Protective Effect of $CD4^+CD25^+$ Regulatory T Cells on Mice Model of Rheumatoid Arthritis

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Abstract

Naturally occurring $CD4^+CD25^+$ regulatory T (T_{reg}) cells, a component of the innate immune response which play a key role in the maintenance of self-tolerance, have become the focus of numerous studies over the last decade. These cells have the potential to be exploited to treat autoimmune disease. These cells inhibit the immune response in an antigen-nonspecific manner by interacting with other T cells. These T cell populations actively control the properties of other immune cells by suppressing their functional activity to prevent autoimmunity but also influence the immune response to allergens as well as against tumor cells and pathogens. In this experiment we showed that induced regulatory T cells have a protective effect on mice model of rheumatoid arthritis (RA). RA mice which were injected intraperitoneally with *Andrographis paniculata* substrate or injected with induced regulatory T cells showed the effects of recovery. We further showed that the generation of leukocyte including B cells can be promoted by the administration of *A. paniculata* substrate. Tissue damage from free radicals that arise due to imperfect metabolism can be prevented by such treatment in RA model mice. Recovery effects occurred in RA model mice involves the increasing number of $CD4^+CD25^+$ regulatory T cells.

Keywords: $CD4^+CD25^+$, Regulatory T cells, Rheumatoid Arthritis, *Andrographis paniculata*, Autoimmune

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Introduction

$CD4^+CD25^+$ regulatory T cells can protect an individual from autoimmune diseases. There are some evidence that $CD4^+CD25^+$ regulatory T cells have an ability to prevent the development of autoreactive cells (Amelsfort *et al.,* 2001; Bleesing *et al.,* 2001; Chatenoud *et al.,* 2001; Chen *et al.,* 2003; Malek *et al.,* 2004; Seddon *et al.,* 2000; Wan *et al.,* 2005). Rheumatoid arthritis (RA) is a chronic inflammatory disorder that ultimately leads to the destruction of joint architecture. The pathogenic events that lead to the development of RA are not fully understood, although the presence of inflammatory cytokines has been well documented to play a key role in the induction and maintenance of this disease (Wan *et al.,* 2005; Yudoh *et al.,* 2000; Zittermann, 2003).This disease attacked around 1% population of the world that appear in people in the aged of 25-50 years, but did not close the possibility that people in any ages can suffer that disease.

RA is a degenerative disease that mostly attacks women. It is thought that various factors including genetic elements have a significant contribution to the emergence of this disease. RA treatment is usually done by resting the affected joint and consume synthetic drugs. Treatment is often only relieving pain and preventing inflammation. Drugs that have been used for pain relieve and prevention of inflammation is non-steroidal drug, anti-inflammatory (aspirin and ibuprofen), slow-acting drugs, penicillamine, corticosteroids, and immunosuppressive drugs. The use of these drugs in the long term will be very harmful and cause various side effects that involve almost every organ (McGargill *et al.,* 2005; Najafian *et al.,* 2003; Papiernik *et al.,* 1998; Rifa'i *et al.,* 2004; Seddon *et al.,* 2000; Suciu-Foca *et al.,* 2003; Takahashi *et al.,* 2000; Thornton *et al.,* 2000; Von Herrath *et al.,* 2003a, 2003b; Wan *et al.,* 2005; Yudoh *et al.,* 2000; Zittermann *et al.,* 2003).

Because of serious impacts resulting from the rheumatoid arthritis treatment, it is necessary to include prevention efforts that

can normalize cell homeostasis. In this study we tested herbs to cure the RA as a safe choice to replace synthetic drugs.

Materials and Methods

Induction of rheumatoid arthritis in BALB/c mice. Mice were intravenously injected with CFA (complete Freund's adjuvant) of 75 µl, then performed a second booster in mice feet by 75 µl (each leg of 35.5 µl). Observations were made every day to see the swelling of the feet (Mengyue *et al.*, 2011).

Induction of $CD4^+CD25^+$ regulatory T cells from naive $CD4^+CD25^-$. Spleen cells were cultured at 3 cm well plate in RPMI medium in the presence of anti-CD3 stimulation. Andrographis paniculata substrate is added to each well (2 µl/ml) to drive the differentiation of $CD4^+CD25^-$ to $CD4^+CD25^+$ T cells. A. paniculata substrate was obtained by boiling 3 g dried leaves and stems with 100 ml of PBS. When the volume reached volume of 50 ml, boiling was discontinued. The substrate was then sterilized before being used to induce the development of regulatory cells (Battaglia *et al.*, 2005).

Test of the effectiveness of induced $CD4^+CD25^+$ regulatory T cells in vitro. Effectiveness of regulatory T cells developed *in vitro* can be tested by their suppression to other cells. Suppression can be measured by looking at the proliferation resistance of tested cells. In this experiment, total cells from lymph node, mostly T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured in 24 well plates with anti-CD3 stimulation in the presence of induced $CD4^+CD25^+$ regulatory T cells. The effectiveness of induced regulatory cells developed *in vitro* was assessed by measuring inhibition of responder cell proliferation. Proliferation can be observed by monitoring the intensity of CFSE in flow cytometry using CellQuest software.

Adoptive transfer therapy using induced $CD4^+CD25^+$ regulatory T cells in mice model of RA. Induced $CD4^+CD25^+$ regulatory T cells (5×10^6) were intraperitoneally injected in RA mice model. Analysis was done 7 weeks after injection.

Therapy using substrate of *A. paniculata* in mice model of RA. Mice model of RA were intraperitoneally injected with 100 µl or 400 µl *A. paniculata* substrate.

Assessment of superoxide dismutase (SOD) and malon dialdehyde (MDA). SOD and MDA were analyzed by standard method (Firdaus *et al.*, 2010; Hai-feng *et al.*, 2011)

Results and Discussion

A. paniculata is a medicinal plant which is able to stimulate the expression of CD25 molecules on T cells. CD25 molecule expression on CD4 T cells is one marker of the cell with the nature of the regulatory function. In normal individuals $CD4^+CD25^+$ regulatory T cells generally range between 3-10% of the population of CD4 T cells (Figure 1). In this study we proved that the addition of *A. paniculata* substrates *in vitro* was able to elicit the differentiation of CD4 T cells from the spleen to regulatory cells. Regulatory T cells developed by *in vitro* system have an ability to suppress proliferation of other cells that can be observed in cell culture by looking at the intensity of CFSE dilution (Figure 2).

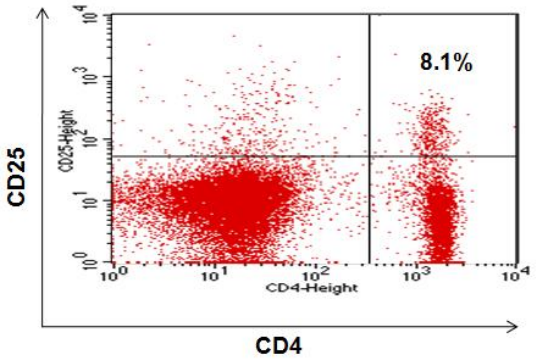

Figure 1. $CD4^+CD25^+$ regulatory T cells observed in mice spleen. Spleen cells were obtained from 7 week old wild-type BALB/c mice, stained with indicated fluorescence conjugated antibodies, and analyzed by flow cytometry. Percentages of $CD25^+$ cells in $CD4^+$ cells are shown in panel. Data are representative of three experiments.

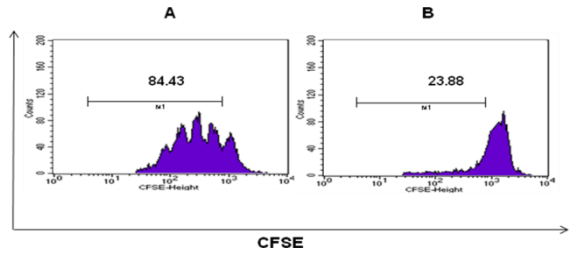

Figure 2. Induced regulatory T cells *in vitro* potentially inhibit spleen cell proliferation. A) CFSE labeled spleen cells were cultured for three days and analyzed by flow cytometry. B) Spleen cells as seen in panel 'A" but added with regulatory cells by a ratio of 1:1. Results are representative of three experiment.

Intraperitoneally injection in RA mice with regulatory T cells, showed the effect of suppression of T cell proliferation. In this experiment, we clearly showed the influence of regulatory T cells in the development of CD4 and CD8 T cells. Injection of regulatory T cells developed *in vitro* was able to inhibit CD4 and CD8 T cell proliferation (Figure 3). The mouse model of RA showed a decrease in CD62L levels, which indicated that the cells in RA were activated and developed into memory state so that the examination of the CD4 T cells showed a significant decrease in the number of naive $CD4^+CD62L^+$ cells (Figure 4). Activation of lymphocytes is closely associated with the inflammation in mice that had suffered from rheumatoid arthritis. In this study, swelling of the soles of the feet was examined by measuring volume expansion or inflammation (Figure 5).

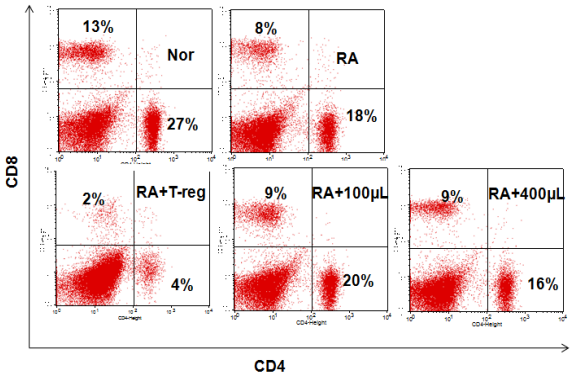

Figure 3. Induced regulatory T cells *in vitro* potentially inhibits the development of T cells. Spleen cells were labeled with anti-CD4-FITC and anti-CD8-PE antibodies. Upper left panel (Nor = normal individuals), and the upper right panel (RA = rheumatoid arthritis models). The numbers of $CD4^+$ and $CD8^+$ T cells are decreased when obtained injection of induced regulatory T cells. Adding *A. paniculata* substrate (100 µl and 400 µl) in mice did not affect the amount of $CD4^+$ and $CD8^+$ T cells in RA mice models. Data are representative of three independent experiment.

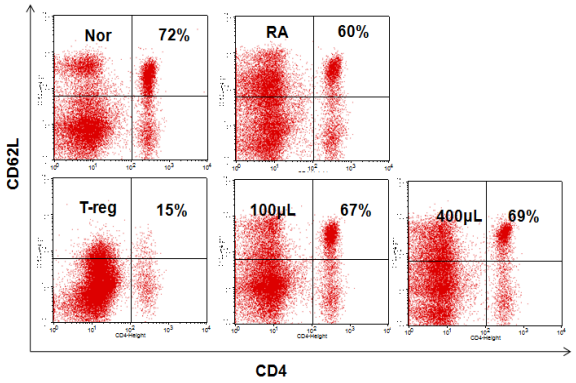

Figure 4. The expression of CD62L is decreased in mice model of RA. Spleen cell was obtained from RA mice seven weeks after treated with *A. paniculata* substrate or regulatory T cells. Upper left panel is a control that shows the expression of $CD4^+CD62L^+$ molecules and the right panel showed the decreased level of $CD4^+CD62L^+$ in RA mice model. Injection of regulatory T cells and *A. paniculata* substrate (100 µl and 400 µl) were shown in the bottom panel, respectively. Data are representative of three independent experiments.

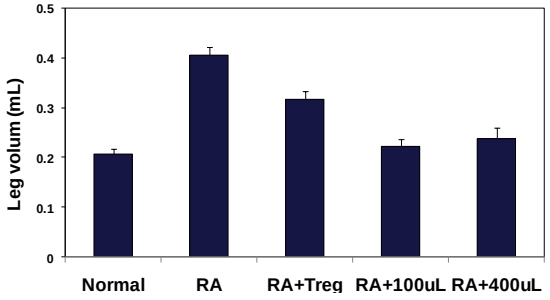

Figure 5. Mice with rheumatoid arthritis (RA) experienced swelling in the joints. These bars are the average volume of RA mice foot treated with induced regulators or *A. paniculata* substrate (100 µl and 400 µl). The two bars on the left are control for comparison. It is shown that RA mice feet have swollen twice larger compared with normal mice. Injection of *A. paniculata* substrate (100 µl and 400 µl) can cure RA mice to become normal phenotype. Data are mean ± SD values of three independent experiments *(P<0.01*).

In this experiment we found that injection of *A. paniculata* substrate was able to improve the health condition of RA mice. Interestingly, injection with regulatory T cell suppressed T cells proliferation and more than 80% of CD4 T cells lose CD62L expression. In this experiment we can not yet explain why the RA mice injected with regulatory T cell were more

healthy than those of without treatment, even though most of T cells loss in this mice. In this study the doses of *A. paniculata* substrate 100 µl and 400 µl was entered in the normal physiological range with the proof that there was no toxic effect either directly or indirectly. *A. paniculata* injection for seven weeks of treatment do not affect the body weight compared with normal mice (Figure 6). The relative ratio between B cells and T cells, which is represented by CD4, did not appear to change at all circumstances. Injection of *A. paniculata* 400 µl, gave a trend in increase in the number of B cells in RA patients mice. The growing cell will synthesize IL-2 and IL-4. In this mechanism we suspected that the excess of systemic IL-2 and IL-4 are used for B cells development and proliferation. Injection of 400µl of *A. paniculata* increased the number of B cells significantly compared with controls (Figure 7). In RA mice when viewed from the absolute number of leukocytes, it will be described an increasing number of B cells. This can be understood as in the RA state, the cell damage will occur to form auto-DNA. This auto-DNA will be responded by specific B cells. *A. paniculata* injection has no significant effect on increasing the number of leukocytes in RA mice (Figure 8). However, these injections have an effect to increase the number of cells that express CD62L molecules as markers of naive T cells as shown in Figure 4. It is suspected that intraperitoneally injection of *A. paniculata* can increase the number of precursor cells through the intervention of leukocytes in the bone marrow. Another possibility that the active compound in *Andrographis paniculata* has a target of the memory cells or activated cells.

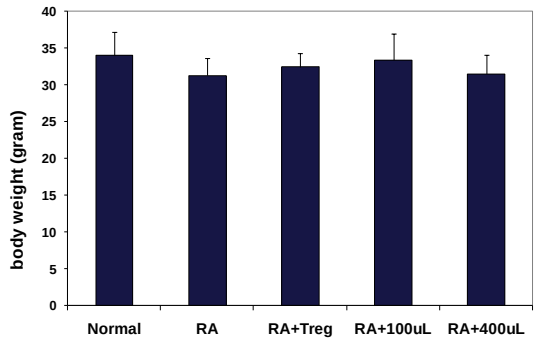

Figure 6. *A. paniculata* substrat (100 µl and 400 µl) did not show toxic effects in animal models of RA. The body weight of mice are measured 7 weeks after treatment. Data are mean ± SD values of three independent experiments *(P<0.01)*.

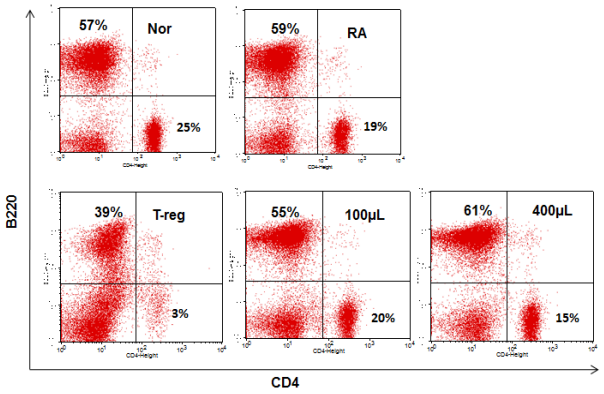

Figure 7. *A. paniculata* substrate injection (400µl) can increase the absolute number of B cells. Spleen was obtained from RA mice seven weeks after treated with *A. paniculata* substrate or regulatory T cells. Spleen cells were labeled with anti-CD4-FITC and anti-B220-PE to assess cell surface molecules. Upper panels are controls that showed positive expression of B220 cells and the right panel shows RA mice that showed positive B220 cells. Additions of regulatory T cells or *A. paniculata* substrate (100 µl, and 400 µl) were shown in the bottom panel. This image shows the relative ratio among control and treated one. Data are mean ± SD values of three independent experiments *(P<0.01)*.

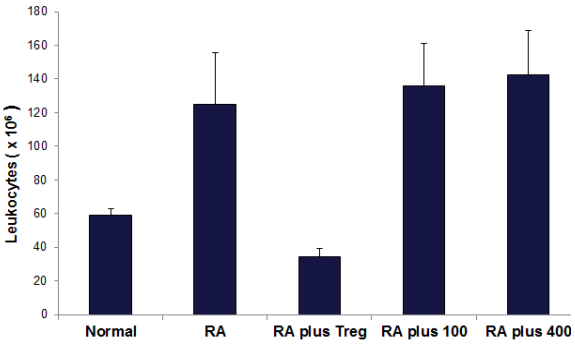

Figure 8. In RA mice the number of leukocytes increased dramatically. The bars show the calculation of the number of leukocytes in the spleen of mice that had been treated with *A. paniculata* substrate (100 µl and 400 µl). Analysis was carried out seven weeks after treatment. The first two bars on the left are controls for comparison. RA mice showed increasing number of leukocytes, two fold compare with normal. When injected with $CD4^{+}CD25^{+}$ regulatory T cells the leukocytes dropped dramatically. Data are mean ± SD values of three independent experiments *(P<0.01)*.

Mechanisms which lead to a decrease number of activated memory cannot be

explained in this study. It is suspected that inflammation occurs in RA is one of the effects of activated T cells. Activated cells from CD4 and CD8 populations have the potential to produce pro-inflammation molecules such as TNF-α and IFN-γ. Pro-inflammatory molecules are in turn influencing the capillary walls that increase the permeability of endothelial cells (Zittermann, 2003). This mechanism will cause effectors molecules and plasma to easily penetrate capillary walls. This condition, although the physiology is required but will worsen the situation if not controlled. Cured phenotypes seen in mice that had received *A. paniculata* injection is associated with cell regeneration after damage by free radicals. In mice that received injection of *A. paniculata* showed increased superoxide dismutase (SOD) activity when compared with RA mice without treatment. RA mice have SOD 21.130 U/ml, while RA mice that received *A. paniculata* 400 µl and 100 µl become 24.435 and 35.290 U/ml, respectively. The results of malon dialdehyde (MDA) analysis in mice that received *A paniculata* (100 µl and 400µl) are 0.507 and 0.603 ng/ml. An increase in SOD activity and decreased MDA in RA mice treated with *A paniculata* substrate indicates a cellular level repair. This study showed that there is an increase of free radicals in RA mice, while *A. paniculata* injection cause the reduction of free radicals. *A. paniculata* substrate injection contribute greatly to increase the number of regulatory cells, $CD4^+CD25^+$.

In this study, it is clear that $CD4^+CD25^+$ regulatory cells increased significantly in RA mice that received injections of *A. paniculata* substrate (Figure 9). An increasing number of regulatory cells is thought to help recovery in mice undergoing RA inflammation. When compared with the normal mice, the number of Treg cells in RA mice increased twice. Treg cell of RA mice that received injections of *A. paniculata* substrate 100 µl and 400 µl increased 4 and 5 fold, respectively. We suspected that $CD4^+CD25^+$ cells in RA mice were activated cells which were not part of the professional regulatory cells. The cells undergone activation can express CD25 molecule that is actually a T cell receptor α (Papiernik *et al.*, 1998; Zittermann, 2003).

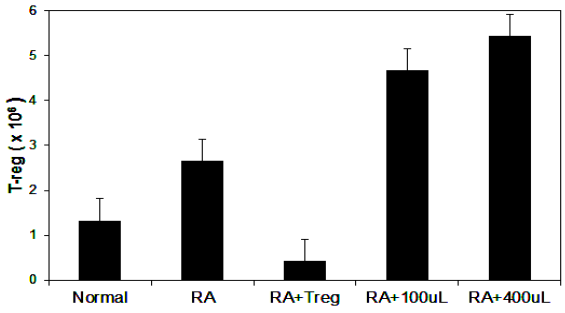

Figure 9. Treg ($CD4^+CD25^+$) cells increased in RA mice that received injections of *A. paniculata* substrate (100 µl and 400 µl). The bars are calculation of the number of CD4 T cells expressing positive CD25 molecules on the spleen cells of mice that had been injected with *A. paniculata* substrate (100 µl and 400 µl). Analysis was carried out seven weeks post treatment. The two first bars on the left are controls for comparison. Data are mean ± SD values of three independent experiments *(P<0.01)*.

Conclusion

A. paniculata has significant benefits for increasing the number of sets of $CD4^+CD25^+$ regulatory T cells. Increased $CD4^+CD25^+$ regulatory T cells will help to cure rheumatoid arthritis. In mice model of rheumatoid arthritis, $CD4^+CD25^+$ regulatory T cell play important role to inhibit the development of effector T cells and prevent the development of inflammation in the joints. The generation of $CD4^+CD25^+$ regulatory can be induced *in vivo* and *in vitro*. Induced $CD4^+CD25^+$ regulatory T cell both *in vivo* and *in vitro* can ameliorate mice model of rheumatoid arthritis.

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