored by repeating the experiment described in **Figure 1E** using DQ-OVA instead of OVA.

Uptake of synthetically glycosylated antigen by APCs is mediated through the carbohydrate binding domain of various C type lectin and scavenger receptors and can be inhibited by the addition of free sugars in media (19). We analyzed the immgen database (http://www.immgen.org/) for the expression of several scavenger and lectin receptors involved in the uptake of carbohydrates, including GluNAc-terminated residues, among APCs targeted by OVA-p(GluNAc), and found that they were broadly expressed, but to different extents on these cell types (**Figure S1B**). We identified Asgr1 and 2 to be only minor players in LN APCs compared to hepatic APCs (48). Clec4g (LSECtin) was found to be highly expressed exclusively on LECs, justifying their high uptake of OVA-p(GluNAc) and their similarity in scavenging profile to liver sinusoidal endothelial cells (49). Other receptors found highly expressed by the hematopoietic APCs were DEC-205 (Ly75), which has been explored as an antigen target for the induction of tolerance (50) and Clec9a, primarily found as apoptotic scavenger receptor by cross-presenting DCs (51). LECs and macrophages share MARCO expression, which has been used for antigen targeting in tolerance induction (17, 52). The mannose receptor (Mrc1), which can promiscuously bind GluNAc glycosylated antigen, was also highly expressed on LECs (53). This analysis also revealed shared receptors between LECs and macrophages, which reflects their synergy in scavenging in the LN subcapsular sinus, similar to the parallels between sinusoidal endothelial cells and Kupffer cells in the liver (54). Thus, by virtue of size, retention, expression of C-type lectin and scavenger receptors, LN APCs are able to effectively take up synthetically glycosylated antigen.

Compared to the liver or oral mucosa, where immune responses are skewed toward tolerance because of the abundance of oral or gut antigen that need to be interpreted in an innocuous manner, immune responses to exogenous antigens in the peripheral lymphatics usually aim to generate an inflammatory response in the context of an infection. However, LNs under homeostasis do continually drain self-antigen from the local tissue, and this constant antigen exposure may be important in maintaining peripheral tolerance. For example, in mice lacking skin-draining lymphatics, skin-specific autoimmunity was observed to develop (55). Furthermore, LECs have an essential role in the maintenance of tolerance to peripheral tissue-transcribed antigens *via* the deletion of autoreactive cells or the generation of autoantigen-specific $CD4^+$ Tregs, thereby acting as an additional mechanism to compensate for potentially autoreactive T cells that escape central tolerance (56–58). LECs can also induce tolerance to exogenous antigens draining from peripheral sites of immunization, inflammation and tumors, through direct antigen presentation to both naïve $CD8^+$ and $CD4^+$ T cells (59, 60). This tolerogenic antigen presentation is accompanied by the up-regulation of co-inhibitory molecules, as well as soluble mediators such as IDO that can directly suppress T cells and prevent APCs from maturing and presenting antigen to produce effectors (61).

Canonical $Foxp3^+CD25^+$ Tregs play a crucial role in ensuring the maintenance of tolerance and, more recently, antigen-specific Tregs induced in the periphery are being increasingly recognized as important regulators (62, 63). We have also shown the dependence of LN-targeted suppression on long-lived $CD8^+$ regulatory T cell subsets (**Figures 3I–M**). These constitute an

important arm in the natural control of autoimmunity (64) but can also be induced under different treatment conditions that have mostly been investigated in immune-privileged sites (65) and in the context of transplantation and peptide immunotherapy in lupus (66). The ability of antigen-p(GluNAc) to result in broad antigen-specific regulatory and suppressor subsets of T cells would be a highly desirable property.

Memory has been found to contribute beneficially or harmfully to the maintenance of tolerance in a context-dependent manner. In type 1 diabetes, lower avidity auto-reactive clones have been shown to adopt a central memory phenotype that serves to regulate antigen presentation and activation of destructive high-avidity autoreactive clones in the pancreatic dLNs (38). Memory $CD8^+$ T cells have also been shown to promote tolerance to graft through nitric oxide production (37). We see a similar phenomenon at play where antigen-specific $CD8^+$ T cells that survived deletion post-antigen-p(GluNAc)-mediated abortive proliferation preferentially differentiate into a central memory state (**Figures 3C, D**) where they can mediate suppression to future antigenic challenge (**Figures 2**, **S3C**). In future mechanistic studies, it will be of interest to evaluate the contribution of $TCF1^+$ stem-cell like memory to the central memory compartment and tolerogenic state induced by s.c. antigen-p(GluNAc) administration (67).

There were noticeable differences in the response of antigen-specific $CD4^+$ and $CD8^+$ T cells to blockade of the distinct co-inhibitory pathways (Lag-3, PD-1 and CTLA-4). $CD8^+$ T cell tolerance was significantly more ablated when these signaling pathways were disrupted, indicating a higher dependence on these signaling pathways for tolerance induction (**Figure 4**). All three pathways were found to be important to some extent for $CD4^+$ and $CD8^+$ T cell tolerance, a result that did not surprise us given that many of these co-inhibitory molecules form part of an immunosuppressive module co-regulated by overlapping signaling such as IL-27 (33). Lag-3 was found to be an essential suppressive pathway responsible for inducing deletional tolerance in $CD8^+$ T cells in both the dLNs and spleen and in $CD4^+$ T cells in the spleen (68). This also suggested to us that other signaling axes exist to ensure $CD4^+$ T cell peripheral tolerance is maintained. One example is considering how Lag-3 expressed on $CD4^+$ T cells interacts with its ligands in the LN microenvironment. We have shown that OVA-p(GluNAc)-educated OTII cells express higher Lag-3 levels (**Figure 2M**). Lag-3 binds to MHCII on various APCs, an interaction that contributes to $CD4^+$ T cell activation and is not blocked by the αLag-3 (C9B7W) antibody that we used in our experiments (69). Lag-3 on T cells has also been reported to interact with LSECtin that is highly expressed on LN-LECs (**Figure S1B**) (40).

This is the first report of local LN macrophage depletion using a s.c. injection of CSF-1R depleting antibody, but s.c. administration is a recently validated strategy for the locoregional enrichment of blocking antibodies such as checkpoint antibodies in the sentinel LNs for tumor control (70). Francis et al. demonstrated that s.c. administration of αPD-1 or αCTLA-4 antibodies ipsilateral to the primary tumor results in accumulation in the local dLNs and anti-tumor efficacy but also a systemic abscopal effect. While we observed a robust decrease in macrophage subsets in the dLNs, we did not suppress macrophages in the spleen, indicating that antibodies rapidly drain to and are retained in the dLNs where they exert a local effect, leading to a systemic immunological response. While our data showed a dispensable role for macrophages in glycoconjugate-medicated antigen priming, it is possible that macrophages relay the acquired antigen to DCs for further processing and presentation onto MHC, as has been reported (71). This coordinated effort and transfer of antigen between different APC subsets through vesicular routes has been evidenced under steady-state (72) and, more recently, elegantly demonstrated in the context of sentinel LN priming in cancer (73).

Dendritic cells have unique and varied intrinsic pathways of antigen presentation but can also be highly cooperative, depending on context (74). For example, mannose receptor-directed antigen is channeled to early endosomes and the cross-presentation pathway (75). Even though the current paradigm is that DC1s (LN-resident $CD8^+$ or migratory $CD103^+$) are specialized in cross-presenting antigen to $CD8^+$ T cells, while DC2s (LN-resident or migratory $CD11b^+$) are better equipped to present to $CD4^+$ T cells, all DCs are capable of presenting to both $CD4^+$ and $CD8^+$ T cells given the right circumstances dictated by location (both anatomically and within the LN), antigen dose and administration route, and inflammatory stimulus (24, 76–79). Consistent with this, we found that *ex vivo* priming with OVA-p(GluNAc) by DC1s and DC2s resulted in both OTI and OTII expansion and activation but primarily a $CD8^+$ T cell response with at least a two-fold difference in OTI proliferation, compared with OTII (**Figures 5D–F**).

The divergence in $CD4^+$ and $CD8^+$ T cell proliferation is not surprising given that they have very different activation requirements (80). For instance, $CD4^+$ T cell proliferation is more dependent on prolonged antigen exposure compared to $CD8^+$ T cells (81). The APC antigen uptake and distribution landscape is also instrumental to regulating differential priming (82). Furthermore, while $CD4^+$ T cells are required for optimal $CD8^+$ T cell activation during a primary activation or memory recall response and for survival (83), $CD8^+$ T cell memory formation has been shown to be intrinsic and $CD4^+$ T cell independent (84). In the context of peripheral tolerance, $CD4^+$ T cell help is usually an instigator of autoreactive $CD8^+$ T cell effector function in several autoimmune conditions such as in type 1 diabetes and is undesirable in transplant tolerance (85–87). Since the antigen-specific $CD4^+$ and $CD8^+$ T cells were both in contact with the sorted DCs at the same time in our *ex vivo* sorting and priming experiment, the $CD4^+$ T cell help provided by OTII cells could be an additional factor that contributed to the OTI proliferation (**Figures 5D, E**). The OTI and OTII proliferation was elicited by both sorted DC1 and DC2 populations, especially LN-resident subsets, which is what we expected given that OVA-p(GluNAc) drains rapidly to the LN and is not retained at the s.c. site of injection 72 h post-injection, which is the timeframe for when migratory DCs make their way to dLNs with captured antigen (**Figure S1A**).

In conclusion, in this work, we present a novel approach of inducing antigen-specific tolerance using synthetically glycosylated antigen *via* peripheral s.c. routes of targeting. We leverage the biophysical, biochemical and immunological environment of the LN and its cellular players to induce robust and lasting prophylactic

tolerance to an exogenous antigen. This strategy has powerful implications in the prophylaxis and treatment of autoimmune and inflammatory diseases.

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 animal study was reviewed and approved by Institutional Animal Care and Use Committee at the University of Chicago.

AUTHOR CONTRIBUTIONS

JH and MS oversaw all research. CM, EW, MS, and JH designed the research strategy. DW conceptualized materials. DW and MR synthesized materials. CM, SC, EW, AS, MN, JR, and HN-S performed experiments. CM analyzed experiments. CM and JH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.714842/full#supplementary-material

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Human Wharton's Jelly-Derived Mesenchymal Stromal Cells Primed by Tumor Necrosis Factor-α and Interferon-γ Modulate the Innate and Adaptive Immune Cells of Type 1 Diabetic Patients

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The unique immunomodulation and immunosuppressive potential of Wharton's jelly-derived mesenchymal stromal cells (WJ-MSCs) make them a promising therapeutic approach for autoimmune diseases including type 1 diabetes (T1D). The immunomodulatory effect of MSCs is exerted either by cell-cell contact or by secretome secretion. Cell-cell contact is a critical mechanism by which MSCs regulate immune-responses and generate immune regulatory cells such as tolerogenic dendritic cells (tolDCs) and regulatory T cell (Tregs). In this study, we primed WJ-MSCs with TNF-α and IFN-γ and investigated the immunomodulatory properties of primed WJ-MSCs on mature dendritic cells (mDCs) and activated T cells differentiated from mononuclear cells (MNCs) of T1D patient's. Our findings revealed that primed WJ-MSCs impaired the antigen-mediated immunity, upregulated immune-tolerance genes and downregulated immune-response genes. We also found an increase in the production of anti-inflammatory cytokines and suppression of the production of pro-inflammatory cytokines. Significant upregulation of *FOXP3, IL10* and *TGFB1* augmented an immunosuppressive effect on adaptive T cell immunity which represented a strong evidence in support of the formation of Tregs. Furthermore, upregulation of many critical genes involved in the immune-tolerance mechanism (*IDO1* and *PTGES2/PTGS)* was detected. Interestingly, upregulation of *ENTPD1/NT5E* genes express a strong evidence to switch immunostimulatory response toward immunoregulatory response. We conclude that WJ-MSCs primed by TNF-α and IFN-γ may represent a promising tool to treat the autoimmune disorders and can provide a new evidence to consider MSCs-based therapeutic approach for the treatment of TID.

Keywords: type 1 diabetes, Immunomodulation, priming, Wharton's jelly-derived mesenchymal stromal cells, regulatory T cells, tolerogenic dendritic cells

INTRODUCTION

Type 1 diabetes (T1D) is a T-cell mediated autoimmune disorder in which insulin-secreting β-cells of the pancreas are selectively destroyed. The mechanisms involved in the β-cell destruction are still not understood. Currently, tyrosine kinase, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), insulinoma-associated protein 2 (IA-2), zinc transporter (ZnT), and glutamic acid decarboxylase 65 (GAD65) are all considered to be involved in the β-cell specific autoimmune process (1, 2). However, GAD65 is identified as the major antigen targeted by T-cell autoantibodies in T1D (3).

Mature dendritic cells (mDCs) and T cells are implicated in the pathogenesis of T1D. Mature dendritic cells play a key role in all stages of β-cells destruction due to their immunostimulatory effect on naïve T cells (4). As a result, mDCs trigger two important functions in controlling T-cell immunity; T-cell activation by the expression of T-cell antigen and secretion of specific cytokines which determine the nature of T-cell responses (5).

To date, the most reliable approach for the management of T1D remains the islet or pancreas transplantation but there are many obstacles in their use that include; allo-immune graft rejection, recurrence of autoimmunity, as well as inadequate supply of donor tissues (6–8). Hence, there is a need for an efficacious alternative approach to control T1D.

In the past few decades, human mesenchymal stromal cells (MSCs) presented a promising strategy for treating various immune-mediated diseases including T1D due to their ability to interact with many types of immune cells. Furthermore, MSCs exert immunomodulatory and anti-inflammatory effects on the adaptive and innate immune system both *in vivo* and *in vitro* (9, 10), through their secretion of chemokines, cytokines, growth factors, and biological active substances (11, 12). Unfortunately, many limitations have threatened the use of MSCs-based therapies such as, cell senescence, loss of function after cryopreservation, and unpredictability of MSCs behavior *in vivo* (13, 14). On the other hand, various studies demonstrated that under normal conditions, MSCs express low or insufficient levels of immunomodulatory and immunosuppressive factors (15–17). For these reasons, many ongoing researches proposed the requirement of a practical approach to improve MSCs survival, differentiation potential, therapeutic efficacy, and immunomodulatory functions (18–20). Recently, cell priming or cell activation is considered one of the most interesting functional approaches (21–23). "Human mesenchymal stromal cells Priming" or "MSCs licensing" is defined as the exposure of MSCs to pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-1α and IL-1β, to increase their anti-inflammatory and immunomodulatory effects on the innate and adaptive immunity (24, 25).

Human mesenchymal stromal cells can strongly interact with many types of immune cells either by cell-cell contact or by their secretome (26). Cell-cell contact is a critical immunosuppressive mechanism of MSCs due to their ability to express a wide range of chemokine receptors and surface adhesion molecules, such as CXCR3, CCR5, CXCL9, CXCL10, CXCL11, VCAM, ICAM-1 and ICAM-2, which enhance their affinity to bind to immune cells and exert their immunomodulatory functions (27–31).

We hypothesized that preconditioning Wharton's jelly-derived mesenchymal stromal cells (WJ-MSCs) with IFN-γ and TNF-α may lead to robust immunomodulatory effects on immune cells leading to the induction of immunoregulatory cells such as tolDCs and Tregs. Wharton's jelly-derived mesenchymal stromal cells are optimal candidates for cellular therapies in allogeneic transplantation due to their low immunogenicity and their immunomodulatory properties in addition to their ability to release large amounts of tolerogenic factors by direct and indirect contact with immune cells. Therefore, this study aimed to investigate the effect of primed WJ-MSCs on the profile and functions of mDCs and activated T cells that differentiated from T1D patients.

MATERIALS AND METHODS

Human Platelet Lysate Preparation

According to Awidi et al. (32), human platelet lysate (PL) was obtained from different platelet apheresis collections at blood banking unit in the Jordan University Hospital, Jordan. The count of platelets was performed using automated hematology analyzer. Pooled samples were subjected to three repeated temperature cycles, frozen at -80°C, then heated at 37°C, then frozen and stored at - 20°C until experimental use.

Isolation and Culture of WJ-MSCs

Human WJ-MSCs were obtained and processed immediately after cesarean section (n=5). All donors signed a consent form before delivery. The protocol was approved by Institutional Review Board (IRB) committee of the Cell Therapy Center, Jordan. (IRB NO. 07-11-2019). Briefly, MSCs were isolated from the Wharton's Jelly region of the Umbilical Cord (UC). The cords were washed twice with PBS supplemented with 100u/ml Penicillin-Streptomycin, cut into 2 mm^2 pieces and placed into 148 cm^2 tissue culture plates containing α-MEM supplemented with 10% PL, 100u/ml penicillin/streptomycin and 2mM/ml L-Glutamine. Plates were incubated in a 5% CO2 incubator at 37°c and 95% relative humidity for 7 to 9 days. The medium was changed every 2–3 days. After that, the pieces were removed and the cells were harvested and seeded into T-175 flask for 4 passages. For all experiments, MSCs were used at passages 3-4.

To induce primed cells, WJ-MSCs were cultured in 6- well plates (1x10^5/well) and stimulated with recombinant human 50ng/ml TNF- α and 50ng/ml IFN-γ(R&D Systems, USA) for 48 hours. then cells were washed and stromal cell markers were characterized using hMSCs Analysis kit (BD, UK). The differentiation potential was determined by using StemPro® Differentiation Kit (GIBCO, USA). In parallel experiment, WJ-MSCs were cultured in 6- well plates (1x10^5/well) for 48 hours, then the morphological features of unprimed and primed WJ-MSCs were assessed using Evos Cell Imaging System AMEX1200 (Life Technologies, USA) and the morphological differences were analyzed by Imagej software. The concentration of stimulants

was chosen depending on WJ-MSCs morphological changes and based on the expression of immunomodulatory factors.

Generation and Culture of Human mDCs

All patients signed a consent form before blood samples collection. The data of patients groupes were documented before samples collection (**Table 1**). Fresh heparinized peripheral blood was obtained from five newly onset T1D patients recruited from the Jordan University Hospital, Jordan. In short, mononuclear cells (MNCs) were separated by a Ficoll-Paque gradient centrifugation (specific gravity, 1.077g/ml- Sigma-Aldrich, UK) and cultured in 6-well plates with RPMI 1640 supplemented with 10% PL, 100u/ml penicillin/streptomycin and 2Mm/ml L-Glutamine for two hours in a 5% CO2 incubator at 37°c and 95% relative humidity. Non-adherent cells, T lymphocytes, were collected and tested for CD3+ expression (86.9 ± 5.9) by Flow cytometry using CD3-APC (Biolegend, USA). Adherent cells, monocytes, were washed twice with PBS and were then evaluated for CD14+ expression (92.3± 2) by flow cytometry using CD14-FITC (BD Bioscience, USA). Monocytes were differentiated into immature DCs (iDCs) using 100 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) and 50 ng/ml IL-4 (R&D Systems, USA) and incubated for 5 days. Immature DCs were pulsed at a concentration of $1x10^6$ cells/ml with 10μg/ml GAD65 (Abcam, UK), and cultured with 50 ng/ml IL-1β and 50 ng/ml TNF- α (R&D Systems, USA) for 48 hours to generate mDCs. The concentrations were chosen according to Favaro et al. (33). All cells were cultured in 6-well plates with RPMI 1640 supplemented with 10% PL, 100u/ml penicillin/streptomycin and 2Mm/ml L-Glutamine.

T Cells Activation and Proliferation Assay

T lymphocytes were stained with carboxyfluorescein succinimidyl ester (CFSE). The concentration of CFSE fluorescent dye is reduced by cell division. During cell division, CFSE is distributed among daughter cells. Hence, proliferation is assessed by dilution of the CFSE dye (34). Briefly, $1x10^6$ lymphocytes were incubated with CFSE (10mM; Abcam, UK) for 10 minutes in a 5% CO2 incubator at 37°c and 95% relative humidity. The reaction was stopped with RPMI supplemented with 10%PL. T Lymphocytes were co-cultured with GAD65 pulsed mDCs (mDCs: T cell ratio, 1:10) in 6-well plates with RPMI 1640 supplemented with 10% PL, 100u/ml penicillin/streptomycin, 2Mm/ml L-Glutamine, and pulsed at concentration of $1x10^6$ cells/ml with 10μg/ml GAD65 (Abcam, UK) for 4 days (referred to as activated T cells). After that, activated T cells (CD4+ and CD8+) were tested using flow cytometry. In a parallel experiment, T lymphocytes were cultured alone under the same conditions without activation (referred to as inactivated T cells).

WJ-MSCs Co-Cultured With GAD65 Pulsed mDCs and T Cells

Primed WJ-MSCs were cultured overnight in 6-well plates (1x10^5 cells/well) with α-MEM media (Gibco, UK) supplemented with 10% platelet lysate (PL), 100u/ml penicillin/streptomycin and 2Mm/ml L-Glutamine. The medium was replaced by RPMI 1640 supplemented with 10% PL, 100u/ml penicillin/streptomycin and 2Mm/ml L-Glutamine. After that, GAD65-pulsed mDCs were co-cultured with primed WJ-MSCs monolayer (mDCs: WJ-MSCs, ratio 1:1) for 48hours. The ratio was chosen according to Favaro et al. (33). In parallel experiment, primed WJ-MSCs were cultured overnight in 6-well plates (1x10^5 cells/well) with α-MEM media (Gibco, UK) supplemented with 10% platelet lysate (PL), 100u/ml penicillin/streptomycin and 2Mm/ml L-Glutamine. The medium was replaced with RPMI 1640 supplemented with 10% PL, 100u/ml penicillin/streptomycin and 2Mm/ml L-Glutamine. Then, activated T cells were co-cultured with primed WJ-MSCs monolayer (WJ-MSCs: T cells, ratio 1:5) for 48hours. The ratio was chosen according to Liu et al. (35).

Flow Cytometry Analysis of DCs and T Cells

The expression of co-stimulatory molecules and maturation surface markers protein of iDCs, mDCs and mDCs co-cultured with primed WJ-MSCs were analysed by flow cytometer using the following antibodies: CD14- PE-CY7, CD83- PE-CY5, CD80-FITC, CD86- BV421, CD1a-PE, CD40-BV510, CD209-APC and HLA-DR- Percp-CY5.5, all antibodies are purchased from (BD Bioscience, USA). Cell proliferation and cell-surface marker expression of activated T cells (CD4+ and CD8+) were detected using the following antibodies: CD4-APC and CD8-PE-CY7 (Biolegend, USA). Activation of T cells was detected using CD69-PE (Biolegend, USA).

Enzyme- Linked Immunosorbent Spot (ELISPOT)

T lymphocytes (2x 10^5) were pulsed with GAD65 and were then cultured under the following conditions: inactivated T cells, activated T cells cultured with or without primed WJ-MSCs.

TABLE 1 | Data of the study groups of T1D patients.

Patient no	Gender	Age	C-Peptide (NR 1.1-4.4ng/ml)	GAD65	HbA1c (NR 4.8%-5.8%)
1	Male	34	0.83 ng/ml	Positive	6.4%
2	Male	18	0.56 ng/ml	Positive	8.1%
3	Female	21	0.79 ng/ml	Positive	7.4%
4	Male	17	2.8 ng/ml	Positive	9.5%
5	Female	22	1.1 ng/ml	Positive	5.2%

NR, Normal Range.

IFN-γ ELISPOT analysis was performed as previously described (36, 37) and according to the manufacturer's instructions (Abcam, UK). All patients were positive for GAD65 antigen.

Cytokine Quantification by ELISA

Levels of secreted IL-10, IL-6, and TGF-β in mDCs were measured in cell-free culture supernatant under the following conditions: primed WJ-MSCs alone, mDCs alone, and primed WJ-MSCs co-cultured with mDCs. Regarding T cells cytokines, levels of secreted IL-10, IL-6, IFN-γ, IL-17 and TGF-β were measured in cell-free culture supernatant under the following conditions: inactivated T cells alone, activated T cells alone and primed WJ-MSCs co-cultured with activated T cells. All cytokine kits were purchased from Abcam, UK.

qPCR for Quantification of Gene Expression

To determine the expression of the target genes at the mRNA level, qPCR was performed. Shortly, mDCs and activated T cells were collected from culture by gentle pipetting followed by centrifugation, then the cell pellets were lysed by Trizol-hybrid method for RNA extraction using RNeasy micro kit (Qiagen, USA). The extracted RNA was quantified by a Nanodrop (Thermofisher, USA). To synthesize cDNA, 0.5 µg of total RNA was reverse transcribed by using the PrimeScript RT Master Mix (Takara, China) using T100™ Thermal cycler PCR instrument (BioRad, USA). Primers were designed using Primer-BLAST (RRID : SCR- 003095) and obtained from IDT (USA) (**Table 2**). The qPCR reaction mix was prepared by mixing 25 ng of cDNA with and 200 nM of gene-specific forward and reverse primers (IDT), 7.2 µL of free nuclease water, and10 µL of SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara, China). The amplification was performed on CFX96 C1000 Touch thermal cycler (BioRad, USA) with the following temperature setting: (i) 95°C for 3 minutes, (ii) 40 cycles 95°C for 5 seconds and 61°C for 30 seconds. 18s rRNA was used as a reference gene. Each sample was performed in triplicate, and a mean value was calculated. Data were analyzed according to 2–ΔΔCT method using CFX Maestro™ Software - Bio-Rad.

Statistical Analysis

Kolmogorov-Smirnov test was used to verified the normal distribution of data. Graph Pad Prism 6 was used for statistical analysis. Data were presented as Mean ± Standard Deviation (SD). One-way ANOVA, unpaired *t*-test, and two-way ANOVA with Tukey's *post hoc* analysis were performed to analyze the differences between experimental points. qPCR data were analyzed according to 2–ΔΔCT method using CFX Maestro™ Software - Bio-Rad. In all analyses P values <0.05 were considered significant. In qPCR Data Analyses P values <0.01 were considered significant. The used test was described in the figure legends.

RESULTS

Characterization and Differentiation of Primed WJ-MSCs

Morphology and surface marker expression of WJ-MSCs were evaluated at passage 3. Primed WJ-MSCs expressed typical MSCs surface markers CD90 (100% ± 0.0%), CD73 (99.7% ± 0.2%), CD44 (100% ± 0.0%), and CD105 (90.1% ± 4%), and did not express hematopoietic markers; CD34, CD11b, CD19, CD45 and HLA-DR (0.1% ± 0.05%) (**Figure 1A**). Primed WJ-MSCs exhibited unique morphological features after their priming with TNF- α and IFN-γ compared with unprimed WJ-MSCs (**Figures 1B, C** and **Table 3**). The change of MSCs morphology upon TNF- α and IFN-γ stimulation strongly correlated with a previous study that investigated the morphological features of bone marrow derived MSCs (BM-MSCs after being primed with IFN-γ (38).

Primed WJ-MSCs were grown in adipogenic and osteogenic induction media for 14-21 days. All primed WJ-MSCs demonstrated the multilineage differentiation potential (**Figures 1D, E**).

Effect of Primed WJ-MSCs on GAD65 Pulsing mDCs Profile and Function

The phenotypic analysis of monocyte-generated mDCs cultured in the presence of primed WJ- MSCs showed skewing of mDCs

TABLE 2 | qPCR primer sequences of immunostimulatory and immunomodulatory genes that are involved in the immunoregulatory effect of primed WJ-MSCs.

Gene	Forward Primers Sequence	Reverse Primers Sequence
IL10	5′ GCTGAGAACCAAGACCCAGA3′	5′ AAGAAATCGATGACAGCGCC3′
IL6	5′GGCACTGGCAGAAAACAACC3′	5′GCAAGTCTCCTCATTGAATCC3′
TGFB1	5′GGAAATTGAGGGCTTTCGCC3′	5′CCGGTAGTGAACCCGTTGAT3′
IL17A	5′CGGACTGTGATGGTCAACCT3′	5′TCCTCATTGCGGTGGAGATT3′
TNFA	5′CCTGTAGCCCATGTTGTAGCAAA3′	5′TTATCTCTCAGCTCCACGCCA3′
IFNG	5′GAGTGTGGAGACCATCAAGGA3′	5′TGGACATTCAAGTCAGTTACCGAA3′
FOXP3	5′GAACCTTCCAGGGCCGAGAT3′	5′ATGGTGGCATGGGGTTCAAG3′
IL2	5′TTTTACATGCCCAAGAAGGCCA3′	5′TCCTCCAGAGGTTTGAGTTCT3′
PTGES2	5′GGTGCCTGGTCTGATGATGT3′	5′GATTAGCCTGCTTGTCTGGAAC3′
IDO1	5′CTCTGCCAAATCCACAGGAAA3′	5′CAACTCTTTCTCGAAGCTGGC3′
NT5E	5′CGCTCAGAAAGTGAGGGGTG3′	5′GGAAGGTGGATTGCCTGTGTA3′
ENTPD1	5′CTCAGCCTTGGGAGGAGATAA3′	5′ATGTGCTCCCAGGAATCAGC3′
PTGS1	5′AGCCCTTCAATGAGTACCGC3′	5′TGCCATCTCCTTCTCTCCTAC3′
18S rRNA	5′CGGCGACGACCCATTCGAAC3′	5′GAATCGAACCCTGATTCCCCGTC3′

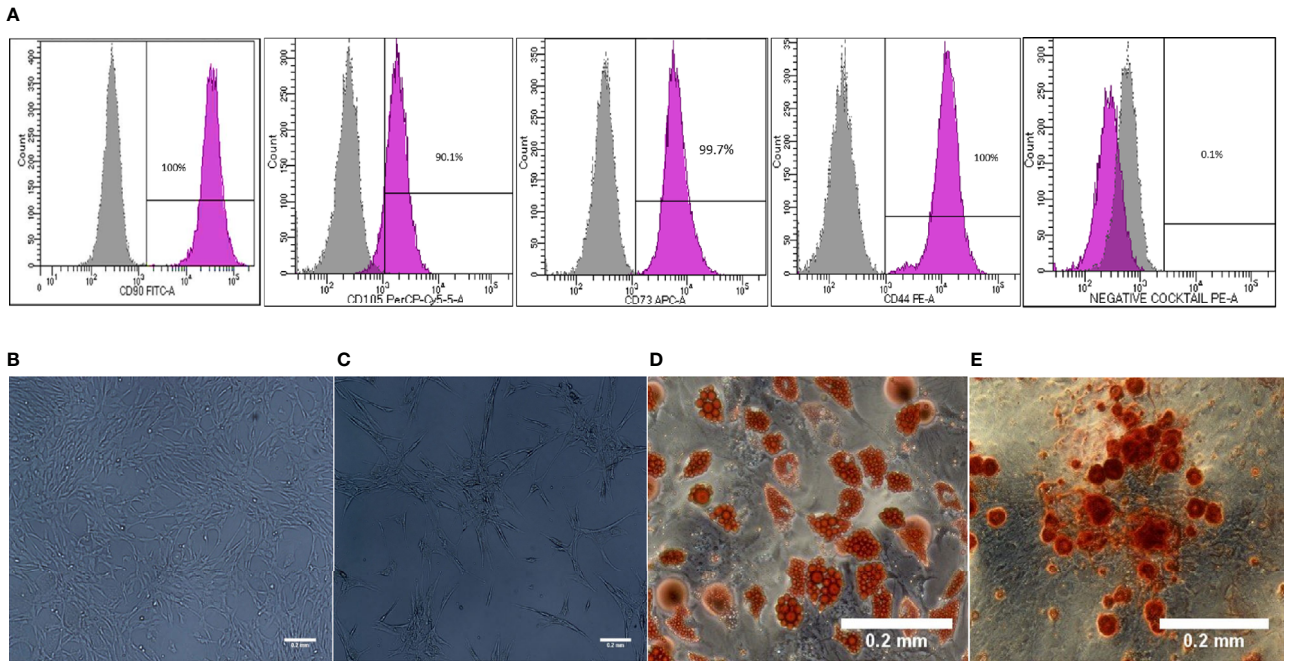
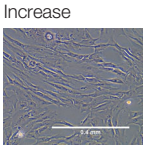
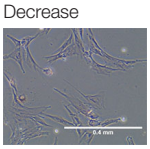


FIGURE 1 | Characterization and differentiation potential of WJ-MSCs priming with IFN-γ and TNF-α **(A)** Representative flow cytometry histograms showing high expression of CD90, CD105, CD73 and CD44 and lack the expression of negative MSCs cocktail (CD34, CD11b, CD19, CD45 and HLA-DR). Gray peak corresponds with isotype control and the violet peak corresponds with the antibodies. **(B)** Unprimed WJ-MSCs. **(C)** Primed WJ-MSCs. **(D)** Oil red staining of primed WJ-MSCs after culture in adipogenic differentiation media. **(E)** Alizarin red staining of primed WJ-MSCs after culture with Osteogenic differentiation media. Data were calculated from five samples. Experiments performed in triplicate for each sample. Data were analyzed using FACS canto II. Magnification = 100x.

TABLE 3 | Morphological features of WJ-MSCs before and after priming with IFN-γ and TNF-α.

WJ-MSCs morphological features	Unprimed WJ-MSCs	Primed WJ-MSCs
Irregularity	Decrease	Increase
Range	Increase	Decrease
Circumferences	Decrease	Increase
Form factor	Increase	Decrease
Eccentricity	Decrease	increase
Rigidity	Increase	Decrease
Aspect ratio	Decrease	Increase
Nucleus-cytoplasm ratio	Increase	Decrease
Morphological response		

Morphological features were investigated using Evos Cell Imaging System at 20X magnification and the morphological variations were analyzed by ImageJ software.

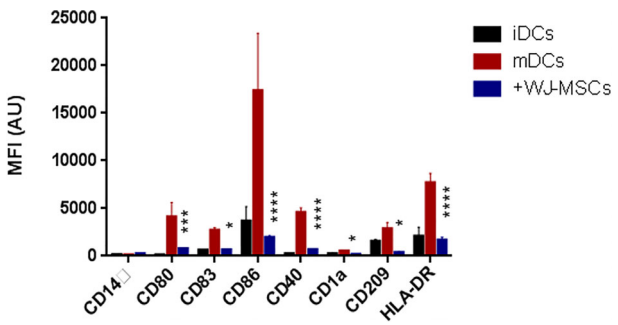


FIGURE 2 | Phenotype of DCs. Mean fluorescence intensity (MFI) ± SD for the surface intensity of co-stimulatory molecules and maturation markers on iDCs (black), GAD65 pulsed mDCs (red), and GAD65 pulsed mDCs co-cultured with primed WJ-MSCs (blue). Data were calculated from three different experiments for each patient. Experiments performed in triplicate for each patient. DCs from five patients were used. Statistical significance was tested using a two-way ANOVA with Tukey's post hoc for multiple comparisons. In all analyses *p < 0.05, ***p < 0.001 and ****p < 0.0001.

toward an immature state with decreased expression of all co-stimulatory molecules and maturation markers (CD80, CD84, CD86, CD40, CD1a, CD209, and HLA-DR) (**Figure 2**).

Effect of Primed WJ-MSCs on Activated T Cells Profile and Function

No proliferation was observed in quiescent or inactivated T cells while activated T cells showed high proliferation rate which decreased significantly when co-cultured with primed WJ-MSCs (**Figures 3A, B**). Activated T cells also exhibited a significant decrease in activated CD69+ T cells. Furthermore, the percentage of CD4+ and CD8+ T cells was also significantly decreased from 21% to 5% of CD4+ and from 17% to 8% of CD8+ (**Figure 3C**).

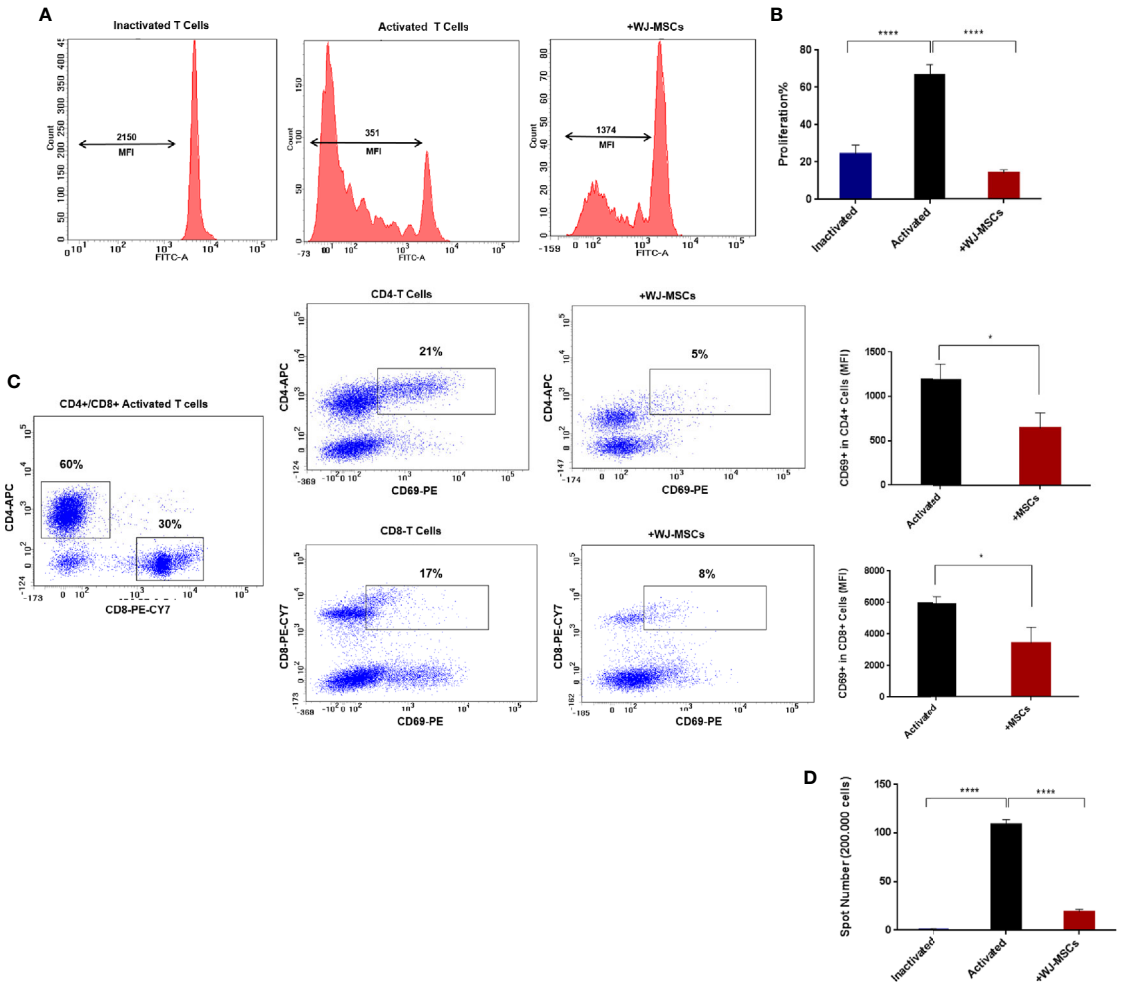


FIGURE 3 | T cells proliferation and activation and IFN-γ ELISPOT analysis **(A)** Representative flow cytometry analysis and mean fluorescent intensity (MFI) the dilution of CFSE fluorescent dye **(B)** Percentage of T cell proliferation under the following conditions: inactivated T cells (blue), activated T cells cultured without (black) and with primed WJ-MSCs (red). Statistical significance was tested using a one-way ANOVA with Tukey's post hoc for multiple comparisons **(C)** Representative flow cytometry analysis of CD4+/ CD8+ activated T cells and activated CD69+ T cells cultured with and without primed WJ-MSCs and MFI ± SD of CD69+ T cell in CD4+ and CD8+ activated T cells under the following conditions: activated T cells alone (black) and activated T cells co-cultured with primed WJ-MSCs (red). An unpaired *t*-test was performed to test statistical significance **(D)**. Mean ± SD of IFN-γ spots per well (200.000 cells) under the following conditions: inactivated T cells (blue), activated T cells cultured without (black) and with primed WJ-MSCs (red). Statistical significance was tested using a one-way ANOVA with Tukey's post hoc for multiple comparisons. Data were calculated from three different experiments for each patient. Experiments performed in triplicate for each patient. T cells from five patients were used. For all analyses *$p < 0.05$, and ****$p < 0.0001$.

Moreover, ELISPOT analysis showed significant inhibition of positive IFN-γ response to GAD65 when activated T cells were co-cultured with primed WJ-MSCs (**Figure 3D**).

Effect of Primed WJ-MSCs on the Secretion Level of Pro-Inflammatory and Anti-Inflammatory Cytokines

The secretion levels of anti-inflammatory cytokines (IL-6, IL-10 and TGF-β) were significantly increased when mDCs were co-cultured with primed WJ-MSCs (**Figure 4A**). Furthermore, the secretion levels of anti-inflammatory cytokines (IL-6, IL-10 and TGF-β1) were significantly increased when activated T cells were co-cultured with primed WJ-MSCs. Activated T cells secreted larger amounts of IFN-γand IL-17 than quiescent or inactivated T cells. The levels of IFN-γand IL-17 were significantly decreased when activated T cells were co-cultured with primed WJ-MSCs (**Figure 4B**).

TNF-α and IFN-γ Priming WJ-MSCs Enhances the Expression of Immunomodulatory Genes and Impair the Expression of Immunostimulatory Genes in mDCs and Activated T Cells

As shown in (**Figure 5**), gene expression profile was evaluated in mDCs. The results showed significant increase in the expression of immunoregulatory genes *(IL10, IDO1, NT5E/ENTPD1,*

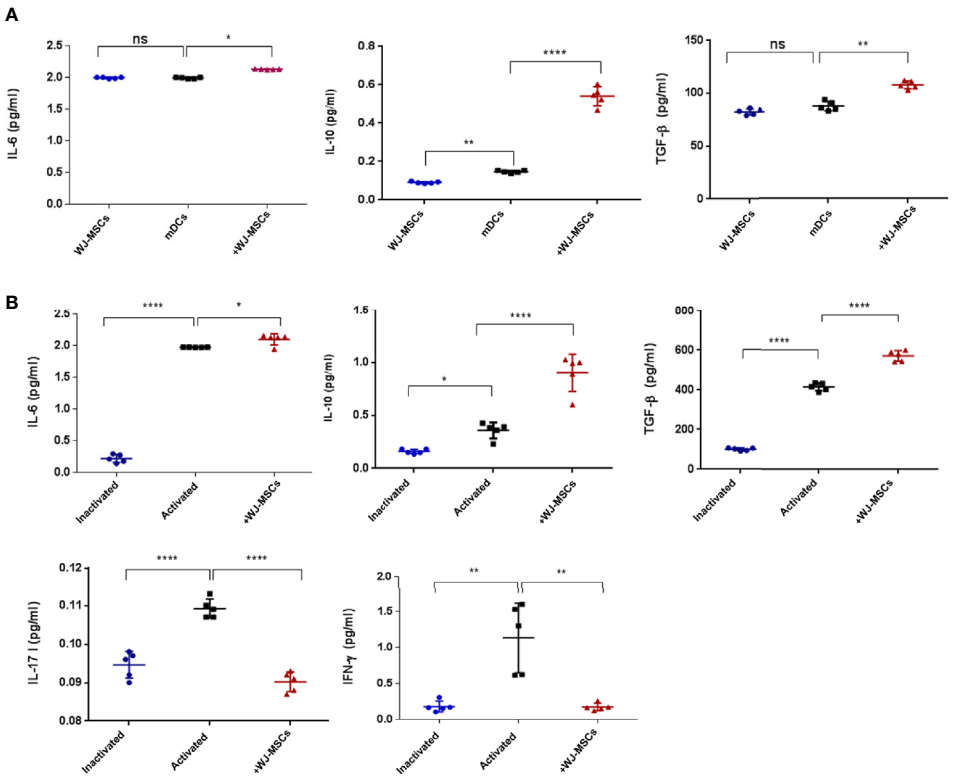


FIGURE 4 | Cytokines secretion level. **(A)** Mean ± SD levels of secreted IL-6, IL-10, and TGF-β in cell free culture supernatant under the following culture conditions: primed WJ-MSCs alone (blue), GAD65- pulsed mDCs co-cultured without (black) and with primed WJ-MSCs (red). **(B)** Mean ± SD levels of secreted IL-6, IL-10, TGF-β, IL-17 and IFN-γ in cell free culture supernatant under the following culture conditions: inactivated T cells (blue), activated T cells without (black) or with primed WJ-MSCs (red). Data were calculated from five patients. Experiments performed in duplicate for each patient. In all analyses *p < 0.05, **p < 0.01 and ****p < 0.0001. ns, not significant.

FOXP3, IL6, and PTGES2/PTGS1). In addition, they showed insignificant increase in the expression of *TGFB1*. Furthermore, mDCs showed significant increase in the expression of *TNFA*. Fold regulation results confirmed the upregulation and downregulation of mDCs target genes. Also, no expression of *IFNG, IL-17A* and, IL-*2* was detected in mDCs (**Table 4**).

As shown in (**Figure 6**), activated T cells showed significant increase in the expression of immunoregulatory genes *(IL10, IDO1, NT5E/ENTPD1, FOXP3, IL6, and PTGES2/PTGS1)*. Significant increase in the expression of *TGFB1* was detected in activated T cells compared to mDCs. Moreover, significant downregulation of immunostimulatory genes (*TNFA, IFNG, IL-17A*, and *IL-2)* was also detected. Fold regulation results confirmed the upregulation and downregulation of activated T cells target genes. However, T cells exhibited more upregulation and downregulation in gene expression than mDCs (**Table 5**).

DISCUSSION

Type 1 diabetes is a well-known autoimmune disease characterized by specific adaptive immunity against β-cell antigens. Type 1 diabetes occurs when the balance between the regulatory and inflammatory T-cells is lost. To date, there is no effective therapeutic approach for the management of T1D but recently, MSCs have been reported as a promising immunosuppressant in various autoimmune diseases including T1D (39, 40). However, successful MSCs-based therapy still faces obstacles due to the high sensitivity of MSCs to the environment of immune-mediated diseases, the differences of culturing protocols, and the cell senescence that results from overexpansion of cells. Therefore, a current concern is how to enhance the immunomodulatory effects of MSCs.

In this study, the immunomodulatory effects of primed WJ-MSCs were investigated. The results showed that high differentiation potential and unique morphological features were obtained when WJ-MSCs were primed with both IFN-γ and TNF-α. Accordingly, our findings provide an additional evidence that the morphological appearance can be used to predict the function of MSCs *in vitro* when pre-conditioned with both *TNF-α and IFN-γ.*

Primed WJ-MSCs exerted an immunomodulatory effect on mDCs by skewing toward tolerogenic or immature phenotype. Tolerogenic DCs express low amounts of co-stimulatory molecules on their surfaces and display increased production of anti-inflammatory cytokines including IL-6, IL-10, and TGF-β1. Furthermore, tolDCs are capable of driving T cells to

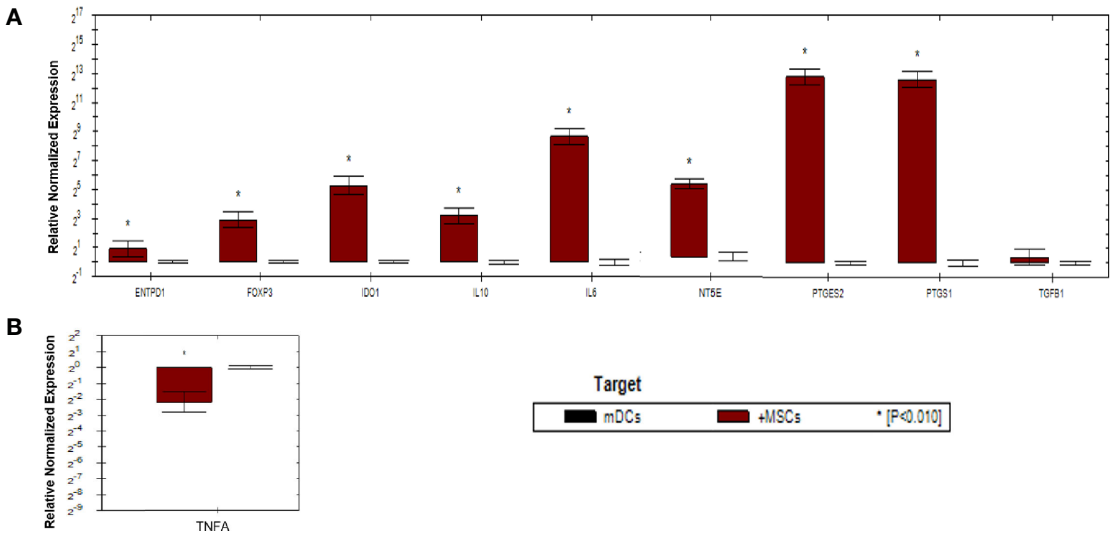


FIGURE 5 | Relative normalized expression of immunomodulatory and immunostimulatory genes of mDCs after co-cultured with primed WJ-MSCs. **(A)** upregulated genes. **(B)** downregulated gene. Mature dendritic cells from four patients were used. Results were normalized to 18S rRNA Each sample was performed in triplicate, and a mean value was calculated. Data were analyzed according to 2−ΔΔCT method using CFX Maestro™ Software - Bio-Rad. *$p \leq 0.01$ and fold change ≥ 1.5.

TABLE 4 | Upregulated and downregulated genes in mDCs after co-cultured with primed WJ-MSCs.

Gene symbol	Fold regulation 2−ΔΔCT	P value
TGFB1	1.3	0.010785
IL10	9	0.000062
IL6	402	0.000028
IDO1	38.4	0.000778
FOXP3	7.5	0.000175
PTGS1	10.2	0.007765
ENTPD1	1.9	0.000871
NT5E	12.4	0.000961
PTGES2	7106	0.000088
IFNG	NA	NA
TNFA	-4.5	0.000562
IL17A	NA	NA
1L2	NA	NA

Results were normalized to 18S rRNA Each sample was performed in triplicate, and a mean value was calculated. Data were analyzed according to 2−ΔΔCT method using CFX Maestro™ Software - Bio-Rad. $p \leq 0.05$ and fold change ≥ 1.5.
NA, not applicable.

differentiate into Tregs. This increase is a result of the overexpression of *IL6, IL10*, and *FOXP3*.

Primed WJ-MSCs exhibited immunomodulatory effect on CD4+ and CD8+ T cells by producing tolDCs which inhibit antigen-specific T cell responses through induction T cell anergy.

The levels of IFN-γ were significantly decreased when activated T cells were co-cultured with primed WJ-MSCs. This led us to conclude that primed WJ-MSCs suppress T cells-mediated autoimmunity. It is well known that IFN-γ are produced extensively by activated T cells and it is a key moderator of T cells-mediated immunity (41, 42).

As for the production of immunomodulatory factors by mDCs and by activated T cells after co-culturing with primed WJ-MSCs, significant upregulation of *IDO1* expression in both mDCs and activated T cells was detected. This provides a potent evidence which confirms that priming WJ-MSC with TNF-α and IFN-γ promotes the immunosuppressive potential of these cells. This can be considered a critical finding because quiescent MSCs are unable to express *IDO1* (43). However, *IDO1* is considered one of the key modulators of the immune tolerance mechanism (43, 44), and is involved in the tryptophan catabolites or depletion (45–47) which is responsible for the inhibition of T cell activation and proliferation *via* the induction of T cell anergy (48, 49).

Both TGF-β1 and IL-10 are critical anti-inflammatory cytokines for Treg formation (50, 51). Moreover, they are capable of inhibiting T cells proliferation and activation as well as suppressing of Th17 generation. These results are consequences of the increase in the production of TGF-β1, IL-10, IFN-γ, and IL-17 after activated T cells are co-cultured with primed WJ-MSCs. Furthermore, TGF-β1 is a key anti-inflammatory cytokine which is responsible for the formation of Tregs due to its ability to upregulate *FOXP3*.

The downregulation of *IFNG, IL2* and IL*17A* expression and the significant reduction of IFN-γ and IL-17 cytokines after activated T cells were co-cultured with primed WJ-MSCs represent a strong evidence of the suppression of T cells proliferation and activation. In addition, the downregulation of *IL2* represents a strong evidence of the inhibition of T cells-mediated autoimmunity because *IL2* are produced only by CD4+ and CD8+ T cells. Moreover, IL2 plays a pivotal role in T cells-receptor signaling pathway (52).

Significant expression of *PTGS1* and *PTGES2* was observed when primed WJ-MSCs were co-cultured with mDCs and activated T cells. *PTGES2* and *PTGS1* genes are responsible for

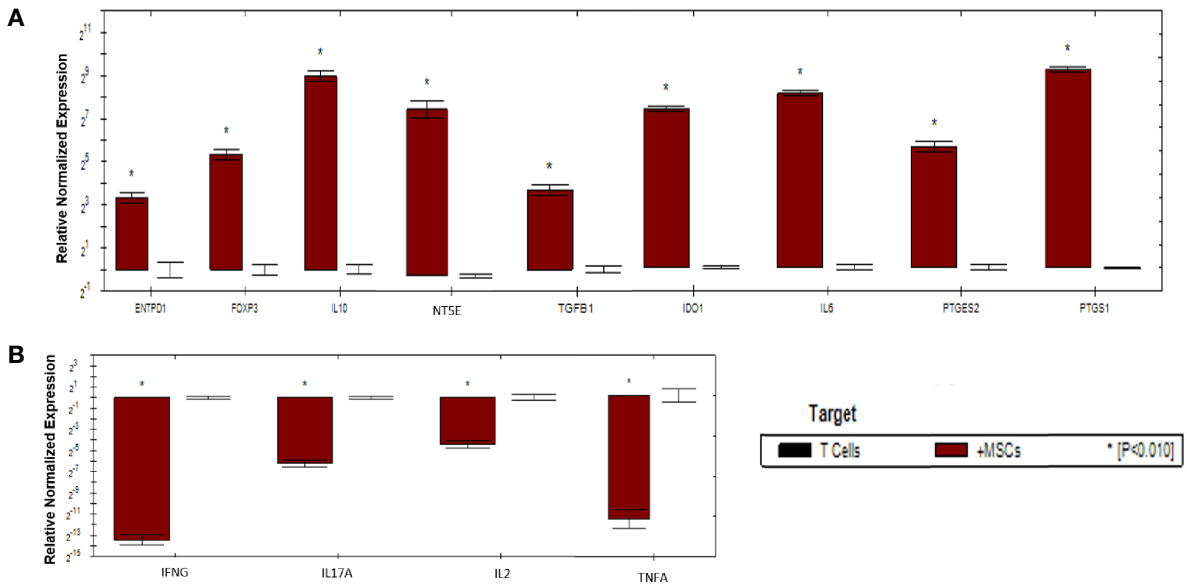


FIGURE 6 | Relative normalized expression of immunomodulatory and immunostimulatory genes of activated T cells after co-cultured with primed WJ-MSCs. **(A)** upregulated genes. **(B)** downregulated genes. T cells from four patients were used. Results were normalized to 18S rRNA Each sample was performed in triplicate, and a mean value was calculated. Data were analyzed according to 2−ΔΔCT method using CFX Maestro™ Software - Bio-Rad. *$p \leq 0.01$ and fold change ≥ 1.5.

TABLE 5 | Upregulated and downregulated genes upon priming WJ-MSCs with TNF-α and IFN-γ and cultured with activated T cells.

Gene symbol	Fold regulation 2−ΔΔCT	P value
TGFB1	13	0.000567
IL10	499.1	0.002305
IL6	177.5	0.000057
IDO1	112	0.000089
FOXP3	40.5	0.000541
PTGS1	369	0.000001
ENTPD1	6151.5	0.001495
NT5E	1639.7	0.000197
PTGES2	36	0.002198
IFNG	-11191	0.002797
TNFA	-8.3	0.000545
IL17A	-74.8	0.000117
1L2	-21.1	0.008139

Results were normalized to 18S rRNA Each sample was performed in triplicate, and a mean value was calculated. Data were analyzed according to 2−ΔΔCT method using CFX Maestro™ Software - Bio-Rad. $p \leq 0.05$ and fold change ≥ 1.5. NA, not applicable.

the production of prostaglandin E2 (PGE2) which is involved in the immune-suppressive mechanism of MSCs and it increases the expression of anti-inflammatory factors (53–55). The upregulation of *IL10*, and *TGFB1* as well as the significant production of TGF-β1 and IL-10 might be explained by the overexpression of PTGS1 and PTGES2.

Interleukine-6 (IL-6) is a key factor in the formation of Tregs and in the suppression of pro-inflammatory responses (56, 57). The upregulation of *IL6* gene and the increase of IL-6 cytokine may explain the inhibition of pro-inflammatory responses of mDCs and activated T cells which is caused by primed WJ-MSCs. Moreover, a previous study illustrated that immunosuppressive properties of amnion-derived MSCs are not constitutive, but require a supportive signal to produce PGE2, IDO1, and a high level of IL-6 (51). Our findings may support the immunomodulatory effect of primed WJ-MSCs on mDCs and activated Tcells.

Destructed β cells play a key role in the progression of T1D through the release of dangerous extracellular ATP signal which acts as a potent immune-stimulator to enhance inflammatory responses. So, we investigated the effect of primed WJ-MSCs on the expression of *ENTPD1/NT5E* genes which play a pivotal role in the production of extracellular adenosine through ATP hydrolysis. However, extracellular adenosine act as a potent immune-regulator signal that modulate innate and adaptive immunity. Moreover, adenosine can prevent the activation, proliferation and cytokine production in CD4+ and CD8+ T cells (58–60). ENTPD1/NT5E are considered the most important genes in the immunosuppressive mechanisms that attribute to Treg formation and T cell anergy (61, 62). Furthermore, they maintain the balance between ATP/adenosine to increase immune hemostasis (63). Significant increase in the expression of *ENTPD1/NT5E* may be considered a robust finding because of their role in maintaining balance between anti-inflammatory and pro-inflammatory factors.

Although the direct contact with MSCs is critical for the immunomodulatory effects, the paracrine effects of MSCs cannot be ignored. The Increase in the production of anti-inflammatory cytokines (IL-10, IL-6 and TGF-β1) and the suppression in the production of pro-inflammatory cytokines (IL-17 and IFN-γ) in conditioned media suggest that the immunomodulatory effects of primed WJ-MSCs after being co-cultured with mDC and T cells seems to be a consequence of a synergic effect mediated by both the direct contact and secretome of primed WJ-MSCs.

Collectively, this study confirmed that WJ-MSCs primed by IFN-γ and TNF-α modulated mDCs-mediated antigen presentation through the induction of tolDCs in addition to the modulation of antigen-specific-T cell responses through the induction of T cell anergy. More importantly, this study paves the road to utilizing primed WJ-MSCs-based transplantation therapies. Further study of primed WJ-MSCs cellular therapies in an animal model of T1D is recommended before attempting in humans. Successful treatment in humans would involve establishing safety first, then optimizing administration in respect to disease stage. Premature of primed WJ-MSCs therapies in human would not only risk safety and efficacy, but also provide false hope to patients.

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 protocol was approved by Institutional Review Board (IRB) committee of the Cell Therapy Center, The University of Jordan, Jordan (IRB NO. 07-11-2019). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MA-M designed and conducted the study. MA-M and SM analyzed and interpreted data. MA-M wrote the original manuscript. NA revised the manuscript. HJ and AA were the administrators of the project. AA provided the advices for study. All authors contributed to the article and approved the submitted version.

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How to Prevent and Mitigate Hypersensitivity Reactions to Biologicals Induced by Anti-Drug Antibodies?

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Biologicals are widely used therapeutic agents for rheumatologic diseases, cancers, and other chronic inflammatory diseases. They are characterized by complex structures and content of variable amounts of foreign regions, which may lead to anti-drug antibodies (ADA) development. ADA onset may limit the clinical usage of biologicals because they may decrease their safety. In fact they are mainly associated with immediate hypersensitivity reactions (HSRs). Development of ADAs is reduced by concomitant immunosuppressive treatment, while it is increased by longer intervals between drug administrations; thus, regular infusion regimens should be preferred to reduce HSRs. Once ADAs have formed, some procedures can be implemented to reduce the risk of HSRs. ADAs may belong to different isotype; the detection of IgE ADA is advisable to be assessed when high and early ADAs are detected, in order to reduce the risk of severe HRs. In patients who need to reintroduce the biological culprit, as alternative therapies are not available, drug desensitization (DD) may be applied. Desensitization should be conceptually dedicated to patients with an IgE-mediated HSR; however, it can be performed also in patients who had developed non-IgE-mediated HSRs. Although the underlying mechanisms behind successful DD has not been fully clarified, the DD procedure is associated with the inhibition of mast cell degranulation and cytokine production. Additionally, some data are emerging about the inhibition of drug-specific immune responses during DD.

Keywords: anaphylaxis, immunogenicity, hypersensitivity reactions, drug desensitization, anti-drug antibodies, IgE

INTRODUCTION

Biologicals are indispensable therapeutic agents in immunological, oncological, and inflammatory diseases, and their application in clinical practice is increasing. However, despite the therapeutic benefits, biologicals may cause hypersensitivity reactions (HSRs) that represent a major safety concern and a significant challenge for clinicians. Hypersensitivity reactions may occur during the

first lifetime exposure or after repeated administrations and different pathogenic mechanisms are involved. In addition, immediate and delayed HSRs have been conveniently described; immediate reactions occur during the drug administration or within 1 h after the end of infusion, whereas delayed reactions appear from 1 h to several days after (1, 2).

The potential risk of biologicals' immunogenicity is an important issue in clinical practice, leading to the development of anti-drug antibodies (ADA). Immunogenicity is expected following treatment with non-human sequence proteins, but it is now well established that the immune response may be elicited also by fully human sequences. The unexpected and unpredictable unwanted immunogenicity may impact both the efficacy and the safety of the drug. The induced ADA may compromise the clinical efficacy by altering the circulating drug levels and/or neutralizing its biological functions; furthermore, ADA development has been associated with the onset of HSR, ranging from mild to severe grade. Patients with ADA, as IgG or IgE developed during treatment with biologicals or preexisting, are more likely to have increased risk of immediate HSRs (1).

In patients with HSR toward a biological, approaches for avoiding future adverse events differ depending on the mechanism of reactions. These strategies are very important when no alternative therapies are available. Similarly, it was shown that in some cases, reactions against the biological could be predicted by the presence of ADA.

In this review we discuss the clinical and diagnostic strategies to prevent and mitigate HSR toward biologicals, especially when related to the development of immunogenicity.

MECHANISMS OF HYPERSENSITIVITY REACTIONS TO BIOLOGICALS

Taking into account the structural characteristics of biologicals, which differ from traditional drugs, and their ability to elicit ADA, the mechanisms underlying HSR to biologicals can be divided into ADA and non-ADA mediated. Additionally, ADA may belong to different isotypes, so ADA-mediated HSR may be classified as IgE- and non-IgE-mediated reactions. According to clinical manifestations, immediate HSRs are classified as mild, moderate, or severe, and in some cases even life-threatening reactions have been described (3). Hypersensitivity reactions may have similar clinical presentation in ADA-positive and ADA-negative patients.

IgE-Mediated Reactions to Biologicals

The development of IgE ADA has been described in patients treated with different types of biologicals (4–9). IgE binds to mast cells' and basophils' surface receptors (FcεRI, high-affinity receptor), thus initiating immediate HSRs sustained by the release of vasoactive mediators such as histamine, tryptase, leukotrienes, prostaglandins. IgE ADA are detectable in a subgroup of patients with HSR to biologicals; in patients suffering from immunomediated inflammatory diseases and treated with infliximab who had experienced a previous reaction, about 20% of them tested positive for IgE ADA (10). The onset of an anti-drug humoral response of IgE isotype is facilitated by the repeated administrations of biologicals, so IgE-mediated reactions occur during or after the second drug administration. Cetuximab, largely used in head and neck cancers, represents an exception in this field, because most of the cetuximab-induced reactions occur within minutes upon first treatment exposure (11). Cetuximab-specific IgE antibodies pre-exist to the first drug administration, and their production has been shown to be triggered by tick bites (12). IgE response to cetuximab is different from typical IgE responses specific for protein epitopes expressed by other biologicals; in fact, it is directed towards a mammalian oligosaccharide epitope, galactose-alpha-1,3-galactose (alpha-gal), present in the Fab portion of this monoclonal antibody, as well as present on non-primate mammalian proteins (13). IgE sensitization towards biologicals is associated with a higher severity of reactions, as shown for both infliximab and cetuximab (10, 14), and at least for infliximab, it has been shown more frequently at re-exposure after a period of drug interruption (10). Finally, IgE ADA are more frequently developed in patients with higher ADA levels and earlier ADA onset, but their rate of negativization is faster (15).

Non IgE-Mediated Reactions to Biologicals

The IgE antibody pathway had been universally accepted as the only pathogenic explanation of anaphylaxis until more recent observations obtained in mouse models suggest the existence of a non-classical (non-IgE-mediated) pathway for anaphylaxis (16). Taking into account that the majority of ADA belong to the IgG isotype, and that the majority of patients tested negative for IgE ADA, the existence of IgG-mediated HSR to biologicals has been hypothesized. Animal models revealed that anaphylaxis may occur through an IgE-independent manner, involving specific IgG, FcγRIII, macrophages, basophils, and the Platelet Activating Factor (PAF) as major mediator (17). The development of drug-specific IgG may lead to the formation of immunocomplexes between biological and ADA with subsequent Complement activation and production of anaphylatoxins (C3a and C5a), which directly activate mast cells, expressing C3a and C5a receptors. Additionally, FcγR-mediated activation of basophils, neutrophils, monocyte/macrophages induced by ADA may be involved in systemic immediate HSR (18). ADA may directly activate neutrophils and basophils (expressing FcγRIIA and FcγRIIIB receptors) and monocyte/macrophages (expressing FcγRIIA and FcγRIIIA receptors), leading to their release of PAF. In addition, FcγRIIA-expressing platelets may be involved by ADA-drug immunocomplexes, leading to their release of pathogenic serotonin (19).

PREVENTION OF HSR TO BIOLOGICALS

Identification of Patients With Clinical Risk Factors for HSR

The identification of those patients with higher risk for ADA development and HSR onset represents an important clinical issue.

Risk assessment for biologicals-related HSR requires accurate evaluation of risk factors associated with immunogenicity.

Female patients seem to show a higher rate of biological-induced HSR, although this point remains a matter of debate (20). Atopic status, as well as previous adverse drug reactions, do not appear to be a clinical risk factor. In a multicenter cohort prospective study including 560 patients with different immune-mediated diseases and treated with eight different biologicals, immune-suppressants and antibiotics were associated with a decreased risk of ADA development, whereas smoking and infections during the study were associated with increased risk. Additionally, HLA-DQA1*05 was associated with a significantly increased rate of immunogenicity, although evidence to support genotyping strategy are lacking (21). The underlying disease itself, and in particular the highly activated B-lymphocyte status and the high expression of costimulatory molecules on dendritic cells in patients with immune-mediated diseases, can be an important factor in the development of an unwanted immune response towards the biological, as shown by the higher incidence of infliximab reactions in patients with rheumatoid arthritis than in those suffering from seronegative spondiloarthritis and vasculitis (22), or by the higher detection rate of anti-rituximab antibodies in autoimmune than in lymphoma patients (23–25).

Finally, taking a thorough history, including the course of treatment, represents the most useful risk-assessment tools in HSR to biologicals related to immunogenicity. In fact, intermittent therapy or re-exposure after a long treatment-free interval may be associated with an enhanced immune response (or loss of tolerance) to the biological agent, and thus, re-treated patients must be considered at risk of reactions (26–30).

Assessment of Immunogenicity to Prevent Hypersensitivity Reactions to Biologicals

Using current technology and reagents, it is possible to develop highly sensitive ADA screening assays capable of detecting the most prevalent classes of ADA (IgM, IgG, and IgA); however, a separate IgE ADA assay may be required for IgE detection, due to the low circulating levels of specific IgE. It has been shown that ADA may be present in serum before therapy as in the case of cetuximab (pre-existing ADA), or developed during the course of treatment (induced ADA). Anyway, also in the case of induced antibodies, the development of ADA precedes the onset of reactions. In a longitudinal study performed in 91 infliximab-treated patients, assayed for ADA and drug levels by enzyme-linked immunosorbent assay and for IgE by ImmunoCAP system, it has been shown that the HSR tends to be preceded by ADA development, which in turn is associated with the reduction in drug serum levels (15). Specifically, all HSRs that occur after a period of drug interruption are preceded by ADA development.

Data from literature clearly show that the detection of pre-existing ADA IgE, in the serum of patients at baseline, is helpful to identify patients at risk of (severe) cetuximab-induced HSR (13). Finally, analyses stratified by ADA titer may identify patient subpopulations more at risk for clinical events (1). Overall, both ADA evaluation and therapeutic drug monitoring may have a relevant impact on clinical practice to prevent HSR to biologicals.

The Role of Immunomodulatory Therapies to Block ADA Formation

Compared to biologic monotherapy, concomitant use of immunomodulators often increases the systemic exposure of the biologicals and decreases the formation of anti-drug antibodies, consequently enhancing clinical efficacy (31).

The use of methotrexate (MTX) may attenuate the frequency of ADA in patients suffering from rheumatoid arthritis, spondiloarthritis, and inflammatory bowel diseases. Azathioprine, also an immunosuppressive drug with a similar effect to MTX, has been observed with azathioprine usage in the management of Crohn's disease, where it can be given in combination with infliximab or adalimumab to improve treatment and reduce immunogenicity and ADA formation. Some studies looked at concomitant therapy with leflunomide and mycophenolate that have also been shown to be associated with lower ADA prevalence, suggesting that all DMARDs may be associated with benefits against drug-induced immunogenicity (32). However, the definition of the impact of individual DMARDs on immunogenicity is still an unmet need in inflammatory arthritis because of small numbers of patients on DMARDs other than MTX, and because some patients were treated with more than one conventional DMARD. Of note, there have been studies indicating that addition of immunomodulators to the TNF-α inhibitors not only prevents the immunogenicity but also helps in the elimination of existing ADA, thus improving treatment and its safety (33–36). More research should be undertaken to identify and validate prognostic markers for predicting patients who would benefit the most and those who are at greater risk from combination therapy with immunomodulators and biologicals.

The Role of Premedication

Antihistamines, corticosteroids, and acetaminophen are commonly used in premedication protocols to prevent HSR towards biologicals, with a high variability of protocols among centers (37). Among antihistamines, diphenhydramine and cetirizine are the most frequently administered, the latter favored by a lower degree of induced sedation. Most of clinicians use hydrocortisone before biological infusion, but 6-metyl-prednisolone is also administered. A placebo, randomized, controlled trial has previously shown that 200 mg hydrocortisone intravenously administered is able to reduce ADA development in a significant manner, although without totally abrogating HSR (38). Several studies have demonstrated a reduction in the number of HSR with the use of premedication, especially in cancer patients (39). However, in different clinical setting, the use of premedication is still controversial; despite some initial favorable results (40), most recent data obtained in both adult and pediatric patients suggest that premedication would not change the incidence of infliximab-related HSR (41–44). To date, there are no definitive data because a specific schedule has never been validated in controlled studies.

LONG-TERM MANAGEMENT OF PATIENTS WITH HSR TO BIOLOGICALS

Hypersensitivity reactions to biologicals may be characterized by severe anaphylaxis that can be rapidly progressing and fatal, and therefore establishing its cause is pivotal to long-term risk management, but more importantly, the optimal management of reactive patients must ensure the patient the most effective therapy for the treatment of the underlying disease in a safe manner.

Definition of the Pathogenic Mechanism of HSR

Both skin testing and detection of serum ADA may be useful to define the pathogenic mechanisms in HSR patients. Skin testing represents the gold standard for the identification of true allergy (IgE-mediated) towards biologicals and are safe procedures also in patients who had experienced severe reactions (10). However, some main limitations and unmet needs are recognized: the low availability of test solutions, the lack of standardization of drug concentrations, and the unknown negativization rate (45).

Although they are not widely used, several commercial tests are available for the assay of ADA (CE marked), able to detect non-isotype-specific ADA. On the other hand, commercially available tests for IgE ADA detection are lacking, thus representing a crucial unmet need in the diagnostic workup of immediate HSR to biologicals (45). Other challenges in the IgE assay are represented by the low concentration of IgE compared with IgG antibodies, also with the same specificity, which may interfere with the IgE ADA assessment (46). Basophil activation test (BAT) could be included in the diagnostic workup of immediate HSR to show the ADA-mediated mechanism (both IgG and IgE) (47). However, studies in larger series of patients are needed to confirm the findings and establish BAT as a diagnostic tool, taking into account some technical limitations of BAT, mainly related to the existence of patients with non-responsive cells.

Drug Desensitization to Induce Tolerance in Patients With HSR

Re-treatment with the same biological represents an option following an HSR, and drug desensitization (DD) is a therapeutic approach to safely administer biologicals causing an HSR. It is able to provide a temporary immune tolerance to drugs, and it is highly recommended when switching to alternate products with equal efficacy is not possible. Drug desensitization is a method largely applied for chemotherapy (48) and more recently used also for a safe reintroduction of biologicals in reactive patients; DD allows the administration of the full therapeutic dose in relatively short time (4–12 h), during which the biological is administered at increasing concentrations and increasing rate of infusion. Although different protocols have been published until now, the most frequently applied is the 12-step protocol (49). However, additional steps may be added, as well as other modifications may be performed regarding the time intervals between doses and the final rate of infusion. DD procedure has been demonstrated as a safe option for patients who have experienced HSR to cetuximab, rituximab, trastuzumab, anti-TNF blockers, and tocilizumab (50, 51). DD may be complicated by breakthrough reactions (45) that occur during the last steps and are usually mild and less severe than initial HSR (3). A correlation between breakthrough reactions and positivity of skin tests has been reported; in fact it has been described that a positive skin test result is the main predictor for breakthrough reactions (49).

Although DD is conceptually dedicated to patients in which an IgE-mediated mechanism is demonstrated by positive skin testing or serum IgE for culprit drug, patients with immediate HSR to chemotherapy (taxanes and platins) in which the IgE mechanism cannot be demonstrated have also been successfully desensitized (52).

There is as yet no consensus in literature about the underlying mechanisms operating in DD; the majority of data focused on the role of mast cells showing that desensitization procedure is associated with the inhibition of mast cell degranulation and cytokine production (53). Patients with IgE-dependent HSR displayed negative skin testing after DD, suggesting inhibition of the mechanisms that induce cell activation. These data have been extensively described in DD for chemotherapeutics and more recently for biological agents (54, 55) and show that DD is an antigen-specific process. Recent studies have shown that antigen/IgE/FcεRI surface expression do not change during the DD procedure and that mast cells' hyporesponsiveness is attributable, at least partially, to abrogation of Ca++ mobilization, a critical determinant of both degranulation and cytokine production responses in mast cells (53). The adaptive immune response sustained by drug-specific T cells and its modification during DD procedures have been scarcely evaluated until now. Results obtained in patients submitted to DD for biologicals highly suggest that DD procedure may be able to modulate the adaptive immune response, with a decrease of ADA, including IgE isotype (53). More importantly, the modulation of humoral immune response is accompanied by the reduction of drug-specific T-cell proliferation to the biological (53, 54). Furthermore, the involvement of regulatory mechanisms, such as activation/expansion of drug-specific IL-35-producing T cells, may occur during the procedure and participate in the modulation of effector drug-specific response (56, 57).

CONCLUSIONS

Biologicals are structurally immunogenic and are able to elicit a complete adaptive immune response, which negatively impacts their safety and may limit their clinical use. Specific diagnostic workup and a modified method of drug delivery (drug desensitization) are available in the clinical setting to manage patients with HSR. The knowledge of the mechanisms underlying HSR and biologicals' immunogenicity is useful to increase the safety profile of current and novel biologicals.

AUTHOR CONTRIBUTIONS

AV and AM wrote the manuscript. EM, MP, FN, and EV revised the manuscript. All authors contributed to the article and approved the submitted version.

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Polyclonal Regulatory T Cell Manufacturing Under cGMP: A Decade of Experience

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We report on manufacturing outcomes for 41 autologous polyclonal regulatory T cell (PolyTreg) products for 7 different Phase 1 clinical trials over a 10-year period (2011-2020). Data on patient characteristics, manufacturing parameters, and manufacturing outcomes were collected from manufacturing batch records and entered into a secure database. Overall, 88% (36/41) of PolyTreg products met release criteria and 83% (34/41) of products were successfully infused into patients. Of the 7 not infused, 5 failed release criteria, and 2 were not infused because the patient became ineligible due to a change in clinical status. The median fold expansion over the 14-day manufacturing process was 434.8 -fold (range 29.8-2,232), resulting in a median post-expansion cell count of 1,841 x 10^6 (range 56.9-16,179 x 10^6). The main correlate of post-expansion cell number was starting cell number, which positively correlates with absolute circulating Treg cell count. Other parameters, including date of PolyTreg production, patient sex, and patient age did not significantly correlate with fold expansion of Treg during product manufacturing. In conclusion, PolyTreg manufacturing outcomes are consistent across trials and dates of production.

Keywords: regulatory T cell manufacturing, cGMP, *ex vivo* expansion, cellular therapy, regulatory T cells

INTRODUCTION

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells that suppress excessive immune activation and prevent autoimmunity (1–4). Adoptive cell therapy with polyclonal Tregs (PolyTregs) shows a favorable safety profile in patients with autoimmune disease, solid organ transplant, and graft versus host disease (5–13).

Efficient clinical-scale manufacturing of Treg products requires isolation of Tregs and adequate *ex-vivo* expansion while maintaining Treg cell identity. Several different clinical scale methods for Treg product manufacturing have been published that use different Treg sources, isolation methods, expansion methods, and dose formulations (14). However, the overall approach to *in vitro* Treg manufacturing is broadly similar. Most Treg manufacturing approaches start with autologous

patient peripheral blood, which contains a small percentage of Tregs. Tregs are then selected by one or more methods such as magnetic-activated cell sorting (MACS) for $CD25^+$ cells or fluorescence activated cell sorting (FACS) for $CD4^+CD25^+CD127^{lo/-}$ cells (6–8, 11–18). Tregs are then activated with potent stimulation through the T cell receptor and CD28 in the presence of high levels of exogenous interleukin-2 (IL-2). Beads conjugated with anti-CD3 and anti-CD28 are commonly used, although other activation reagents such as stimulated B cells or artificial APCs have also been described (19, 20). Rapamycin is sometimes added to the culture to prevent outgrowth of contaminating effector cells. Expansion methods vary greatly in the cell culture media, length of culture, and type of antigen receptor stimulus, and frequency of that stimulus. Most Treg manufacturing methods have been developed and validated using blood from healthy donors or with blood from a single patient population.

Here we describe our experience manufacturing autologous polyclonal Treg products for patients with autoimmune diseases or transplantation in multiple clinical trials over a 10-year period. The approach to Treg manufacturing used for these patients involves sorting of $CD4^+CD25^+CD127^{lo/-}$ Treg from peripheral blood mononuclear cells using FACS, following by expansion for 14 days in medium containing IL-2. Stimulation with anti-CD3/CD28 beads is provided on days 0 and 9 of the expansion. These data provide insight into the effects of intrinsic patient variability and patient disease status on Treg manufacturing outcomes.

We provide data on 41 *in vitro* expanded polyclonal $CD4^+CD127^{lo/-}CD25^+$ PolyTreg products manufactured for 7 clinical trials in patients with autoimmune conditions, patients who have undergone kidney transplantation, or patients receiving *de novo* pancreatic islet transplant. We quantify manufacturing outcomes and describe significant correlations using detailed records of patient characteristics, manufacturing parameters, and cell manufacturing outcomes.

MATERIALS AND METHODS

Manufacturing of Treg Products for Clinical Trials

Isolation, *ex-vivo* expansion, and quality control testing of Treg products was performed at the Human Islet and Cellular Transplantation Facility (HICTF) and GMP Facility, an FDA-registered cellular therapy facility at the University of California San Francisco (UCSF), using methods as previously described (21). Briefly, peripheral blood was collected in Anticoagulant Citrate Phosphate Dextrose Solution, USP (CPD) Blood Pack Units (Fenwal, Lake Zurich, IL) and processed within approximately 24 hours of collection. Peripheral blood mononuclear cells (PBMCs) were collected following density gradient centrifugation using Ficoll-hypaque solution (Amersham/GE Healthcare, Piscataway, NJ). Cell number and viability were assessed. Tregs were then isolated by fluorescence activated cell sorting (FACS) using fluorescent labeled anti-CD4, -CD127 and -CD25 antibodies. Cells were gated on $CD4^+$ $CD25^+$ $CD127^{lo/-}$. An aliquot of sorted cells was run through the flow cytometer again to assess purity. Following isolation, Tregs were plated at 0.25×10^6 cells per mL in a 24-well plate (Thermo Fisher; Waltham, MA) in X-VIVO 15 media (Lonza) containing 10% human AB serum from qualified donors. Tregs were activated with Dynabeads ClinExVivo anti-CD3/CD28 coated microbeads (Invitrogen; Carlsbad, CA) at a 1:1 bead to cell ratio. On day 2 of culture, the culture volume was doubled and IL-2 (Proleukin; Chiron Therapeutics, Emeryville, CA and others) was added to a final concentration of 300 IU/mL. Cells were resuspended, counted and fresh media containing IL-2 was added on days 5, 7, 9 and 12. IL-2 was maintained at 300 IU/mL assuming total consumption from media at each feeding. On days 5, 7, and 9, cell concentration was maintained between 0.2×10^6 and 0.3×10^6 cells per mL in appropriately sized plastic plates or flasks (Costar; Cambridge, MA). On day 12, cell concentration was maintained at 0.5×10^6 in culture bags (Saint-Gobain; Gaithersburg, MD), cell number permitting. On day 9, cells were restimulated with fresh anti-CD3/CD28 coated beads at a 1:1 ratio (**Figure 1**). Cells were harvested on day 14, counted, and analyzed for CD4, CD8, CD25 and FOPX3 expression using flow cytometry. Treg specific demethylated region (TSDR) methylation status was determined by Epiontis (Berlin, Germany). Products meeting release criteria were released for infusion. Method adjustments over the period described in this study included: qualification and use of new lots of human serum when the initial lots were used up, and a change in release criteria. The release criterion for $CD8^+$ cells was changed from <5% $CD8^+$ to <5% $CD8^+CD4^-$ since we occasionally observed $CD4^+FOXP3^+$ Treg that expressed low levels of CD8. These cells were present in only a small subset of patients and may reflect an underlying genetic de-repression of CD8 in cells committed to the CD4 lineage.

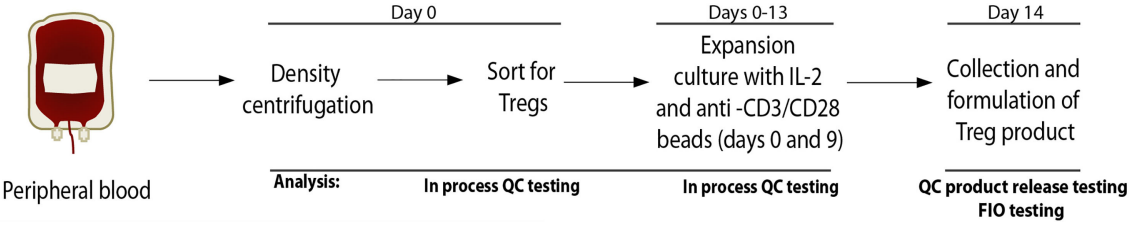

FIGURE 1 | Outline of Treg cell collection and manufacturing process. QC, quality control; FIO, for information only.

FOXP3 Locus Methylation Analysis

TSDR methylation assays were performed by Epiontis using an established protocol (22). In brief, methylation status at the FOXP3 enhancer CNS2 region was determined using bisulfite treatment of genomic DNA extracted from Treg product cell samples, followed by real-time PCR analysis using methylation-specific primers. The percentage of unmethylated DNA was calculated using the following formula: unmethylated DNA/(unmethylated DNA + methylated DNA). For female patients, the results were multiplied by 2 due to the presence of a fully methylated, inactivated X chromosome in female patients.

Data Collection and Storage

Manufacturing batch records from each Treg product were obtained and data were manually extracted and recorded in Research Electronic Data Capture (REDCap, v9.5.25), a secure and Health Insurance Portability and Accountability Act–compliant web-based system for building and managing surveys and databases.

Analysis

Means of continuous variables were compared using ANOVA and Kruskall-Wallis test. Where only two categories existed, means were compared by Student's two-tailed t-test. Linear regression models were constructed for assessment of correlation between variables. For categorical variables, post-expansion cell number was classified by level and means were compared using ANOVA and Kruskall-Wallis test. All data analysis was performed using Prism (v9.1.0, GraphPad Software).

Clinical Trials for which Treg products were manufactured are: NCT01210664 (T1D), NCT 02772679 (TILT), NCT02428309 (ALE08; "Lupus"), NCT03239470 (APG01; "Pemphigus"), NCT02088931 (TASKp), NCT02711826 (TASK), NCT03444064 (pTregITX).

RESULTS

Autologous polyclonal Treg products manufactured for clinical trials between 2011 and 2020 were included in this analysis. During that period, a total of 43 polyclonal Treg products were manufactured for 7 different clinical trials. **Figure 2** includes the trial NCT numbers, the years of production, and the clinical indication for enrollment in the clinical trial. Data extracted from manufacturing batch records were entered into a secure

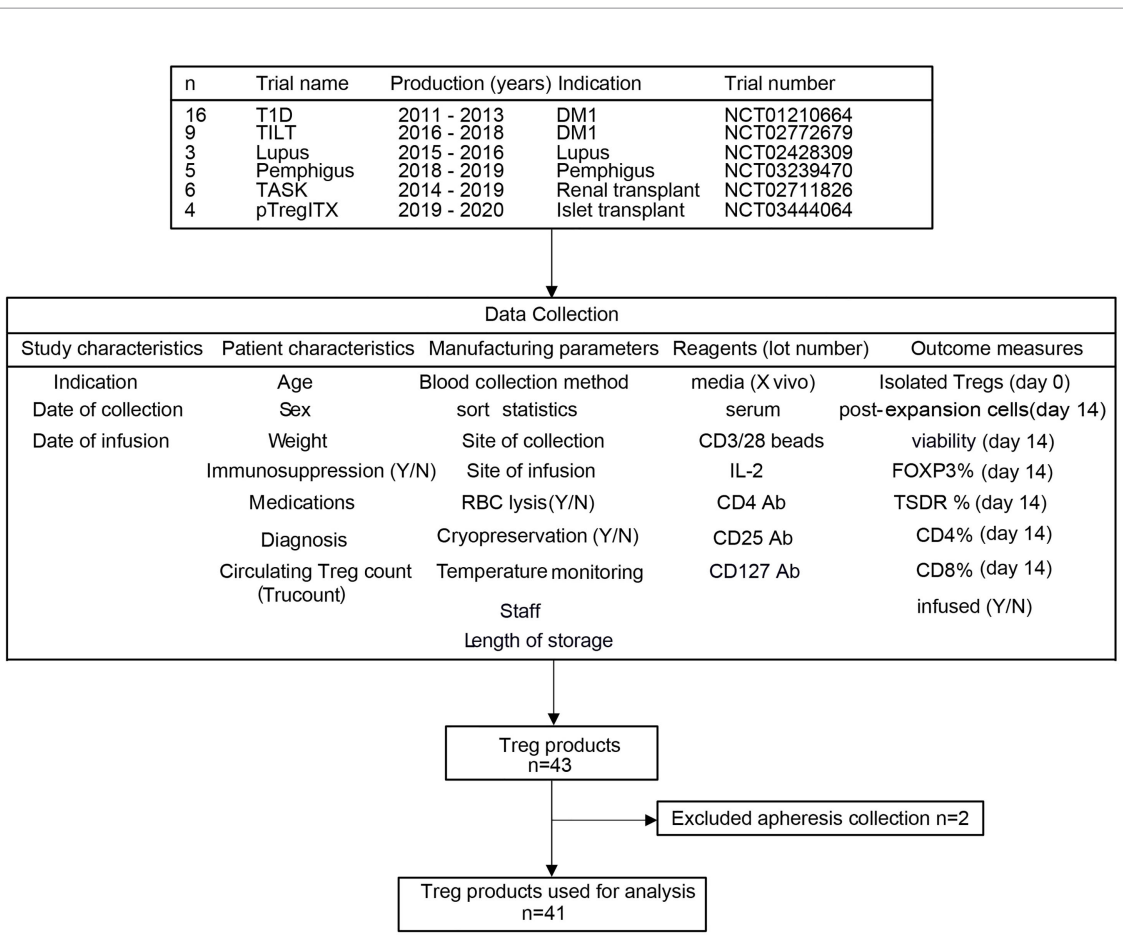

FIGURE 2 | Study diagram. Schematics of PolyTreg clinical trials, data collection, and analysis.

database. Data analyzed included study characteristics, patient characteristics, manufacturing parameters, reagent lots and outcome measures. Two products were excluded from further analysis because they were manufactured from autologous apheresis collections and were therefore not comparable to all the other products, which were manufactured from whole blood.

Key manufacturing parameters including the number of Treg isolated at the beginning of the manufacturing process, fold expansion of Treg during manufacturing, and percentage of $FOXP3^+$ cells by flow cytometry in the final product are summarized in **Table 1**.

The median peripheral blood Treg number in patient blood was 56.1 cells/mL (range 23.0-115.5). A median of 462.5 mL (range: 367-532.5) of whole blood was used as a starting material. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient, then stained with GMP-grade fluorochrome-conjugated anti-CD4, anti-CD25, and anti-CD127 antibodies, and $CD4^+CD25^+CD127^{lo/-}$Tregs were then sorted using FACS. The sorts yielded a median of $4.2x10^6$ Tregs (range: 0.7-11.8). Median post-sort purity was 99.1% (range: 96.3-100), based on the markers used for sorting. A representative purity check is shown (**Figure 3A**). Median fold expansion during the 14-day manufacturing period for all products was 434.8-fold (range: 29.8-2232). This fold expansion was sufficient for infused products to meet the minimum required dose, as defined by the various clinical trial protocols. Median cell viability post-expansion was 99.3% (range: 90.5-100). We did not have any product failures due to low Treg viability.

Cell growth over the 14-day manufacturing period for all 41 PolyTreg products is shown (**Figure 3B**). Flow cytometry analysis of the final Treg product showed a median of 96.8% (range: 86.6-98.9) $CD4^+$ (>95% required for product release, **Figure 3C**), 93.9% (range: 20.9-98.6) $FOXP3^+$ (>60% required for product release, **Figure 3D**) with representative flow cytometry (**Figure 3E**), and 0.37% (range: 0.03-7.6) $CD8^+$ cells (<5% required for product release, **Figure 3F**). Methylation analysis of intron 1 of the *FOXP3* gene shows consistently high levels of demethylation (median 90.8%, range: 73.62-128.4), which confirms the identity of the cells as Treg (**Figure 3G**). Calculated demethylation results were slightly over 100% in a few samples. These were all samples from female patients having more than 50% demethylated TSDR resulting in >100% after doubling to account for X chromosome inactivation. The higher than 50% demethylation in female samples may be due to over conversion of methylated cytosine to uracil in these samples.

Out of a total of 41 PolyTreg products produced, 34 were infused (34/41 = 83%). Of the infused products, the leading indication was Type 1 diabetes mellitus, totaling 23 products. Five infused products were manufactured for other autoimmune indications (systemic lupus erythematosus, pemphigus vulgaris), and 6 were made for patients with solid organ or tissue transplant (kidney, islet transplant). The remaining 7 products were not infused. Of the products not infused, 4 did not meet release criteria. Specifically, products were not released due to low percentage of $CD4^+$ cells (n=2), of which one also had a high percentage of $CD8^+$ T cells. One additional product had a low percentage of $FOXP3^+$ cells (n=1) in the final product. These manufacturing failures were due to outgrowth of non-Treg T cells in the cultures. The fourth product that did not meet release criteria contained 7.36% $CD4^+CD8^+$ cells that were later determined to be $FOXP3^+$ and suppressive *in vitro*. Subsequently the release criterion was changed from <5% $CD8^+$ to <5% $CD8^+CD4^-$ to account specifically for contamination by non CD4-expressing cells. Finally, one product was not infused due to a false positive in-process fungal culture, which was later determined to be due to a microbiology lab error. Overall, 88% (36/41) of products met release criteria.

TABLE 1 | Manufacturing outcomes of PolyTregs by infused (first column), not infused (second column) or total (third column) products.

	PolyTreg manufactured		Total
	PolyTreg infused	PolyTreg not infused	
Number products	34	7	41
Age (min-max)	40 (22-69)	47.8 (41-64)	42 (22-69)
Sex: F, M	12, 22	4, 3	16, 25
Indication			
Type 1 DM	23	2	25
Lupus	1	2	3
Pemphigus	4	1	5
Renal transplant (TASK)	4	0	4
Allogeneic islet transplant	2	2	4
Treg/μL blood (Trucount) (min-max)	56.9 (23.0-115.5)	43.4 (32.4-64.8)	56.1 (23.0-115.5)
Isolated Treg number ($x10^6$) (min-max)	4.3 (1.1-11.8)	1.6 (0.7-7.8)	4.2 (0.7-11.8)
Isolated Treg purity % (min-max)	99.0 (96.3 - 99.9)	99.2 (97.6-100)	99.1 (96.3-100)
Post-expansion Treg number ($x10^6$) (min-max)	2031 (186-12920)	215 (37.9-16179)	1841 (56.9-16179)
Fold expansion (min-max)	511.5 (29.8-1704)	150.7 (37.9-2232)	434.8 (29.8-2232)
Final $CD4^+$ % (min-max)	97.1 (95.0-98.9)	95.6 (86.6-98.5)	96.8 (86.6-98.9)
Final $FOXP3^+$ % (min-max)	94.0 (73.0-98.6)	90.2 (20.9-93.3)	93.9 (20.9-98.6)
Final $CD8^+$ % (min-max)	0.31 (0.03-3.3)	0.91 (0.1-7.6)	0.37 (0.03-7.6)

Number of products is indicated in following rows: number products, sex, and indication. Remaining rows indicate median, with minimum and maximum values in parentheses.

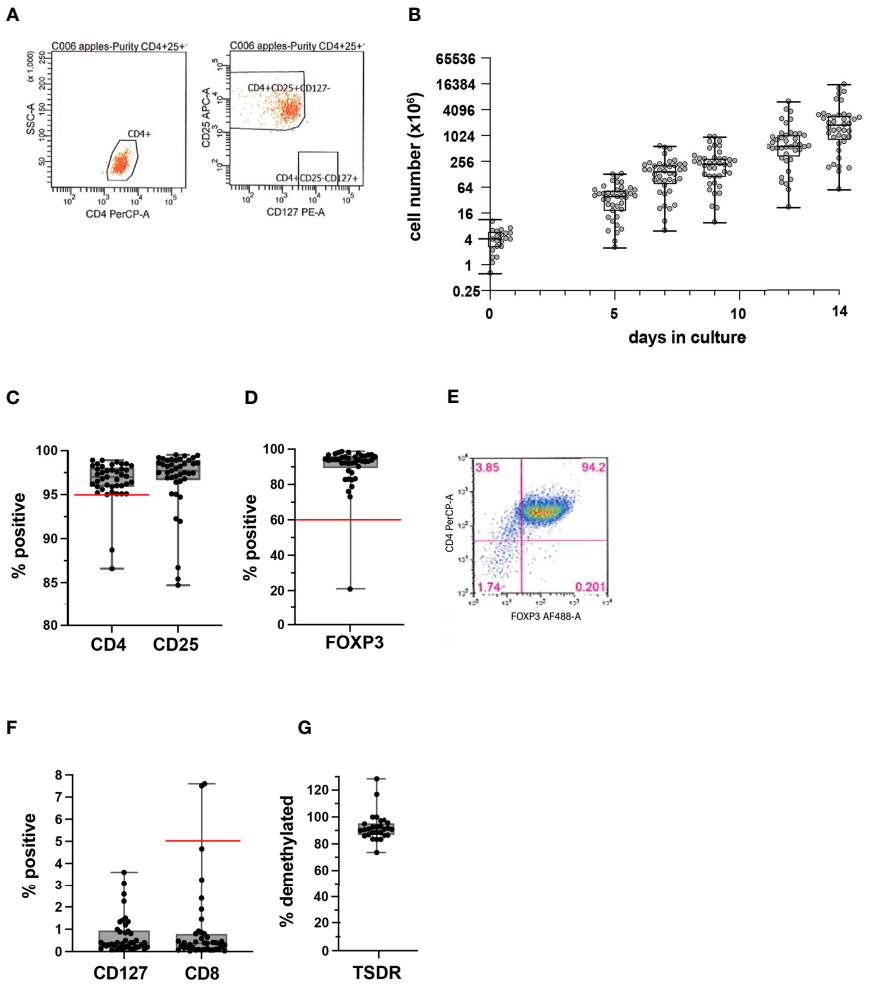

FIGURE 3 | Quality assessment of isolated Tregs and PolyTreg products. Representative flow cytometric plots of post-sort purity check **(A)**. Cell numbers over the 14-day manufacturing period for all products (n=41) **(B)**. Post-expansion % CD4+, CD25+ **(C)**, and FOXP3$^+$ in PolyTreg products analyzed by flow cytometry **(D, E)**. CD127, or CD8 expression in Treg products by flow cytometry **(F)**. TSDR methylation of PolyTreg products, expressed as % methylated sites **(G)**. Release criteria cutoffs are highlighted as a red line in each plot, as applicable.

Interestingly, we observed a high variability in Treg product yield (**Figure 3B**). We thus explored to determine if patients and manufacturing parameters may be associated with product yield. There was no clear correlation between the number of Treg isolated from the starting material and patient age (**Figure 4A**). Treg numbers isolated from starting material were similar between male and female patients (**Figure 4B**).

A modest correlation between circulating Treg number in patients' whole blood and Treg number isolated at the beginning of manufacturing (**Figure 4C**) was observed. Further analysis of different patient populations showed significant differences in the number of Treg isolated from starting material between patients with different indications for Treg therapy (**Figure 4D**). These results may reflect differences in previous treatment or in disease state that could affect Treg numbers in the peripheral blood. When analyzed by individual trial, initial isolated Treg number differed by trial and patient population (**Figure 4E**).

We then sought to explore associations with total cell yield at the end of manufacturing. Age and sex were not associated with a statistically significant difference in Treg fold expansion (**Figures 5A, B**). Post expansion cell number generally corresponded with different trials and populations (**Figures 5C, D**). Because products failing release criteria contained contaminating cells, post-expansion cell number and fold-expanded values would be skewed and therefore these datapoints were excluded from further analysis. The remaining products were analyzed for significant associations (n=36). A significant correlation was observed between Treg number isolated at the start of manufacturing and post-expansion cell yield (**Figure 5E**). The five data points with the highest post-expansion cell yield for isolated Treg number were from type 1 diabetes patients. Consistent with this, individually plotted isolated and post-expansion cell numbers show a generally similar trajectory with highest cell yield from type 1 diabetes patients (**Figure 5F**).

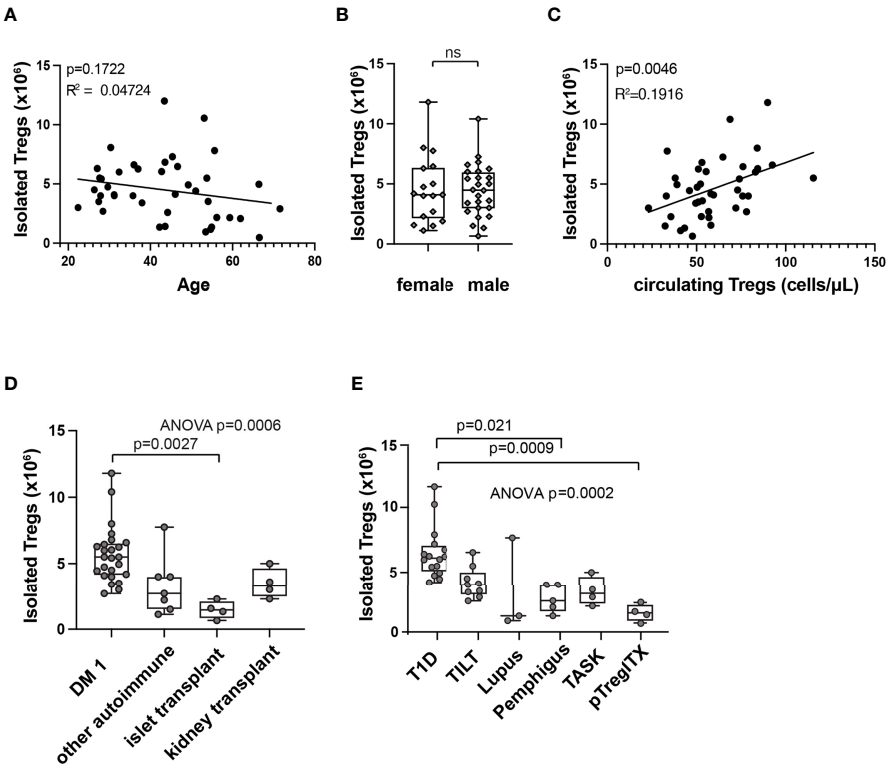

FIGURE 4 | Correlations with isolated Treg numbers at start of manufacturing (day 0). Correlation between patient age **(A)**, sex **(B)**, or circulating Treg number **(C)** and isolated Treg number on day 0 of manufacturing by simple linear regression. R squared (R^2) values and p-value assess goodness of fit, and significance of slope not equal to zero. Isolated Treg number on day 0 of manufacturing, subcategorized by patient indication **(D)** or by clinical trial **(E)**. Means comparison, ns, not significant. In box and whisker plots, the box shows mean and quartiles, whiskers show minimum and maximum values.

There was marked variability in Treg number sorted from patient blood (**Figure 4C**). To normalize for isolated cell number, fold expansion was calculated (fold expanded). Fold expansion also varied in different patient populations, and the difference did not reach statistical significance (**Figure 5G**).

We next sought to explore potential determinants of this variability. To discover whether any independent variables (patient demographics, pre-expansion cell descriptors, manufacturing parameters) impacted manufacturing outcomes, unbiased correlation analysis was performed (**Figure 6A**). Variable definitions are found in **Supplementary Table 1**. Correlation analysis revealed several expected relationships, such as correlation between Treg numbers at the beginning and the end of the manufacturing process. Among continuous variables, the number of Treg isolated from whole blood at the beginning of the manufacturing process was significantly correlated with circulating Treg number, and with final cell yield. The impact of categorical variables on post-expansion cell number was calculated separately, which included patient demographics (sex), and manufacturing parameters (personnel, reagent lot number). Although the difference in post-expansion cell number across a few lots of reagents reached significance (**Figure 6B**), the correlations may be confounded by preferential use of lot numbers within trials. Fold expansion among type 1 diabetes patients was plotted over time and no clear correlation was seen (**Figure 6C**).

DISCUSSION

In this summary of manufacturing outcomes for autologous polyclonal Treg products in 7 clinical trials, we show a high rate of success in manufacturing products from different patient populations over a 10-year period. The median 434.8-fold expansion over a 14-day manufacturing period is higher than many other clinical Treg protocols that have been published (7, 13, 15, 18) This higher fold expansion may be in part due to the manufacturing protocol described here does not require rapamycin to prevent overgrowth of effector T cells. However, we did see rare manufacturing failures due to outgrowth of non-Treg cells, either $CD4^+$ or $CD8^+$.

Variability in total yield of Treg during the culture period significantly correlated with patient disease status and number of Treg in the patient's peripheral blood, but showed weak or no correlation with reagent lots, manufacturing personnel, or other manufacturing parameters. This result underscores the importance of patient disease state in contributing to variability in cGMP

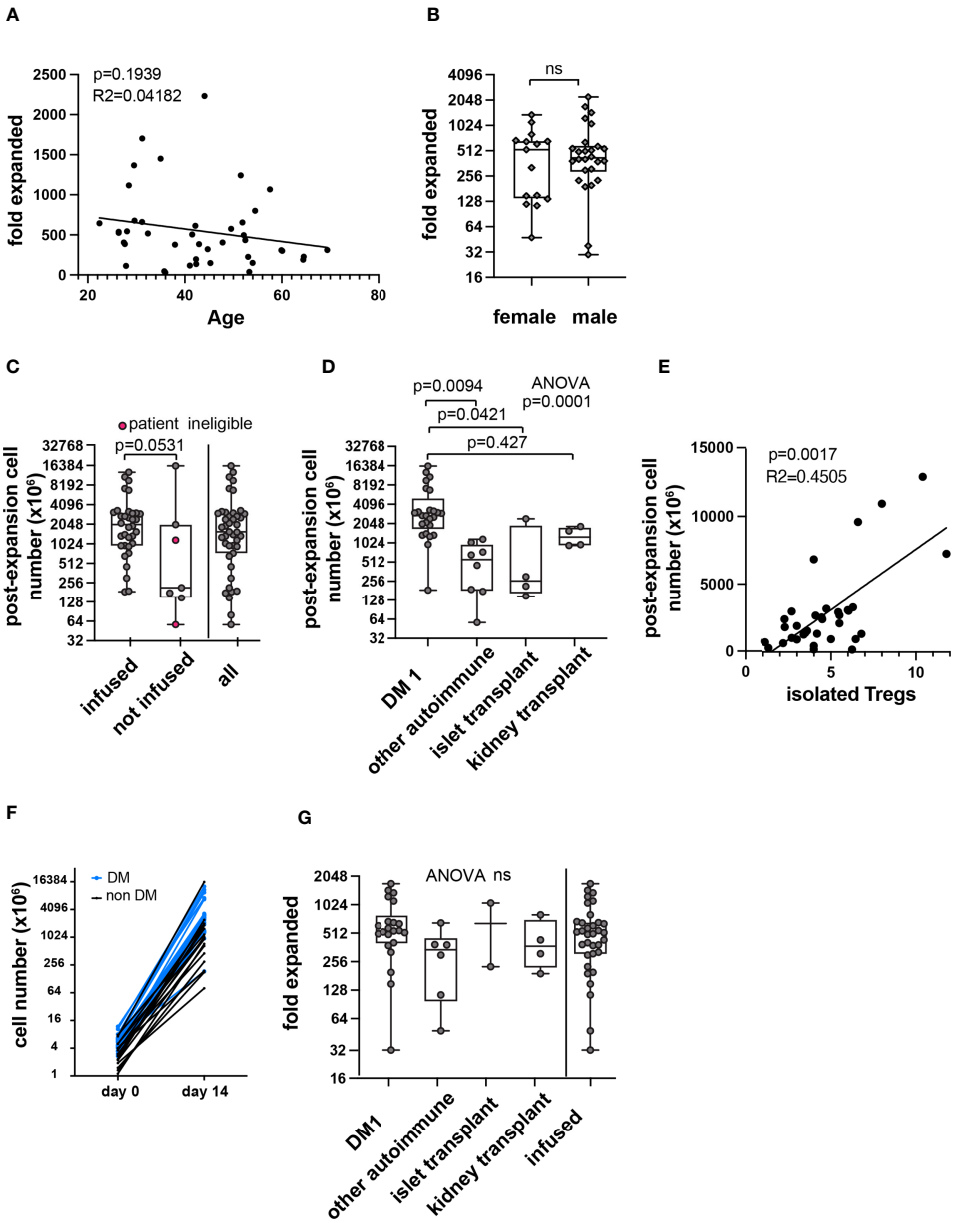

FIGURE 5 | Correlations with PolyTreg products *in-vitro* expansion. Correlation between age **(A)** or sex **(B)** and Treg fold expansion. Post expansion Treg number in all products (n=41) in millions by infusion status or total **(C)**. Pink circles in the second column indicate patient ineligibility for infusion. The remaining data points in the "not infused" column represent manufacturing failures. Post-expansion cell number subcategorized by indication (n=41) **(D)**. Post-expansion cell number plotted against isolated Treg number **(E)** with simple linear regression (n=36). Isolated and post-expansion cell number plotted individually by patient (n=36) **(F)**. Blue lines and symbols represent patients with type 1 diabetes, black lines and symbols represent all other indications. Fold-expansion by indication or among all infused products (n=36) **(G)**. R squared (R^2) values and p-value assess goodness of fit, and significance of slope not equal to zero. In box and whisker plots, the box shows mean and quartiles, whiskers show minimum and maximum values. ns, not statistically significant.

manufacturing outcomes and the importance of including patient material, if possible, during process development of manufacturing protocols. Batch-to-batch variability observed among the reagents used in this study was small and did not have important manufacturing impacts. However, we have observed significant batch-to-batch variability during our qualification process across lots of human serum. This result underscores the importance of a formal qualification program for materials and reagents such as human serum. Although our clinical trials did not include or exclude patients based on peripheral blood Treg counts, this clinical parameter could be useful in the future for predicting manufacturing outcomes. Importantly, existing clinical assays for peripheral blood Tregs are often not comparable, so a standard assay is required to compare data between different trials or

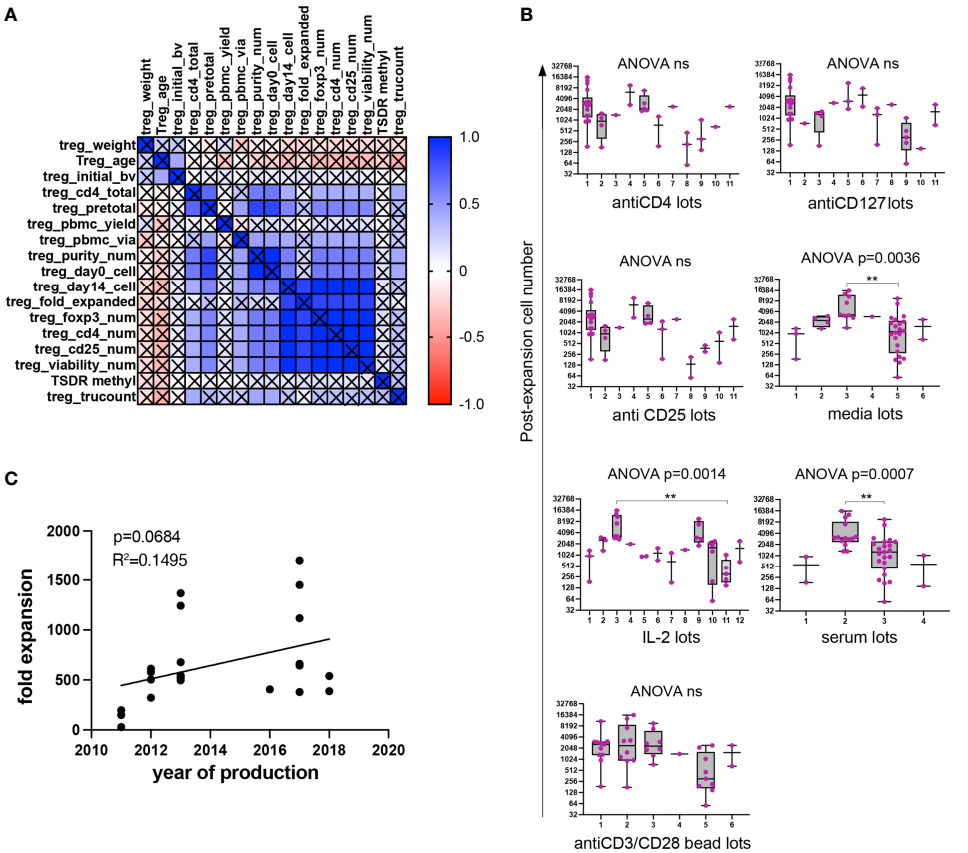

FIGURE 6 | Unbiased correlation analyses. Unbiased correlation analysis of all continuous variables **(A)**. Pseudocolor; red represents negative correlation, blue represents positive correlation. Intensity of color is proportional to size of correlation. Significance threshold set at p=0.01. Black cross over cell represents nonsignificant correlation. Analysis of impact by categorical variable (material lots) on post-expansion cell number **(B)**. In box and whisker plots, the box shows mean and quartiles, whiskers show minimum and maximum values. Fold expansion by year of production among DM1 patients (n=22) **(C)**. R squared (R^2) values and p-value assess goodness of fit, and significance of slope not equal to zero. In box and whisker plots, the box shows mean and quartiles, whiskers show minimum and maximum values. **p < 0.01. ns, not significant.

different centers. Although the age of patients did not significantly correlate with initial Treg numbers or final Treg yield, we cannot completely exclude a role for patient age in manufacturing outcomes.

Treg manufacturing cost is mostly contributed by the cost of the GMP grade materials and salary of highly skilled personnel. The costs changed over time due to increases in the costs of materials and labor. To choose a point in time relevant to this report, materials for a single Treg product in 2016 was estimated at approximately $26,500. Labor costs are much more difficult to calculate given that the manufacturing personnel performed many other duties during the period under consideration.

This study is limited by its relatively small number of Treg products. Excluding DM1, relatively few products were made in each trial. Polyclonal Treg products were not systematically evaluated for *in vitro* suppression capacity, and in-depth cell profiling was not performed. Greater insight into correlations among *in vitro* Treg expansion, *in vitro* suppression, and cellular manufacturing parameters could help guide future Treg therapy efforts.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JE, QT, and JAB designed the analyses. BRS designed and built the RedCap database. AP, LM, AL, LA, FK, ML, VN, WL, SP, JX,

and ASL executed production and collected data. LA and BS entered data in database. JB, JE, and QT analyzed data and wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.744763/full#supplementary-material

Supplementary Table 1 | Correlation variables and units.

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REVIEW
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Mechanistic and Biomarker Studies to Demonstrate Immune Tolerance in Multiple Sclerosis

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The induction of specific immunological tolerance represents an important therapeutic goal for multiple sclerosis and other autoimmune diseases. Sound knowledge of the target antigens, the underlying pathomechanisms of the disease and the presumed mechanisms of action of the respective tolerance-inducing approach are essential for successful translation. Furthermore, suitable tools and assays to evaluate the induction of immune tolerance are key aspects for the development of such treatments. However, investigation of the mechanisms of action underlying tolerance induction poses several challenges. The optimization of sensitive, robust methods which allow the assessment of low frequency autoreactive T cells and the long-term reduction or change of their responses, the detection of regulatory cell populations and their immune mediators, as well as the validation of specific biomarkers indicating reduction of inflammation and damage, are needed to develop tolerance-inducing approaches successfully to patients. This short review focuses on how to demonstrate mechanistic proof-of-concept in antigen-specific tolerance-inducing therapies in MS.

Keywords: multiple sclerosis, peripheral tolerance, mechanistic studies, antigen-specificity, autoreactive cell, regulatory T cells, biomarkers, tolerance induction

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INTRODUCTION

Multiple sclerosis (MS) is considered a prototypic organ-specific autoimmune disease that affects the central nervous system (CNS; brain and spinal cord) of young adults and particularly women. In most cases MS begins between 20-40 years of age but may also start in childhood or later in life. There are two main forms with respect to clinical course. Relapsing-remitting MS (RMS) is characterized by bouts of disease activity in different CNS areas that might affect vision, sensation, motor-, bladder-, bowel and sexual function. Initially, the deficits are only transient and often completely recover. RMS is diagnosed based on clinical presentation as well as typical magnetic resonance imaging (MRI) lesions and signs of inflammation in the cerebrospinal fluid (CSF) (1). Pre-stages of RMS are the so-called clinically isolated syndrome (CIS), i.e. a first clinical event with suggestive MRI and CSF findings, or even the accidental discovery of MRI lesions without any prior clinical symptoms, which is referred to as radiologically isolated syndrome (RIS). Prior to the era of effective disease-modifying immunomodulatory treatments, RMS usually evolved into secondary progressive MS (SPMS) after 10-20 years. At this stage, neurological deficits and disability of the

patients steadily worsen with or without superimposed relapses, which later completely stop. 80-85% of patients show one of these stages of RIS-CIS-RMS-SPMS. A smaller fraction (10-15%) shows progressive increase of disability from the beginning usually with insidious onset of walking problems. This form is referred to as primary progressive MS (PPMS) and affects women and men equally. Besides these different forms of MS with respect to disease course, there is substantial variation in how quickly neurological deficits develop. Few patients have a benign course (approximately 5%), the majority will develop disabilities over 2-3 decades, if they are not treated, and another, smaller portion (approximately 5-10%) shows rapid disease progression with severe deficits in a few years. In addition to variation in disease course, heterogeneity is also seen with respect to clinical presentation, neuropathological findings, distribution of lesions in the brain and spinal cord and response to treatment.

Although the etiology and pathomechanisms of MS are not yet completely understood, enormous progress has been made during the last 20 years. Genome-wide association studies have characterized the complex genetic trait that confers MS risk with now more than 240 single nucleotide polymorphisms (SNPs) across the genome (2). The vast majority of these and particularly the most important MS risk genes, i.e. the two HLA-DR15 alleles (3), are immune function-related (4). Environmental risk factors include Epstein Barr virus (EBV), low vitamin D3, smoking, obesity during early adolescence (5) and imbalances of gut microbiota (6–8). Differences in the interplay of genetic and environmental risk factors are likely responsible for the heterogeneity of MS with respect to clinical course and involvement of different functional systems of the CNS, imaging findings, pathology and response to treatment.

The development of treatments for MS has been very successful during the last 25 years. More than 20 treatments are now approved including variations in dosing or application forms. They reach from moderately effective injectables and small molecules (IFN-β, glatiramer acetate, teriflunomide) to highly effective biologics such as anti-CD20, anti-VLA4, anti-CD52 and the small molecule cladribine. All act by immunomodulation and/or -suppression, but by different mechanisms (9). The most important effects target autoreactive CD4+ T cells and/or B cells, but to various degrees also innate immune cells. Autologous hematopoietic stem cell transplantation (aHSCT), which is only approved in some countries, is an exception. It primarily acts by completely abrogating the patient's adaptive immune system and then forming a new one from autologous CD34+ hematopoietic stem cells (10).

In contrast to the above, antigen-specific tolerance induction aims at a subtle readjustment of perturbed immune reactivity, which harms CNS tissue. The currently pursued approaches employ mechanisms of peripheral immune tolerance in the physiological situation, and they are therefore expected to be very safe and not to impair protective immunity against infections and tumors. If immune tolerance-inducing approaches shall successfully enter the clinic and hopefully acquire a firm place in our treatment armamentarium, clinical efficacy, i.e. the reduction of relapses and/or the attenuation of disease progression need to be shown (11). Development of treatments towards approval follows certain standards in MS. Clinical efficacy needs to be documented by two positive phase III trials, which are very costly and usually performed at up to 100 or more sites. For immune tolerance induction, stopping the disease evolution at very early stages, i.e. RIS or CIS, is of particular interest. If robust predictive biomarkers were available, antigen-specific tolerance induction would be even more interesting as a true prophylactic measure to prevent the development of MS. So far, it has, however, been very difficult to overcome the hurdles during the earlier clinical trial stages, i.e. phase IIa and -b.

Below we will outline which immunological mechanisms contribute to MS, how peripheral immune tolerance is generated and maintained, at which aspects of these tolerizing strategies aim, and which factors need to be considered to demonstrate at the mechanistic level whether tolerance induction has been achieved in patients. The challenges of gathering evidence for mechanistic proof-of-concept particularly in early-stage clinical trials will be discussed including which methodologies are currently available.

PATHOMECHANISMS OF MS

Understanding the autoimmune process and target antigens are required for measuring changes after tolerization. Below, we will summarize these, but only briefly mention target antigens in MS, since these has been covered in detail elsewhere recently (11). Also, while it is clear that innate immune cells such as dendritic cells and microglia are involved at different steps of the pathogenesis of MS, we will focus on adaptive immune mechanisms, since these are most relevant for antigen-specific tolerization.

The pathomechanisms of MS involve autoreactive CD4+ T cells with specificity for myelin- and a few other proteins and peptides thereof (4, 12–14), proinflammatory B cells (15) and possibly also autoantibodies (16), but likely also other cell types including CD8+ T cells (17), microglia and other innate immune cells (18). The strong association with a specific HLA-DR haplotype (3), the large body of evidence from experimental autoimmune encephalomyelitis (EAE) studies (19), and also the studies of immune mechanisms in MS patients underscore the central role of autoreactive CD4+ T cells (4, 20). Consistent with the fact that MS only affects the CNS and that demyelination is a key aspect of MS lesions, but also with data from EAE studies, autoreactive CD4+ T cells recognize peptides from several myelin proteins including myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and a few others [recently summarized in detail in (11, 21)]. Based on T cell recognition with higher antigen avidity (22), data from humanized transgenic mouse models (23, 24) and from epitope mapping studies, a few immunodominant epitopes of MBP, PLP and MOG appear particularly important (11), but non-myelin antigens including alpha-B crystallin (25), GDP L-

fucose synthase (GDPLFS) (13), and RAS guanyl-releasing protein 2 (RASGRP2) (14, 26) should also be considered. Both GDPLFS and RASGRP2 have been discovered by searching for the specificity of CD4+ T cells that were clonally expanded in active MS brain lesions (13, 14). Further, MS patients with intrathecal T cell reactivity against human GDPLFS also recognized homologue bacterial peptides from a gut bacteria that is overrepresented in MS patients, *Akkermansia muciniphila* (13). RASGRP2 is not only expressed by cortical neurons in the brain, but also by proinflammatory B cells that activate autoreactive T cells (14). The antigen-specific T cell response may broaden over time, a phenomenon that is referred to as epitope spreading (27) and means that additional antigen specificities emerge and/or prior ones are lost. Spreading can be intramolecularly, i.e. to a new peptide of the same protein, or intermolecularly, i.e. a peptide from another target protein. Epitope spreading has been examined in detail in EAE (28), but only few studies have addressed it in MS patients (29). In the context of tolerance induction, it implies that not only the antigens contained in the tolerance-inducing approach, but also other candidate targets should be assessed.

With respect to their functional phenotype, autoreactive CD4+ T cells in MS express T helper 1 (Th1; produce IFN-γ), Th1*- (produce IL-17 in addition to IFN-γ) or Th17- (express IL-17) phenotypes and furthermore markers that are important for brain homing such as VLA-4, and the chemokine receptors CXCR3 and CCR6 (30). In our studies of both peripheral blood- and cerebrospinal fluid (CSF)-derived T cell clones in MS, the hierarchy of importance is Th1>Th1*>Th17 cells.

Besides T cell-mediated autoreactivity, autoantibodies have long been considered important in MS pathogenesis. Antibodies that are produced in the CSF as oligoclonal bands (OCBs) are known for more than 70 years and are a diagnostic hallmark in MS, but the pathogenic importance of both OCBs and in general autoantibodies in MS remains controversial (16). However, there is a pathologically defined pattern II MS, in which immunoglobulin and complement factor deposition in the brain (31), the therapeutic responsiveness to plasmapheresis (32), and the recent demonstration of autoreactive Th2 CD4+ T cells (33), all support that antibodies play a role. To our knowledge, no biomarker in the blood and CSF has been identified that allows the identification of pattern II MS patients.

While autoantibody production is probably less important overall in MS, there is no doubt that B cells play an important role. B cell-depleting therapies with anti-CD20 monoclonal antibodies, but also with anti-CD52 and cladribine, which in addition to B cells eliminate other immune cells, are among the most effective therapies for MS (34, 35). The observation that disease activity decreased much earlier after anti-CD20 treatment than expected from removal of antibodies, stimulated the search for additional roles of B cells in MS, including cytokine/chemokine mediated regulation of inflammation and antigen-presentation. During the last years, several studies have shown increased frequencies of B cells that secrete GM-CSF and IL-6 (15) and express other proinflammatory cytokines and chemokine receptors involved in brain homing and interaction with autoreactive T cells (36). Further, proinflammatory memory B cells appear to be involved in presenting antigen to autoreactive T cells, in their activation, and priming for brain homing (14). Peptides derived from the MS-associated HLA-DR15 molecules and upregulation of DR15 itself on the surface of B cells are involved in cross-talk and increased autoproliferation of both B- and autoreactive CD4+ T cells (26), however, it is not clear yet whether the activation occurs first in the B- or T cell. Furthermore, RASGRP2, one of the novel autoantigens is upregulated in proinflammatory B cells in MS and can be cross-recognized by autoreactive T cells that also respond to EBV- and *Akkermansia*-derived peptides (26). In this context it is important to note that EBV, a key environmental risk factor of MS, infects B cells (37), and the risk to develop MS is increased several-fold after symptomatic EBV infection, i.e. infectious mononucleosis (38). EBV infection of B cells and also T cell reactivity to EBV have been implicated in multiple ways both in the peripheral immune compartment and also the CNS (39, 40). Tertiary lymphoid structures in the meninges, which contain B- and T cells, have been linked to cortical lesion formation in MS and also to progressive disease (41, 42). At the latter stage, a compartmentalized chronic immune response in the CNS/meninges is suspected to drive the disease process (43).

In summary, it is clear that autoreactive CD4+ T cells with a Th1- or Th1*-, and, in pattern II MS patients, also Th2 cells as well as proinflammatory B cells play key roles in several steps of the autoimmune pathogenesis of MS. For more detail, the reader is referred to reviews on this topic (4, 44).

PERIPHERAL IMMUNE TOLERANCE MECHANISMS

The multitude of tolerance mechanisms in humans in both health and disease are incompletely understood. Below, we will mention key aspects that are relevant for characterizing these before and after attempts of tolerance induction.

Immunological tolerance to self-antigens results from both central and peripheral mechanisms. The elimination of strongly self-reactive lymphocytes is controlled by central tolerance mechanisms in the thymus and bone marrow for T and B cells, respectively. During thymic development, T cells which recognize self-antigens with high avidity, undergo negative selection *via* clonal deletion, whereas those recognizing antigens with low avidity are positively selected and constitute the peripheral immune repertoire. T cells bearing TCRs for antigens not expressed in the thymus may, however, not be deleted (45, 46), and low avidity autoreactive T cells responding to myelin antigens can be found in both healthy individuals MS patients (47–50). Such potentially pathogenic cells are controlled by several checkpoints that operate at different stages to avoid the development of autoimmunity.

Among peripheral tolerance mechanisms, one is ignorance of self-antigens, either because anatomical barriers limit accessibility (for example the blood-brain-barrier), or because

it is present at too low concentrations, or the expression of HLA molecules is limited or absent (51). Although CD4+ MBP-specific T cells in MS primarily derive from the naïve repertoire and show higher antigen avidity (52), they lack adhesion molecules and chemokines receptors that are necessary for organ homing. T cell activation in the absence of costimulatory signals results in a state of unresponsiveness, referred to as anergy. Certain tolerizing approaches induce anergy (53), which can be overcome by IL-2 and therefore is not durable. T cell responsiveness can further be controlled when antigen recognition occurs in the context of a growing family of so-called co-inhibitory molecules including CTLA-4, PD1, TIGIT, BTLA4, LAG-3, TIM-3 (54). Different from anergy, apoptotic deletion of autoreactive cells by activation-induced cell death (AICD), is triggered when already activated cells are newly stimulated with antigen. AICD is mediated by Fas-Fas ligand interaction (55). Additional peripheral cell death checkpoints other than apoptosis also play a role in maintaining tolerance. Further, antigen-induced T cell exhaustion and senescence are non-deletional mechanisms which limit T cell responses at the effector level. Finally, immune deviation or phenotypic skewing of the effector cells toward a non-pathogenic cytokine profile may also contribute to immune tolerance [reviewed in (54, 56–58)] (**Figure 1**).

The above mechanisms act on the autoreactive T cell itself to avoid its activation or dampen an already initiated immune response. T cell reactivity can also be indirectly modulated by other cell subsets with regulatory properties. Among these, regulatory T cells (Tregs) are a key component to maintain tolerance and towards active suppression of unwanted immune responses (**Figure 1**). Circulating regulatory T cells consist of different cell populations including naturally occurring CD4+ regulatory T cells (nTregs) (59) and type 1 regulatory T cells (Tr1) (60).

nTregs maintain self-tolerance and immune homeostasis. They are characterized by expression of the transcription factor Forkhead-Box-Protein P3 (FOXP3) and they are referred to as FOXP3+ Tregs. Most FOXP3+ Tregs arise in the thymus (thymus-derived Tregs or tTregs), but they can also be generated in the periphery (peripherally derived Tregs or pTregs) through conversion of conventional T cells (61, 62). During thymic selection, T cells recognizing self-antigens with intermediate avidity in between the range of positive and negative selection are not deleted and differentiate into tTregs (63). They enter the peripheral immune system in an already antigen-primed, activated, and functionally competent state. Additional markers of thymic/naive FOXP3+ Tregs like GPA33 have been identified (64), and the stability of these cells in the peripheral immune system is being investigated. Whether FOXP3+ Tregs need to be antigen-specific to exert their suppressive action remains to be clarified.

Tr1 cells are induced in the periphery after activation by specific antigen and characterized by secreting large amounts of

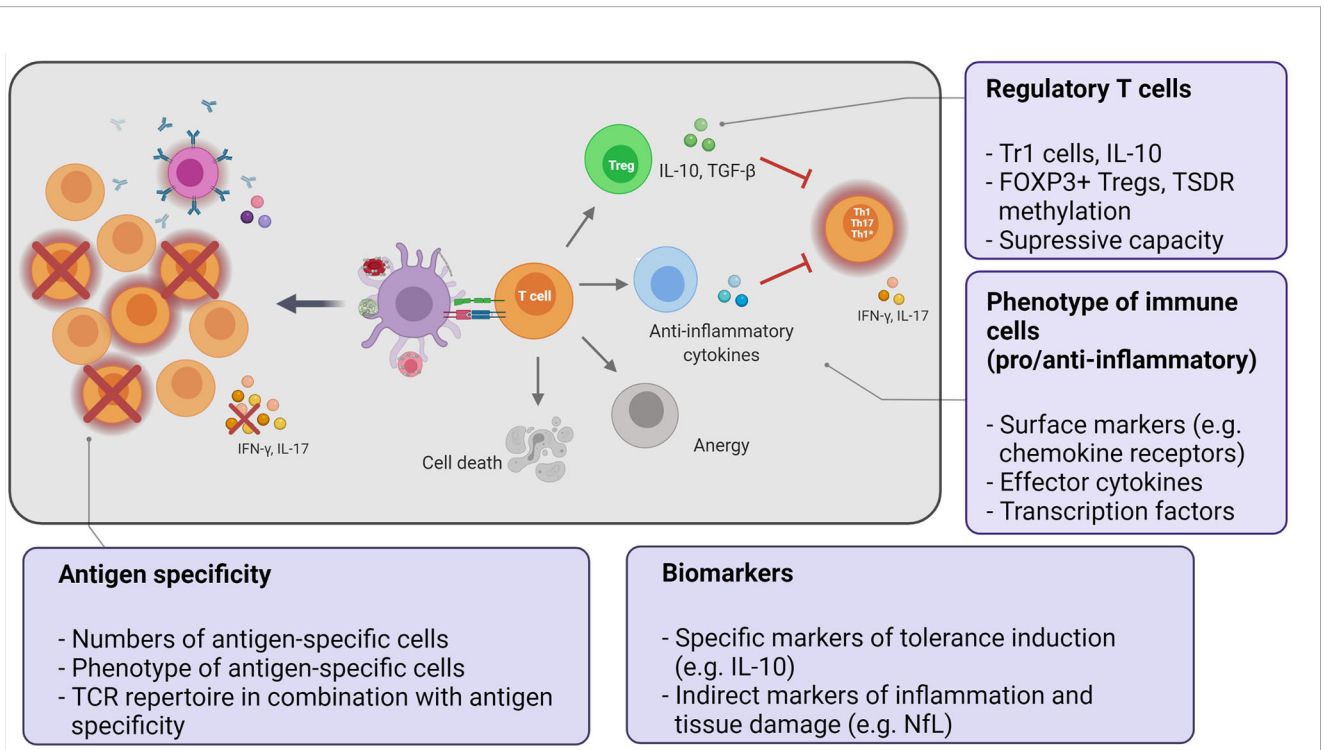

FIGURE 1 | Key aspects of the mechanistic program to monitor immune tolerance. Tolerization strategies employ different approaches to deliver the autoantigen to tolerogenic antigen-presenting cells in order to induce or enhance peripheral tolerance mechanisms. The potential mechanisms of tolerance induction can directly delete or silence autoreactive cells or indirectly suppress them by induction of regulatory T cells or effector T cells producing immunomodulatory cytokines (immune deviation). The final outcome is the reduction of autoreactive, pathogenic effector cells (Th1/Th17/Th1*). Text bubbles summarize the key aspects of the mechanistic studies.

IL-10, which constitutes their main suppressive effector function (65). Both FOXP3+ Tregs and Tr1 cells act through a range of mechanisms to regulate cells in close proximity and thereby can mediate bystander suppression against cells with different antigen specificities. For more detailed information on markers of Treg differentiation, function, tissue homing, and potential activation markers the reader is referred to reviews (62, 65, 66).

In summary, tolerance to self-antigens should be viewed as a complex and dynamic process resulting from several mechanisms acting in concert simultaneously or in a sequential manner to keep autoreactive cells under control (58). While mechanisms such as anergy or apoptosis are triggered at the initial phases after antigen encounter, regulatory T cells are presumed to contribute to long-term and stable tolerance induction and maintenance. Failure of these mechanisms leads to tolerance breakdown, enabling the development of autoimmune diseases.

TOLERANCE-INDUCING APPROACHES

A wide range of antigen-specific tolerization approaches have been tested in animal models of autoimmune diseases and particularly in EAE. The majority of these employed putative target antigens *via* different routes of administration, as peptides, proteins in free form, coupled to cells, MHC molecules or in the context of nanoparticles, *via* liposomes, or *via* expression vectors (11, 54, 67–69) and more recently as modified RNAs (70). Furthermore, they varied with respect to targeted organ and putative mechanism(s) of action, and their putative mechanisms of action, advantages and disadvantages have been reviewed elsewhere (11, 67, 68, 71). We have recently also reviewed those that have been tested already in MS (11) and will therefore focus on the principles here.

The ideal therapeutic approach for MS should aim to specifically silence the imbalanced self-directed immune responses by inducing long-lasting, stable immune tolerance against the target antigen (antigen-specific tolerization). Such an approach should leave immune effector functions, e.g. against infectious agents and tumors, intact and restore immune homeostasis. The ultimate goal is to deliver the autoantigen in a non-immunogenic context in order to exploit and enhance peripheral tolerance regulatory mechanisms and induce a durable state of immune tolerance (**Figure 1**). In the EAE model, antigen-coupled, cell-induced tolerance with myelin-derived antigens has consistently shown high efficacy both prophylactically and therapeutically and also prevented epitope spreading (72), but many other methods have been tested successfully as well (67, 68, 70–73). Although their mechanisms are not completely elucidated yet, free peptides or APLs are presumed to induce direct tolerance through anergy or phenotypic skewing (68), while tolerization by antigen-coupled cells or antigen-loaded nanoparticles involves more complex mechanisms. These include early PD-L1-mediated anergy of autoreactive cells, followed by induction of regulatory T cells for long-term tolerance maintenance, with IL-10 playing a key role in both processes (68, 73, 74).

In conclusion, major hurdles in translating these into patients include the differences of immune mechanisms between rodents and humans, e.g. with respect to the complexity of the MHC/HLA, the fact that EAE is an induced model, while MS arises spontaneously and probably long time before it becomes clinically manifest, the complexity and heterogeneity of disease mechanisms that contribute to MS, the still incomplete knowledge about target antigens, and, as will be detailed below, the difficulties of demonstrating tolerance induction and the underlying mechanisms in humans.

MECHANISTIC TESTING ALONG CLINICAL TRIALS AIMING AT TOLERANCE INDUCTION

For successful translation it is paramount not only to choose the target patient population, clinical trial design and outcomes well, but also to include a carefully designed mechanistic program that shows that antigen-specific immune tolerance has been achieved during the early clinical development stage. The form and stage of MS, ideally early RMS patients, who did not fail multiple prior treatments, the extent of disease activity, the reactivity against important target antigens and whether there is already epitope spreading or not, the HLA background, the presumed mechanisms, by which the respective tolerance-inducing strategy is supposed to work, and of course the safety of the approach, i.e. that it does not suppress the immune system or even lead to immune activation, all have to be considered. Most of the early tolerance trials in MS have not invested sufficient efforts to demonstrate that tolerance has been achieved at the mechanistic level. Furthermore, the high cost and the fact that a growing number of effective therapies are available have been reasons why such trials have been difficult to conduct. Below, we will address key mechanistic aspects to improve in this area in the future (for summary see also **Figure 1**).

Immunosafety

The mechanistic studies performed along any tolerizing approach should first of all show that the treatment is not causing unwanted immune activation or signs of immunosuppression, i.e. that it is safe from an immunological point of view. Besides the standard hematology and blood chemistry analyses to check the general health status of the patients and identify potential adverse effects, flow cytometry is very useful to monitor immune cell composition after applying a tolerizing therapy. Current multicolor flow cytometry techniques allow the quick assessment of multiple parameters in parallel, thus enabling a comprehensive characterization of numerous immune cell subsets. Moreover, the use of immune cell profiling from fresh blood identifies potential changes in non- or minimally manipulated samples, thus getting a glimpse at the *in vivo* immunological status. Important aspects to take into account are the use of optimized and standardized methods of sample collection and processing, the timing of sample preparation, instrument settings, inclusion of counting beads

and parallel measurement of absolute mononuclear blood cell numbers, in order to reduce variability between analyses as much as possible (75).

Phenotypic Changes of Immune Cells

Several immune cell populations, including pro-inflammatory CD4+ Th1, Th1*, and Th17, memory B cells, CD8+ T cells, regulatory T- and B cells and others, are involved in the pathogenesis of MS. Disturbances in circulating immune cells have been reported in MS, and some of these alterations reflect those observed in the CNS (76). Detailed immune profiling of peripheral blood therefore not only provides information about the safety, but could also in principle be used to monitor changes related to disease activity and response to treatment.

In the context of tolerance induction the aim is to detect a shift in the pathogenic immune response from the Th1/Th1*/Th17 towards a "normal" one including the disappearance of pathogenic cells, appearance/activation of regulatory cell populations, or changes in markers indicative of tolerance. Multi-parametric flow cytometry techniques combining different surface markers (lineage markers, chemokine receptors, activation- and migration markers, antigen-induced T cell exhaustion and senescence markers) and intracellular staining to detect cytokines and transcription factors provide detailed information about phenotype, activation status, and functional profile of immune cells. Even more detailed analyses can be achieved with high resolution (up to 40 colors) flow cytometry, spectral cytometry (77, 78) and mass cytometry (CyTOF) (79, 80), but they are not used yet in the routine setting and, to our knowledge, have also not yet been employed in tolerance trials. Another recent development, the combination of oligonucleotide-bar-coded monoclonal antibodies against a wide range of surface markers of immune cells with single-cell RNAseq (referred to as Cellular Indexing of Transcriptomes and Epitopes by Sequencing; CITE-Seq), opens an entirely new level of information on immune cell composition, differentiation, functional phenotypes, and even TCR α/β expression. These methods are increasingly applied to characterize immune cell infiltrates in a variety of infectious-, inflammatory-, and autoimmune diseases and also in tumors (81–83). Since they have only recently been introduced and their bioinformatics analyses are very demanding, they have, to our knowledge, not been used in tolerance trials yet, but we find them very promising.

Despite these powerful analytical tools, the hypothetical and expected changes, which might be induced by a tolerization strategy, may still escape detection at the level of bulk PBMCs. Important reasons for this are the low precursor frequency of antigen-specific, proinflammatory T cells (see below) and the fact that MS patients are immunologically healthy, i.e. the disease-specific abnormalities are very subtle and do not lead to easily discernible alterations or general compromises of protective immune function.

Finally, a comprehensive mechanistic program should include the phenotyping and functional profiling of immune cells in the target organ, i.e. CNS-infiltrating cells. However, the low numbers of cells that can be obtained from CSF after a lumbar puncture, the obvious limitations for repeated spinal taps at different time points (compared to peripheral blood) and the fact that autoreactive T cells are expected to enter and leave the CSF and brain compartment, i.e. that they will not be there all the time, limits the usefulness, even if multiple spinal taps could be performed.

From a technical point of view, important aspects to consider are the use of fresh versus frozen material for the immune phenotyping, since sample processing and cryopreservation may impact the expression of several markers such as chemokine receptors or activation markers. Further, intracellular staining for cytokine detection requires the use of activators and fixation procedures, and cytokine production may be influenced by the activation method.

In summary, the comprehensive phenotypic characterization of immune cells before and after tolerization should at a minimum include multi-color flow cytometry panels to capture changes in the main immune cell populations and, in more detail, the phenotypes, migration markers and activation states of CD4+ T cells and B cells. We anticipate that the abovementioned novel techniques that combine surface markers with RNAseq will allow much more detailed analyses in the near future.

Measuring Antigen-Specific T Cells

Documenting the effects of the respective tolerizing approach on the numbers and phenotype of autoreactive T cells is a prerequisite for demonstrating that it indeed induces antigen-specific tolerance. This aspect is currently one of the least well developed and most challenging. Below, we will cover important points that need to be considered.

There is solid evidence that certain immunodominant peptides of MBP, MOG, PLP and a few other non-myelin/non-CNS antigens appear involved in MS, and CD4+ T cells against are increased in MS, show higher antigen avidity, express proinflammatory phenotypes, and are frequently restricted by MS-associated HLA-DR molecules (4, 20–22, 26, 84). It is therefore important to document that a tolerizing approach either silences/anergizes these cells, deletes them or induces Tregs that control them. While this is obvious in theory, translating it into practice poses enormous challenges from several reasons including: a) the very low precursor frequency of autoreactive CD4+ T cells in the range of 10^{-4} - 10^{-7} depending on the assay (85–87), b) the methods that are available for reliably detecting these rare cells before tolerization or, even worse, their reduction after tolerization, remain poorly developed, c) demonstrating a change in phenotype of such rare cells in conjunction with their antigen specificity is also very difficult. Different from vaccination approaches, where one may start with a low precursor frequency T cell population, but wants to demonstrate its increase, the opposite, i.e. that very infrequent autoreactive T cells decrease or disappear, is a major challenge. Several methods are in principle available, and we list their main characteristics in **Table 1**. In short, they include detecting antigen-specific T cells by proliferative testing [3H-thymidine incorporation (88, 95); CFSE dilution (89)], ELISpot and FluoroSpot (90, 91), and FACS-based methodologies that measure the upregulation of CD154 or other surface markers

TABLE 1 | Assays for testing the frequency of autoantigen-specific T cells.

Assay methodology	Advantages	Disadvantages	Reference
(3H)-thymidine incorporation	Easy, high dynamic range, sensitive, easy to quantitate, well established, inexpensive	Requires radioactivity, takes several days, detected precursor frequencies low*	(88)
CFSE dilution	Easy, well established, allows characterization of the viability, phenotype and functional status by flow cytometry	Difficult to quantitate, insensitive, narrow, dynamic range, less data for use with autoantigens	(89)
ELISpot/ FluoroSpot	Easy to use, relatively more reliable/standardized, detects frequency of cells with a specific functional phenotype (based on the detected cytokine/s), detects higher frequencies than proliferation, relatively robust	Overall less experience than with e.g. thymidine incorporation	(90, 91)
Upregulation of CD154	Fast (few hours), easy to quantitate, preferentially detects proinflammatory cells	Relatively insensitive, less data for use with autoantigens**, requires freshly isolated cells	(92, 93)
HLA-class II/ peptide tetramers	In principle suited for direct detection of antigen-specific T cells, allows isolation of cells	Narrow dynamic range, insensitive, overall poorly developed for autoantigens***, few DR/peptide tetramer combinations available, promising in combination with TCR sequencing once available	(94)

In the range of 10^{-4} to 10^{-7} for myelin-specific CD4+ T cells. Therefore, it is important to seed sufficient numbers of cells, i.e. minimally 5 and better 10 or more wells with $2x10^5$ cells/well. We have obtained better results with respect to background and number of positive wells with seeding CD45RA- cells, which contain memory T cells and monocytes/macrophages, but no B cells, which are often responsible for high background stimulation.

**Works best with freshly isolated cells; an assay that measures the upregulation of CD137 after antigen stimulus preferentially detects activated Tregs*

***In our hands no HLA-DR/peptide tetramer from commercial- and academic sources has given reliable results so far; due to the fact that only few DR/peptide tetramers are available, even if they did work, one would have to use multiple ones to capture T cells restricted by all or most HLA-DR/DQ combinations that the patient expresses.*

after short-term, antigen-specific activation (92, 93), or using antigen-loaded HLA-class II tetramers (94). Besides the detection method, it is important to consider the organ compartment (peripheral blood lymphocytes versus CSF-infiltrating T cells), further the cell types (whole PBMC or memory T cells, freshly isolated versus frozen cells), the stimulating antigens (peptides versus whole proteins including controls to rule out effects on viral and bacterial recall antigens), and finally also to include antigens that might be important to capture effects on epitope spreading (29). After testing multiple different assay types and variables, we find the considerations that are summarized in **Table 2** helpful. May be with the exception of the FluoroSpot assay, which was recently introduced by Bronge et al. (90), none of these assay platforms is sufficiently well established and standardized to measure the frequency of autoantigen-specific T cells. Most require experience in cellular immunology techniques, and some should be performed preferentially with freshly isolated cells (96). HLA-DR/peptide tetramers have in our experience so far not worked at all for measuring autoreactive, peptide-specific T cells in bulk peripheral blood lymphocytes. The reasons probably include the low precursor frequency of autoreactive T cells, their too low antigen avidity, and the fact that only few specific HLA-DR/peptide tetramers are available. Recent developments enhancing tetramer binding and affinity may help to overcome these problems (97, 98), and combinations of tetramer staining and TCR sequencing to track antigen-specific T cells before and after tolerization could greatly improve it further.

Defining clear response criteria, e.g. a 50% reduction of T cells with a given specificity, is important as is the timing, when responses are examined. Measurements at two time points prior to and also after tolerization are preferable, and, whenever frozen cells can be used, cells from time points before and after tolerization should be measured together to minimize inter-assay variations. Regarding the compartment from which the cells are derived, CSF appears preferable, since CSF-infiltrating T cells are likely important for the pathogenesis of the disease, however, several points need to be considered. Incorporating CSF testing and repeat spinal taps is rarely possible. Since the patient may refuse a second spinal tap, it carries the risk that no measurement after tolerization is available. Furthermore, the number of patients, who show positive responses of CSF CD4+ T cells to the abovementioned antigens, is in the range of 10-20% (99), probably because autoreactive T cells enter and leave the CSF compartment.

In summary, we currently favor the Fluorospot assay (90) or proliferation of CD45RA- memory T cells from the peripheral blood after pre-enrichment of these cells (14, 26, 88). It is currently open whether the rapidly improving single cell technologies, which can incorporate isolation of certain cell types or bar-coded antibody tagging and single cell RNA sequencing, or affinity-matured tetramers will resolve some of the above issues. While the single cell methods offer clear advantages such as information about the gene expression profile and TCR expression, their costs are still very high, bioinformatics are demanding, and, since only between 10.000-20.000 cells can be analyzed, their application for low frequency autoreactive T cells needs further refinements.

Regulatory T Cells

The induction of stable regulatory cell populations that sustain a robust state of immune tolerance and over long periods of time or forever is an important goal of tolerizing approaches. Both FOX3-expressing Tregs and Tr1 are thought to be important in MS, although the data on circulating Tregs in MS and their involvement in its pathogenesis are conflicting. The paragraph below will summarize findings about Tregs with a focus on MS and how this information is relevant for antigen-specific tolerization.

Tregs are usually identified and their frequency assessed by flow cytometry. Their heterogeneity regarding phenotypes,

TABLE 2 | Important considerations for measuring the frequency of autoantigen-specific CD4+ T cells.

1. Testing memory T cells (e.g. CD45RA- cells) is preferable over whole PBMCs
2. Seed sufficient numbers of cells/well and replicates in order to detect a meaningful number of autoreactive T cells before and after tolerance induction
3. Protein antigens that cover all potential epitopes are preferable to peptides
4. If peptides are used, focus on immunodominant epitopes and those peptides that are used in the respective tolerization approach
5. Stimulate cells with antigen at a low/intermediate concentration to increase the chance to measure high avidity T cells (e.g. 1-5 μM peptide)
6. Include peptides that are not part of the tolerization approach to detect epitope spreading and the influence of the tolerizing approach on these specificities
7. Include foreign antigen-derived peptides as controls, ideally peptides or proteins to which most humans react, e.g. tetanus toxoid, viral peptides from CMV, EBV, influenza (like CEFII)
8. Include sufficient numbers of cells/well and replicates that are not stimulated (negative control)
9. Define response criteria
10. Consider HLA-class II types of patients in the context of peptides in the tolerization approach and their known HLA-class II restriction of CD4+ autoreactive T cells

differentiation and stability complicates Treg characterization before and after tolerization. Further, clear criteria for combinations of markers that unequivocally identify the different types of Tregs and address their functionality are still lacking.

FOXP3+ Tregs are defined as CD4+ CD25+ cells with low or no CD127 expression and expressing FOXP3. In MS, reduced (100), unaltered (101–103), but also increased (104, 105) numbers of Tregs have been reported. Other studies showed impairments of Treg function in MS patients (106, 107). These discrepancies are to some extent due to differences in the criteria for characterizing Tregs. Despite the archetypical high CD25 and low CD127 expression, there are multiple phenotypic subsets reflecting distinct differentiation- and activation states based on surface marker expression (like CD45 isoforms, CCR7 or HLA-DR) and with different suppressive capacities, which are, however, also not well characterized. FOXP3+ Tregs exert their suppressive effect by different mechanisms including IL-2 deprivation *via* CD25 capture, CTLA-4 expression, generation of immunosuppressive metabolites, release of immunosuppressive cytokines, and cytotoxic activity *via* perforin and granzyme, among others (62). Some of the surface markers used for their characterization are related to these mechanisms of action, yet none of them is specific for Tregs. CD39 is an ectoenzyme involved in ATP catabolism into cAMP and adenosine, which are immunosuppressive. CD39 identifies cells with an effector-memory phenotype that control Th17 responses, and CD39+ cells have been described to be decreased and functionally impaired in MS patients (108, 109), but increased levels have also been reported in RMS patients during relapses (110, 111).

FOXP3, the master regulator for the development and function of Tregs, remains their core marker (112, 113). Several studies have linked low FOXP3 expression levels with MS (100, 114), which were restored after vaccination with peptides of TCRs that are expressed by myelin-autoreactive cells (115). However, FOXP3 expression alone is not sufficient to identify Tregs, as the transcription factor is also transiently expressed in activated cells lacking suppressive ability (116). Stable FOXP3 expression in Tregs, and Treg lineage stability in general, is epigenetically regulated and relies on the presence of DNA demethylation in non-coding regions of the FOXP3 locus [Treg-specific demethylated region (TDRS)] and other Treg-associated genes such as IL2RA, CTLA4, IKZF2, and IKFZ4 (117–119). This epigenetic signature and not only FOXP3 expression marks *bona fide* Tregs and allows discrimination from activated CD4+ CD25+ FOXP3+ conventional T cells (120). Accordingly, it has been proposed that the term Treg should only be used for cells exhibiting the epigenetic Treg signature or with proven suppressive ability (61).

Therefore, the assessment of the methylation status of FOXP3 and the above genes could in principle give information about a favorable outcome of a tolerization strategy. However, these techniques are demanding and not established for routine enumeration of Tregs. In this regard, GPA33 (Glycoprotein A 33), a member of the immunoglobulin superfamily, has recently been reported to be a reliable marker of stable Tregs of thymic, but not peripheral origin, which show the epigenetic signature (64) and thus could be useful for the quantitative analysis of tTregs.

The identification of Tr1 cells is similarly complicated due to their low frequencies in peripheral blood, the lack of specific surface markers, and the inconsistent nomenclature in the literature. Like FOXP3+ Tregs, Tr1 cells display different suppressive mechanisms to regulate cells in their vicinity, such as IL-10 and TGF-β secretion, granzyme B- and perforin-mediated cytotoxicity, cell-contact dependent mechanisms or ATP catabolism. IL-10 is the Tr1 signature cytokine and critical both for their generation and function (65). Hence, Tr1 cells are best identified by their high IL-10 secretion. Functional defects of Tr1 cells have been described in MS patients, using IL-10 secretion *ex vivo* as indirect assessment of their suppressive function (121, 122), and during several tolerization trials in MS patients increased levels of Tr1 cells have been reported (123–125). However, the assumption that any IL-10-producing cell with suppressive capacity should be considered Tr1 is not correct (65), as other T cells can also release IL-10.

Gagliani and coworkers reported that the simultaneous expression of the surface markers CD49b and LAG-3 (Lymphocyte-Activation Gene 3) on memory CD4+ memory T cells (gated as CD3+ CD4+ CD45RA-) identifies Tr1 cells (126). This marker combination may not detect all Tr1 subsets, but only activated memory Tr1 cells. However, although some authors have argued that IL-10-producing CD4+ T cells constitute a heterogeneous cell population and that IL-10 is not an ideal marker for Tr1 cells (127), the current consensus is that CD4+ memory CD49b+ LAG3+ T cells, which produce high amounts of IL-10 and have regulatory activity independent from FOXP3, can be defined as CD4+ Tr1 cells (65).

The suppressive function of Tregs can be assessed with *in vitro* suppression assays (128, 129), which show the ability of a regulatory population to suppress the proliferation of conventional T cells in co-culture experiments. Suppression assays are relatively easy to perform, sensitive and inexpensive, and allow the parallel detection of cytokines in the supernatant. The use of CFSE to measure proliferation has advantages over the classical [H^3]thymidine incorporation-based methods. It is not radioactive, more precise in assessing Treg function, since proliferation of Tregs cannot be excluded in [H^3]thymidine incorporation assays, and shows the number of cell divisions and furthermore allows the phenotypic and functional characterization of the proliferating cells by flow cytometry (130). However, the *in vitro* conditions may not truly replicate the *in vivo* situation. Another drawback is the above-mentioned lack of well-defined surface markers for Tregs, which prevents the purification of homogeneous populations for the assays. Also, FOXP3 staining requires fixation of the cells which renders them useless for functional tests. Furthermore, is it not clear whether lack of suppression in MS is due to functional Treg defects and/or an increased resistance of autoreactive cells to suppression (44). Finally, due to the heterogeneous composition of both regulatory- and effector T cells, differences in their activation and state in the cell cycle, senescence and plasticity, these assays are very difficult to standardize, particularly if one uses bulk Treg and effector cell populations.

In summary, the identification of markers that are constitutively expressed by Tregs and reliably identify them as well as the development of a standardized functional assay would greatly help to assess Treg physiology and their role in MS, particularly in tolerization trials. It is possible that changes in the composition of Tregs rather than in the overall frequencies occur at different stages of the disease. In this context, FOXP3+ Tregs are believed to be essential for the initial phase of tolerance induction at the target organ, while Tr1 cells are key for the maintenance of long-term tolerance (65). Consequently, not only how they are assessed but also the time point of sampling after tolerization can influence the results of Treg analyses.

Soluble Biomarkers of Inflammation and Reduced Target Organ Damage

We will briefly outline, which types of biomarkers exist and how they may aid mechanistic studies during tolerance induction.

According to standard pharmacology terminology, one can consider three types of biomarkers. a) Pharmacodynamic markers that are directly related to the mechanism of action of the tolerization approach. The induction of Tregs and their stability (e.g. TSDR demethylation), reduction of autoreactive T cells and respectively the induction of regulatory- (e.g. IL-10) or decrease of proinflammatory cytokines (IFN-γ, IL-17, GM-CSF) fall in this category. b) Pharmacokinetic markers, which allow determining the onset and duration of the effects. The same set of markers could be applied here. The fact that both proinflammatory and anti-inflammatory cytokines are found in serum or plasma at low picogram levels poses another hurdle to demonstrate differences from before to after tolerization. For conventional ELISAs these values are at the detection limits, and they are thus usually not suited. However, newer, more sensitive techniques as for example the single molecule array (SIMOA) methodology, or electrochemiluminescence-based assays, allow the reliable detection of cytokines even at these values. c) Finally, there are biomarkers that serve as an indirect readout for reduced target organ damage or inflammation, but are not specific for tolerization. As markers of target organ damage, i.e. damage of neurons and axons, and indirectly also an indicator of reduced tissue inflammation in the brain, neurofilament light chain (NfL), that can be measured in serum or plasma and CSF, is probably the best examined and validated (for serum) biomarker (131, 132). Chitinase 3-like protein 1 (CH3L1) reflects innate immune activation and inflammation (133), but is relatively unspecific. Other analytes reflect different aspects of the pathomechanisms of MS such as de- and remyelination (myelin proteins, oligodendrocyte differentiation markers), microglia and astroglia activation (e.g. GFAP), metabolic changes, and adaptive immune cell infiltration/activation. For detailed discussion, the reader is referred to reviews of biomarkers in MS (132, 134, 135). Since protein-measuring methodologies are robust and likely more relevant than changes in gene expression, which can be detected by multiplex quantitative PCR or RNAseq methodologies, they should be included in the mechanistic program. During early stage trials, a broader panel that is less hypothesis-driven and instead discovery-oriented is of interest. Several platforms offer the measurement of large(r) panels (up to 100 and more) analytes including mesoscale discovery, flow cytometry, bead-based methods, OLINK, SIMOA, and others. Some offer preset collections of analytes that are known to be related to inflammatory-, neurological-, or autoimmune conditions and can be analyzed in very small sample volumes. Performing genome-wide RNAseq with cells of interest, e.g. CD4+ T cells or B cells, at the bulk or single cell level affords an even broader look with the caveat that increased gene expression not necessarily translates into increased protein expression and that quantitation remains more difficult. The latter methodologies can be combined with stimulation by global- or antigen-specific activation, but such steps have so far not been applied in tolerance approaches. Further, a number of proteomics technologies that measure either large sets of defined markers (e.g. SOMAscan; SomaLogic) or even broader sets (e.g. SWATH-MS; Creative Proteomics), that aim to overcome one core issue, i.e. the very low concentrations of immunologically relevant analytes in serum/plasma, have been developed and are beginning to be tested. Similar to the above single cell RNAseq methodologies, proteomics- and also epigenetic profiling methods will likely not only be applied alone, but also in combination in the future.

In summary, soluble analytes that can serve as pharmacodynamic and pharmacokinetic measures are currently very scarce. Biomarkers of target damage or -inflammation are not specific for tolerance induction and probably only change with some delay. Despite these problems carefully collected, processed and cryopreserved samples from tolerization trials are not only an invaluable resource for

exploratory studies that are already feasible, but furthermore can be used in the future, if improved methods become available.

Other Important Aspects

Dose finding: Mechanistic studies along tolerance-inducing therapies (for a summary of suitable assays see **Table 3**) can assist in identifying the best dose of the respective regimen. In some previously tested approaches in animal models, the dose range appeared critical for achieving the desired effect, and in MS trials with different tolerizing therapies such as oral tolerance (136), altered peptide ligand vaccination (95, 137), and also subcutaneous administration of peptides (124), there was no linear relationship between dose and effect, but rather a critical/optimal or even damaging dose range. Tolerization with peptide-coupled splenocytes in EAE and other animal models of autoimmune diseases showed a threshold dose, below which no effect was observed (138), but an upper limit had not been seen with the relatively narrow span of doses that had been tested. There is no well-established formula that would allow to extrapolate dose ranges from animal testing to humans in the field of tolerance induction. One should therefore try to gather as much information as possible from mechanistic studies in parallel to surrogate outcomes such as magnetic resonance imaging lesions during early clinical testing. In addition to capturing tolerance-related effects, it is also imperative throughout the clinical development program to assure that the respective, supposedly tolerance-inducing therapy is safe and does not induce rather than attenuate proinflammatory autoimmune reaction. The latter has been observed with the highest dose of an altered peptide ligand of MBP 83-99 (95) underscoring that the documentation of immunosafety is a key goal.

Duration of tolerization effects: Mechanistic readouts, for example the reduction of autoreactive T cells or the induction of different types of Tregs, can also help in determining how long a tolerizing effect lasts and when retreatment may be needed (see above for pharmacokinetic markers). A pertinent example is again tolerization with peptide-coupled splenocytes in EAE (68), which is not only very effective when applied prophylactically and therapeutically, but also shows remarkably long-lasting effects. A single tolerization is usually sufficient in autoimmune models to protect the animal lifelong from re-induction of the autoimmune disease (68). In humans, we do not know if tolerization effects, if they can be induced, will last equally long, but assume that periodic re-treatment will be necessary even with peptide-coupled cell-based tolerization. Accordingly, periodic testing of the putative tolerizing effects should be incorporated particularly during phase II testing.

Patient selection: Autoreactive CD4+ T cells recognize peptides in the context of specific HLA-class II molecules, for example MBP 111-129 together with HLA-DRB1*04:01 (139), or GDP L-fucose synthase with DRB3*02:02/03:01 (13, 99) while others, for example MBP 83-99 is a promiscuous HLA-class II binder and immunodominant not only in the context of the DR15 alleles DR2a and DR2b, but also other DR alleles (140). Assuring that the patient population of a tolerance trial is representative with respect to HLA-class II types in the context of the tolerizing antigens is therefore important. Futhermore, patients at early stages of CIS or RMS with inflammatory disease activity as measured by MRI are probably the ideal group for early-phase tolerization trials and most informative. Patient selection should therefore consider inclusion of the most prevalent, MS-associated HLA-class II alleles, robust reactivity to at least one of the tolerizing autoantigens to be able to measure changes, and patients with early active MS rather than in the progressive stage and after failing multiple MS drugs before. For further details see (11).

CONCLUSIONS AND OUTLOOK

Incomplete understanding of peripheral immune tolerance and how specific tolerance approaches work in humans, the frequent

TABLE 3 | Important components of a mechanistic studies/biomarker program to test immune tolerance in multiple sclerosis.

Goal	What should be measured/method	Comments
Immunosafety	Increase of autoreactive T cells Change to more proinflammatory phenotype Exclusion of immunosuppression and major alterations of immune cell composition	Multiple time points and at least two complementary methods
Reduction of autoreactive T cells	Decrease of autoreactive T cells ELISpot/FluoroSpot with whole antigen Proliferation assay with peptides	For details, see **Tables 2**, **3**
Effects on FOXP3+ Tregs, Tr1 cells	Flow cytometry protocols using several markers Suppressive function of Tregs	Consider epigenetic modifications of TSDR*
Pharmacodynamic soluble biomarkers	IL-10	Use highly sensitive assay; made by multiple cell types besides Tr1 cells
Biomarkers for tissue damage	Neurofilament light chain	Use highly sensitive assay
Biomarkers for inflammation	Chitinase 3-like protein 1, others	As above
Exploration of previously unknown mechanisms and cell types	RNAseq in single cells, ideally in combination with methods that allow measuring transcription in defined cells (e.g. by bar-coded antibodies against immune cell surface markers)	Data analysis still challenging; several methods in development
	Proteomics techniques suited to measure large numbers of analytes	Several methods and approaches; technically demanding to measure low-abundance molecules in serum/plasma

**TSDR, Treg-specific demethylated region.*

omission to include mechanistic studies along early clinical trials and also the lack of reliable methods to measure for example the reduction of autoantigen-specific T cells or the induction of Tregs are possible reasons why prior efforts failed. This short review emphasizes the importance of mechanistic- and biomarker studies for the clinical development of tolerance-inducing approaches. These should be tailored to the respective approach and its putative mechanism(s) of action. Carefully developed standard operating procedures and validation of different methods are necessary. Similar to the use of imaging parameters, which have been accepted as surrogates for clinical efficacy in phase II clinical trials in MS, a core set of mechanistic studies and biomarkers should be incorporated. This should at least include measuring the reduction of antigen-specific T cells, the changes of natural- and induced Tregs and pharmacodynamic biomarkers such as IL-10, but also markers depicting damage of the target tissue as for example NfL. Further, it is desirable that these are measured in a standardized fashion across clinical trials and different approaches to reach a consensus on methods and analyses, which in turn should help in understanding and comparing immunologic effects of therapeutic approaches and support clinical development and interaction with regulators. In the US, the Immune Tolerance Network (ITN), which exists for more than a decade and is jointly sponsored by the National Institutes of Health and the Juvenile Diabetes Research Foundation, has invested a lot of effort in developing standardized assay protocols, pursued some of the above aspects already along trials, e.g. in type I diabetes (54), and also sponsored tolerization trials (https://www.immunetolerance.org/). Their efforts have been instrumental in systematically addressing several of the challenges that we mention, but since the pathogenic mechanisms differ between diseases as well as the knowledge on target antigens and tolerance mechanisms, it would be highly desirable to intensify international exchange and collaborations further in specific disease areas in the future to harmonize mechanistic studies along tolerization trials.

AUTHOR CONTRIBUTIONS

All authors declare that they have substantially participated in the preparation and writing of the manuscript and have taken due care regarding their contribution to ensure the integrity of the work. All authors contributed to the article and approved the submitted version.

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Mapping the Mechanical and Immunological Profiles of Polymeric Microneedles to Enable Vaccine and Immunotherapy Applications

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Biomaterials hold great promise for vaccines and immunotherapy. One emerging biomaterials technology is microneedle (MNs) delivery. MNs are arrays of micrometer-sized needles that are painless and efficiently deliver cargo to the specialized immunological niche of the skin. MNs typically do not require cold storage and eliminate medical sharps. Nearly all materials exhibit intrinsic properties that can bias immune responses toward either pro-immune or inhibitory effects. Thus, because MNs are fabricated from degradable polymers to enable cargo loading and release, understanding the immunological profiles of these matrices is essential to enable new MN vaccines and immunotherapies. Additionally, understanding the mechanical properties is important because MNs must penetrate the skin and conform to a variety of skin or tissue geometries. Here we fabricated MNs from important polymer classes – including extracellular matrix biopolymers, naturally-derived polymers, and synthetic polymers – with both high- and low-molecular-weights (MW). We then characterized the mechanical properties and intrinsic immunological properties of these designs. The library of polymer MNs exhibited diverse mechanical properties, while causing only modest changes in innate signaling and antigen-specific T cell proliferation. These data help inform the selection of MN substrates based on the mechanical and immunological requirements needed for a specific vaccine or immunotherapy application.

Keywords: microneedles, immunomodulation, nanotechnology, biomaterials, vaccines, intrinsic immunogenicity

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1 INTRODUCTION

Existing pathogens and diseases continue to create challenges for current vaccine and immunotherapy technologies. The challenges span not only efficacy and selectivity, but also distribution, storage, and compliance (1, 2). Most recently, for example, the COVID-19 pandemic highlights the need for vaccines that can be easily disseminated without the need for refrigeration or complex cold chains (3). Also evident is the need for vaccines that generate potent and durable responses (4). Likewise, in cancer immunotherapy – where the target antigens are on

cancerous host cells or tissues, there is a great need for safe and selective approaches that generate strong responses against difficult-to-detect tumor antigens. Integrating new engineering technologies to improve the distribution, storage, and performance characteristics of vaccines and immunotherapies could enable next-generation vaccines that are easily deployed and generate strong and selective outcomes.

Biomaterials – including polymer and lipid nanoparticles, engineered scaffolds, and biodegradable materials – are being intensely investigated for vaccines and immunotherapies across infectious disease (5, 6), cancer (7–9), and autoimmunity (10–12). These materials can be either naturally occurring, fully synthetic, or hybrid in composition. Across these categories, a generally attractive feature is the ability for improved levels of control. For example, many biomaterials provide tunable control over loading and release of multiple immune cues, targeting specific cells or tissues, cargo protection, and control over release kinetics (13). The ability to chemically modify and functionalize the surface of nano- or microparticles also offers a modular ability that is particularly attractive in displaying immune cues to direct immune processes (14–16).

As alluded to earlier, one key ability of biomaterials is targeting specific tissue niches. In this context, one emerging approach particularly relevant for vaccines and immunotherapies are microneedles (MNs). MNs are small arrays of micrometer-sized needles made of synthetic or natural matrices. The design and length scale of these technologies ensure delivery of cargo across the skin barrier, and efficient access to the unique immunological niche within the skin (17). Because the skin is immunologically rich in specialized antigen presenting cells (APCs) – such as dendritic cells (DCs) and Langerhans cells (LCs), this organ is an important target for vaccine and immunotherapy (18). One design requirement for MNs is lengths sufficient to penetrate the skin, typically in the range of 25-500μm.

Beyond skin targeting, MNs provide other advantages including painless delivery – since the needles are too short to reach pain receptors, elimination of medical sharps, and incorporation and release of multiple immune cargos. Importantly, because the synthetic or natural polymer matrices used to synthesize MNs typically stabilize biological cargo, MNs often eliminate the need for refrigeration or cold-chain distribution (19). MNs can be either solid, coated, degradable, or hollow, depending on the application. Further, they can exhibit a range of mechanical properties – stiffness or flexibility, for example - that can determine the applications and features (e.g., skin penetration). Further, biomaterials can exhibit intrinsic immune profiles that can be immune activating or even anti-inflammatory (14, 20, 21). Thus, as with all biomaterials, the potential of MNs also requires additional attention to understanding how these matrices might interact with skin and immune cells, along with the other vaccine or immunotherapy components.

Toward this need, here we focused on understanding the mechanical and immunological profiles of key classes of polymer matrices used to form degradable MNs. Degradable or dissolvable designs are particularly relevant for immune applications because this strategy enables the encapsulation of cargo and degradation or dissolution to deliver cargo with controlled-release kinetics (22–24). Some of the key degradable polymers employed for MNs include gelatin, carboxymethyl cellulose (CMC), polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), and hyaluronic acid (HA) (25–31). Of note, gelatin-based MNs recently cleared phase I clinical trials for influenza (32). For the current studies, we selected six degradable polymer matrices from both natural and synthetic origins, testing high and low molecular weight (MW) formulations for each matrix type. The immunomodulatory properties of these MNs were characterized using DC activation studies, T cell co-cultures, and gene expression studies. In parallel, key mechanical characteristics of these degradable - polymer MNs including fracture force and stiffness - were characterized to assess the ability of MNs to penetrate the skin and conform to different locations and organ geometries (i.e., stiffness). These studies revealed the MN matrices had diverse mechanical properties and caused modest – though statistically significant – changes to immune signaling as a result of intrinsic immune profiles. These studies contribute to strategies for selecting MN matrices appropriate for specific immune engineering applications with respect to both mechanical and immunological performance characteristics.

2 MATERIALS AND METHODS

2.1 Microneedle Matrices Used for the Experiments

Low Bloom and High Bloom Gelatin, Sodium Carboxymethyl Cellulose (90kDa and 700kDa), Dextran (9-11kDa and 150kDa), PVA (13-23kDa and 85-124kDa), and PVP (10kDa and 1300kDa) were purchased from Sigma Aldrich, U.S.A. HA (<10kDa and 100-150kDa) was purchased from Lifecore Biomedical, USA.

2.2 Fabrication of MNs

MNs were fabricated using a solvent casting process using an MN master and polydimethylsiloxane (PDMS) (SYLGARD Kits, DOW, 184 SIL ELAST KIT) molds. This method was adapted with modifications from our lab's previous work on MNs (33), including careful considerations to the purity and avoiding endotoxin. A 5% w/w of the polymer solution was pipetted into the PDMS mold. The PDMS mold was then centrifuged at 4000g for 10min to fill the tines. This centrifuged PDMS mold was dried for 24-48h followed by releasing carefully to obtain the degradable polymer MNs.

2.3 Scanning Electron Microscopy

All MNs were imaged using a Phenom XL G2 Desktop SEM at 180x magnification, high vacuum, accelerating voltage of 15kV, and using a backscattered electron detector (BSD). To get a complete picture of the tines, the MNs were mounted on a 45° stud.

2.4 Mechanical Properties

To characterize the mechanical properties (stiffness and fracture force) of the MNs, a Dynamic Mechanical Analyzer (DMA) TA Q800 was used in the static stress-controlled mode using a compression clamp. In brief, the MN array was fixed on the lower plate, and the upper plate was moved towards the MN array in the strain ramp mode of the instrument at the speed of 0.01mm/s. The compression was stopped once the loading displacement reached 300μm. For approximating the stiffness of the MNs, blocks of polymers (cuboidal shapes 1cm x 1cm x ~3mm) were used. Qualitatively, stiffness was also measured by compressing the MNs using a tweezer and taking images before and after compression.

2.5 Characterizing Immunomodulatory Properties

2.5.1 DC Activation Studies

For DC Activation studies, primary DCs were isolated from spleens of naïve C57BL/6 mice using CD11c+ magnetic isolation beads (Miltenyi, 130-108-338). Spleens were isolated, minced, and incubated in Spleen Dissociation Media (StemCell Technologies, 07915), dissociated using a 16G needle, passed through a 40μm strainer, resuspended in MACS buffer containing CD11c+ magnetic isolation beads, and passed through an LS column in a magnet, with CD11c+ cells being collected in a final wash. Isolated DCs were plated at a density of 100,000 cells per 200μL in wells of a 96-well plate. These CD11c+ DCs were stimulated with MN tines dissolved in DC Media. Cells treated with LPS (1μg/mL), and cells treated with PBS were used as a positive and negative control, respectively. Polymer concentrations ranging from 0.0001-100μg per well for each lower and higher MW polymer were used for the treatment groups. After incubation with the groups for 24h, the DCs were washed twice with FACS buffer and then blocked using Fc Block (25X dilution, BD biosciences) for 10min at room temperature. The cells were then stained with antibodies for CD80, CD86, CD40, and viability. All antibodies were fluorescent conjugates and were used by staining for 20min at a 1:100 dilution in FACS buffer (for CD80, CD40, and CD86) and 1:200 dilution for Viability dye. Cells were then washed twice with FACS buffer for analysis by flow cytometry. Flow cytometry was performed on a FACS Celesta (BD Biosciences) and CytoFLEX flow cytometer (Beckman Coulter), and analyzed using FlowJo. For gene expression analysis, DCs were cultured for 24h with LPS and MN substrate or MN substrates alone prior to isolation and analysis.

2.5.2 T Cell Co-Culture

To see the effect of the MN matrices on T cell proliferation and expansion, CD11c+ DCs isolated as previously mentioned were treated with the MN solution (0.0001-100μg), LPS, and PBS. Soluble SIINFEKL (5μg/mL) was also pulsed into the wells along with the MN substrates and controls. After 48h, T cells isolated from OT-1 mice using CD8+ T cell negative selection kits (StemCell Technologies, 19852) were stained with cell proliferation dye eFluor 670 (0.5μM/well) during a 5 min incubation at room temperature. T cells were then co-cultured with each DC sample by adding 3x 10^5 T cells per well (making the ratio 1:3 DC to T cells). T cell proliferation was determined by the mean fluorescence intensity of the eFluor 670 signal and compared with the positive and negative controls.

2.5.3 Gene Expression Analysis

For gene expression analysis, RNA was isolated using the Quick-RNA Microprep Kit (Zymo Research, R1050), where cells were lysed in their wells using a lysis buffer, genomic material was captured in a silica-based matrix, and DNA was degraded with DNase I. RNA was diluted to 20ng/μL in RT-qPCR grade water (Thermo Fisher, AM9935). cDNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, 4368813). The qPCR reaction mix was made using TaqMan Gene Expression Assay probes in TaqMan Gene Expression Master Mix (Thermo Fisher, 4369016). Taqman probes utilized were: glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), Mm99999915_g1; actin beta (*Actb*), Mm00607939_s1; 18s rRNA (*18s*), Mm00434228_m1; interleukin 6 (*Il-6*), Mm00446190_m1; interleukin 10 (*Il-10*), Mm99999067_m1; interferon-gamma (*Ifn-γ*), and Mm00441891_m1; tumor necrosis factor (*Tnf-α*). qPCR was performed in a MicroAmp Optical 384-well reaction plate (Applied Biosystems, 4309849) with optical adhesive film (Applied Biosystems, 4360954) on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, 4485701).

2.6 Animal Care

All animal care and experiments were carried out in compliance with federal, state, and local guidelines and using protocols reviewed and approved by the University of Maryland's Institutional Animal Care and Use Committee (IACUC).

2.7 Statistical Analysis

All characterization studies were replicated at least three times, and all data points, along with mean ± standard deviation, were reported. Cellular analyses were replicated at least twice to ensure reproducibility of biological effects. For DC activation, T cell studies, and RT-qPCR studies, one-way ANOVA with Tukey post-test corrections for multiple comparisons were used to compare groups. Analysis and hierarchal clustering were done in MATLAB v.R2019b using the clustergram function, where the data were standardized for each gene to compare across multiple groups, and clustering was performed using a single linkage (nearest neighbor). Statistical calculations were performed using GraphPad Prism v.9.1.0.

3 RESULTS

We began by assessing polymers with three different origins 1) those derived from the extracellular matrix - gelatin and hyaluronic acid (HA), 2) naturally-derived polymers - carboxymethyl cellulose (CMC) and dextran, and 3) synthetic polymers - polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP). MNs were prepared from each material class by a solvent casting fabrication method involving a MN master and PDMS mold (**Figure 1A**). This process allowed facile manufacturing of MNs irrespective of matrix type. Scanning electron microscopy (SEM) revealed well-defined geometries that maintained the

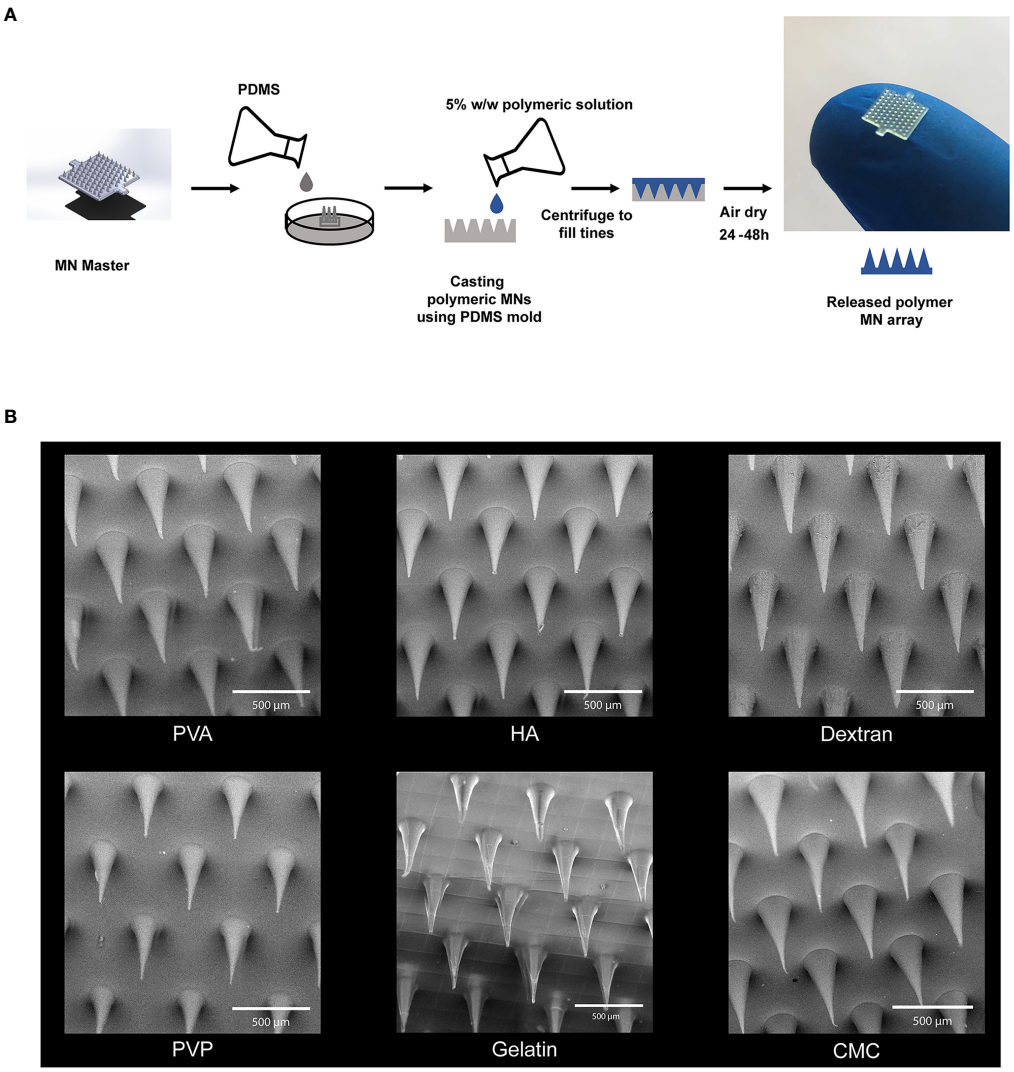

FIGURE 1 | Microneedles were fabricated using six different polymers. **(A)** Schematic of fabrication scheme for making the MNs. **(B)** SEM Images of the MNs fabricated using high MW degradable polymers. Scale bar = 500µm.

fidelity of the PDMS molds (**Figure 1B**). These data also confirmed the expected length scale for each matrix, with MNs exhibiting lengths of 500µm-520µm and base diameters of 200µm-220µm.

Next, we assessed important mechanical properties of MNs using dynamic mechanical analysis, including fracture force, the force required for mechanical fracture of the MNs (34). This parameter determines if a particular MN can support the force needed to penetrate the skin. Additionally, we assessed stiffness, the extent to which an object resists deformation in response to an applied force. Thus, stiffness determines if MNs are stiff enough to support higher pressure contact with skin– such as during a transient application with a quick dissolution design, or flexible enough to be applied to non-flat geometries with conformal contact, such as a slow release application requiring skin contact for longer durations (e.g., hours, days). We measured fracture force and stiffness using compressive forces applied during DMA (**Figure 2A**). Fracture force and stiffness studies revealed dramatic differences in the properties of MNs formed in this library, both as a function of polymer structure, and in some cases, also as a function of MW (e.g., CMC) (**Figures 2B–D**). Notably, most matrices exhibited fracture forces greater than 4N (**Figure 2B**), the minimum force required to penetrate the skin for these geometries (24). In contrast, gelatin (low MW) and PVP MNs exhibited fracture forces < 4N (**Figure 2B** and **Supplementary Table 1**), suggesting these designs might fracture before penetration. The stiffness varied over several orders of magnitude, an important finding since different applications may require MN patches that are either rigid or flexible (**Figure 2C** and **Supplementary Table 2**). Interestingly, some of the matrices afforded high fracture forces at both MWs but allowed stiffness to be controlled to achieve

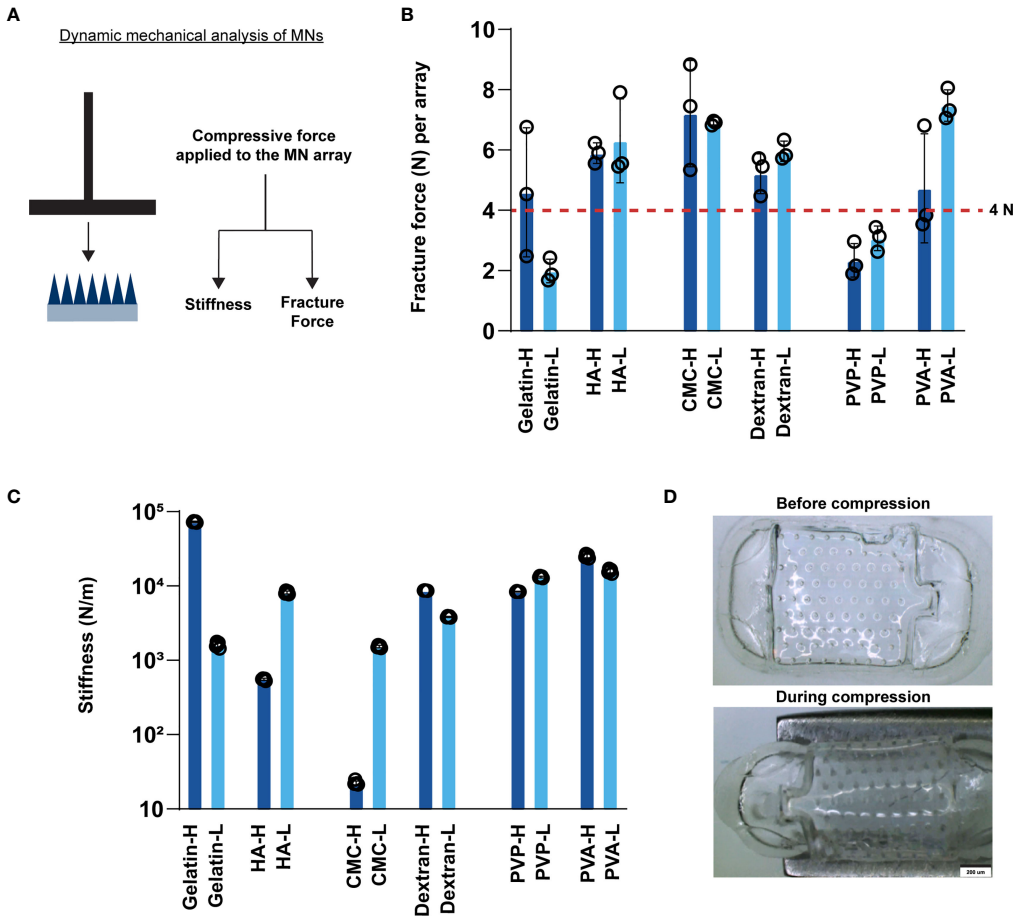

FIGURE 2 | MNs exhibit varying stiffness and fracture forces as a function of matrix type. **(A)** Schematic showing the experimental setup for measuring fracture force and stiffness. **(B)** Fracture force of the fabricated MN arrays. The red horizontal line at 4N indicates the minimum force required to penetrate the skin per array. **(C)** Stiffness of the fabricated MNs. **(D)** Images of high MW CMC MNs before and during compression.

either flexible or rigid materials that both support skin penetration (e.g., CMC). **Figure 2D** demonstrates the flexibility of CMC MN arrays using images obtained before and during compression. In general, lower stiffness values corresponded to flexible MNs. This flexibility, while maintaining sufficient fracture force limits on the actual needles, could be useful when applying MNs to geometries or skin that are not flat, facilitating contact and adhesion (35, 36).

After testing the mechanical properties of the MNs, we carried out a series of studies to characterize the intrinsic immune profiles of the MN matrices. We began these studies with viability assessment in primary mouse DCs (**Figure 3A**). All of the MN matrices generally afforded good viability for both high MW (**Figures 3B, C**) and low MW (**Figures 3D, E**) designs. Relative to positive and negative controls, there were statistically significant decreases, though these were modest at 5-9%. We also varied the dose to determine how sensitive DCs might be to the mass of MN matrix encountered during the application of MNs loaded with immune signals. However, viability was not significantly impacted as a function of dose across three orders of magnitude (i.e., 1ng *vs.* 1μg) (**Figures 3B** *vs.* **3D**; **Figures 3C** *vs.* **3E**).

Next, we tested if these MN designs alter DC activation profiles. These studies were initially conducted by incubating cells with the low dose (1ng) of MN matrices. Generally we found that the matrices did not increase CD80 relative to PBS, for either low or high MW polymers (**Figures 4A, B**). One exception was a modest increase observed during treatment with low MW versions of the natural polymers, CMC and dextran. For CD86, nearly all polymers led to a small, but statistically significant increase in activation relative to PBS (**Figures 4C, D**). In contrast, CD40 expression was significantly higher for all the MN matrices when compared to the PBS group (**Figures 4E, F**). However, in all cases the LPS positive control led to much higher activation than relative to the MN matrices (**Figure 4**). This indicates that at low doses, the MN substrates have some immune-activating properties, which are significantly less than LPS. Next, we tested a higher dose of the MN matrices (1μg) with DCs. Similar to the low dose treatment, most MN matrices either did not increase activation of CD80, CD86, and CD40, or led to very small increases (**Figure 5**).

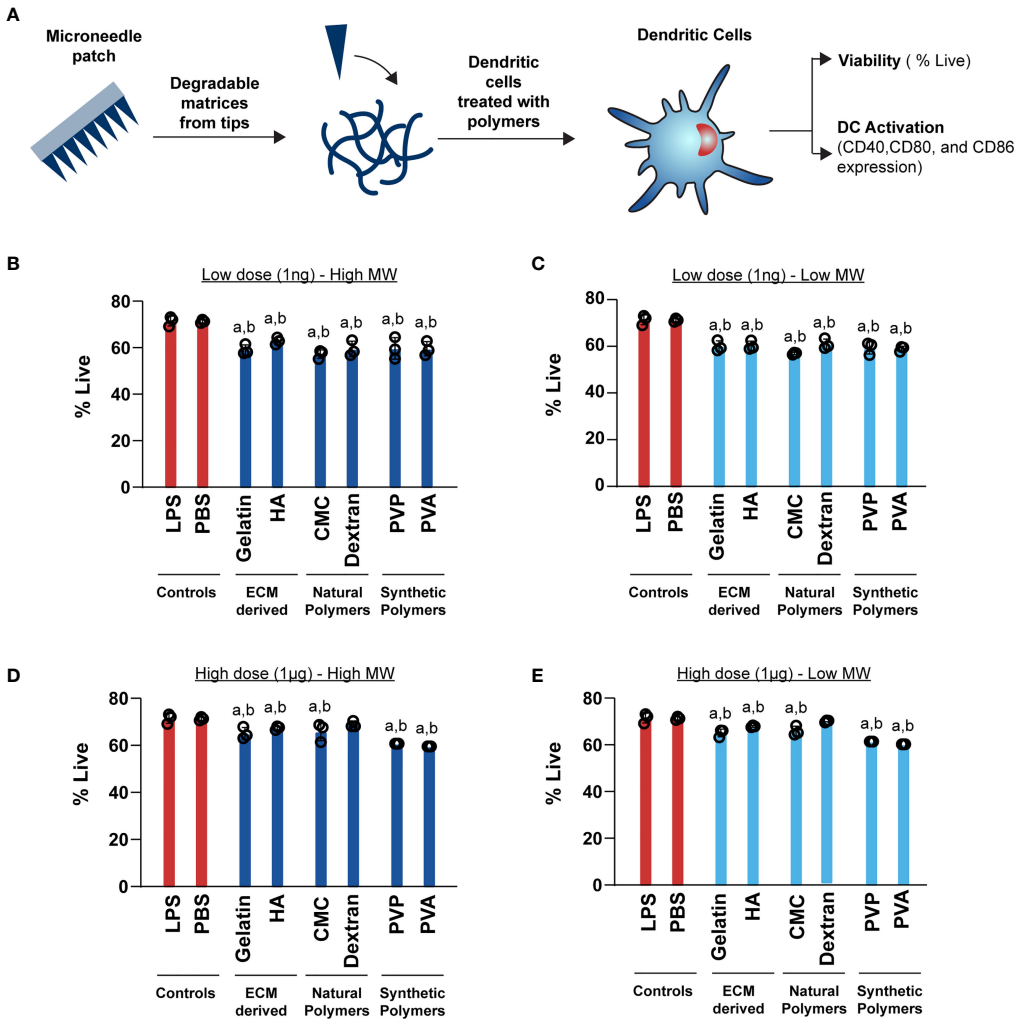

FIGURE 3 | High (1µg) and Low (1ng) doses of both high and low MW polymers have a moderate effect on viability. **(A)** Schematic showing the experimental setup for DC activation **B**) Viability of cells treated with 1ng of high MW polymers **(C)** Viability of cells treated with 1ng of low MW polymers **(D)** Viability of cells treated with 1µg of high MW polymers **(E)** Viability of cells treated with 1µg of low MW polymers. For panels **(B–D)**, "a" and "b" represents significant statistical differences ($p < 0.05$) when compared with LPS and PBS group, respectively.

Interestingly, although the MN matrices slightly increased CD40 expression at the higher dose (**Figures 5E, F**), the magnitude was less than that observed for the low dose treatments (**Figures 4E, F**). This may indicate engagement of feedback mechanisms during encounter of high or persistent polymer doses (37, 38). However, for all doses and all polymers, activation was modest relative to LPS. Even so, the variation in expression reveals the unique immune-activating properties that vary as a function of both composition and dose; the latter is driven by proximity of cells to the MN insertion site and the diffusion or clearance of degradation byproducts.

We next used RT-qPCR to measure gene expression changes in DCs as a function of MN matrix type and MW. To assess the intrinsic immune profiles of these materials, we measured the gene expression after 24h of cell incubation with MN matrices. We selected genes *Tnf-α*, *Ifn-γ*, and *Il-6*, which are common innate inflammatory cytokines, and *Il-10*, a common regulatory cytokine. As expected, stimulation with LPS significantly increased the expression of *Tnf-α*, *Ifn-γ*, *Il-6*, and *Il-10* when compared to treatment with PBS or MNs matrices. This was evident in unsupervised clustering of the gene expression heat map (**Figure 6A**). In assessing *Ifn-γ*, we discovered a modest activating effect relative to PBS - except for PVP, consistent with the prior studies using polymers in soluble non-MN form that natural and synthetic matrices activate innate inflammatory pathways (**Figure 6B**). When LPS was also present, the addition of MN matrices generally did not further increase expression of *Ifn-γ*, though one notable exception was dextran (**Figure 6B**). For *Tnf-α*, MN matrices alone did not meaningfully alter gene expression compared to PBS (**Figure 6C**). However, with LPS also present, there was a small but significant decrease in *Tnf-α* expression relative to LPS alone for most of the MN matrices (**Figure 6C**). MN treatment caused only very small perturbations

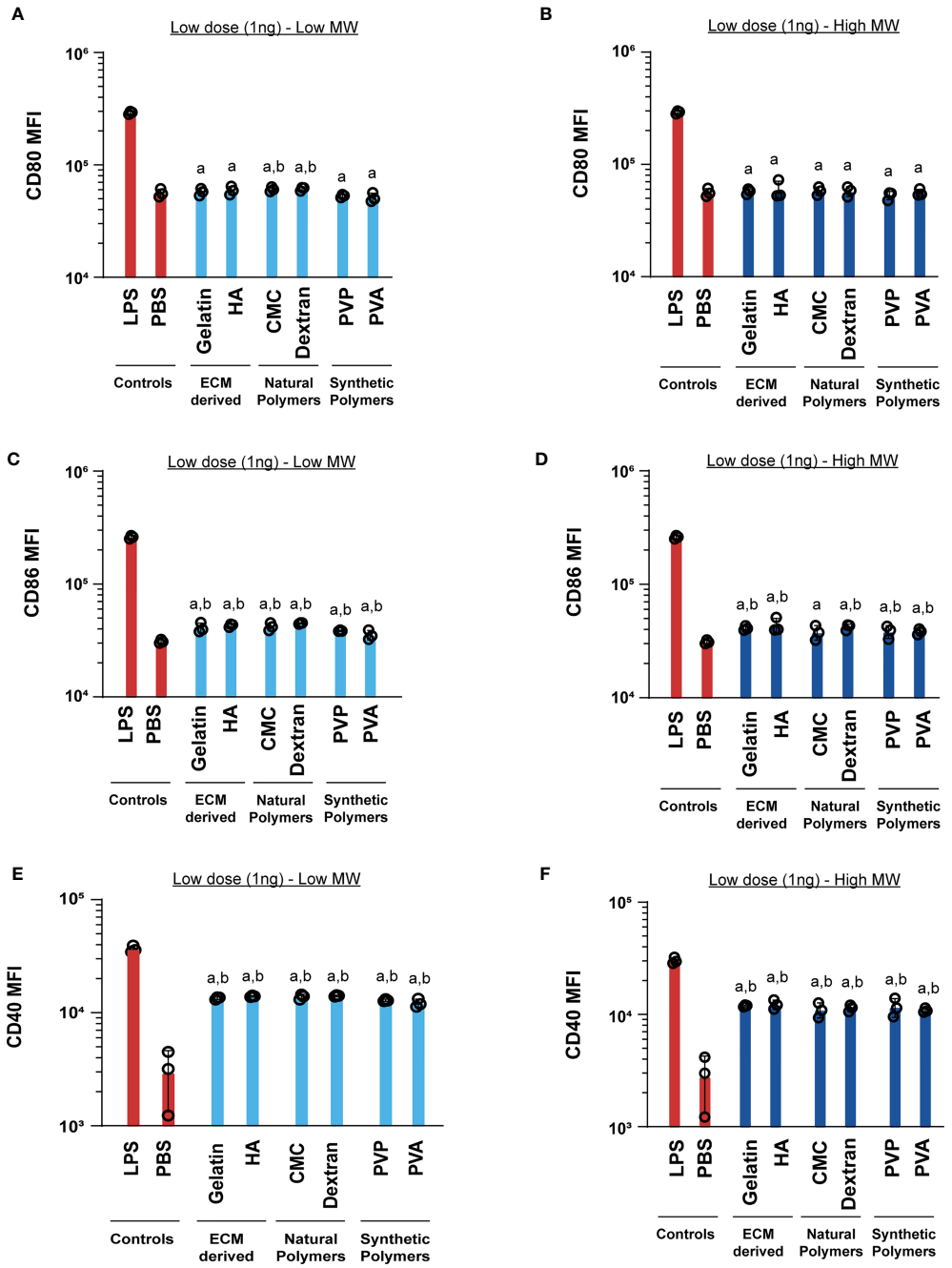

FIGURE 4 | Low (1ng) doses of both high and low MW polymers lead to low activation levels. Activation of DCs treated with low or high MW polymers at low doses, respectively, as indicated by expression of **(A, B)** CD80, **(C, D)** CD86 and **(E, F)** CD40. "a" and "b" represents significant statistical differences ($p < 0.05$) when compared with LPS and PBS group respectively.

in *Il-6* gene expression, with or without the presence of LPS (**Figure 6D**). Likewise, for *Il-10*, we saw some statistically significant decreases for MN matrices in both the absence and presence of LPS, though these were also modest in magnitude (**Figure 6E**). Taken together, this gene expression analysis is consistent with the DC activation studies, suggesting the MN matrices have intrinsic immune properties that modestly impact signaling and activation, despite creating opportunities for vastly different mechanical properties to support specific design applications.

We next investigated whether MNs enhance or inhibit the ability of T cells to engage their cognate antigen and proliferate. These studies were carried out by treating DCs with MN matrices, adding in a model antigen – SIINFEKL, then co-culturing these DCs with T

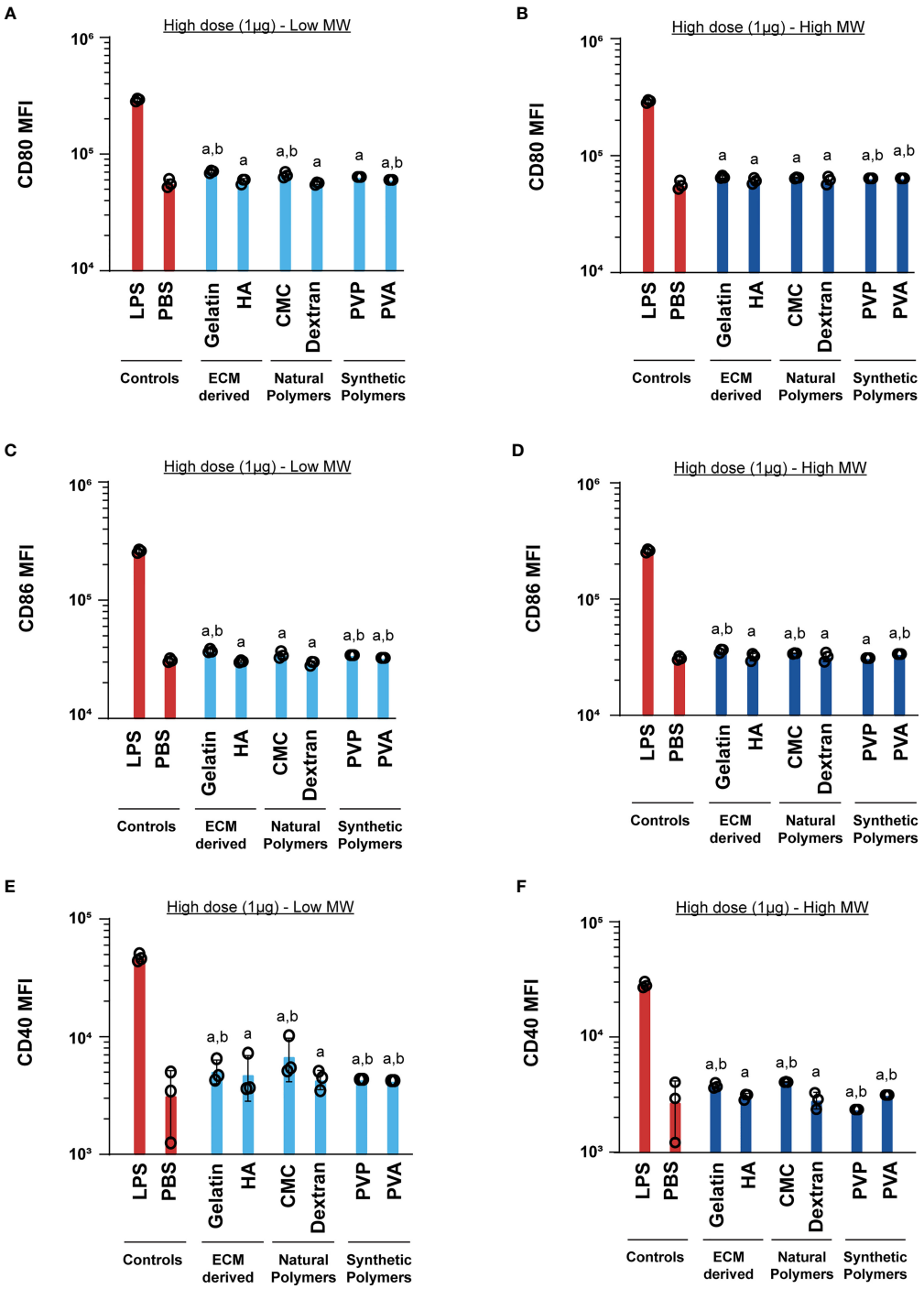

FIGURE 5 | High (1µg) dose of both high and low MW polymers leads to activation similar to the negative control. Activation of DCs treated with low or high MW polymers at high doses, respectively, as indicated by expression of **(A, B)** CD80, **(C, D)** CD86 and **(E, F)** CD40. "a" and "b" represents significant statistical differences ($p < 0.05$) when compared with LPS and PBS group respectively.

cells from OT-I transgenic mice. OT-I T cells are specific for SIINFEKL, causing proliferation and activation when T cells encounter SIINFEKL displayed in MHC-I by DCs (**Figure 7A**). We choose to use the high dose (1µg) as this dose is representative to the cargo: matrix ratio used in immune engineering applications. In all cases, we observed T cell proliferation was unaffected by the presence of MN matrices when compared to SIIN only and SIIN+LPS group (**Figures 7B, C** and **Supplementary Figure 1**).

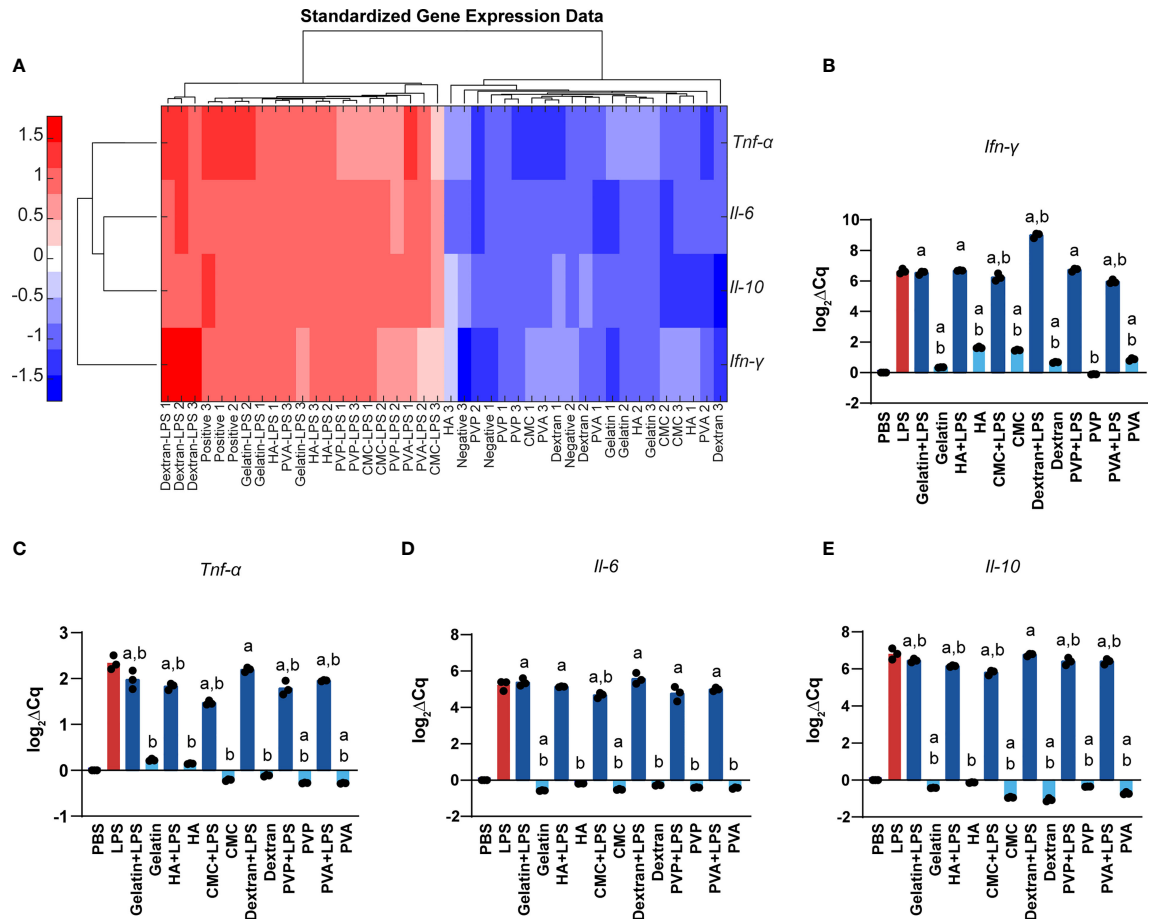

FIGURE 6 | Polymer MNs have significant but modest differences in gene expression of inflammatory as well as regulatory cytokines. **(A)** Heat map and log2 gene expression data for **(B)** *Ifn-γ*, **(C)** *Tnf-α*, **(D)** *Il-6*, and **(E)** *Il-10*. "a" and "b" represent significant statistical differences ($p < 0.05$) when compared with PBS and LPS group respectively.

These proliferation levels were also much higher than those observed during the treatment of cells with LPS and an irrelevant antigen (Irr. Antigen). Overall, these data indicate that these MN matrices do not alter the ability of T cells to engage with a cognate antigen.

4 DISCUSSION

As the applications of MNs increase there is a greater need to understand the immunomodulatory properties of the materials used to fabricate them. Here we fabricated MNs from polymers with diverse origins, assessed their suitability to penetrate the skin by measuring important mechanical properties, and characterized their intrinsic immunomodulatory properties. Creating these types of profiles is needed to enable robust MN technology platforms for a variety of applications. For example, in cancer and infectious disease, strong pro-immune outcomes are desired, whereas in tolerance or allergy applications, anti-inflammatory features may be useful. Likewise, different mechanical properties are amenable to different applications and wear-times; longer durations, for example, require increased flexibility to maintain adhesion to complex tissue or body geometries.

For the reasons just mentioned, translating MNs from bench to clinic also requires attention to fabrication processes. We used a solvent casting method for fabricating the MNs (39–41), which is more reproducible and robust compared to other techniques like microlithography and laser ablation (42). The manufacturing technique can also impact the mechanical properties, including key parameters, such as fracture force and stiffness: with insufficient fracture force values, MNs could fracture during insertion; with inappropriate levels of stiffness/flexibility the MN patch application, adhesion, and durability may be mismatched with the intended application (43, 44). We found most of the matrices tested could suitably penetrate skin, but interestingly, that some materials - such as CMC, could be prepared over a large range of flexibilities.

MNs are specialized for skin delivery, which creates unique relevance for immunoengineering applications. DCs, for

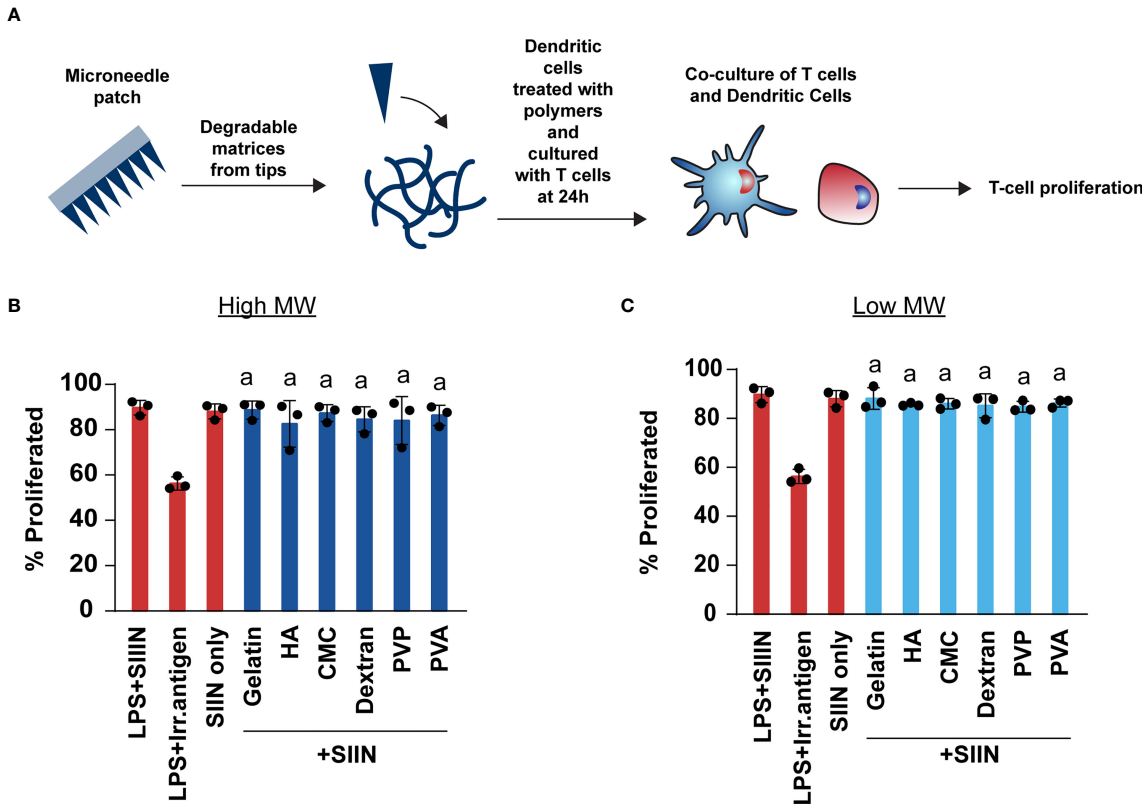

FIGURE 7 | Polymer MNs do not limit the ability of T cells to engage with their antigen or proliferate. **(A)** Schematic showing experimental set-up for T cell co-culture experiments. **(B, C)** Proliferation of T cells when treated with high and low MW polymer MNs, respectively. "a" represents significant statistical differences ($p < 0.05$) when compared with LPS+ irrelevant antigen (irr. antigen). The comparison between LPS+SIIN and SIIN with the polymer MNs was not significant.

example, are a major type of skin-resident immune cells involved in presenting antigen and co-stimulatory cues T cells. When T cells bind antigen and costimulatory signals on the surface of DCs, these cells differentiate and proliferate to mount immune responses against that antigen (45). This is one of the basic premises for vaccines, and also for many antigen-specific immunotherapies (46). We used splenic DCs in our experiments to facilitate the large number of primary cells needed to screen the library of matrices and doses. These cells do share important broad activating characteristics with skin-derived APCs. For example, Langerhans Cells and splenic DCs upregulate MHC, CD40, and CD80 during expression (47, 48). However, directly assessing the unique features of specific skin-resident populations will be important in future studies centered on specific immunological applications. Across the matrices tested, we generally found good viability profiles, and low levels of intrinsic inflammatory activity associated with the matrices. Although there were some increases in activation as a function of MN composition, these increases were always small relative to LPS. One interesting result was the lower activation of CD40 for high doses, compared to low MN doses. This could be due to the engagement of immunological feedback mechanisms at these higher doses. Along similar lines, in our gene expression studies, we observed a few instances where specific materials modestly dampened the activating ability of LPS. However, none of these changes limited the ability of T cells to engage their cognate antigen. Because each matrix has a slightly different immunomodulatory profile – and these profiles are generally mild in either pro- or anti-inflammatory nature - this creates opportunity for design. For example, these subtle changes might help bias responses toward a desired outcome – immunity or regulation – based on the other components in a vaccine or immunotherapy.

In summary, our studies reveal that these polymer MN substrates have profiles varying in both mechanical and immunological properties, allowing selection of MN substrates for different applications based on a combination of requirements in each of these areas. Our work is distinct from past MN studies in that we have characterized a range of matrices in these area, rather than focus on a specific vaccine candidate. This type of comparative benchmarking of intrinsic properties is important to support MN technology development and also for new vaccines and immunotherapies (49). However, understanding how the subtle changes we observed in innate signaling connect to downstream outcomes – such as T cell polarization and phenotype, is an important next step. Likewise, extending these types of studies to other skin APC types, including LCs will also be important to

broaden the relevance. Lastly, a goal is to use the profiles generated in these and other studies with MN designs integrating immune signals, rather than just the matrix. This will reveal and help isolate the interplay between background intrinsic matrix effects, and the impact of the active immune cues included in emerging vaccines and immunotherapy candidates.

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 animal study was reviewed and approved by University of Maryland Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

SS, RO, SK, and CJ conceived the studies. SS and RO carried out the studies. SS, RO, and CJ carried out the data analysis. SS and CJ wrote and revised the manuscript with input from all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.843355/full#supplementary-material

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REVIEW
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Overcoming the Challenges Imposed by Humoral Immunity to AAV Vectors to Achieve Safe and Efficient Gene Transfer in Seropositive Patients

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One of the major goals of *in vivo* gene transfer is to achieve long-term expression of therapeutic transgenes in terminally differentiated cells. The extensive clinical experience and the recent approval of Luxturna® (Spark Therapeutics, now Roche) and Zolgensma® (AveXis, now Novartis) place vectors derived from adeno-associated viruses (AAV) among the best options for gene transfer in multiple tissues. Despite these successes, limitations remain to the application of this therapeutic modality in a wider population. AAV was originally identified as a promising virus to derive gene therapy vectors because, despite infecting humans, it was not associated with any evident disease. Thee large proportion of AAV infections in the human population is now revealing as a limitation because after exposure to wild-type AAV, anti-AAV antibodies develops and may neutralize the vectors derived from the virus. Injection of AAV in humans is generally well-tolerated although the immune system can activate after the recognition of AAV vectors capsid and genome. The formation of high-titer neutralizing antibodies to AAV after the first injection precludes vector re-administration. Thus, both pre-existing and post-treatment humoral responses to AAV vectors greatly limit a wider application of this gene transfer modality. Different methods were suggested to overcome this limitation. The extensive preclinical data available and the large clinical experience in the control of AAV vectors immunogenicity are key to clinical translation and to demonstrate the safety and efficacy of these methods and ultimately bring a curative treatment to patients.

Keywords: AAV vectors, gene therapy, immunogenicity, humoral response, B-cells, neutralizing antibodies

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INTRODUCTION

Adeno-associated viruses (AAV) are constituted by a 25-nanometer protein icosahedral capsid containing a single-stranded DNA genome flanked by two palindromic inverted terminal repeats (ITR). The 4.7 Kb AAV genome encodes for four different Rep proteins (Rep78, Rep68, Rep52 and Rep40), three Cap proteins (VP1, VP2 and VP3), the assembly activating protein (AAP) and the newly identified membrane-associated accessory protein (MAAP) (1–3). Cap proteins constitute the

capsid of the virus and mediate the interaction with the host. The capsid proteins VP1 and VP2 share most of the sequence with VP3 that is the major component of the AAV capsid with 50 out of 60 capsid subunits being VP3 (4). At the time of writing, 13 different AAV serotypes and more than hundred isolates, distinguished by amino acid modifications in the capsid proteins have been identified in different species (1, 5–8).

After its isolation as a contaminant of adenovirus preparations in 1965 (9–11), it took almost 20 years for molecular cloning of the AAV genome thus opening the way to the generation of recombinant AAV (rAAV) vectors from AAV by encapsidating a transgene expression cassette flanked by the ITRs from serotype 2 (12–15). Importantly, through this process, the same transgene expression cassette can be pseudo-typed by virtually any of the natural AAV serotypes. As for the natural virus, capsid composition affects the tissue tropism and the intracellular trafficking of the recombinant virus (1, 16).

The adenovirus-free method of rAAV production is based on transient transfection of mammalian cells with three plasmids (17). Two of the plasmids provide in trans the *rep* and *cap* genes and the helper genes, typically from adenovirus (18, 19). A third plasmid contains the transgene expression cassette flanked by the two ITRs. Recombinant AAV vectors can be produced in mammalian cells also through the infection with adenovirus (20) or herpes simplex virus (21). Finally, rAAV can be produced in insect cells infected with baculoviruses carrying all the components necessary for vector production (22). Regardless of the production method, and differently from the wild-type virus (23), rAAV vectors are produced as a mix of full capsids, containing the genomic material, and empty capsids. Several distinct natural serotypes isolated in humans and other mammalian species were produced as well as chimeric AAV obtained through different techniques [recently reviewed in (24)].

As previously mentioned, the transduction properties of rAAV vectors are a direct consequence of the capsid composition. Surface-receptors binding, endocytosis and intracellular trafficking as well as the escape of the vector from the late endosome/lysosomal compartments and the nuclear import contributes to the preference of rAAV vectors for a certain cell type/tissue [reviewed in (25)]. After nuclear translocation, the genome of rAAV do not integrate efficiently and remains in the episomal form (26).

AAVs infect humans and other mammalian species starting from the first years of life (27–33), but are not associated to any known disease (34). Infection with AAVs results in the formation of a humoral response against the virus. Although the frequency of individuals seropositive to AAV may vary, large portions of the human population are infected, with an estimated seroprevalence for neutralizing antibodies (NAb) for the different AAV serotypes in the range of 30-60% (27–29, 35–37). The presence of NAbs due to exposure to the wild-type AAV reduce the number of patients that may benefit from the treatment.

Although multi-year transgene expression was reported in large animals and patients treated with rAAV (38–43), loss of expression at long-term is still possible as a consequence of slow cell replication or mechanisms of inactivation acting on the vector genome (44). The formation of anti-AAV NAbs with long persistence and wide specificity after injection of rAAV (45) represents an important limitation to re-administration of the vectors in patients that have received the rAAV and experienced an expression loss.

This review will then focus on the methods that overcome the limitations imposed by the humoral response to gene transfer with rAAV (**Figure 1**) perceived as fundamental to expand patients access to therapy and allow for life-long treatment of genetic diseases.

HUMORAL IMMUNITY TO AAV

AAVs naturally infect humans and other mammalians starting from infancy. Although the natural history of AAV infection is still poorly characterized, data indicated that, after infection, a large proportion (ranging from 30% to 60%) of the infected population developed cross-reactive anti-AAV NAbs possibly due to successive infections and/or broad cross-reactivity between AAV serotypes (46). The proportion of seropositive individuals increased from early childhood to peak at the age of adolescence (27–29). Infection with wild-type AAV resulted in antibodies from all IgG subclasses with a preference for IgG1. The levels of IgG, in general, correlated with the neutralizing antibody titers measured by an *in vitro* neutralization test (35, 36) although it was reported the presence of IgG that while binding to AAV vectors did not neutralize their capacity to enter the cells *in vitro* and *in vivo* (47). NAbs against AAV2 and AAV1 were reported as the most prevalent in the human population (48) starting from three years of age. Importantly, the occurrence of maternal transfer of Nabs, with neonates being seronegative only between 7 and 11 months, leaves a short time window for AAV gene transfer (27).

At the very beginning of gene therapy with AAV vectors, the role of pre-existing immunity in gene transfer efficacy was uncertain. The presence of neutralizing antibodies to AAV2 was not among the exclusion criteria in the first clinical trial of liver gene transfer after systemic infusion with this vector (49). Although the choice was possibly motivated by the use of a "local" administration of the vector, i.e. portal vein infusion, data showed a robust reduction in transgene expression in one patient having low anti-AAV neutralizing titers (49). Preclinical data obtained in mice and published in the same year supported this finding (50). Later on, experiments in non-human primates (NHP) clearly showed neutralization due to low-titer anti-AAV pre-existing antibodies (51). Based on this experience, exclusion criteria for seropositive patients were included in most of the clinical trials.

In humans, administration of AAV vectors resulted in a fast and robust rise in IgM, followed by IgG with a high neutralizing titer either after intramuscular (52) or portal vein infusion (49). Similarly to the wild-type virus infection, the neutralizing titers in general correlate with the levels of binding antibodies as measured by ELISA (53). Long term follow-up of patients

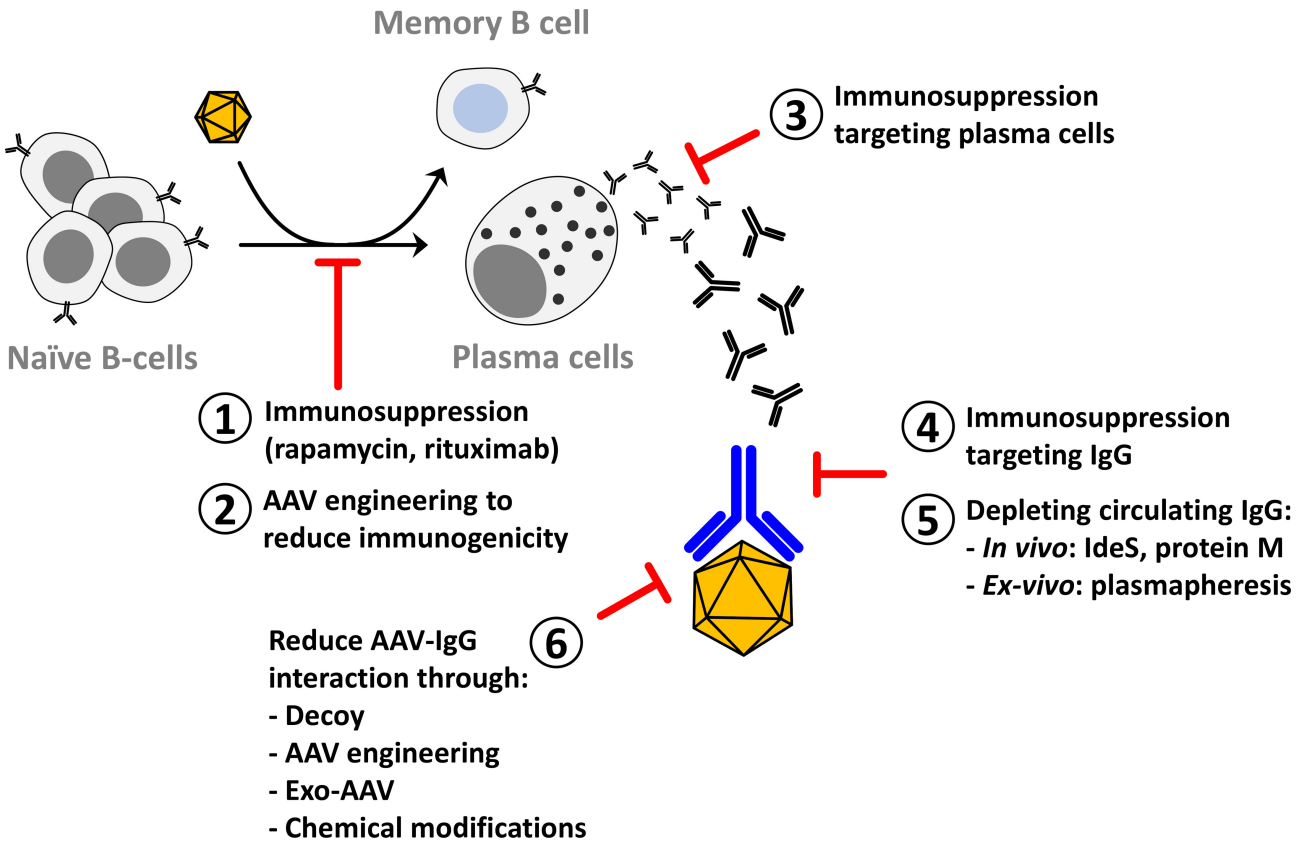

FIGURE 1 | Methods to reduce the impact of anti-AAV neutralizing antibodies on AAV gene transfer. After AAV gene transfer in seronegative individuals naïve B-cells are activated to plasma cells that are specialized in antibody secretion. Part of the activated B-cells become memory B-cells that participate in the long-term stability of the humoral response. Methods to reduce the activation of B-cells include immunosuppression to target the CD4-mediated helper function (1) or to reduce the viability of plasma cells (3) and AAV engineering to reduce the activation of the immune system (2). Immunosuppression to reduce circulating IgG has been proposed in autoimmune diseases and can be applied in AAV gene therapy (4). Circulating IgG can also be reduced through *in vivo* or *in vitro* depletion (5). Another method to reduce the impact of anti-AAV IgG on gene transfer efficacy is to modify the vector to evade neutralization (6).

receiving treatment for hemophilia B indicates up to fifteen years of stability of neutralizing anti-AAV antibody titers for the injected AAV (43, 45). Importantly, while higher titers were measured against the injected serotype, the antibodies were neutralizing also against other serotypes although with lower titers (45).

Neutralizing factors, capable of binding to AAV vectors thus reducing their efficacy *in vitro* were identified as elements of the complement system (54, 55). Important and open questions about neutralizing factors against AAV are their exact nature and role in the immune response and gene transfer efficacy *in vivo*. Complement activation was reported in ongoing clinical trials for Duchenne muscular dystrophy and spinal muscular atrophy type 1, in which patients administered with high-dose AAV vectors developed serious adverse events including liver dysfunction, acute kidney injury or thrombocytopenia (56–58). More recently, LogicBio reported two cases of thrombotic microangiopathy following the administration of 5 x 10^{13} vg/kg of AAV-LK03 (59). Both complement proteins and antibodies are likely being investigated for their role in those adverse events.

In animal models, neuronal degeneration in the dorsal root ganglia (DRG) were observed few weeks after high-dose intravenous or intrathecal administration in piglets and NHPs (60–62). This neuronal toxicity was not prevented by conventional steroid regimens and was dependent on overabundance of the transgene product and subsequent activation of cellular stress pathway (63). Beside this DRG toxicity, acute liver damage occurred in NHPs 3-4 days after high-dose systemic delivery of AAV9 or AAV-PHP.B, with acute elevations in liver enzymes, thrombocytopenia as well as acute hemorrhage (60, 64). Interestingly, prophylactic steroid treatment mitigates the increase in liver enzymes but not the thrombocytopenia suggesting that these two events may not correlate. Moreover, selection of seronegative NHPs for the studies, timing of this acute toxicity as well as activation of alternative complement pathway support a working hypothesis where classical complement pathway activated by antigen-antibody immune complexes is likely not involved in these deleterious events. Poor availability of predictive animal models to study complement activation and limited clinical

experience with AAV vectors administration in seropositive patients may be two of the reasons why, so far, a mechanistic proof of the involvement of these factors in AAV toxicity is still missing.

Regardless of their involvement in unwanted toxicities, the long-term stability and the wide neutralization potential of the humoral immune response to AAV vectors are important limitations of this gene therapy modality as they preclude both vector infusion in seropositive patients and re-administration in patients that received a sub-optimal dose or in whom the efficacy of gene therapy waned overtime.

AAV CAPSID MODIFICATIONS TO REDUCE ANTIBODY-MEDIATED NEUTRALIZATION

Early attempts to prevent capsid neutralization were based on the use of empty capsids as decoys to shield the full particles from neutralization (65). This approach required large amounts of empty AAV capsids thus increasing total vector load. Liver toxicity associated with the death of four patients was recently reported in a clinical trial for X-linked myotubular myopathy (NCT03199469) and likely linked to the high vector dose and pre-existing liver disease. Another potential limitation in the decoy strategy is the lack of efficient methods for the production and purification of empty capsids with limited encapsidation of random cellular components and genomic and plasmid DNA.

The relatively low complexity of the capsid structure and AAV virus genome has allowed for the creation of a multitude of engineered capsids with improved properties. Rational design as well as random mutagenesis and capsid shuffling were used to derive novel serotypes. Peptide insertion in specific VP3 protein position combined with directed evolution, provided a powerful tool to improve AAV capsids biodistribution (66–68). Structure-guided evolution was also used to modify the epitopes recognized by antibodies thus evading neutralization. In particular, the resolution of the structure of AAV2 complexed with a monoclonal antibody (69) enabled rational mutation of the AAV2 sequence to avoid neutralization from this specific clone (70). An evolution of this approach is based on the rational mutation of the regions of AAV capsids involved in the interactions with polyclonal human serums (71). Through this approach, novel AAV capsids were identified with improved evasion of NAbs without compromising vector productivity or biodistribution. Another method to isolate neutralization-resistant AAV vectors is the directed evolution of AAV libraries in an *in vitro* neutralization setting. Although limited by the poor *in vitro* transduction of AAV vectors, the use of selective pressure on randomly mutated or shuffled AAV capsid libraries led to the isolation of serotypes that were less neutralized by purified intravenous immunoglobulins (IVIg) (72–74). The use of immune-orthogonal orthologues of AAV has been proposed to overcome the constraints imposed by the humoral response to AAV vectors (75). This approach is limited by the higher complexity of the human immune system that reduces the chance to identify orthologs (76). However, after confirmation in relevant preclinical models, this approach may represent an option to dose seropositive patients.

Another interesting approach to evade antibody-mediated neutralization is the use of AAV vectors co-purified with exosomes derived from the producer cell line. Exosomes are extracellular vesicles naturally produced by various types of cells both in culture and *in vivo* and used by the cells for communication or exchange of proteins and genetic material (reviewed in (77)). Importantly, some viruses showed the ability to hack this system and acquire a membrane shield to the immune system. Similarly, AAV vectors associated with membranes of the producer cell lines can be purified as exosome-associated AAV vectors or exo-AAV (78). Exo-AAV showed improved cell transduction and reduced sensitivity to neutralization in an *in vivo* neutralization assay (79–81). One potential limitation associated to the clinical translation of exo-AAV is related to the potential adjuvant effect of the proteins and nucleic acid co-packaged with the exo-AAV and to the challenges regarding the development of a clinical grade manufacturing process and the associated analytical methods.

The high stability of the AAV capsid in extreme conditions led to the development of methods to modify the surface of AAV capsids by cross-linking different chemical molecules. RGD-containing peptides as well as PEG were originally proposed to improve AAV tropism but also to reduce the impact of neutralizing antibodies on transduction (82–86). More recently, amino sugars, known to increase liver targeting were chemically linked to the AAV capsid (87). Although the chemical alteration of AAV vectors may, in principle, extensively modify the capsid surface, only partial evasion of NAbs was reported so far (87). One important limitation of the chemical modification of the AAV capsid and more generally of proteins is that drastic conditions are required to obtain extensive modification of the surface, and this may not be compatible with the stability of the 3D structure of the virus.

Although different methods were proposed to modify the AAV capsid and reduce the impact of neutralizing antibodies, so far, there is no clinical proof of the possibility to administer those modified AAV vectors in seropositive individuals. Perhaps in this context are worth mentioning the recent clinical results suggesting that AAV5 is less sensitive to neutralization in humans (88). Administration of an AAV5 vector expressing human coagulation factor IX in patients who have retrospectively been shown to have significant NAb titer resulted in similar expression levels of the transgene with a lower impact on the transduction compared to what was previously reported for AAV2 (49). Those results were confirmed in a large study in NHPs were different doses of AAV5-hFIX demonstrated efficacy irrespective of the presence of pre-existing anti AAV5 NAb at titers up to 1:1030 (88). The absence of standardized tests to compare neutralizing titers measured in the different clinical trials limits any further conclusion. However, these results suggest inconsistency between the neutralization titers measured with an *in vitro*

assay and the neutralization *in vivo* after administration of the AAV5 vector in humans. If confirmed in a larger number of patients, the use of AAV5 for liver gene transfer may represent a valid option to treat seropositive patients.

IMMUNOSUPPRESSION TO PREVENT ANTI-AAV CAPSID ANTIBODY FORMATION

The formation of anti-AAV NAbs following AAV gene transfer is dependent on the serotype used and on the route of administration which may influence the presentation of the AAV capsid proteins on antigen-presenting cells (APCs) (89–91). Blocking of classical costimulatory pathways, implicated in B-cell activation, demonstrated efficacy on the inhibition of humoral response and allowed AAV re-administration in mice (92, 93). Early findings supported the involvement of the innate signaling in the formation of the humoral immune response to AAV vectors (94). In particular, loss of MyD88, a central node for the signaling downstream of Toll-like receptor (TLR) and interleukin-1 (IL-1) receptor pathways, significantly reduced anti-AAV NAb titers (95, 96). An isotype switch from IgG2c to IgG1 was also observed in MyD88 deficient mice possibly due to a switch to a Th2-type immune response in the absence of Th1-polarizing stimuli. In human peripheral blood mononuclear cells (PBMCs), stimulation with AAV2 capsid resulted in an interleukin-1b (IL-1b) -dependent B-cell maturation and anti-AAV antibody secretion (97). Importantly, the inhibition of IL-1b through monoclonal antibodies resulted in decreased anti-AAV antibody formation both *in vitro*, in human PBMCs and *in vivo* in C57Bl6 mice challenged with an AAV8 vector (97). Based on these data, the inhibition of innate signaling seems to be a promising approach to control adaptive immunity to AAV vectors. Another relevant target for the suppression of the anti-AAV humoral response is the inhibition of the CD4 co-receptor. Both the antioxidant MnTBAP and a non-depleting anti-CD4 antibody were used to reduce the activation of CD4+ T-cells, thus decreasing the anti-AAV humoral response (90, 98). As an alternative, rapamycin (Sirolimus), an immunosuppressive drug largely used in transplantation, was shown to reduce anti-AAV immune response (99). Interestingly, rapamycin formulated in nanoparticles showed greater efficacy in small and large preclinical models and allowed to re-administer an AAV vector in primates while reducing the dose of rapamycin by specific targeting APCs (100–102). B-cell depletion with an anti-CD20 antibody (Rituximab) has also shown efficacy in the reduction of anti-AAV humoral immune response in preclinical models (103, 104). The combination of Sirolimus and Rituximab is being investigated in a clinical protocol for AAV vector re-administration in Pompe disease (NCT02240407). The clinical protocol involves the repeated intramuscular administration of an AAV9 vector expressing GAA in the presence of an immunosuppression regimen combining Rituximab and Sirolimus. Although the results of this trial are not public, a single-case report indicated that simultaneous treatment with these two drugs prevented the formation of anti-AAV1 antibodies when the vector was administered into the diaphragm (105).

One important limitation of the immunosuppression methods described above is that proof of their efficacy was obtained mainly in naïve animals that received the AAV vector for the first time and their efficacy is likely very limited in primed animals were the B-cells are already activated and differentiated in both memory B-cells and plasma cells (PCs). Since PCs are responsible for the maintenance of high levels of circulating antibodies, different strategies, derived from those used in autoimmune diseases and myeloma, were attempted to substantially reduce the number of active PCs. Targeting PCs may result in toxicity and the debate on the risk/benefit ratio of such approaches in AAV gene therapy is still open. Among them, we may cite the use of bortezomib, a proteasome inhibitor approved for the treatment of mantle cell lymphoma and myeloma, that despite its toxicity, has been proposed for the control of anti-AAV immune response (106). Although the combination of bortezomib with AAV vectors was also tested in dogs (107), no significant effect was reported on the pre-existing humoral immunity to AAV vectors and the strategy was never moved used in the clinic. Novel approaches are being developed for the control of PCs proliferation with improved selectivity and reduced toxicity [reviewed in (108)] and based on their improved risk/benefit ratio they may be tested as a pretreatment to reduce the humoral immune response to AAV vectors and allow for vector administration in seropositive patients.

METHODS TO REDUCE THE LEVELS OF CIRCULATING ANTIBODIES

As already discussed, plasma cells are a relevant target to reduce the impact of neutralizing antibodies on gene transfer. However, the current approaches to drastically reduce the number of antibody-secreting cells suffer from toxicity and poor selectivity.

A strategy to reduce the impact of NAbs on AAV transduction relies on the isolated perfusion of the liver with a catheter to flush the blood from the liver (109, 110). Although this method allows to reduce the titers of NAbs and increase liver transduction in seropositive monkeys, its clinical translation may prove complex and potential inflammation of the liver related to the procedure may threaten the outcome of gene transfer in humans.

As an alternative, reduction of the circulating levels of IgG is an ideal strategy to reduce the impact of anti-AAV NAbs on AAV gene transfer. Among those methods, the use of blockers of neonatal Fc receptors (FcRn) was proposed for AAV vector administration in seropositive patients (111), although it was never tested in this setting. FcRn receptors have a profound effect on the levels of circulating IgG by increasing their recycling (112). Blocking the action of FcRn led to increased antibody trafficking to lysosomes and degradation and reduced the levels of circulating IgG in both NHPs and humans (113, 114). FcRn-targeting therapeutics demonstrated efficacy in preclinical models of autoimmunity such as antibody-induced arthritis,

experimental autoimmune encephalomyelitis or immune thrombocytopenia [reviewed in (115)].

A more invasive alternative to FcRn blockers is the use of plasmapheresis. Plasmapheresis is widely used in autoimmune diseases to reduce the negative effects of auto-antibodies. Early findings indicated that multiple cycles of plasmapheresis were needed in humans to significantly reduce the level of anti-AAV NAbs in humans (116). In NHPs, two cycles of plasmapheresis allowed for muscle targeting after administration of AAVrh74 through isolated limb perfusion (117). More recently, a second study in NHPs demonstrated that three cycles of plasmapheresis reduced the levels of neutralizing antibodies to AAV5 of at least ten-fold on average and allowed for repeated administration of the same vector bearing two distinct transgenes (118). In the same publication, the authors showed that two cycles of plasmapheresis in humans led to a three to five-fold decrease in the anti AAV2 and AAV9 NAb titers (118). Although these results seem to support the use of plasmapheresis for AAV administration in seropositive individuals or for vector re-administration, the procedure is burdensome and results in transient suppression of the immunoglobulin in circulation. For this reason, two specific approaches were recently developed (119, 120). Both of them were based on the immobilization of an AAV capsid (AAV8 and AAV9 respectively) on a chromatography resin then used to specifically sequester anti-AAV antibodies from serum. These proof-of-concept studies clearly demonstrated the advantages of antigen-specific IgG depletion over plasmapheresis. However, the clinical development of this technology may prove complex and the demonstration of efficacy in large animal models is still missing.

An alternative to the use of plasmapheresis could be the reduction of circulating IgG with a bacterial protein, the IgG-degrading enzyme of Streptococcus pyogenes (IdeS) (121, 122). This cysteine endopeptidase is part of the bacterial arsenal to defend themselves from IgG-mediated opsonization (121). IdeS was originally proposed to prevent kidney rejection in HLA-sensitized individuals (123–125), and is now approved for use in this patients' population (Idefirix®, Hansa Biopharma). The molecular mechanism of action is based on the very fast and efficient cleavage of human IgG into F(ab')2 fragments and Fc (126, 127). Although the F(ab')2 fragments still retains some neutralizing activity, in the absence of the Fc portion, they are rapidly eliminated from the circulation. One of the biggest limitations for the identification of the potential of IdeS in gene therapy with AAV is the lack of preclinical models to demonstrate its efficacy. IdeS is highly specific for human IgG, it does not cut murine IgG and it is only partially efficient towards monkey IgG. *In vitro* digestion of monkey IgG with IdeS resulted in the degradation of the vast majority of the IgG. However, single chain IgGs, intermediates of the digestion reaction with a neutralizing potential and not easily cleared from the circulation, were still present after overnight digestion (128). Recently, we demonstrated the efficacy of IdeS for the degradation of anti-AAV antibodies and the successful administration of AAV vectors in seropositive NHPs (128). In particular, IdeS pre-treatment improved liver transduction efficacy in seropositive NHPs both in the context of first administration in monkeys naturally exposed to AAVs and in a re-administration setting (128). Confirmation of the potential of IdeS in AAV gene therapy was further provided by the use of IdeZ, an IdeS homolog (111). Importantly, although the injection of the bacterial protein induced the formation of anti-IdeS antibodies, they were not neutralizing the protein function, and IdeS was able to degrade IgG in the presence of anti-IdeS antibodies both *in vivo* and *in vitro* (128). Another interesting property of IdeS is its capacity to cleave B-cell receptor and temporarily inhibit memory B-cell activation (129) thus providing a further advantage when it comes to the administration of vectors in non-naïve individuals. In conclusion, IdeS has the potential to enable AAV vectors administration in seropositive patients even in the context of repeated administration of the same vector. One limitation toward the clinical use of IdeS in this setting could be that the repeated administration of IdeS may trigger a hypersensitivity reaction. Ongoing work is trying to address this potential issue by developing new IdeS molecules with reduced immunogenicity to unlock the potential of this approach in autoimmune diseases, chronic transplant rejection, oncology and gene therapy.

An orthogonal approach to IdeS, based on a distinct bacterial protein, is the use of protein M, an IgG binding protein isolated in human mycoplasma (130). Very positive preliminary data on the use of this protein to reduce the neutralizing titers against AAV were communicated (131) although no published data are available so far.

CONCLUSIONS

The overcoming of humoral immune response to AAVs is key to unlock the full potential of gene transfer with this vector.

The exclusion of patients from clinical trials based on the presence of antibodies against the gene therapy vector raises also important ethical questions. Different methods were developed to evaluate the neutralization activity against AAV vectors in patients (**Table 1**) , however limitations still exist and validation of those methods across laboratories is required to allow comparison of the data and equal access to the treatment starting from early-stage clinical research.

Different methods were developed in the past to reduce the impact of anti-AAV NAbs (**Figure 2**). Among them, immune suppression strategies inspired to those developed in transplantation seem to be the one with the highest potential in the clinic. So far, clinical proof of their efficacy in the reduction of anti-AAV antibody titers has been obtained only in individuals that, being seronegative, were likely naïve for AAVs.

The modification of the surface of AAV through direct engineering of the capsid sequence, or vector shielding with empty particles, exosomes and through chemical modifications was also proposed to escape neutralization. These techniques can be used to prevent neutralization in seropositive individuals. However, after the first injection the generation of antibodies specific for those modified capsids is likely to prevent vector re-administration. AAV-exosome have the potential to achieve re-

TABLE 1 | Methods currently used to evaluate antibody response to AAV vectors.

Method	Neutralizing factor detected	Unit	Sensibility	Standardization possible	Advantages	Limitations	Used in clinical trials
ELISA	Binding Abs	Concentration or titer (1:x) where x corresponds to the higher dilution giving greater OD than cut-off	+	+	• AAV serotype-independent • Detection of different Ab classes	• Do not reflect neutralizing activity • Saturation effect; need to test several sample dilutions • Availability of specie-specific secondary antibodies	Yes
Dot-blot	Binding Abs	% of max intensity signal	–	–	• AAV serotype-independent • Detection of different Ab classes	• Complex assay • Few samples tested	No
***In vitro* NAb**	Neutralizing activity	Titer (1:x) where x corresponds to the first dilution at which at least 50% inhibition of the reporter gene expression is measured	+/- Depending on reporter gene, cell line and multiplicity of infection (MOI) used	+/-	• Detects neutralizing activity *in vitro* • Can be used with any AAV serotype • Can be used with any species (do not require a secondary antibody)	• Low transduction efficacy may affect the results • Do not discriminate IgG classes or neutralizing factors other than Ig	Yes Routinely
***In vivo* NAb**	Neutralizing activity	% of inhibition of reporter gene expression compared to control mice	–	–	• Detect neutralizing activity *in vivo* • Can be used with any AAV serotype • Can be used with any species (do not require a secondary antibody)	• Low throughput • High variability • Do not detect low levels/ low affinity neutralizing factors	No

administration although data in relevant preclinical models are still missing.

A more specific alternative to immune suppression is to transiently reduce the levels of IgG, known to be the more important class of antibodies neutralizing AAV vectors, to create a window for the delivery of AAV vectors to target organs. The possibility to administer AAV vectors in seropositive monkeys through IdeS pre-treatment supports the hypothesis that by clearing circulating IgG a safe and efficient gene transfer can be achieved in the liver. Similar results were obtained with plasmapheresis in primates further confirming AAVs as vectors with a relatively low immunogenicity also in individuals with pre-existing immunity. Proof-of-concept of the efficacy of these approaches required adaptation due to the low activity of IdeS on monkey IgG and the small size of the animals. A clinical demonstration is likely required to demonstrate the full potential of these approaches.

Intravenous infusion is probably the route of administration where NAbs have the largest negative impact. Other routes of administration of AAV vectors, e.g. intravitreal or intrathecal are being tested in the clinic. For these routes, further studies are needed to better understand the impact of pre-existing NAbs on AAV gene transfer. The spatial constrains, the different composition and density of the fluids in these compartments and potential differences in the antibody composition of vitreous or cerebrospinal fluid may impact the efficacy of the different methods described in this review and they will possibly need to be adapted to the specificities of those compartments.

The administration of AAV vectors in non-naïve individuals is a potential concern when one of these strategies will reach the clinic. Immune responses to AAV vectors are unique in humans and it cannot be excluded that pre-exposed and naïve individuals may react differently to AAV vectors. This is particularly relevant for studies involving large doses of AAV vectors where toxicity was observed, possibly dependent on complement or platelets activation. Importantly, future studies of AAV administration in seropositive individuals will take advantage of the large clinical experience available on the control of AAV vector immunogenicity.

In conclusions, although all the approaches described in this review have the potential to reduce the impact of NAbs on tissue transduction, their efficacy was frequently demonstrated either with low neutralizing titers or through partial reduction of higher titers. Lower neutralizing titers are observed in individuals naturally infected by AAV and this is possibly the first population that will benefit from these approaches. In case of vector re-administration, the titers are in general more elevated and a combination of orthogonal techniques for the reduction of the impact of NAbs is potentially needed.

Despite the challenges imposed by the humoral immune response to AAV gene transfer, the knowledge on the immune response to AAV vectors and the technological advances make possible the clinical validation for some of these approaches. As we learned through the extensive clinical experience with AAV gene transfer, only a confirmation of safety and efficacy in humans could open the way to a generalized use of techniques to decrease the impact of humoral immune response on AAV gene transfer. The stakes are extremely high. On one hand, pre-existing immunity prevents seropositive patients to access a life-changing treatment. On the other hand, setting-up of protocols for safe and efficient AAV vector re-administration will completely change the paradigm of gene

	Method	Clinical readiness	PoC	Immunogenicity /toxicity	Pre-existing Ab	Post-treatment Ab
IS	Inhibition of B-cells activation	+	+	-	-	+
	Block plasma cells	+	-	+	+	+
	Empty capsid decoy	+	+	+/-	+	+
	AAV capsid engineering	+/-	+	?	+	-
	AAV exosome	-	+	?	+	+/-
	Chemically modified AAV	-	+/-	?	+/-	-
	FcRn inhibitors	+	-	-	+	+
	IdeS	+	+	+/-	+	+
	Plasmapheresis	+	+	+/-	+	+

FIGURE 2 | Comparative analysis of the different methods to reduce the anti-AAV humoral response. The last two columns refer respectively to the efficacy of the method in the reduction of the impact of pre-existing neutralizing antibodies or in the inhibition of the anti-AAV humoral immunity after treatment with recombinant vectors. PoC, proof-of-concept with AAV vector gene transfer; Ab, antibodies; FcRn, neonatal Fc-receptors; IdeS, Immunoglobulin G-degrading enzyme of S. Pyogenes.

therapy, moving from a once-in-a-life treatment to a treatment on-demand. This is a fundamental step to ensure durability of the treatment and provide a better alternative to other treatment like small-molecules or protein replacement therapy.

AUTHOR CONTRIBUTIONS

D-AG, NT, CL, and GR wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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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REVIEW
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Autoimmunity and Cancer—Two Sides of the Same Coin

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Autoimmune disease results from the immune response against self-antigens, while cancer develops when the immune system does not respond to malignant cells. Thus, for years, autoimmunity and cancer have been considered as two separate fields of research that do not have a lot in common. However, the discovery of immune checkpoints and the development of anti-cancer drugs targeting PD-1 (programmed cell death receptor 1) and CTLA-4 (cytotoxic T lymphocyte antigen 4) pathways proved that studying autoimmune diseases can be extremely helpful in the development of novel anti-cancer drugs. Therefore, autoimmunity and cancer seem to be just two sides of the same coin. In the current review, we broadly discuss how various regulatory cell populations, effector molecules, genetic predisposition, and environmental factors contribute to the loss of self-tolerance in autoimmunity or tolerance induction to cancer. With the current paper, we also aim to convince the readers that the pathways involved in cancer and autoimmune disease development consist of similar molecular players working in opposite directions. Therefore, a deep understanding of the two sides of immune tolerance is crucial for the proper designing of novel and selective immunotherapies.

Keywords: immune tolerance, autoimmune diseases, cancer immunology, tumor microenvironment, regulatory cells

INTRODUCTION

Immune tolerance is a state of unresponsiveness of the immune system to self-tissues with a concomitant ability to identify and respond against non-self and dangerous antigens. Multiple mechanisms shape and control this state, including the elimination of autoreactive receptors from the system in bone marrow and the thymus (central tolerance). However, not all autoreactive cells are deleted in the primary lymphoid organs. For example, the naive T-cell repertoire that leaves the thymus contains up to 40% of low-avidity self-reactive T cells. These cells can potentially trigger an autoimmune response; therefore, several mechanisms of peripheral tolerance evolved to prevent their activation (1). Specialized cell subsets, such as regulatory T (Tregs) and B cells (Bregs),

tolerogenic dendritic cells (tolDCs), and M2 macrophages, participate in keeping the balance between tolerance and activation. However, genetic predispositions and epigenetic modifications combined with exposure to environmental factors can disrupt this status, resulting in the development of autoimmunity. Therefore, an increasing number of approaches that boost the immune tolerance have been evaluated and were already implemented for the treatment of autoimmune diseases in humans. On the other hand, the same mechanisms can be exploited by cancer to set up cancer tolerance (2). In fact, the attraction of tolerogenic cell subsets and evading immune response is considered as one of the hallmarks of cancer. The malignant cells used to express immune checkpoint proteins show impaired antigen presentation, undergo epithelial-to-mesenchymal transition (EMT), or present alterations in RNA editing. In consequence, the presence of a tumor-specific antigen (TSA) or tumor-associated antigen (TAA) does not elicit immune responses to malignant cells (3). Therefore, multiple approaches have been already made to break cancer tolerance and awaken the immune system for the fight against cancer. These strategies were based on monoclonal antibodies, adoptive cell therapies, or therapeutic anti-cancer vaccines. Nevertheless, there is still a lack of full understanding of the complex network of mechanisms leading to tolerance induction or its breakdown. Therefore, with the current review, we aim to discuss the mechanisms involved in the development of autoimmunity and cancer, shedding a light simultaneously on two sides of the same coin. We hope that our paper will sort out the current knowledge in the field and inspire future studies on immune tolerance.

MICROBIOME AND IMMUNE RESPONSE

Gut microbiota imbalance is associated with the development and progression of multiple diseases, such as gastrointestinal cancers or inflammatory bowel disease. The link between gut dysbiosis and tumor development has been already reported with *Helicobacter pylori* being the best studied pathogen in this context (4, 5). However, this is definitely not the only component of the digestive tract microbiome involved in carcinogenesis. However, not only the composition of microbiota but also its activity have an impact on cancer development. Microbial metabolites, such as short-chain fatty acids (SCAFs) or N-nitroso compounds (NOCs) showed anti- and procarcinogenic effects, respectively (6, 7). The microbiome, as well as its metabolites, also affects the function of the immune system and, in this way, may contribute to cancer tolerance or the stimulation of anti-cancer responses. For instance, the fungal genus *Candida*, which is detected in 74% of oral cancer patients, was reported to increase the proliferation of myeloid-derived suppressor cells (MDSCs) known to dampen the anti-cancer response (8). Therefore, not surprisingly, gut microbiota may affect the efficacy of anti-cancer management as it was reported for immunotherapy with immune checkpoint inhibitors. For instance, the abundance of *Bifidobacterium* species or *Akkermansia muciniphila* (next-generation probiotic bacteria) was associated with slow tumor growth and beneficial responses to anti-PD-1 (programmed death receptor 1) therapy (9–11). Therefore, the modulation of gut microbiota may positively affect treatment efficiency and thus patient survival.

On the other hand, the interactions between immunological, microbial, and environmental factors in genetically susceptible individuals are involved in the etiopathogenesis of Crohn's disease (12, 13). Dysbiotic microbial alterations, such as low gut microbiota diversity, as well as a decreased amount of bacteria belonging to the *Firmicutes* phylum, are observed in patients with Crohn's disease (14). The link between mutations in TLR4 (Toll-like receptor 4) (rs4986790) and the IL-10 receptor with *Mycobacterium avium* subspecies paratuberculosis in these patients was also noted (15).

The nucleotide-binding oligomerization domain-containing protein 2/caspase recruitment domain-containing protein 15 *(NOD2/CARD15)* gene located on chromosome 16q12 was the first described gene connected with Crohn's disease pathogenesis (16, 17). It encodes the NOD2 protein, which is mainly expressed not only by dendritic cells (DCs) and monocytes but also enterocytes and Paneth cells. The molecule is known to play a significant role in the intestinal innate immune response against the bacterial cell wall. More than 30 variants of the *NOD2/CARD15* gene have been identified, while an increased risk of Crohn's disease development was connected to R702W, G908R, and L1007fs variants, as well as P268S and IVS8+158 polymorphisms (17).

The role of microorganisms in autoimmunity development was also extensively studied for type 1 diabetes (T1D). Molecular mimicry is described as the structural similarity between self- and foreign (microbial) antigens and has been connected with the break of tolerance to pancreatic beta cells in T1D (18). Researchers described a number of homologies between the antigens of beta cells and microorganisms such as Coxsackievirus (19) or Rotaviruses (20). These data demonstrate a big dynamism of the immune status and suggest that tuning the microbial repertoire may skew the immune response to the desirable profile to fight the cancer or restore immune tolerance to self-antigens.

ESCAPE FROM CENTRAL TOLERANCE MECHANISMS AND CANCER IMMUNE EVASION

There is a considerable body of literature presenting the different genetic factors that are associated with specific disease phenotypes as well as with the risk of the disease occurrence (21). Various alleles of human leukocyte antigen (HLA) class I and class II molecules were reported to be associated with a particular autoimmune disease occurrence, including T1D, multiple sclerosis (MS), rheumatoid arthritis (RA), or celiac disease (22, 23). The exact mechanism of how HLA polymorphisms predispose to autoimmunity remains poorly understood. However, it is suggested that differences in the binding affinity of HLA molecules to autoantigens might be involved (24). Nevertheless, the association between autoimmune

disorders and the polymorphisms of other genes involved in immune cell antigen recognition and activation like protein tyrosine phosphatase non-receptor type 22 (PTPN22), cytokines, chemokine receptors, costimulatory molecules, and inhibitory checkpoints were also identified (25).

The hallmark of autoimmunity is the presence of autoreactive T and B cells that were not deleted by the mechanisms of central tolerance (26). One of the most studied defects of T-cell-negative selection is mutations in the transcriptional autoimmune regulator gene (*AIRE*). *AIRE* is mainly expressed by the thymic medullary epithelial cells (mTECs) and is responsible for the expression of tissue-restricted antigens within the thymus. The T cells responding to these antigens are considered self-reactive and eliminated through negative selection. Thus, when *AIRE* is defective, the T cells specific to self-antigens leave the thymus and enter circulation. This results in a variety of autoimmune disorders (27, 28). The mouse models of *Aire* knockout showed that the *AIRE* expression prevents multiorgan lymphocyte infiltration, various organ-specific autoantibodies, and infertility (29). In humans, *AIRE* mutations lead to a severe condition called autoimmune polyendocrinopathy syndrome type 1 (APS1) (30, 31). In addition, it was observed that *AIRE* expression is regulated by sex hormones, leading to sexual dimorphism in autoimmune diseases (32, 33). For example, the castration of male animals led to a lower thymic expression of AIRE, while estrogen treatment resulted in the downregulation of AIRE in cultured human thymic epithelial cells (TECs). In addition, AIRE levels in the human thymus grafted into immunodeficient mice differed according to the sex of the recipient (32, 33). Therefore, AIRE has also been extensively studied in the context of reproductive system cancers. Kalra et al. reported that the AIRE expression in prostate cancer is responsible for resistance to anti-cancer therapy and increased invasiveness. $AIRE^{+}$ prostate cancer cells were shown to secrete increased levels of IL-6 and prostaglandin 2 (PGE2), which polarized the tumor-associated macrophage toward the M2 phenotype with an increased expression of CD206 and CD163 antigens. In addition, prostate cancer growth and lymphadenopathy after subcutaneous tumor engraftment were only observed in the $AIRE^{+/+}$ animal model. On the contrary, $AIRE^{-/-}$ mice showed small benign tumors (33).

The defects of the central tolerance mechanism of B cells, observed in a number of autoimmune diseases, result in the accumulation of autoreactive B cells in the periphery. The mutations of PTPN22, Bruton's tyrosine kinase (BTK), adenosine deaminase (ADA), impaired BCR light-chain rearrangements, and Toll-like receptor (TLR) alterations were observed to contribute to the increase in autoreactive B cells (34). Recently, PTPN22 also emerged as a potential target for cancer immunotherapy. It is not surprising as PTPN22 plays an inhibitory role in the antigen-specific responses of both T and B cells; dectin-1 signaling in DCs; the development and function of Tregs; the macrophage functions mediated *via* TLRs, NOD2, and NLRP3; and neutrophil adherence and mast cell activation in an IgE-dependent manner (35). Several single-nucleotide polymorphisms in the *PTPN22* gene were identified. The most extensively studied is a missense mutation at position 1858 (C3T), resulting in the substitution of an Arg (R) at position 620 to Trp (W). The generation of the Lyp620W variant (also identified as rs2476601) of the protein was found to impair the negative selection of autoreactive T and B cells during their development in the thymus and bone marrow, respectively, and the generation of self-reactive antibodies (36, 37). In consequence, the Lyp620W variant of PTPN22 was identified in multiple autoimmune diseases, including T1D, RA, systemic lupus erythematosus (SLE), Graves' disease, and myasthenia gravis (38–41). On the other hand, the same variant of PTPN22 was reported to augment antitumor responses and be associated with lower cancer incidence (35, 42). For example, the carriers of the *PTPN22(C1858T)* variant have a lower risk of non-melanoma skin cancer, while the homozygotes for the *PTPN22(C1858T)* have improved survival when treated with atezolizumab (anti-PDL1 antibody). These data underline again that immune tolerance is indispensable for preventing autoimmunity, but lowering the threshold of T-cell activation can improve tumor control and the efficacy of anti-cancer treatment.

Cancer immune evasion and autoimmunity prevalence can also be affected by sex hormones. Differences in the male and female endocrine systems lead to discrepancies in the quality and quantity of their immune responses. It was reported that while the female immune system provides better antimicrobial and anticancer responses, it is also more prone to autoimmune diseases (43). Estrogen levels are higher during pregnancy and are correlated with an increased proportion of Tregs in peripheral blood (44). Accordingly, the incidence of relapses of MS in pregnant women decreases significantly (45). Both innate and adaptive immune cells express estrogen receptors α and β (higher expression was observed in B cells than T cells, NK cells, and monocytes) that activate protolerogenic effects (46). Estrogens drive the polarization of T cells into Th2 and Treg cells; increase the production of IL-4, IL-10, and transforming growth factor-β (TGF-β); induce the expression of GATA-3, FoxP3, PD-1, and CTLA-4 (cytotoxic T-lymphocyte antigen 4) on T cells; and reduce the Tfh (T follicular helper cell) response (47, 48). On the other hand, SLE patients experience more flares during pregnancy (49). Interestingly, B-cell tolerance is regulated by estrogens at the maturation stage by engaging estrogen receptor α. Estradiol was shown to be responsible for decreased B-cell lymphopoiesis while expanding the population of splenic marginal-zone B cells through the increase of BAFF concentration (50). Estrogens were also shown to influence immune cells in the tumor microenvironment (TME). Certain mutations in the estrogen receptor result in an increase of tumor-infiltrating Tregs and T helper cells (51). It was also reported that estrogens influence tumor-associated macrophages, directing their polarization into the M2 phenotype and thus promoting their immunosuppressive activity (52, 53).

IMMUNE CHECKPOINTS

Immune checkpoints are inhibitory receptors that convey negative signals to immune cells, preventing autoimmunity (54). The importance of immune checkpoints in supporting

tolerance and preventing autoimmunity development is best observed in knockout mice models. For instance, the lack of CTLA-4, PD-1, BTLA (B- and T-lymphocyte attenuator), TIGIT (T-cell immunoreceptor with immunoglobulin and ITIM domain), and VISTA (V-domain Ig suppressor of T-cell activation) was shown to cause massive lymphoproliferation, an onset of autoimmune diseases, or fatal multiorgan tissue destruction (notably CTLA-4 deficiency) (55–61). In humans, several polymorphisms of immune checkpoint genes were identified and reported to be associated with susceptibility to autoimmune diseases (62–70).

CTLA-4 is a critical regulator of T-cell responses expressed by Tregs and activated conventional T cells. The main role of the receptor is to inhibit antigen presentation and the following activation of naive T cells by competitive binding to costimulatory receptors CD80 and CD86 on antigen-presenting cells (APCs) (71, 72). It was reported that CTLA-4 not only binds its ligands but also captures and removes them from APCs by a process of trans-endocytosis. In consequence, these costimulatory molecules are degraded inside CTLA-4-expressing cells resulting in a temporary lack of CD80/CD86 on APCs and thus impaired costimulation *via* CD28 (73). CTLA-4 is indispensable for preventing autoreactivity (74, 75). Its deficiency in humans is a common hallmark of primary immune deficiencies associated with immune dysregulation and prominent autoimmunity with highly variable features. The clinical symptoms probably result from the aberrant activation of polyclonal T cells. In addition, the deficiency of CTLA-4 results in increased CD28 co-stimulation that triggers self-reactive T cells against a variety of tissues. Treg dysfunction plays a vast role in the immune activation associated with CTLA-4 loss-of-function mutations (75). On the contrary, CTLA-4 expression on tumor cells was recognized as a prognostic factor of poor outcome in breast, pancreatic, and nasopharyngeal cancers (76–78). The application of therapeutic antibodies targeting CTLA-4 such as ipilimumab became a breakthrough in cancer therapy. Anti-CTLA-4 antibodies were shown to unlock the immune response to cancer, as well as lead to the depletion of tumor-infiltrating Tregs *via* antibody-dependent cell-mediated cytotoxicity. This way, anti-CTLA-4 demonstrated durable clinical activity in a subset of patients with solid malignancies including advanced melanoma (79–81).

Programmed cell death receptor 1 (PD-1) is another immune checkpoint significant for self-tolerance and the cessation of the immune response that became a target of cancer immunotherapy. Upon engagement by its ligand (PD-L1, Programmed cell death ligand 1), PD-1 acts as a brake to the immune system that induces the apoptosis of activated T cells (82). PD-L1 expression can be detected in pancreatic islets, vascular endothelial cells, and placenta where it is responsible for tissue protection from autoimmune responses (83). For example, in T1D, PD-L1 was observed to be upregulated in insulin-producing beta cells under an autoimmune attack and correlated with the intensity of $CD8^+$ T-cell infiltration in the pancreas (84, 85). In addition, PD-1/PD-L1 interaction was reported to be involved in the generation of inducible Tregs (iTregs). Francisco et al. showed that PD-L1-negative APCs had an impaired ability to generate Tregs, either *in vitro* or *in vivo* (86). The failure of APCs isolated from SLE patients to upregulate PD-L1 expression validates these findings in humans (87). The blockade of PD-1 or PD-L1 in experimental models of autoimmunity led to disease onset and exacerbation (88, 89), indicating the essential role of these immune checkpoints in tolerance and, specifically, in Treg maintenance. Recent reports on autoimmune-related adverse events in oncologic patients treated with PD-1/PD-L1 axis blockers support these findings (90, 91).

In cancer, effector T cells, which are persistently exposed to antigen stimulation in TME, express PD-1 at high levels, in the long term, causing T-cell functional exhaustion. It results in the inability of T cells to eliminate tumor cells and facilitates cancer progression (34, 92). Additionally, cancer cells actively exploit PD-L1 to evade the immune system and hijack the immunosurveillance mechanisms with PD-L1 expression (93). Moreover, the results presented by Chen et al. (2018) revealed that apart from cell surface expression, PD-L1 was present in extracellular vesicles (exosomes) produced by melanoma cells, suggesting its systematic immunosuppressive impact (94). As a result, it leads to the transcriptomic changes and the exhaustion of $CD4^+$ (95) and $CD8^+$ (96) T cells that are unable to eliminate cancer cells effectively. In a vast number of cancers, lymphocyte infiltration is in positive correlation with PD-L1 expression, which is simply an adaptive mechanism of the tumor to escape an immune response. Even though tumor PD-L1 expression usually suggests poor prognosis, then higher levels of tumor PD-L1 expression correlate with a better efficiency of immunotherapy (97).

Another molecule involved in central and peripheral tolerance is Fas. Fas/FasL ligation on TCR-stimulated lymphocytes restricts the overactivation of immune cells after an antigenic challenge, called activation-induced cell death (AICD). It is one of the main mechanisms in restoring immune homeostasis (98). The Fas/FasL-induced apoptosis of B cells was shown to be important in germinal center reactions (98). FasL can be expressed on non-immune cells in immune-privileged sites such as the eye, brain, and placenta, restricting the access of activated immune cells to these tissues (99). Alterations in Fas-mediated apoptosis were implicated in the pathogenesis of autoimmune diseases. Mutations in Fas/FasL axis-related genes lead to a striking lymphoproliferation with autoimmune cytopenias in humans termed autoimmune lymphoproliferative syndrome (ALPS) (100, 101). An interesting feature of ALPS is an accumulation of double-negative T cells that are terminally differentiated, with the markers of immune exhaustion (102). On the other hand, increased expression of FasL was observed in T1D (103), autoimmune thyroid diseases (104), and in MS (105, 106). An interesting feature of Fas/FasL signaling is the opposite outcome of ligation with membrane-bound versus soluble forms of these molecules where the soluble Fas and FasL do not induce apoptosis (107, 108). This discovery prompted studies investigating the levels of serum Fas/FasL molecules in autoimmune diseases, revealing elevated levels in SLE patients (107, 109) and Sjögren's syndrome (SS) (110). Excessive Fas signaling in the tumor microenvironment, majorly caused by high levels of the Fas ligand released by myeloid-derived suppressor cells (MDSCs), leads to the apoptosis of tumor-infiltrating lymphocytes (TILs) and was described as one of the core reasons for the failure of

cancer immunotherapy (111). In addition, FasL was reported to be expressed in numerous cancer types with a potential to induce the apoptosis of immune cells in the TME and was associated with poor prognosis. On the other hand, there is still controversy when it comes to the role of Fas/FasL axis in cancer cells. Several *in vitro* studies suggest that the ultimate effect may depend on the level of FasL expression by tumor cells. As elevated levels of FasL cause neutrophil-mediated inflammation that leads to tumor rejection, surprisingly low levels of FasL seemed to facilitate tumor growth. The Fas/FasL role in cancer is still not fully understood and brings a lot of controversies but surely requires further investigation as targeting Fas may significantly improve the efficiency of immunotherapy and tumor rejection (112, 113).

Other known immune checkpoints include BTLA, T-cell immunoglobulin and mucin domain-3 (TIM-3), and TIGIT (114, 115). In general, all were shown to inhibit the responses of activated T cells, while BTLA also demonstrated an impact on B cells (116). It was observed that patients with SLE and MS present a low expression of BTLA on B and T cells (117–119). Its decreased expression on naïve B cells was associated with increased IFN-γ and autoantibody levels in SLE patients that could suggest alterations in B-cell activation during the course of the disease (118). In conditions where Th17/Treg balance is shifted, the involvement of immune checkpoint signaling pathways was also implicated. A study by Wu et al. described a lower frequency of TIM-3 positive T cells together with increased IL-17 levels in patients suffering from autoimmune hepatitis, and experiments on mice confirmed that the blockade of TIM-3 signaling aggravated liver injury (120). TIGIT has been recently associated with Treg biology through the transcriptional profiling of these cells. It was suggested to be a marker of natural thymus-derived Tregs (tTregs) with strong suppressive activity and lineage stability (121). It competes with the CD226 molecule for binding a costimulatory poliovirus receptor (PVR) CD155 and inhibitory CD112 (Nectin-2) expressed on DCs (121). TIGIT-CD226 signaling in T cells was shown to be implicated in the pathogenesis of experimental autoimmune encephalomyelitis (EAE). CD226 knockout EAE mice showed favorable Th17/Treg proportion and increased TIGIT and CTLA-4 expression on Tregs (122). On the other hand, the lack of TIGIT resulted in increased levels of proinflammatory cytokines and hindered IL-10 production by T cells (61). Recently, a novel ligand for TIGIT was discovered on cancer cells. Nectin4 was reported to bind exclusively to the TIGIT molecule (123). TIGIT-Nectin4 interaction inhibits natural killer (NK) cell activity, which is a crucial element of the anti-cancer immune response. In addition, antibodies blocking Nectin4 induced enhancement of tumor killing in vitro and *in vivo* (123).

REGULATORY T CELLS

Central tolerance is crucial for the development of a small subset of intermediate-affinity, self-reactive T-cell clones that are rescued from deletion and become (tTregs) (124, 125). Apart from tTregs, Tregs can be induced on the periphery from naïve or effector T cells, becoming peripheral Tregs (pTregs). In addition, specific Treg subpopulations can be distinguished based on secreted cytokines, such as type 1 regulatory T cells (Tr1), T-helper type 3 cells (Th3), and IL-35-producing regulatory T cells (iTr35). They secrete IL-10, TGF-β, and IL-35, respectively (126–128). Functionally, follicular Tregs (Tfr) can also be distinguished within the FoxP3$^+$ population (129). Tfr cells have a TCR repertoire resembling tTregs and were shown to be able to control germinal center reactions and antibody production (130, 131).

Tregs exert their immune-suppressive effects using diverse mechanisms. The most important are (1) a high expression of immune checkpoint inhibitors; (2) infectious tolerance, where Tregs exert and transfer suppressive activity toward other immune cells when activated by autoantigens (132); (3) the secretion of anti-inflammatory cytokines (133), (4) IL-2 deprivation, and (5) adenosine accumulation *via* CD39 and CD73 activities (134). Apart from cytokines, extracellular vesicles are recently gaining attention as a way of efficient intercellular communication with a significant role in the regulation of the immune system (135, 136).

Tregs are crucial for preventing autoimmune reactions (**Figure 1**). They play an important role in immune tolerance maintenance, as their deficiency causes immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome, leading to multiorgan autoimmune damage when not treated (137, 138). Numerous studies described quantitative Treg changes in autoimmune diseases. A decrease in the Treg population was shown in juvenile idiopathic arthritis (139) and RA (140). However, in some diseases, such as systemic sclerosis (SSc), Tregs were shown to be increased (141). The results from SLE patients regarding Treg frequencies are conflicting, which may arise from differences in the analyzed phenotypes of Tregs (142). Numerous studies suggested the decreased immunosuppressive potential of Tregs in autoimmune diseases (143–147). The main limitation of studying Tregs in human organ-specific diseases is usually the lack of insight into the damaged tissue, as systemic and local immune responses may differ dramatically. Nevertheless, several studies pursued this problem. For instance, Marazuela et al. reported lower numbers of Tr1 and higher proportions of tTregs in the thyroid glands of patients with autoimmune thyroid disease (AITD) as compared with peripheral blood (145, 146). In patients with relapsing–remitting MS (RR-MS), higher frequencies of Tregs were present in cerebrospinal fluid (CSF) rather than in the peripheral blood. The same group of patients had decreased peripheral blood Treg levels compared to the patients with secondary-progressing MS and other neurological diseases, suggesting the migration of Tregs to the site of autoimmune inflammation (148). In addition, the primary role of tTregs, as opposed to pTregs, was demonstrated to control T1D development. However, the deficiency in pTregs increased the incidence of insulitis (149). In the synovial fluid of arthritis patients, high frequencies of iTregs and tTregs were present; however, tTregs presented an unstable FoxP3 expression. Moreover, FoxP3$^-$ Tregs were converted to IL-17-producing cells under the environment of the inflamed joint (150, 151). The Th17 cytokine profile (IL-17, IL-12, IFN-γ) influences the organ tissue environment, causing chronic inflammation and, ultimately, organ failure (152). Considering the close transcriptional programs of

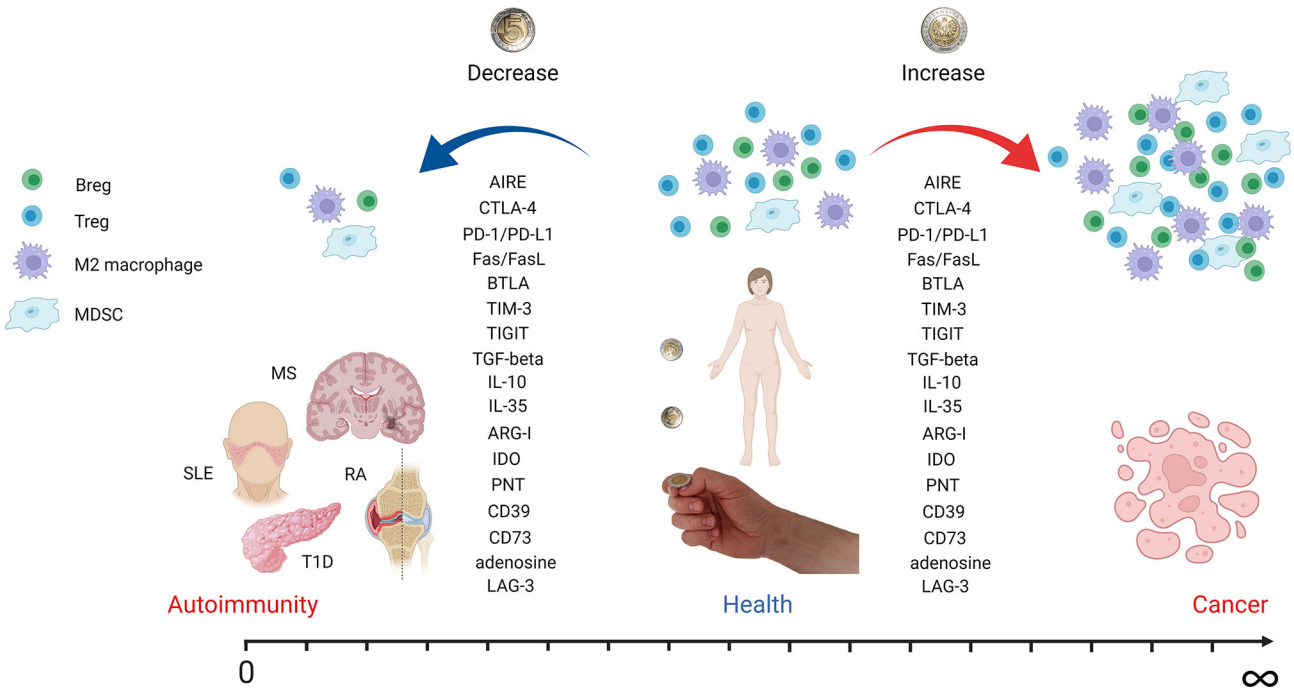

FIGURE 1 | Autoimmunity and cancer as two sides of the same coin. The figure depicts how tuning of immune system regulatory mechanisms can contribute to autoimmunity, health, or cancer development. A decrease in regulatory cell populations like Tregs, Bregs, M2 macrophages, and MDSCs leads to autoimmune disease onset. However, an increase in the same cell subsets is associated with cancer development and progression. Effector molecules involved in immune tolerance induction are downregulated in autoimmunity but overexpressed in cancer. The most important molecules mentioned in the text are listed. AIRE, autoimmune regulator; CTLA-4, cytotoxic T-lymphocyte antigen 4; PD-1, programmed cell death receptor 1; PD-L1, programmed cell death ligand 1; BTLA, B- and T-lymphocyte attenuator; TIM-3, T-cell immunoreceptor with immunoglobulin and ITIM domain; TIGIT, T-cell immunoglobulin and ITIM domain; TGF-beta, transforming growth factor beta; IL, interleukin; ARG-I, arginase I; IDO, indoleamine-pyrrole 2,3-dioxygenase; PNT, peroxynitrite; LAG-3, lymphocyte-activation gene 3 (figure created with BioRender.com).

Th17 and Tregs, both depending on TGF-β, Tregs in the presence of IL-6 were shown to be converted into Th17 cells, or IL-17+ ex-regulatory T cells (exTregs). This plasticity of Tregs results in the blunting of suppressive capacity and the secretion of proinflammatory IL-17 and IFN-γ (153–155). On the other hand, cytokines IL-10 and TGF-β enable the differentiation of immune cells into anti-inflammatory Tregs, Bregs, tolDCs, and M2 macrophages (155).

Indisputably, within the TME, Tregs are present in high frequencies. Treg presence is accommodated by the immunosuppressive cytokine milieu at the site as well as the chemotactic factors produced in TME. High numbers of FoxP3$^+$-expressing Tregs infiltrating TME in lung, breast, and pancreatic cancers were associated with poor prognosis (156). Tregs express various chemokine receptors, like CCR4 and CCR5, that allow migration to TME more efficiently (157, 158). TME is rich in TGF-β and promotes the differentiation of conventional CD4$^+$ T cells into pTregs (159). Resting Tregs are not immunosuppressive unless they become activated through TCR engagement and signaling molecules. The Tregs found in TME are, however, highly activated and immunosuppressive, characterized by upregulated levels of the master regulatory transcription factor FoxP3 (160). This subsequently leads to the suppression of CD8$^+$ T cells, NK cells, NKT cells, and M1 macrophages and the maturation of DCs through IL-10, TGF-β, and indoleamine-pyrrole 2,3-dioxygenase (IDO) (161–163). In addition, Tregs not only bind IL-2 competitively to conventional T cells but also release soluble CD25 (IL-2R subunit) that eliminates IL-2 and alters cytotoxic T-cell functions. Tregs in TME may also release IL-35 that increases the expression of inhibitory receptors like PD1, TIM-3, and lymphocyte-activation gene 3 (LAG-3). This leads to the exhaustion of TILs (164–166). Interestingly, Treg elimination that was followed by cancer antigen vaccination generated effective anti-tumor CD4$^+$ and CD8$^+$ T-cell responses in cancer patients with advanced malignancies (167). However, as mentioned before, systemic Treg depletion would lead to severe autoimmune disorders, emphasizing the need for more selective methods that would specifically target intratumoral Tregs.

REGULATORY B CELLS

B-cell maturation mechanisms require consecutive checkpoints to develop tolerance: clonal deletion, receptor editing, and anergy. Immature B cells transmitting an overly strong signal

through the B-cell receptor (BCR) in response to self-antigen undergo clonal deletion. A tolerance mechanism unique to B cells is the possibility of repeated immunoglobulin light-chain gene recombination. Such rearrangements lead to alterations in BCR specificity to ideally avoid the formation of self-reactive B-cell clones (168, 169). The subsets of B cells expressing PD-1 (170), TIM-3 (171), and BTLA (117) were described as Bregs, an important element for the maintenance of peripheral tolerance (**Figure 1**). However, a consensus regarding the definition and detailed phenotype of Bregs has not yet been reached. The distinct methods for identification in various disease models and different tissues complicate the general classification. IL-10, TGF-β, and IL-35 have been identified as the main suppressive cytokines produced by Bregs; thus, some authors used to classify the cells into IL-10^+, TGF-β^+, and IL-35^+ Bregs (172). Among IL-10^+ human Bregs, the following phenotypes of Bregs were reported: $CD1d^{hi}$ $CD5^+$ (173), $CD5^+$ (174), $CD24^{hi}CD27^+$ (175), $CD24^{hi}CD38^{hi}$ (176–178), $CD25^+CD71^+CD73^-$, and $CD25^+CD71^+CD73^{low}$PD-L1$^+$ (179), $CD154^+$ (180), $CD5^{hi}CD38^{low}$PD-1^{hi} (181), $CD27^{int}CD38^+$ (182). Up to now, 2 subsets of TGF-β^+ Bregs have been identified in humans: $CD25^{hi}CD27^{hi}CD86^{hi}$ $CD1d^{hi}$ (183) and $CD24^{hi}CD38^{hi}$ (178). Despite the fact that IL35^+ B cells have been identified in humans, up to now, specific surface markers have not been reported for these cells in men (172, 184). The manipulation of the Breg compartment through the adoptive transfer of isolated or *ex vivo*-induced cells was explored in the murine models of autoimmune diseases. For example, IL-10^+ Bregs were shown to suppress inflammation in the mice models of RA, EAE, and SLE. The most prominent therapeutic effects were observed when Bregs were administered early in the disease course (183, 185–188). The mechanisms used by Bregs have not been studied extensively. Nevertheless, *in vitro* studies performed by Kessel et al. resulted in several interesting observations. Human Bregs defined as $CD25^{high}$ $CD27^{high}$ $CD86^{high}$ $CD1d^{high}$ IL-10^{high} TGF-β^{high} cells were shown to significantly decrease the proliferation of autologous conventional $CD4^+$ T cells in a dose-dependent manner. In addition, Bregs were found to upregulate FoxP3 and CTLA-4 expression in Tregs in cell-to-cell-dependent contact. The effect was even stronger when Bregs were pretreated with a TLR-9 agonist (oligodeoxynucleotide) and CD40L (183). The other groups also reported the suppressive effects of Bregs on DC and macrophage cytokine production and antigen presentation (175, 189). Increased frequencies of IL-10^+ B cells and their progenitors were found in patients with various autoimmune diseases, such as SLE, RA, SS, autoimmune vesiculobullous skin disease, and MS. However, the significance of Bregs in the pathogenesis of human autoimmune diseases is yet to be determined (175).

The impact of B cells in cancer is still unclear and ambiguous as they were shown to play a role in both cancer promotion and anti-cancer responses (190). Significant B-cell infiltration was found in breast cancer, non-small cell lung cancer (NSCLC), ovarian cancer, melanoma, and renal cell carcinoma. Bregs have been also identified in a number of cancers including lung (191), gastric (192), and breast cancers (193). Increased infiltration with Bregs results in the inhibition of effector T-cell responses and their impaired proliferation. It was suggested that the tumor and TME can direct tumor-infiltrating B cells into tumor-induced Bregs (tBregs) (194) by the direct tumor cell: B-cell contact (195). Lindner et al. reported that tumor-infiltrating Bregs use Granzyme B for the degradation of the CD3 ζ-chain in $CD4^+$ T cells. The phenomenon results in a limited proliferation of the target $CD4^+$ T cells (196). Interestingly, tBregs were also shown to play a substantial role in the education of MDSCs, enhancing cancer-induced immune suppression (197). In addition, Breg-derived IL-10 leads to the conversion of conventional B cells into Bregs and contributes to Treg expansion (183). tBregs were also found to direct conventional $CD4^+$ T cells into Tregs in breast and gastric cancers (177, 198). Another study utilizing a mouse model showed that tumor-educated Bregs suppress not only the proliferation of helper and cytotoxic T cells but also the secretion of Th-1 cytokines and the expansion of NK cells in a TGF-β- or PD-L1-dependent manner (195). A similar immunosuppressive activity was reported for IL-35^+ Bregs. Breg-derived IL-35 was shown to stimulate cancer (199), as well as convert both T and B cells into Tregs and Bregs, respectively. Several surface molecules have been identified to be involved in direct cell-to-cell interactions between Bregs and the target immune cells, like Bregs CD40/CD40L, CTLA-4/CD80 and CD86, PD-L1/PD-1, or Fas/FasL (200–203).

MYELOID-DERIVED SUPPRESSOR CELLS

A significant population of cells identified within the tumor was described as activated immature myeloid cells with immunosuppressive function, termed myeloid-derived suppressor cells (MDSCs). These cells, in general, can be divided into 2 populations: mononuclear (M-MDSCs; $CD11b^+Ly6G^-Ly6C^{hi}$) and polymorphonuclear/granulocytic MDSCs (PMN-MDSCs; $CD11b^+Ly6G^{+/hi}Ly6C^{low/int}$) (204). The granulocyte monocyte-colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), stem cell factor (SCF), prostaglandins, TNF-α, IFN-γ, and IL-18 were shown to promote the differentiation of functional MDSCs that contributed to the establishment of immunosuppressive niche and tumor progression (205–209). MDSCs were shown to be engaged in the suppression of TIL activity, EMT, and angiogenesis and participate in establishing a pre-metastatic niche (210, 211). In addition, the increased production of nitric oxide (NO) by MDSCs resulting from inducible nitric oxide synthase (iNOS) overexpression was reported to be responsible for T-cell apoptosis and proliferation suppression, as well as the inhibition of antigen presentation by DCs (212, 213). Moreover, MDSCs isolated from tumor-bearing animals showed significantly higher levels of reactive oxygen species (ROS) than the cells isolated from healthy controls. Further studies demonstrated that ROS are crucial for the MDSC suppression of T-cell proliferation, survival, and TCR signaling (214–216). It was also reported that MDSCs express elevated levels of arginase I (ARG-I; **Figure 1**). This way, they can deplete

TME from indispensable amino acids, such as L-arginine or cysteine affecting T-cell activation and proliferation (217, 218). One of the mechanisms that stands behind this T-cell suppression is the downregulation of the CD3 ζ-chain of the TCR complex (219). Tumor-derived MDSCs are also a potent source of IDO, an L-tryptophan-degrading enzyme that induces the suppression of T-cell proliferation and survival, as well as promotes Treg induction (220–222). Another important effector molecule used by MDSCs is peroxynitrite (PNT). The production of PNT in TME was shown to nitrate the TCR complex, leading to the unresponsiveness of tumor-infiltrating cytotoxic T lymphocytes to the specific antigens presented by MDSCs (223–225).

It is also recognized that MDSCs participate in the generation of immunosuppressive adenosine (226, 227). MDSCs express the ectoenzymes triphosphate diphosphohydrolase 1 (NTPDase 1/CD39) and ecto-5'-nucleotidase (5'-NT/CD73). The first ectoenzyme is responsible for the hydrolysis of extracellular ATP or ADP into AMP, which is then degraded by CD73 into adenosine. Adenosine is known to inhibit the activation and effector function of T cells, mainly by A2A and A3 adenosine receptors (228). However, these receptors can also be found at the surface of MDSCs. The blockade of the A2B receptor was shown to reduce the secretion of IL-10 and monocyte chemoattractant protein 1 (MCP-1) by MDSCs in mice with melanoma (229). Aside from IL-10, TGF-β is another cytokine important for MDSC function. MDSC-derived IL-10 and TGF-β promote the differentiation of T cells into Tregs and suppress T- and NK-cell activation as well as DC function (230, 231). TGF-β was reported to induce EMT in cancer cells (211, 232), generate pro-tumorigenic M2 macrophages (233), and drive pro-tumorigenic neutrophil polarization (234). In NSCLC, higher levels of TGF-β were associated with an increased expression of inhibitory molecules such as CTLA-4 and TIM3 on cancer cells (235). It was suggested that MDSCs are responsible for the induction and recruitment of the Treg population in the TME. While the process of Treg induction is not fully elucidated and was suggested to depend on cytokine milieu and cell-to-cell contact, the Treg recruitment was shown to be largely dependent on the production of CCL2 and CCL5 chemokines (236, 237). On the other hand, MDSCs may also limit the T-cell infiltration of the tumor by metalloproteinase 17 (ADAM17), which cleaves L-selectin (CD62L) present on the surface of naïve T cells. In consequence, T cells are not able to infiltrate tumor or enter peripheral lymph nodes (238).

The growing body of research on MDSCs and their suppressive capacity in TME sparked interest for the exploration of their role and potential therapeutic use in autoimmune diseases. In the aim to diminish the heterogeneity of studied MDSCs, they used to be divided into M-MDSC ($CD11b^+Ly6G^-Ly6C^{hi}$) and PMN-MDSC ($CD11b^+Ly6G^{+/hi}Ly6C^{low/int}$) subsets as in cancer studies (239, 240). Multiple studies on the animal model of MS have pointed to the beneficial role of MDSCs in autoimmunity. Moliné-Velázquez et al. identified ARG-I positive MDSCs in the spinal cord during the course of EAE. The cells showed tropism to demyelinated areas in CNS. The density of ARG-I^+ MDSC infiltrate, as well as the local proportion of the apoptotic T cells, correlated with the disease course and clinical state. They peaked in parallel with the clinical score, which were decreased significantly during remission, and was not detectable in the chronic phase (240). These data correspond with the previous studies that reported the presence of ARG-I^+ cells exclusively when the switch from proinflammatory to anti-inflammatory conditions occurred and the active phase was about to end (241–243). These data indicate that MDSCs are involved in limiting inflammatory damage in MS and contribute to relative recovery in the remitting phase of the disease.

In humans, as in previously described animal studies, the numbers of MDSCs were found to be an indicator of the disease phase. For example, RR-MS was characterized by significantly higher levels of the PMN-MDSC subset in the peripheral blood at relapse than in the remission period or in healthy individuals. Experiments *in vitro* revealed that PMN-MDSCs from patients with RR-MS suppress autologous T-cell proliferation, suggesting their beneficial role for remission induction (244).

However, higher proportions of M-MDSCs were observed to be positively correlated with proinflammatory Th17 and Th1 cells, as well as with a worsened metabolic profile in the patients with T1D and their relatives at elevated risk for the disease (245). Similar patterns were described in RA (246) and SLE (247). These data indicate that a detailed characterization of MDSC subsets and their further stratification is inevitable if MDSCs are planned to be harnessed to stop autoimmune diseases. Nonetheless, the idea of utilizing the suppressive activity of MDSCs in therapy prompted the experiments of adoptive transfer of MDSCs to diabetes-prone mice that successfully prevented the onset of autoimmune diabetes and established tolerance to self-antigens *via* Treg induction (248).

MACROPHAGES

Macrophages can be divided into two main groups, classically activated, proinflammatory macrophages (M1) and alternatively activated macrophages (M2) with anti-inflammatory and regenerative properties. M1 and M2 cells can be distinguished by secreted cytokines, for example, INF-γ, IL-1, IL-6, IL-12 and IL-10, and TGF-β, respectively. However, macrophages exhibit exceptional plasticity depending on the microenvironment (249). It has been reported that tumor-associated macrophages (TAMs) are recruited to TME by chemokines, such as CCL2 in different tumors, including glioblastoma and breast and lung cancers (250–252). Moreover, TAMs start to produce CCL2 and thus recruit more macrophages and stimulate their polarization toward a pro-tumoral M2 phenotype (253–256). Targeting TAMs in pancreatic ductal adenocarcinoma by inhibiting CCR2 has shown a therapeutic benefit by restoring anti-tumor immunity in preclinical models (257). Although TAMs can produce IL-8, a chemotactic factor for T cells, high levels of IL-8 in plasma, peripheral mononuclear cells, and TAMs were negatively correlated with clinical prognosis regardless of high $CD8^+$ T-cell infiltration in the tumor (258). TAM-derived cytokines include IL-6, IL-10, and TGF-β.

IL-6 combined with IL-6R can activate anti-apoptotic pathways in tumor cells and prolong their survival (259). A meta-analysis revealed that the serum levels of IL-10 are positively correlated with tumor progression, showing the importance of TAMs in the promotion of tumor development (**Figure 1**) (260). Additionally, TAMs secrete inflammatory mediators, including prostaglandin E2 (PGE2) and matrix metalloproteinase-7 (MMP-7). These molecules interfered with TLR-mediated or IFN-γ-mediated DC and macrophage activation. In addition, a direct induction of genes that suppress APC function was observed. Thus, TAMs indirectly impair the T-cell recognition of tumor antigens (261).

Macrophages are constantly present in peripheral tissues, where they can rapidly act as APC, as shown in the T1D animal model (262). In autoimmune diseases, the overreaction of the immune system and the resulting highly proinflammatory environment lead to tissue damage. Therefore, the imbalance in M1/M2 macrophage subsets was observed in several autoimmune diseases, both organ specific (MS) (263) and systemic with in-tissue manifestations (RA, SLE, SSc; **Figure 1**) (264). Recent studies on human pancreata from T1D patients, using multiparametric analyses, revealed the presence of macrophages of mixed M1/M2 characteristics, confirming the high plasticity of these cells (265, 266). Studies on EAE showed that the polarization of macrophages follows the natural pattern of the disease with the increase of M2 macrophages during the remission phase (263). The adoptive transfer of M2 macrophages in the mouse model of SLE decreased the disease severity score (267) and prevented diabetes in NOD mice (268). Importantly, these transferred cells were homed to the site of ongoing insulitis (268). These results suggest an attractive therapeutic opportunity.

FIBROBLASTS

TME contains a special subpopulation of fibroblasts with a myofibroblastic phenotype. Cancer-associated fibroblasts (CAFs) are activated, but unlike in a physiological wound-healing process and tissue repair, they remain constantly activated, leading to pathological fibrosis. Active fibroblasts and myofibroblasts are the main effectors involved in the initiation of fibrosis due to excessive collagen deposition and the modulation of extracellular matrix (ECM) (269, 270). Multiple mechanisms can be involved in their activation, like the composition of the (ECM), DNA damage, physiological stress (mediated by ROS), inflammatory signals (e.g., IL-1 and IL-6), and growth factors, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) (271–273). Once activated, they are sufficient not only to promote tumor growth but also to further model ECM; produce proinflammatory cytokines, proangiogenic VEGF, and the chemokine ligand CXCL12 that is responsible for attracting immunosuppressive cells into TME that indirectly assist in immune tolerance establishment" as this part of the sentence is continuation of the role of CXCL12 (274). It was reported that throughout the secretion of TGF-β, CAFs induce the occurrence of EMT and promote lung metastasis in breast cancer (275). Moreover, the cytokine is involved in the synthesis of collagen and matrix modification by macrophages and fibroblasts, leading to local tissue scarification, like pulmonary fibrosis (276). Tissue fibrosis and the contractile properties of myofibroblasts stiffen ECM subsequently, lowering blood circulation and leading to local tissue hypoxia (277). These effects also reduce the possibility of cytotoxic effectors to reach cancer cells, therefore reducing immune surveillance and therapy efficacy. While using the combinations of multiple biomarkers to help identify cell subsets in TME, it has been found that the presence of CAFs is negatively correlated with the prognosis in patients receiving PD-1 immunotherapy in metastatic melanoma (266). This shows that the combination of different biomarkers can not only help us target CAFs as a potential clinical marker for the success of therapy, but targeting CAFs can also improve the efficacy of immunotherapy. Inhibiting the growth and proliferation of CAFs and preventing or reversing their activation status are potential ways to target CAFs in cancer therapy.

The therapeutic application of fibroblasts in autoimmune diseases has not been extensively studied. Jalili et al. reported tolerance induction by fibroblasts in the animal model of T1D and pancreatic islet transplantation. However, the therapeutic fibroblasts were transduced with a lentiviral vector carrying IDO cDNA. Thus, the cells artificially overexpressed IDO and efficiently suppressed immune responses (278, 279) Nevertheless, the data of Khosravi-Maharlooei et al. suggest the potential therapeutic use of fibroblasts in autoimmune diseases. They showed that fibroblasts can condition DCs to express higher levels of co-inhibitory molecules and anti-inflammatory cytokines. In addition, fibroblasts arrested the ability of DCs to induce the proliferation of T cells in both direct and indirect pathways. Fibroblast-primed DCs were also reported to migrate to the regional lymph nodes and present fibroblast-derived antigens. This study sheds light on the role of fibroblasts in the maintenance of self-tolerance and regulation of immune responses (280). Finally, the data provide inspiration for the future therapeutic approaches.

EPITHELIAL-TO-MESENCHYMAL TRANSITION IN CANCER AND AUTOIMMUNE DISORDERS

Another complex phenomenon modulating immunity is EMT, which induces morphological changes in epithelial cells, after which, they start to resemble mesenchymal cells—fibroblasts (281–284). As a result, cells undergoing EMT show increased motility and invasiveness due to the degradation of extracellular matrix, but it can also acquire other features, like stem cell properties or the ability to escape the immune system, which overall contributes to the aggressive phenotype of cancers (281, 285, 286). A direct connection between immunotolerance and EMT was shown in breast and lung cancer *in vitro* studies, where upon EMT induction, the expression of PD-L1 in cancer cells increased (287, 288). Moreover, cells with a mesenchymal phenotype showed higher levels of PD-L1 than cells of epithelial phenotype (288). Hypoxic hepatoma cells, which undergo EMT, induce IDO

expression in monocyte-derived macrophages and further suppress the proliferation of T cells as well as promote the expansion of Tregs (289). Pancreatic tumors with EMT features co-express PD-L1, and melanoma cells with EMT features show increased NK immunosuppressive function in comparison to epithelial melanomas (290), which overall indicates that EMT in cancer cells leads to a decreased immune response. On the other hand, the EMT inducers present in the tumor microenvironment can modify the activity and composition of the immune cells in the tumor niche. TGF-β, a potent inducer of EMT in multiple cancers, including breast (291, 292), lung (235, 293, 294), and colon (295, 296) cancers, exerts immunosuppressive function (235). In lung adenocarcinoma, the EMT signature of the tumor was associated with increased infiltration by $CD4^+$ $FoxP3^+$ Tregs (297), a decreased infiltration of activated effector T cells (including Th17 cells), and higher levels of activated B cells and γδ T-cells (235). Similarly, in patients with pancreatic ductal adenocarcinoma, tumors with mesenchymal features have decreased the number of $CD8^+$ T cells and increased the frequencies of Tregs (298).

EMT develops in response to chronic inflammation where it can lead to pathological fibrosis-the generation of myofibroblasts, which actively deposit ECM, leading to a decreased functionality of the affected organs (299–301). The triggers for EMT and fibrosis are overlapping; most importantly, both require TGF-β (302, 303). Chronic inflammation in autoimmune disorders such as RA, Crohn's disease, SLE, or scleroderma have been associated with fibrotic tissue remodeling (300, 304, 305). The local proinflammatory environment is not neutral for tissue-resident mesenchymal cells/fibroblasts that become activated and, as ECM-producing cells, exacerbate fibrosis. Signaling through the proinflammatory IL-17A receptor was responsible for fibroblast activation and the fibrosis of lung tissue in RA-associated lung disease and idiopathic pulmonary fibrosis (306). It seems that during chronic inflammation, overridden tolerance mechanisms interfere in the natural process of healing and repair mediated by fibroblasts, which can additionally support inflammation.

RNA EDITING

One of the mechanisms used by the innate immune response for self- vs. non-self-recognition is the RNA-editing process. There are two main types of RNA editing: (i) adenosine-to-inosine (A-to-I) conversion catalyzed by adenosine deaminases acting on RNA (ADAR) enzymes and (ii) cytidine to uridine (C-to-U) deamination by apolipoprotein B mRNA-editing catalytic polypeptide-like (APOBEC) family. A-to-I RNA editing allows cells to mark the host RNA as self. This way, the cell is able to recognize and tolerate edited self-RNAs with viral dsRNA sensors (such as PKR, MDA5, and RIG-I) and simultaneously discriminate non-edited dsRNAs present in the cells as viral genetic material (307). This launches an innate immune response, and results in death of cells where non-edited dsRNA was detected. Defects in RNA editing may contribute to autoimmune diseases and are observed in various cancers (308, 309).

The role of RNA editing and the enzymes involved in this process in cancer are currently being explored (308). Potentially, RNA editing may lead to presentation of edited and thus changed peptides by the MHC class I molecules of malignant cells. This phenomenon was recently shown in melanoma, where TILs were able to recognize the peptides derived from the ADAR1-edited form of cyclin I (CCNI) presented on the surface of cancer cells (310). These findings suggest that either the absence of or a higher expression of ADAR1 can result in novel ADAR1-dependent neoantigens that may be used as biomarkers in cancer or as potential targets for cancer immunotherapy. The study of Asaoka et al. supports this hypothesis. The increase of APOBEC3-mediated RNA editing in breast cancer was correlated with a higher T-cell infiltration of the tumor, improved survival, and better prognosis (311). The role of RNA editing in immune regulation is also proven by the fact that the expression of some RNA-editing enzymes is dependent on IFN (312). The knockdown of *Adar1* in mouse B16 melanoma cells was shown to increase the susceptibility of the tumor cells to anti-PD1 therapy after engraftment to animal model (313). Interestingly, *Adar1* knockout does not disturb growth of B16 cells in culture but mediates killing of B16 $Adar1^{-/-}$ cells by T lymphocytes *in vivo*. This effect is determined by abnormal activation of the intracellular dsRNA sensors (Mda5 and PKR) by unedited intracellular dsRNA mimicking virus infection (314). In contrary to B16 mouse melanoma cells, in many human cancer cell lines, loss of *ADAR1* results in cell death, even in the absence of innate immune cells. These ADAR1-dependent tumors usually show high IFN induction, probably through the innate immune DNA sensor STING (315) and have a higher expression of both: IFN-stimulated genes (ISGs, including *ADAR1*) and innate immune sensors for dsRNA, than other types of tumor cells. In addition, they are sensitive to elevated levels of dsRNAs while *ADAR1* knockdown is lethal for these cells through the Mda5/MAVS and PKR pathways (315).

RNA editing is also involved in autoimmune diseases connected to the dysregulation of IFN signaling. For instance, mutations in the *ADAR1* gene were identified to be involved in the development of type I interferonopathies, including Aicardi–Goutieres syndrome (316), dyschromatosis symmetrica hereditiaria (317), bilateral striatal necrosis (318), and spastic paraplegia (319). ADAR1 expression was shown to be also involved in RA or SLE (320, 321). The enzyme was over-expressed in synovium of RA patients regardless of the disease duration. In addition, the ADAR1p150 isoform was found to be elevated in the blood of the patients with active RA. Interestingly, decreased baseline ADAR1p150 expression and the individual adenosine RNA editing rate of cathepsin S $AluSx^+$ in RA were indicators of a good clinical response to the treatment (320).

DISCUSSION

Immune response and tolerance are vital for proper reaction against pathogens and maintaining internal homeostasis. For years, immunologists have been studying the mechanisms' underlying tolerance to fight autoimmune diseases. However, a

deeper understanding of immune tolerance in TME as well as the mechanisms underlying autoimmunity may help to generate an antitumor response and break tolerance to cancer. Phenomena, such as the generation of tolerogenic immune cell populations or EMT, are revealing pathways that lead to immunological changes in the tumor milieu. Anti-cancer immunotherapies should attempt to break immune tolerance toward the tumor; otherwise, the efficacy of such treatments is greatly limited. On the other hand, the immunotherapies aiming to combat autoimmune diseases seek to induce immunological tolerance, therefore, to limit the pathological immune reaction against self-antigens. As potent tolerance to cancer and the lack of self-tolerance in autoimmune diseases stand on two sides of the same coin (**Figure 1**; **Table 1**), certain lessons can be learned from the understanding of these two fields of medicine. We believe that combining knowledge from research on autoimmune diseases and cancer therapies will lead to a considerable progress in both areas. The advantages of exchanging knowledge between these two research fields can already be observed in the therapeutic strategies that are being developed. For instance, while genetically engineered super-activated CAR T cells have been successfully applied for the therapy of non-solid malignancies (322), the depletion of autoreactive immune cells gives promising results in the treatment of autoimmune diseases (323, 324). Moreover, the therapeutic potential of CAR Tregs is being explored in the context of autoimmunity (325), as antigen-specific Tregs proved to have better control over autoreactive effector cells than polyclonal Tregs (326). The strategy has already proved its efficacy in the animal models of MS (327), colitis (328), and T1D (329). Another example of a similar therapeutic approach in cancer and autoimmune diseases are adoptive cellular therapies, such as those that use mature DCs in cancer and tolDCs in autoimmune diseases. In cancer research, DCs loaded with tumor antigens are used as a cancer vaccine (330). In the therapy of autoimmune diseases, tolDCs presenting synovial fluid-derived peptides have been recently tested in a phase I clinical trial in RA patients (331). Many of the immune regulatory axes can be targeted in both autoimmune diseases and cancer, usually in an opposite manner—targeting different cytokines (including IL-2, IL-6, IL-10, IL-15, IL-17, and TNF-α) to manipulate the tolerance and increasing or decreasing the regulatory populations of the cells. As presented in this review, cytokine imbalance is a vital component of TME or autoimmune disorders that creates an opportunity for therapeutic intervention. On the other hand, therapies depleting or promoting the expansion of effector subsets of immune cells are also valid therapeutical strategies, for example, the depletion of effector cells in autoimmune diseases and adoptive cell therapy in cancer patients (322).

Immune checkpoint inhibitors were found to be a milestone in cancer therapy. Ipilimumab, the first immune checkpoint-blocking antibody targeting CTLA-4, was approved by the FDA. It was used for the first time in 2002 and later approved in 2011 for treating unresectable melanoma (332). PD-1-inhibiting antibodies have been also successfully used for the treatment of multiple cancer types as they are at least partially able to reinvigorate exhausted T-cells that regain the cytotoxicity against the cancer (333). Mechanistically, PD-1 signaling acts as a brake to the immune system but it can be stopped by implementation of either PD-1 or PD-L1 blocking monoclonal antibodies that are able to directly inactivate the PD-L1 inhibitory signaling in TME, reverse T-cell exhaustion, and ultimately induce tumor regression (334, 335). Nivolumab, pembrolizumab, and cemiplimab are FDA-approved PD-1-blocking antibodies for the treatment of various cancers including melanoma, renal cell carcinoma, NSCLC, and squamous cell carcinoma. However, many other indications are waiting for the approval (336). When it comes to PD-L1 inhibitors, currently, FDA has approved the following three: atezolizumab, durvalumab, and avelumab (337). At the same time, immune checkpoint fusion proteins are arising as a tool in the treatment of autoimmune diseases. The first promising results of exploiting the inhibitory activity of CTLA-4 in animal models of autoimmune diseases were presented over 25 years ago (338, 339). Successful clinical trials in human patients with psoriasis vulgaris, RA, and juvenile idiopathic arthritis led to the FDA approval of abatacept in 2005 (340–342). CTLA-4Ig is also tested in MS (343) and T1D (344, 345); however, these organ-specific diseases were far less responsive to this therapeutic agent. Experimental studies revealed that CTLA-4Ig induced the suppression of tolDCs (346) and Treg differentiation (347), improved the Treg function (348), and decreased the numbers of Th2 cells (349). The fusion proteins of PD-1 also convey immunomodulatory properties (350). Consequently, other immune checkpoint fusion proteins or agonistic antibodies, such

TABLE 1 | Mechanisms involved in breaking tolerance to self-tissues and in induction of cancer tolerance.

	Autoimmunity	Cancer-induced tolerance
General tolerance mechanism	Escape from central tolerance and impaired peripheral tolerance	Escape from immune recognition and induction of peripheral tolerance
Subsets of regulatory cells	↓ Function and/or quantity of tTregs and pTregs ↑ MDSCs during active disease	↑ Tregs, induction of pTregs and Bregs in tumor microenvironment ↑ Suppressive activity of MDSCs in tumor microenvironment
Activity of cells	↓ Migration of regulatory cells ↓ Immune checkpoint expression by immune cells	↑ Migration of regulatory cells ↑ Immune checkpoint expression by immune cells and tumor cells
Cytokines	↑ Proinflammatory cytokines	↑ Immunosuppressive cytokines
Chronic effects on immune cells	Differentiation of Tregs into inflammatory IL-17^{+} exTregs	Exhaustion of TILs

MDSC, myeloid-derived suppressor cell; Tregs, regulatory T cells; pTregs, peripheral Tregs; TIL, tumor-infiltrating lymphocyte; tTregs, thymus-derived Tregs; Bregs, B regulatory cells. ↓, decrease; ↑, increase.

as TIGIT-Fc, TIGIT mAb, and VISTA mAb, are evaluated in pre-clinical and clinical trials (351–353).

A particularly attractive therapeutic approach is the generation of an antigen-specific response with antigen-based and cell-based anti-cancer vaccines (354). These type of vaccines also constitute an extensively investigated strategy to induce tolerance in autoimmune diseases (355). Noteworthy, the combined use of different therapeutic strategies proved to be a valid option for enhancing the response to therapy in both—cancer and autoimmune disease (356, 357). However, therapeutic strategies need to be focused on restoring balance in the immune system and be applied with caution, as the overstimulation of the immune system in cancer may lead to the development of autoimmune disorders (358, 359). On the other hand, over-suppression in the treatment of autoimmune diseases might create a window of opportunity for cancer growth and progression (360, 361).

We hope that with the current paper, we were able to give a glimpse into the mechanisms that regulate tolerance to self-tissues and cancer. A dynamic balance between the resting and activation states is crucial to keep the organism safe from external and internal threats like pathogenic microorganisms, cancer cells, or hypersensitivity. We believe that a better understanding of these mechanisms opens the opportunities for novel and selective immunotherapies.

AUTHOR CONTRIBUTIONS

JS, ŁA, MJ, NM-T, and PT contributed to the concept of the review. All authors wrote the manuscript. NM-T revised the manuscript with the contribution of all authors. All authors read and approved the manuscript.

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Conflict of Interest: NM-T and PT are the co-authors of 2 patents related to the presented content and are shareholders of PolTREG S.A. company.

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