Building Better Biotherapeutics and Vaccines by Design: EpiVax, Inc., an Immunology Company

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ABSTRACT

EpiVax, Inc., is an early-stage informatics and immunology biotechnology company in Providence, Rhode Island. It applies computational tools to harness immunity in three major areas: immunomodulation, biotherapeutic immunogenicity risk assessment and de-risking, and vaccine development. Immunotherapy, bio-better and vaccine candidates under development at EpiVax promise to improve the health outcomes of millions of people affected by devastating immune-related diseases.

KEYWORDS: vaccines, immunoinformatics, immunotherapy, immunomodulation, autoimmune diseases

A BRIEF HISTORY OF EPIVAX

A talented post-baccalaureate, a statistics major, and a professor who aspired to develop an HIV vaccine are at the root of EpiVax. Gabriel Meister, Bill Jesdale and Anne S. De Groot, MD, were members of the TB/HIV Research Lab team in Brown University's BioMed Center that created two novel computer-driven tools, EpiMer and EpiMatrix, between 1992 and 1996. These 'epitope discovery' algorithms generated the foundation for a whole suite of advanced in-silico tools that now form the core of EpiVax, Inc., a privately held immunoinformatics company in Providence. The late Michael Lysaght, an approachable and optimistic Brown professor of biotechnology, was another instrumental person in the company's establishment; he recognized the promise of EpiVax and connected the founders to the Slater Center for Biotechnology, a source of funding that brought the technology out of the academe into the entrepreneurial world in 1998. With the addition of a programming expert (Bill Martin) and a formidable lawyer (Fred Stolle), a company was born.

Fifteen years later, EpiVax has evolved into a powerhouse of ideas that is changing the way that we think about vaccines and biotherapeutics. EpiVax has also been the source of an unusual spin-out, the Institute for Immunology and Informatics (established in 2008) at the University of Rhode Island, which has exclusive access to the EpiVax technology to research and develop vaccines for neglected tropical diseases and other targets. Team members at EpiVax are now working on a second spin-out devoted to another promising technology that may change the treatment of autoimmune disease.

URI

Dr. Anne S. De Groot, at URI's Institute for Immunology and Informatics at the University of Rhode Island (URI), is the CEO of EpiVax, Inc.

IN-SILICO DESIGN FOR VACCINES AND PROTEINS

Vaccines are among the most important inventions of modern medicine, but the technology for making vaccines was based on empirical rather than hypothesis-driven science until 1996, when molecular biology made bacterial and viral proteins interpretable by computers. EpiVax has harnessed the availability of whole genomes to develop bioinformatics algorithms and apply them to a four-point vaccine design strategy. Immunoinformatics tools are first used to sort through thousands of potential vaccine candidates in a pathogen's genome, comparing those sequences to similar pathogens and identifying sequences that would trigger a human immune response. Protein sequences are then mapped for short, linear, putative T cell epitopes. These epitopes are synthesized as peptides and evaluated in vitro and in vivo for human leukocyte antigen (HLA) binding and antigenicity in survivors of infection or vaccinees. Finally, the optimal composition of immunogenic sequences to drive an effective human immune response is computationally derived (iVAX software suite), and prototype epitope-based vaccines are

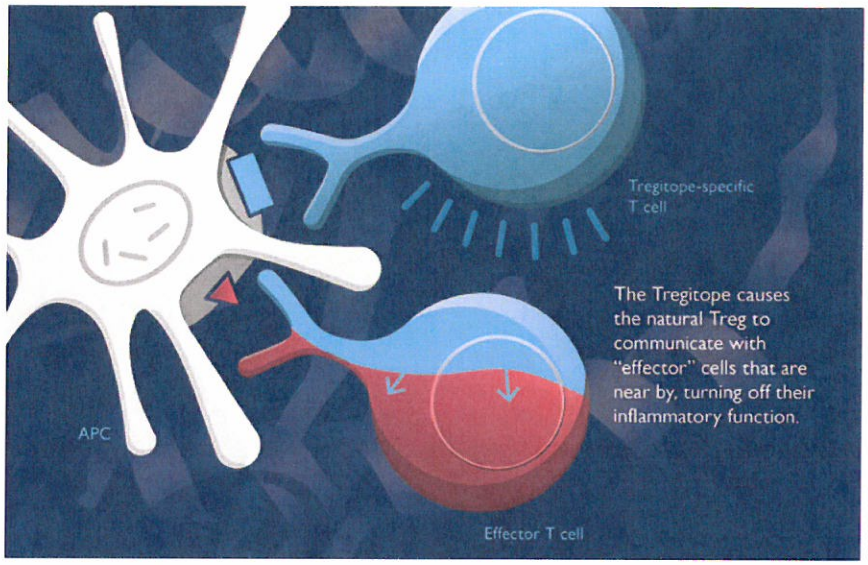
evaluated for immunogenicity and efficacy in humanized transgenic mice. Using this approach, we have demonstrated pre-clinical proof-of-concept for smallpox and tularemia prophylactic vaccination and therapeutic immunization for *H. pylori* infection.[1-4]

The genomes-to-vaccine strategy has two important advantages. First, it strips a pathogen down to the minimal essential antigens, eliciting robust and sustained protective immunity while eliminating non-essential information that could lead to diminished protective immunity and/or immunopathology sometimes associated with whole organism vaccines. This approach may appear to handicap vaccine design because vital elements (i.e., adjuvant, carrier structure) that are normally part of a pathogen are removed, but it creates a valuable opportunity that forms the second advantage. One can combine this novel approach with best-in-class adjuvant and delivery technologies for optimal vaccine construction.

This methodology also forms the core of immunogenicity screening, the process by which protein therapeutics are evaluated for their potential to elicit harmful responses that would impede their effectiveness. Non-vaccine protein therapeutics risk causing harmful immunoreactions, which can render a biologic ineffective and severely compromise patient health. For example, the induction of antibodies known as "inhibitors" against factor VIII in the treatment of hemophilia is a sign of therapeutic protein immunogenicity.[5,6] In 2001, antibodies to a commonly used therapeutic protein drug Erythropoietin, were linked to transfusion dependent anemia.[7] Consequently, unwanted immunoreactions to biologics are a major concern for physicians and drug developers.

EpiVax has thus developed an entire suite of immunoinformatics tools for prospectively identifying and reducing protein therapeutic immunogenicity "in silico," a process that dramatically reduces the time and effort involved, allowing drug developers to accelerate the pre-clinical development of their protein products. The tools are organized in an interactive website called the ISPRI (Interactive Screening and Protein Reengineering Interface) system. Using the ISPRI system, researchers have the ability to screen the protein sequences of product candidates for the presence and immunogenic potential of putative T cell epitopes (EpiMatrix) and epitope clusters (ClustiMer). Protein sequences can be ranked for immunogenic potential in comparison to known proteins on a normalized scale, and an interactive protein reengineering tool (OptiMatrix) allows researchers to modify, or deimmunize, T cell epitope clusters in real time by optimizing the amino acid sequence so that it is no longer able to interact with T cells.

The EpiMatrix toolkit has been extensively validated internally and externally, with several key publications demonstrating the technology and rigorous testing procedures using known protein therapeutic targets.[8,9] In addition, EpiVax incorporates exclusive knowledge of the impact of Tregitopes (T cell regulatory epitopes) on the immunogenicity of protein therapeutics in clinical use, leading to higher accuracy in immunogenicity predictions.



TREGITOPES: AN EPIVAX DISCOVERY AND IMMUNOMODULATION POWERHOUSE

The discovery of Tregitopes, or "T Regulatory Epitopes" in one of the most common proteins found in blood (immunoglobulin G, or IgG) can be attributed to keen observation on the part of the scientific team at EpiVax. Tregitopes turned up regularly in the immunogenicity screens that were performed by the scientists at EpiVax as soon as the ISPRI tools were being applied to monoclonal antibody therapeutics, but were only recognized for their regulatory potential by De Groot and Martin in 2008.[10] Tregitopes act as a natural 'off switch' for the immune system. They are naturally part of the arms (Fab) and stem (Fc) of human IgG and are thought to balance the inflammatory triggers that are present in the re-arranged, or hypervariable segments (variable loops) of the antibody arms. Tregitopes are also found in Intravenous IgG (IVIG), a blood-derived product that is used clinically to control autoimmune conditions.[11] Indeed, some of the anti-inflammatory activity of IVIG may be due to the presence of Tregitopes.[12]

The Tregitope discovery has been validated in a range of standard preclinical models and by collaborating laboratories, where Tregitopes have been shown to suppress and treat autoimmune disease and allergies,[13] and to effectively suppress the immunogenicity of co-administered proteins.[14,15] In addition, Tregitopes have been shown to modify immune

responses to biotherapeutics, such as FVIII. In vitro, co-incubation of proteins with Tregitopes leads to suppression of effector cytokine and chemokine secretion, reduced proliferation of effector T cells, and expansion of antigen-specific adaptive Tregs. In vivo, co-administration of Tregitopes with a wide range of proteins (i.e., FVIII, ovalbumin, and autoantigens) leads to antigen-specific suppression of T cell and antibody responses.

Funding for research on Tregitopes has been flowing. For example, EpiVax recently received a Small Business Innovation Research (SBIR) Phase I grant for $600,000 to explore the use of Tregitope in facilitating tolerance to the lifesaving enzyme replacement therapy for Pompe's disease.[16] In 2012 alone, EpiVax scientists were able to obtain $3.4 million in National Institutes of Health (NIH) funding for development of Tregitope therapies; the group has been awarded more than $6 million in grants to develop Tregitopes over the past few years. Once the right formulation of Tregitopes is identified, and they pass the usual regulatory hurdles, their use is expected to have a radical impact on the clinical management of autoimmunity, transplant rejection, and protein replacement therapies.

CONCLUSION

EpiVax will continue to apply the experience gained from these basic research efforts to practical problems in immunotherapy and vaccine design. In the field of protein therapeutics, we are broadly recognized as thought leaders, and we expect to maintain this position through our discovery work on Tregitopes and tolerance. In addition, our work on epitope-driven vaccines – such as the smallpox, Tularemia, and *H. pylori* vaccines in our pipeline – has begun to demonstrate the power of T cell epitopes to generate protective immune responses. We will combine these breakthroughs with advancements in delivery and formulation to bring novel immunomodulatory therapies and vaccines to market.

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Clinical validation of the "*in silico*" prediction of immunogenicity of a human recombinant therapeutic protein

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Abstract Antibodies elicited by protein therapeutics can cause serious side effects in humans. We studied immunogenicity of a recombinant fusion protein (FPX) consisting of two identical, biologically active, peptides attached to human Fc fragment. EpiMatrix, an *in silico* epitope-mapping tool, predicted promiscuous T-cell epitope(s) within the 14-amino-acid carboxy-terminal region of the peptide portion of FPX. On administration of FPX in 76 healthy human subjects, 37% developed antibodies after a single injection. A memory T-cell response against the above carboxy-terminus of the peptide was observed in antibody-positive but not in antibody-negative subjects. Promiscuity of the predicted T-cell epitope(s) was confirmed by representation of all common HLA alleles in antibody-positive subjects. As predicted by EpiMatrix, HLA haplotype DRB1*0701/1501 was associated with the highest T-cell and antibody response. In conclusion, *in silico* prediction can be successfully used to identify Class II restricted T-cell epitopes within therapeutic proteins and predict immunogenicity thereof in humans.

Introduction

Virtually all therapeutic proteins induce some level of antibody response. Protein-induced immune responses can vary from low-level, low-affinity and transient IgM antibodies to high-level, high-affinity IgG antibodies. Antibody-related clinical sequelae also vary from none to severe, and can occasionally be associated with life threatening side effects [1]. Antibody analyses from a number of clinical studies strongly suggest that serious side effects are associated with high levels of IgG antibodies [2–6]. Such an antibody response is T-lymphocyte driven and includes isotype switching and affinity maturation [7]. T-helper cells, a subset of T-lymphocytes that specifically recognize epitopes presented by antigen presenting cells (APCs) in the context of MHC (major histocompatibility complex) Class II molecules, are the major drivers of the mature antibody response. Protein therapeutics that express MHC Class II

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restricted T-helper epitopes are likely to elicit more frequent and mature antibody responses with IgG as predominant isotypes. These T-helper epitopes can be represented as linear sequences comprising 8 to 12 contiguous amino acids that fit into the MHC Class II binding groove. Over the last 10 years, a number of computer algorithms have been developed and used for detecting Class II epitopes within protein molecules of various origins (De Groot and Berzofsky [8] and the accompanying issue of *Methods* [9]). Such "*in silico*" predictions of T-helper epitopes have already been successfully applied in attempts to increase immunogenicity and efficacy of vaccines [10–12].

The relationship between T-cell epitopes and immune response has also been the subject of a number of investigations in the field of protein therapeutics. In some cases, therapeutic proteins have also been screened for T-helper epitopes in an attempt to evaluate their potential immunogenicity [13–16]. Obviously, reliable *in silico* prediction of helper epitopes would be of significant value in development of protein therapeutics. Such predictions would make it possible to meaningfully rank candidates at the pre-clinical stage of drug development or to reengineer proteins to make them less immunogenic. Furthermore, individuals at higher risk of developing T-cell-driven antibody responses to the protein therapeutic could be identified prospectively using HLA (human leukocyte antigen) typing, if certain HLA can be associated with T-cell response and higher neutralizing antibody titers, as recently described by Barbosa et al. [14]. The use of these methods in the context of clinical trials of protein therapeutics is rather recent and deserves further exploration.

In this study, we describe the *in silico* evaluation of a protein therapeutic in terms of its T-helper epitope content. At the time of the epitope analysis, this protein had also been administered to human subjects in a clinical trial. We were therefore, able to test subsequent immune responses *in vivo* and compare them with the EpiMatrix predictions of immunogenicity.

Materials and methods

Human fusion protein

A recombinant human fusion protein (FPX) consisting of a human Fc fragment fused with two identical 24-amino-acid peptides was generated as described previously [17]. Briefly, phage display peptide libraries (Dyax Corp.) were employed and panned against the targets. After several rounds of selection, the resulting target specific-binding clones were recovered and converted into peptide-Fc fusion proteins (by expressing the active peptides in *Escherichia coli* as fusions to the Fc portion of human IgG1). One of the peptide-Fc fusion proteins (termed FPX) with two identical 24-amino-acid peptides attached to the amino-terminal end of the human germ line Fc-γ fragment was chosen for the study.

Generation of FPX peptide fragments

Solid-phase peptide synthesis

In addition to the whole fusion protein, three peptides spanning amino acids 1–10 (aa 1–10), 11–24 (aa 11–24) and 1–24 (aa 1–24) of the FPX peptide portion were synthesized. These peptide fragments were prepared at 0.2-mmol scale on a Symphony peptide synthesizer (Protein Technologies Inc., Tucson, AZ) or Odessey peptide synthesizer (CEM, Matthews, NC) employing Fmoc/OtBu protection strategy. All Fmoc amino acids were purchased from Midwest Biotech (Fishers, IN). Preloaded Fmoc-Glu (OtBu), Fmoc-Trp (Boc), Fmoc-Pro, Fmoc-Leu HMP resins were purchased from Midwest Biotech (Fishers, IN).

Purification

Crude peptide and HPLC pooled fractions were analyzed by analytical RP-HPLC with a Vydac (Hesperia, CA) 214TP™ C18 column using linear gradients of 0–60% ACN in 0.1% aqueous TFA over 30 min, and a flow rate of 0.6 ml/min. The large-scale RP-HPLC was carried out with a Vydac 218TP C18 column (Vydac) by using a linear gradient of 5 to 40% ACN in 0.1% aqueous TFA over 50 min with a flow rate of 20 ml/min.

Mass spectral analysis

HPLC-MS was performed with an API 150 (PerSeptive Biosystems) in conjunction with Waters analytical HPLC system (Waters Corporation, Milford, MA) by using YMC ODS-AQ C18 column, with a linear gradient of 0% to 60% ACN in 0.1% aqueous TFA over 12 min with a flow rate of 0.6 ml/min.

Human subjects and FPX dosing

The primary objective of this study was to assess the safety and tolerability of FPX. The study included standard safety monitoring (e.g., collection of adverse experience reports; repeated clinical chemistry, hematology, urinalysis, electrocardiograms and other assessments typical for this stage of development) as well as assessment of the pharmacokinetic properties of FPX. Healthy human female and male subjects were dosed with FPX in a Phase 1, single dose, and placebo-controlled, randomized, blinded, sequential dose escalation study. All subjects provided informed consent for participation in the study including HLA typing, anti-FPX antibody assays and T-cell assays. The study enrolled 76 subjects aged 18 to 55 years. In total, 36 subjects received a single dose of FPX intravenously and 40 subjects received FPX subcutaneously. Blood samples for analysis of anti-FPX antibodies were collected from all subjects on day 1 prior to dosing and on day 42 after dosing. In addition, eleven antibody-positive and 4 antibody-negative subjects agreed to provide additional blood samples for the *in vitro* PBMC studies with the FPX peptide fragments and HLA typing at the 6 months follow-up visit.

Prediction and characterization of T-helper epitopes

The peptide portion of the FPX fusion protein (FPX peptide) was screened for potential immunogenicity using previously published EpiMatrix System [18,19]. Briefly, the 24-amino-acid sequence was parsed into overlapping 9-mer frames where each frame overlaps the last by eight amino acids. Each frame was then scored for predicted binding to each of eight common Class II HLA alleles (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301, and DRB1*1501). Due to their prevalence and their difference

from each other, these eight alleles cover around 97% of human populations worldwide [20].

EpiMatrix raw binding score predicted for each 9-mer sequence was normalized with respect to a distribution of scores derived from a very large set ($N>10,000$) of randomly generated 9-mer sequences. This results in a "Z" score for each analyzed 9-mer. The *Z* score determines the position of a 9-mer relative to the distribution of all binding scores generated for the random 9-mer sequences. Any peptide scoring above 1.64 on the EpiMatrix "Z" scale (approximately the top 5% of the random peptide set) has a significant chance of binding to the MHC molecule for which it was predicted. Peptides scoring above 2.32 on the scale (the top 1%) are extremely likely to bind; most published T-cell epitopes fall within this range of scores. Therefore, the higher the *Z* score, the higher is the probability that a peptide will be presented to T-cells by the antigen presenting cells (APCs). Previous studies have demonstrated that EpiMatrix accurately predicts published MHC ligands and T-cell epitopes [21–23].

Antibody detection and characterization

Antibody detection

Antibodies directed against FPX were detected using a validated surface plasmon resonance (SPR)-based biosensor immunoassay using the Biacore 3000™ instrument (Biacore, Inc., Uppsala, Sweden) as previously described [24,25]. In brief, FPX peptide was covalently immobilized onto the flow cell of a CM-5 sensor chip using standard amine coupling chemistry. Human serum samples were injected across the FPX surface followed by the goat anti-human IgG $F(ab')_2$ antibody to confirm the antibody nature of the material bound to the FPX surface. The change in the SPR signal resulting from the bound human antibody and anti-human secondary antibody was reported in relative response units (RU). Serum samples were reported "reactive" for anti-FPX antibodies if the combined sample and confirmatory antibody binding (in RU) was greater than or equal to the validated threshold value (in RU). Samples below this threshold value were reported antibody-negative. All "reactive" samples were reported as positive if the combined sample and confirmatory binding of the post-dose sample was at least 2-fold greater than the combined sample and confirmatory binding of the pre-dose sample.

Antibody characterization

Serum samples from eleven antibody-positive (5 IV and 6 SC dosed) subjects who agreed to provide additional blood samples for the *in vitro* T-lymphocyte studies with the FPX peptide fragments and HLA typing were characterized to determine the antibody isotype.

Relative antibody concentration

A rabbit polyclonal affinity-purified anti-FPX antibody (prepared at Amgen) was spiked into normal human serum and serially diluted to achieve calibrator concentrations ranging from 0.2 to 50 μg/ml. The binding of each calibrator to the immobilized FPX was analyzed as described, expressed in RUs and plotted against the antibody concentration. The relative antibody concentration for each clinical sample was extrapolated from the calibrator curve and expressed in μg/ml of anti-FPX antibody. The limit of detection of this assay was validated at 800 ng/ml of anti-FPX antibodies in neat human serum. Concentrations below 800 ng/ml were not detectable due to the background observed in drug-naive normal human sera.

Antibody isotype determination

The commercial (ICN Biomedicals; anti-human IgG, 8029-F-ICN; anti-human IgM M2110168; anti-human IgE, 8646F-ICN; anti-human IgA, 8647-F-ICN) isotype reagents were used. Each clinical sample was injected across the immobilized peptide portion of FPX on the sensor chip followed by the sequential injection of the anti-IgA, IgE, IgM and then the IgG antibody. Report points were taken before and after each injection and the difference was recorded in relative RUs. Binding of each isotype-specific reagent (in RU) greater than 100 RU from the RUs recorded after sample binding, was reported as positive for the qualitative identification of the specific antibody isotype.

HLA typing

HLA typing was carried out by the National Histocompatability Laboratory division of the American Red Cross using IsoCards according to the protocol supplied by the Red Cross. Thirty to forty microliters of blood was obtained from each subject by finger prick. Blood samples were placed on the IsoCard using a pipette. The samples were then allowed to air dry and individually sealed in plastic bags. Each sample was then shipped to the American Red Cross (National Histocompatibility Laboratory at the University of Maryland Medical System) for processing and HLA typing.

ELISPOT assays

Peripheral blood mononuclear cells (PBMC) were isolated from FPX-treated subjects' heparinized peripheral blood samples by Ficoll separation and cryopreserved in 10% DMSO (dimethyl sulfoxide) in liquid nitrogen. For the assay, the PBMCs were quickly thawed at 37 °C, diluted in 15 ml of RPMI supplemented with 10% heat-inactivated human serum (Valley Biomedical), spun at 300×*g* for 20 min, decanted, and resuspended in RPMI supplemented with 10% heat-inactivated human serum. The cells were then plated at 1×10^7 cells/well in a 12-well tissue culture dish (Corning) and placed in a 37 °C incubator with 5% CO_2. After 24 h, 10 μg/ml of the study peptides was added to the wells. PHA (10 μg/ml) was added to positive control and the culture medium to the negative control wells. On Day 2, 10 U/ml IL-2 and 20 ng/ml IL-7 (R&D Systems) were added to each of the wells. Cells were incubated for 7 days, with half the media being replaced on Day 4 with fresh media containing the interleukins.

On Day 7, the PBMCs were harvested and plated at 2×10^5 cells per well in separate ELISPOT plates pre-coated with monoclonal antibodies to INF-γ and IL-4 respectively (MabTech). Target peptide was added to each test well at 10 μg/ml and the final volume was adjusted with medium to 200 μl/well. This was followed by the 48-h incubation at 37 °C and 5% CO_2. Positive control wells treated with 10 μg/ml PHA and negative controls with 10 μl PBS were incubated under identical conditions. Cells were discarded

Activation of natural regulatory T cells by IgG Fc–derived peptide "Tregitopes"

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We have identified at least 2 highly promiscuous major histocompatibility complex class II T-cell epitopes in the Fc fragment of IgG that are capable of specifically activating $CD4^+CD25_{Hi}FoxP3^+$ natural regulatory T cells (nT_{Regs}). Coincubation of these regulatory T-cell epitopes or "Tregitopes" and antigens with peripheral blood mononuclear cells led to a suppression of effector cytokine secretion, reduced proliferation of effector T cells, and caused an increase in cell surface markers associated with T_{Regs} such as FoxP3. In vivo administration of the murine homologue of the Fc region Tregitope resulted in suppression of immune response to a known immunogen. These data suggest that one mechanism for the immunosuppressive activity of IgG, such as with IVIG, may be related to the activity of regulatory T cells. In this model, regulatory T-cell epitopes in IgG activate a subset of nT_{Regs} that tips the resulting immune response toward tolerance rather than immunogenicity. (Blood. 2008;112:3303-3311)

Introduction

Induction of specific tolerance to self- or to foreign antigens is the goal of therapy for autoimmunity, transplant rejection, and allergy; unresponsiveness is also desirable in the context of therapy with potentially immunogenic autologous proteins (such as factor VIII) and nonautologous proteins (such as botulinum toxin). Until recently, therapeutic tolerance induction relied on broad-spectrum interventions that resulted in widespread effects on immunity, rather than on strategies directed toward restoring a balance between effector immune responses and regulatory immune responses to a specific protein.

Natural means of controlling autoimmune responses (natural tolerance) and of inducing tolerance (adaptive tolerance) are known to exist. For example, suppression of inflammation by $CD4^+CD25_{Hi}FoxP3^+$ natural regulatory T cells (nT_{Regs}) is an important mechanism of effector T-cell regulation, and may represent one of the critical forms of autoregulatory response to self-antigens. Upon antigen-specific activation through their TCR, nT_{Regs} are able to suppress bystander effector T-cell responses to unrelated antigens by contact-dependent and -independent mechanisms. Adaptive T_{Reg} (aT_{Reg}) induction is one outcome of a T-regulatory immune response, and sustained tolerance (to grafts, to allergens, and to autologous proteins) probably requires the existence of aT_{Regs} with the same antigen specificity as the self-reactive T cells.[1-3] Adaptive T_{Regs} are also known as induced T_{Regs} (iT_{Regs}). However, despite extensive efforts and with few exceptions,[4,5] the antigen specificity of nT_{Regs} is still unknown.

Natural T_{Regs} may also control immune responses to autologous proteins to which central tolerance may not exist. For example, it has been suggested that T cells need to be rendered tolerant to the variable regions of antibodies that have undergone somatic hypermutation.[6] To date, no natural T_{Regs} that respond to IgG epitopes have been identified nor have adaptive T_{Regs} to hypervariable IgG regions been identified.

We scanned the Fc region of IgG for natural T_{Reg} epitopes that may explain (1) tolerance to antibody variable regions and (2) the induction of tolerance to selected antigens after administration of therapeutic immunoglobulins or Ig fusion proteins.[7,8] Using peripheral blood mononuclear cells (PBMCs) from individuals allergic to either house dust mite *Dermatophagoides pteronyssinus* (HDM) or to the major birch tree allergen, Bet v $1_{141-155}$, we evaluated the effect of these IgG T_{Reg} epitopes ("Tregitopes") in a standard 2-step "bystander suppression" assay. We explored whether the Tregitopes induced aT_{Reg} to Bet v $1_{141-155}$ using HLA DR*1501 tetramers to the Bet v $1_{141-155}$ epitope. We also coadministered HDM lysate and Tregitopes to HLA transgenic mice and observed suppression of immune response to HDM as measured by whole-antibody enzyme-linked immunosorbent assay (ELISA) and IL-4 enzyme-linked immunosorbent spot (ELISpot). Further studies need to be performed, but these Tregitopes may provide an explanation for the limited immunogenicity of Fc fusion proteins, the expansion of $CD4^+CD25_{Hi}$ regulatory T cells after administration of therapeutic IVIG,[8] and the observed effect of immunoglobulin therapy on autoimmune diseases and other medical conditions.

Methods

Computational epitope mapping

To determine whether T_{Reg} epitopes exist in immunoglobulin G, we used the EpiMatrix and ClustiMer epitope-mapping algorithms (EpiVax) to scan the complete amino acid sequence of human IgG sequences derived from the human IgG germ-line heavy and light chain sequences (GenBank accession J00228 and J00241, respectively[9]). The EpiMatrix system is a suite of epitope-mapping tools (including EpiMatrix, ClustiMer, and BlastiMer) that has been validated over the course of more than a decade, both in vitro and in vivo (for example, see De Groot et al[10] and Koita et

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An Inside *Blood* analysis of this article appears at the front of this issue.

al[11]). For this evaluation of IgG sequences, we used EpiMatrix to identify 9-mer peptides likely to bind to at least 1 of 8 common class II alleles (DRB1*0101, *0301, *0401, *0701, *0801, *1101, *1301, and *1501).[10] Then, using the ClustiMer algorithm, we mapped the EpiMatrix motif matches (for these 8 alleles) along the length of IgG and calculated the density of motifs for the panel of 8 HLA alleles. Epitope-dense regions, or clusters of HLA-binding potential, are usually highly immunogenic in vitro and in vivo.[12,13]

Peptide synthesis

Peptides were synthesized using solid-phase Fmoc chemistry. Automated production was carried out on Gilson, Applied Biosystems, and Advanced Chemtech peptide synthesizers, initially at SynPep (Dublin, CA) and subsequently at New England Peptide (Gardner, MA). Tregitope 289 was amidated on the C-terminus. Peptides were purified via reverse phase high-performance liquid chromatography (HPLC) to more than 80% purity and characterized by mass spectrometry.

HLA-binding assay

Soluble MHC-binding assays were performed as previously described.[11,14] Nonbiotinylated test peptide was suspended in a 96-well polypropylene plate for binding measurements at concentrations ranging from 0.1 μM to 400 μM in triplicate wells. Purified recombinant HLA class II monomers (Benaroya Research Institute, Seattle, WA), in a solution containing 1 mM PefaBloc, 0.75% n-Octyl-B-D-glucopyranoside in 150 mM citrate-phosphate buffer (pH 5.4), were then added to a final concentration of 200 ng/well. The 96-well plates were incubated at 37°C in 5% CO_2 for 45 minutes. Biotinylated influenza hemagglutinin peptide 307-319 (a known HLA DRB*0101 binder) was added to a final concentration of 0.1 μM per well and incubated at 37°C for 20 hours. The contents of each well were then added to a 96-well high-binding ELISA plate previously coated with the antihuman HLA-DR L243 capture antibody (Becton Dickinson, San Jose, CA) and incubated at 4°C for 20 hours. The plate was then developed by addition of 100 μL europium-labeled streptavidin at 10 μg/mL (Perkin-Elmer, Waltham, MA) and 100 μL enhancement buffer (Perkin-Elmer) to each well. After incubation in the dark at room temperature for 15 to 30 minutes, fluorescence was measured on a Wallac Victor 3-V time-resolved fluourometer (Turku, Finland). Binding curves were fitted by nonlinear regression analysis and concentration that inhibits binding by 50% (IC_{50}) values calculated (SigmaPlot, San Jose, CA). Binding strength was categorized based on comparisons with known peptides; an IC_{50} of 250 μM or more is indicative of a weak or nonbinding interaction.

PBMC isolation

Peripheral blood samples were obtained from 1 of 3 sources for this study. Normal donor blood was purchased by EpiVax from the Rhode Island Blood Center in Providence, RI. Citrated peripheral blood was obtained from birch pollen–allergic patients recruited at Hospital Béclère (Clamart, France), after informed consent was obtained according to the Declaration of Helsinki and under the approval of the Comité Consultatif pour la Protection des Patients dans la Recherche Biomédicale (CCPPRB, Béclère). Protocols for blood draws observed US federal and French guidelines and were approved by the respective institutional review boards (CCPPRB and Independent Review Consulting [IRC], Corte Madera, CA). PBMCs were isolated by Ficoll density gradient centrifugation, according to the Accuspin protocol (Sigma-Aldrich, St Louis, MO). Cryopreserved PBMCs isolated from dust mite–allergic individuals were purchased from Cellular Technologies (Cleveland, OH).

T_{Reg} depletion

To evaluate the role of $CD4^+CD25_{Hi}$ cells in the indirect suppression assay, cells were surface stained with anti-CD4 and anti-CD25 fluorescence-labeled antibodies (BD Biosciences, San Jose, CA) and run on a 3-laser fluorescence-activated cell sorting (FACS) Aria high-speed cell sorter (BD Biosciences). $CD4^+$ cells with a CD25 mean fluorescence intensity greater than 100 were sorted and discarded. Cells from all remaining gates were combined and used in the assay.

Suppression assays

HDM lysate. To test for direct (adaptive) suppression of immune response, sorted and unsorted PBMCs were plated at 5×10^6 cells/well in a 12-well tissue culture dish (Corning, Corning, NY) and stimulated with 2 μg/mL *D pteronyssinus* (HDM) lysate (Greer Labs, Lenoir, NC), HDM lysate with 10 μg/mL Tregitopes, or no peptide and placed in a 37°C incubator with 5% CO_2. After 24 hours, 10 U/mL IL-2 and 20 ng/mL IL-7 (R&D Systems, Minneapolis, MN) were added to each of the wells. The cells were fed every 2 days by half media replacement containing the same concentration of cytokines. On day 8, all the PBMCs were collected, washed, and restimulated with 2 μg/mL HDM lysate. On day 8, all the PBMCs were collected, washed, and restimulated with 2 μg/mL HDM lysate in IFN-γ or IL-4 ELISpot kits (Mabtech, Nacka Strand, Sweden) according to manufacturer's instructions. Culture supernatants were analyzed by Searchlight multiplex ELISA assay (Pierce, Woburn, MA).

C3d peptides. Suppression was also tested with a second antigenic system, C3d. The complement component C3d has recently been established as an autologous T-helper cell target.[15] Human PBMCs were cultured for 8 days as described for HDM lysate with 3 different sets of stimuli: 1 control and 2 experimental. The control was a pool of immunogenic C3d peptides alone. The 2 stimulation groups were (1) a pool of immunogenic C3d peptides with hTregitope 167, and (2) a pool of immunogenic C3d peptides with hTregitope 134. Cells were harvested and washed with PBS, and 2×10^5 cells/well were plated into a 96-well plate and restimulated with the immunogenic peptide pool alone, the immunogenic peptide pool and Tregitopes, or no peptide (negative control) for 65 hours. Supernatants were analyzed by multiplexed ELISA analysis.

Tetramer studies. PBMCs from 3 different HLA-DRB1*1501 birch pollen–allergic donors were stimulated weekly with autologous PBMCs pulsed with Bet v $1_{141\text{-}155}$ peptide (10 μg/mL) with or without Tregitopes (10 μg/mL). After the third stimulation, cells were stained with the HLA-DRB1*1501 Bet v $1_{141\text{-}155}$ tetramer for 45 minutes. The cytokine secretion profile of $CD4^+$ Bet v $1_{141\text{-}155}$ tetramer–positive cells was subsequently determined using a cytokine surface-capture assay.

To establish the phenotypic profile of Bet v $1_{141\text{-}155}$–specific T cells, PBMCs from an HLA-DRB1*1501 birch pollen–allergic donor were stimulated weekly with autologous PBMCs pulsed with Bet v $1_{141\text{-}155}$ peptide (10 μg/mL) with or without Tregitopes at 10 μg/mL. After the second stimulation, cells were stained with the HLA-DRB1*1501 Bet v $1_{141\text{-}155}$ tetramer and with a combination of antibodies directed against the indicated surface markers. For control purposes, a subset of cells was also stained with irrelevant tetramers. PBMCs (10^6) were stained with Fl-labeled antibodies as described[16] according to manufacturers' protocols (eBioscience [San Diego, CA] and BD Biosciences). All stained cells were run on a FACSCalibur (BD Biosciences), and data were analyzed using FlowJo software (TreeStar, Eugene, OR). In addition, cytokine secretion was measured by ELISA using PC-conjugated anti–IL-5 and anti–IL-10 antibodies (Cytokine Secretion Assay kit from Miltenyi Biotech, Auburn, CA).

Coadministration in HLA transgenic mice

We used HLA-DRB1*0401 transgenic mice produced by Dr Chella David (Mayo Clinic, Rochester, MN)[17] for the studies described here. The lines were generated by coinjection of DR alpha and DRB1*0401 beta gene fragments into mouse embryos to produce knockin transgenes. The DR4 genes were subsequently introduced individually into class II–negative $H2^{Abo}$ mice to produce strains expressing class II HLA and no mouse class II MHC, as determined by FACS analysis.

HLA DR4 transgenic mice (4- to 6-week-old females) were injected weekly 3 times subcutaneously (scruff of the neck) with (1) 50 μg HDM alone, (2) 50 μg HDM and 50 μg murine homologue of Tregitope 289, or (3) PBS sham control. In a fourth arm, mice were first sensitized to HDM through 3 weekly injections of 50 μg HDM and then treated with coinjections of HDM (50 μg) and Tregitope 289 (50 μg). One week after the final injections, mice were killed, and splenocytes harvested and plated in murine IL-4 ELISpot plates as described in "HDM lysate." The following were added to the plated cells (in triplicate): PBS (no-stimulus control), HDM lysate, purified HDM antigen DerP2, or PHA.

Figure 1. EpiMatrix analysis of human Tregitopes. EpiMatrix Z scores for eight common HLA alleles are shown, for each of the overlapping 9 mer frames for hTregitope 289 (A) and 167 (B). The EpiMatrix Z score indicates the potential of a 9-mer frame to bind to a given HLA allele; the strength of the score is indicated by the shading. The top 5% of scores are shaded medium and the top 1% of scores are shaded darkest. All scores in the Top 5% (Z-Score ≥ 1.64) are considered "Hits." Scores in the top 10% (shown but not highlighted) are considered elevated; other scores are masked for simplicity. Frames containing four or more alleles scoring above 1.64 are referred to as EpiBars and are highlighted. These frames have an increased likelihood of binding to HLA. Flanking amino acids, added to stabilize the cluster during in vitro testing, are underlined.

A

Frame Start	AA Sequence	Frame Stop	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits
1	YNSTYR	9									0
2	YNSTYPV	10	1.67		1.44	1.64		1.31			1
3	YNSTYRVV	11									0
4	YNSTYRVVS	12	1.69	1.33	1.59	1.72	2.14	2.72	1.74	1.29	5
5	NSTYRVVSV	13									0
6	STYRVVSVL	14									0
7	TYRVVSVLT	15				1.70					1
8	YRVVSVLTV	16	2.56		2.65	3.04	1.47	2.02		2.00	5
9	RVVSVLTVL	17									0
10	VVSVLTVLH	18		1.74	2.21	1.49	1.35	2.15	1.55	1.55	3
11	VSVLTVLH	19			2.08						1
12	SVLTVLH	20									0
13	VLTVLH	21		1.72							1
Summarized Results			**DRB1*0101**	**DRB1*0301**	**DRB1*0401**	**DRB1*0701**	**DRB1*0801**	**DRB1*1101**	**DRB1*1301**	**DRB1*1501**	**Total**
Maximum Single Z score			2.56	1.74	2.65	3.04	2.14	2.72	1.74	2.00	--

B

Frame Start	AA Sequence	Frame Stop	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits
1	LQSSGL	9	1.33			1.39					0
2	LQSSGLY	10		1.44					1.31		0
3	LQSSGLYS	11	1.41		1.58					1.75	1
4	LQSSGLYSL	12		1.70		1.72				1.68	3
5	QSSGLYSLS	13									0
6	SSGLYSLSS	14			1.52						0
7	SGLYSLSSV	15									0
8	GLYSLSSVV	16				1.66					1
9	LYSLSSVVT	17	1.98		1.60	1.95				1.35	2
10	YSLSSVVTV	18	1.74		1.90	2.87					3
11	SLSSVVTVP	19									0
12	LSSVVTVPS	20		1.30	1.87			1.82		1.48	2
13	SSVVTVPSS	21									0
14	SVVTVPSSS	22									0
15	VVTVPSSSL	23	1.71			2.37				2.44	3
16	VTVPSSSL	24									0
17	TVPSSSL	25			1.37	1.84					1
18	VPSSSL	26									0
Summarized Results			**DRB1*0101**	**DRB1*0301**	**DRB1*0401**	**DRB1*0701**	**DRB1*0801**	**DRB1*1101**	**DRB1*1301**	**DRB1*1501**	**Total**
Maximum Single Z score			1.98	1.70	1.90	2.87		1.82	1.31	2.44	--

Serum was obtained by cardiac puncture. Quantification of IgG antibody to HDM antigen was determined by antibody-capture ELISA. HDM antigen DerP2 (10 μg/mL) was placed into a 96-well microtiter plate overnight at 4°C. The plates were then washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST) and blocked for 3 hours at room temperature with 5% fetal bovine serum (FBS; Gibco, Carlsbad, CA) in PBS. Serial dilutions of sera in 0.5% FBS/PBS were added to the plates and incubated at room temperature for 2 hours. The microtiter plates were then washed with PBST and 100 μL goat anti–mouse IgG (gamma-chain–specific) conjugated to horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL) diluted 1:10 000 in 0.5% FBS/PBS was added to each well. Microtiter plates were washed in PBST and then developed with 3,3′,5,5′-tetramethylbenzidine (TMB; Moss, Pasadena, MD). Absorbances were read at a wavelength of 450 nm measured on a Wallac Victor 3-V time-resolved fluourometer. Correction for optical imperfections in the plate was made by subtraction of intensities at 540 nm from the 450-nm values. Response to positive control PHA was robust after both immunization conditions and both assay readouts (data not shown). The animal studies protocols were approved by Biomedical Research Models (BRM docket 07-11) and were carried out under Office of Laboratory Animal Welfare assurance number A-4234-01 to BRM.

Results

Using EpiMatrix and ClustiMer, we analyzed human Fc and defined 2 clusters of MHC-binding motifs. Human Tregitope 289 (hTregitope 289, Figure 1A) located in the Fc sequence of IgG, contains 2 frames with EpiBars, features characteristic of promiscuous epitopes. Interestingly, the predicted epitope is located in the region known to be involved in FcR binding and is conserved in all IgG allotypes but is not conserved between isotypes. Human Tregitope 167 (hTregitope 167, Figure 1B) does not contain a classic EpiBar; however, it does contain a high number of T-cell epitopes compared with expectation based on similar protein sequences.

hTregitope 289 and hTregitope 167 were synthesized and evaluated in soluble HLA-binding assays for their ability to compete with high-affinity HLA-specific biotinylated peptides. IC_{50} values were calculated by nonlinear regression analysis using the SigmaPlot analysis program. As predicted, the peptides bound to multiple HLA molecules with high affinity (Figure 2); Tregitope 167 bound and with high affinity to all 5 HLA class II alleles, and hTregitope 289 bound with moderate affinity to HLA DRB1*0101 and DRB1*0301, and with high affinity to HLA DRB1*0401 and 0701, but did not bind to HLA DRB1*1501.

Activation of nT_{Regs}

To determine whether the predicted peptides could activate a subset of nT_{Regs}, we added the hTregitopes (167 and 289), tetanus toxin peptide, or solvent control (no peptide) to a culture of freshly isolated human PBMCs in vitro. After 4 days, the cells were collected and analyzed by flow cytometry. We found similar increases in CD25 expression in both the tetanus- and hTregitope-stimulated samples, indicating T-cell activation by both peptides (Figure 3A; results shown for hTregitope 289). However, expression of FoxP3 within the $CD4^+CD25_{Hi}$ subset differed significantly depending on the stimulus used. Tetanus stimulation led to an expected decrease in expression of FoxP3, suggesting that the $CD4^+CD25_{Hi}$ subset was primarily T effector cells (Teffs), whereas Tregitope stimulation led to a more than 2-fold increase in expression of FoxP3, suggesting activation of nT_{Regs} (Figure 3B), thereby confirming the expected activity of the Tregitopes.

ID	AA Sequence	DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0701	DRB1 *1501
hTregitope 289	EEQYNSTYRVVSVLTVLHQDW	Moderate	Moderate	high	high	non-binder
hTregitope 167	PAVLQSSGLYSLSSVVTVPSSSLGTQ	high	high	high	high	high

Figure 2. HLA binding results. The IC_{50} value (μM) and affinity interpretation for each of the epitopes is shown. Based on comparisons with known peptides IC_{50} scores lower than 25 μM indicate high binding, while an IC_{50} above 400 μM is indicative of a weak or nonbinding interaction.

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Review

Tregitope update: Mechanism of action parallels IVIg

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ABSTRACT

In the course of screening immunoglobulin G (IgG) sequences for T cell epitopes, we identified novel Treg epitope peptides, now called *Tregitopes*, contained in the highly conserved framework regions of Fab and Fc. Tregitopes may provide one explanation for the expansion and stimulation of Treg cells following intravenous immunoglobulin (IVIg) therapy. Their distinguishing characteristics include in silico signatures that suggest high-affinity binding to multiple human HLA class II DR and conservation across IgG isotypes and mammalian species with only minor amino acid modifications. Tregitopes induce expansion of $CD4^+/CD25^{hi}/FoxP3^+$ T cells and suppress immune responses to co-incubated antigens in vitro. By comparing the human IgG Tregitopes (hTregitopes 167 and 289, located in the IgG CH1 and CH2 domains) and Fab to murine sequences, we identified class II-restricted murine Tregitope homologs (mTregitopes). In vivo, mTregitopes suppress inflammation and reproducibly induce Tregs to expand. In vitro studies suggest that the Tregitope mechanism of action is to induce Tregs to respond, leading to production of regulatory signals, followed by modulation of dendritic cell phenotype. The identification of Treg epitopes in IgG suggests that additional Tregitopes may also be present in other autologous proteins; methods for identifying and validating such peptides are described here. The discovery of Tregitopes in IgG and other autologous proteins may lead to the development of new insights as to the role of Tregs in autoimmune diseases.

Contents

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1. Introduction

IVIg is considered to be a low-risk form of treatment for inflammatory disorders and autoimmune diseases. Although it is only approved for use in a handful of diseases [1], IVIg is also used off-label for more than 100 conditions. Recent reports indicating that polyclonal immunoglobulin therapies induce Treg expansion and IL-10 secretion in vivo in animals and humans [2–4] have improved our understanding of the mechanism of action of IVIg. As a result, research on the effects of IVIg in autoimmune disease models has expanded, and more off-label uses are being reported [5–10]. In addition to its application in autoimmune diseases, IVIg is used as a component of the "Bonn-Malmo protocol" therapy for acquired inhibitors to Factor VIII [11,12] and for suppressing immunological rejection after transplantation [13]. Although initially used to protect against infection, IVIg is now used in combination with other medications (Rituximab) for tolerance induction in Pompe disease [14]. The link between IVIg and induction of tolerance in these clincal settings and in autoimmune disease therapy sparked our interest in the potential for parallel mechanisms of action between IVIg and Tregitopes, novel peptides that were previously described by our group.

Tregitope peptides were discovered because they contain in silico signatures that suggested high-affinity binding to multiple human class II Major Histocompatibility Complexes (class II MHC; HLA-DR) [15]. We previously published the sequences of two Tregitopes identified in the constant fragment (Fc) region of immunoglobulin G (IgG) that are highly conserved across IgG Fc sequences. In previous work, we demonstrated that these Fc-derived T regulatory epitopes (Tregitopes) are able to (i) bind to many different HLA with high affinity and (ii) cause expansion of $CD4^+/CD25^{hi}/FoxP3^+$ regulatory T cells (Treg) both in vitro and in vivo. Taking together their location in (and conservation across) IgG, their ability to bind to MHC class II, and their observed stimulation of Treg, we speculated that the purpose of these Tregitopes was to help to suppress human anti-human (anti-idiotypic) immune responses targeting neo-epitopes contained in antibody hyper-variable regions (see Fig. 1). In addition to suppressing immune responses to adjacent complimentarity determining region (CDR) epitopes, we reasoned that the peptides might also suppress immune responses to other co-administered antigens in vitro and in vivo. We were able to confirm this hypothesis, and in the original publication, we described Tregitope-mediated suppression of immune responses to allergens such as dust mite lysate and birch pollen in vitro and in vivo. The incubation of human T cells with Tregitope peptides was also associated with activation of Tregs (as measured by expression of FoxP3 and IL-10 up-regulation) and a numerical expansion of Tregs. Induction of natural Tregs in this manner also increased expression of adaptive Treg cell surface proteins (GITR and CTLA-4) [15]. Elyaman et al. have further explored the induction of antigen-specific adaptive tolerance to the $MOG_{35–55}$ epitope using human Tregitopes 167 and 289 in vivo [16].

Another validation of the original Tregitope hypothesis emerged from clinical studies of monoclonal antibodies. We analyzed a wide range of commercially available monoclonal antibodies for the presence of Tregitopes. Consistent with the original hypothesis that these epitopes may be associated with regulation of anti-idiotypic responses, we reported that monoclonal antibodies (used in the clinic) that contained more Tregitopes were less immunogenic in human subjects than those that contained few Tregitopes [17].

Moving beyond their natural function and applications to co-administered antigens, we have hypothesized that the presence of these epitopes in human IgG might explain at least one mechanism of action of IVIg in humans and animals, and, in part, how IVIg acts to suppress autoimmune diseases [18,19]. In this summary of recent work, we describe the process used to identify additional human Tregitopes and the murine homologs of human Tregitopes. We report the MHC binding affinity of human and murine Tregitopes (hTregitopes and mTregitopes, respectively) to murine MHC and review the mechanism(s) of action (MOA) by which Tregitopes engage Treg populations to induce immune tolerance. The purpose of this article is to highlight our rationale for proposing that the MOA of Tregitopes closely parallels certain aspects of the MOA established for IVIg.

2. Identification and validation of Tregitopes

Human Tregitopes 167 and 289 were originally identified while screening for monoclonal antibody immunogenicity using the EpiMatrix immunoinformatics tools developed by Martin and De Groot [15,20,21]. The EpiMatrix tools are designed to predict the probability that peptides will bind to particular MHC class II alleles. This analysis is typically carried out by first parsing the complete amino acid sequence of a protein (such as FVIII, IgG or monoclonal antibody sequences) into overlapping 9-mer frames. Then, each frame is evaluated for binding potential to a panel of eight common HLA alleles (DRB1*0101, *0301, *0401, *0701, *0801, *1101, *1301, and *1501) using the EpiMatrix T cell epitope prediction tool [20,21]. The protein sequences are then re-examined with the ClustiMer algorithm [22], which identifies regions of epitope clustering. The best-defined human Tregitopes and a brief description of their MHC-binding characteristics are summarized in Table 1.

2.1. In silico identification of Tregitopes

We have now developed a consistent protocol for in silico identification of candidate Tregitopes. We start by downloading human IgG sequences from Genbank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Downloaded sequences are aligned, and CDR regions are identified through conventional means. We then identify all 9-mer peptides that are conserved in at least 10% of the antibodies in our sample. These relatively conserved peptides are subsequently scored for HLA binding affinity using the EpiMatrix and ClustiMer algorithms, searching for affinity to the eight common HLA alleles mentioned above [20–22]. IgG peptides that are both highly conserved and high-scoring (in terms of both epitope content and strong epitope clusters) are evaluated for their potential to induce Tregs, as described below.

In Table 1, we list the two previously published Tregitopes (hTregitope 167 and 289) and three additional highly conserved (in more than 30% of published IgG sequences) epitopes that we classify as Tregitopes. The five Tregitopes each contain at least one EpiBar, which is defined as a single 9-mer frame that contains the potential to bind to four or more HLA alleles (for a comprehensive discussion of EpiBars and their relevance to T cell response, see reference [22]). These putative Tregitopes, listed in Table 1, are derived from *both* the Fc region and from the framework regions of variable domains (Fab). Together, they broadly cover heterogeneity in the HLA repertoire of humans, and thus they comprise our list of Tregitopes that may be clinically useful for induction of tolerance.

We do not believe that Tregitopes are confined to IgG. Thus, we have compiled a list of an additional 35 candidate Tregitopes, including sequences from other prevalent autologous proteins such as collagen and albumin, that have also been identified using the method described above (not shown). These include unique peptides as well as several families of frequently occurring variant sequences derived

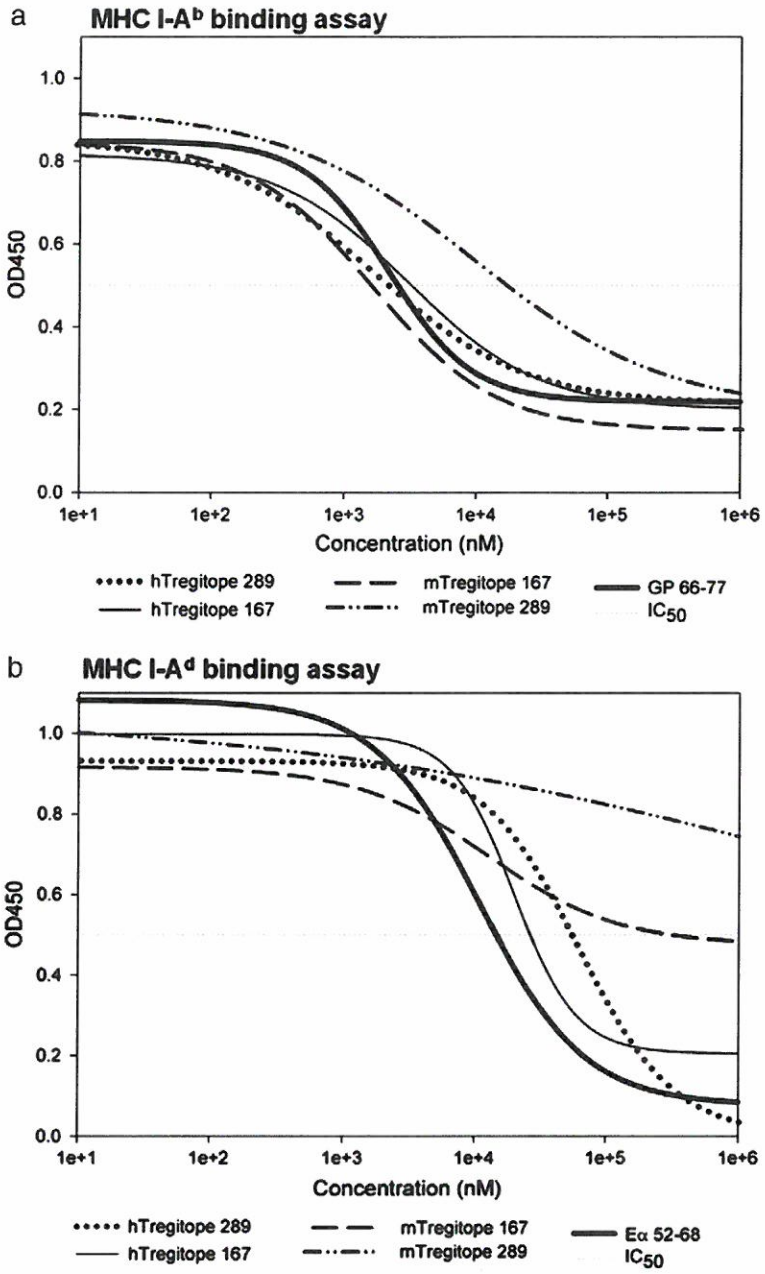

Fig. 1. a and b. Binding of human Tregitopes and murine Tregitopes to murine MHC I–A^b and I–A^d. Four Tregitope peptides provided by EpiVax Inc. (hTreg167, hTreg289, mTreg167 and mTreg289) were tested for affinity to I–A^b and I–A^d, two common murine MHC types. For predicted affinity, see Table 2. MHC is diluted into a citrate phosphate buffer (pH 5) containing fluorescently labeled peptides known to bind to the MHC of interest under these conditions (FITC–GP66 for I–A^b and FITC–Eα52 for I–A^d) at 100 nM along with concentrations of Tregitope peptides ranging from 100 to 72,900 nM. Unlabeled versions of these ligands are titrated in parallel for reference (dark solid lines). After 48 h at 30 °C, the reaction is neutralized to pH 7 and the MHC-FITC complexes are captured on an ELISA plate.

from IgG and other proteins. Validation studies of these putative Tregitopes in vitro, ex vivo, and in vivo using reproducible testing procedures are ongoing in the EpiVax laboratory.

2.2. Identification of murine homologs

To support in vivo testing of the Tregitope hypothesis in various murine-based disease models and TCR-transgenic mice (BALB/c, C57BL/6, NOD, DO11.10, OT-II), we defined murine homologs of the human Tregitopes. To accomplish this, we searched murine IgG constant domain sequences and identified regions corresponding to the human Tregitopes described above; see sequences in Table 2 below. Retention of MHC binding affinity was estimated using the EpiMatrix prediction tools designed for C57BL/6 and BALB/c mice (alleles I–A^b and I–A^d, respectively).

As human IVIg has been shown to induce the expansion and activation of Tregs in C57BL/6 mice [18], we also evaluated whether hTregitopes (derived from human IgG) were predicted to bind to murine MHC. As shown in Table 2a, hTregitope 167 has a total of three murine MHC motifs; one high-scoring and one moderate-scoring murine motif for

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Research Article

Application of IgG-Derived Natural Treg Epitopes (IgG Tregitopes) to Antigen-Specific Tolerance Induction in a Murine Model of Type 1 Diabetes

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HLA class II-restricted regulatory T cell (Treg) epitopes in IgG (also called "Tregitopes") have been reported to suppress immune responses to coadministered antigens by stimulating the expansion of natural Tregs (nTregs). Here we evaluate their impact on human immune responses to islet cell antigens *ex vivo* and on the modulation of type 1 diabetes (T1D) in a murine model *in vivo*. Co-administration of Tregitopes and T1D antigens delayed development of hyperglycemia and reduced the incidence of diabetes in NOD mice. Suppression of diabetes could be observed even following onset of disease. To measure the impact of Tregitope treatment on T cell responses, we evaluated the effect of Tregitope treatment in DO11.10 mice. Upregulation of FoxP3 in KJ1-26-stained OVA-specific $CD4^+$ T cells was observed following treatment of DO11.10 mice with Tregitopes, along with reductions in anti-OVA Ig and T effector responses. In *ex vivo* studies of human T cells, peripheral blood mononuclear cells' (PBMC) responses to GAD65 epitopes in the presence and absence of Tregitope were variable. Suppression of immune responses to GAD65 epitopes *ex vivo* by Tregitope appeared to be more effective in assays using PBMC from a newly diagnosed diabetic subject than for other more established diabetic subjects, and correlation of the degree of suppression with predicted HLA restriction of the Tregitopes was confirmed. Implementation of these defined regulatory T cell epitopes for therapy of T1D and other autoimmune diseases may lead to a paradigm shift in disease management.

1. Introduction

Induction of antigen-specific tolerance is a logical strategy for immunological therapy for type 1 diabetes (T1D). T1D is an organ-specific autoimmune disease resulting from the destruction of insulin-producing pancreatic islet beta cells. In nondiabetics, islet antigen-specific T cells are deleted during thymic development, rendered anergic, or converted to regulatory T cells (Tregs) that actively suppress effector responses to islet cell antigens. In persons with T1D and in the non-obese diabetic (NOD) mouse model of T1D, these tolerance-inducing mechanisms do not function properly. In humans, defects in Tregs have been proposed as one mechanism by which individuals develop T1D, and this defect is said to be both functional and quantitative [1]. In the absence of effective regulatory suppression, $CD8^+$ and $CD4^+$ auto-reactive T cells respond to islet antigens presented by human leukocyte antigen (HLA) molecules. The gradual destruction of islet cells by these auto-reactive cells eventually leads to glucose intolerance.

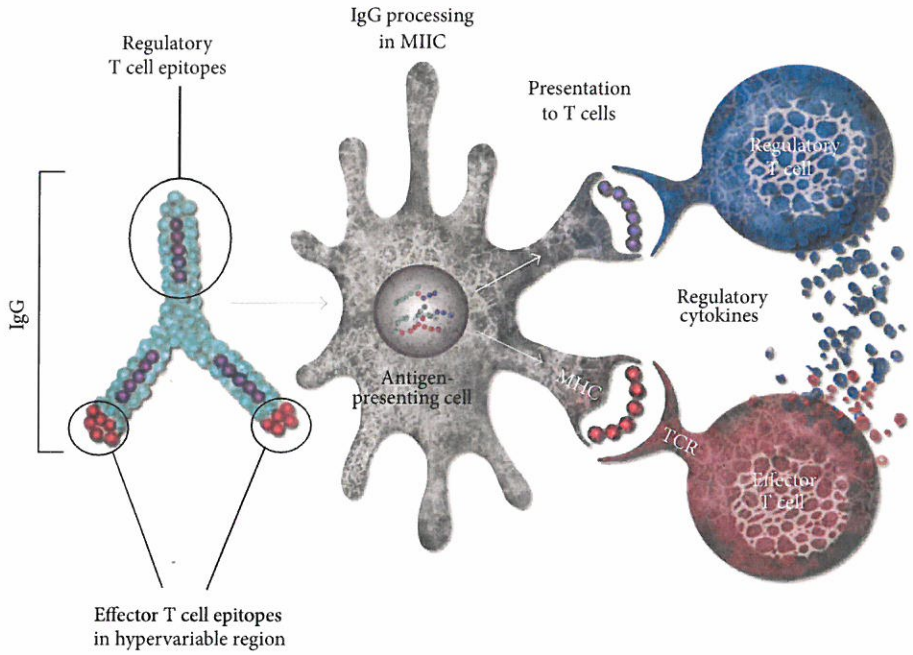

FIGURE 1: Postulated Tregitope mechanism of action. Tregitopes are highly conserved Treg epitopes found in human and other species' IgG. They are postulated to reduce the immunogenicity of neo-epitopes in the hypervariable region of IgG CDR; they also suppress immune response to other co-delivered T cell epitopes. (A version of this figure was originally published in Blood. De Groot A. S., Moise L., McMurry J. A., Wambre E., Van Overvelt L., Moingeon P., Scott W., Martin W. Activation of natural regulatory T cells by IgG Fc-derived peptide "Tregitopes". Blood 2008; 112 : 3303.)

Natural Tregs (nTregs) are an important component of immune regulation in the peripheral circulation, suppressing auto-reactive T cell responses to unrelated antigens by both contact-dependent and -independent mechanisms [2]. Expansion of $CD4^+CD25^{hi}FoxP3^+$ nTregs is being considered as a potential novel therapy for treatment of diabetes [3]. Ideally, as described here, administration of nTregs or nTreg-inducing therapies in conjunction with islet cell "target antigen(s)" would convert auto-reactive T cells to adaptive Tregs, restoring antigen-specific tolerance. Expansion of nTregs is generally performed through non-specific means (with IL-2) since, with few exceptions [4, 5], the antigen specificity of nTregs is still unknown. Autoimmune responses can be modulated by nTregs by inhibiting the antigen-specific activity of nearby auto-reactive effectors and/or by changing the phenotype of the effectors to an induced Treg (iTreg) phenotype.

We previously identified a set of Treg epitopes derived from immunoglobulin G (IgG) that induces Tregs to expand and leads to antigen-specific tolerance [6] (**Figure 1**). We hypothesized that these Tregitopes (T regulatory cell epitopes) are natural T cell epitopes contained in IgG; their presence in the highly conserved domains of IgG may explain why intravenous immunoglobulin therapy is associated with the expansion of nTregs in mice and in humans [7, 8]. Tregitopes can be defined as peptides that (a) bind to multiple MHC class II molecules, (b) induce Tregs to suppress effector T cell responses to co-delivered antigen, and (c) upregulate Treg-associated cytokines and chemokines. T cells that expand in response to Tregitopes exhibit a T regulatory phenotype ($CD4^+CD25^{hi}FoxP3^+$) [6]. In addition to the Tregitopes that were first described in 2008, several Tregitopes that fit the above criteria have been identified in the Fab (framework) and Fc regions of IgG. We and others have suggested that Tregitopes may occur in other common serum proteins such as albumin [9]. The Tregitopes used in the present study include two previously described human Tregitopes (hTregitopes), 167 and 289, and two murine homologs of hTregitopes 167 and 289 (mTregitopes), which are located in the CH1 and CH2 domains of IgG Fc, respectively.

Tregs are known to suppress T cell responses directly and indirectly [10]. In concordance with existing theories about nTregs, direct suppression of bystander T cells may be mediated by Tregitope-specific nTregs [10, 11] through expression of certain cytokines and/or by modulation of the antigen-presenting cell (APC) towards a tolerance-inducing phenotype. Indirect suppression (not specific to the T cell epitope or Tregitope recognized by the nTreg) would be mediated by adaptive Tregs, also known as iTregs [2, 10]. In the present study, we tested the ability of Tregitopes to suppress immune responses *in vivo* with and without murine preproinsulin (PPI, islet cell antigen) epitopes in the NOD model of T1D. We also treated DO11.10 mice with Tregitopes, so as to determine the effect on bystander effector cells *in vivo*, and utilized well-defined reagents to examine antigen-specific T cells from these mice. The DO11.10 studies permitted an examination of Tregitope effects elicited solely due to induction of nTregs

and the subsequent induction of iTregs that, in the case of DO11.10, would be OVA antigen specific.

If, as demonstrated here, activation of nTregs by Tregitopes and the subsequent induction of iTregs could slow or halt the immune destruction of islet beta cells in the pancreas, Tregitopes might be used early in diabetes as a treatment for preserving endogenous insulin production. Moreover, the potential for Tregitopes to induce antigen-specific tolerance (as compared to more broadly immunosuppressive treatments) could contribute to a shift in clinical management of autoimmune disease, away from immunosuppression and towards antigen-specific immunomodulation.

2. Materials and Methods

2.1. In Silico Immunoinformatics Methods

2.1.1. EpiMatrix. EpiMatrix is a T cell epitope-mapping algorithm that is used to identify putative HLA ligands/T cell epitopes, such as Tregitopes, contained within protein sequences (e.g., epitopes identified from pathogen genomes in references [12, 13]). Computation is performed by comparing peptide sequences with a set of HLA allele-specific coefficient matrices. To complete an analysis using the EpiMatrix algorithm, target protein sequences are parsed into overlapping 9-mer frames in which each frame overlaps the last by eight amino acids. Each amino acid in the 9-mer is then assigned a positive or negative coefficient based on its previously determined propensity to positively or negatively impact peptide binding when located at that amino-acid position within the HLA-binding groove [14]. The coefficients are then summed to produce a raw score for each 9-mer. Raw scores are normalized with respect to a distribution derived from a large set of randomly generated peptide sequences. The resulting Z-scores from this distribution are directly comparable across predictions for different alleles.

2.1.2. ClustiMer. By performing this operation repeatedly on a large range of protein sequences, from pathogens to autologous proteins, we have determined that T cell epitopes are not randomly distributed throughout protein sequences but instead tend to "cluster" together within a protein sequence [15]. T cell epitope "clusters" range from 12 to roughly 25 amino acids in length and can contain anywhere from four to 40 binding motifs in the overlapping 9-mer frames. ClustiMer preferentially identifies epitope clusters restricted by multiple HLA class II alleles covering 95% of human populations [16]; clusters selected in this manner demonstrate strong HLA binding affinity and T cell responses [13, 17–19]. Many of the most immunogenic T cell epitope clusters contain a feature we now refer to as an EpiBar [20]. An EpiBar is a single 9-mer frame that is predicted to be reactive to at least four different HLA alleles. Peptides containing EpiBars appear to have a greater-than-expected ability to induce T cell responses of either a stimulatory or suppressive phenotype. This may be due to the co-location of strong HLA binding motifs [12, 13].

2.1.3. Selection of GAD65 Epitopes and Clusters. Based on EpiMatrix analysis of GAD65, we selected 14 peptides containing EpiBars for *in vitro* HLA binding assays and T cell assays using peripheral blood mononuclear cells (PBMC) from T1D subjects. The human GAD65 sequence was obtained from the GenBank sequence database at the National Center for Biotechnology Information (NCBI: Accession Q05329). This sequence was parsed into 577 overlapping 9-mers and scored for predicted HLA binding affinity using the EpiMatrix algorithm as previously described [21, 22]. **Figure 2** illustrates the locations of GAD65 clusters (highlighted in shades of red that correspond with overall EpiMatrix score) selected by EpiMatrix, as well as a comparison between EpiMatrix predictions and T1D epitopes published in a recent compendium (in blue [23]). Details of the sequences tested *in vitro* are also provided in greater detail in **Table 1**.

Figures **3(a)** and **3(b)** provide more detailed EpiMatrix cluster reports for two GAD65 peptides. "EpiBars" are present in these peptides. For the cluster located at amino acid position 450 (GAD65 450, **Figure 3(a)**), one EpiBar begins at Frame 455, a second starts at 456, and a third (weaker due to lower overall scores) at 459. This GAD65 sequence has not previously been identified or published as an epitope. As illustrated in Figures **2** and **3(b)**, there are published GAD65 epitopes located in peptide GAD65 550 at frame 557; the same sequence contains an EpiBar.

2.1.4. iTEM Analysis. The iTEM (Individualized T cell Epitope Measure) tool provides an estimate as to whether a given individual will develop a T cell response to a protein antigen, based on HLA binding predictions. We use a mathematical formula that converts DRB1 allele binding predictions generated by EpiMatrix into an allele-specific scoring system. Thus, iTEM can be used to define an HLA binding threshold, above which immune response is likely to be present and below which immune response is likely to be absent for individual study subjects. iTEM scores have been shown to correlate with immune responses to peptides in T cell assays *in vitro* [24]. An iTEM score was calculated for each of the Tregitope peptides, and the result was compared to *in vitro* responses generated in the two-step ELISpot assay.

2.2. Peptides and Peptide Synthesis. All peptides used in this study were synthesized by 9-fluoronylmethoxy-carbonyl (Fmoc) synthesis to a purity of >80% as determined by HPLC by New England Peptide (Gardner, MA, USA) and 21st Century Biochemicals (Marlborough, MA, USA). The peptide masses were confirmed using either a Q-Star nanospray Mass Spectrometer (New England Peptide) or by MALDI-TOF mass spectrometry (21st Century Biochemicals). Third party QC was performed by Cell Essentials (Cambridge, MA, USA). Tregitope peptides are as previously described [6]; murine Tregitopes 167 and 289 are as described in Elyaman et al. [25]. Tregitope sequences, murine PPI peptide sequences, and class II-binding control peptide sequences are listed in **Table 2**. Murine Tregitopes 167 and 289 each contain a single significant score (1.98 and 1.73, resp.) for the murine class II MHC allele I-Ag7 (NOD) based on *in silico*

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ORIGINAL ARTICLE

Adeno-associated virus mediated delivery of Tregitope 167 ameliorates experimental colitis

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Author contributions: van der Marel S carried out experiments; van der Marel S and Ferreira V performed literature searches, analyzed the data and wrote the article; Majowicz A, Kwikkers K and van Logtenstein R participated in the experiments; Meijer SL performed histological analysis of colon section; van der Marel S, Majowicz A, Kwikkers K, Logtenstein R, te Velde AA, De Groot AS, Meijer SL, van Deventer SJ, Petry H, Hommes DW and Ferreira V contributed conceptually to the work, reviewed the manuscript and assisted with the editing of the paper.
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Abstract

AIM: To explore the anti-inflammatory potential of adeno-associated virus-mediated delivery of Tregitope 167 in an experimental colitis model.

METHODS: The trinitrobenzene sulfonate (TNBS) model of induced colitis was used in Balb/c mice. Subsequently after intravenous adeno-associated virus-mediated regulatory T-cell epitopes (Tregitope) delivery, acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol followed by a second treatment with TNBS (0.75 mg in 20% ethanol) 8 d later. Control groups included mice not treated with TNBS (healthy control group) and mice treated by TNBS only (diseased group). At the time of sacrifice colon weight, the disease activity index and histology damage score were determined. Immunohistochemical staining of the colonic tissues was performed to asses the cellular infiltrate and the presence of transcription factor forkhead Box-P3 (Foxp3). Thymus, mesenteric lymph nodes, liver and spleen tissue were collected and the corresponding lymphocyte populations were further assessed by flow cytometry analysis for the expression of CD4+ T cell and regulatory T cell associated markers.

RESULTS: The Tregitope 167 treated mice gained an average of 4% over their initial body weight at the time of sacrifice. In contrast, the mice treated with TNBS alone (no Tregitope) developed colitis, and lost 4% of their initial body weight at the time of sacrifice ($P < 0.01$). The body weight increase that had been observed in the mice pre-treated with Tregitope 167 was substantiated by a lower disease activity index and a decreased colon weight as compared to the diseased control group ($P < 0.01$ and $P < 0.001$, respectively). Immunohistochemical staining of the colonic tissues for CD4+ showed that inflammatory cell infiltrates were present in TNBS treated mice with or without administration with tregitope 167 and that these cellular infiltrates consisted mainly of CD4+ cells. For both TNBS treated groups CD4+ T cell infiltrates were observed in the sub-epithelial layer and the lamina

propria. CD4+ T cell infiltrates were also present in the muscularis mucosa layer of the diseased control mice, but were absent in the Tregitope 167 treated group. Numerous Foxp3 positive cells were detected in the lamina propria and sub-epithelium of the colon sections from mice treated with Tregitope 167. Furthermore, the Foxp3 and glycoprotein A repetitions predominant markers were significantly increased in the CD4+ T lymphocyte population in the thymus of the mice pre-treated with adeno-associated virus serotype 5 (cytomegalovirus promoter-Tregitope 167), as cytomegalovirus promoter compared to lymphocyte populations in the thymus of diseased and the healthy control mice ($P < 0.05$ and $P < 0.001$, respectively).

CONCLUSION: This study identifies adeno-associated virus-mediated delivery of regulatory T-cell epitope 167 as a novel anti-inflammatory approach with the capacity to decrease intestinal inflammation and induce long-term remission in inflammatory bowel disease.

Key words: Adeno-associated virus; Regulatory T cell epitope; Inflammatory bowel diseases; Adeno-associated virus

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INTRODUCTION

Inflammatory bowel diseases (IBD) are inflammatory diseases that affect mostly young adults[1,2]. Although the precise pathogenesis has not been identified, it is generally accepted that IBD result from inappropriate mucosal immune system responses against intestinal flora and other luminal antigens[3-5]. IBD are associated with a reduction in quality of life[6-8] and no curative treatments are available.

Despite the fact that novel treatment strategies, including tumor necrosis factor (TNF)-neutralizing antibodies, have greatly expanded the therapeutic armamentarium, these therapeutics do not prevent complications in IBD and many patients still have to undergo surgery[9]. New treatment strategies that would prevent the initiation of inflammation and enable long-term remission would improve the lives of millions of individuals who are affected by IBD world-wide[10,11].

Recently, biological therapies that target immune pathways have emerged as a new therapeutic approach for the treatment of immune-mediated diseases. They include administration of monoclonal antibodies against inflammatory cytokines[12] and those that influence immune responses such as certain small molecules, Helminths and stem cells[10,13,14]. Since IBD are immune-mediated diseases, these biological therapies are highly promising treatment approaches and have the potential to achieve mucosal tolerance and long-term remission in IBD[10,12-14]. Here, we introduce regulatory T-cell epitopes (Tregitopes)[15,16] as novel biological agents that could create new possibilities for the regulation of inflammation and postulate that Tregitopes, delivered by adeno-associated virus (AAV), could be developed as a new therapeutic modality for the treatment of IBD.

Tregitopes are a set of putative regulatory T cell epitopes present in the immunoglobulin G molecule, which have been identified by using computational epitope mapping[15,16]. Tregitope sequence 167 (Tregitope 167) and an additional sequence (Tregitope 289) were synthesized and shown to bind to multiple Major Histocompatibility complex (MHC) class II molecules and to suppress immune response when co-administered with an antigen. Tregitopes 167 and 289 were also shown to expand natural occurring regulatory T (nTreg) cells and to induce a regulatory phenotype and function in peripheral T (iTreg) cells[15,16]. Harnessing the potential of Treg cells activated or induced by Tregitopes to regulate pathological immune responses in IBD may reduce the requirement for systemic immunosuppressive therapies. However, the use of immunomodulatory peptides in clinical applications for IBD so far have shown that the *in vivo* delivery of these peptides for therapeutic purposes is hindered by difficulties in obtaining sufficient and stable peptide concentrations[17-19]. Therefore, novel means for stable delivery of regulatory peptides have to be explored. AAV present a good safety profile and have been shown to be effective as gene delivery vectors in the clinic for the treatment of a broad range of diseases[20-22]. Therefore, AAV-mediated delivery represents an attractive approach to deliver the immuno-modulatory Tregitope peptides.

In the present study, the potential of AAV-mediated gene therapy for the therapeutic delivery of Tregitope 167 was explored. Systemic AAV-mediated administration of Tregitope 167 was shown to ameliorate the clinical and histo-pathological severity of trinitrobenzene sulfonate (TNBS) induced inflammatory colitis in mice. Hence, AAV-mediated delivery of regulatory T-cell epitopes appears to be a promising novel therapeutic approach for the treatment of IBD and could represent an alternative or adjunct to the use of immunosuppressive drugs.

MATERIALS AND METHODS

AAV vector production and characterization

Mouse Tregitope cDNA was synthesized (Integrated

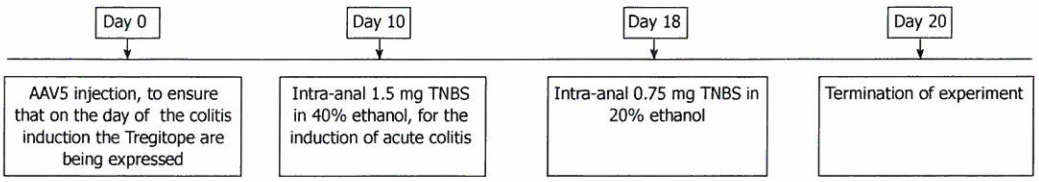

Figure 1 Schematic overview of the trinitrobenzene sulfonate induced colitis model. Mice were injected intravenously with either phosphate-buffered saline (PBS) or adeno associated virus (AAV) 5 [cytomegalovirus (CMV) promoter T-cell epitopes (Tregitope) 167]. Ten days after AAV-mediated Tregitope delivery acute colitis was initiated by intra-rectal administration of 1.5 mg trinitrobenzene sulfonate (TNBS) in 40% ethanol followed by a second TNBS treatment (0.75 mg in 20% ethanol) 8 d later. Control groups consisted of mice not treated by TNBS (healthy control group) and mice treated by TNBS only (diseased control group).

DNA Technologies, IDT, Inc) according to the published corresponding sequence[15,16] and cloned into the plasmid pPSC10[23] under the control of the cytomegalovirus (CMV) promoter. The Woodchuck hepatitis virus post-transcriptional enhancer was incorporated behind the Tregitope 167 cDNA to further optimize gene expression[24]. The AAV vector, AAV5 (CMV-Tregitope 167) was produced according to a technology adapted from Negrete *et al*[23]. The AAV vector was purified with an anion column using the ÄKTA explorer system (GE-Healthcare). After purification, the concentration of AAV vector genomes copies (genome copies/mL) was determined at 9×10^{13} genome copies/mL by Taqman qPCR amplification. The biological infectivity of AAV5 (CMV-Tregitope 167) was demonstrated *in vitro* by PCR amplification of the "CMV-Tregitope 167" DNA fragment (product size 402 bp) on DNA isolated from HEK293T transduced with AAV5 (CMV-Tregitope 167). Primers designed and synthesized for Tregitope 167 and the CMV promoter were used.

Induction of colitis and study design

Balb/c mice (males, age 6-8 wk) were obtained from Harlan Laboratories, the Netherlands. The experimental protocol was approved by the ethical committee for animal welfare of the AMC (Academic Medical Center, Amsterdam, the Netherlands). Colitis was induced in mice by administration of TNBS (TNBS, Sigma-Aldrich), as described previously[25]. The general procedure is summarized in Figure 1.

Mice were injected intravenously with either phosphate-buffered saline or AAV5 (CMV-Tregitope) 10 d before acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol. Consecutively, a second TNBS treatment (0.75 mg in 20% ethanol) was done 8 d after the first TNBS treatment as described precedently[25]. Mice not treated with TNBS (healthy control group) and mice treated with TNBS only (diseased control group) were used as references to monitor colitis development. A concomitant sham AAV control vector was not used in this study as this control has been shown to be equivalent to saline control[26,27]. Even though AAV-mediated gene transfer leads to the development of neutralizing antibodies against the vector capsid[28], preventing vector re-administration, no inflammatory responses against the AAV capsid were documented in *in vivo* gene transfer mice models using AAV vectors[27,29].

Assessment of inflammation

The body weights of the mice were recorded daily, and wasting disease progression was expressed by the percentage of weight loss as compared to body weight at the day of initiation of TNBS treatment (day 10, Figure 1). Animals were withdrawn from the study when their weight loss was > 25% of their original body weight. At the time of sacrifice, colons were collected and presence of loose stool and visible fecal blood was assessed.

At the time of sacrifice, a composite score [disease activity index (DAI)] was established as described previously[25]. Body weight loss was scored on a scale of 0-4 (0, < 1%; 1, 1%-5%; 2, 5%-10%; 3, 10%-15%; 4, > 15%). Loose stool was scored on a scale of 0-4 (0, normal; 1, loose droppings; 2, loose stools, colon filled with feces; 3, loose stool, feces only near cecum; 4, empty bowel). Visible fecal blood was scored on a scale of 0-4 (0, negative; 2, positive; 4, gross bleeding). The DAI consists of a combination of body weight loss, loose stool and visible fecal blood scores divided by 3 as described previously[25].

Colon tissue weights were recorded and used as an indicator of disease-related intestinal wall thickening. Increased colon weight has been shown to correlate with increased colon inflammation[25]. Colons were first divided longitudinally into two parts: one part was immediately frozen in liquid nitrogen for protein extraction and cytokine determination, while the second part was stored in formalin and embedded in paraffin for (immuno-) histological evaluation. Blood was collected by orbital puncture immediately following sacrifice and plasma was separated by centrifugation (5000 r/min for 5 min). Plasma samples were stored at -80 ℃ until analysis.

Histological analysis

Colonic segments were fixed in 10% formalin overnight and thereafter stored in 70% ethanol before embedding in paraffin. Tissues sections (7 μm thick) were stained with haematoxylin and eosin (HE) for histology scoring. The histology damage score was calculated using the following criteria: percentage of area involved, number of follicle aggregates, edema, fibrosis, erosion/ulceration, crypt loss, and infiltration of mononuclear and polymorphonuclear cells, as described previously[30]. The percentage of area involved and crypt loss were scored on a scale

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Human Vaccines: News and Views

Of [hamsters] and men

A new perspective on host cell proteins

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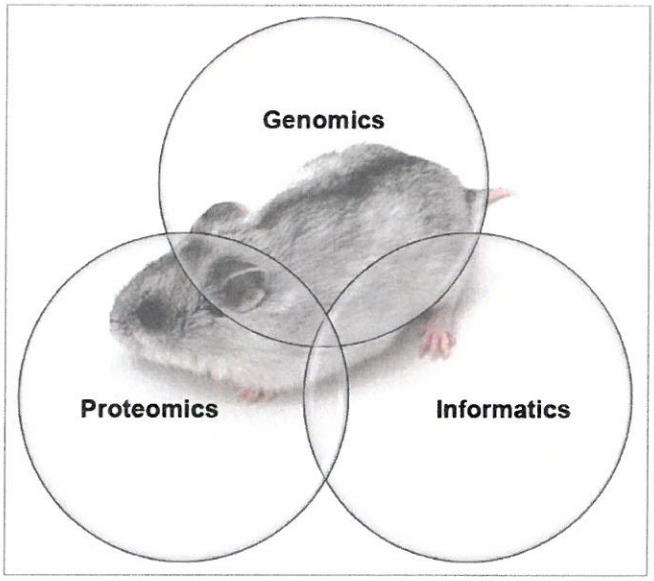
Over many years, recombinant protein biologics developers have addressed product immunogenicity with a focus on the active pharmaceutical ingredient. Recently, immune responses to the native host cell proteins (HCP) have gained attention, as they too may have an effect on the immune response to the formulated drug, namely diminished drug safety and efficacy. The recent suspension of two clinical trials due to the presence of antibodies to Chinese Hamster Ovary (CHO) HCPs in subjects treated with a recombinant biologic clearly reveals the serious concern regulatory agencies attribute to contaminating HCPs. It appears that even minor amounts of CHO-derived HCP in the final formulation of therapeutics can potentially stimulate an immune response to these contaminants; of even greater concern are immune responses that may be cross-reactive with human proteins. Publication of the CHO-K1 genome and transcriptome provides an opportunity to gain insight into one of the most commonly used expression systems in recombinant protein production. We recently applied immunoinformatics tools to evaluate the immunogenic potential of CHO HCP. Rather than evaluate HCP for their intrinsic potential immunogenicity, we suggest that we should estimate their immunogenicity on a fine-tuned scale that accounts for regions that are homologous to human sequences. As more information on the exact identity of the HCP that drive immunogenicity emerges, the accuracy of this approach is likely to improve.

Bio-process engineers are scrambling to identify means for reducing host cell protein (HCP) content and ways to identify HCP that have the potential to raise antibody responses following the cancellation of two phase III clinical trials. The trials were evaluating the safety and efficacy of Inspiration's IB1001, a recombinant factor IX produced in CHO cells; the development of anti-Chinese hamster ovary (CHO) antibodies at higher levels than expected in patients treated with IB1001 triggered the FDA ruling.[1] Anti-CHO antibodies did not reduce F.IX efficacy, thus the ruling was presumably not related to drug efficacy, but rather to drug safety.

The discovery of anti-CHO antibodies in F.IX-treated patients and the FDA ruling is likely to have a chilling effect on the recombinant protein industry. Fortunately, a number of tools have been developed in the past decade that dramatically accelerate immunogenicity screening, whether for the 'protein of interest' (POI) or for HCP. Here we describe the recent application of existing immunogenicity screening tools to evaluate the potential immunogenicity of CHO proteins. The availability of the CHO genome[2] and transcriptome[3] has made it possible to apply these validated immunoinformatics tools to HCP analysis, significantly accelerating research on the impact of HCP on immunogenicity.

Recombinant protein therapeutics have revolutionized present day medicine. Currently, more than 165 biotherapeutic agents are licensed for treatment of a wide variety of illnesses and generate over $99 billion in global sales.[4,5] These proteins are generally produced by expressing the gene in mammalian cell lines, which can be cultured to high density in large bioreactors (**Fig. 1**). Since the early days of recombinant protein production in the 1980s, protein engineers have expanded the number of CHO-derived and other mammalian cell lines that are available for protein expression. In the vaccine context, animal cells such as chicken embryo fibroblasts, dog kidney cells, monkey kidney cells, rabbits and hamsters have been used for production of poliovirus, mumps virus, rubella virus, measles virus, influenza and many others.[6,7] In the biologics field, Chinese hamster ovary (CHO) cells are the most commonly used expression systems.[8] About 70% of recently approved recombinant proteins are expressed in CHO cells. CHO cell lines are the preferred choice for recombinant protein expression due to their capacity to tolerate genetic engineering and their ability to produce complex therapeutics.[9] A key advantage of CHO cells is their ability to perform human-compatible post-translational modifications (e.g., glycosylation), an aspect of protein production that is believed to be relevant to the efficacy of the protein product.[10,11] Methods for cell transfection, gene amplification and clone selection are also well characterized in CHO cells, further adding to their value in the biopharmaceuticals market.



The field of CHO-based protein production has been marked by several major milestones. For example, CHO cells have been subjected to genetic engineering, improving their ability to grow to high density in culture;[8] RNA silencing has also been used in CHO cells,[12] as has treatment with drugs that are normally intended for the treatment of human diseases.[13] Industry-wide conferences such as "Cell Culture Engineering" (run by Engineering Conferences International) currently devote several entire days to CHO engineering. Thus, it is not surprising that CHO was one of the first cell lines for which the entire genome was sequenced[2,14] and for which the proteome is also being determined.[3]

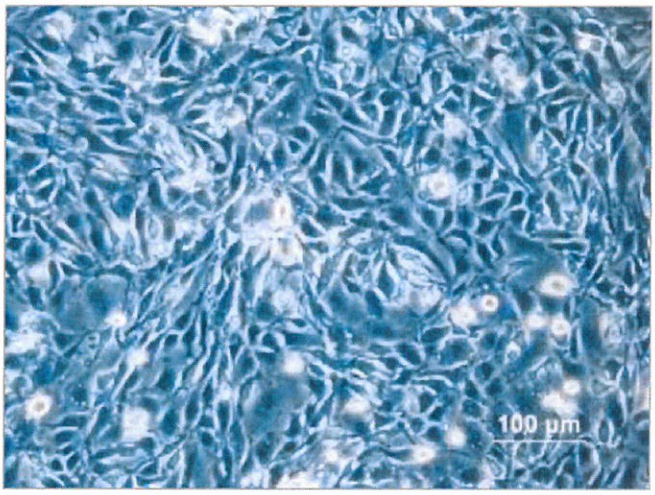


Figure 1. CHO K1 (Chinese hamster ovary) cells at 17 h post plating (image is from the Health Protection Agency Culture Collections website: http://www.hpacultures.org.uk /products/celllines/generalcell/detail.jsp?refId = 85051005&collection = ecacc_gc

The widespread use of CHO cells for recombinant protein production has not escaped the attention of regulatory bodies such as the FDA. The presence of cell-culture-derived "process impurities" such as "Host Cell Proteins" (HCP) is discussed in European Medicines Agency (EMA) and US Food and Drug Agency (FDA) guidances.[15,16] One issue that is receiving increased attention is the potential for HCP to contribute to the immunogenicity of biologics.[17,18]

Like other process-related contaminants, HCP are readily found in final products, even at levels as low as 1–100 ppm, regardless of the close monitoring and purification standards throughout downstream processing.[19] At the same time, HCP comprise a unique and complex group of impurities. Their composition and abundance is dependent on factors that include not only host expression system, but also subcellular localization of expression, culture conditions, the purification process, and the protein produced.

One of the driving forces behind concern about HCP immunogenicity is similarity with human proteins, and the inherent risk of anti-self (auto-immune) responses. There are a number of animal models in which imperfect homology between an antigen and the host-origin protein contributes to the development of antibody responses to the protein (human TSH-R,[20] to human diabetes antigens[21]); epitope spreading from the original non-homologous epitope to other conserved epitopes has been described.[22] Regulatory guidelines from the FDA and European Commission regulations require that the level of HCP in protein therapeutics be identified and quantified during manufacturing and before approval.[23] Extremely low levels of HCP may still be present, especially in final bioproducts, due to the propensity of some HCP to 'hitchhike' on the protein of interest. Currently, the most common techniques for HCP detection and quantification involve protein separation techniques followed by analytical assays, such as enzyme-linked immunosorbent assay (ELISA), western blotting, and mass spectrometry.[24] However, detecting nanograms of HCP in milligrams of protein therapeutics may require use of even more sensitive methods. It is also worth noting that the HCP content is highly dependent ELISA assay system. For example, a commercial assay (multiproduct immunoassay) may estimate low HCP content. But product-specific immunoassays may estimate high HCP content. CHO cell culture supernatant and partially purified HCP are used as the antigen, respectively.[19] Establishment of precise analytical methods that can detect all or the majority of the HCP in the biologic represents a challenge, due to the vast molecular divergence and complexity of HCP.

So as to be able to help the biologics (and vaccine) industry reduce immunogenicity related to HCP, our immunoinformatics research team has begun to examine constitutively expressed CHO proteins as described in the CHO-K1 genome (and transcriptome) and to evaluate their T-cell epitope content using EpiMatrix.[25] This algorithm takes overlapping 9-mer frames derived from CHO protein sequences and scores them for potential binding affinity against a panel of class II HLA alleles; each frame-by-allele assessment that scores highly and is predicted to bind HLA is a putative T cell epitope. Immunogenic potential is assessed by epitope density and ranked on an immunogenicity scale, which was developed to contrast therapeutic proteins of interest against recognized immunogenic and non-immunogenic proteins. In parallel, EpiMatrix evaluates the aggregate epitope density of a given protein with respect to the aggregate epitope density of a set of randomly-generated pseudo-protein sequences of similar size.[26] Correcting for size and expected epitope density allows the potential immunogenicity of a candidate protein to be determined. The epitope prediction tools have been benchmarked, and we have observed that both pathogen and biologic proteins (such as proteins used in coagulation factor deficiencies) that have higher epitope densities as predicted by EpiMatrix tend to be more immunogenic (EpiMatrix whole protein score > 20), while low-density proteins tend to be immunologically inert.[27] High-scoring proteins may stimulate unwanted immune responses.

The observed correlation between pathogen proteins and immunogenicity score has been helpful for vaccine development. However, we believe that the overall HCP immunogenicity score for individual CHO proteins may need to be adjusted for regional homology to human proteins, because immune responses to these homologous regions may be muted or absent due to endogenous tolerance. In general, the identity of human proteins to CHO-expressed proteins is greater than 77%, thus CHO HCP do not pose as great an immunological threat of foreignness as would *E. coli* HCP. However, the high similarity with self does contribute to concern that epitope cross-reactivity with a human homolog may stimulate autoreactive T cells to break tolerance and lead to autoimmunity. On the other hand, sequence differences on the 9-mer level that are HLA-restricted require careful consideration because of their potential for immunogenicity. Immunogenicity may initially arise in response to the foreign epitope, which then leads to epitope spreading, involving autoreactive T cells, or autoimmunity. Thus once immunogenicity is established, epitope spreading may lead to more serious sequelae.[28,29]

When identifying CHO proteins to evaluate, one of the problems that protein manufacturers face is the variability of protein expression from run to run and product to product. The range of proteins produced by CHO cells depends entirely on the cell line, conditions for growth, and growth factors that are administered to the culture or engineered into the host cell.[30] In a pilot project to be published separately, we have initially focused on secreted proteins (Gutierrez et al., manuscript in revision[31]), but, as CHO cells may be apoptotic in the final stages of protein

Murine response to TSH-R	Mouse Sequence same as Hu	Mouse Sequence Different
T cell Epitope Present	Tolerance	Immunogenicity
T Cell Epitope Absent	No Response	Absent epitope, no response

Human response to HCP	Human Sequence Same as CHO	Human Sequence Different
T cell Epitope Present	Tolerance	Immunogenicity
T cell Epitope Absent	No Response	Absent epitope, no response

Figure 2.

production, non-secreted internal proteins should also be evaluated for potential immunogenicity. While the relative abundance of secreted and intracellular CHO proteins in growth medium is not known, the importance of intracellular proteins to potential immunogenicity is underscored by a study showing that intracellular human proteins, as a group, contain greater numbers of putative HLA class II restricted epitopes in comparison with extracellular proteins.[32]

Notably, it is not the mere presence of T-cell epitopes in HCP that poses a risk. A combination of factors associated with the drug therapy, including route of delivery, vehicle, the presence of aggregates, the presence of innate immune system triggers, frequency of dosing and the ability of the protein to interface with the humoral (B cell) and cellular (T cell) immune systems, all impact potential immunogenicity. Even if not sufficient, T cell epitopes are necessary for stimulation of pro-inflammatory T cells and are certainly present in HCP.

While some level of HCP contamination may be inevitable, it is nonetheless crucial to minimize its impact. Products that were previously thought to contain "undetectable" amounts of HCP have been shown to contain contaminants using new analytical technologies. In general, however, immune response to HCP, as compared with immune response to the therapeutic drug are not considered to be critical safety concerns. There has yet to be any evidence of "anti-self" immune response or autoimmunity caused by HCP contaminants, however, based on ample evidence of anti-self immune responses in the literature (**Fig. 2**), the presence of these HCP contaminants may lead to autoreactivity. Thus, it is important to consider the potential for HCP immunogenicity, and new immunoinformatics tools, such as those described here, make it both feasible and relatively easy to evaluate HCP for immunogenicity.

The propensity for CHO proteins to induce anti-autologous protein responses may be directly related to the presence of epitopes that are significantly different from human. Examples can be abundant in the literature. Given any human protein, injected to an animal that has a similar autologous protein, any epitope that is significantly different in terms of its T-cell-receptor facing residues but still is able to bind to the animal's MHC will induce an immune response particularly when a 'danger signal' is present, immune response to the 'foreign' epitope then leads to the spread of immune responses to other epitopes that may be homologous, in the same protein, to host T cell epitopes.[17,18] One example illustrated above comes from the Graves' disease literature (see Inaba H. et al.).[20,21] Murine immune response to human FVIII (used to study Hemophilia A in vivo) also illustrates this principle.[33]

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Immunoinformatic Analysis of Chinese Hamster Ovary (CHO) Protein Contaminants in Therapeutic Protein Formulations

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ABSTRACT

Over many years, therapeutic protein manufacturers have addressed the issue of product immunogenicity focusing on the active pharmaceutical ingredient. Recently, concerns about immune responses to contaminating host cell proteins (HCPs) have emerged, mainly due to concerns about their contribution to anti-drug immune responses and drug safety. The presence of Chinese hamster ovary (CHO) -derived HCPs contributed to the cancellation of two clinical trials in 2012. CHO cells are the most commonly used mammalian cell expression systems in recombinant protein production. The publication of the widely-used CHO-K1 genome and transcriptome provides an opportunity to analyze CHO HCPs and better understand their potential to drive immune responses. Of greatest concern are immune responses that may be cross-reactive with human T cell epitopes. We analyzed CHO-derived protein sequences from three overlapping datasets. These datasets consisted of (1) expressed; (2) putatively secreted; and (3) experimentally determined CHO HCPs. After arriving at a final subset of 1757 constitutively expressed CHO proteins, we used our T cell epitope prediction tool, EpiMatrix, to explore CHO HCP immunogenicity as defined by their relative epitope abundance. Each protein received an immunogenicity score reflecting T cell epitope content, and the results revealed 26% of the 1757 proteins analyzed were above a pre-defined threshold for potential immunogenicity, containing an abundance of predicted T cell epitopes. A subset of these proteins was then evaluated for epitopes that were substantially different from the human genome. This is the first step in the important process of uncovering the potential for HCPs to contribute to unwanted immunogenicity.

Categories and Subject Descriptors

J.3.A [Life and Medical Sciences]: Biology and Genetics

General Terms

Algorithms, Design, Experimentation

Keywords

Immunology, informatics, T cell, epitope, recombinant protein, process impurity, Chinese hamster ovary cells, host cell proteins.

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1. INTRODUCTION

Recombinant protein therapeutics have revolutionized present day medicine. Currently, greater than 100 biotherapeutic agents are licensed for treatment of a wide variety of illnesses and generate nearly 100 billion dollars in global sales [1]. These proteins are generally manufactured by means of genetically engineering host cells to produce the proteins of interest [2]. Chinese hamster ovary (CHO) cells serve as one of the most common expression systems employed in recombinant protein production. About 70% of recently approved therapeutic glycoproteins are expressed in CHO cells [3]. CHO cell lines are the preferred choice for protein expression due to their capacity for genetic engineering, proper folding, post-translational modifications, secretion, and volumetric scalability in the production of complex therapeutics. Methods for cell transfection, gene amplification and clone selection are also well characterized in CHO cells, further adding to their value in the biopharmaceuticals market. A key advantage of CHO cells in comparison to other mammalian cell expression systems is their ability to perform human-compatible post-translational modifications (e.g. glycosylation), thereby improving therapeutic efficacy and protein longevity, as well as reducing safety concerns [4].

Despite the many advantages of protein production in CHO, the final product can be affected by impurities that may contribute to undesired effects. Impurities may be product-related such as aggregates, oxidized forms and unwanted glycoforms, or process-related, including co-purifying host cell proteins (HCPs), DNA, endotoxins, and cell culture components. Like other process-related contaminants, HCPs are readily found in final products, even at levels as low as 1–100 ppm, regardless of the close monitoring and purification standards throughout downstream processing [5]. At the same time, HCPs comprise a unique and complex group of impurities. Their composition and abundance is dependent on factors that include not only host expression system, but also subcellular localization of expression, culture condition, the purification process, and the protein produced. Thus, therapeutic proteins produced by the same CHO cell line may be contaminated with a variety of CHO proteins that differ in each production run of the same protein.

The discovery of anti-CHO antibodies in biologics-treated patients recently led to the cancellation of two Phase III trials. This FDA ruling has had a dramatic effect on the recombinant protein industry. Regulatory bodies such as the EMA and FDA have already issued guidelines on the topic of cell-culture-derived "process impurities" such as HCPs [6, 7]. Fortunately, the availability of the CHO genome and transcriptome has made it possible to apply immunoinformatics tools to HCPs analysis, significantly accelerating research on HCPs immunogenicity.

Immunogenicity screening tools based on epitope density have been described [8-11]. De Groot and Martin have benchmarked a

set of immunogenicity screening tools for protein therapeutics. The relationship between epitope density and immunogenicity has been validated previously, both retrospectively (comparing predicted immunogenicity scores to published data) and prospectively (forecasting immunogenicity of protein therapeutics in clinical trials) [8, 9, 12, 13]. In the research reported here, we applied these and additional tools to the CHO genome, so as to advance research on CHO HCPs and their impact on drug safety.

Here we have focused on the potential for immune-mediated adverse effects related to the presence of CHO HCPs in biologics. Individually analyzing proteins derived from CHO cells enables us to establish some preliminary predictions for HCPs that may be present in biologic products. We have focused on HLA (human leukocyte antigen) class II-restricted T cell epitopes since they serve as a major driving force behind CD4+ T-helper cell responses leading to inflammatory processes and antibody production. T helper (Th) epitopes are short linear sequences (8 to 15 amino acids in length) that are easily compared across the genomes of different species, and the relationship between CHO epitopes and human genome-derived epitopes is of greatest interest in this context. Slight differences between CHO and human proteins could lead to cross-reactive Th cell responses and epitope spreading as is frequently observed in animal models of human disease where human proteins are used as immunogens (TSH-R, Graves disease; human FVIII; inhibitors to FVIII). Additionally, antibodies raised against CHO proteins may cross-react with their human homologs and interfere with their normal function (for example, antibodies against human hormones or cytokines driven by immune responses to CHO proteins that are similar to, but not exactly identical with to the human homolog).

To investigate the immunogenic potential of CHO HCPs, we identified constitutively expressed CHO proteins using the recently published CHO-K1 genome and transcriptome, and evaluated their Th epitope content using immunoinformatics tools. EpiMatrix, a T cell epitope prediction algorithm, has been validated extensively [14-18] and showed higher accuracy compared to other available tools [9, 19]. We performed both a comprehensive analysis of the epitope content of expressed proteins, as well as a more focused study of secreted proteins that would be present in all CHO cell cultures. Both the expressed protein analysis and the secreted protein analysis were performed to illustrate the overall potential of CHO proteins to drive immune responses. In addition, we performed an analysis of a specific set of previously identified and published CHO HCPs [20, 21] so as to provide a real-life example of the immunogenic potential of HCPs that have been found in CHO cell cultures.

2. METHODS

2.1 Databases

CHO genome. The central data set we used in our analysis was the genomic sequence information for the commonly used CHO-K1 cell line, which has only recently been made available. This genomic sequence data was created through a combination of de novo gene-prediction programs as well as homology-based methods to predict genes in the CHO-K1 genome. This gene set is comprised of 24,383 predicted genes, 29,291 transcripts and 416 noncoding RNAs. Many of the predicted 24,383 genes have homologs in human (19,711), mouse (20,612) and rat (21,229) [22].

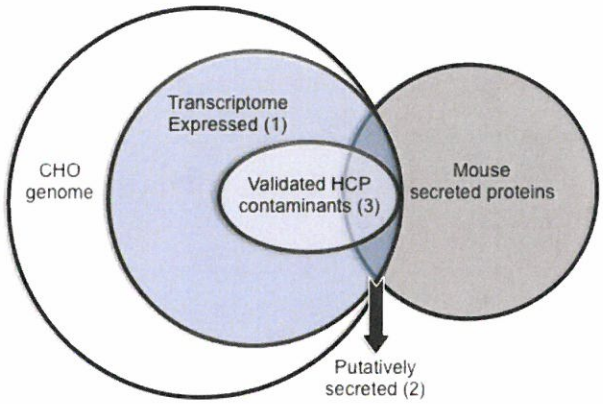

Figure 1. Databases and CHO HCP datasets. Illustration of the sources used to define the CHO protein datasets analyzed. Sources include CHO genome, CHO transcriptome, published CHO proteins identified in biotherapeutic products (Validated HCP contaminants), and mouse secreted proteins downloaded from LOCATE and UniProtKB/Swiss-Prot. Three datasets of proteins were analyzed: (1) Expressed (Transcriptome), (2) Putatively secreted (intersection between transcriptome and mouse secreted proteins), and (3) Validated HCP contaminants.

2.2 Datasets

Three datasets were analyzed:

1) *Expressed CHO proteins.*

Proteins derived from the CHO genome were screened for similarity to CHO transcriptome contigs using the BLAST algorithm. Full-length highly similar hits (above 95% sequence identity) were included in this dataset (Figure 1).

2) *Putatively secreted CHO proteins.*

A set of secreted CHO proteins was discovered by determining the intersection of expressed CHO proteins, as defined above, and homologous proteins in mice that were experimentally shown to be secreted, given the phylogenetic proximity of Chinese hamsters and mice. Secreted mouse proteins were obtained from two databases, UniProtKB/Swiss-Prot and LOCATE [23]. Proteins from each database were individually BLASTed against proteins derived from the CHO genome (Figure 1). CHO homologs of mouse proteins (full-length hits that had greater than 30% sequence identity [24]) were then BLASTed against translated CHO transcriptome contigs to identify potentially expressed/secreted CHO proteins. Duplicate hits from both datasets were merged. Since a higher similarity was expected between CHO proteins derived from the genome and proteins from the CHO transcriptome, hits with identity greater than 50% were further analyzed. Presence of signal peptide was confirmed using SignalP [25].

3) *Validated HCP contaminants.* This dataset was comprised of 28 CHO proteins that were previously identified and quantified in biotherapeutic protein products [20, 21] (Figure 1).

2.3 Immunogenicity screening

Using EpiMatrix, protein sequences from the three datasets described above were parsed into overlapping nine-mer frames, each of which were evaluated for binding potential to eight common class II HLA alleles (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*1101, DRB1*1301, DRB1*1501) that "cover" the genetic backgrounds of most humans worldwide [26]. The standard approach, which captures

binding of peptides to the most prevalent MHC binding pockets in the human population, has been previously described [16, 27-29]. EpiMatrix raw scores are normalized to a "Z" scale. Peptides scoring above 1.64 on the EpiMatrix "Z" scale generally comprise the top 5% of any given peptide set, and it is these peptides that are defined as "hits" and considered potentially immunogenic. Z-scores above 2.32 are in the top 1% and are extremely likely to bind the HLA molecule for which they are predicted. The EpiMatrix algorithm has been compared to other online prediction tools, including SMM-align, IEDB ARB, TEPITOPE, MHCPRED among others, and shown to have a sensitivity rating, on average, for HLA Class II predictions of 77%, which is 5-17% greater than the others [9, 19].

Immunogenic potential was assessed by epitope density and ranked on an immunogenicity scale, which was developed to contrast therapeutic proteins of interest against recognized immunogenic and non-immunogenic proteins. EpiMatrix evaluates the aggregate epitope density of a given protein with respect to the aggregate epitope density of a set of 10,000 randomly generated pseudo-protein sequences of similar size [30]. This provides a normalized distribution of scores, against which new proteins (such as those found in the CHO genome) can be compared. Correcting for size and expected epitope density allows the potential immunogenicity of a candidate protein to be determined. In general, proteins having higher epitope densities tend to be more immunogenic (EpiMatrix whole protein score >20), while low-density proteins tend to be immunologically inert [8, 9]. The accuracy of the EpiMatrix immunogenicity scale has been tested in several prospective and retrospective analyses of recombinant proteins (such as, but not limited to, monoclonal antibodies [9] fusion proteins [12], and GDNF [13]. In a previous publication, we also provided distributions for intracellular versus secreted human proteins and found a significant difference in terms of Th epitope content [31].

3. RESULTS

Immunogenicity scores of all proteins analyzed (1757) showed a non-normal distribution (Jarque-Bera statistic 710.5, $p < 0.001$) with a mean of 2.01 and standard deviation of 39.80 (Figure 2). Proteins scoring above 53.26 represented the top 10%. The range of overall immunogenicity scores of the 3 datasets of proteins

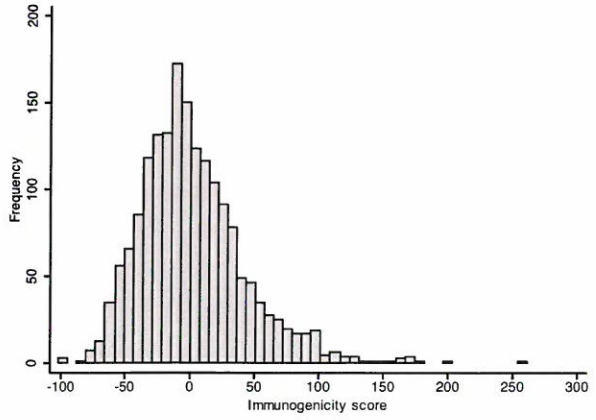

Figure 2. Protein immunogenicity score distribution. Immunogenicity scores of the proteins analyzed from the tree datasets followed a non-normal distribution with a mean of 2.01 and standard deviation of 39.80.

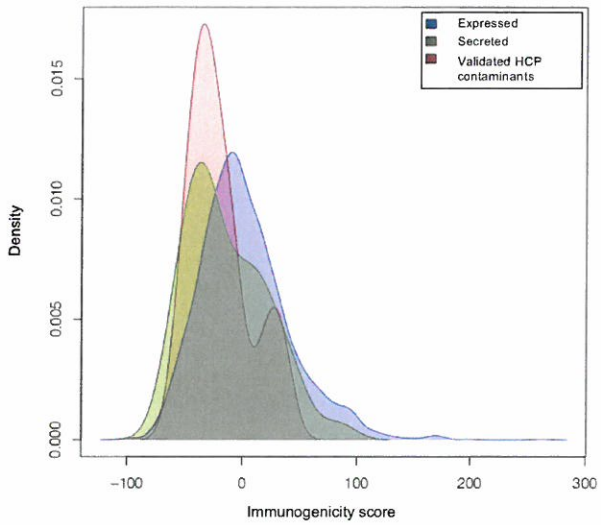

Figure 3. Kernel density estimate of Protein immunogenicity scores from tested proteins. Scores for the three datasets partially overlap. Shoulders in validated HCP contaminants (red) and in putatively secreted protein (green) sets arise due to higher scores in subpopulations. Area under each curve is 1.

tested is shown in Figure 3. Secreted proteins peak at lower immunogenicity relative to all expressed proteins and their average immunogenicity value is below 0 i.e. non-immunogenic. This is consistent with our previous analysis of extracellular and intracellular proteins, which showed that secreted proteins contain fewer epitopes [31]. These proteins, in general, appear to have naturally reduced their T-cell epitope content over the course of evolution. Subtraction of the secreted protein density from the transcribed set would reveal a profile for intracellular proteins that peaks at higher immunogenicity and includes values at the higher end of the scale never reached by secreted proteins.

The major validated HCP contaminant protein peak co-locates with the peak for secreted proteins, suggesting lower potential immunogenicity for this set but a minor peak higher on the scale suggests that contaminating HCPs do carry a risk of immunogenicity. As well, a rightward shoulder on the secreted proteins peak, suggests that these proteins also carry some risk and that there are two distinct populations in the secreted class. An explanation for this observation may be that secreted proteins acting locally may be more immunogenic than those that travel greater distances to exert their effects and are therefore more at risk of immune surveillance.

These results are described in greater detail below:

1) *Expressed CHO* proteins: 1690 full-length expressed proteins showing sequence identity above 95% were identified from CHO transcriptome contigs. 454 of 1690 (27%) of these proteins were predicted as immunogenic (score >20) (Table 1).

2) *Putative secreted CHO proteins*:

***UniProtKB/Swiss-Prot database*:** 65 (85.5%) CHO proteins homologous to 76 secreted mouse proteins from UniProtKB/Swiss-Prot were identified. From those 65 proteins, 14 (18.4%) had above 50% sequence identity with proteins found in CHO transcriptome contigs; 9 (11.8%) of them were predicted to be secreted based on SignalP results.

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Teaching tolerance

New approaches to enzyme replacement therapy for Pompe disease

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Babies born with Pompe disease require life-long treatment with enzyme-replacement therapy (ERT). Despite the human origin of the therapy, recombinant human lysosomal acid α glucosidase (GAA, rhGAA), ERT unfortunately leads to the development of high titers of anti-rhGAA antibody, decreased effectiveness of ERT, and a fatal outcome for a significant number of children who have Pompe disease. The severity of disease, anti-drug antibody (ADA) development, and the consequences thereof are directly related to the degree of the enzyme deficiency. Babies born with a complete deficiency GAA are said to have cross-reactive immunologic material (CRIM)–negative Pompe disease and are highly likely to develop GAA ADA. Less frequently, GAA ADA develop in CRIM-positive individuals. Currently, GAA-ADA sero-positive babies are treated with a combination of immunosuppressive drugs to induce immunological tolerance to ERT, but the long-term effect of these regimens is unknown. Alternative approaches that might redirect the immune response toward antigen-specific tolerance without immunosuppressive agents are needed. Methods leading to the induction of antigen-specific regulatory T cells (Tregs), using peptides such as Tregitopes (T regulatory cell epitopes) are under consideration for the future treatment of CRIM-negative Pompe disease. Tregitopes are natural T cell epitopes derived from immunoglobulin G (IgG) that cause the expansion and activation of regulatory T cells (Treg). Teaching the immune system to tolerate GAA by co-delivering GAA with Tregitope peptides might dramatically improve the lives of CRIM-negative babies and could be applied to other enzyme replacement therapies to which ADA have been induced.

Keywords: Pompe Disease, Anti-drug Antibodies, ADA, Lysosomal acid alpha glucosidase (GAA), Enzyme Replacement Therapy, ERT, Tregitope, Treg, Regulatory T cell, tolerance

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Introduction

Pompe disease is a lysosomal storage disorder caused by a deficiency in the enzyme acid α-glucosidase (GAA). The consequence of GAA deficiency is muscle inflammation, disruption of muscle tissue, and impaired function of heart and skeletal muscle. Although the advent of enzyme replacement therapy (ERT) for Pompe disease has had a dramatic impact on the life expectancy of babies who are born with this disorder, treatment advances are still needed. Current therapy for Pompe disease is based on early detection of the genetic defect and infusions of the recombinant human enzyme acid α-glucosidase (rhGAA) to prevent glycogen accumulation. Pompe-affected children who do not express endogenous GAA (cross-reactive immunologic material; CRIM) and undergo myozyme treatment develop high-titer anti-drug-antibodies (ADA) because they are not immunologically tolerant to GAA. ADA decrease GAA enzyme uptake by muscle and/or inhibit its activity. High ADA titers correlate with poor outcomes, and even though ERT has prolonged the life of Pompe disease babies,

most CRIM-negative Pompe infants who have complete GAA deficiency will eventually succumb to the disease if they are not treated with tolerance-inducing drugs.

The development of experimental immune tolerance regimens to inhibit ADA against life-saving enzyme replacement therapy is an active area of investigation. Current approaches for mitigating GAA-ADA are based on treatment with methotrexate (MTX) to inhibit the proliferation of lymphocytes and Rituximab (Rituxan) to deplete antibody (Ab)-producing B cells.[1-3] These approaches, however, share significant limitations. Namely, these treatments are not effective in eliminating long-lived plasma cells, thus the timing of intervention in patients experiencing an ADA response is critical. Additional pharmacological agents that suppress antibody production by long-lived plasma cells might be of use, such as a drug currently in use for plasma cell leukemia and multiple myeloma, Bortezomib (Velcade).[4] However, the long-term effects of Rituximab and Bortezomib, that both suppress the immune system systemically, are as yet unknown. Finally, the association between the use of Rituximab and development of certain infections has been reported.[5]

In addition to these strategies aimed at inhibiting proliferation or eliminating lymphocyte subsets participating in the ADA response are those aimed at modulating the immune system to become tolerant to the therapeutic protein. IVIG has been shown to be associated with modulation of the regulatory T cell axis, including induction of nTregs;[6] reduction of IL-17,[7] and by enhancing the suppressive function of Tregs.[8] It has thus been applied with much success in a number of autoimmune diseases. A recent report describes the clinical outcomes in two Pompe patients who had received prolonged Rituximab therapy for ADA who were also placed on chronic IVIG in an effort to decrease infectious complications. The addition of IVIG may have provided an additional immunomodulatory benefit in promoting tolerance to the GAA therapy.[9]

Therapies that safely and permanently harness the immune system to induce long-lasting and specific tolerance in Pompe disease children will address a critical unmet medical with broad-reaching implications for other replacement-protein therapies that are also limited by ADA (the Lysosomal Storage Disorders, Hemophilia A and B, etc.).[10-12]

Natural Regulatory T cells, Tregitopes and Tolerance Induction

Autoreactive T cells with moderate T cell receptor (TCR) affinity are known to escape deletion in the thymus to circulate in the periphery where they function as 'natural' regulatory T cells (nTreg) by suppressing immunity against self-antigens.[13] Induced Tregs (iTregs), also known as adaptive Tregs, are generated from circulating T effector cells; these cells perform similar functions but have more plasticity. It has become increasingly clear that both nTregs and iTregs contribute to immune regulation in the periphery and that their presence, or absence, contributes to the induction of tolerance and the development of autoimmunity and inflammation, respectively.

One of the most fundamental questions about nTreg cells has been their antigen-specificity. We surmised that autologous proteins, such as immunoglobulin G (IgG), contain nTreg epitopes. The presence of nTreg epitopes in IgG might explain why immunoglobulins, which undergo somatic hypermutation in the periphery, do not elicit the expected immune response against the new 'foreign' hypervariable sequences. After discovering highly promiscuous MHC class II epitopes in the constant region of IgG, we proposed that these epitopes were nTreg epitopes (Tregitopes)[14] that provide inherent inhibitory signals to counter-balance any stimulatory signals that might result from neo-epitopes expressed in Ab hypervariable region.[15] Two independent publications support our hypothesis: Ephrem and colleagues showed that intravenous immunoglobulin G (IVIg) induces nTreg,[6] and Anthony and Ravetch demonstrated the a linkage between immunoglobulin binding to surface receptors that are associated with antigen-processing pathways, and Treg induction by IVIg.[16,17] We describe here our acquired knowledge on the ability of Tregitope peptides to reduce T cell and T cell-dependent antibody responses, induce regulatory T cells, and lessen disease scores in animal models of inflammatory disease.[18]

Tregitopes have the following four characteristics: 1) Their sequences are highly conserved in IgG sequences; 2) They bind to MHC class II promiscuously; 3) In response to Tregitopes, T cells that exhibit a T regulatory phenotype ($CD4^+CD25^+$ $FoxP3^+$) expand in vitro and in vivo; and 4) Co-incubation of Tregitopes with target autoantigens such as pre-proinsulin inhibits effector T cell (Teff) proliferation in vitro and suppresses the secretion of effector cytokines (De Groot et al., unpublished and 18).

We have more recently demonstrated that APCs present Tregitopes to Treg, engage feedback mechanisms promoting a tolerogenic APC phenotype, induce Treg expansion, and modulate antigen-specific effector T cell responses (De Groot De Groot et al., unpublished). Proportions of APC expressing MHC II, CD80, and CD86 are suppressed, consistent with reported effects of IVIg[19] and of the IgG-derived peptide hCDR1.[20] Moreover, we have observed significant increases in proportions of IL-10-producing CD4+CD25+ FoxP3-expressing Treg in the presence of Tregitopes. The basic mechanism of Tregitope tolerance induction is currently proposed to be as follows: 1) APC present Tregitopes to nTreg, 2) nTreg are activated to proliferate and produce IL-10, 3) nTreg provide tolerogenic feedback signals to APC, modulating the APC phenotype, and 4) nTreg and tolerogenic APC together suppress antigen-specific T cell responses (De Groot et al., unpublished).

In Vivo Studies with Tregitopes

Modulation of T cell responses with Tregitopes may contribute to the design of new approaches for the treatment of autoimmune and inflammatory diseases via expansion of Tregs in vivo or ex vivo. Experience with IVIg gives an example of the therapeutic potential of this approach, and evidence is accumulating that Tregitopes provide beneficial immunomodulatory effects that in many respects parallel IVIg. Within our collaborative

Table 1. Overview of Tregitope in vivo experiments and results

Experimental Model	Finding	Human Disease Parallel	Current Clinical Therapy	In vivo controls	Source
OVA-specific tolerance in C57BL/6 mice	Suppression of T cell proliferation; Suppression of OVA-antibody titer	Enzyme Replacement Therapy	Immune Tolerance Induction	Influenza HA (Negative CTR) No effect; IVIG CTR similar to Tregitope	L. Cousens, A. De Groot (EpiVax)
OVA-specific tolerance in DO11.10 TCR Transgenics	Suppression of T cell proliferation; induction of antigen-specific Tregs	Enzyme Replacement Therapy	Immune Tolerance Induction	Influenza HA peptide: No effect	D.Scott, Y. Su (USUHS)
EAE prevention with Tregitope In C57Bl/6	Reduction of EAE symptoms; induction of Tregs	Multiple Sclerosis	Copaxone; Interferon β Tysabri; Campath	OVA peptide (Negative CTR) No effect; IVIG CTR similar to Tregitope	S.Khoury; W. Elyaman (Brigham)
OVA Allergy Model; Therapy with Tregitope vs. IVIG	Reduction of allergic response to OVA and airway reactivity, increase in Treg Induction	Allergy	Anti-histamines, Immune Tolerance Induction	Albumin (Negative CTR) No effect; IVIG CTR similar to Tregitope	B. Mazer, A. Massoud (McGill)
Tregitope in AAV; TNBS-induced model of inflammatory colitis	Reduction of clinical and histological severity, increased Treg infiltration in the colon	Crohn's Disease	Azathiaprine, Anti-TNF Mabs, Steroids	Saline; No effect	V. Ferreira, S. van der Marel (UniQure)
Tregitope in AAV for Gene Transfer	Suppressed CD8+-T cell response to AAV epitope in gene transfer model	Gene Transfer	Gene Transfer Vectors currently induce CTL response	Scrambled Tregitope peptide (Negative CTR) No effect	F. Mingozzi (CHOP)
Tregitope in T1D (NOD model) with insulin peptides	Suppressed development of diabetes; effect enhanced when Tregitope co-delivered with insulin	Type I Diabetes	Insulin Therapy	PPI peptides, TetTox peptide (Negative CTR) No effect	L. Cousens, A. De Groot (EpiVax)

Tregitope network, we have performed a number of studies to probe potential therapeutic applications of Tregitopes in mouse models of MS (EAE), cardiac transplant, diabetes (NOD), antigen-induced airway hyper-responsiveness, and modulation of viral vector immunogenicity in adeno-associated virus (AAV)-mediated gene transfer.[21] Together, the results obtained in these models show that Tregitopes co-administered with proteins suppress antigen-specific T cell and antibody responses, and induce expansion of functional Tregs. Side-by-side in vivo comparisons of Tregitope with IVIg have been performed in the autoimmune EAE model [Khoury and Elyaman, personal communication] and antigen-induced allergic airway disease [Mazer and Massoud, personal communication], demonstrating that IVIg effects can be replicated by Tregitope administration. Adaptation or incorporation of Tregitopes in drug design may aid in the design of improved tolerance-inducing therapies, and safer, more effective protein therapeutics.

Natural (n)Treg participate in central tolerance by controlling immune responses to autologous proteins, and in peripheral tolerance by stimulating induced (i)Treg cells.[22] Induction of iTreg is associated with sustained tolerance to transplants, allergens and autologous proteins.[13,23,24] Tolerance can be induced with non-depleting anti-CD4 antibodies[25,26] and with AAV vector-mediated expression of antigens in the liver,[27] including rhGAA.[28-30] IVIg, a source of Tregitopes, induces tolerance in a number of different settings such as solid organ transplant,[31] eradication of FVIII and FIX inhibitory antibodies in hemophilia patients,[32-34] and autoimmune diseases such as systemic lupus erythematosus (SLE[35]), idiopathic thrombocytopenic purpura (ITP) and chronic inflammatory demyelinating polyneuropathy (CIDP).[36-41] Of relevance to the goal of inducing tolerance to rhGAA, IVIg has been used to reduce ADA in Pompe patients undergoing Myozyme treatment.[9] See **Table 1** for a list of experiments that have been performed with Tregitope, the human disease parallel, and a comment on current therapy for that condition (highlighting the potential role of Tregitope in the future therapy of the condition).

How Could Tregitopes be Applied to Pompe Disease Therapy?

We believe Tregitopes are natural, human regulatory T cell epitopes ("Tregitopes")

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Research Paper

The two-faced T cell epitope: Examining the host-microbe interface with JanusMatrix

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Keywords: T cell epitope, T cell receptor, TCR, agretope, computational Immunology, cross-reactivity, epitope, immunodominance, immunoinformatics, regulatory T cell, vaccine

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Abstract:

Advances in the field of T cell immunology have contributed to the understanding that cross-reactivity is an intrinsic characteristic of the T cell receptor (TCR), and that each TCR can potentially interact with many different T cell epitopes. To better define the potential for TCR cross-reactivity between epitopes derived from the human genome, the human microbiome, and human pathogens, we developed a new immunoinformatics tool, JanusMatrix, that represents an extension of the validated T cell epitope mapping tool, EpiMatrix. Initial explorations, summarized in this synopsis, have uncovered what appear to be important differences in the TCR cross-reactivity of selected regulatory and effector T cell epitopes with other epitopes in the human genome, human microbiome, and selected human pathogens. In addition to exploring the T cell epitope relationships between human self, commensal and pathogen, JanusMatrix may also be useful to explore some aspects of heterologous immunity and to examine T cell epitope relatedness between pathogens to which humans are exposed (Dengue serotypes, or HCV and Influenza, for example). In Hand-Foot-Mouth disease (HFMD) for example, extensive enterovirus and human microbiome cross-reactivity (and limited cross-reactivity with the human genome) seemingly predicts immunodominance. In contrast, more extensive cross-reactivity with proteins contained in the human genome as compared to the human microbiome was observed for selected Treg epitopes. While it may be impossible to predict all immune response influences, the availability of sequence data from the human genomes, the human microbiome, and an array of human pathogens and vaccines has made computationally-driven exploration of the effects of T cell epitope cross-reactivity now possible. This is the first description of JanusMatrix, an algorithm that assesses TCR cross-reactivity that may contribute to a means of predicting the phenotype of T cells responding to selected T cell epitopes. Whether used for explorations of T cell phenotype or for evaluating cross-conservation between related viral strains at the TCR face of viral epitopes, JanusMatrix further studies may contribute to developing safer, more effective vaccines.

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A comparison of two methods for T cell epitope mapping: "cell free" in vitro versus immunoinformatics

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Abstract

Background

Methods for identifying physiologically relevant T-cell epitopes are critically important for development of vaccines and the design of therapeutic proteins. As the number of proteins that are being evaluated for putative immunogenicity expands, rapid and accurate tools are in great demand. Several methods to identify T-cell epitopes have been developed, the most recent of which is a cell free system consisting of a minimal set of proteases incubated with HLA DRB1*0101, HLA-DM and whole antigen. Isolation and sequencing of the HLA bound peptides using mass spectrometry allows for the prospective identification of immunodominant T-cell epitopes.

Results

We present here, a comparison of this cell free in vitro antigen processing system to an immunoinformatics approach using the EpiMatrix algorithm. Our comparison reveals that in addition to identifying a similar set of epitopes to the cell-free system, the immunoinformatics approach prospectively identifies more HLA-DRB1*0101 epitopes and can simultaneously analyze multiple HLA alleles.

Conclusions

Although the cell-free system incorporates antigen processing and MHC binding, the immunoinformatics approach identifies many validated epitopes with a very high degree of accuracy and can be performed much faster with far fewer resources.

Background

Methods for the prospective identification of physiologically relevant T-cell epitopes are critically important for development of vaccines and for the design of therapeutic proteins. A cell free system (CFS) for prospectively identifying T-cell epitopes from whole antigens was recently described and applied to the identification of influenza epitopes [1]. As described by Hartman et al. in their publication, CFS epitope mapping was performed by pre-incubating whole antigens with HLA-DRB1*0101 and HLA-DM, and then exposing the mixture of antigen and HLA DR/DM to a minimal set of proteases, followed by isolation and sequencing of the HLA-bound peptides using mass spectrometry. The CFS was initially validated using two model antigens (HA1 from influenza A/Texas/1/77 and type II collagen) as positive controls and then applied prospectively for the discovery of new HLA -DRB1*0101 immunodominant epitopes from a recombinant liver-stage antigen of malaria falciparum (LSA-NRC) and HA1 from H5N1 influenza (Viet Nam).

The publication of the CFS method provided an opportunity for comparing a purely immunoinformatics approach based entirely on MHC binding affinity (EpiMatrix) to an in vitro system that involves both antigen processing and presentation [2]. We hypothesized that predicted MHC binding (as performed in silico) would provide results that were at least equivalent to the more laborious CFS approach. As the identification of T cell epitopes using the CFS approach. requires a significant amount of laboratory effort, reagents, and specific expertise in the use of MALDI mass-spectrometry, the immunoinformatics approach might, in addition, offer significant time and cost savings. As is described here, our detailed comparison reveals that the immunoinformatics method correctly identified four of the six epitopes identified by the CFS method, at lower cost and with greater time efficiency, and, in addition, identified other potential epitopes that appear to have been missed by the CFS. Neither of the two CFS epitopes that were missed by EpiMatrix were validated in follow up assays. In the brief report below, we provide a detailed comparison of the *in silico* approach using EpiMatrix and the CFS approach, in terms of epitopes identified and the relative speed, effort required and cost of the two methods.

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Results

CFS reductionist method

The CFS approach to finding immunodominant epitopes, as published in reference 1, is described here for comparison with the EpiMatrix method. The cell free system (CFS) is restricted to evaluations of a single HLA at a

time. The assay requires combining a minimal set of components for antigen processing (full length antigen, human MHC Class II HLA-DRB1*0101, HLA-DM, and Cathepsins S, B, and H) under both endosomal and lysosomal conditions. Hartman et al. describe the application of the CFS method to four proteins: (1) an artificial construct of influenza H1N1 (A/PR/8/34) HA with a single, well-known DR1-restricted epitope (A/Texas/1/77 $HA_{306-318}$) appended to the C-terminus; (2) Collagen type II; (3) influenza H5N1 (A/Vietnam/1203/2004) HA and (4) Liver stage malaria antigen. The resulting peptide-DR1 complexes were isolated by immunoprecipitation and the bound peptides were eluted under acidic conditions. These eluted peptides were then analyzed on a matrix-assisted laser desorption ionization (MALDI) mass spectrometer.

Results for the CFS method were obtained using a single allele (HLA-DRB1*0101) [1]. The eluted epitopes were validated in vitro using T cell proliferation, cytokine induction, tetramer staining, or some combination of the three following immunization of HLA-DRB1*0101 mice with the whole protein antigen.

For example, recombinant HA1 (rHA1), engineered to include a published epitope, was incubated in the cell free system. After isolating HLA DRB1*0101 complexes, the genetically-linked A/Texas/1/77 known immunodominant epitope and only one other peptide (A/PR/8/34 $HA_{298-317}$) were eluted from peptide-DR1 complexes. T cell proliferation assays using peripheral blood mononuclear cells (PBMC) obtained from HLA DRB*0101 transgenic mice immunized with rHA1 showed a strong dose dependent response to the A/Texas/1/77 single epitope identified by the cell free assay and a weaker but still significant response to the A/PR/8/34 peptide. The sum of proliferative responses (ex vivo) to these two epitopes approached the magnitude of the response to whole rHA1.

As a second example, bovine type II collagen (CII) was used to test the epitope identification system. CII is a major component of cartilage and is the main suspected auto-antigen in rheumatoid arthritis in DR1+ individuals [4]. A core DR1 restricted immunodominant epitope, $CII_{282-289}$, has been identified in CII in mouse studies. Following enzymatic digestion and incubation in the CFS, one peptide was eluted, $CII_{273-305}$, as well as variants of that peptide that share the same core epitope. Proliferation studies performed with T cells from CII-immunized mice validated the eluted $CII_{273-305}$ epitope.

The CFS was also used to prospectively identify immunodominant DR1 epitopes from HA1 protein of influenza A/Vietnam/1203/2004 (H5N1) and LSA-NRC, a recombinant modified version of a protein expressed exclusively in malaria-infected hepatocytes at a preerythrocytic stage, which was designed as a vaccine against preerythrocytic stage malaria. Several new epitopes (Figure 1) were identified in these previously unmapped proteins and subsequently validated in T cell assays following immunization of HLA-DR1 transgenic mice with the whole antigen in CFA.

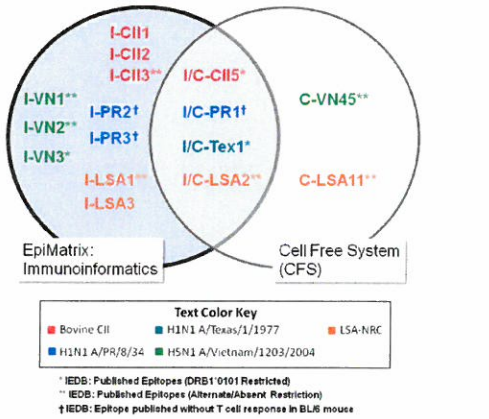

Figure 1 - EpiMatrix prospectively identifies more potential epitopes than the CFS.
Epitopes identified by the CFS are labelled C, epitopes discovered by Immunoinformatics are labelled I and epitopes discovered by both are labelled I/C; the rank in the HLA DR1 EpiMatrix analysis for the antigen is indicated by the number 1-5; note that only one epitope from influenza A Texas was used (recombinantly fused to the PR protein) rather than the whole protein and therefore this single epitope is given the first rank (I/C-Tex1).

Immunoinformatics method

The sequences of the four antigens evaluated in the CFS were obtained from GenBank and then analyzed using EpiMatrix [EpiVax, Providence USA]. Standard criteria (EpiMatrix score in top 5% of scores on a Z scale) were used for epitope identification [2]. A list of the EpiMatrix-predicted HLA DRB1*0101 epitopes was created and compared to the epitopes identified by the CFS. EpiMatrix prospectively identified four of the six epitopes that were identified by the CFS (67%), and one epitope from each of the antigens; I/C-CII3, I/C-PR1, I/C-Tex1, and I/C-LSA2 (Table 1). These epitopes are shown at the intersection of the Venn diagram in Figure 1 and labelled I/C to denote that they were identified by both the Immunoinformatics (I) and the CFS (C) approaches.

All four I/C epitopes scored among the top 5 HLA DRB1*0101 EpiMatrix scores for the antigens (the EpiMatrix rank is indicated by the numerical suffix in Table 1 and Figure 1). Both of the "C" epitopes, which were discovered by the CFS but not by EpiMatrix, scored within the top 10% of EpiMatrix scores, which is below the top 5% cut-off that would normally be used for the selection of T cell epitopes by EpiMatrix analysis. We note that one of these epitopes, selected by the CFS (C-VN45) has been reported to be HLA DRB1*0401- and HLA DRB1*1101-restricted according to IEDB, but not HLA DRB1*0101-restricted. Consistent with the IEDB-reported findings, DRB1*0401 and DRB1*1101 EpiMatrix scores for this epitope are in the top 1% of predicted binders. The second epitope identified by the CFS and not by EpiMatrix (C-LSA11) fell just below the EpiMatrix cut-off for a positive score (1.58, rather than 1.64, see Methods).

Code (Fig.1)	AA Sequence	Core EpiMatrix Epitope	DRB1*0101 Z-Score	IEDB Reference ID
C-VN45	SNGNFIAPEYAYKIVKKGDS	IAPEYAYKI	1.53	1009685**
C-LSA11	YDNFQDEENIGIYK	FQDEENIGI	1.58	1002410**
I/C-CII5	QTGEPGIAGFKGEQGPKGEPGPAGVQGAPGPAG	FKGEQGPKG	2.66	1007108*
I/C-PR1	AINSSLPYQNIHPVTIGECPK	YQNIHPVTI	2.72	1000157†
I/C-Tex1	SRGACPKYVKQNTLKLATGMRK	YVKQNTLKL	3.06	1000083*
I/C-LSA2	EDITFMKLGGSGSPHHHH	FMKLGGSGS	2.62	1002410**
I-CII1	YRSQKTSRL	YRSQKTSRL	3.14	N/A
I-CII2	FLRLLSTEG	FLRLLSTEG	2.98	N/A
I-CII3	FTGLQGLPG	FTGLQGLPG	2.75	1014795**
I-PR2	YQNENAYVS	YQNENAYVS	2.54	1000157†
I-PR3	WTLLKPGDT	WTLLKPGDT	2.53	1000157†
I-VN1	FHNIHPLTI	FHNIHPLTI	2.76	1018856**
I-VN2	LKHLLSRIN	LKHLLSRIN	2.74	1018856**
I-VN3	YIVEKANPV	YIVEKANPV	2.64	1009685*
I-LSA1	FKSLLRNLG	FKSLLRNLG	3.09	1002322**
I-LSA3	IKSNLRSGS	IKSNLRSGS	2.03	N/A

Table 1 - Epitope Comparison
Summary of epitopes identified by EpiMatrix and the CFS. In the case of I/C-PR1, I/C-Tex1, and C-VN45, multiple overlapping peptides were eluted in the CFS; those peptides sharing the same core 9-mer identified by EpiMatrix are considered one epitope. Variable flanking resides are indicated in grey text. Refer to Figure 1 for nomenclature and annotation.

As compared to the CFS, the EpiMatrix immunoinformatics approach identified many more prospective epitopes for each of the antigens. Performing the analysis as described here, a total of 13 epitopes were identified based on their HLA DRB1*0101 score: these included the three highest-scoring epitopes for each of four antigens that were evaluated by the CFS and the one high-scoring HLA DRB1*0101 epitope from A/Texas (only one published epitope was included in the rHA protein tested in the CFS). Of these 13 epitopes, eight (62%) were previously confirmed as human T cell epitopes, according to IEDB; validation is not yet recorded the remaining five epitopes.

Detailed comparison of CFS and EpiMatrix results

Epitope sequences identified by both the CFS and EpiMatrix method were cross-referenced against the Immune Epitope Database (IEDB) of published T cell epitopes and MHC ligands using the substring parameter. Further, all T cell responses and MHC binding results available on IEDB were compiled for each input antigen. If IEDB is taken as the reference standard for validation of epitopes predicted by either method, EpiMatrix prospectively identified five more 'validated' epitopes that the CFS method did not identify. CFS identified two epitopes that were not identified by EpiMatrix, and although these are published in IEDB, they have been published for alternative alleles. If more stringent criteria are applied, (restriction by HLA DRB1*0101 and publication in IEDB), EpiMatrix correctly identified three of the three (100%) HLA DRB1*0101-restricted epitopes. This significant reduction is due in part to the limited HLA-restriction references available on IEDB; of the four test antigens, only H5N1 Vietnam had references qualified as HLA-DRB1*0101-restricted. As we will discuss in greater detail below, the single HLA DRB1*0101-restricted epitope prospectively identified by the CFS (I/C-CII5), was only validated as a result of querying the EpiMatrix-identified core.

Influenza PR antigen. The single PR epitope that was identified by both the CFS and EpiMatrix, I/C-PR1, was ranked first of all PR-derived HLA DRB1*0101 epitopes by EpiMatrix, and was extracted from the same amino acid locus (306-318) as the influenza HA epitope control peptide (I/C-Tex1) that was fused to the A/PR/8/34 antigen. The two epitopes also identified by EpiMatrix have high DRB1*0101 scores (I/C-PR1 and I/C-Tex1) and contain an epitope bar (or EpiBar) [6,7], a feature that is often present in promiscuous epitopes [5], and that has been associated with immunogenicity in human studies [8,9]. This important feature of promiscuous, immunogenic epitopes is not detectable using the CFS. EpiMatrix also identified two additional epitopes in influenza A/PR/8/34 HA (Table 1).

Influenza A Viet Nam. When the full length HA1 from H5N1 A/Vietnam/1203/2004 was analyzed in the CFS, two unique peptide species were identified; both shared the same core sequence (C-VN45). The authors selected the shorter of these two peptides ($HA_{259-274}$) to test for immunodominance in T cell proliferation assays, cytokine production assays, and tetramer staining assays. EpiMatrix analysis shows that the shorter HA most likely has a truncated terminal HLA DRB1*0101 HLA binding motif. The peptide only has one EpiBar at the C-terminal end of the sequence and the N-terminal end of the peptide, which is devoid of predicted HLA binding motifs, would interfere with binding, *in vitro*, and with immunogenicity. The properly centered peptide would have been identified prospectively using EpiMatrix.

Three other epitopes were predicted by EpiMatrix in the same protein to be better HLA DRB1*0101 binders, but they were not identified by the CFS. All three have been experimentally validated and published; only one was confirmed for HLA DRB1*0101. Based on the fact that the third-ranked EpiMatrix epitope is published and found to be an HLA DRB1*0101 epitope in IEDB, the other two (ranked 1 and 2) are equally likely if not more likely to be HLA DRB1*0101-restricted, however this would have to be tested prospectively in T cell assays as was done for the CFS epitopes.

Collagen. It is notable that the single collagen epitope (I/C-CII5) identified by the CFS was also confirmed by EpiMatrix. It was the 5th ranked peptide for HLA DRB1*0101, which is not unexpected considering the greater length of the CII sequence compared to other proteins examined in the CFS. The peptide has a