# Combination peptide immunotherapy suppresses antibody and helper T-cell responses to the RhD protein in HLA-transgenic mice

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## ABSTRACT

The offspring from pregnancies of women who have developed anti-D blood group antibodies are at risk of hemolytic disease of the newborn. We have previously mapped four peptides containing immunodominant T-helper cell epitopes from the RhD protein and the purpose of the work was to develop these into a product for suppression of established anti-D responses. A panel of each of the four immunodominant RhD peptides was synthesized with modifications to improve manufacturability and solubility, and screened for retention of recognition by human T-helper cells. A selected version of each sequence was combined in a mixture ($RhDP_{mix}$), which was tested for suppressive ability in a humanized murine model of established immune responses to RhD protein. After HLA-DR15 transgenic mice had been immunized with RhD protein, a single dose of $RhDP_{mix}$, given either intranasally ($P$=0.008, Mann-Whitney rank sum test) or subcutaneously ($P$=0.043), rapidly and significantly suppressed the ongoing antibody response. This was accompanied by reduced T-helper cell responsiveness, although this change was less marked for subcutaneous $RhDP_{mix}$ delivery, and by the recruitment of cells with a regulatory T-cell phenotype. The results support human trials of $RhDP_{mix}$ peptide immunotherapy in women with established antibody responses to the RhD blood group.

## Introduction

The RhD antigen is a clinically important blood group, and the major target for maternal alloantibodies that mediate destruction of fetal red blood cells (RBC) in hemolytic disease of the newborn (HDN).[1,2] RhD-negative women pregnant with an RhD-positive child are at risk of alloimmunization due to fetomaternal hemorrhage, and exposure to RhD-positive RBC at birth. Prophylaxis using passive anti-D immunoglobulin to block maternal immunization has reduced the incidence of HDN, but the treatment provides only temporary protection and fails in 0.5-2% of susceptible women.[3,4] At present, once RhD alloimmunization has occurred, it cannot be reversed. Subsequent pregnancies are at risk of HDN and require intensive monitoring, which may include invasive fetal blood sampling,[5] and treatment of serious cases relies on intrauterine transfusion, which carries an up to 26% risk of adverse outcomes and 3% risk of fetal loss.[6,7] The challenges of managing mismatched pregnancies in women who have developed anti-D antibodies have prompted a search for novel therapies that are able specifically to suppress established alloimmune responses to the RhD antigen.

The manipulation of $CD4^+$ T-helper (Th) and T-regulatory (Treg) cell subpopulations offers a potentially effective strategy for treating the underlying responses in a wide variety of immune-mediated disease,[8-10] including those in which pathology is caused by antibodies.[11-13] The vast majority of IgG antibody responses are dependent on T-cell help, and the production of antibodies specific for RBC, including anti-D, is no exception.[1,2,14,15] An attractive therapeutic approach is to deliver synthetic peptides containing the dominant epitopes recognized by Th-cells in such a way as to induce tolerance, rather than effector immune responses.[2,16,17] For example, mucosal administration of the relevant peptides can protect mice from experimental autoimmune encephalomyelitis (EAE)[16] and other inflammatory diseases, and is also beneficial in models associated with pathogenic antibodies such as autoimmune hemolytic anemia (AIHA),[11,18] myasthenia gravis,[19] and allergy.[20] However, it has been common practice to test peptide therapy given before or soon after onset of disease, and it remains to be established how effectively and rapidly antibody levels can be suppressed once responses have been established.

The most robust peptide immunotherapies induce forms of active tolerance mediated by Treg cells[16,21] that have the potential to control established pathogenic responses,[22] in addition to preventing them. Treg cells are characterized by expression of the transcription factor Foxp3,[23,24] and suppress *via* a number of poorly defined mechanisms, including those dependent on direct cell-to-cell contact.[25] Although presentation of peptides *via* the mucosae was originally thought to be advantageous in imparting tolerance and inducing Treg cells, it is now suggested that delivery of soluble peptides systemically, such as by the subcutaneous route, can have similar therapeutic effects.[26] Recently, it has also been reported that mice are better protected in a model of allergy if multiple immunodominant peptides are administered together,[20] and, given the variation in peptide binding preferences of different MHC molecules, such combination therapy would also improve coverage of an HLA-dis-

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parate population when translated to patients.[1,2,27,28]

We have previously mapped peptides containing dominant helper epitopes from the RhD protein, which carries the RhD blood group.[1] In particular, peptides $RhD_{52-66}$, $RhD_{97-111}$, $RhD_{117-131}$ or $RhD_{177-191}$, are each able to stimulate Th-cells *in vitro* from more than 50% of RhD-negative donors who have been alloimmunized with RhD-positive RBC, with responses to at least one sequence in every donor tested.[1] To evaluate whether these peptides have the *in vivo* tolerogenic properties required for development as immunotherapy to prevent HDN, we generated a humanized murine model of responsiveness to the RhD protein, since the antigen is not immunogenic in wild-type mice.[2] As predicted, transgenic expression of HLA-DR15, a major restricting allele for RhD epitope-specific Th-cells,[1] conferred on mice the ability to respond to purified RhD protein.[2] When each of the four peptides we had mapped was given by an intranasal route to the transgenic mice, prior to immunization with RhD protein, both Th and antibody responses were prevented.[2] However, the unmet clinical need, and initial indication for RhD peptide therapy, is the treatment of women who have existing anti-D antibodies, and so the question now arises as to whether administration of these peptides can also suppress responses to the RhD protein once these have been established *in vivo*. It is also desirable to establish whether subcutaneous delivery, which raises fewer issues for the approval of eventual human clinical trials, is as effective as the intranasal route.

The purpose was to develop a product for suppression of RhD immunity, based on the sequences of the four immunodominant peptides we have identified,[1] and to test its efficacy *in vivo* in a pre-clinical model of established responses to the RhD protein. The first step was to select soluble forms of each of the four peptides that retain human T-cell recognition, since solubility is a key tolerogenic property. We then wished to test these in combination to verify whether they could inhibit established antibody and Th responses to the RhD protein in our HLA-transgenic immunization model, and to induce Treg cells, comparing mucosal and subcutaneous routes of administration. The results identify a tolerogenic peptide product and simple dosing regimen, suitable for translation to human trials as the first specific treatment for women at risk of HDN due to existing anti-D antibodies.

## Methods

### Donors

RhD-negative patients with anti-D antibodies, following incompatible pregnancy, were recruited by the Scottish National Blood Transfusion Service, and samples for preparation of serum or peripheral blood mononuclear cells (PMBC) taken by venipuncture respectively into plain or lithium heparin Vacutainers (Becton Dickinson, Oxford, UK) (patient information is summarized in Table 1). The Grampian Health Board and the University of Aberdeen Joint Ethical Committee approved the study and all donors gave informed consent.

### Mice

Mice transgenic for HLA-DRA1*1010 and HLA-DRB1*1501, which express HLA-DR15 but not murine MHC class II,[2,29] were originally supplied by Professor Daniel Altmann, Imperial College London, and maintained at the University of Aberdeen. PCR and flow cytometry confirmed presence and expression of HLA-DR15, but not wild-type, genes.[2] The work was approved by the UK Home Office.

**Table 1.** Details of human blood donors.

| Patient | Age on sample date | Years since last incompatible pregnancy | Anti-D antibody level at recruitment (iu/mL) |
|---|---|---|---|
| 1 | 46 | 13 | 131.8 |
| 2 | 35 | 1 | 13.7 |
| 3 | 31 | <1 | 4.1 |
| 4 | 37 | 2 | 11.3 |
| 5 | 29 | <1 | 33.5 |
| 6 | 34 | 3 | 18.8 |
| 7 | 39 | 4 | 23.0 |
| 8 | 31 | <1 | 173.0 |
| 9 | 35 | <1 | 0.9 |
| 10 | 32 | <1 | 67.7 |
| 11 | 38 | <1 | 1.1 |
| 12 | 34 | 10 | 2.6 |
| 13 | 24 | 2 | 2.8 |

*The table shows the age, the approximate date of previous exposure to RhD through pregnancy and the anti-D levels at recruitment for each blood donor.*

### Antigens

The four 15-mer peptides from the RhD protein sequence that we have previously demonstrated to contain immunodominant Th epitopes,[1,2] together with extended or modified sequences, were manufactured by standard Fmoc chemistry and supplied at over 90% purity by GL Biochem, Shanghai, China (Table 2). To determine solubility, peptides were added to $dH_2O$ at 5 mg/mL and the percentages entering solution or remaining in a precipitate determined by bicinchoninic acid (BCA) assay (Thermo Scientific, UK). $RhDP_{mix}$ comprised equal concentrations (see below) of each of the four selected versions of the immunodominant sequences. Human RhD protein was purified from $R_2R_2$ RBC by immunoprecipitation.[2,28]

### Mouse immunization and peptide treatment

As previously described,[2] immune responses to the RhD protein were induced in HLA-DR15 transgenic mice, by a subcutaneous and two intraperitoneal injections, each of 400 μg affinity purified RhD protein, two weeks apart. A single dose of $RhDP_{mix}$, containing 100 μg of each of the four RhD peptides, was delivered in 50 μL or 200 μL of sterile saline by intranasal or subcutaneous route, respectively, six weeks after the first immunization.

### Murine antibody quantification

Blood was collected from the tail vein and serum IgG antibody capable of binding human RhD-positive RBC ($R_2R_2$) measured by a sensitive indirect enzyme-linked antiglobulin test[2] with data normalized to a standard negative control value.[30-32]

### Cell culture

As previously described, $1.25x10^6$ human PBMC/mL, isolated by density gradient centrifugation (Lymphoprep 1077; Nycomed Denmark)[1,28] or $2x10^6$ murine splenic mononuclear cells (SMC)/mL[2] were cultured in alpha modification of Eagles medium (Gibco/Invitrogen, Paisley, UK) supplemented with 1% 2 mM L-glutamine (Invitrogen), 2% 20 mM HEPES buffer (Sigma, Poole, UK) and 2% penicillin streptomycin (Invitrogen), at 37°C in a humidified atmosphere of 5% CO2/95% air. PBMC or SMC cul-

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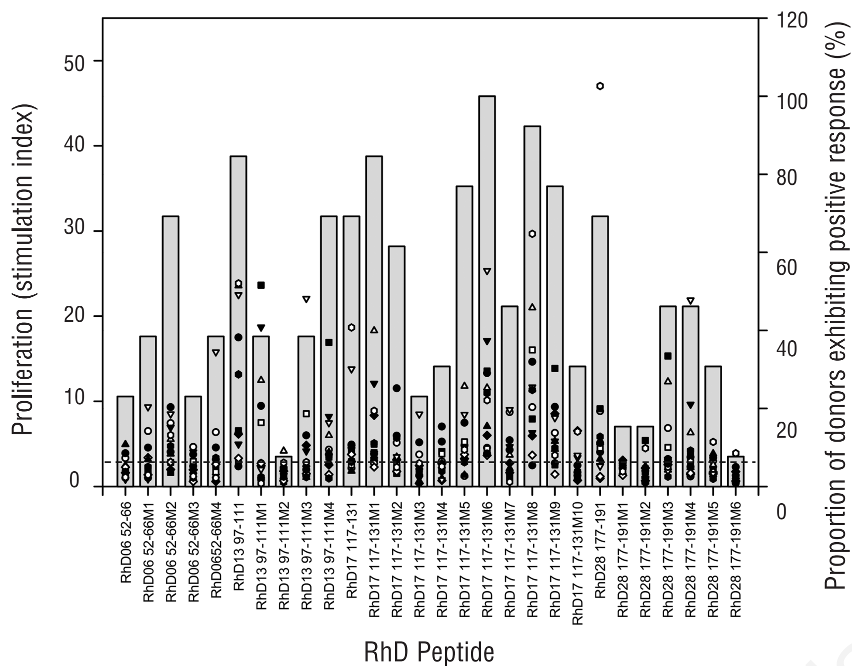


**Figure 1.** Identification of modified RhD peptides capable of eliciting proliferative responses in alloimmunized donors. The proliferative responses to the modified RhD peptides by PBMC taken from female donors, alloimmunized to RhD through pregnancy, are represented by a different symbol for each donor. These results are summarized by the gray bars and right-hand side axis, which shows the percentage of patients that exhibited a positive response (SI>3) to each peptide. SI: stimulation index (ratio of stimulated against unstimulated proliferative responses).

tures were also supplemented respectively with 5% autologous serum, or 1% syngeneic serum plus 5 μM 2-mercaptoethanol (Sigma). Cultures were stimulated with antigen for five days and then analyzed by proliferation assay, flow cytometry or cELISA as previously described (*Online Supplementary Appendix*).[2,33]

### Statistical analyses

Statistical differences were analyzed by parametric two-tailed t-test when similar variances were observed, or the non-parametric Mann-Whitney rank sum test, using SigmaPlot (SyStat Software).

## Results

### Solubility of immunodominant RhD peptides

The solubility of peptides in aqueous media is important for the ability to induce immunological tolerance, and for efficiency of manufacture.[10] The four immunodominant RhD peptides we mapped[1] had proved suitable for small-scale manufacture, and sufficiently soluble in a standard laboratory diluent containing 10% dimethyl sulfoxide (DMSO) for initial *in vitro* and *in vivo* characterization, which included demonstration of the ability to prevent antibody and T-cell responses when given to HLA-DR transgenic mice before immunization with RhD protein.[2] However, bioinformatic analyses predicted low solubility in aqueous media, with grand mean of hydropathicity (GRAVY) scores of 1.280 (RhD6$_{52-66}$), 1.147 (RhD13$_{97-111}$), 1.827 (RhD17$_{117-131}$) and 0.933 (RhD28$_{177-191}$) (Table 2), suggesting that modification of the sequences may benefit development into a product for large-scale manufacture and clinical use. In particular, it was considered desirable to demonstrate solubility without addition of DMSO, which is unlikely to be an acceptable excipient for human use, and there may be more stringent requirements for peptide solubility when attempting to suppress established responses, rather than prevent them.[10] Extension to include hydrophilic residues at the termini of peptides, or selected amino acid substitutions, can improve solubility, without necessarily losing Th-cell recognition of the core epitope.[10] Therefore, a panel of modified peptides, based on the four immunodominant RhD sequences, with additional hydrophilic residues was manufactured and characterized (Table 2). Of the original immunodominant peptides, only RhD$_{97-111}$ was 100% soluble when added at 5 mg/mL in water, but modified versions of each of the other three sequences that were soluble at this concentration could also be identified.

### Human Th-cell recognition of RhD peptides

A key question was whether the modified RhD peptides retained Th-cell recognition. Each peptide was predicted to bind the exemplar human MHC class II molecule HLA-DR (Table 2), but such data alone are unreliable indicators of Th-cell responses.[34] The panel of modified and original peptides was, therefore, tested for the ability to stimulate proliferation by PBMC from 13 RhD-negative donors (Table 1) who had developed anti-D antibodies following incompatible pregnancy (Figure 1). As expected, there was a high rate of responsiveness to each of the original peptides.[1] Although most analogs were less stimulatory than the parental version, at least one modified peptide derived from each of the four original sequences induced a significant proliferative response in over 60% of donors tested (Figure 1).

### Selection of RhD peptides for therapeutic product (RhDP$_{mix}$)

It was intended to combine peptides derived from each of the four immunodominant RhD sequences into a single therapeutic product for women with anti-D antibodies, since this would maximize both efficacy and coverage of the target population.[1,2,27] The criteria used to select which version of each peptide should be chosen were a low GRAVY score, high measured solubility, retention of T-cell recognition as well as minimal changes from wild-type sequence. On this basis, RhD6$_{52-66}$M1, RhD13$_{97-111}$, RhD17$_{117-131}$M1 and RhD28$_{177-191}$M4 were chosen to comprise the RhD peptide mixture (RhDP$_{mix}$).

### Treatment with RhDP$_{mix}$ suppresses established immune responses to the RhD protein in DR transgenic mice

The next aim was to determine whether administration

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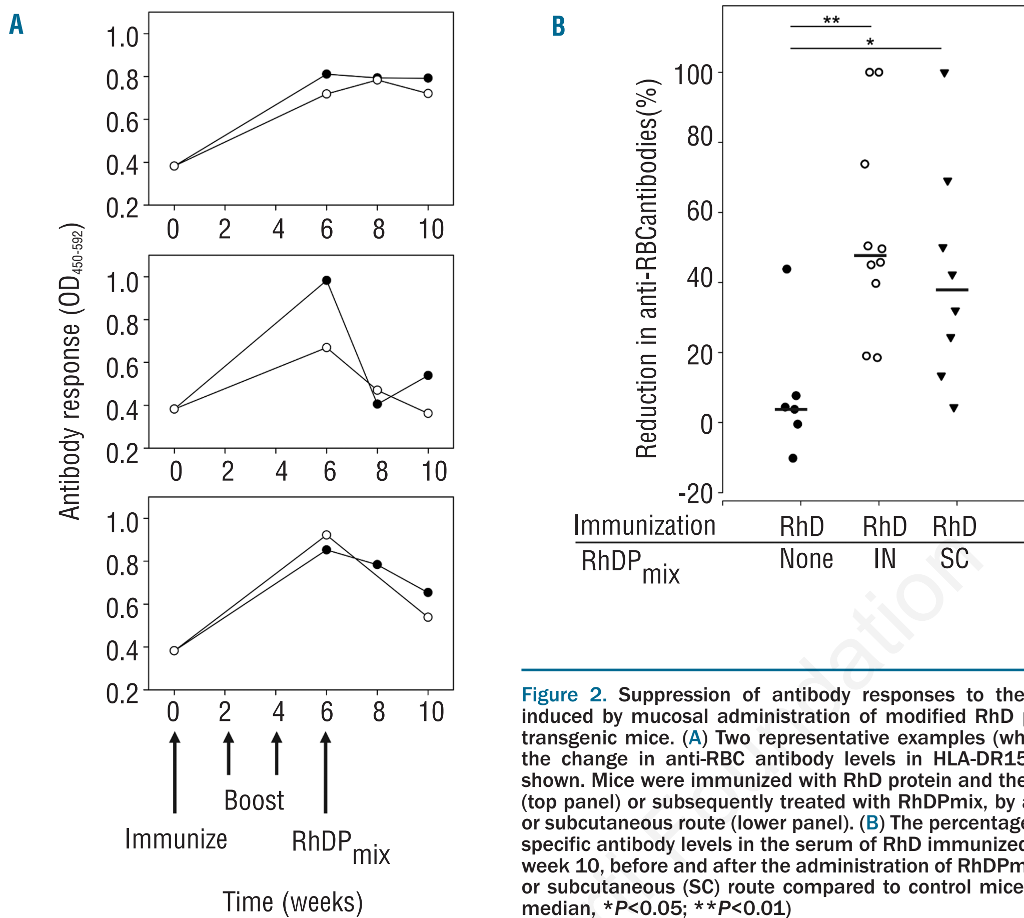


Figure 2. Suppression of antibody responses to the RhD protein can be induced by mucosal administration of modified RhD peptides in HLA-DR15 transgenic mice. (A) Two representative examples (white or black circles) of the change in anti-RBC antibody levels in HLA-DR15 transgenic mice are shown. Mice were immunized with RhD protein and then either left untreated (top panel) or subsequently treated with RhDPmix, by a nasal (middle panel) or subcutaneous route (lower panel). (B) The percentage reduction in anti-RBC specific antibody levels in the serum of RhD immunized mice, from week 6 to week 10, before and after the administration of RhDPmix by an intranasal (IN) or subcutaneous (SC) route compared to control mice is shown (n≥6, line = median, **P*<0.05; ***P*<0.01)

of $RhDP_{mix}$ would suppress established immune response to the RhD protein in a pre-clinical model. Conventional mice are refractory to RhD immunization, and so we exploited the HLA-DR15 transgenic strain that mounts both Th and antibody responses to the RhD protein.[2]

HLA-DR15 transgenic mice were immunized with purified RhD protein, boosted twice and, six weeks after initiation of the immunization schedule, received $RhDP_{mix}$. As previously reported[2] the immunization protocol induced IgG antibodies that bind RhD-positive RBC, and serial sampling of mice revealed that, without further intervention, the levels remained constant for at least six weeks after the final booster (Figure 2). The effects of nasal and subcutaneous $RhDP_{mix}$ on the established antibody response were compared, since, although mucosal administration was originally thought necessary to confer tolerogenic properties on peptides,[11,16,18,19] the novelty of the route may impede regulatory approval of any subsequent human trial, and there is now evidence in other systems that soluble peptides may also be suppressive *via* more conventional delivery.[35] Although tolerance in other models has been induced by repeated low doses of peptide, a single, larger administration can also be effective, and the $RhDP_{mix}$ was given using the latter approach, since it represents the more practical regimen for human use. The dosage chosen, 100 μg of each peptide, reflects experience elsewhere.[16,36,37] The kinetics of changes in antibody levels between serial blood samples from individual mice are illustrated in Figure 2A, and the results are summarized in Figure 2B. It can be seen that antibody levels declined significantly within four weeks after dosing with peptide by either route of administration (intranasal *P*=0.008; subcutaneous *P*=0.043, Mann-Whitney rank sum test). These rapid falls are in contrast to the persistent elevation seen in immunized controls that had received no $RhDP_{mix}$. The response was dominated by IgG1 rather than IgG2a, and this bias was retained in any residual antibody levels detected after peptide treatment (*Online Supplementary Figure S1*). Although there was a trend for larger reductions in antibody response after intranasal *versus* subcutaneous peptide dosing, this difference was not significant.

Antibody production by HLA-DR15 transgenic mice after immunization with RhD protein is accompanied by Th activation,[2] and so we also determined the effects of $RhDP_{mix}$ treatment by both routes on splenocyte Th proliferation and IFN-γ secretion in response to purified RhD protein since tolerance to the entire protein was the therapeutic goal (Figure 3 and *Online Supplementary Figure S2A*). These responses were significantly reduced in RhD immunized mice that had been given intranasal $RhDP_{mix}$ compared to those receiving no peptide (proliferation *P*=0.028; IFN-γ *P*=0.037, Mann-Whitney rank sum test). Inhibition of the IFN-γ responses in peptide-treated mice was manifest not only by reductions in the secreted cytokine, but also by lower numbers of splenic $CD4^+$ T cells producing IFN-γ (*Online Supplementary Figure S2C*). Mean responses, particularly IFN-γ secretion, were also lower when $RhDP_{mix}$ was administered subcutaneously, but these reductions were less than for the intranasal route, and not significant.

Table 2. Modifications to immunodominant RhD peptides.

| Peptide ID | Peptide sequence | GRAVY score | Binding prediction ($IC_{50}$) | % soln*** (BCA Assay) |
|---|---|---|---|---|
| RhD06$_{52-66}$ | QDLTVMAAIGLGFLT | 1.28 | 427 | 0.0 |
| RhD06$_{52-66}$ M1 | **K**QDLTVMAAIGLGFLT**K** | 0.671 | 348 | 100.0 |
| RhD06$_{52-66}$ M2 | **pgQ**DLTVMAAIGLGFLT**SSFRR** | (0.570)* | (75.2)** | 36.8 |
| RhD06$_{52-66}$ M3 | **pgD**LTVMAAIGLGFLT**KK** | (0.931)* | (509.2)** | 100.0 |
| RhD06$_{52-66}$ M4 | **pgD**LTVMAAIGLGFLTK**KK** | (0.647)* | (509.2)** | 86.9 |
| RhD13$_{97-111}$ | FLSQFPSGKVVITLF | 1.147 | 192.7 | 100.0 |
| RhD13$_{97-111}$ M1 | KFLSQFPSGKVVITLF**K** | 0.553 | 121.3 | 100.0 |
| RhD13$_{97-111}$ M2 | KKFLSQFPSGKVVITLF**KK** | 0.084 | 76.9 | 99.1 |
| RhD13$_{97-111}$ M3 | FLSQFPSGKVVITLF**KK** | 0.553 | 192.7 | 100.0 |
| RhD13$_{97-111}$ M4 | FLSQFPSGKVVITLFK**KK** | 0.306 | 192.7 | 100.0 |
| RhD17$_{117-131}$ | TMSALSVLISVDAVL | 1.827 | 201.4 | 0.0 |
| RhD17$_{117-131}$ M1 | **KK**TMSALSVLISVDAVL**GKK** | 0.57 | 189.6 | 100.0 |
| RhD17$_{117-131}$ M2 | **KKK**TMSALSVLISVDAVL**GKKK** | 0.164 | 189.6 | 100.0 |
| RhD17$_{117-131}$ M3 | TMSALSVLISVDAVL**GKKK** | 0.805 | 189.6 | 100.0 |
| RhD17$_{117-131}$ M4 | TMSALSVLISVDAVL**GKKKK** | 0.570 | 189.6 | 100.0 |
| RhD17$_{117-131}$ M5 | **KSIRLAT**MSALSV**K** | 0.457 | na | 100.0 |
| RhD17$_{117-131}$ M6 | **SIRLAT**MSALSV**KK** | 0.457 | na | 100.0 |
| RhD17$_{117-131}$ M7 | **K**TMSALSVLISVD**K** | 0.700 | na | 55.6 |
| RhD17$_{117-131}$ M8 | **KK**TMSALSVLISVD**KK** | 0.125 | 637.1 | 100.0 |
| RhD17$_{117-131}$ M9 | TMSALSVLISVD**KKK** | 0.393 | 620 | 100.0 |
| RhD17$_{117-131}$ M10 | TMSALSVLISVD**KKKK** | 0.125 | 513.9 | 100.0 |
| RhD28$_{177-191}$ | AYFGLSVAWCLPKPL | 0.933 | 103.4 | 98.5 |
| RhD28$_{177-191}$ M1 | **K**AYFGLSVAWCLPKPL**K** | 0.365 | 87.6 | 100.0 |
| RhD28$_{177-191}$ M2 | **K**AYFGLSVAWSLPKPL**K** | 0.171 | 38.6 | 100.0 |
| RhD28$_{177-191}$ M3 | AYFGLSVAWCLPKPL**KK** | 0.365 | 103.4 | 100.0 |
| RhD28$_{177-191}$ M4 | AYFGLSVAWSLPKPL**KK** | 0.171 | 38.6 | 100.0 |
| RhD28$_{177-191}$ M5 | AYFGLSVAWCLPKPLKKK | 0.128 | 103.4 | 100.0 |
| RhD28$_{177-191}$ M6 | AYFGLSVAWSLPKPLKKK | -0.056 | 38.6 | 100.0 |

*The table shows the predicted affinity of binding to HLA-DR15, grand average of hydropathicity (GRAVY) score and measured solubility for the wild-type and modified RhD peptide sequences. MHC binding predictions of modified versus parent peptides with a ∅IC50>100 were considered to indicate a change in the likelihood of presentation. A reduction in GRAVY score >0.5 is suggestive of decreasing solubility. Measured solubility was determined from the percentage of the peptide measured by BCA assay in the supernatant at 5mg/ml in dH20. The selected peptides used to generate RhDPmix are highlighted in gray. Protparam and NN-align were accessed on 11th November 2012. Sequence selected for RhDPmix shaded Alterations from parent sequence in bold. *Protparam software, http://web.expasy.org/protparam, values in brackets are based on the sequence without the addition of pyroglutamate (which cannot be analyzed by the software). **NN-Align software, http://tools.immuneepitope.org/analyze/html/mhc II_binding.htm, values in brackets are based on the sequence without the addition of pyroglutamate (which cannot be analyzed by the software). ***Percent in solution at 5 mg/mL (in dH2O) measured by BCA assay.*

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### *Treatment with RhDP$_{mix}$ induces expansion of Foxp3$^{+}$ Treg cells*

The final question was whether the suppression of responses to RhD protein in mice that had received the RhDP$_{mix}$ was accompanied by expansion of Treg cell numbers. Splenocytes were isolated from mice that been immunized with RhD protein and then received RhDP$_{mix}$ either intranasally or subcutaneously, or from unimmunized controls, and the numbers of cells with a CD3$^{+}$CD4$^{+}$CD25$^{+}$Foxp3$^{+}$ Treg phenotype enumerated by flow cytometry after no further *in vitro* stimulus. To determine whether the Treg population was capable of expanding further in response to specific antigen, parallel cultures were also stimulated with RhD protein. Representative analyses are illustrated in Figure 4A, and the data summarized in Figure 4B. Immunization alone, or followed by RhDP$_{mix}$ treatment of mice *via* either route, had no significant effect on the proportions of Treg phenotype cells unless splenocytes were further stimulated with RhD protein *in vitro*. RhD stimulation of cultures elicited striking, significant expansions of the Treg phenotype population from mice that received both immunization and either intranasal ($P$=0.013, Mann-Whitney rank sum test) or subcutaneous ($P$=0.024) RhDP$_{mix}$ treatment. It was confirmed that the Treg population in these stimulated cultures increased not only in proportion to the CD4$^{+}$ fraction, but also in absolute numbers (*Online Supplementary Figure S3*). There was no Treg expansion when splenocytes from unimmunized mice were stimulated, and although the proportions of Treg cells increased in stimulated cultures ($P$=0.001) when mice had been immunized but not RhDP$_{mix}$ treated, this expansion was not necessarily reflected in absolute numbers, and was significantly lower than for animals that also received the peptides (intranasal $P$=0.012; subcutaneous $P$=0.039). Although Foxp3 is commonly accepted as a reliable marker for Treg cells,[24] it can also be induced in activated Th-cells, with or without the acquisition of regulatory ability.[23,38] We, therefore, further characterized the expansion of Foxp3$^{+}$ T cells after RhD stimulation of splenocytes from RhD immunized mice to determine whether it was due to recruitment

of existing Treg or *de novo* induction of Foxp3. The transcription factor Helios has been reported to be expressed by thymically committed natural Treg, but not by T cells with induced Foxp3.[39,40] In cultures taken from animals that had been treated with RhDP$_{mix}$, there was an increase in the population of CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ cells that expressed high levels of Helios after stimulation with the RhD protein ($P$<0.01, two-tailed t-test) (Figure 5), consistent with the expansion being largely attributable to a natural Treg phenotype. Regulatory cells of this type have been consistently described to inhibit *in vitro via* mechanisms that are independent of cytokine secretion,[41,42] and there was no increase in the levels of the archetypal suppressive cytokine IL-10 in the stimulated cultures from RhD-immunized mice that had received peptide therapy compared to those that were untreated (respective median IL-10 concentrations 107 pg/mL *vs.* 102 pg/mL, n>12). Finally, we confirmed the suppressive function of the putative Treg population that was expanded in the RhDP$_{mix}$ tolerized mice, by demonstrating their ability to inhibit effector T-cell responses to RhD protein in a dose dependent manner (Figure 5C).

Our interpretation of these data is that treatment with

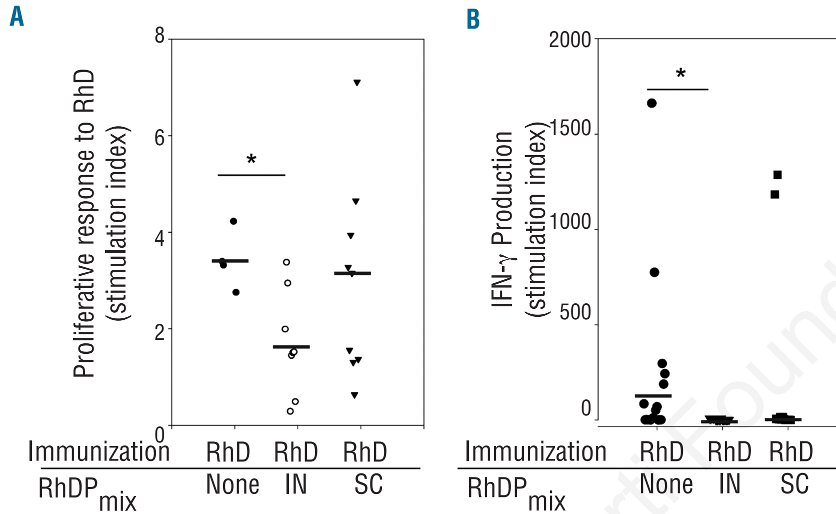


**Figure 3.** Treatment of RhD immunized mice with RhDP$_{mix}$ is associated with a decrease in Th1 responses. Proliferative (A) and IFN-γ (B) responses to RhD protein by splenocytes that have been isolated from RhD immunized mice given RhDP$_{mix}$ by intranasal or subcutaneous routes (n≥4, line = median, *$P$<0.05)

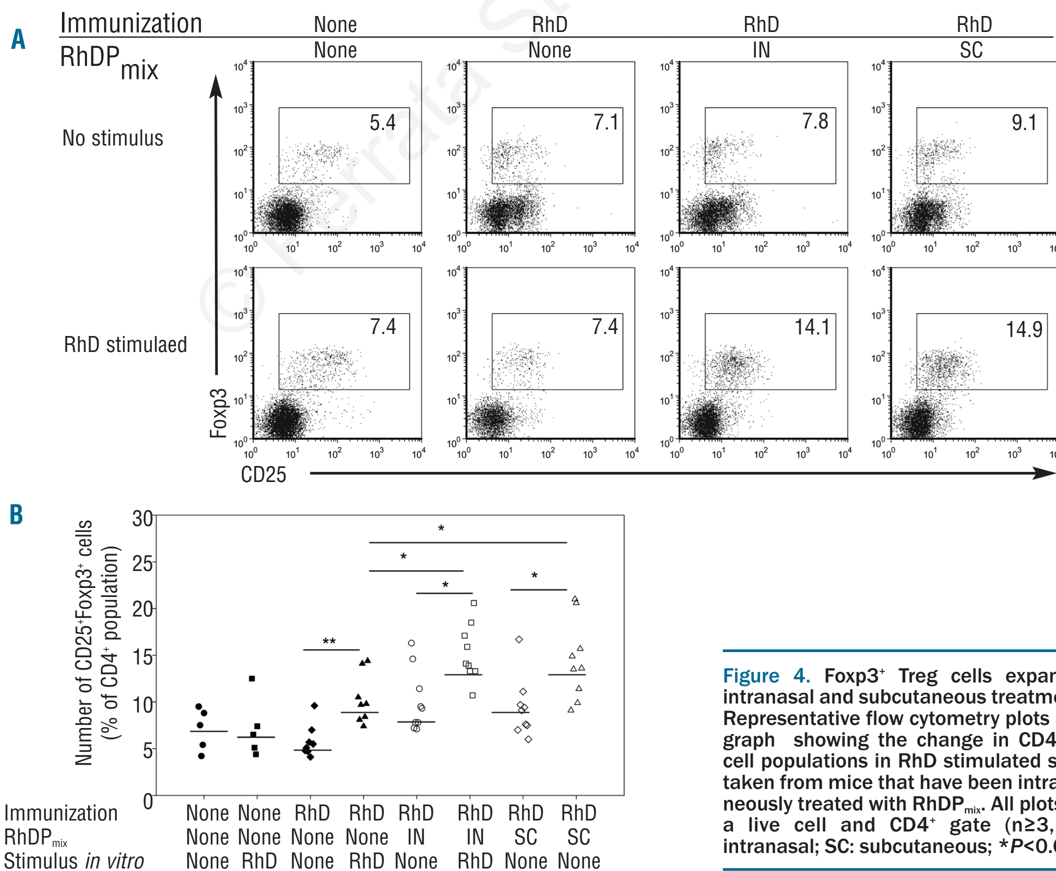


**Figure 4.** Foxp3$^+$ Treg cells expand in response to intranasal and subcutaneous treatment with RhDP$_{mix}$ (A) Representative flow cytometry plots and (B) a summary graph showing the change in CD4$^+$CD25$^+$Foxp3$^+$ Treg cell populations in RhD stimulated splenocyte cultures, taken from mice that have been intranasally or subcutaneously treated with RhDP$_{mix}$. All plots were restricted by a live cell and CD4$^+$ gate (n≥3, line: median; IN: intranasal; SC: subcutaneous; *$P$<0.05; **$P$<0.01).

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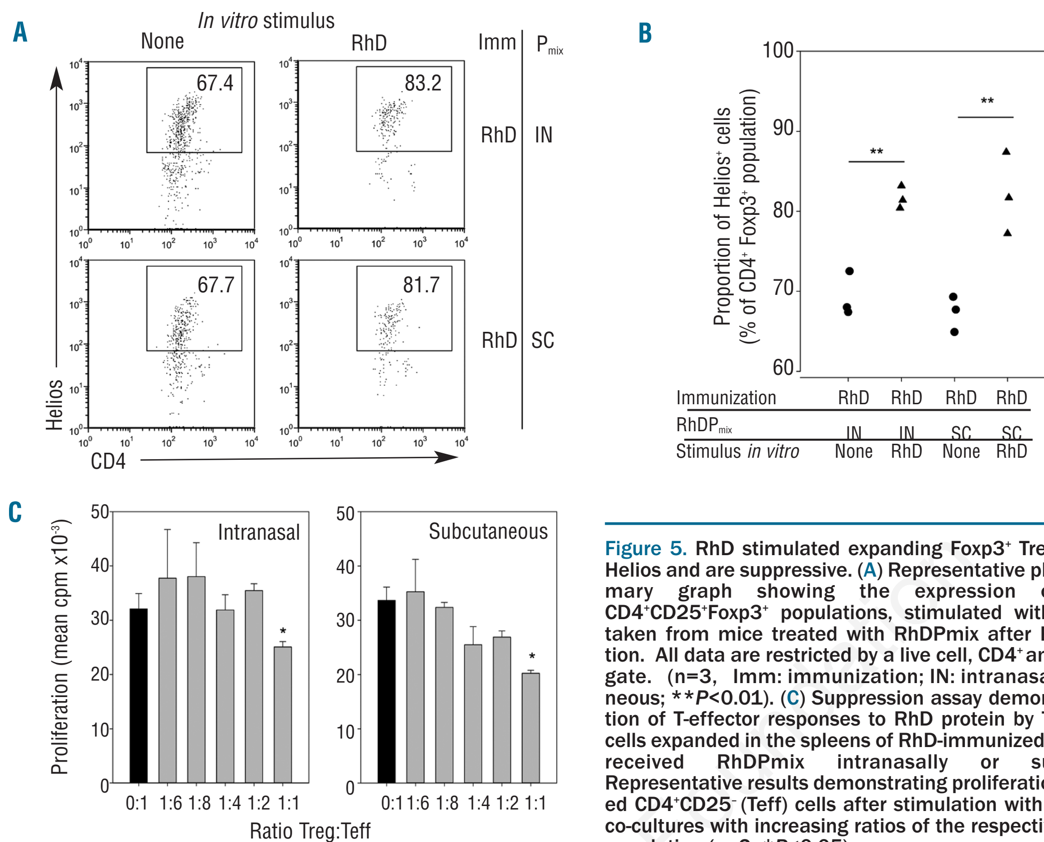


**Figure 5. RhD stimulated expanding Foxp3+ Treg cells express Helios and are suppressive.** (A) Representative plot and (B) summary graph showing the expression of Helios in CD4+CD25+Foxp3+ populations, stimulated with RhD protein, taken from mice treated with RhDPmix after RhD immunization. All data are restricted by a live cell, CD4+ and CD25+Foxp3+ gate. (n=3, Imm: immunization; IN: intranasal; SC: subcutaneous; **$P<0.01$). (C) Suppression assay demonstrating inhibition of T-effector responses to RhD protein by Treg phenotype cells expanded in the spleens of RhD-immunized mice that have received RhDPmix intranasally or subcutaneously. Representative results demonstrating proliferation of fractionated CD4+CD25- (Teff) cells after stimulation with RhD protein in co-cultures with increasing ratios of the respective CD25+ (Treg) population (n=2, *$P<0.05$).

$RhDP_{mix}$ inhibits responses to prior immunization with RhD protein immunization, and that either intranasal or subcutaneous administration of a single dose can have suppressive effects and recruit specific Treg cells. In comparisons of efficacy, there are no clear advantages in using the intranasal route.

## Discussion

The main finding reported here is that treatment with peptides containing immunodominant helper epitopes can suppress established immune responses to the RhD protein in a pre-clinical murine model. A combination of four previously identified peptides was modified to improve solubility and manufacturability, whilst retaining human alloreactive Th-cell recognition, and successfully tested for suppressive ability in humanized HLA-DR transgenic mice that had responded to immunization with RhD protein. These results support the case for human clinical trials of this peptide combination as the first specific treatment for women with anti-D alloantibodies. It is now well established that prior administration of peptides corresponding to the sequence of immunodominant antigens, by a mucosal route, can prevent induction of respective immune responses in animal models,[16,19,43] and we have previously reported that pre-treatment with each of the four dominant RhD peptides can block subsequent immunization of mice with RhD protein.[2,16,19,43] However, there is surprisingly little evidence that tolerance can be established to an existing immune response, with isolated reports of peptide therapy ameliorating ongoing EAE22 or AIHA in the NZB mouse.[11] It was, therefore, important to determine whether the response to prior immunization with RhD protein could also be suppressed by peptide therapy, because the unmet clinical need and lead indication for human trials is the treatment of women with existing anti-D antibodies due to failure of the current passive prophylaxis.

The product developed here comprised a mixture of four immunodominant peptides derived from the sequence of the RhD protein,[1] each modified if necessary to improve solubility whilst preserving the Th epitopes they contain. Solubility is a key feature in the efficient manufacture of pure peptides, and in their ability to induce tolerance.[10,11] Importantly, the peptides we designed and selected for the $RhDP_{mix}$ product require no solubilizing excipients such as DMSO, which are commonly used and convenient for laboratory work,[2] but which introduce a barrier to regulatory approval for human use. Delivered in a simple single dosing regimen, $RhDP_{mix}$ significantly inhibited an ongoing antibody response to RhD protein in HLA-DR15 transgenic mice, resolving the issue as to whether such peptide therapy can rapidly lower antibody levels. Although peptide treatment has also been reported to alter the ratio of IgG subclasses produced in an antibody response, and thereby modify disease,[32] no evidence of such an effect was seen in the current work. There was no significant difference in suppression of the antibody response between peptide delivered intranasally or subcutaneously, providing support for an injected route that may be more acceptable to the regulatory authorities for human treatment.

This study also provides evidence as to how $RhDP_{mix}$ exerts tolerogenic effects. Peptide administration, most

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markedly *via* the nasal route, resulted in reductions in the Th response to RhD protein by immunized mice, including effector cytokine secretion. Such deprivation of help would be expected to curtail ongoing B-cell activation and the production of further antibody, but does not fully account for the rapidity with which antibody levels declined. Furthermore, subcutaneous peptide suppressed antibody more completely than Th responses, adding to the examples of peptide therapies that are effective despite not abrogating entirely all arms of the specific immune response.[11,18] One explanation for these effects would be the induction of Treg cells able to suppress not only Th, but also B-cell responses directly.[11,19,20,44] Delivery of $RhDP_{mix}$ by either route was associated with a population of cells expressing the $CD3^{+}CD4^{+}CD25^{+}Foxp3^{+}$ Treg phenotype, which could be expanded by RhD protein stimulation *in vitro*. This expansion was greatest in mice that had both been immunized with RhD protein and received $RhDP_{mix}$, consistent with the recruitment of a specific Treg population. Although Treg cells are often considered anergic, they can proliferate after activation *via* the T-cell receptor,[45-47] and human Treg cells responsive to the RhD protein have been cloned.[13] IL-2 is well-known for its ability to reverse T-cell anergy, and could have been produced in our cultures by effector cells prior to their suppression, but may not be an absolute requirement for the propagation of all Treg types, particularly those of thymic origin.[48-50] Predominant expression of Helios within the expanded population suggests that it did contain a majority of committed, thymically derived Treg cells, rather than reflecting *de novo* induction of Foxp3.[39,42] The reliability of Helios in the identification of such cells has been questioned,[51] but it remains a widely used marker.[40,50] Classically, natural Treg inhibition *in vitro* is not mediated by secreted cytokine, but *via* poorly defined contacts with other cells, and the cells induced by peptide conformed to this pattern, since they did not secrete the key inhibitory cytokine IL-10.

The induction of specific Treg cells underlies the inhibitory effects of peptide therapy in many other systems,[10,16,20,21,26,27,35] and appears to be a major mechanism of tolerance in the current study. An alternative explanation could be that RhD peptides given to mice at the peak of response provoked activation-induced cell death, or deletion, of the corresponding effector T-cell populations. However, peptide treatment of RhD-immunized mice induced T cells that not only had a regulatory phenotype, but also a suppressive function *in vitro*, and there was no lack of effector T cells responsive to RhD protein in the spleens of these animals. We cannot exclude the possibility that effector T cells were to some degree depleted after peptide administration *in vivo*, but this would not necessarily be inimical to regulation, since transient proliferation and activation-induced cell death can presage Treg development.[52] Reports of cellular immunotherapy for autoimmune disease and transplant rejection[53-56] confirm that tolerance transferred by Treg populations is optimal if specific cells are activated by cognate antigen in association with MHC class II,[53] and our data illustrate that this effect can be achieved by the simple delivery of relevant peptides *in vivo*.

The current work focuses on the development of peptide therapy for women with anti-D antibodies who are at risk of pregnancies affected by HDN. Parallel approaches have been taken in other immune-mediated diseases, and human clinical trial data are emerging in allergy,[57] rheumatoid arthritis,[58] multiple sclerosis,[59] and type 1 diabetes[60] that support short immunotherapeutic peptides as a viable and successful treatment modality.[10] Thus, although the translation of advances in our understanding of immune tolerance and regulation to human therapies has been protracted,[61] treatments based on peptide immunotherapy now show considerable promise.


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