Mol. Cells, Vol. 25, No. 1, pp. 64-69

Molecules and Cells

$CD4^+CD25^+$ Regulatory T Cells Selectively Diminish Systemic Autoreactivity in Arthritic K/BxN Mice

Sang Mee Kang†, Eunkyeong Jang†, Doo-Jin Paik, Young-Ju Jang[1], and Jeehee Youn*

Department of Anatomy and Cell Biology, College of Medicine, Hanyang University, Seoul 133-791, Korea;
[1] Laboratory of Immunology, Institute for Medical Science, Ajou University, Suwon 443-749, Korea.

(Received June 7, 2007; Accepted September 4, 2007)

Although the arthritis symptoms observed in the K/BxN model have been shown to be dependent on the functions of T and B cells specific to the self antigen glucose-6-phosphate isomerase, less is known about the *in vivo* roles of $CD4^+CD25^+$ regulatory T (T_{reg}) cells in the pathology of K/BxN mice. We determined the quantitative and functional characteristics of the T_{reg} cells in K/BxN mice. These mice contained a higher percentage of $Foxp3^+$ T_{reg} cells among the $CD4^+$ T cells than their BxN littermates. These T_{reg} cells were anergic and efficiently suppressed the proliferation of naïve $CD4^+$ T cells and cytokine production by effector $CD4^+$ T cells *in vitro*. Antibody-mediated depletion of $CD25^+$ cells caused K/BxN mice to develop multi-organ inflammation and autoantibody production, while the symptoms of arthritis were not affected. These results demonstrate that despite the inability of the T_{reg} cells to suppress arthritis development, they play a critical role protecting the arthritic mice from systemic expansion of autoimmunity.

Keywords: Autoimmunity; K/BxN Model; Regulatory T Cells; Rheumatoid Arthritis.

Introduction

Autoimmune disease can develop not only because of the generation of self-reactive T cells, but also due to the inability to maintain the appropriate number of functional regulatory cells. $CD4^+CD25^+Foxp3^+$ regulatory T (T_{reg}) cells are a unique population of professional suppressor cells that constitute 5−10% of peripheral $CD4^+$ T cells in both mice and humans (Miyara and Sakaguchi, 2007; Shevach, 2002). They are naturally anergic to stimulation via the T cell receptor (TCR) and inhibit the proliferation and function of conventional T cells by a contact-dependent mechanism. Evidence suggests that a failure to regulate the number of T_{reg} cells can cause some types of autoimmune disease, such as autoimmune diabetes and collagen-induced arthritis (Morgan et al., 2003; Salomon et al., 2000; Wu et al., 2002). In addition, functional defects in T_{reg} cells have been found in human autoimmune diseases such as multiple sclerosis, autoimmune polyglandular syndrome and rheumatoid arthritis (RA) (Ehrenstein et al., 2004; Kriegel et al., 2004; Viglietta et al., 2004). While T_{reg} cells have been implicated in autoimmune diseases, their roles are still obscure. T_{reg} cells from patients with RA are found to be unable to suppress pro-inflammatory cytokine production by activated T cells, unlike normal functioning T_{reg} cells (Ehrenstein et al., 2004). However, another study demonstrated that patients with RA actually contained higher numbers of T_{reg} cells with increased suppressive activity (van Amelsfort et al., 2004). Furthermore, a study of a proteoglycan-induced arthritis model concluded that T_{reg} cells are not involved in controlling autoimmune arthritis, while another report suggested that collagen-induced arthritis is regulated by suppression by T_{reg} cells (Bardos et al., 2003; Morgan et al., 2003). Thus, the role of T_{reg} cells in the pathogenesis of RA remains the subject of considerable debate.

The K/BxN murine model of RA has many of the clinical and histological features of the human disease. K/BxN mice were generated by crossing KRN TCR transgenic mice with NOD mice. In the context of MHC $I\text{-}A^{g7}$, the KRN TCR, which is composed of $V_\alpha4$ and $V_\beta6$ chains, recognizes peptide 281-293 of glucose-6-phosphate isomerase (GPI), a ubiquitous glycolytic enzyme that is also present on the surface of inflamed joints (Kouskoff et al., 1996). Because activation of autoreactive $CD4^+$ T cells is

† These authors contributed equally to this work.

* To whom correspondence should be addressed.
Tel: 82-2-2220-0604; Fax: 82-2-2281-7841
E-mail: jhyoun@hanyang.ac.kr

actively suppressed by T_{reg} cells in normal conditions, some abnormality of T_{reg} cells is suspected to be the cause of autoimmunity in this model. The observation that these mice have reduced numbers of thymic emigrants that give rise to a skewed TCR repertoire (Jang et al., 2006) prompted us to investigate whether functional T_{reg} cells are present in the periphery of these mice. A recent study proposed that the over-expression of a peptide mimic of the GPI epitope under the control of the MHC class II promoter in K/BxN mice resulted in thymic deletion of GPI-specific T_{reg} cells, and led to systemic autoimmunity (Shih et al., 2004). However, the entire thymic repertoire is lost in this double transgenic system; both GPI-specific effector T (T_{eff}) cells and T_{reg} cells are deleted and GPI-nonspecific T_{eff} cells are enhanced. Therefore, the autoimmune response might not be caused by T_{reg} cell deletion alone.

In the present study we investigated whether $CD4^+$ $CD25^+$ T_{reg} cell function is associated with the pathogenesis of joint-restricted disease in K/BxN mice, by determining the number, phenotypes and *in vitro* functions of T_{reg} cells derived from these mice. In addition, antibody (Ab)-mediated *in vivo* depletion of $CD25^+$ T_{reg} cells allowed us to determine the *in vivo* role of T_{reg} cells when autoimmune inflammation develops systemically but genetically programmed arthritogenicity is not implicated.

Materials and Methods

Mice A cross between KRN TCR-transgenic mice on a C57BL/6 background (K/B) and NOD mice generated arthritic transgenic progeny (K/BxN) and nontransgenic littermates (BxN). All mice were maintained under specific pathogen-free conditions in the animal facility at Hanyang University in accordance with the guidelines established by Hanyang University.

Flow cytometry Single cell suspensions of spleen and draining (axillary, inguinal and popliteal) lymph nodes were surface or intracellularly stained by standard protocols. The following monoclonal Abs (mAbs) were used for flow cytometry: PE- or APC-conjugated anti-CD25 (PC61.5), FITC-conjugated-anti-CD27 (LG.7F9), and FITC-conjugated anti-Foxp3 (FJK-16) Abs purchased from eBioscience, and PerCP-conjugated-anti-CD4 (RM4-5) and PE-conjugated anti-Vβ6 (RR4-7) Abs obtained from BD Pharmingen.

***In vitro* suppression assays** $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified by MACS (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the resulting cell fractions was around 90–95%. $CD4^+CD25^-$ responder T cells from BxN mice (5×10^4/well) were placed in 96-well plates with 3,000 rad-irradiated $CD4^+$ T cell-depleted splenocytes from BxN mice as antigen-presenting cells (APC, 5×10^4/well), soluble 1 μg/ml anti-CD3 Ab (BD Pharmingen), and the indicated numbers of $CD4^+CD25^+$ cells from either BxN or K/BxN mice. To examine suppression of self antigen-specific proliferation, $CD4^+CD25^-$ responder cells from K/BxN mice (10^5/well) were co-cultured with $CD4^+CD25^+$ cells from either K/BxN or BxN mice in the presence of APC and the indicated concentrations of GPI peptide (Matsumoto et al., 1999). After pulsing with [^{3}H]-thymidine (0.5 μ*Ci*/well; Amersham Biosciences) for the last 6 h of a total of 72 h of culture, [^{3}H]-thymidine incorporation was assayed. All assays were conducted in triplicate.

Cytokine assays $CD4^+CD25^-$ cells purified from BxN mice were stimulated with 10 μg/ml anti-CD3 and 2 μg/ml anti-CD28 Abs in the presence of 10 ng/ml IL-12 and 5 μg/ml anti-IL-4 mAb (all from BD Pharmingen). After 5 days, the cells were washed and restimulated for 72 h with 1 μg/ml anti-CD3 Ab and APCs in the presence or absence of $CD4^+CD25^+$ cells purified from either BxN or K/BxN mice. The concentrations of IFN-γ and TNF-α in the culture supernatants were determined by sandwich ELISA (R&D Systems).

RT-PCR The levels of Foxp3 mRNA in purified $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were measured by semi-quantitative RT-PCR with the following primers: 5′-CTT CAT GCA TCA GCT CTC CAC-3′ and 5′-AGA CTC CAT TTG CCA GCA GTG-3′. The relative quantity of Foxp3 cDNA was normalized by the amount of CD4 cDNA measured with the following primers: 5′-AAC GCT CCC ACT CAC CCT CAA GAT AC-3′ and 5′-CCA AGG AAA CCC AGA AAG CCG AAG-3′.

***In vivo* $CD25^+$ cell depletion and clinical and histopathological analyses** The PC61.5.3 (anti-murine CD25) hybridoma was purchased from the American Type Culture Collection and the Ab was purified from culture supernatants using a protein G column (Amersham Biosciences). IgG purified from normal mouse serum (Sigma-Aldrich) was used as a control. K/BxN mice were injected intraperitoneally with 0.5 mg/mouse/day purified anti-CD25 mAb or IgG on two consecutive days starting on day 21 after birth. The extent of $CD25^+$ cell depletion in peripheral blood was determined by FACS analyses. Following blind inspection, the severity of the disease was evaluated using the scoring system previously described (Jang et al., 2006). Kidney, lung, and liver were removed postmortem on day 28 after Ab injection, fixed and embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Antibody measurement by ELISA. Sera were collected from K/BxN mice on days 7, 14, and 21 after anti-CD25 or IgG injection to measure the level of anti-double stranded (ds) DNA and anti-GPI IgG_1 Abs by ELISA, as described previously (Jang et al., 2006; Park et al., 2001). In brief, for anti-dsDNA ELISA, sera were diluted 1:100 in PBS and applied to immunosorbent plates (Nunc) coated with 1 μg/ml thymus DNA (Sigma-Aldrich). Mouse anti-dsDNA mAb (G1-5) produced previously from MRL-*lpr/lpr* mice (Park et al., 2001) was purified by affinity chromatography and used as standard. Goat anti-mouse Ig-horseradish peroxidase (R&D Systems) was used as secondary

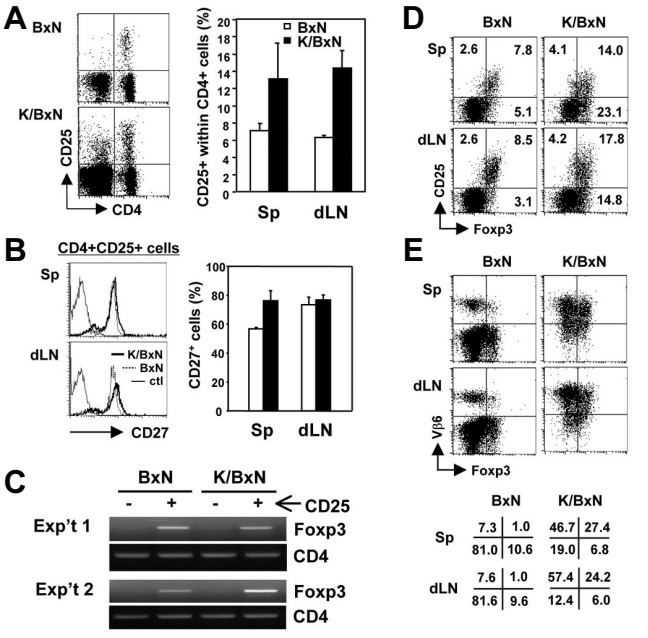

Fig. 1. Identification of T_{reg} cells in K/BxN mice. A and B, spleen (Sp) and draining lymph node (dLN) cells from 5- to10-week-old K/BxN and BxN mice were stained with anti-CD4-PerCP, anti-CD25-PE, and anti-CD27-FITC, followed by FACS analysis. **A.** a representative CD4/CD25 profile of spleen cells (left) and the percentage of $CD25^+$ cells among $CD4^+$ cells in spleen and draining lymph node from K/BxN (■) and BxN (□) mice (right) are shown. **B.** representative CD27 profiles on gated $CD4^+CD25^+$ cells in spleen and draining lymph node (left) and the percentage of $CD27^+$ cells among $CD4^+CD25^+$ cells in spleen and draining lymph node from K/BxN (■) and BxN (□) (left) mice are shown. **C.** RT-PCR analysis of Foxp3 expression, in comparison with CD4 levels, in purified $CD4^+CD25^-$ or $CD4^+CD25^+$ cells from K/BxN and BxN mice. **D** and **E.** spleen and draining lymph node cells were stained with anti-CD4-PerCP, anti-CD25-APC, anti-Foxp3-FITC, and anti-V_β6-PE. Figures are FACS plots on gated $CD4^+$ T cells; they are representative of five independent experiments. The mean percentages of cells in each quadrant are indicated.

Ab. For GPI-specific IgG_1 ELISA, sera diluted 1:5000 were compared with standard serum collected from seven-week-old K/BxN mice and serially diluted.

Results

Higher percentages of $CD4^+CD25^+$ T_{reg} cells in peripheral lymphoid organs of K/BxN mice As shown previously by us and other investigators, K/BxN TCR transgenic mice exhibit $CD4^+$ T lymphopenia in their peripheral lymphoid organs (Jang et al., 2006; Shih et al., 2004), implying that the normal thymic selection which occurs during development is perturbed by the expression of TCR transgenes. K/BxN mice contain an approximately 2-fold higher proportion of $CD25^+$ cells within $CD4^+$ T cell population in their spleens and draining lymph nodes than their nontransgenic BxN littermates (Fig. 1A). Because CD25 is not only a marker for T_{reg} cells but is also transiently up-regulated in non-T_{reg} cells upon activation, this increase may in part be due to the emergence of T_{eff} cells activated during disease progression. To discriminate T_{reg} cells from T_{eff} cells, we determined the CD27 positivity of $CD4^+CD25^+$ T cells, since it has been shown that CD27 is lost on terminally differentiated T_{eff} cells but retained on T_{reg} cells after activation and expansion (Ruprecht et al., 2005). The majority of $CD4^+CD25^+$ cells in both spleen and draining lymph node from both strains of mice expressed CD27 on their cell surface, suggesting a T_{reg} cell phenotype (Fig. 1B). More critically, the mRNA level of Foxp3, a T_{reg} cell-specific marker, in $CD4^+CD25^+$ T cells from K/BxN mice was comparable to that from those of BxN mice (Fig. 1C). In cytofluorometric analyses by intracellular Foxp3 staining, K/BxN mice exhibited approximately 3-fold higher fraction of $Foxp3^+$ cells among the $CD4^+$ T cells than did their BxN littermates; increases of approximately 2- and 4.7-fold were observed in $CD25^+Foxp3^+$ and $CD25^-Foxp3^+$ cells, respectively (Fig. 1D). Regardless of strain, 75–81% of the $CD4^+CD25^+$ T cells were $Foxp3^+$ and only 2.6–4.7% of the $CD4^+$ T cells were $CD25^+Foxp3^-$; thus, the majority of $CD4^+CD25^+$ cells from K/BxN mice were Foxp3-expressing T_{reg} cells rather than conventionally activated T_{eff} cells. Approximately 80% of the $Foxp3^+$ $CD4^+$ cells were TCR $V_\beta6^+$ in both spleen and draining lymph nodes (Fig. 1E), a similar percentage to that among the conventional $Foxp3^-CD4^+$ T cells in K/BxN mice. This result demonstrates that most T_{reg} cells express the TCR encoded by the transgene rather than by the endogenous pool of TCR genes.

Normal *in vitro* anergic/suppressive activity of T_{reg} cells from K/BxN mice To determine whether the $CD4^+CD25^+$ cells from K/BxN mice retain normal T_{reg} cell functions, we subjected $CD4^+CD25^+$ cells purified from K/BxN mice to *in vitro* proliferation and suppression assays. Like T_{reg} cells from normal BxN mice, $CD4^+CD25^+$ cells from K/BxN mice did not proliferate upon stimulation with anti-CD3 Ab plus APCs, and efficiently suppressed the proliferation of $CD4^+CD25^-$ T cells obtained from BxN mice (Figs. 2A and 2B). $CD4^+CD25^-$ cells from K/BxN mice proliferated in response to the GPI peptide in a concentration-dependent manner, and this effect was suppressed by $CD4^+CD25^+$ cells from K/BxN mice at a ratio of responders: suppressors of 8:1, but not by those from BxN mice even at a 1:1 ratio (Fig. 2C). Moreover, the ability of $CD4^+CD25^+$ cells from K/BxN mice to suppress the production of TNF-α and IFN-γ by $CD4^+$ type 1 helper T cells from BxN mice was equal to that of the cells from BxN mice (Fig. 2D). Thus, $CD4^+CD25^+$ T cells from K/BxN

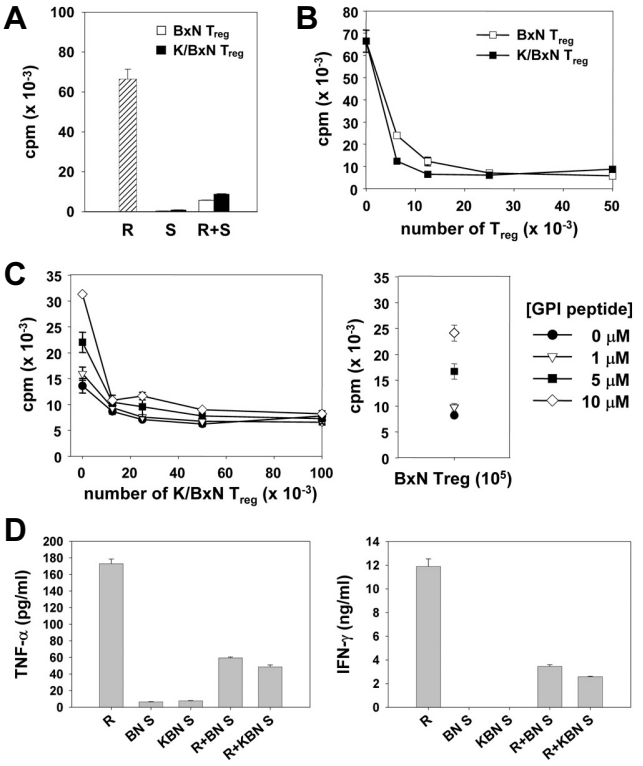

Fig. 2. *In vitro* function of T_{reg} cells from K/BxN mice. **A.** $CD4^+CD25^-$ responder T cells (R) from BxN mice, $CD4^+CD25^+$ T cells (S) from either BxN (□) or K/BxN (■) mice, and a mixture of equal numbers of R and S were stimulated with anti-CD3 and APC for 3 days, followed by [^{3}H]-thymidine incorporation assays. **B.** $CD4^+CD25^-$ responder T cells from BxN mice (5×10^4/well) were cultured with the indicated numbers of $CD4^+CD25^+$ cells from either BxN (□) or K/BxN (■) mice in the presence of anti-CD3 and APC. **C.** $CD4^+CD25^-$ responder T cells from K/BxN mice (10^5/well) were cultured with the indicated numbers of $CD4^+CD25^+$ cells from K/BxN mice (left) or 10^5/well of $CD4^+CD25^+$ cells from BxN mice. The cells were stimulated with APC and GPI peptide as indicated. **D.** $CD4^+$ Th1 cells (R) were stimulated in the presence or absence of $CD4^+CD25^+$ cells purified from either BxN (BN S) or K/BxN (KBN S) mice for 72 h. The concentrations of IFN-γ and TNF-α in the culture supernatants were determined by ELISA. Figures are representative of at least 3 independent sets of experiments.

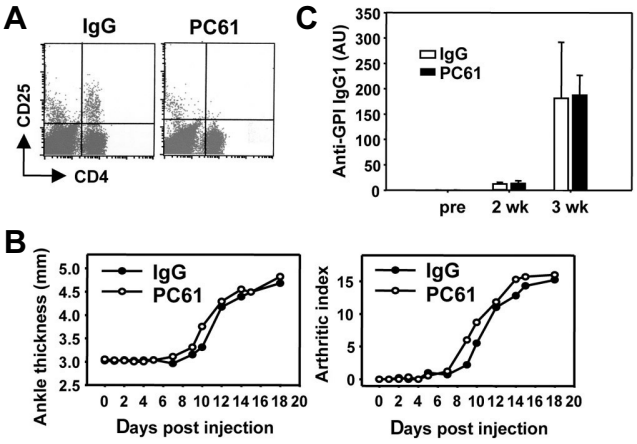

Fig. 3. PC61-mediated depletion of $CD25^+$ cells in K/BxN mice. K/BxN mice were injected with PC61 anti-CD25 Ab or control IgG. **A.** Depletion of $CD4^+CD25^+$ T cells was checked in the blood weekly by FACS with anti-CD4 and anti-CD25 Abs. A representative staining pattern on day 21 is shown. **B.** ankle thickness of hindpaws and mean arthritis index of PC61- and IgG-injected K/BxN mice are shown. **C.** Serum was collected before and two and three weeks post-injection from PC61 (■)- and IgG (□)-injected mice and the level of GPI-specific IgG_1 was determined by ELISA. Figures are representative of at least 3 independent sets of experiments (n = 3–4 per group in each set of experiments).

mice appear to be functionally intact T_{reg} cells retaining anergic properties and suppressor activities.

Multi-organ inflammation and autoantibody production in $CD25^+$ cell-depleted K/BxN mice To investigate the *in vivo* role of T_{reg} cells in K/BxN mice, T_{reg} cells were depleted by injection with PC61 anti-CD25 mAb. $CD4^+CD25^+$ T cells were effectively depleted for at least 3 weeks after Ab injection (Fig. 3A). Unexpectedly, PC61 injection did not accelerate nor exacerbate the onset and progression of arthritis, compared to control IgG injection (Fig. 3B). Consistent with this, serum levels of GPI-specific IgG_1 did not differ between the two groups of mice (Fig. 3C). However, importantly, the PC61-injected K/BxN mice exhibited perivascular and interstitial inflammation in their kidney, lung and liver tissues 4 weeks after Ab injection, whereas IgG-injected K/BxN mice did not (Fig. 4A). In addition, serum levels of anti-dsDNA Abs were significantly higher in the PC61-injected mice (Fig. 4B). Other symptoms indicating wasting disease were not evident up to 4 weeks after Ab injection and control IgG injection did not alter the physiology of the mice in any of the tests to which they were submitted (data not shown). Thus, $CD25^+$ T_{reg} cell depletion resulted in systemic autoimmunity, as manifested by multi-organ inflammation and autoantibody production.

Discussion

The anti-GPI transgenic K/BxN mice develop autoimmune disease that is restricted to joints where anti-GPI IgG produced through GPI-specific $CD4^+$ T cell help coordinates diverse inflammatory responses. Therefore, the balance between the two counteracting populations, GPI-specific T_{eff} cells and their suppressors, would be expected to be important for the maintenance of self tolerance in this arthritis model. However, our results indicate this is not the

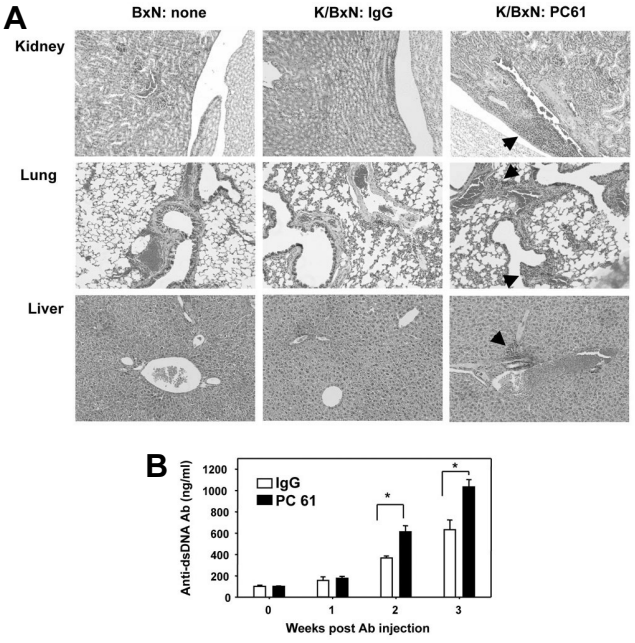

Fig. 4. Effect of $CD25^+$ cell depletion in K/BxN mice. K/BxN mice were injected with PC61 anti-CD25 Ab or control IgG. **A.** kidney, lung, and liver were removed postmortem on day 28 after Ab injection. Hematoxylin and eosin staining of the tissues from untreated BxN (left), IgG- (middle) and PC61-treated K/BxN (right) mice. Arrows indicate inflamed sites. **B.** the serum level of anti-dsDNA Ab was determined by ELISA. * $P < 0.05$ by Student's *t*-test. Figures are representative of at least 3 independent sets of experiments (n = 3-4 per group in each set of experiments).

case. We have demonstrated here that, despite the presence of an increased proportion of $CD4^+CD25^+$ T_{reg} cells with intact suppressor activity, autoimmune arthritis in the K/BxN model is not suppressed. In accordance with the spontaneous development of arthritis despite the presence of T_{reg} cells, *in vivo* depletion of these cells did not affect the onset or course of joint disease. However, depletion of T_{reg} cells did induce autoantibody production and substantial levels of inflammation in several organs. These results, taken together, suggest that the effect of the T_{reg} cells is not sufficient to block the genetically programmed arthritogenicity, but that they actively engage in the maintenance of systemic self-tolerance. It still remains enigmatic how arthritogenic T cells escape suppression by the T_{reg} cells, even though the mice contain both TCR $V\beta6^+$ transgenic and endogenous TCR^+ T_{reg} cells.

The lack of susceptibility of autoimmune arthritis to suppression by T_{reg} cells is intriguing since several studies in animal models including K/BxN mice, and in patients with RA, have noted enrichment of T_{reg} cells in the inflamed joint cavities (Cao et al., 2004; Nguyen et al., 2007). This may be partly due to the unique characteristics of the joint microenvironment. Joints have been previously shown to be the locations where vascular permeability is greatly enhanced, circulating Abs accumulate, and the alternative complement pathway is preferentially activated by immune complexes (Binstadt et al., 2006; Ji et al., 2002). These characteristics may provide a mechanism that bypasses T_{reg} cells and initiates autoimmune arthritis.

It is also notable that K/BxN mice contain an enhanced proportion of $CD25^-CD4^+Foxp3^+$ cells. This phenomenon may be related to their lymphopenic state, since $CD25^+CD4^+$ T_{reg} cells may cease to express CD25, especially when they are subjected to homeostatic proliferation in a lymphopenic environment (Nishimura et al., 2004). It is thus likely that the Foxp3-expressing $CD25^+$ and $CD25^-$ T_{reg} cells present in K/BxN mice are functionally similar and developmentally equivalent. Depletion of $CD25^+$ T cells may result in only partial ablation of the T_{reg} cell population. This may explain the discrepancy between our findings and the recent report of Nguyen et al. (2007) concerning the influence of T_{reg} cell depletion on arthritis development. Nguyen and coworkers showed that the absence of T_{reg} cells in Foxp3 mutant K/BxN mice led to faster and more aggressive arthritis. Nevertheless, this discrepancy does not alter our conclusion that K/BxN mice develop arthritis in the presence of intact T_{reg} cells.

In summary, we have demonstrated that T_{reg} cells in arthritic K/BxN mice play a critical role in inhibiting the systemic spread of autoimmunity, although the activities of these cells are not sufficient to suppress the genetically programmed arthritogenicity. Our demonstration of a T_{reg} cell-bypassing mechanism that initiates autoimmune arthritis may provide an alternative interpretation for the joint restriction of autoimmune disease.

Acknowledgments We thank Drs. Diane Mathis and Doo Hyun Chung for providing K/B mice, Dr. Moon-Hyang Park for histopathologic interpretation, Dr. Michael A. Lynes for valuable discussion, Mr. Chul-Shik Lim for technical assistance, and Ms. Jocelyn Graf for editorial assistance. This work was supported by MOST/KOSEF grants (R01-2005-000-10107-0 and R11-2002-098-05001-0) and a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A050445).

References

Bardos, T., Czipri, M., Vermes, C., Finnegan, A., Mikecz, K., and Zhang, J. (2003). $CD4^+CD25^+$ immunoregulatory T cells may not be involved in controlling autoimmune arthritis. Arthritis Res. Ther. *5*, 106–1013.

Binstadt, B.A., Patel, P.R., Alencar, H., Nigrovic, P.A., Lee, D.M., Mahmood, U., Weissleder, R., Mathis, D., and Benoist, C. (2006). Particularities of the vasculature can promote the organ specificity of autoimmune attack. Nat. Immunol. *7*, 284–292.

Cao, D., van Vollenhoven, R., Klareskog, L., Trollmo, C., and

Malmstrom, V. (2004). $CD25^{bright}CD4^{+}$ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease. Arthritis Res. Ther. *6*, R335–R346.

Ehrenstein, M.R., Evans, J.G., Singh, A., Moore, S., Warnes, G., Isenberg, D.A., and Mauri, C. (2004). Compromised function of regulatory T cells in RA and reversal by anti-TNFα therapy. J. Exp. Med. *200*, 277–285.

Jang, E., Kim, H.R., Cho, S.H., Paik, D.J., Kim, J.M., Lee, S.K., and Youn, J. (2006). Prevention of spontaneous arthritis by inhibiting homeostatic expansion of autoreactive $CD4^{+}$ T cells in the K/BxN mouse model. Arthritis Rheum. *54*, 492–498.

Ji, H., Ohmura, K., Mahmood, U., Lee, D.M., Hofhuis, F.M., Boackle, S.A., Takahashi, K., Holers, V.M., Walport, M., Gerard, C., et al. (2002). Arthritis critically dependent on innate immune system players. Immunity *16*, 157–168.

Kouskoff, V., Korganow, A.S., Duchatelle, V., Degott, C., Benoist, C., and Mathis, D. (1996). Organ-specific disease provoked by systemic autoimmunity. Cell *87*, 811–822.

Kriegel, M.A., Lohmann, T., Gabler, C., Blank, N., Kalden, J.R., and Lorenz, H.M. (2004). Defective suppressor function of human $CD4^{+}CD25^{+}$ regulatory T cells in autoimmune polyglandular syndrome type II. J. Exp. Med. *199*, 1285–1291.

Matsumoto, I., Staub, A., Benoist, C., and Mathis, D. (1999). Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. Science *286*, 1732–1735.

Miyara, M. and Sakaguchi, S. (2007). Natural regulatory T cells: mechanisms of suppression. Trends Mol. Med. *13*, 108–116.

Morgan, M.E., Sutmuller, R.P., Witteveen, H.J., van Duivenvoorde, L.M., Zanelli, E., Melief, C.J., Snijders, A., Offringa, R., de Vries, R.R., and Toes, R.E. (2003). CD25+ cell depletion hastens the onset of severe disease in collagen-induced arthritis. Arthritis Rheum. *48*, 1452–1460.

Nguyen, L.T, Jacobs, J., Mathis, D., and Benoist, C. (2007). Where FoxP3 dependent regulatory T cells impinge on the development of inflammatory arthritis. Arthritis Rheum. *56*, 509–520.

Nishimura, E., Sakihama, T., Setoguchi, R., Tanaka, K., and Sakaguchi, S. (2004). Induction of antigen-specific immunologic tolerance by *in vivo* and *in vitro* antigen-specific expansion of naturally arising $Foxp3^{+}CD25^{+}CD4^{+}$ regulatory T cells. Int. Immunol. *16*, 1189–1201.

Park, J.S., Kim, Y.T., Chung, H.Y., Baek, K., and Jang, Y.J. (2001). Primary structures and chain dominance of anti-DNA antibodies. Mol. Cells *11*, 55–63.

Ruprecht, C.R., Gattorno, M., Ferlito, F., Gregorio, A., Martini, A., Lanzavecchia, A., and Sallusto, F. (2005). Coexpression of CD25 and CD27 identifies $Foxp3^{+}$ regulatory T cells in inflamed synovia. J. Exp. Med. *201*, 1793–1803.

Salomon, B., Lenschow, D.J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A., and Bluestone, J.A. (2000). B7/CD28 costimulation is essential for the homeostasis of the $CD4^{+}CD25^{+}$ immunoregulatory T cells that control autoimmune diabetes. Immunity *12*, 431–440.

Shevach, E.M. (2002). $CD4^{+}CD25^{+}$ suppressor T cells: more questions than answers. Nat. Rev. Immunol. *2*, 389–400.

Shih, F.F., Mandik-Nayak, L., Wipke, B.T., and Allen, P.M. (2004). Massive thymic deletion results in systemic autoimmunity through elimination of $CD4^{+}CD25^{+}$ T regulatory cells. J. Exp. Med. *199*, 323–335.

van Amelsfort, J.M.R., Jacobs, K.M.G., Bijlsma, J.W.J., Lafeber, F.P.J.G., and Taams, L.S. (2004). CD4+CD25+ regulatory T cells in RA. Arthritis Rheum. *50*, 2775–2785.

Viglietta, V., Baecher-Allan, C., Weiner, H.L., and Hafler, D.A. (2004). Loss of functional suppression by $CD4^{+}$ $CD25^{+}$ regulatory T cells in patients with multiple sclerosis. J. Exp. Med. *199*, 971–979.

Wu, A.J., Hua, H., Munson, S.H., and McDevitt, H.O. (2002). Tumor necrosis factor-α regulation of $CD4^{+}CD25^{+}$ T cell levels in NOD mice. Proc. Natl. Acad. Sci. USA *99*, 12287–12292.