

Published in final edited form as:

*Clin Immunol.* 2012 March ; 142(3): 291–295. doi:10.1016/j.clim.2011.11.004.

## Tolerance induced by anti-DNA Ig peptide in (NZB × NZW)F<sub>1</sub> lupus mice impinges on the resistance of effector T cells to suppression by regulatory T cells

Yiyun Yu<sup>a,b</sup>, Yaoyang Liu<sup>a</sup>, Fu-Dong Shi<sup>c</sup>, Hejian Zou<sup>b</sup>, Bevera H. Hahn<sup>a</sup>, and Antonio La Cava<sup>a,\*</sup>

<sup>a</sup>Division of Rheumatology at the David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA 90095

<sup>b</sup>Division of Rheumatology, Huashan Hospital, Fudan University, Shanghai, China

<sup>c</sup>Barrow Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ 85013

### Abstract

We have previously shown that immune tolerance induced by the anti-DNA Ig peptide pCons in (NZB × NZW)F<sub>1</sub> (NZB/W) lupus mice prolonged survival of treated animals and delayed the appearance of autoantibodies and glomerulonephritis. Part of the protection conferred by pCons could be ascribed to the induction of regulatory T cells (T<sub>Reg</sub>) that suppressed the production of anti-DNA antibodies in a p38 MAPK-dependent fashion. Here we show that another effect of pCons in the induction of immune tolerance in NZB/W lupus mice is the facilitation of effector T cell suppression by T<sub>Reg</sub>. These new findings indicate that pCons exerts protective effects in NZB/W lupus mice by differentially modulating the activity of different T cell subsets, implying new considerations in the design of T<sub>Reg</sub>-based approaches to modulate T cell autoreactivity in SLE.

### Keywords

Systemic lupus erythematosus; immune tolerance; anti-DNA antibodies; regulatory T cells; effector T cells; tolerogenic peptide

## 1. Introduction

Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disease characterized by the presence of dysregulated mechanisms of immune tolerance that include autoantibodies, autoreactive CD4<sup>+</sup> T cells, and reduced numbers and/or function of regulatory T cells (T<sub>Reg</sub>) that suppress CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>-</sup>effector T cells (T<sub>Eff</sub>) [1]. Although it has long been held that the inability of T<sub>Reg</sub> to control autoimmune reactivity in SLE could be either secondary to their decreased frequency or abnormal suppressive capacity [2], some studies have suggested that another possibility could be that T<sub>Eff</sub> can become resistant to T<sub>Reg</sub>-mediated suppression [3-5]. This phenomenon prompted our interest in revisiting the role of T<sub>Reg</sub> in

© 2011 Elsevier Inc. All rights reserved.

\*Corresponding author: Department of Medicine at the University of California Los Angeles, 1000 Veteran Avenue 32-59, Los Angeles, California 90095-1670; Tel. 1 310 267-4975; Fax: 1 310 206-8606; alacava@mednet.ucla.edu .

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

the mechanisms that control immune tolerance in SLE. In particular, we had previously shown that the treatment of (NZB  $\times$  NZW) $F_1$  (NZB/W) lupus-prone mice with the anti-DNA Ig peptide pCons effectively delayed lupus-like disease and prolonged mice survival [6]. We also showed that part of the protection had to be ascribed to pCons induced  $T_{Reg}$  that suppressed autoimmune responses in a p38-dependent fashion [7]. However, the effects of pCons on  $T_{Eff}$  remained elusive. Here we report that the threshold for  $T_{Eff}$  suppression by  $T_{Reg}$  in untreated NZB/W mice is lowered by pCons in a p38-independent fashion, and this favors the inhibition of autoimmune reactivity. These findings can have implications for the design of targeted interventions in the inhibition of autoimmune T cell reactivity in SLE.

## 2. Materials and Methods

### 2.1 Mice

Female NZB/W mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and treated according to the National Institutes of Health guidelines for the use of experimental animals, with the approval of the Institutional Animal Research Committee.

### 2.2 Peptides

pCons has T cell determinants from different J558  $V_H$  regions of NZB/W anti-dsDNA Ig, while the negative control peptide pNeg has similar MHC binding motifs but it does not activate  $CD4^+$  T cells [6]. Peptides were synthesized at Chiron Mimotopes (San Diego, CA), purified to a single peak by HPLC, and analyzed by mass spectroscopy for expected amino acid content before use.

### 2.3 Tolerance induction and cell preparation

For tolerance induction, 10- to 12-wk-old NZB/W mice received a single i.v. dose of 1 mg pCons dissolved in saline [6]. Control mice received either a similar amount of pNeg or saline. Ten days after treatment, single cell suspensions of splenocytes were prepared by passing cells through a sterile wire mesh. After lysis of red blood cells with ACK lysing buffer (Sigma-Aldrich, St. Louis, MO), cells were centrifuged, washed and resuspended in HL-1 medium (Lonza, Walkerville, MD).  $T_{Reg}$  or  $T_{Eff}$  were purified from splenocytes using the  $CD4^+CD25^+$  Regulatory T cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions.

### 2.4 p38 inhibition

The p38 inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580) and negative control molecule 4-ethyl-2-(p-methoxyphenyl)-5-(4'-pyridyl)-1H-imidazole (SB202474) were purchased from Calbiochem (San Diego, CA) and dissolved in saline. For p38 inhibition *in vivo*, mice were injected i.p. daily for 2 wk with 2 mg/kg SB203580 or SB202474 or equal volumes of saline [7].

### 2.5 Western blot

Total cell lysates from sorted  $T_{Eff}$  were obtained in 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM sodium fluoride, 1mM sodium orthovanadate, 2 $\mu$ g/ml aprotinin, 2 $\mu$ g/ml leupeptin, 2 $\mu$ g/ml pepstatin. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes (BioRad Laboratories, Hercules, CA). Membranes were blocked in 5% nonfat milk/PBS, 0.5% Tween 20 (PBST) at 4°C for 2 h, and then incubated with anti-phospho Abs from Cell Signaling Technology (Danvers, MA) (anti-p-ZAP-70 $^{Tyr319}$ , anti-p-ERK1/2 $^{Thr202/Tyr204}$ , anti-p-STAT1 $^{Ser727}$ , anti-p-STAT3 $^{Ser727}$ , anti-p-STAT6 $^{Tyr641}$ , anti-p-SAPK $^{Thr183/Tyr185}$ , anti-p-p38 $^{Thr180/Tyr182}$ ) or Santa Cruz Biotechnology (Santa Cruz, CA) (anti-p-JNK $^{Thr183/Tyr185}$ , anti-p-p38 $^{Tyr182}$ ). After wash in

PBST, membranes were incubated with peroxidase-conjugated secondary Ab. After additional wash, peroxidase activity was detected with the ECL system (Amersham, Piscataway, NJ) or Femto system (Thermo Scientific, Rockford, IL). Membranes were subsequently stripped and reprobbed with Ab for the corresponding non-phosphorylated protein purchased from Cell Signaling Technology: anti-ZAP-70, anti-p27<sup>kip1</sup>, anti-ERK1/2, anti-STAT1, anti-STAT3, anti-STAT6, anti-SAPK and anti-p38, or Santa Cruz Biotechnology: anti-JNK, anti-p38, before peroxidase detection using secondary Ab. Finally, membranes were stripped for final reprobing with anti- $\beta$ -actin Ab (Cell Signaling) to determine protein equivalency. Exposed films were quantified by densitometric analysis using ScionImage program (Scion Corporation, Frederick, MD).

## 2.6 Suppression assay

After cell sorting, T<sub>Eff</sub> were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA) by incubation in 0.5  $\mu$ M CFSE at 37°C for 10 min, then placed in HL-1/5% FCS and incubated in ice for 5 minutes before extensive washes. Mixtures of CFSE-labeled T<sub>Reg</sub> and T<sub>Eff</sub> at ratios of 1:1 or 1:4 or T<sub>Eff</sub> alone were plated in round-bottom 96-well plates and stimulated with Dynabeads mouse anti-CD3/CD28 Ab (Invitrogen) at a ratio of 0.5 bead/cell. Since T<sub>Reg</sub>:T<sub>Eff</sub> ratios of 1:1 or 1:4 ratios gave comparable results, the manuscript only reports data on 1:1 ratios. Flow cytometry was performed after three days using a FACSCalibur™ instrument (BD Biosciences, San Jose, CA) and analysis was done using FlowJo software (Tree Star Inc., Ashland, OR). Cells were analyzed for CFSE dilution and percent suppression was determined based on the percentage of dividing CFSE-labeled cells in the coculture as compared with CFSE-labeled cells cultured alone.

## 2.7 Statistical analyses

Statistical analyses were done using Prism 4 software (GraphPad, La Jolla, CA). Comparisons between two groups were done by Mann-Whitney *U* test, and ANOVA for more than two groups. *P*<0.05 was considered statistically significant.

## 3. Results

### 3.1 Effects of pCons on T<sub>Eff</sub> pathways

To study the effects of pCons on T<sub>Eff</sub>, we analyzed molecular pathways related to cell cycle, energy and T cell receptor signaling in sorted T<sub>Eff</sub> from pCons-treated animals versus controls. No differences were observed in the activation of ZAP-70, p27, ERK, STAT1, STAT3, STAT6, JNK, SAPK and p38 in T<sub>Eff</sub> from tolerized mice and controls (Fig.1).

### 3.2 pCons facilitates T<sub>Eff</sub> suppression by T<sub>Reg</sub>

Although intracellular signaling in the pathways tested in T<sub>Eff</sub> was not influenced by pCons, the suppression of T<sub>Eff</sub> by T<sub>Reg</sub> was more effective in pCons-tolerized mice as compared to mock-treated controls (Fig. 2). Since it has been shown that T<sub>Eff</sub> can acquire resistance to T<sub>Reg</sub> suppression in autoimmune conditions including SLE [2-4], we tested the possibility that pCons could modulate this aspect of the mechanisms of T<sub>Reg</sub>-mediated suppression in NZB/W mice. In cocultures of CFSE-labeled T<sub>Eff</sub> plus T<sub>Reg</sub> from pCons-tolerized or control (pNeg-treated) mice, T<sub>Reg</sub> more effectively suppressed T<sub>Eff</sub> from pCons-tolerized than from control mock-treated mice, whether the Treg were derived from either tolerized or control NZB/W mice (Fig. 2). Conversely, T<sub>Eff</sub> from tolerized mice were suppressed more than T<sub>Eff</sub> from control mice independently of whether T<sub>Reg</sub> were derived from pCons-treated or control mice (Fig. 2). Thus, pCons increased the sensitivity of T<sub>Eff</sub> to T<sub>Reg</sub> suppression in NZB/W mice. The observed effects were not due to altered T<sub>Eff</sub> responsiveness after peptide

treatment, since proliferative responses of  $T_{\text{Eff}}$  after polyclonal stimulation were similar between control and pCons-tolerized mice (Fig. S1).

### 3.3 pCons effects on $T_{\text{Eff}}$ resistance are p38-independent

We previously showed that a modulation of p38 activity in  $T_{\text{Reg}}$  contributed to the protection induced by pCons in NZB/W mice [7]. Although here we did not find differences in major signaling events (Fig. 1) or  $T_{\text{Eff}}$  proliferation (Fig. S1) after pCons-induced tolerance, it could still be possible that p38 might influence  $T_{\text{Eff}}$  activity. To address this possibility, NZB/W mice were injected with p38 inhibitor SB203580 or with control SB202474 or saline for 14 days. On day 7, mice were tolerized with pCons or left untreated, and on day 15  $T_{\text{Eff}}$  and  $T_{\text{Reg}}$  were isolated for functional studies. The proliferation of  $T_{\text{Eff}}$  from mice treated with SB203580 or SB202474 (or saline, not shown) was similar when  $T_{\text{Eff}}$  were suppressed by  $T_{\text{Reg}}$  from mice treated with SB203580 or SB202474 (Fig. 3), suggesting that the increased sensitivity of  $T_{\text{Eff}}$  to  $T_{\text{Reg}}$  suppression after pCons treatment was independent of the p38 pathway in  $T_{\text{Eff}}$ .

## 4. Discussion

In SLE, dysregulated T cell responses include an inadequate control of the activity of  $T_{\text{Eff}}$  by  $T_{\text{Reg}}$  [8]. Functional deficits and/or abnormal numbers of  $T_{\text{Eff}}$  and  $T_{\text{Reg}}$  have both been proposed as mechanisms that contribute to the loss peripheral immune tolerance in SLE [2]. For example, lupus  $T_{\text{Eff}}$  could differentiate into pathogenic T cell subsets and proliferate excessively [9], or  $T_{\text{Reg}}$  could be numerically reduced and/or be functionally deficient (and thus favor T cell autoreactivity) [10]. The findings reported in this manuscript might explain the conflicting data in the literature that reported abnormal or normal  $T_{\text{Reg}}$  number and/or function in SLE [2], since  $T_{\text{Eff}}$  resistance to suppression by  $T_{\text{Reg}}$  could influence outcomes. Importantly, our results also show that  $T_{\text{Eff}}$  resistance to  $T_{\text{Reg}}$  suppression can be modulated by the induction of immune tolerance. We acknowledge that additional factors that influence  $T_{\text{Reg}}$  suppression and  $T_{\text{Eff}}$  susceptibility, such as distinct TCR ligands or cytokines or environmental factors, could all contribute to the  $T_{\text{Reg}}$  suppression of  $T_{\text{Eff}}$  [11-14]. Yet the finding that pCons operates at multiple levels that include a modulation of  $T_{\text{Eff}}$  resistance to suppression imply that a manipulation of  $T_{\text{Reg}}$  in therapeutic settings might be complicated by the responsiveness of target cells. For example, if  $T_{\text{Eff}}$  resistance occurred in the setting of a numeric increase of  $T_{\text{Reg}}$ , those  $T_{\text{Reg}}$  might not be clinically effective. Conversely, the induction of a reduced threshold of  $T_{\text{Eff}}$  suppression by  $T_{\text{Reg}}$  (such as after tolerance), could result in beneficial effects on the disease progression and outcome.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The work was supported in part by the National Institutes of Health (AI095921 to A. L. C. and AI083294 to F-D. S.) and the Muscular Dystrophy Association (to F-D. S.).

## Abbreviations

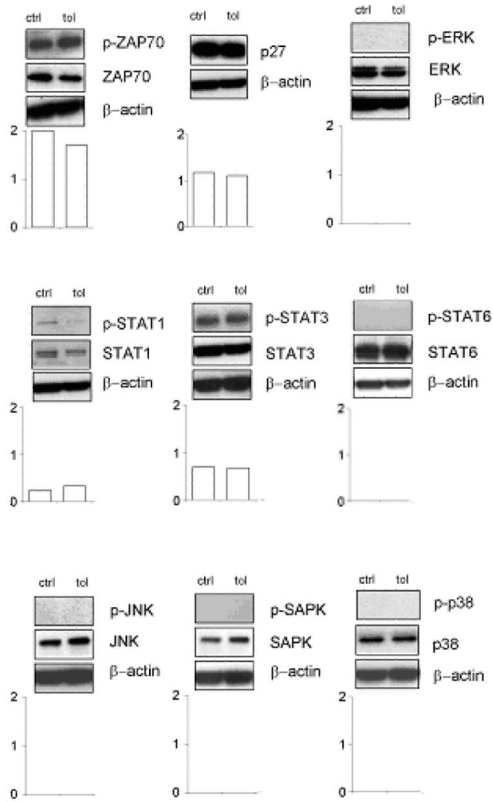
<b>SLE</b>	systemic lupus erythematosus
<b><math>T_{\text{Eff}}</math></b>	T effector cells
<b><math>T_{\text{Reg}}</math></b>	T regulatory cells

## References

1. Hahn, BH. An overview of the pathogenesis of systemic lupus erythematosus. In: Wallace, DJ.; Hahn, BH., editors. *Dubois' Lupus Erythematosus*. Lippincott Williams & Wilkins; Philadelphia: 2002. p. 87-96.
2. La Cava A. T-regulatory cells in systemic lupus erythematosus. *Lupus*. 2008; 17:421–425. [PubMed: 18490420]
3. Monk CR, Spachidou M, Rovis F, Leung E, Botto M, Lechler RI, Garden OA. MRL/Mp CD4<sup>+</sup>, CD25<sup>-</sup> T cells show reduced sensitivity to suppression by CD4<sup>+</sup>, CD25<sup>+</sup> regulatory T cells in vitro: a novel defect of T cell regulation in systemic lupus erythematosus. *Arthritis Rheum*. 2005; 52:1180–1184. [PubMed: 15818683]
4. Venigalla RK, Tretter T, Krienke S, Max R, Eckstein V, Blank N, Fiehn C, Ho AD, Lorenz HM. Reduced CD4<sup>+</sup>, CD25<sup>-</sup> T cell sensitivity to the suppressive function of CD4<sup>+</sup>, CD25<sup>high</sup>, CD127<sup>-/low</sup> regulatory T cells in patients with active systemic lupus erythematosus. *Arthritis Rheum*. 2008; 58:2120–2130. [PubMed: 18576316]
5. Vargas-Rojas MI, Crispin JC, Richaud-Patin Y, Alcocer-Varela J. Quantitative and qualitative normal regulatory T cells are not capable of inducing suppression in SLE patients due to T-cell resistance. *Lupus*. 2008; 17:289–294. [PubMed: 18413409]
6. Hahn BH, Singh RR, Wong WK, Tsao BP, Bulpitt K, Ebling FM. Treatment with a consensus peptide based on amino acid sequences in autoantibodies prevents T cell activation by autoantigens and delays disease onset in murine lupus. *Arthritis Rheum*. 2001; 44:432–441. [PubMed: 11229475]
7. Lourenço EV, Procaccini C, Ferrera F, Iikuni N, Singh RP, Filaci G, Matarese G, Shi FD, Brahn E, Hahn BH, La Cava A. Modulation of p38 MAPK activity in regulatory T cells after tolerance with anti-DNA Ig peptide in (NZB × NZW)F<sub>1</sub> lupus mice. *J. Immunol*. 2009; 182:7415–7421. [PubMed: 19494264]
8. La Cava A, Ebling FM, Hahn BH. Ig-reactive CD4<sup>+</sup>CD25<sup>+</sup> T cells from tolerized (New Zealand Black × New Zealand White)F<sub>1</sub> mice suppress in vitro production of antibodies to DNA. *J. Immunol*. 2004; 173:3542–3548. [PubMed: 15322219]
9. Shin MS, Lee N, Kang I. Effector T-cell subsets in systemic lupus erythematosus: update focusing on Th17 cells. *Curr. Opin. Rheumatol*. 2011; 23:444–448. [PubMed: 21720245]
10. Scalapino KJ, Daikh DI. Suppression of glomerulonephritis in NZB/NZW lupus prone mice by adoptive transfer of ex vivo expanded regulatory T cells. *PLoS One*. 2009; 4:e6031. [PubMed: 19551149]
11. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4<sup>+</sup>CD25<sup>+</sup> T cell-mediated suppression by dendritic cells. *Science*. 2003; 299:1033–1036. [PubMed: 12532024]
12. De Rosa V, Procaccini C, Cali G, Pirozzi G, Fontana S, Zappacosta S, La Cava A, Matarese G. A key role of leptin in the control of regulatory T cell proliferation. *Immunity*. 2007; 26:241–255. [PubMed: 17307705]
13. Yates J, Rovis F, Mitchell P, Afzali B, Tsang JY, Garin M, Lechler RI, Lombardi G, Garden OA. The maintenance of human CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cell function: IL-2, IL-4, IL-7 and IL-15 preserve optimal suppressive potency in vitro. *Int. Immunol*. 2007; 19:785–799. [PubMed: 17545278]
14. Hänig J, Lutz MB. Suppression of mature dendritic cell function by regulatory T cells in vivo is abrogated by CD40 licensing. *J. Immunol*. 2008; 180:1405–1413. [PubMed: 18209035]

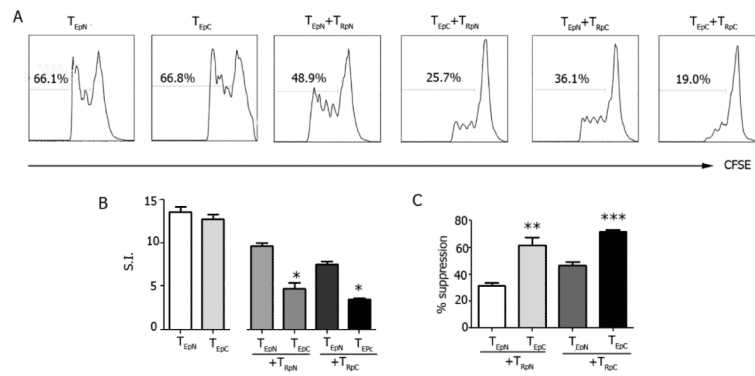
### Highlights

- We studied the effects of the tolerogenic peptide pCons on  $T_{\text{Eff}}$  in NZB/W lupus mice
- We found that pCons decreased the resistance of  $T_{\text{Eff}}$  to suppression by  $T_{\text{Reg}}$
- The findings are relevant in the design of  $T_{\text{Reg}}$ -based therapies in SLE



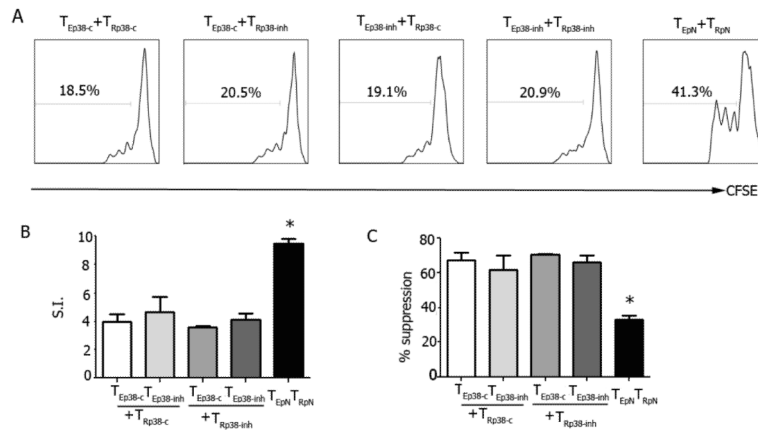
**Figure 1. Signaling pathways in T<sub>Eff</sub> after tolerization with pCons**

Western blots for phosphorylated (p-) and non-phosphorylated ZAP-70, p27, ERK, STAT1, STAT3, STAT6, JNK, SAPK and p38 in sorted T<sub>Eff</sub> from mice tolerized with pCons and control mice receiving pNeg (saline gave identical results, not shown). Graphs show the densitometric quantitation of each protein to its non-phosphorylated form. One representative experiment of four is shown.



**Figure 2. pCons reduces T<sub>Eff</sub> resistance to suppression by T<sub>Reg</sub> in NZB/W lupus mice**  
 CFSE-labeled T<sub>Eff</sub> (T<sub>E</sub>) were cocultured with T<sub>Reg</sub> (T<sub>R</sub>) from pCons-tolerized (p<sub>C</sub>) or pNeg-treated control (p<sub>N</sub>) NZB/W mice in the presence of CD3/CD28 Ab for 3 days before flow cytometry. Representative (A) and cumulative (B) results including the percent of T<sub>Eff</sub> suppression by T<sub>Reg</sub> (C). \*P<0.004; \*\*P<0.009; \*\*\*P<0.007.





**Figure 3. pCons reduces T<sub>Eff</sub> suppression by T<sub>Reg</sub> in a p38-independent fashion**

Groups of 7-8 NZB/W mice each were injected daily with the p38 inhibitor SB203580 (p38-inh) or control SB202474 (p38-c) for 14 days. On day 7, mice were tolerized with pCons or given control pNeg. After one week, *ex vivo* sorted T<sub>Eff</sub> (T<sub>E</sub>) were CFSE-labeled and cocultured with T<sub>Reg</sub> (T<sub>R</sub>) in the presence of CD3/CD28 Ab for 3 days before flow cytometry. Representative (A) and cumulative data of T<sub>Eff</sub> suppression by T<sub>Reg</sub> (B-C). \*P<0.002 by ANOVA.