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Prophylactic immune tolerance induced by changing the ratio of antigen-specific effector to regulatory T cells

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Summary

Background—Gene and protein replacement therapies for inherited protein deficiencies such as hemophilia or lysosomal storage disorders are limited by deleterious immune responses directed against their respective therapeutic protein. Therefore, the development of protocols preventing such responses is key to providing successful long-term therapy.

Objectives—We sought to develop a protocol, utilizing a drug/peptide cocktail, that would effectively shift the antigen-specific CD4⁺ T cell population; tipping the balance from effector T cells (Teff) towards regulatory T cells (Treg).

Methods—Treg-deficient (DO11.10-tg Rag2^{−/−}) BALB/c mice were used to screen for an optimal protocol addressing the aforementioned goal and to study the mechanisms underlying *in vivo* changes in T cell populations. Muscle directed gene transfer to hemophilia B mice was also performed in order to test the optimal protocol in a therapeutically relevant setting.

Results—Specific antigen administration (4-week repeated dosing) combined with rapamycin and IL-10 led to substantial reductions in Teff, via activation-induced cell death, and induced CD4⁺CD25⁺FoxP3⁺ Treg to high frequency in multiple organs. The proportion of apoptotic T cells also increased over time, while Teff and Treg were differentially affected. When applied to a model of protein deficiency (gene therapy for hemophilia B), the protocol successfully prevented inhibitor formation whereas non-specific immune suppression was only marginally effective.

Conclusions—It is feasible to provide a short-term, prophylactic protocol allowing for the induction of immune tolerance. This protocol may provide a marked advance in efforts seeking to improve clinical outcomes in disorders involving therapeutic protein replacement.

Keywords

rapamycin; tolerance; factor IX; regulatory T cells; gene therapy; hemophilia

Introduction

Treatment of inherited protein deficiencies such as hemophilia, lysosomal storage diseases (LSDs), and others are constrained by unwanted immune responses, including formation of antibodies against the therapeutic protein. Treatment of hemophilia is currently based on intravenous infusion of coagulation factor concentrate. Several clinical trials have been

performed to develop a gene therapy capable of providing a continuous supply of clotting factor [1]. In subjects with hemophilia B (i.e., a deficiency of factor IX, F.IX), underlying mutations with a greater loss of coding information are more likely to be associated with an adaptive immune response against infused functional protein [2,3]. In 9-23% of patients with severe disease (i.e., 3-4% of all hemophilia B patients) receiving protein therapy, inhibitory antibodies against F.IX develop [2]. The incidence of inhibitor formation in hemophilia A is higher (i.e., approximately 20% overall and in 40% of subjects with severe disease, with an even higher prevalence for some ethnic groups), and is influenced by additional genetic factors such as polymorphisms in genes encoding cytokines [4]. Unfortunately, treatment of subjects with inhibitors is complicated and expensive, and immune tolerance protocols based on frequent high dose infusion of clotting factor are less effective for F.IX.

Therapies for other genetic diseases encounter similar problems. For example, antibody formation is frequent in enzyme replacement therapies for LSDs such as Pompe disease or mucopolysaccharidosis I [5]. In Pompe disease and hemophilia B, anaphylactic responses against the therapeutic protein can occur. Interestingly, some gene therapy protocols for hemophilia may be viewed as favorable due to certain safety aspects or simplicity of vector administration; but may, for example in muscle-directed gene transfer, pose an increased risk of immune responses against the clotting factor gene product [6]. In sum, there are a variety of diseases, treatment approaches, and patient populations that would benefit tremendously by the development of a prophylactic, antigen-specific protocol capable of inducing immune tolerance to the therapeutic protein prior to the onset of therapy, allowing for avoidance of subsequent complications and immunotoxicities.

From a mechanistic perspective, antibodies formed against clotting factors are typically dependent on T cell help. In addition, studies on gene transfer-induced tolerance to F.IX uncovered a critical role of regulatory CD4⁺CD25⁺ T cells (Treg) [7]. Therefore, we sought to develop a protocol that would direct a shift in the antigen-specific CD4⁺ T cell population from conventional T cells to Treg. We considered rapamycin (sirolimus), a drug used to prevent transplant rejection, as a potential tool for this purpose given its ability to suppress T cell signaling in response to growth factors through binding to intracellular protein FKBP12, which in turn inhibits the protein kinase mammalian Target Of Rapamycin (mTOR). At the same time, this compound does not interfere with the initial steps of T cell activation (T cell receptor (TCR) signaling or calcineurin activity). The consequence is a block in cell cycle progression, leading to inhibition of proliferation and potentially to activation induced cell death (AICD) [8]. Importantly, antigen presentation in the presence of rapamycin leads to selective expansion of Treg *in vitro* [9]. Stable systemic expression of the cytokine IL-10 has also been shown to impart an increase in Treg frequency [10]. Administration of rapamycin combined with IL-10 prevented type 1 diabetes via an induction of Treg and T Regulatory Type 1 (Tr1) cells in non-obese diabetic mice [11].

This current study introduces an *in vivo* screening strategy for drug combinations that promotes antigen-specific CD4⁺ T cell tolerance. A 1-month protocol involving repeated co-administration of rapamycin, IL-10, and specific antigen directed a substantial shift in CD4⁺ T cells by deleting effector T cells (Teff) while inducing Treg, and successfully prevented inhibitor formation in gene therapy for hemophilia B.

Methods

Reagents

Peptide A2-54 containing the dominant CD4⁺ T cell epitope for human F.IX (hF.IX) in C3H/HeJ mice and chicken ovalbumin (ova) peptide amino acids 323 to 339 were from

Anaspec (San Jose, CA) [12]. Murine IL-10 (Sigma Aldrich, St. Louis, MO) and rapamycin (LC Laboratories, Woburn, MA) stock solutions were made in 0.02% CMC and 0.25% Tween-80. AAV-CMV-hF.IX vector was as previously described [13]. It contains the hF.IX cDNA (plus a 1.4-kb portion of the first intron) expressed from the cytomegalovirus immediate-early enhancer/promoter. Adeno-associated virus (AAV) serotype 1 vector was produced by triple transfection of HEK-293 cells, purified by gradient centrifugation, and titered by quantitative slot-blot hybridization as described [14].

Animal studies

BALB/c mice transgenic for ovalbumin-specific DO11.10 CD4⁺ TCR and having Rag-2 deletion (DO11.10-tg Rag-2^{-/-}) were obtained from Taconic (Germantown, NY). C3H/HeJ were from Jackson Laboratories (Bar Harbor, ME). F.IX knockout mice on a C3H/HeJ background (C3H/HeJ-F9^{-/-}) were as published [15]. All mice were male and 6-8 weeks old at the onset of experiments. Drug cocktails were administered by intraperitoneal (IP) injection in 200 µl of sterile PBS, 3-times per week. Ova or hF.IX-specific peptide (2A-54) were given at 100 µg/dose, rapamycin at 4 mg/kg/dose, and IL-10 at 50 ng/kg/dose. Viral vector was administered at 1×10¹¹ vector genomes [vg]/mouse by intramuscular (IM) injection into quadriceps and tibialis anterior muscles [13]. Blood samples were obtained using microhematocrit capillary tubes via the retro-orbital plexus for ELISA or via tail-bleed into 3.8% sodium citrate buffer for clotting assays (or into EDTA tubes for whole blood analysis). Animals were housed in specific pathogen free conditions.

Analyses of plasma samples

Levels of hF.IX were determined by ELISA as previously published [7]. Activated partial thromboplastin time (aPTT) was measured with a fibrometer (Fibrosystem; BBL, Cockeysville, Maryland). Bethesda assay for measuring the titer of inhibitory antibodies was performed as published [13]. One Bethesda unit (BU) represents a residual hF.IX activity of 50%.

Flow cytometry

Cells were isolated from the spleen, inguinal, popliteal lymph nodes, thymus and peripheral blood using standard protocols. Viable cells were enumerated using a hemocytometer and trypan blue. Staining for surface and intracellular molecules was carried out according to manufacturers' protocols. Antibody-fluorochrome combinations are listed in Supplementary Data. Apoptosis was measured with the AnnexinV-PE Apoptosis detection kit (BD Biosciences/Pharmingen, USA). Data acquisition was carried out using the BD LSR II and BD FACSCalibur system. Data analysis was done with the FACS DIVA 6.1 and CellQuest software.

Cytokine secretion assay

Mouse Cytokine Secretion Assay-Detection Kit (PE) (Miltenyi Biotech, Auburn, CA) was used as per the manufacturer's recommendations. Briefly, splenocytes from DO11.10-tg Rag^{-/-} mice were re-suspended in 5-mMLC medium at 1 × 10⁷ cells/ml and added to a round-bottom 96-well plate (1 × 10⁶/well in triplicate) for *in vitro* stimulation with ova peptide (final concentration of 10 µg/ml) followed by IL-4/IL-10 secretion assay. IL-4 and IL-10 expressing CD4⁺ T cell were identified by flow cytometry using anti-murine CD4-FITC, anti-murine IL4-PE, and anti-murine IL-10-APC.

In vitro and in vivo suppression assays

Splenic CD4⁺ cells were isolated using negative selection, CD25⁺ T cells were isolated from the enriched CD4⁺ population by positive selection using magnetic cell sorting (Miltenyi

Biotech). Cells were plated in triplicates in flat-bottom 96-well plates. The Celltiter96 Aqueous Non-Radioactive Cell Proliferation assay (Promega, Madison, WI) was used to assess suppression of effector T cell proliferation in the presence of Treg cells. The plate was read at 490nm using an ELISA reader (Biorad, Hercules CA). For adoptive transfer studies, CD4⁺CD25⁺ splenocytes from tolerized C3H/HeJ F9^{-/-} mice were purified by magnetic sorting, pooled and injected at 1×10⁶ cells/mouse into tail veins of wild-type C3H/HeJ mice [12]. Recipient mice received IM injection of AAV-CMV-hF.IX (1×10¹¹ vg/mouse) and were followed for anti-hF.IX formation.

Statistical analysis

Results are represented as mean values ± SD. Assessment of significant differences between means was done by unpaired student's t-test. *P* values of <0.05 were considered significant.

Results

Rapamycin in combination with IL-10 and a specific peptide depletes Teff cells and induces CD4⁺CD25⁺FoxP3⁺ Treg

CD4⁺ T cells of DO.11.10-tg RAG-2^{-/-} BALB/c mice are entirely specific for ova antigen and lack Treg because of a lack of endogenous rearranged TCRs. The transgenic TCR is specific for ova peptide-I-A^d MHC II complex [16]. We had used these mice previously to demonstrate induction of Treg by hepatic ova gene transfer [7]. Here, we tested whether this strain could be useful to screen for antigen/drug combinations that may be optimal for induction of CD4⁺ T cell tolerance. DO.11.10-tg RAG-2^{-/-} mice were injected IP 3-times per week for 4 weeks with a) rapamycin and ova, b) IL-10 and ova, or c) a combination of rapamycin, IL-10, and ova (n=4 per experimental group). Control animals received rapamycin, IL-10, and an irrelevant hF.IX peptide. When splenocytes were analyzed 1 day after the last dosing, IL-10/ova-treated mice had slightly reduced CD4⁺ T cell numbers compared to control treated and naïve animals, which gave similar results (Fig. 1 A, B, and data not shown). However, rapamycin/ova-, and even more so, rapamycin/IL-10/ova-treated mice, showed a substantial reduction (up to 5.5-fold) compared to controls (Fig. 1A, B). Control mice treated with rapamycin/IL-10/irrelevant peptide did not show induction of CD4⁺CD25⁺FoxP3⁺ T cells (<1% of splenic CD4⁺ T cells by flow cytometry). The frequency of Treg was increased to ~3% in IL-10/ova-treated mice, to ~8% in rapamycin/ova-treated, and to ~14% in rapamycin/IL-10/ova-treated mice (Fig. 1C). Induced Treg also stained positive for CD62L and CTLA-4 (data not shown).

Upon *in vitro* stimulation with ova, we were unable to detect IL-10⁺ or IL-4⁺ T cells in any experimental group, except for IL-10/ova-treated mice, which showed an ova-specific CD4⁺IL-10⁺IL-4⁺ response, indicating Th2 activation (Fig.1D). A substantial decrease in CD4⁺ T cells was again seen in this assay for rapamycin/IL-10/ova-treated mice.

Next, we investigated the effect of the most optimal protocol on different lymphoid organs. Rapamycin/IL-10/ova was administered IP 3-times per week for 3 weeks to DO11.10-tg Rag-2^{-/-} mice. Control animals received an irrelevant hF.IX peptide instead of ova. Subsequent flow cytometric analysis showed that CD4⁺ cell frequencies were markedly reduced in peripheral blood and secondary lymphoid organs (spleen, inguinal and popliteal lymph nodes) and slightly reduced in the thymus (Fig. 2B-E). Mice receiving an irrelevant peptide showed minor reduction (spleen) or no change (lymph nodes and thymus) in CD4⁺ T cell numbers compared to naïve mice (Fig. 2B-E). Mice treated with rapamycin/IL-10/irrelevant peptide, similar to naïve mice, had again no detectable CD4⁺CD25⁺FoxP3⁺ cells (<0.4%) in any of the organs analyzed. In contrast, a substantial induction of Treg to ~20% of CD4⁺ T cells was observed in peripheral blood and all secondary lymphoid organs in

rapamycin/IL-10/ova-treated mice (Fig. 2A, F, G, I). Treg were also detectable in the thymus, albeit less consistent and with lower frequency (~0.9%, with one animal achieving 7% of CD4⁺ T cells, compared to 0.1-0.3% in control mice, Fig. 2A, H).

Activation and apoptosis of ova-specific CD4⁺ T cells

DO.11.10-tg RAG-2^{-/-} mice were injected IP 3-times per week for 1 week with rapamycin/IL-10/ova. When analyzed 1 day after the last drug administration, splenic CD4⁺ T cells showed an up-regulation of activation markers CD25, CD44, and CD69, and a down-regulation of CD62L compared to naive mice or mice receiving an irrelevant hF.IX peptide (Fig. 3A-E).

Rapamycin/IL-10/ova-treated mice also showed a 2- to 3-fold increase in Fas (CD95)⁺ and in apoptotic (Annexin V⁺) splenic CD4⁺ T cells compared to controls (Fig. 4A,B). These cells stained double positive for CD90 and annexin V (Fig. 4C). Taken together, the data demonstrate induction of AICD. As a result, the portion of apoptotic CD4⁺ T cells increased with time during treatment, causing a decline of CD4⁺ T cell numbers over time (Fig. 4D,E).

Induced Treg suppress Teff and show lower rate of apoptosis

While antigen presentation in the presence of rapamycin and IL-10 induced apoptosis of CD4⁺ T cells and consequently a decline in CD4⁺ T cell numbers, Treg were induced to high frequency; therefore, there had to be a differential effect on Teff vs. Treg. To test this hypothesis, we measured the frequency of apoptotic cells among Treg and Teff 3 weeks after treatment with rapamycin/IL-10/ova cocktail. In 3 of 4 mice, splenic CD4⁺CD25⁻FoxP3⁻ Teff clearly contained a higher proportion of annexin V⁺ cells compared to CD4⁺CD25⁺FoxP3⁺ Treg (Fig. 5A). These data indicate a higher resistance of Treg to antigen/rapamycin-induced apoptosis. As expected, rapamycin/IL-10/irrelevant peptide treated animals showed only a low level of apoptotic CD4⁺ cells (which are CD25⁻FoxP3⁻).

To demonstrate the suppressive phenotype of induced Treg, an *in vitro* suppression assay was performed on splenocytes isolated from DO.11.10-tg RAG-2^{-/-} mice after 3 weeks of treatment with rapamycin/IL-10/ova. While the tolerized splenocytes failed to proliferate upon stimulation with ova, T cell proliferation was observed in splenocyte cultures depleted for CD4⁺CD25⁺ cells (Fig. 5B). When purified Treg were added back to the depleted cells, proliferation was again suppressed (Fig. 5B).

Prevention of inhibitor formation in gene therapy for murine hemophilia B

Muscle-directed F.IX gene transfer has been tested in clinical trial and, given ever improving vectors and delivery techniques combined with non-invasiveness and safety of the procedure, would be a highly attractive treatment of hemophilia B if it was not for an increased risk of inhibitor formation [17]. We therefore decided to test our protocol in this therapeutic setting. A hF.IX-specific peptide (2A-54, containing the dominant CD4⁺ T cell epitope in C3H/HeJ mice) was co-administered with rapamycin and IL-10 3-times per week for 4 weeks to C3H/HeJ hemophilia B mice (n=4 per experimental group). These mice have a targeted F9 gene deletion and show stronger adaptive immune responses to F.IX than other inbred strains [15]. From previous studies on nasal tolerance, we knew that the peptide is effective over a wide dose range [12]. Furthermore, use of a peptide avoids the risk of hF.IX-specific B cell activation before establishment of tolerance.

After the third week of treatment, AAV1-CMV-hFIX vector was IM injected (Fig. 6A). Control mice received vector only, or received identical treatment, except that an irrelevant (ova) peptide was used instead of A2-54 (n=4 per experimental group). Without immune

suppression, mice developed inhibitors of 4-19 BU within 1 month after gene transfer (Fig. 6B). Mice treated with the hF.IX tolerance protocol low to undetectable inhibitors (<2 BU) for the duration of the experiment (5.5 months, Fig. 6B). Of importance, non-specifically immune suppressed mice formed low-titer inhibitors of 4-5 BU, and therefore showed only partial suppression of the inhibitor response. Systemic hF.IX levels in tolerized mice were stable, and on average 200-250 ng/ml (4-5% of normal human levels), while both control groups showed substantially lower expression of on average 0-50 ng/ml (Fig. 6C). Clotting times (aPTTs) were partially corrected (~50 sec) in the hF.IX-tolerized group compared to an average of 65-80 sec in the control groups, which was identical to untreated mice (Fig. 6D).

While hF.IX levels and aPTTs did not improve, inhibitory antibody titers in animals without immune suppression were unchanged for 3.5 months and subsequently declined to lower titers, similar to published data [13]. In the non-specifically suppressed group, 3 of 4 animals also showed a decline in Bethesda titers by 6 months, resulting in increased hF.IX expression and improvement of the aPTTs, which may reflect an additional tolerizing effect of hF.IX expression over time. However, one animal still showed 5 BU and lacked systemic expression (Fig. 6D and data not shown). Elimination of IL-10 from the rapamycin/A2-54 cocktail resulted in less effective blockage of inhibitors, but still resulted in high hF.IX levels, and average aPTTs were somewhat lower or similar to the non-specifically suppressed group, depending on the time point (Fig. 6B-D).

In order to demonstrate Treg induction in tolerized C3H/HeJ F9^{-/-} mice, CD4⁺CD25⁺ splenocytes were adoptively transferred to wild-type C3H/HeJ mice followed by vector administration, and found to suppress antibody formation to hF.IX (Fig. 6E). Gene transfer to mice that received control cells (CD4⁻ splenocytes, which are not expected to have suppressive properties) formed significantly higher titers of IgG1 anti-hF.IX (Fig. 6E).

Preliminary data suggest that the protocol is also effective in protein therapy. After 4 weekly administration of 1 IU recombinant hF.IX (Benefix; one IP followed by 3 IV injections), 3/5 hemophilia B mice died shortly after the last injection, presumably because of anaphylactic shock, as we had repeatedly observed (unpublished data). The remaining 2 mice had inhibitors of 5-7 BU (Fig. 7). The rapamycin/IL-10/2A-54 protocol suppressed inhibitor formation and desensitized the mice from the hypersensitive response (Fig. 7). Non-hF.IX specific effects in rapamycin/IL-10 treated C3H/HeJ mice included transient reduction in B and T cell frequencies and transient neutropenia (see Supplementary Data).

Discussion

Among the many benefits of immunity, the immune system itself has evolved to protect the host against infections. In order to achieve an effective and specific response and to create memory, antigen presentation to T lymphocytes results in the generation of effector and memory T cells. CD4⁺ T cells provide help for activation of B cells, which subsequently form antibodies. However, if the adaptive response is directed against a therapeutic protein, it can become a major complication and source of immunotoxicity in gene and protein replacement therapies for genetic disease. Hemophilic patients with inhibitor formation are at higher risk for bleeding-related morbidity and mortality. Patients at high risk for antibody formation in protein therapy for hemophilia and LSD would benefit from prophylactic immune tolerance protocols. Furthermore, safety of gene therapies, which offer the chance of long lasting cures for genetic disease, would increase if such protocols were developed.

Creating an optimal shift from an effector to a regulatory T cell population

Experiments presented here illustrate the usefulness of the highly defined DO.11.10-tg Rag2^{-/-} mouse model to screen for drugs (and define their mechanisms of action) that are effective in directing a shift from a Teff to a Treg response upon specific antigen presentation to CD4⁺ T cells. Changing this balance in favor of regulation is critical for tolerance. Rapamycin, and even more so the combination of rapamycin and IL-10, had a substantial effect by deleting ova-specific CD4⁺ T cells while allowing induction of Treg. In the absence of the specific antigen, the drugs did not change the ova-specific T cell population. Mechanistically, our data demonstrates activation of CD4⁺ T cells upon *in vivo* antigen presentation in the presence of rapamycin/IL-10, resulting in apoptotic cell death. At the same time, we found a greater resistance to antigen/rapamycin-induced apoptosis in Treg compared to Teff, which explains why Treg induction and expansion was successful. The tolerized T cell population regained responsiveness to their specific antigen when Treg were removed. Rapamycin/FKBP12 complex inhibits IL-2 stimulated signal transduction by preventing phosphorylation of p70 S6 kinase and of eIF-4E BP1, which are both regulated by mTOR, leading to a block in cell cycle progression. A recent study has shown that IL-2 stimulation of Treg, compared to Teff, leads to preferential use of the STAT5 rather than the mTOR pathway, resulting in resistance to inhibition of mTOR [18]. Compared to hepatic ova gene transfer, here we achieved a 2- to 8-fold higher frequency of Treg among splenic CD4⁺ T cells, which may reflect more robust Treg induction or more deletion of Teff [7].

Treg are critical for many tolerance induction protocols

Usefulness of the rapamycin/IL-10/antigen cocktail for therapeutic application was demonstrated in the hemophilia B mouse model, where inhibitor formation was effectively blocked using a short (4-week) tolerance protocol starting 3 weeks prior to muscle-directed gene transfer. Immune tolerance protocols that utilize immune suppressive drugs will only be acceptable for treatment of hemophilia, if they are limited in time. Recent study accomplished this goal in hemophilia A mice by short-term administration of low doses of a non-Fc-receptor binding anti-CD3 monoclonal antibody or transient blockade of ICOS signaling, a co-stimulatory pathway for T cell activation [19,20]. In contrast to these studies, utilizing more recently developed and still experimental reagents, our investigation established the usefulness of a drug, rapamycin, which is well established in clinical practice. Therefore, translational research could take advantage of a wealth of experience in humans (including pediatric patients). Furthermore, this protocol causes only moderate general immune suppression. Nonetheless, patients will have to be monitored weekly for rapamycin levels, lipid profile (in particular cholesterol levels), general health, and signs of opportunistic infection. The effect of the route of drug administration also has to be further investigated, since rapamycin is given orally in humans. Oral administration may also result in Tr1 activation, which could add another useful subset of regulatory T cells [11].

Implications for translational research

Prevention of inhibitor formation upon muscle gene transfer required co-administration of the immune modulatory drugs rapamycin/IL-10 and the hF.IX-specific peptide. Without the specific antigen, the drugs were only partially effective in blocking the response to muscle-derived hF.IX. These findings argue for antigen-specific immune tolerance protocols that are initiated prior to the onset of therapy rather than general immune suppression applied at the time of gene transfer. In this regard, peptide administration is highly effective, because the peptide can be directly loaded onto MHC molecules without a need for antigen processing and because the risk of inducing an adaptive response against the cognate protein antigen in the early phase of the protocol (before tolerance is established) is substantially reduced [12]. However, translation to clinical use would require knowledge of CD4⁺ epitope in the diverse human patient population. Therefore, it is desirable to develop and optimize the protocol

using the entire protein as the antigen for induction of T cell tolerance. Elimination of IL-10 was partially effective in the hemophilia B model, suggesting that a rapamycin/antigen cocktail may be sufficient following optimization of dosing. Induction of long-term tolerance to hF.IX in protein therapy may also require additional optimization of the protocol. One advantage of gene therapy is the continuous persistence of the antigen, which should aid in maintaining tolerance. This is further supported by the deferred down-regulation of the initially high inhibitor response in non-immune suppressed mice.

In conclusion, antigen administration, when combined with a suitable cocktail of immune modulatory drugs, can create a powerful shift from Teff to Treg populations. This concept can be exploited for the development of prophylactic immune tolerance protocols for treatment of inherited protein deficiencies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Flow cytometric analysis of splenocytes from DO.11.10-tg Rag^{-/-} BALB/c mice injected IP 3-times/week for 4 weeks with rapamycin/IL-10/specific peptide (ova), rapamycin/ova, IL-10/ova or control rapamycin/IL-10/irrelevant (2A-54; “irr”) peptide. **A, B.** Percent CD4⁺ T cells of total gated lymphocytes as a function of treatment (**A.** representative examples; **B.** Averages $\pm$ SD and fold-decline compared to controls). **C.** Frequency of CD25⁺FoxP3⁺ Treg of total CD4⁺ cells and fold-increase compared to control group. **D.** IL-4 and IL-10 cytokine release upon *in vitro* stimulation using ova-containing or mock media. Cytokine capture in treatment groups. **A-C.** *** significant difference with $P < 0.0001$.

Fig. 2.

Flow cytometric analysis of lymphoid organs in DO.11.10-tg Rag^{-/-} mice treated for 3 weeks with rapamycin/IL-10/ova or rapamycin/IL-10/A2-54 peptide or in naïve mice. **A.** Representative examples of CD4⁺CD25⁺FoxP3⁺ Treg frequencies (% Treg of total CD4⁺ cells). **B-E** Frequency of CD4⁺ cells of total gated lymphocytes in spleen (**B**), pooled inguinal and popliteal lymph node cells (**C**), thymus (**D**), and peripheral blood (**E**). Summary of CD4⁺CD25⁺FoxP3⁺ Treg frequencies in spleen (**F**), lymph nodes (**G**), thymus (**H**), and peripheral blood (**I**). **B-I.** Data are averages $\pm$ SD; * significant difference with $P < 0.05$.

Fig. 3.

Measurement of T cell activation in splenocytes from DO.11.10-tg Rag^{-/-} mice treated for 1 week with rapamycin/IL-10 and ova (or irrelevant) peptide and from naïve mice. **A.** Overlay of representative examples of CD62L, CD69, and CD44 expression in CD4⁺ T cells from a single treated and or naïve mouse. Graphs show summaries of results (averages $\pm$ SD, n=4 per group) for percent CD69⁺ (**B**), CD44^{high} (**C**), CD4⁺CD62L⁺ (**D**), and CD25⁺ (**E**) of CD4⁺ cells. * significant difference with $P < 0.05$.

Fig. 4.

Measurement of apoptosis and Fas (CD95) expression in DO.11.10-tg Rag^{-/-} mice treated for 1 week with rapamycin/IL-10/ova, rapamycin/IL-10/irrelevant peptide, or untreated (naïve) mice. **A.** Percent CD95⁺ of CD4⁺ T cells. **B.** Percent AnnexinV (AV)⁺PI⁻ of CD4⁺ T cells. **C.** Percent AnnexinV⁺PI⁻CD95⁺ of CD4⁺ T cells. **D.** Increase in apoptotic cell numbers as a function of time during treatment with rapamycin/IL-10/ova. **E.** Change in ova-specific CD4⁺ T cell frequency as a function of time during treatment with specific or non-specific protocol, and comparison to untreated mice.

Fig. 5.

Differential effects of treatment on Teff and Treg. **A.** Percent apoptotic (Annexin V⁺) cells among splenic Teff (CD4⁺CD25⁻FoxP3⁻) and Treg (CD25⁺CD25⁺FoxP3⁺) from DO. 11.10-tg Rag^{-/-} mice after 3 weeks of treatment with rapamycin/IL-10/ova compared to CD4⁺ T cells from control rapamycin/IL-10/irrelevant peptide treated mice. Shown are results for individual mice. **B.** Ova-specific T cell proliferation using complete splenocyte cultures (“total CD4⁺”), CD25⁺ cell-depleted cultures (“Teff”), and depleted cultures reconstituted with CD4⁺CD25⁺ T cells at a ratio of 0.5:1 when compared to CD4⁺CD25⁻ cell number (“Teff : Treg; 1 : 0.5”). Splenocytes were from DO11.10-tg Rag^{-/-} mice treated for 3 weeks with rapamycin/IL-10/ova. Results are average $\pm$ SD for quadruplicate cultures.

Fig. 6. Prevention of inhibitor formation in hemophilia B mice receiving gene therapy. C3H/HeJ $F9^{-/-}$ mice were treated with rapamycin/IL-10 and 2A-54 or irrelevant peptide for 4 weeks. After the third week of treatment, AAV1-CMV-hF.IX was IM injected (**A**). A third group received IM gene transfer only, and a fourth group received rapamycin/2A-54 plus gene transfer. Shown are average results $\pm$ SD ($n=4$ per group) for inhibitory antibody titers (in BU; **B**), hF.IX plasma levels (ng/ml; **C**), and clotting times (**D**; aPTT in sec, which is 25-35 sec for wild-type mice and >60 sec for untreated hemophilia B mice) as a function of time after vector administration. **E**. Anti-hFIX IgG1 formation in wild-type C3H/HeJ mice after adoptive transfer of splenic $CD4^{+}CD25^{+}$ T cells or $CD4^{-}$ control cells from specifically tolerized C3H/HeJ $F9^{-/-}$ mice, followed by AAV1-CMV-hF.IX administration. ** significant difference with $P \leq 0.005$.

Fig. 7.

Prevention of inhibitor formation in hemophilia B mice receiving protein therapy. C3H/HeJ F9^{-/-} mice were treated with rapamycin/IL-10/2A-54 for 4 weeks. **A.** After the third week of treatment, weekly administration of Benefix (arrows) was initiated. **B.** Inhibitor titers (average ±SD; n=5) after the 4th protein injection were compared to control mice that had received protein therapy only (results shown for 2 surviving individual mice). **C.** Survival of tolerized and control C3H/HeJ F9^{-/-} mice after 4 injections of Benefix (n=5 per group).