Published in final edited form as:

J Autoimmun. 2008 June ; 30(4): 257–265. doi:10.1016/j.jaut.2007.10.005.

RDP58 inhibits T cell-mediated bladder inflammation in an autoimmune cystitis model

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Abstract

Interstitial cystitis (IC) is a chronic inflammatory condition of the urinary bladder with a strong autoimmune component. Currently, the major challenge in IC treatment is the development of effective therapies. RDP58 is a novel D-amino acid decapeptide with potent immunosuppressive activity. In this study, we investigated whether RDP58 was effective as an intravesical agent for treating bladder autoimmune inflammation in a transgenic mouse model (URO-OVA mice). URO-OVA mice were adoptively transferred with syngeneic activated splenocytes of OT-I mice transgenic for the OVA-specific CD8⁺ TCR for cystitis induction and treated intravesically with RDP58 at days 0 and 3. Compared with controls, the RDP58-treated bladders showed markedly reduced histopathology and expressions of mRNAs and proteins of TNF- α , NGF and substance P. To determine whether the inhibition of bladder inflammation by RDP58 was due to the interference with effector T cells, we treated the cells with RDP58 in vitro. Cells treated with RDP58 showed reduced production of TNF- α and IFN- γ as well as apoptotic death. Collectively, these results indicate that RDP58 is effective on treating T cell-mediated experimental autoimmune cystitis and may serve as a useful intravesical agent for the treatment of autoimmune-associated bladder inflammation such as IC.

Keywords

Autoimmunity; Cystitis; Inflammation; RDP58; Urinary Bladder

1. Introduction

The urinary bladder is known to be an immunoreactive organ and often develops infectious and non-infectious inflammation. Non-infectious cystitis can be a complication of autoimmune diseases outside the bladder [1-6]. Bladder-originated autoimmunity is also apparent among non-infectious cystitis. For instance, interstitial cystitis (IC) is a chronic inflammatory condition of the bladder with a strong autoimmune component while the etiology of IC remains to be elucidated [7-9]. Antinuclear IgG and anti-epithelial cell autoantibodies have been found

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in the majority of IC patients [10-14]. Abnormal urothelial HLA-DR expression has also been reported in IC patients [15,16]. Autoimmune disorders such as bronchial asthma, systemic lupus erythematosus, Sjogren's syndrome and rheumatoid arthritis have been found to coexist in some IC patients [8,9,17-23]. Considerable data have been published on the histopathology of bladder specimens demonstrating a role for cell-mediated immunological mechanisms in IC [24-26]. Of special relevance is the convergence of the neurological pain pathway with inflammatory mediators through a possible link with mast cells [7,27-30]. Since the causative factors are obscure, no cure has yet been found for IC. The current therapies are mainly aimed at symptomatic relief, especially for chronic pain management. However, relapse, persistence and deterioration are common. Thus, the major challenge in IC treatment remains the development of effective modalities that can provide a long-term beneficial clinical outcome and an improved quality of life.

To facilitate the study of bladder autoimmunity, we recently developed a novel line of transgenic mice, designated as URO-OVA mice, that expresses a membrane form of the model antigen ovalbumin (OVA) as an autoantigen on the bladder epithelium [31]. We have demonstrated that URO-OVA mice develop IC-like histological bladder inflammation at day 7 after adoptive transfer of OVA-specific T cells from OT-I mice, a transgenic line expressing the OVA-specific CD8⁺ T cell receptor (TCR) [31]. The inflamed bladder showed clear histopathology including cellular infiltration and epithelial hyperplasia [31]. Increased mast cells were also observed in the inflamed bladder at later timepoints [31]. In addition to histopathology, the inflamed bladder also showed increased mRNA expression of mast cell inflammatory mediators tumor necrosis factor (TNF)- α and nerve growth factor (NGF) as well as sensory nerve neurotransmitter substance P (SP) precursor [31]. Thus, URO-OVA mice provide an ideal model for the study of bladder-originated autoimmunity and for testing of potential therapeutic agents for autoimmune-associated bladder disorders.

RDP58 is a novel D-amino acid decapeptide (MW: 1380.76; chemical formula: NH₂-arg-norleucine(nle)-nle-nle-arg-nle-nle-nle-gly-tyr-CONH₂) and was initially developed by using a computer-assisted, rationally designed approach [32]. RDP58 was derived from the heavy chain of the HLA class I molecules and screened by a transplantation model [32]. Accumulating evidence supports RDP58 to be a potent anti-inflammatory agent. RDP58 has been demonstrated to work favorably in treating human ulcerative colitis and chemotherapy-induced diarrhea [33,34]. RDP58 has also been shown to be effective in treating rodent colitis and other inflammatory and autoimmune disease models [35-41]. Reduction of pro-inflammatory cytokines after RDP58 treatment has been observed in immune and epithelial cells including TNF- α , interferon (IFN)- γ , interleukin (IL)-2 and IL-12 [32,35-41]. The mechanisms of RDP58 action are thought to be associated with the disruption of the TRAF6-MyD88-IRAK protein complex involved in the signal transduction responsible for the expression of TNF- α and other inflammatory cytokines [42]. Recently, RDP58 was tested as an intravesical agent for treating lipopolysaccharide (LPS)-induced experimental cystitis and found to be effective [43]. In this study, we used intravesical RDP58 to treat experimental autoimmune cystitis (non-infectious, autoantigen-mediated cystitis) and found that RDP58 was also effective in treating this type of bladder inflammation.

2. Materials and Methods

2.1. Mice

URO-OVA mice (B6 and Thy1.2 background) are a novel urothelial OVA transgenic line developed in our laboratory that provides a unique model for the study of bladder-originated autoimmune inflammation [31]. Female URO-OVA mice (6-8 weeks) were used because of their feasibility for intravesical procedures. OT-I mice expressing the transgenic CD8⁺ TCR specific for OVA₂₅₇₋₂₆₄ peptide epitope (H2-K^b) were kindly provided by Dr. W. R. Heath of

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia [44]. OT-I mice were kept in the Thy1.1 background and used as a source of effector T cells for cystitis induction. All mice were housed in a pathogen-free facility at the University of Iowa Animal Care Facility and studies were conducted with procedures approved by the University of Iowa Animal Care and Use Committee.

2.2. Cystitis induction and RDP58 treatment

Splenocytes of OT-I/Thy1.1 mice were prepared and activated in vitro with OVA_{257–264} peptide for 2 days as described previously [31,45]. Female URO-OVA mice (Thy1.2) were transferred intravenously (i.v.) with the pre-activated OT-I/Thy1.1 splenocytes (1×10^7 cells/mouse) at day 0 for cystitis induction (Fig. 1). Pure RDP58 powder was obtained from Procter & Gamble Pharmaceuticals, Inc. (Mason, OH, USA), dissolved in PBS at appropriate concentrations, and used as an intravesical agent. As described previously [46], mice were anesthetized with a single dose of intraperitoneal mixture solution of ketamine and xylazine. The bladder was then catheterized via the urethra with a 24-gauge plastic intravenous cannula and injected with 50 μ l of RDP58 solution (2.5 μ g/dose, 25 μ g/dose and 250 μ g/dose) through the cannula at days 0 and 3. Mice were sacrificed at day 7 for analysis of bladder inflammation.

2.3. Bladder histological analysis

The standard histological hematoxylin and eosin (H&E) staining was performed as described previously [31]. Briefly, paraffin-embedded bladder sections were prepared. After deparaffin, sections were stained with H&E and photographed using an Olympus microscope. Bladder inflammation was scored according to the criteria used in our previous study: 1+ (mild infiltration with no or mild edema), 2+ (moderate infiltration with moderate edema), and 3+ (moderate to severe infiltration with severe edema) [31].

2.4. Bladder flow cytometric analysis

Bladder single-cell suspensions were prepared through mechanical dispersion as described previously [31]. Cells were washed with staining buffer (1% FBS, 0.09% (w/v) Na₃N in Mg²⁺- and Ca²⁺-free PBS), double stained with a FITC-CD8 antibody (eBioscience, San Diego, CA, USA) and a PE-Thy1.1 antibody (eBioscience), fixed in 2% formalin, and analyzed using a FACScan equipped with CellQuest (BD Biosciences, NJ, USA). Post acquisition analyses were carried out using FlowJo software (Tree Star, Inc, OR, USA).

2.5. Bladder RT-PCR analysis

Bladder total RNAs were extracted using Qiagen RNAeasy Kit (Qiagen, CA, USA). Three micrograms of total RNAs were used for cDNA synthesis using Invitrogen Superscript III RNase H Reverse Transcriptase (Carlsbad, CA, USA) and Oligo dT according to the manufacturer's instruction. Two microliters of cDNA products were then processed for PCR amplification using the sequence-specific primer pairs and Invitrogen Taq DNA polymerase. The following primer pairs were used: 5'-GTTCCAGTATGACTCCACT and 5'-GTGCAGGATGCATTGCTG for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 321 bp); 5'-CGTCAGCCGATTTGCTATCT and 5'-CGGACTCCGCAAAGTCTAAG for TNF- α (206bp); 5'-CACTGAGAACTCCCCCATGT and 5'-CTGTGGACCCCAGACTGTTT for NGF (194 bp); 5'-GCCAATGCAGAACTACGAAA and 5'-GCTTGGACAGCTCCTTCATC for SP precursor (280 bp). PCR kinetics was initially established to achieve a desirable discrepancy between controls and the RDP58-treated bladders. Based on the kinetics, 30 cycles were used for GAPDH and 36 cycles were used for TNF- α , NGF and SP precursor. The DNA fragments were run on a 1% agarose gel and the image was captured by EpiChemi digital image analysis system (UVP, Upland, CA, USA).

2.6. Bladder ELISA analysis

As described previously [31], the bladder was homogenized in 300 μ l of preservative buffer containing 0.2 M Tris-HCl (pH 7.6), 0.5% BSA, 0.01% sodium azide, and Complete Protease Inhibitor Tablet (1/10 volume; Roche Applied Science, Indianapolis, IN, USA). After centrifugation at 12,000 rpm, the supernatants were collected and analyzed using the commercial ELISA kits from eBioscience for TNF- α , from Chemocon (Temecula, CA, USA) for NGF, and from Assay Design (Ann Arbor, Michigan, USA) for SP. ELISA was performed according to the manufacturers' instructions. The log ranges of ELISAs were 8-1000 pg/ml, 10-1000 pg/ml and 10-2560 pg/ml for TNF- α , NGF and SP, respectively.

2.7. In vitro inhibition of effector T cell cytokine production by RDP58

OT-I/Thy1.1 splenocytes were prepared and seeded on 96-well plates at 5×10^5 cells/well. Cells were cultured in the absence or presence of stimulators PMA (100 ng/ml) plus ionomycin (1500 ng/ml) or OVA₂₅₇₋₂₆₄ peptide (10 μ g/ml) alone or in combination with RDP58 at 5 μ g/ml, 50 μ g/ml or 500 μ g/ml for 3 days. After incubation, culture supernatants were collected for ELISA analysis. Paired monoclonal capture and detecting antibodies were purchased from eBioscience for TNF- α and from Endogen (Woburn, MA, USA) for IFN- γ . A sandwich format ELISA was performed according to the manufacturers' instructions. The log ranges of ELISAs were 8-1000 pg/ml and 156-10000 pg/ml for TNF- α and IFN- γ , respectively. The cytokine concentrations were plotted after adjustment of dilution factors.

2.8. Viability of effector T cells after RDP58 treatment in vitro

OT-I/Thy1.1 splenocytes were prepared and incubated with RDP58 at 5 μ g/ml, 50 μ g/ml or 500 μ g/ml for 12 and 24 h in culture. After incubation, cells were double stained with FITC-Annexin V (BD Biosciences) and propidium iodide, and analyzed by flow cytometry using FlowJo software.

2.9. Statistical analysis

All determinations were made in multiple experiments. Statistical significance was determined by the Wilcoxon Rank-Sum Test using GraphPad Prism 5.0 (San Diego, CA, USA) for bladder T cell infiltration and cytokine production and by Student's *t*-Test using SPSS11.0 software (Chicago, IL, USA) for in vitro splenocyte cytokine production. A *p*-value of <0.05 was considered to be significant.

3. Results

3.1. Intravesical RDP58 treatment ameliorates histopathology of bladder inflammation

To evaluate the effect of RDP58 on treating autoimmune cystitis, splenocytes were prepared from OT-I/Thy1.1 mice, activated in vitro with OVA₂₅₇₋₂₆₄ peptide, and injected (1×10^7 cells/mouse) i.v. into URO-OVA mice (Thy1.2) at day 0 (Fig. 1). Under this condition, mice developed histological bladder inflammation at day 7 [31]. Mice were treated intravesically with RDP58 (2.5 μ g/dose, 25 μ g/dose or 250 μ g/dose) at days 0 (right after cystitis induction) and 3 and sacrificed for bladder analysis at day 7. Adoptive transfer of pre-activated OT-I/Thy1.1 splenocytes induced histological bladder inflammation in control group (PBS-treated group) as manifested by cellular infiltration, edema and epithelial hyperplasia (Fig. 2B; score: >2+). Compared with the PBS-treated bladder, the bladders treated with RDP58 showed a dose-dependent reduction of bladder inflammation (Figs. 2C-E). Although the bladders treated with RDP58 at 2.5 μ g/dose showed little change in the intensity of inflammation (Fig. 2C; score: 2+), the bladders treated with RDP58 at 25 μ g/dose showed a clear decrease in numbers of inflammatory cells in both the urothelium and lamina propria (Fig. 2D; score: 1+). All defined histopathological indicators were minimal in the bladders treated with RDP58 at 250

µg/dose (Fig. 2E; score: 0 - <1+). In contrast, a control peptide showed no effect on bladder histopathology (data not shown). In addition to the bladder histology, flow cytometry also showed a dose-dependent reduction of infiltrating effector T cells (Thy1.1⁺CD8⁺ T cells) in the RDP58-treated bladders (Fig. 3A). Compared with the control PBS-treated bladders, the total number of Thy1.1⁺CD8⁺ T cells in the RDP58-treated bladders was reduced significantly ($p < 0.05$ for 2.5 µg/dose and $p < 0.001$ for both 25 µg/dose and 250 µg/dose).

3.2. Intravesical RDP58 treatment reduces bladder expression of neuroinflammatory factors

URO-OVA mice are known to express increased mRNAs of TNF-α, NGF and SP precursor in the inflamed bladders [31]. To investigate whether intravesical RDP58 treatment could reduce mRNA expression of these neuroinflammatory factors, total RNAs of the bladder were extracted and analyzed by RT-PCR (Fig. 3B). The normal bladder expressed no detectable mRNAs under the same condition (data not shown). Induction of cystitis resulted in increased mRNA levels of TNF-α, NGF and SP precursor in the control bladders (the PBS-treated bladders). Although there was no (or a marginal) reduction for the bladders treated with RDP58 at 2.5 µg/dose or 25 µg/dose, the bladders treated with RDP58 at 250 µg/dose showed a clear reduction of mRNAs of these neuroinflammatory factors.

Since intravesical RDP58 treatment reduced mRNA expression of TNF-α, NGF and SP precursor, we suspected that this reduction of mRNAs could lead to reduced protein expression of the corresponding neuroinflammatory factors. To investigate this, bladder lysates were prepared and analyzed using the commercially available high sensitive ELISA kits (Fig. 3C). The normal bladders showed no or minimal expression of these proteins (data not shown). Compared with the control PBS-treated bladders, the RDP58-treated bladders showed a clear reduction of TNF-α, NGF and SP. RDP58 treatment significantly reduced TNF-α at 250 µg/dose ($p < 0.001$), NGF at 25 µg/dose ($p < 0.05$) and 250 µg/dose ($p < 0.001$), and SP at 25 µg/dose ($p < 0.001$) and 250 µg/dose ($p < 0.001$), respectively.

3.3. RDP58 inhibits effector T cells for TNF-α and IFN-γ production in vitro

OVA-specific OT-I/Thy1.1 CD8⁺ T cells served as effector T cells for cystitis induction in URO-OVA mice. To investigate whether the inhibition of bladder inflammation by RDP58 was due to its interference with the effector T cells, we stimulated OT-I/Thy1.1 splenocytes with PMA plus ionomycin (a non-specific stimulation) or OVA₂₅₇₋₂₆₄ peptide (an antigen-specific stimulation) alone or in combination with various doses of RDP58 for 3 days, followed by ELISA analysis of TNF-α and IFN-γ production in culture supernatants. Compared with PMA plus ionomycin stimulation alone, addition of RDP58 reduced TNF-α by 4.3% at 50 µg/ml ($p = 0.008$, $p < 0.05$) and 65.2% at 500 µg/ml ($p = 0.002$, $p < 0.05$) and IFN-γ by 11.7% at 50 µg/ml ($p = 0.01$, $p < 0.05$) and 53.2% at 500 µg/ml ($p < 0.001$), respectively (Fig. 4A). Compared with OVA₂₅₇₋₂₆₄ peptide stimulation alone, addition of RDP58 reduced TNF-α by 59% ($p = 0.004$, $p < 0.05$) and IFN-γ by 24.5% ($p = 0.032$, $p < 0.05$) at 500 µg/ml, respectively (Fig. 4B). In both cases, a control peptide showed no inhibitory effect (data not shown). This observation suggests that the improvement of bladder histopathology by intravesical RDP58 treatment may partially result from its direct inhibition on the effector T cells in vivo.

3.4. RDP58 induces effector T cells for apoptotic death in vitro

To determine whether RDP58 could directly affect the viability of effector T cells, we incubated OT-I/Thy1.1 splenocytes with RDP58 in culture and analyzed their viability after 12 and 24 h (Fig. 5). Compared with PBS and control peptide treated groups, addition of RDP58 increased the double (Annexin V and PI) positive population in a dose-dependent manner (3%, 6% and 70% at 12 h and 9%, 13% and 89% at 24 h for the three indicated doses), suggesting that RDP58 could induce T cell apoptotic death and result in reduced T cell viability in vivo.

Discussion

In this study we used intravesical RDP58 to treat experimental autoimmune cystitis developed in URO-OVA mice and found that this therapeutic approach is effective. The RDP58-treated bladder showed markedly improved histopathology and significantly reduced expression of neuroinflammatory factors TNF- α , NGF and SP. In addition to its effect on the bladder, RDP58 can also act on the effector T cells for cytokine production and apoptotic induction in vitro.

T cells are known to be responsible for the pathogenesis of numerous autoimmune diseases [47-50]. In our autoimmune cystitis model, OT-I CD8⁺ T cells serve as effector T cells for the induction of cystitis [31]. These CD8⁺ T cells are OVA-specific and recognize OVA₂₅₇₋₂₆₄ peptide (H2-K^b) in the context of the major histocompatibility complex (MHC) class I complex [43]. Previous studies showed that up-regulated mRNA expression of TNF- α , NGF and SP was readily detectable as early as day 1 after cystitis induction, much earlier than the development of bladder histopathology at day 7 [31]. This previous observation suggested that the OVA-specific effector T cells likely triggered a process of bladder autoimmunity shortly after being transferred. In this study we performed intravesical RDP58 treatment at days 0 and 3 after cystitis induction and found that URO-OVA mice responded favorably to the treatment. This observation suggests that intravesical RDP58 is effective for bladder autoimmune inhibition. However, the effect of intravesical RDP58 on treating more established autoimmune cystitis remains to be determined.

Increasing evidence suggests that immune/autoimmune mechanisms play an important role in IC pathogenesis. In essence, IC is a complex disease associated with bladder inflammation and sensory nerve innervation. Excessive release of mast cell inflammatory mediators and sensory nerve neurotransmitters is thought to be responsible for the development and propagation of IC symptoms, especially for suprapubic or pelvic pain. Prior studies have demonstrated the elevated expression of certain neuroinflammatory factors in the bladder of IC patients including NGF and SP [51,52]. TNF- α has also been observed to play a role in urothelial inflammation [53]. Our observations that intravesical RDP58 improves bladder histopathology and reduces bladder expression of these neuroinflammatory factors are consistent with those in previously reported LPS-induced cystitis model [43]. These observations strongly suggest that intravesical RDP58 may serve as an effective modality for treating IC.

Mast cells are known to release TNF- α , NGF and other inflammatory mediators through degranulation upon activation. NGF is a neurotrophic factor and causes sensory neurons to release SP and other neurotransmitters [54]. In turn, SP and TNF- α can stimulate mast cells (SP is also a neurotrophic factor and can act on a sensory neuron itself), leading to a positive feedback loop between mast cells and sensory neurons [54-56]. Intravesical RDP58 treatment interferes with this loop as it reduces bladder expression of these neuroinflammatory factors. Although mast cell mediators were readily detected in the inflamed bladder at day 7, increment of mast cells was not detected by Giemsa staining in the same bladder (data not shown). This observation was consistent with our previous observation that mast cell mediators were detected earlier than increment of mast cell number at day 14 [31]. One possible explanation for this inconsistency is the underestimation of mast cells by Giemsa staining due to its intrinsic shortcoming for staining of mucosal mast cells [57]. Alternatively, activation of mast cells may occur prior to increment of mast cell population in this autoimmune cystitis model.

It has been reported that RDP58 has an inhibitory effect on T cells through blocking T cell interaction with the MHC [32]. In this study, we also observed that RDP58 inhibited effector OT-I/Thy1.1 CD8⁺ T cells for TNF- α and IFN- γ production in vitro. We further observed that RDP58 could even induce T cell apoptotic death in vitro. These observations suggest that RDP58 may directly act on the effector T cells and interfere with their functional activity in

vivo. In addition to T cells, RDP58 may also affect other immune cells as well as bladder tissue cells for inhibition of bladder inflammation. In fact, the observed reduction of NGF and SP in the inflamed bladder could suggest the inhibitory effect of RDP58 on mast cells and sensory neurons. Similarly, the reduction of TNF- α could suggest the inhibition of T cells, mast cells or macrophages by RDP58. In addition, bladder epithelial cells and detrusor muscle cells may also be responsive to intravesical RDP58 and contribute to the overall anti-inflammatory effect of RDP58.

Currently, a major focus of IC therapies is on the inhibition of bladder neuroinflammatory responses. Intravesical administration of immune modulators is a common treatment modality for IC at the present time [58]. The results of this study suggest that intravesical administration of RDP58 holds great promise as a possible therapeutic option for IC. Furthermore, because RDP58 has limited systemic bioavailability and minimum side-effects [35,38], it may be particularly useful as an intravesical agent. Further studies are needed to investigate how intravesical RDP58 inhibits bladder immune/autoimmune responses and whether intravesical RDP58 improves voiding dysfunction and other symptoms prior to its use for IC treatment.

Acknowledgments

We thank Procter & Gamble Pharmaceuticals for providing RDP58 and Genzyme Corporation for continued support for this study. We thank Drs. Timothy L. Ratliff and Michael A. O'Donnell for constructive discussion, and Mitchell L. Rotman for helping edit the manuscript. This work was supported in part by National Institute of Health Grant RO1 DK066079 and Department of Defense Peer Reviewed Medical Research Program Award W81XWH-04-1-0070 to Y.L. and by American Urological Association Foundation Research Scholar Grant, with the following contributors Interstitial Cystitis Association and Ortho Women's Health and Urology, to W.L. The authors have no conflict of interest.

Abbreviations

ELISA, enzyme-linked immunosorbent assay
 GAPDH, glyceraldehyde-3-phosphate dehydrogenase
 IC, interstitial cystitis
 IFN- γ , interferon-gamma
 IL, interleukin
 NGF, nerve growth factor
 OVA, chicken ovalbumin
 RDP58, rationally designed peptide 58
 SP, substance P
 TCR, T cell receptor
 TNF- α , tumor necrosis factor-alpha

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Intravesical RDP58 Treatment Schedule

Fig. 1.

Intravesical RDP58 treatment schedule. URO-OVA mice (Thy1.2) were transferred i.v. with in vitro pre-activated OT-I/Thy1.1 splenocytes (1×10^7 cells/mouse) for cystitis induction at day 0, and treated intravesically with RDP58 at days 0 and 3. The bladders were collected for analysis at day 7. SIINFEKL: OVA₂₅₇₋₂₆₄ peptide.

Fig. 2.

Intravesical RDP58 treatment improves histopathology of bladder inflammation. After cystitis induction and treatment, the bladders were collected, prepared for histological cross-sections, and stained with H&E. (A) a normal bladder showing unremarkable mucosa and muscularis. (B) the bladder of a mouse treated with PBS showing inflammation with urothelial hyperplasia, cellular infiltration and edema. (C) the bladder of a mouse treated with RDP58 at 2.5 $\mu\text{g}/\text{dose}$ showing a relative dense inflammatory cell infiltration. (D) the bladder of a mouse treated with RDP58 at 25 $\mu\text{g}/\text{dose}$ showing scattered inflammatory cells within the urothelium and lamina propria. (E) the bladder of a mouse treated with RDP58 at 250 $\mu\text{g}/\text{dose}$ showing minimal

inflammation. The cross-sections shown are representative of 5 animals in each group. Magnification (for all panels): $\times 40$ for the left and $\times 400$ for the right.

Fig. 3.

Intravesical RDP58 treatment reduces effector T cell infiltration and production of neuroinflammatory factors in the inflamed bladders. (A) Decrease of infiltrating effector CD8⁺ T cells in the RDP58-treated bladders. Bladder single-cell suspensions were prepared, stained with anti-Thy1.1 and anti-CD8 antibodies, and analyzed by flow cytometry. Gate was set on lymphocytes according to scatter criteria. The total numbers of double positive T cells are presented for each bladder. Statistical analysis (compared with PBS group): * = $p < 0.05$ and ** = $p < 0.001$. The results are representative of three separate experiments consisting of 3 to 5 animals per group. (B) Reduction of TNF- α , NGF and SP precursor (pre-SP) mRNAs in the RDP58-treated bladders. The bladder total RNAs were extracted and amplified by RT-PCR for the indicated inflammatory mediators. GAPDH was used as an internal control. Three bladders for each group are presented. The normal bladders expressed no detectable mRNAs under the same conditions (data not shown). (C) Reduction of TNF- α , NGF and SP proteins in the RDP58-treated bladders. The bladder lysates were prepared and analyzed by ELISA for the indicated inflammatory mediators. The concentration of the proteins is presented for each bladder. Statistical analysis (compared with PBS group): * = $p < 0.05$ and ** = $p < 0.001$. The normal bladders showed no or minimal expression of the proteins (data not shown). The results are representative of three separate experiments consisting of 3 to 5 animals per group.

Fig. 4.

RDP58 inhibits effector T cells for TNF- α and IFN- γ production in vitro. Splenocytes of OT-I/Thy1.1 mice were prepared and cultured in the absence or presence of PMA (100 ng/ml) plus ionomycin (1500 ng/ml) (for panel A) or OVA₂₅₇₋₂₆₄ peptide (10 μ g/ml) (for panel B) for 3 days. To evaluate the effects of RDP58, cells were also cultured in the presence of the stimulators plus indicated doses of RDP58 (5 μ g/ml, 50 μ g/ml and 500 μ g/ml) for 3 days. Production of TNF- α and IFN- γ in culture supernatants were then analyzed by ELISA. Data are presented as mean $\pm$ SD from two determinations. Statistical analysis (compared with PMA plus ionomycin or OVA₂₅₇₋₂₆₄ peptide group): * = $p < 0.05$ and ** = $p < 0.001$. The results are representative of two separate experiments.

Fig. 5. RDP58 induces effector T cell apoptotic death in vitro. Splenocytes of OT-I/Thy1.1 mice were prepared and incubated with RDP58 or a control peptide (5 µg/ml, 50 µg/ml and 500 µg/ml) for 12 and 24 h. After incubation, cells were double stained with FITC-Annexin V and propidium iodide (PI), and analyzed by flow cytometry. Gate was set on lymphocytes according to scatter criteria. The percentage of stained cells is indicated. The results are representative of two separate experiments.