Immunoregulatory Effects of Extracts from the stem bark of *Albizzia julibrissin* Durazz

Jeong J. Kim[1], Byung E. Lee[1], Dong S. Jung[1], Se-Won Kim[1,2], Seon-Yle Ko[3], Dong-Heon Baek[4],
Sun-Hwa Chang[1], and Youngnim Choi[5]

[1]OCT Inc., Cheonan 330-831, Korea
[2]Dept. of Dental Pharmacology, Dankook University, Cheonan 330-714, Korea
[3]Dept. of Oral Biochemistry, Dankook University, Cheonan 330-714, Korea
[4]Dept. of Oral Microbiology and Immunology, Dankook University, Cheonan 330-714, Korea
[5]Oromaxillofacial Infection & Immunity and Dental Research Institute, College of Dentistry, Seoul National University, Seoul, Korea

Although there are many disease modifying anti-rheumatic drugs (DMARDs) available, more safe and effective reagents have yet to be developed. Activated T cells make good targets for DMARD therapy. Therefore, we screened over fifty herbal extracts, and identified that water extract from the stem bark of *Albizzia julibrissin* selectively killed activated T cells but not naïve splenocytes, *in vitro*. Wb3-3, obtained by further extraction and column chromatography to maximize selective activity, was used for further investigation. Annexin-V and caspase activity assay revealed that WB3-3 killed activated T cells by inducing apoptosis, which was mediated through mainly caspase-8 rather than caspase-9. The effect of WB3-3 *in vivo* was confirmed by the elimination of Vβ8[+]- but no Vβ6[+]-T cells following activation with Streptococcal Enterotoxin B (SEB). Furthermore, WB3-3 inhibited neither CD25 up-regulation nor proliferative response, and rather increased IL-2 secretion during Con A activation of naive T cells. Our results imply the potential of WB3-3 to include a lead compound for a new immunoregulatory drug.

Key words: disease modifying anti-rheumatic drug (DMARD), Albizzia julibrissin, activated T cell, apoptosis, caspase-8.

*Corresponding author: Youngnim Choi, D.D.S., Ph.D. Oromaxillofacial Infection & Immunity and Dental Research Institute College of Dentistry, 28 Yungun-dong, Jongro-gu, Seoul, Republic of Korea. Tel.: +82-2-740-8643, Fax.: +82-2-743-0311, E-mail: youngnim@snu.ac.kr

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory autoimmune disorder that affects 1% of the adult population worldwide (Alarcon *et al.*, 2001). RA is characterized by the inflammation of synovial joints infiltrated by CD4^{+} T cells, macrophages, and plasma cells that play major roles in the pathogenesis of the disease (Feldmann *et al.*, 1996a; Smolen *et al.*, 2003). CD4^{+} T cells responding to an unknown antigen activate macrophages to produce pro-inflammatory cytokines. Among the pro-inflammatory cytokines, TNF-α is on top of a cytokine cascade by inducing the production of IL-1, which in turn induces the production of IL-6 and IL-8 (Feldmann *et al.*, 1996a; Smolen *et al.*, 2003). These cytokines trigger the synthesis of matrix metalloproteinases (MMPs) and osteoclast formation, which ultimately leads to the destruction of joints and the impairment of function (Feldmann *et al.*, 1996b; Billinghurst *et al.*, 1997; Mitchell *et al.*, 1996). Rheumatoid factor secreted by plasma cells aggravates the inflammatory process through complement activation (Feldmann *et al.*, 1996a).

A new guideline for the management of RA from American College of Rheumatology emphasizes early diagnosis and start of disease-modifying anti-rheumatic drug (DMARD) therapy (American College of Rheumatology, 2002). Although there are several DMARDs available, all of them have limited efficacy due to a wide variability between patients (Smolen *et al.*, 2003). Clearly, the introduction of biologic DMARDs that block TNF-α or

IL-1, made a major breakthrough in the treatment of RA last decade. However, more than half of the patients in clinical trials did not achieve ACR50 (improvement of 50 percent or more in the number of tender and swollen joints) and the enormous costs of biologic drugs make them inaccessible to many patients (Paleolog *et al.*, 2003; Olsen *et al.*, 2004). In addition, toxicity is the major concern of most synthetic DMARDs (Smolen *et al.*, 2003; American College of Rheumatology, 2002). Therefore, the need for more effective and less toxic DMARDs of low cost is still high.

Since activated $CD4^+$ T cells have such an important role in the initiation and progression of disease, those make good targets for DMARD therapy (Jung *et al.*, 2004a). Methotrexate (MTX), the gold standard of DMARD therapy, inhibits de novo purine synthesis that leads to the inhibition of clonal expansion and the early deletion of activated peripheral T cells (Genestier *et al.*, 1998; Izeradjene *et al.*, 2001). Similarly, leflunomide inhibits the proliferation of T cells through the irreversible inhibition of dihydro-orotate dehydrogenase, an enzyme that is pivotally involved in de novo pyrimidines synthesis (Ruckemann *et al.*, 1998). In order to minimize damage on protective immune response or other tissues, it is important to eliminate activated T cells selectively. Therefore, we developed a screening system using activated T cells and naïve splenocytes to find a new lead compound as a potential DMARD. Natural herbs used in traditional Korean medicine provide good starting materials for the development of new drugs due to their known effectiveness and safety proved by usage over thousands years. We screened over fifty herbal extracts for selective killing activity and found extracts from the stem bark of *Albizzia julibrissin* Durazz (Albizziae Cortex) to have such an activity. In this study, we investigated various immunoregulatory effects of Albizziae Cortex extract.

Materials and Methods

Preparation of *Albizzia julibrissin* Durazz extract

Three hundred grams of *Albizzia julibrissin* bark was heated in 3 L distilled water at 100°C for 4 h with circulatory shaking. Following filtration and evaporation using a vacuum rotary evaporator (Buchi, Flawil, Switzerland) at below 40°C, 21.9 g of water extract (W) was obtained. W was additionally extracted with n-buatanol (500 ml) at room temperature three times and 7. 9g of WB extract was obtained. Subsequently, WB was subdivided into WB1~WB4 by column chromatography through a silica gel (Merck & Co., Whitehouse Station, NJ) column using the mixture of chloroform/methanol/water 65:35:5 as a solvent. WB3 (1.95 g) with active material was further subdivided into WB3-1~WB3-3 by reverse phase column chromatography through a HP-20 (Supelco, St. Louis, MO) column using 5% acetonitrile (Merck & Co.) as a solvent.

T cell activation and induction of cell death

The preparation of primary activated T cells was performed as described before (Choi *et al.*, 2002). Activated T cells (1×10^6 cells/ml) in fresh medium containing hrIL-2 and fresh isolated splenocytes (2×10^6 cells/ml) in complete medium were plated into 96-well plate at 200 µl/well and treated with media alone, 0.2 µg/ml immobilized anti-CD3e mAb clone 2C11 (Becton Dickinson), MTX (100 nM), or various herbal extracts for 24 h. Cells from triplicate wells were stained with propidium iodide (PI), and viable cells excluding PI were enumerated for 20 s by flow cytometry using a FACSCallibur (Becton Dickinson, San Jose, CA). The percentage of cell death was calculated as: (1-number of viable cells recovered from wells treated with herbal extracts/ number of viable cells recovered from wells treated with media alone) $\times 100$.

Annexin-V assay

Activated T cells at 2×10^5 cells/200 µl/well in 96-well plate were treated with media alone, 0.2 µg/ml immobilized anti-CD3e mAb, or various concentrations of WB3-3 for 4 h. Cells were washed with PBS, stained with Annexin-V-FITC (Becton Dickinson) in 1x binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) for 15 m at room temperature to which PI was added, then analyzed by flow cytometry immediately.

Caspase activity assay

Activated T cells at 1×10^6 cells/ml/well in 24-well plate were treated with media alone, immobilized anti-CD3e Ab (0.2 µg/ml), MTX (100 nM), or various concentrations of WB3-3 for 6 h. Following treatments, cell lysates were prepared and incubated with caspase-3, -8, or -9 substrate (DEVD-AFC, IETD-AFC, LEDH-AFC, respectively from R&D systems, Minneapolis, MN) at 37°C for 1 h. Then, fluorescence from cleaved substrates was measured using a FLUOStar OPIMA (BMG Labtechnologies, Offenburg, Germany).

Selective elimination of activated T cells by RE3WB3-3 *in vivo*

C57BL/6 mice were injected with 50 µg Streptococcal Enterotoxin B (SEB, Sigma, St. Louis, MO) intraperitoneally on day 0. From day 1, mice were treated with 5 mg/kg/day MTX, 6.5 mg/kg/day WB3-3, or PBS by daily intraperitoneal injection. The change of $V\beta8^+$ or $V\beta6^+$ T cell populations in mesenteric lymph node was observed by double staining of $V\beta8^+$-FITC/$CD4^+$-PE, $V\beta8^+$-FITC/$CD8^+$-PE, $V\beta6^+$-FITC/$CD4^+$-PE, and $V\beta6^+$-FITC/$CD8^+$-PE using direct conjugated mAbs. Cells were gated for $CD4^+$ or $CD8^+$ cells and the percentages of $V\beta8^+$ or $V\beta6^+$ cells among gated cells were analyzed. All mAbs were purchased from Becton Dickinson.

CD25 staining

Splenocytes at 2×10^6 cells/ml were activated with 5 µg/ml Con A for 48 h in the absence or presence of WB3-3. Cells

were double stained for CD25 and CD3 using a PE-labeled anti-CD25 clone PC61 and a FITC-labeled anti-CD3 clone 2C11 and analyzed by flow cytometry.

Proliferation assay and IL-2 ELISA

Splenocytes labeled with 1 μM 5-(and-6)-carboxy-fluorescein diacetate, succinimidyl ester (CFSE from Molecular Probe, Eugene, OR) were activated with 5 μg/ml Con A in the absence or presence of WB3-3. At the indicated time points, cells were harvested and supernatants were stored in -70°C until ELISA analysis. Cells were stained with PI and analyzed by flow cytometry. Only viable cells excluding PI were gated and analyzed for proliferation. The amounts of IL-2 in the supernatants were measured using a ELISA kit

(R&D Systems).

Results

Selective killing of activated T cells by Albizziae Cortex extract *in vitro*

First, we tested the validity of our screening system using two known reagents that induce apoptosis of activated T cells but not resting T cells: anti-CD3e mAb and MTX (Fig. 1A). We screened over fifty herbal extracts blindly to find one that killed activated T cells but not naïve splenocytes. Among the extracts screened, the water extract (W) of Albizziae Cortex showed fairly good selective killing
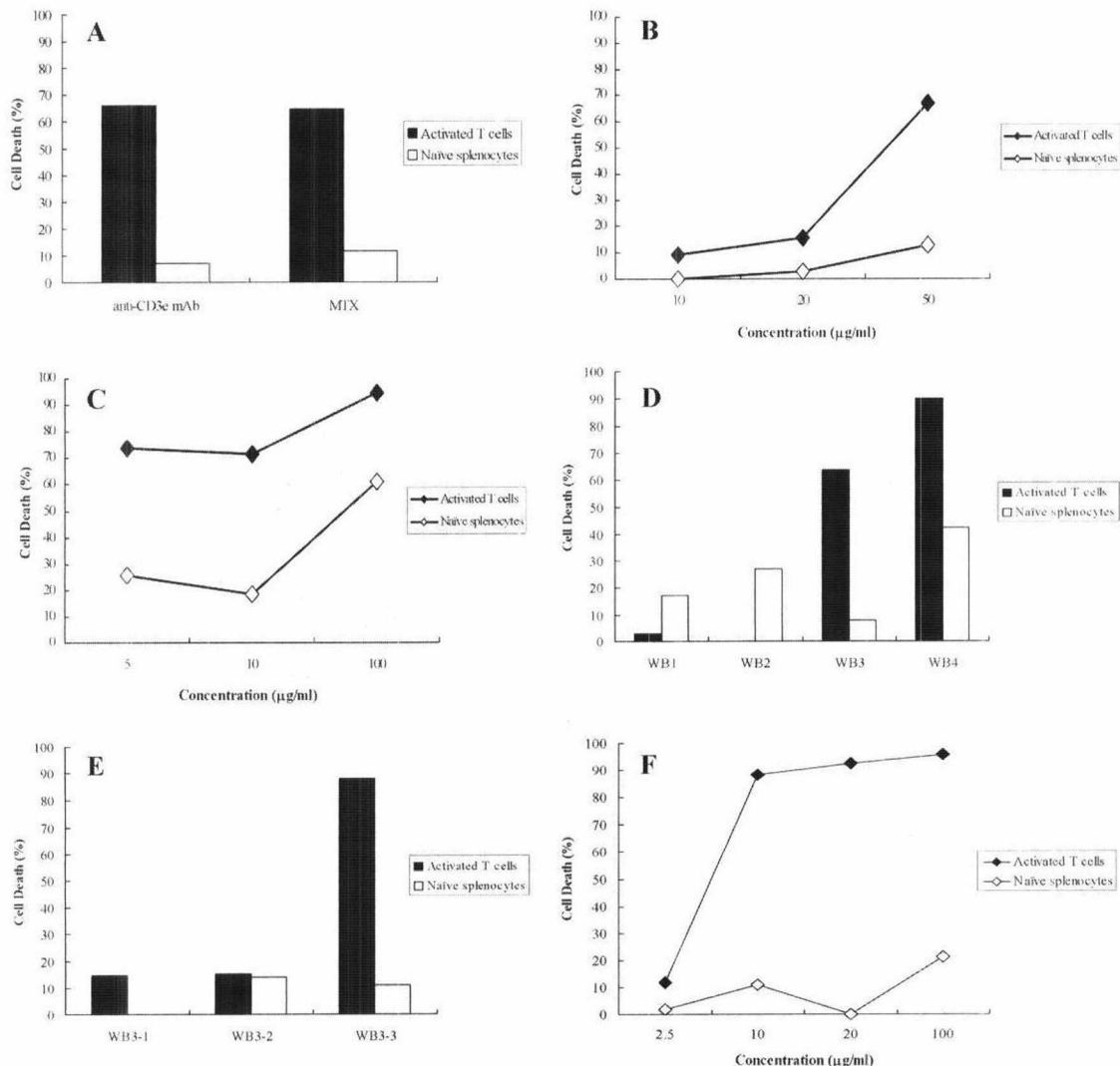

Fig. 1. *In vitro* screening of selective killing activity for activated T cells. Activated T cells (1×10^6 cells/ml) in fresh medium containing hrIL-2 and fresh isolated splenocytes (2×10^6 cells/ml) in complete medium were plated into 96-well plate at 200 μl/well and treated with media alone or various experimental reagents for 24 h. Cells from triplicate wells were stained with PI, and viable cells excluding PI were enumerated for 20 s by flow cytometry. The percentage of cell death was calculated as described in method section. The experimental wells were treated with immobilized anti-CD3e Ab (0.2 μg/ml) or MTX (100 nM) (A), various concentrations of W (B), WB (C), 10 μg/ml WB1~WB4 (D), 10 μg/ml WB3-1~WB3-3 (E), or various concentration of WB3-3 (F). All dada are representatives of at least two different experiments with similar results.

activity at 50 µg/ml (Fig. 1B). By further extracting W with n-butanol, the effective concentration of WB was lowered to around 10 µg/ml (Fig. 1C). WB was fractionated into four fractions by column chromatography. Fraction 3 and 4 showed selective killing activity at 10 µg/ml but fraction 1 and 2 did not (Fig. 1D). Since fraction 3 showed the best selective activity, WB3 was selected and further fractionated into three fractions by reverse phase column chromatography. Among the three fractions, only WB3-3 showed selective killing activity at 10 µg/ml (Fig. 1E). Although the killing activity for the activated T cells at low concentration (2.5 µg/ml) was decreased, cytotoxicity for naïve splenocytes was decreased further, resulting in quite low cell death rate even at 100 µg/ml (Fig. 1F). WB3-3 was used for further investigations.

WB3-3 induced apoptosis of activated T cells.

We studied if WB3-3 killed activated T cells by inducing apoptosis or not. Both Annexin-V and caspase-3 activity assay indicated that WB3-3 induced the apoptosis of the activated T cells in a dose dependent manner (Fig. 2A and

B). Next, we investigated which apoptotic pathway WB3-3 triggered. Anti-CD3e mAb, a reagent that induces apoptosis through death receptors, preferentially activated caspase-8. In contrast, MTX that inhibits DNA replication of activated T cells, induced apoptosis mediated predominantly by caspase-9. WB3-3 induced activation of caspase-8 rather than caspase-9, indicating the involvement of extrinsic apoptotic pathway (Fig. 2C).

Selective killing activity of WB3-3 *in vivo*

In order to confirm selective killing activity *in vivo*, mice were injected with SEB and treated with WB3-3 or PBS as a negative control or MTX as a positive control. In PBS treated animals, the percentage of $V\beta8^+$ cells among $CD8^+$ T cells increased due to clonal expansion that reached a peak on d 3, then decreased by activation induced cell death (AICD). MTX inhibits the clonal expansion of T cells *in vivo* if it is administered before SEB injection (Izeradjene *et al.*, 2001). However, we wanted to simulate the treatment of patients who already have a disease and activated T cells, and started MTX treatment 1 d after SEB injection. In our
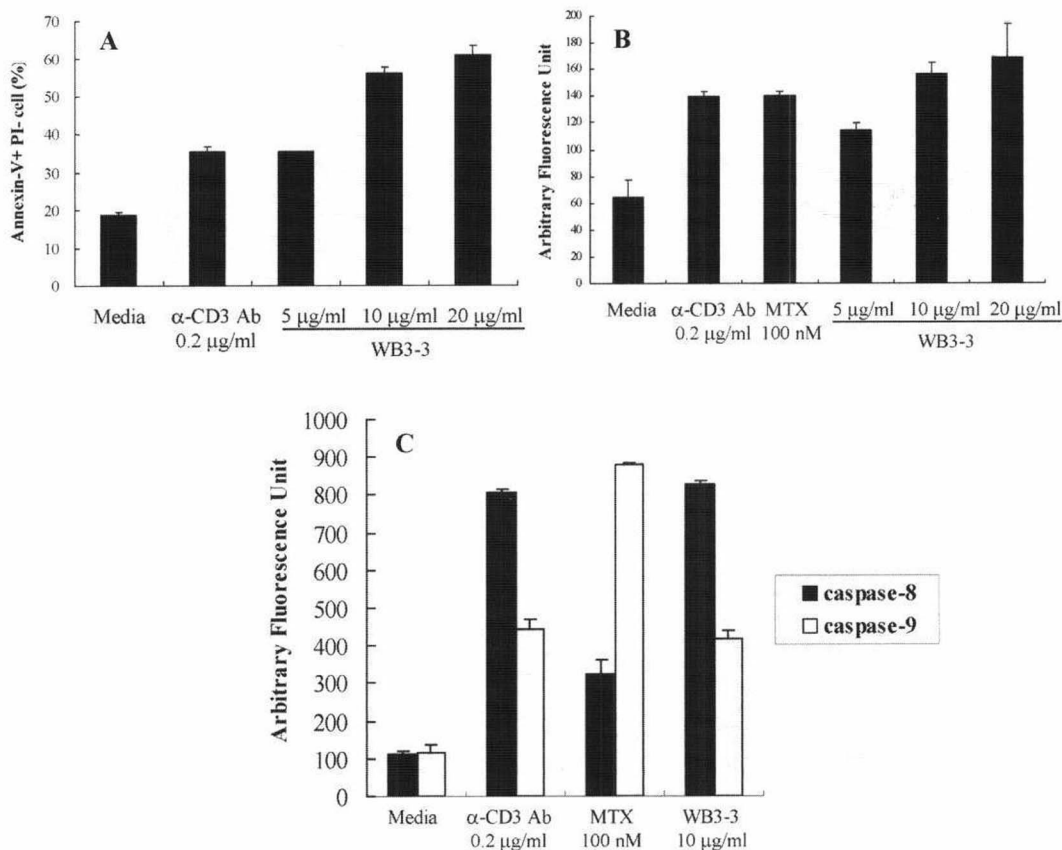

Fig. 2. WB3-3 induced apoptosis in activated T cells. A. Annexin-V assay. Activated T cells at 2×10^5 cells/200 µl/well in 96-well plate were treated with media alone, immobilized anti-CD3e Ab (0.2 µg/ml), or various concentrations of WB3-3 for 4 h. Cells were washed with PBS, stained with Annexin-V-FITC to which PI was added, then analyzed by flow cytometry immediately. Each column represents mean+SEM of triplicate wells. B and C. Caspase activity assay. Activated T cells at 1×10^6 cells/ml/well in 24-well plate were treated with media alone, immobilized anti-CD3e Ab (0.2 µg/ml), MTX (100 nM), or various concentrations of WB3-3 for 6 h. Following treatments, cell lysates were prepared and incubated with caspase-3, -8, or -9 substrate for 1h. The fluorescence from cleaved substrates was measured. Each column represents mean+SEM of triplicate wells.

setting, MTX did not inhibit clonal expansion completely but decreased it on d 3 and resulted in reduced Vβ8⁺ cell population on d 5 and d 7 compared to those in PBS treated animals. WB3-3 treatment induced early deletion of Vβ8⁺ cells. A maximum clonal expansion was reached on d 2 and the percentage of Vβ8⁺ cells was decreased after then, reaching a comparable level to that observed in the MTX treated group on d 7 (Fig. 3A). The percentage of Vβ6⁺ cells did not change in all cases, indicating that non-activated T cells were not affected by either MTX or WB3-3 (Fig. 3B). For CD4⁺ T cells, a similar pattern was observed in WB3-3 treated group but the effect of MTX was negligible (Fig. 3C and 3D).

The effect of WB3-3 on T cell activation

We assessed the effect of WB3-3 on the activation of naïve T cells. First, we measured the expression of IL-2R α chain (CD25) and IL-2 secretion as activation markers. WB3-3 did not affect CD25 up-regulation in response to Con A activation (Fig. 4A). IL-2 secretion during Con A activation

was rather increased by WB3-3 (Fig. 4B). In order to measure the effect of WB3-3 on T cell proliferation, CFSE-labeled splenocytes were cultured with Con A in the absence or presence of WB3-3 for 24, 48, and 72 h, stained with PI, and then analyzed for the proliferation of viable cells by flow cytometry. Most T cells proliferated in the presence of WB3-3 as shown by the decrease of CFSE intensity, although the decrease of total viable cells by WB3-3 was observed after 48 h, especially at 20 μg/ml (Fig. 4C).

Discussion

In the blind screening of various herbal extracts for selective killing activity, we found that the water extract of Albizziae Cortex killed activated T cells efficiently but not naïve splenocytes (Fig. 1A). *Albizzia julibrissin* Durazz grows abundantly in Korea and the water extract of its stem bark has been known to have analgesic, anti-inflammatory, and sedative effects. It has been used commonly to treat
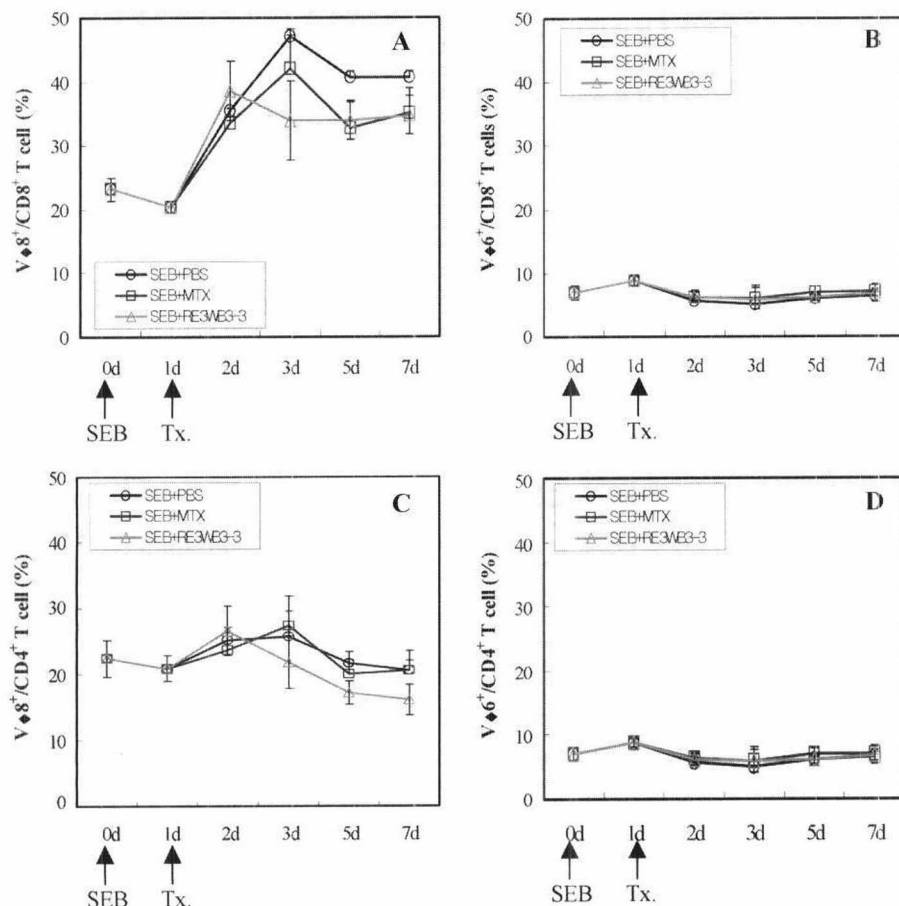

Fig. 3. Selective Elimination of Activated T cells by WB3-3 *in vivo*. C57BL/6 mice were injected with 50 μg Streptococcal Enterotoxin B intraperitoneally on d 0. From d 1, mice were treated with 5 mg/kg/day MTX, 65 mg/kg/day WB3-3, or PBS by daily intraperitoneal injection. The change of Vβ8⁺ or Vβ6⁺ T cell populations in mesenteric lymph node was observed by double staining of Vβ8⁺-FITC/CD4⁺-PE, Vβ8⁺-FITC/CD8⁺-PE, Vβ6⁺-FITC/CD4⁺-PE, and Vβ6⁺-FITC/CD8⁺-PE using direct conjugated mAbs. Cells were gated for CD4⁺ or CD8⁺ cells and the percentages of Vβ8⁺or Vβ6⁺ cells among gated cells were analyzed. Each data point represents mean ± SEM of 5 animals. A. Vβ8⁺/CD8⁺ B. Vβ6⁺⁄ CD8⁺ C.Vβ8⁺/ CD4⁺ D. Vβ6⁺⁄ CD4⁺
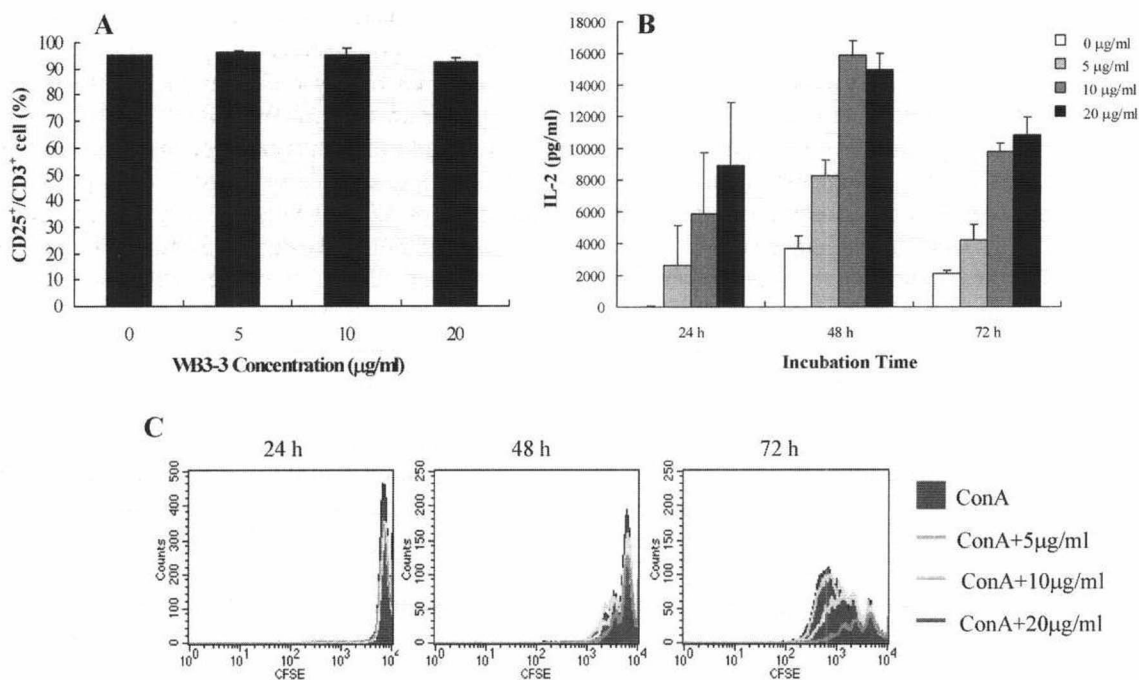
Fig. 4. Eeffects of WB3-3 on T cell activation. A. CD25 up-regulation. Splenocytes were activated with 5 μg/ml Con A for 48 h in the absence or presence of WB3-3. Cells were double stained with a PE-labeled anti-CD25 mAb and a FITC-labeled anti-CD3 mAb, and analyzed by flow cytometry. Cells were gated on CD3$^+$ T cells and the percentage of CD25$^+$ cells among CD3$^+$ T cells was analyzed. Each column represents mean+SEM of triplicate wells. B. IL-2 secretion. Splenocytes were activated with 5 μg/ml Con A for 72 h in the absence or presence of WB3-3. At the indicated time points, supernatants were saved and analyzed using IL-2 ELISA kits. Each column represents mean+SEM of triplicate wells. C. Proliferation assay Splenocytes labeled with 1 μM CFSE were activated with 5 μg/ml Con A in the absence or presence of WB3-3. At the indicated time points, cells were harvested, stained with PI and analyzed by flow cytometry. Only viable cells excluding PI were gated and analyzed for the dilution of CFSE by proliferation.

various diseases including contusion, fracture, furuncle, parasite infection, anxiety, and depression in traditional Korean medicine (Jun, 1999). Saponins named julibrosides (A1-A4, B1, C1, J1, J2, J3, etc.) sapogenins named julibrogenins (A, B, C), and flavonol glycosides have been isolated as major chemical constituents so far (Zou *et al.*, 2004; Chen *et al.*, 1997a; Chen *et al.*, 1997b; Kang *et al.*, 2000; Kinjo *et al.*, 1992). However, the pharmacological effects of *Albizzia julibrissin* are not known much except its anxiolytic-like activity and antioxidant activity (Kang *et al.*, 2000; Kim *et al.*, 2004; Jung *et al.*, 2004b; Jung *et al.*, 2003).

Through stepwise *in vitro* screening and purification, the selective activity for activated T cells was maximized, and WB3-3 that showed the best selectivity (Fig. 1E) was used in further investigations. The selective elimination of activated T cells by WB3-3 *in vivo* was also shown by the elimination of Vβ8$^+$ T cells but not Vβ6$^+$ T cells following the injection of SEB that activate Vβ8$^+$ T cells specifically (Fig. 3).

We could verify that WB3-3 killed activated T cells by inducing apoptosis rather than necrosis, which is important to prevent inflammatory response. WB3-3 treatment induced the increase of Annexin-V$^+$ PI$^-$ cells and caspase-3 activity in a dose dependent manner (Fig. 2A and B).

While WB3-3 killed activated T cells, it did not inhibit the

activation of naïve T cells: T cells expressed CD25 and proliferated in the presence of WB3-3 (Fig. 4A and B). The decrease of viable cells by WB3-3 during Con A activation seems to reflect the killing of activated T cells rather than the inhibition of proliferation because the number of undivided cells was comparable to that observed in the absence of WB3-3 (Fig. 4B). In contrast, proliferation inhibition by nucleotide restriction using MTX let most cells undivided (Quemeneur *et al.*, 2003). Interestingly, WB3-3 increased the accumulation of secreted IL-2 during Con A activation in a dose dependent manner (Fig. 4C). It is unlikely due to the decrease of viable cells that consume IL-2 because the increase of IL-2 was observed also by 5 μg/ml at which the decrease of viable cells was minimal. Collectively, the immunoregulatory effects of WB3-3 are different from those of either MTX or immunosuppressive drugs that inhibit T cell activation such as Cyclosporin A and FK506 (Hamawy., 2003).

There are two known mechanisms that induce the apoptosis of activated cycling T cells but not resting T cells. Nucleotide restriction by de novo synthesis inhibitor such as MTX inhibits T cell proliferation and induces apoptosis by intrinsic pathway, which is mediated by mitochondria and caspase-9 (Fig. 1A, Fig. 2C) (Genestier *et al.*, 1998; Li *et al.*, 2002). Lymphocytes activated by antigen program them-

selves for cell death called AICD, which is mediated by death receptors (extrinsic pathway) and caspase-8 (Fig. 1A, Fig. 2C, Sartorius *et al.*, 2001). WB3-3 seems to trigger the extrinsic pathway since it activated caspase-8 preferentially to caspase-9 (Fig. 2C). In addition, it did not inhibit proliferation (Fig. 4B). The increase of IL-2 secretion by WB3-3 (Fig. 4C) may have contributed to the induction of AICD (Zheng *et al.*, 1998).

AICD is an important mechanism to maintain the homeostasis of immune system that endows potent response to pathogen but prevents autoimmunity. Impaired elimination of activated T cells has been shown to be associated with autoimmune disorders including rheumatoid arthritis in mouse and human (Fisher *et al.*, 1995; Nagata., 1998; Radvanyi *et al.*, 1998; Reap *et al.*, 1995). Furthermore, AICD is a unique phenomenon for lymphocytes, making it a good target for drug development. Although activated T cells are more susceptible to the inhibition of de novo nucleotide synthesis, other proliferating cells are also affected, resulting in adverse effects such as diarrhea, nausea/vomiting, and alopecia. Furthermore, leflunomide is a potent teratogen and MTX is potentially teratogenic (American college of rheumatology, 2002; Ruckemann *et al.*, 1998). Therefore, WB3-3 may have an advantage over either MTX or leflunomide.

In conclusion, WB3-3 extracted from Albizziae Cortex induced the apoptosis of activated T cell selectively *in vitro* and *in vivo*, probably by promoting AICD, and did not inhibit the activation of naïve T cells. Our results show the potential of *Albizzia julibrissin* to include a lead compund for a new immunoregulatory drug and the need for further investigation.

Acknowledgments

Abbreviations

Disease modifying anti-rheumatic drug (DMARD)
Streptococcal enterotoxin B (SEB)
Matrix metalloproteinase (MMP)
Activation induced cell death (AICD)

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