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**BAFF antagonist attenuates the development of skin fibrosis
in Tight-Skin Mice**

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Short title: BAFF in tight-skin mice

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Abstract

The tight-skin (TSK/+) mouse, a genetic model for systemic sclerosis (SSc), develops cutaneous fibrosis and autoimmunity. Although immunological abnormalities have been demonstrated in TSK/+ mice, the roles of B cell activating factor belonging to the tumor necrosis factor family (BAFF), a potent B cell survival factor, have not been investigated. Serum BAFF levels in TSK/+ mice were examined by ELISA. Newborn TSK/+ mice were treated with BAFF antagonist, and then, skin fibrosis of 8-week-old mice was assessed. Serum BAFF levels were significantly elevated in TSK/+ mice. Remarkably, BAFF antagonist inhibited the development of skin fibrosis, hyper- γ -globulinemia, and the autoantibody production in TSK/+ mice. The skin from TSK/+ mice showed up-regulated expressions of fibrogenic cytokines, such as IL-6 and IL-10, while BAFF antagonist significantly suppressed them. Reciprocally, BAFF antagonist augmented anti-fibrogenic cytokines, such as IFN- γ , in the skin of TSK/+ mice. Furthermore, TSK/+ B cells with BAFF stimulation had a significantly enhanced ability to produce IL-6. The results suggest that BAFF/BAFF receptor system is critical for the development of skin fibrosis in TSK/+ mice. The results suggest that BAFF significantly contributes to the development of skin fibrosis and could be a potent therapeutic target.

Key words: Scleroderma, Tight-skin mouse, B cell, BAFF, Cytokine

Introduction

B cell activating factor belonging to the tumor necrosis factor family (BAFF), also known as BLyS, TALL-1, and THANK, is a tumor necrosis factor (TNF) superfamily member (TNFSF13B) best known for its role in the survival and maturation of B cells (Mackay and Browning, 2002). BAFF is produced by several cell types, including monocytes, macrophages, neutrophils, dendritic cells, and T lymphocytes (Schneider *et al.*, 1999), and is a ligand for at least three TNF receptor superfamily (TNFRSF) members: B-cell maturation antigen (BCMA/TNFRSF17), transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI/TNFRSF13B), and BAFF receptor (BAFF-R/BR3/TNFRSF13C) (Gross *et al.*, 2000; Thompson *et al.*, 2001). All three receptors are primarily expressed by B cells (Mackay and Browning, 2002). Among them, BAFF-R plays the central role in the BAFF system (Thompson *et al.*, 2001). BAFF/BAFF receptor family appears to span nearly all stages of B-lineage differentiation, ranging from the development, selection, and homeostasis of naive primary B cells to the maintenance of long-lived bone marrow plasma cells (O'Connor *et al.*, 2004). BAFF also exhibits a strong costimulatory function for B cell activation in vitro (Moore *et al.*, 1999; Schneider *et al.*, 1999). Furthermore, excess BAFF rescues self-reactive B cells from anergy, which may play a crucial role of autoimmune induction and development (Thien *et al.*, 2004). Mice overexpressing BAFF exhibit elevated B cell numbers in spleen and lymph node and characteristics of autoimmune diseases, including spontaneous autoantibody production, immunoglobulin (Ig) deposits in the kidneys, and glomerulonephritis (Batten *et al.*, 2000; Khare *et al.*, 2000; Mackay *et al.*, 1999). Thereby, mice overexpressing BAFF appear to show autoimmune phenotype similar to patients with systemic lupus erythematosus (SLE). Similarly, BAFF is overexpressed in murine models of SLE, such as NZB/NZW F1 mice (Gross *et al.*, 2000), which carry a polymorphism in the BlyS gene (Jiang *et al.*, 2001). Furthermore, inhibition of BAFF by soluble decoy receptor is successful in treating a murine

model of SLE in which serum BAFF levels were elevated (Gross *et al.*, 2000; Kayagaki *et al.*, 2002; Ramanujam *et al.*, 2006). In humans, previous reports have shown elevated serum BAFF levels in patients with SLE, rheumatoid arthritis (RA), and Sjögren's syndrome (Cheema *et al.*, 2001; Groom *et al.*, 2002; Stohl *et al.*, 2003; Zhang *et al.*, 2001).

Systemic sclerosis (SSc, scleroderma) is a connective tissue disease characterized by excessive extracellular matrix deposition in the skin and visceral organs (LeRoy *et al.*, 1988). Although the molecular basis for SSc pathogenesis is unknown, hyper- γ -globulinemia, polyclonal and memory B cell hyperactivity, and altered B cell homeostasis are found in SSc patients (Famularo *et al.*, 1989; Fleischmajer *et al.*, 1977; Sato *et al.*, 2004), with B cell-associated transcripts upregulated in lesional skin (Whitfield *et al.*, 2003). Disease-specific autoantibodies reactive with DNA topoisomerase I, RNA polymerases, and fibrillin-1 further suggests that activated B cells contribute to disease pathogenesis (Kuwana *et al.*, 1993; Steen *et al.*, 1988; Tan *et al.*, 1999). Furthermore, anti-fibrillin-1 autoantibodies could have pathogenic role in SSc via fibroblast activation (Zhou *et al.*, 2005). Recently, we have shown elevated serum levels of BAFF in SSc patients. Serum BAFF levels were associated with skin fibrosis, and B cells from SSc patients exhibited more enhanced responses by BAFF stimulation (Matsushita *et al.*, 2006). These results suggest that BAFF and its signaling in B cells contribute to B cell abnormalities and disease development in SSc.

The tight-skin (TSK/+) mouse serves as a model for SSc (Green *et al.*, 1976), with increased synthesis and accumulation of collagen and other extracellular matrix proteins in the skin (Jimenez *et al.*, 1986). Homozygous mice die in utero, while heterozygous TSK/+ mice survive but develop skin fibrosis (Osborn *et al.*, 1983). A tandem duplication within the fibrillin-1 gene causes tissue hyperplasia and the SSc-like phenotype (Siracusa *et al.*, 1996). However, there is also an immunological component to disease pathogenesis since CD4⁺ T cells, mast cells and other immunocompetent cells contribute to skin fibrosis in TSK/+ mice

(Bocchieri *et al.*, 1993; Everett *et al.*, 1995; Phelps *et al.*, 1993; Wallace *et al.*, 1994). For example, the infusion of bone marrow and spleen cells from TSK/+ mice into normal mice induces a TSK-like cutaneous phenotype and autoantibodies (Phelps *et al.*, 1993). Specifically, type 2 cytokines, such as IL-4, IL-6, IL-10, IL-13, contribute specifically to the fibrotic processes in scleroderma (Bhagal *et al.*, 2005; McGaha *et al.*, 2001; Ong *et al.*, 1999). By contrast, type 1 cytokines inhibits skin sclerosis in TSK/+ mice (Shen *et al.*, 2005; Tsuji-Yamada *et al.*, 2001). TSK/+ B cells also display a hyper-responsive phenotype, with enhanced CD19-induced $[Ca^{2+}]_i$ responses, higher levels of CD19 tyrosine phosphorylation (Saito *et al.*, 2002), impaired CD22 regulation of signal transduction (Asano *et al.*, 2004), and the production of autoantibodies against SSc-specific target autoantigens, such as topoisomerase I, RNA polymerase I, and fibrillin-1. There is also a correlation between the concentration of serum anti-topoisomerase I autoantibodies in TSK/+ mice and histological and biochemical alterations in the skin (Hatakeyama *et al.*, 1996). Thereby, CD19-deficiency in TSK/+ mice down-regulates B cell function, improves skin sclerosis, and inhibits autoimmunity (Hatakeyama *et al.*, 1996; Saito *et al.*, 2002). Thus, B cell is critical not only for induction of autoantibodies, but also for the development of skin fibrosis.

To directly assess roles of BAFF in TSK/+ mice, TSK/+ mice were examined serum BAFF levels and BAFF receptor expression and treated with BAFF antagonist. The current study suggests that BAFF significantly contributes to the development of skin fibrosis and could be a potent therapeutical target.

Results

Elevated serum BAFF levels in TSK/+ mice

Dysregulation of serum BAFF levels in lupus-prone NZB/NZW F1 mice has been demonstrated. Serum BAFF levels in NZB/NZW F1 mice gradually increase with growth and are abundant during the onset (after 20 weeks of age) and progression of SLE (Gross *et al.*, 2000). First, to determine whether/when serum BAFF levels are increased in TSK/+ mice, serum BAFF levels were measured by ELISA kit. Serum BAFF levels were significantly higher in TSK/+ mice than wild type littermates until 8 weeks of age (Figure 1a). In contrast, a significant increase of serum BAFF levels was apparent in NZB/NZW F1 mice compared with wild type mice at 12 weeks of age (Figure 1a). We also examined the expression of three BAFF receptors on B cells in TSK/+ mice using Flow cytometry. The expression levels of BAFF-R, TACI, and BCMA on B cells were similar between TSK/+ and wild type littermates (Figure 1b). Thus, the dysregulated expression of BAFF, but not BAFF receptors, was observed in TSK/+ mice.

BAFF antagonist reduces the development of skin fibrosis in TSK/+ mice

To determine whether BAFF/BAFF receptor interactions affect skin fibrosis in TSK/+ mice, we administrated BAFF-R-Ig, which neutralize BAFF, or Fc control protein into 1-week-old TSK/+ mice thrice a week for 7 weeks. Skin fibrosis was assessed by histopathology of full-thickness skin sections from the back in 8-week-old TSK/+ and wild type littermates. The dermal thickness (the thickness from the top of the granular layer to the junction between the dermis and subcutaneous fat) was similar among each strain (data not shown), consistent with previous reports (Green *et al.*, 1976; Wallace *et al.*, 1994). The hypodermal thickness in 8-week-old TSK/+ mice treated with control protein was increased by ~6-fold compared with wild type littermates ($p < 0.0001$, Figures 2a and b). There was no significant difference in the hypodermal thickness between the treatments with BAFF-R-Ig and control protein in wild type

mice (Figure 2a and b). Remarkably, 8-week-old TSK/+ mice with BAFF-R-Ig treatment showed decreased hypodermal thickness that was significantly 49% thinner than that found in TSK/+ littermates with control protein ($p < 0.0001$, Figure 2a and b).

Cutaneous fibrosis was also assessed by quantifying the collagen content of 6-mm punch biopsies from dorsal skin samples in TSK/+ and wild type littermates (Figure 2c). Although the collagen content in TSK/+ mice treated with control protein was increased by 4.5-fold relative to that in wild type littermates ($p < 0.0001$), BAFF-R-Ig reduced the collagen content by 51% in TSK/+ mice ($p < 0.0001$). Collagen content was comparable between wild type mice with BAFF-R-Ig and those with control protein. BAFF-R-Ig did not significantly affect the development of pulmonary emphysema and cardiac hypertrophy in TSK/+ mice (data not shown) (McGaha *et al.*, 2001). Thus, BAFF-R-Ig reduced skin fibrosis in TSK/+ mice.

BAFF antagonist modifies B cell phenotypes

BAFF has a crucial role during the transitional type 1 (T1)–T2 transition (Gross *et al.*, 2001; Schiemann *et al.*, 2001), and B-cell maturation is blocked at the T1 stage in mice that are treated with BAFF antagonist (Gross *et al.*, 2001; Schneider *et al.*, 2001). B cell phenotypes in BAFF-R-Ig or control protein-treated mice were examined at 8 weeks of age. B cell phenotypes in TSK/+ mice with control protein were similar to wild type littermates with control protein (Figure 3 and Table 1). Total B cells were significantly decreased (~75%) in spleen from TSK/+ and wild type littermates with BAFF-R-Ig. Concerning B cell phenotypes, mature B cells in bone marrow and spleen were significantly decreased by BAFF-R-Ig treatment. In addition, BAFF-R-Ig attenuated T2, marginal zone, and follicular B cells in both TSK/+ and wild type littermates. Conversely, T1 cells were significantly increased by BAFF-R-Ig. Thus, BAFF antagonist modified B cell phenotypes in both TSK/+ and wild type littermates.

BAFF antagonist inhibits autoantibody generation in TSK/+ mice

Although a pathogenic role for autoantibodies in SSc development remains uncertain (Zhou *et al.*, 2005), autoantibody and serum Ig levels were assessed in TSK/+ mice as markers for effective BAFF antagonist and hyperactivity. TSK/+ mice with control protein produced significantly higher levels of anti-topoisomerase I antibody when compared to wild type littermates with control protein ($p < 0.05$; Figure 4a). However, anti-topoisomerase I antibody levels were decreased significantly in TSK/+ mice with BAFF-R-Ig relative to TSK/+ littermates with control protein when assessed by ELISA (Figure 4a).

TSK/+ mice treated with control protein had significantly elevated IgM and IgG2a levels compared to wild type littermates with control protein ($p < 0.05$; Figure 4b). However, TSK/+ mice treated with BAFF-R-Ig had significantly decreased IgM, IgG2a, IgG2b, and IgG3 levels compared to TSK/+ littermates with control protein (Figure 4a). Thus, BAFF antagonist inhibited the autoantibody production and the development of hyper- γ -globulinemia in TSK/+ mice.

BAFF antagonist down-regulates augmented type 2 cytokines expression in the skin of TSK/+ mouse

Cytokines are critical for the development of skin sclerosis in TSK/+ mice (Ong *et al.*, 1999; Tsuji-Yamada *et al.*, 2001). We assessed cytokine expression in the skin by real-time PCR analysis. In the skin, TGF- β mRNA levels were elevated in TSK/+ mice (2.1-fold) relative to wild type littermates ($p < 0.001$; Figure 5). However, BAFF antagonist significantly reduced TGF- β transcript levels in TSK/+ mice ($p < 0.005$) to 1.2-fold of wild type levels, but did not reduce TGF- β levels in wild type mice. IL-6 and IL-10 mRNA levels were increased by 2.2-fold ($p < 0.01$) and 2.1-fold ($p < 0.01$), respectively, in TSK/+ mice with control protein compared to wild type littermates with control protein (Figure 5). By contrast, IFN- γ mRNA levels were tend to be decreased in TSK/+ mice with control protein compared to wild type

with control protein. However, BAFF antagonist significantly reduced IL-6 and IL-10 mRNA levels in TSK/+ mice ($p < 0.005$). BAFF antagonist also significantly increased IFN- γ mRNA levels in TSK/+ mice ($p < 0.005$), but not wild type mice. Thus, the skin from TSK/+ mice exhibited augmented fibrogenic type 2 cytokines, such as IL-6 and IL-10, expression that was down-regulated by BAFF antagonist, while BAFF antagonist up-regulated IFN- γ expression in the skin from TSK/+ mice.

BAFF accelerates IL-6 production by TSK/+ B cells

Recent studies shed light on the B cells as cytokine producing cells (Lipsky, 2001; Mauri *et al.*, 2003). We investigated the role of BAFF in cytokine production by B cells. Splenic B cells were stimulated with BAFF and/or SAC, and culture supernatants were analyzed by ELISA to determine the amount of IL-6 and IL-10. B cells stimulated with BAFF and SAC produced significant amount of IL-6 and IL-10 (Figure 6). IL-6, but not IL-10, production from TSK/+ B cells was significantly higher than those from wild type B cells in the presence of BAFF and SAC. Furthermore, IL-6 and IL-10 production from B cells stimulated with BAFF and SAC were attenuated by BAFF-R-Ig. Thus, TSK/+ B cells had a significantly enhanced ability to produce IL-6 by BAFF stimulation.

Discussion

This is the first study to reveal roles of BAFF on the development of skin fibrosis in TSK/+ mice. The dysregulation of BAFF expression was shown in TSK/+ mice (Figure 1a). Dramatically, the development of skin fibrosis in TSK/+ mice was prevented by treatment with BAFF-R-Ig that blocks BAFF (Figure 2). BAFF antagonist also inhibited the development of hyper- γ -globulinemia and the autoantibody production in TSK/+ mice (Figure 4). TSK/+ skin showed up-regulated expressions of fibrogenic type 2 cytokines, such as IL-6 and IL-10, while BAFF antagonist significantly suppressed them (Figure 5). Reciprocally, BAFF antagonist augmented anti-fibrogenic type 1 cytokines, IFN- γ , in the skin of TSK/+ mice. Furthermore, TSK/+ B cells with BAFF stimulation had a significantly enhanced ability to produce IL-6 (Figure 6). These results suggest that BAFF/BAFF receptor system is critical for the development of skin fibrosis in TSK/+ mice.

Previous studies have shown that B cells contribute to disease pathogenesis in TSK/+ mice (Phelps *et al.*, 1993; Saito *et al.*, 2002; Walker *et al.*, 1989), although some studies have concluded that B cells play no role in skin sclerosis (Dodig *et al.*, 2001; Kasturi *et al.*, 1997; Siracusa *et al.*, 1998). The development of skin fibrosis correlated closely with serum anti-topoisomerase I Ab levels in TSK/+ mice (Hatakeyama *et al.*, 1996). CD19-deficiency in TSK/+ mice down-regulates B cell function, improves skin sclerosis, and inhibits autoimmunity (Saito *et al.*, 2002). In addition, we have recently reported that B cell depletion using anti-mouse CD20 mAb significantly suppressed the development of skin fibrosis, autoantibody production, and hyper- γ -globulinemia in TSK/+ mice (Hasegawa *et al.*, 2006). Depending on similar mechanism, BAFF antagonist prevented the development of skin fibrosis in TSK/+ mice in the current study. Despite down-regulated skin fibrosis, the development of lung emphysema was not affected by blocking of BAFF in TSK/+ mice in the current study (data not shown). Similar dissociation between cutaneous hyperplasia and lung

emphysema has been reported in previous studies (Hasegawa *et al.*, 2006; McGaha *et al.*, 2001; Saito *et al.*, 2002; Wallace *et al.*, 1994).

Type 2 cytokines but not type 1 cytokines are critical for the development of cutaneous fibrosis in TSK/+ mice (Kodera *et al.*, 2002; McGaha *et al.*, 2001; Ong *et al.*, 1999). Consistent with these findings, cytokine balance was skewed to type 2 cytokines rather than type 1 in the skin of TSK/+ mice (Figure 5). Especially, IL-6 induces concentration-dependent increases in the production of collagen and glycosaminoglycans from human dermal fibroblasts in vitro (Duncan and Berman, 1991). In addition, skin fibrosis in TSK/+ mice is improved with a parallel decrease in IL-6 production from B cells (Saito *et al.*, 2002). In the current study, TSK/+ B cells with BAFF stimulation had a significantly enhanced ability to produce IL-6 (Figure 6), and BAFF antagonist suppressed those. Taken together, augmented IL-6 production by TSK/+ B cells with BAFF could play an important role in the development of fibrosis, and BAFF antagonist attenuated skin fibrosis in TSK/+ mice with decrease in IL-6 production from B cells.

BAFF is an essential component of B cell homeostasis and a potent B cell survival factor associated with systemic autoimmune disease in animals (Gross *et al.*, 2000; Khare *et al.*, 2000; Mackay *et al.*, 1999). Previous studies have provided strong evidence that constitutive BAFF overproduction in mice leads not only to polyclonal hyper- γ -globulinemia, but also to spontaneous production of multiple autoantibodies (Gross *et al.*, 2000; Khare *et al.*, 2000; Mackay *et al.*, 1999). Consistent with this, excess BAFF may lead to the autoantibody production and the development of hyper- γ -globulinemia in TSK/+ mice, while it is not clear why BAFF expression is higher in TSK/+ mice. A hypothesis was proposed that altered fibrillin leads to increased TGF- β activity in TSK/+ mice (Lemaire *et al.*, 2006). Like this, dysregulation of BAFF expression may be induced in TSK/+ mice. It is intriguing but remains unsolved why serum BAFF levels in TSK/+ mice return to wild type levels by 12 weeks, which

may indicate that BAFF is essential for the development of skin fibrosis during the early stage of disease, since skin fibrosis in TSK/+ mice establishes until 12 weeks. While we have not identified the factor(s) that induce elevated BAFF levels in TSK/+ mice, we could hypothesize that the “fibrosis-BAFF loop” may exist in TSK/+ mice. Skin fibrosis may activate BAFF-producing cells, such as macrophages, dendritic cells, and T cells, and thereby induce BAFF overproduction. Moreover, BAFF plays an important role in the development of TSK/+ skin fibrosis via cytokine and autoantibody productions from B cells, which have pathogenic roles in fibrosis. Therefore, fibrosis and BAFF could interact with each other in TSK/+ mice. Further studies will be needed to prove this hypothesis formally.

The current study showed that the development of skin fibrosis in TSK/+ mice was significantly attenuated by treatment with BAFF antagonist. Consistent with the positive results in the current study, critical roles of BAFF have also been suggested in various autoimmune mouse models, such as lupus-prone mouse (Gross *et al.*, 2000; Kayagaki *et al.*, 2002; Ramanujam *et al.*, 2006), collagen-induced arthritis (Gross *et al.*, 2001; Wang *et al.*, 2001). Moreover, treatment with BAFF antagonists were already started in SLE patients and showed safety and effectiveness (Baker *et al.*, 2003; Furie *et al.*, 2003; Wallace *et al.*, 2006). Until now, since few therapies have proved to be effective for SSc in control studies, TSK/+ mice could serve as a useful animal model for assessing SSc therapy. However, TSK/+ mice and human scleroderma differs in many ways such as the sites of fibrosis (Green *et al.*, 1976). Therefore, we should be aware that results from TSK/+ mice can not be simply translated into human therapies. Nonetheless, our findings suggest the possibility that BAFF antagonist are potential therapeutic tools in human SSc.

Materials and Methods

Mice

TSK/+ mice with a C57BL/6 genetic background were purchased from The Jackson Laboratory (Bar Harbor, ME). To verify the TSK/+ genotype, PCR amplification of a partially duplicated fibrillin 1 gene was carried out using genomic DNA from each mouse as described (McGaha *et al.*, 2001). NZB/NZW F1 mice were purchased from SLC Japan (Shizuoka, Japan). All mice were housed in a specific pathogen-free barrier facility and screened regularly for pathogens. Female mice were used in these experiments. The Committee on Animal Experimentation of Kanazawa University Graduate School of Medical Science approved all studies and procedures.

In vivo treatment

Murine BAFF-R-Ig (R&D Systems Inc., Minneapolis, MN), which were made by fusing their extracellular domains to the Fc portion of human IgG1 and neutralize murine BAFF, and Fc control protein (Acris Antibodies, Hiddenhausen, Germany) were used in this study. To neutralize BAFF *in vivo*, 1-week-old TSK/+ and wild type littermates received either murine BAFF-R-Ig (2 µg/g *i.p.* 3 times/week) or the same dose of Fc control protein.

Histopathological assessment of skin fibrosis

Morphologic characteristics of skin sections from TSK/+ and wild type littermates were assessed under a light microscope. All skin sections were taken from the para-midline, lower back region (the same anatomic site to minimise regional variations in thickness) as full thickness sections extending down to the body wall musculature. Tissues were fixed in 10% formaldehyde solution for 24 hours and embedded in paraffin. Sections were stained with hematoxylin and eosin. Hypodermal thickness, which was defined as the thickness of a subcutaneous loose connective tissue layer (*i.e.*, the hypodermis or superficial fascia) beneath the panniculus carnosus, was measured for multiple transverse perpendicular sections using an

ocular micrometer. Dermal thickness defined as the thickness of skin from the top of the granular layer to the junction between the dermis and subcutaneous fat was also examined. Ten random measurements were taken per section. Two investigators in a blinded fashion examined all of the sections independently.

Determination of collagen content in the skin tissue

Collagen content was determined by harvesting 6-mm punch biopsies from shaved dorsal skin samples. Skin samples were homogenized by TissueLyser system (QIAGEN Ltd, Crawley, UK), and then skin homogenate were assayed for collagen levels and compared with a standard curve prepared from rat tail collagen using the Sircol assay (Biocolor, Belfast, UK) according to the manufacturer's instructions. The assay was performed in duplicate, and the mean of two data was determined for individual sample.

Enzyme-linked immunosorbent assay (ELISA)

Serum BAFF levels were measured by ELISA kit (Apotech, Epalinges), Switzerland according to the manufacturer's protocol. For BAFF assay, all sera were preabsorbed with protein A (Amersham Biosciences, Piscataway, NJ) to deplete Ig. IL-6 and IL-10 concentrations in the culture medium were measured by ELISA, according to the manufacturer's protocols (BD Biosciences, San Jose, CA). Mouse Ig concentrations in sera were determined by ELISA, using affinity-purified mouse IgM, IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotechnology Associates Inc. LOCATION) to generate standard curves as described (Engel *et al.*, 1995). Anti-topoisomerase I Ab levels were quantified using ELISA kits according to the manufacture's protocol (Medical & Biological Laboratories, Nagoya, Japan). Each sample was tested in duplicate.

Flow cytometry

Cells were stained with the following Abs (obtained from BD Biosciences, unless otherwise noted): IgM (R6-60.2), B220 (RA3-6B2), CD21 (7G6), CD23 (B3B4), CD24 (M1/69),

BAFF-R (7H22-E16; ALEXIS, Lausen, Switzerland), TACI (166010; R&D Systems Inc.), or BCMA (161616; R&D Systems Inc.). For two- or three-color immunofluorescence analysis, single-cell lymphocyte suspensions (1×10^6) were stained at 4°C using predetermined optimal concentrations of mAb for 20 min as described (Sato *et al.*, 1996). Cells with the forward and side light scatter properties of lymphocytes were analyzed on a FACScan flow cytometer (BD Biosciences). Positive and negative populations of cells were determined using unreactive isotype-matched mAbs (BD Biosciences) as controls for background staining.

RNA isolation and real-time RT-PCR

Total RNA was isolated from deep-frozen full-thickness dorsal skin sections or cultured dermal fibroblasts using QIAGEN RNeasy spin columns (QIAGEN Ltd.) and digested with DNaseI (QIAGEN Ltd.) to remove chromosomal DNA in accordance with manufacturer's protocols. RNA was reverse transcribed into cDNA using the Reverse Transcription System (Promega, Madison, WI). Transcript levels were quantified using a real-time PCR method according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Sequence-specific primers and probes were designed by Pre-Developed TaqMan® Assay Reagents or TaqMan® Gene Expression Assays (Applied Biosystems). Real-time PCR (one cycle of 50°C for 2 min, 95°C for 10 min; 40 cycles of 92°C for 15 sec, 60°C for 60 sec) was performed on an ABI Prism 7000 Sequence Detector (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript levels were used as controls to normalize mRNA levels. The relative expression of target transcript PCR products was determined using the $\Delta\Delta C_t$ method (Meijerink *et al.*, 2001). Fold induction = $2^{-[\Delta\Delta C_t]}$, where C_t = the threshold cycle, i.e. the cycle number at which the sample's relative fluorescence rises above the background fluorescence and $\Delta\Delta C_t = [C_t \text{ gene of interest (unknown sample)} - C_t \text{ GAPDH (unknown sample)}] - [C_t \text{ gene of interest (calibrator sample)} - C_t \text{ GAPDH (calibrator sample)}]$. Each sample was run in triplicate, with the mean C_t used in the equation.

B cell culture

Splenic B cells were purified by positive selection with anti-B220 Ab coated magnetic beads (BD Biosciences) and resuspended in RPMI1640 medium containing 5% fetal calf serum. Purified B cells (2×10^5 /well) were cultured in 0.2 ml of culture medium in 96-well flat-bottom plates for 72hr with recombinant mouse BAFF (R&D Systems Inc.), and/or Staphylococcus aureus Cowan strain (SAC; Sigma St. Louis, MO), and/or murine BAFF-R-Ig (R&D Systems Inc.).

Statistical analysis

Data are expressed as mean values \pm SEM. The Mann-Whitney U-test was used for determining the level of significance of differences between sample means and Bonferroni's test was used for multiple comparisons.

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Conflict of interest

The authors state no conflict of interest.

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Figure Legends

Figure 1. BAFF is increased in sera from TSK/+ and NZB/NZW F1 mice.

(a) Serum BAFF levels were determined by ELISA. Horizontal bars represent mean serum BAFF level in each group. * $p < 0.01$, ** $p < 0.05$. vs. wild type. (b) Representative expression of BAFF receptor, including BAFF-R, TACI, and BCMA, on B220⁺ B cells from 8-week-old TSK/+ and wild type littermates. Three BAFF receptor expressions were assessed by 2-color immunofluorescence with flow cytometric analysis. Shaded regions in the histograms represent TSK/+ B220⁺ B cells, boldface lines represent wild type B220⁺ B cells, and dotted lines represent isotype control staining.

Figure 2. Effect of BAFF antagonist on skin fibrosis in TSK/+ mice.

Skin fibrosis in dorsal skin from 8-week-old TSK/+ and wild type littermates treated with BAFF-R-Ig or Fc control protein (control). (a) Skin fibrosis was assessed by quantitatively measuring hypodermal thickness. Representative hematoxylin and eosin stained histological sections ($\times 40$). Dermis is indicated by (d), hypodermis beneath the panniculus carnosus (arrow) indicated by (h). These results represent those obtained with at least 5 mice of each group. Skin fibrosis was further assessed by quantitatively measuring hypodermal thickness (b) and skin collagen content (c). Results from each mouse are represented as single dots. Horizontal bars represent mean hypodermal thickness or collagen content in each group.

Figure 3. BAFF antagonist modifies B cell phenotypes in TSK/+ mice.

B cell phenotypes in 8-week-old TSK/+ and wild type littermates treated with BAFF-R-Ig or Fc control protein (control). Bone marrow and spleen were collected, stained with fluorescently labeled mAbs to a variety of cell surface markers, and analyzed by flow

cytometry. Representative profiles are shown for bone marrow and B220⁺ spleen. **(a)** Bone marrow cells were stained with mAbs to IgM and B220, to identify pro/pre (IgM⁻B220^{low}), immature (IgM⁺B220^{low}), and mature B cells (IgM⁺B220^{high}). **(b)** Splenic B cells (B220⁺-gated) were stained with mAbs to CD21, CD23, and CD24, to identify newly formed (NF; CD21⁻CD23⁻B220⁺), T1 (CD24^{high}CD21⁻ B220⁺), T2 (CD24^{high}CD21⁺ B220⁺), follicular (FO; CD21⁺ CD23⁺ B220⁺), and marginal zone (MZ) B cells (CD21^{high}CD23⁻ B220⁺).

Figure 4. BAFF antagonist inhibits autoantibody generation and hyper- γ -globulinemia in TSK/+ mice.

(a) Anti-topoisomerase I (topo I) Ab and **(b)** antibody levels in sera from 8-week-old TSK/+ and wild type littermates treated with BAFF-R-Ig or Fc control protein (control). Relative serum autoantibody and antibody levels were determined by Ig subclass-specific ELISA. Horizontal bars represent mean autoantibody and antibody levels for each group.

Figure 5. BAFF antagonist down-regulates augmented type 2 cytokines in TSK/+ skin.

Messenger RNA expression of cytokines in dorsal skin from 8-week-old TSK/+ and wild type littermates treated with BAFF-R-Ig or Fc control protein (control). The mRNA levels of TGF- β ₁, IL-4, IL-6, IL-10, and IFN- γ were analyzed by real-time RT-PCR, and normalized with the internal control GAPDH. Each sample was done in triplicate. Data indicate the mean \pm SEM. These results represent those obtained with at least 5 mice of each group.

Figure 6. BAFF accelerates IL-6 production by TSK/+ B cells.

Splenic B cells were purified by positive selection with anti-B220 Ab coated magnetic beads and were stimulated for 72hr with either medium alone or recombinant mouse BAFF and/or Staphylococcus aureus Cowan strain (SAC) and/or BAFF-R-Ig. Culture supernatants were

analyzed by ELISA to determine the amount of IL-6 and IL-10.

Table 1. B cell subsets in spleen

		Wild type	Wild type	TSK/+	TSK/+
		+ Control	+ BAFF-R-Ig	+ Control	+ BAFF-R-Ig
B220 ⁺	B220 ⁺	40.1 ± 2.3	30.5 ± 7.2**	41.4 ± 4.2	31.5 ± 6.5††
T1	CD24 ^{high} CD21 ⁻ B220 ⁺	15.5 ± 3.3	55.5 ± 7.2*	14.0 ± 2.2	54.5 ± 6.1†
T2	CD24 ^{high} CD21 ⁺ B220 ⁺	6.3 ± 1.3	0.4 ± 0.1*	6.5 ± 1.1	0.5 ± 0.2†
Marginal zone	CD21 ^{high} CD23 ⁻ B220 ⁺	5.7 ± 1.0	1.9 ± 0.7*	5.8 ± 0.9	2.2 ± 0.6†
Follicular	CD21 ⁺ CD23 ⁺ B220 ⁺	70.9 ± 3.6	28.6 ± 5.5*	72.0 ± 2.4	30.4 ± 5.7†

Values are mean percent ± SD (n=5). *p<0.01, **p<0.05 vs. wild type littermates treated with control protein, †p<0.01, ††p<0.05 vs. TSK/+ littermates treated with control protein.

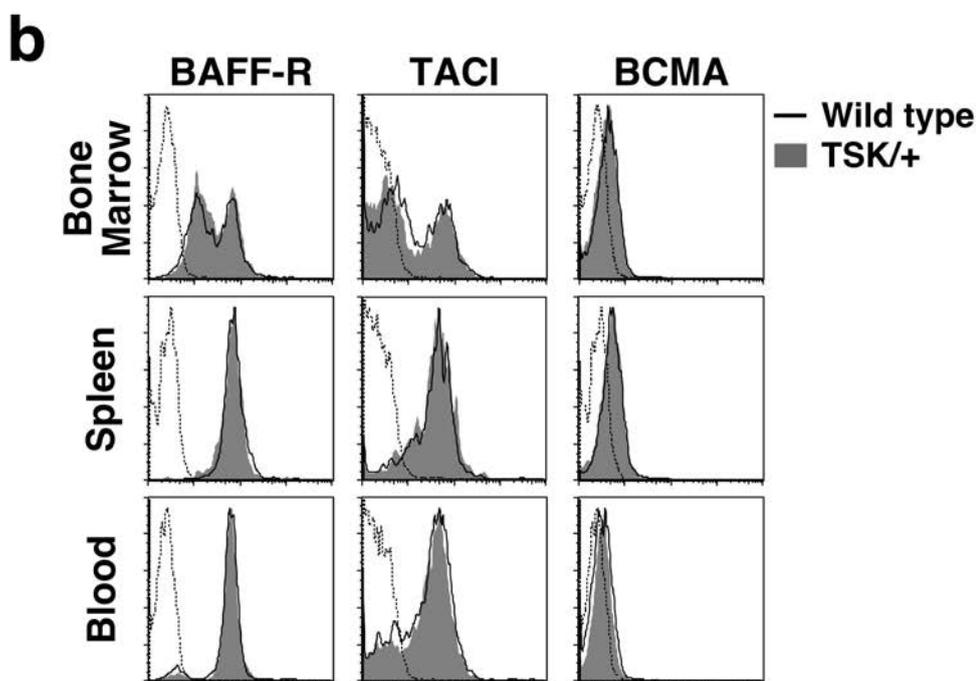
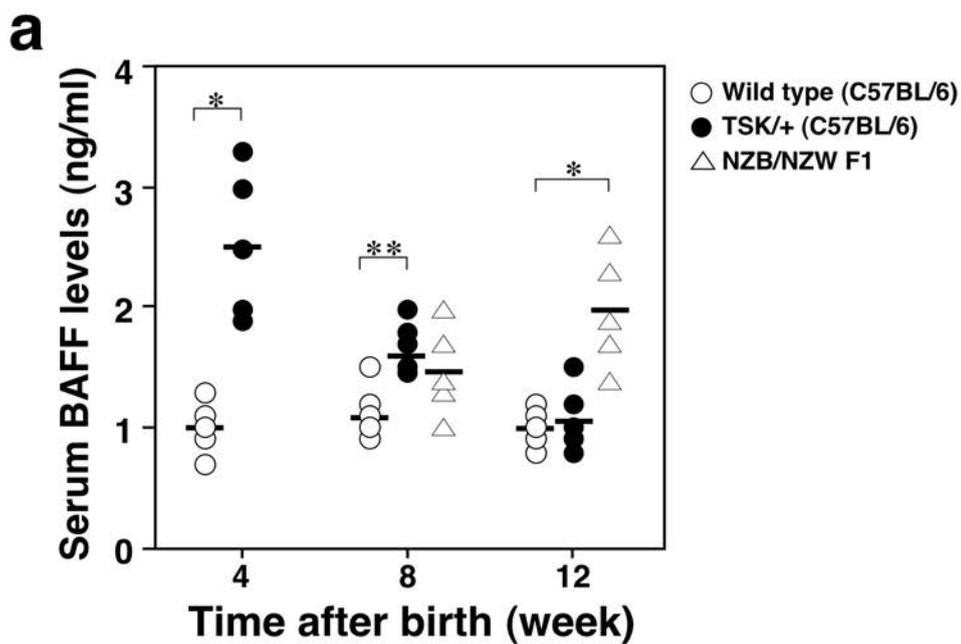


Figure 1
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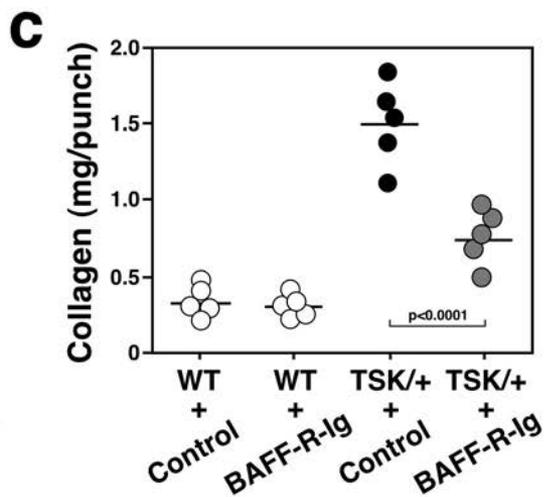
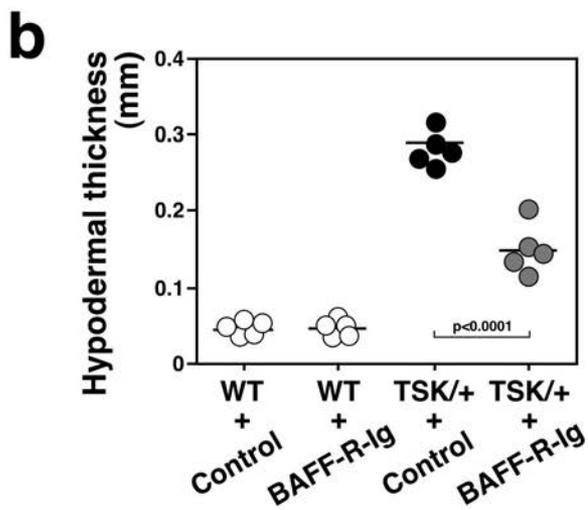
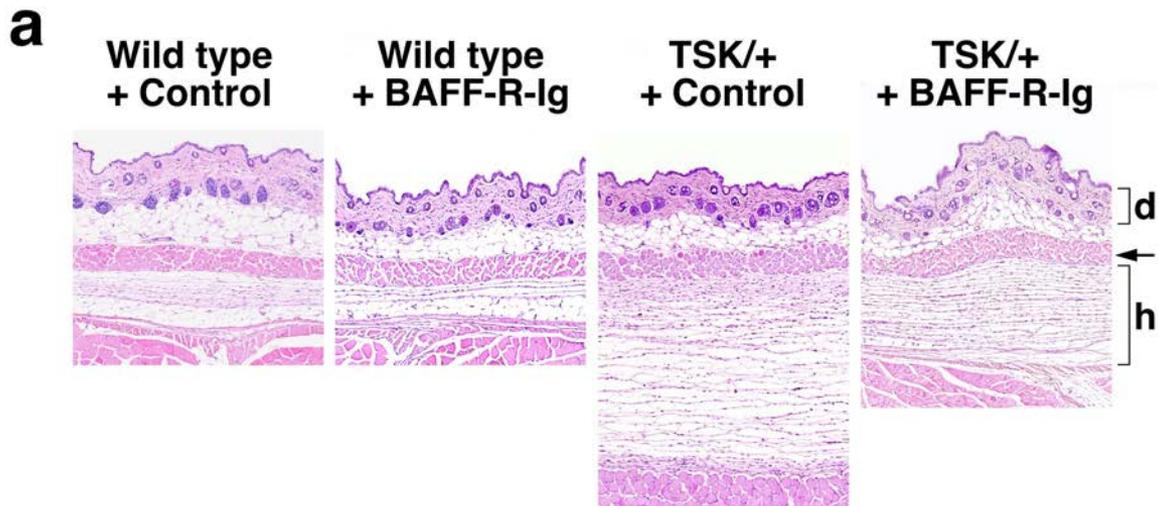


Figure 2
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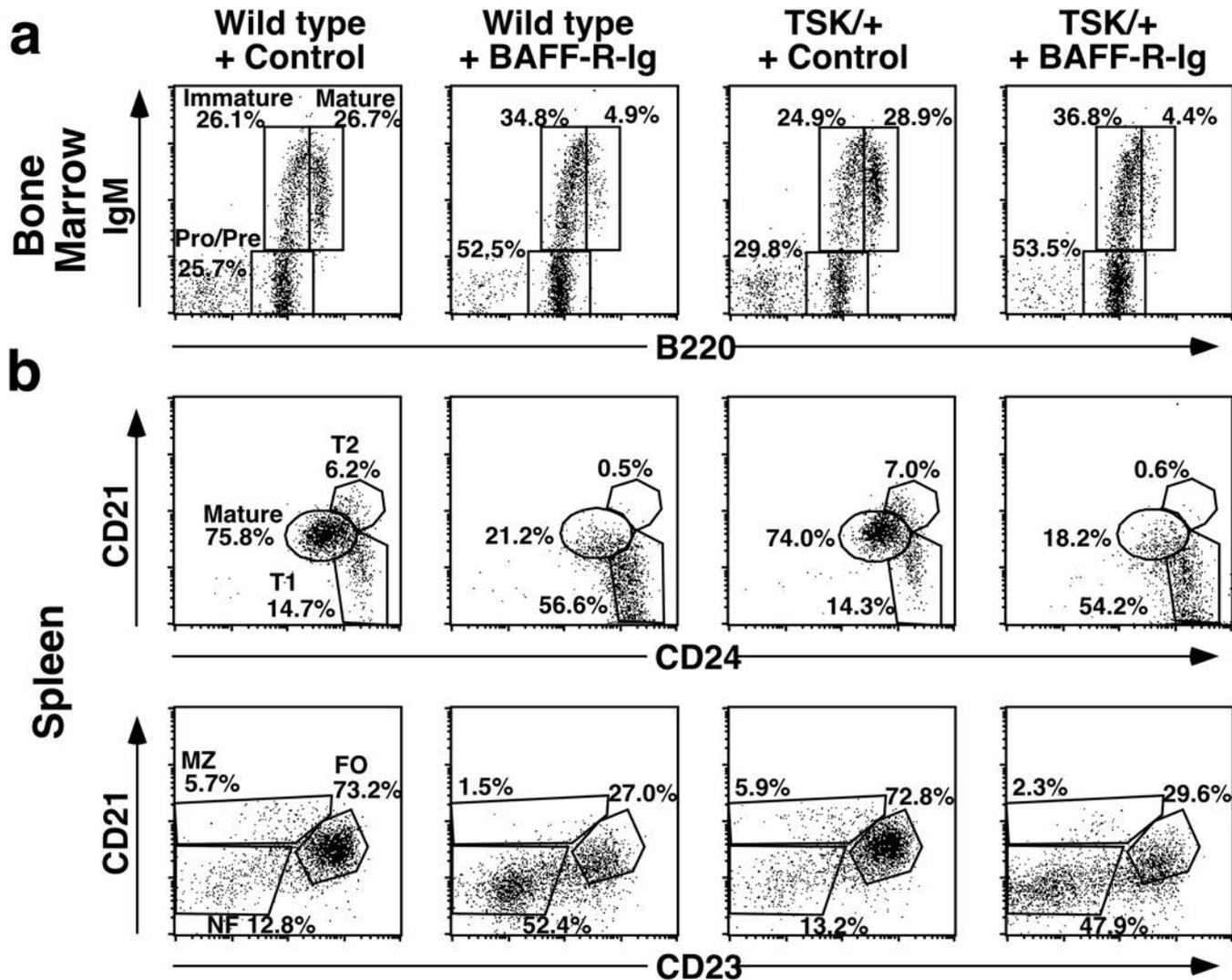


Figure 3
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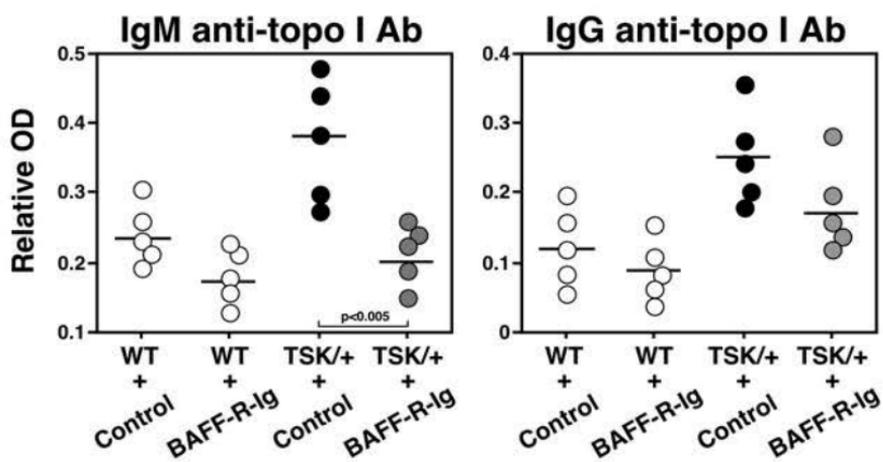
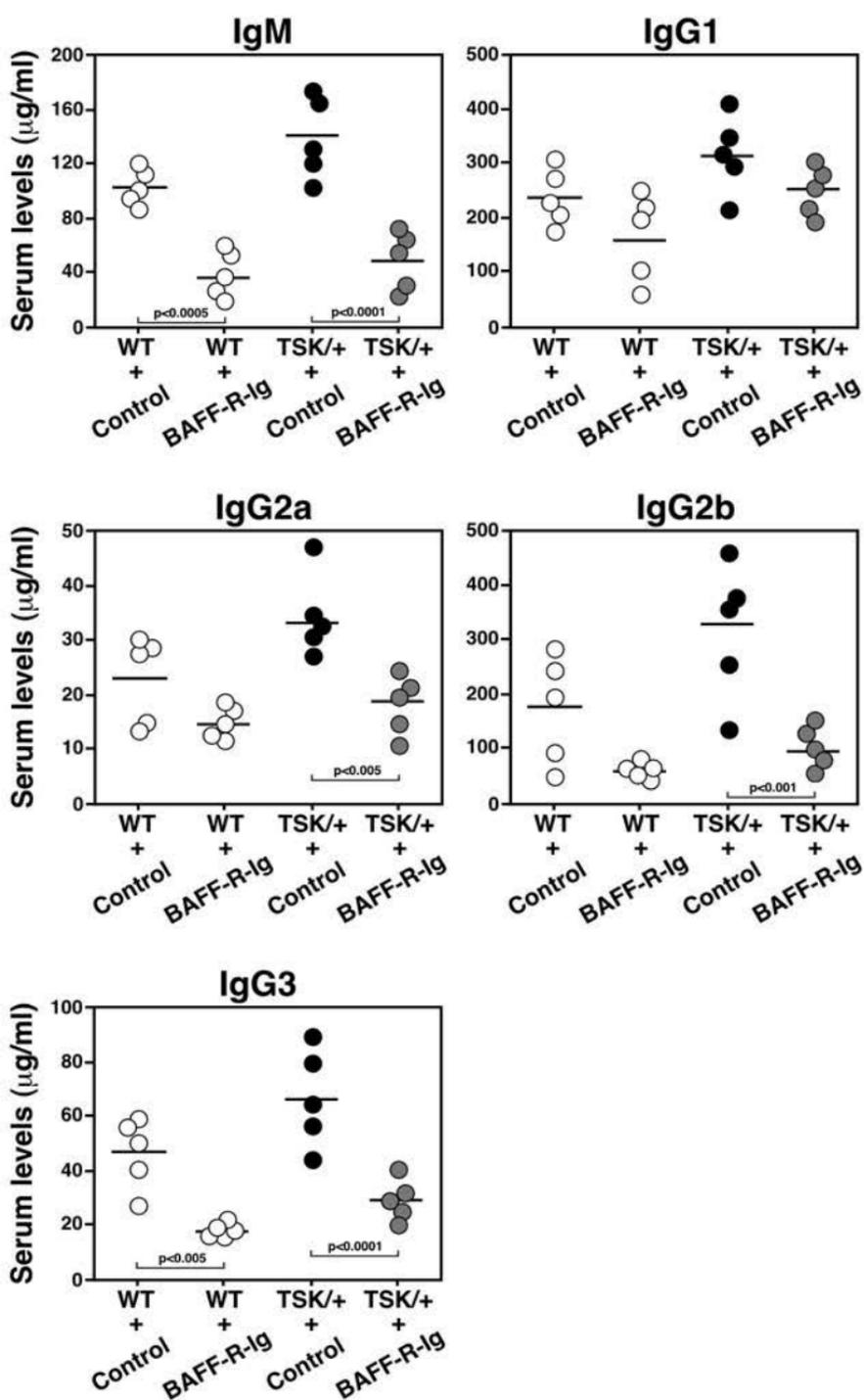
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Figure 4
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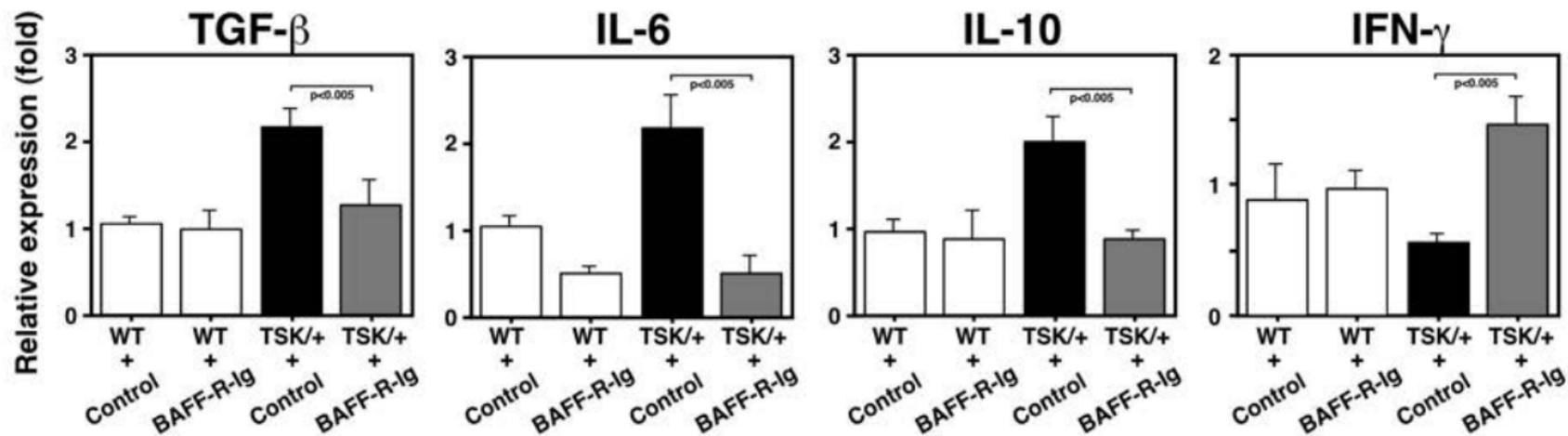


Figure 5
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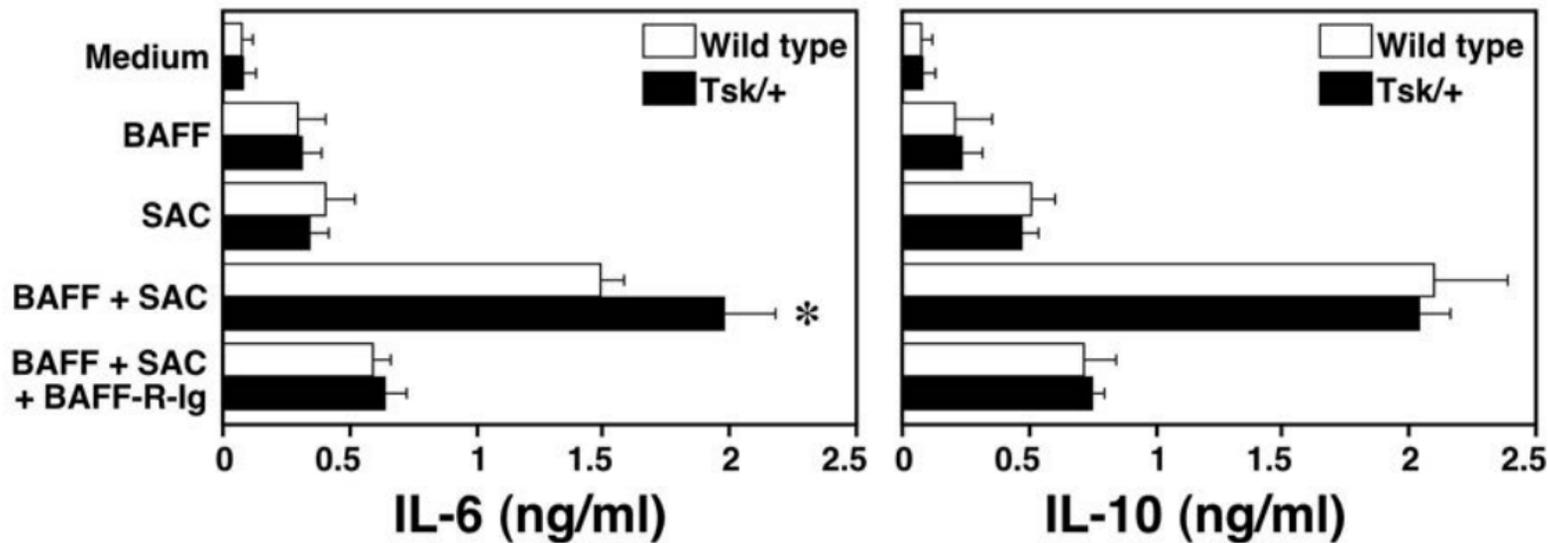


Figure 6
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