

学位論文

「Effects of TNF inhibitors on human monocytes」

(単球に対する TNF 阻害薬の効果の検討)

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## 著者の宣言

本学位論文は、著者の責任において実験を遂行し、得られた真実の結果に基づいて正確に作成したものに相違ないことをここに宣言する。

## Effects of TNF inhibitors on human monocytes

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### Abstract

**Objective.** To explore the effects of TNF inhibitors, etanercept and infliximab on function of human monocytes.

**Methods.** Monocytes from healthy donors were cultured in the presence of staphylococcal enterotoxin B (SEB) with pharmacologically attainable concentrations of biological agents or control IgG. The expression of IL-6 mRNA was determined by quantitative RT-PCR. The expression of CD80 and CD86 and the induction of apoptosis of monocytes were measured by flow cytometry.

**Results.** All of etanercept, infliximab promoted apoptosis of SEB-stimulated monocytes. The induction of apoptosis of monocytes by these biological agents were reversed by addition of IgG, but not IgG F(ab')<sub>2</sub> fragments.

Etanercept and infliximab significantly suppressed the expression of CD80 and CD86 on SEB-stimulated monocytes. Finally, all of etanercept, infliximab suppressed the expression of mRNA for IL-6 of monocytes stimulated with SEB.

**Conclusions.** These results demonstrate that one of the mechanism of action of TNF inhibitors involves the induction of apoptosis of monocytes, which involves interaction with Fc receptor on monocytes. Moreover, the data also indicate that TNF inhibitors strongly inhibit IL-6 production of monocytes, and suppress the expression of costimulation molecules at the same time.

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## Introduction

Increasing attention has been paid to the role of proinflammatory cytokines, including IL-1, IL-6 and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), in the pathogenesis of rheumatoid arthritis (RA) (1, 2). In fact, the agents inhibiting these cytokines are effective in the treatment of RA (3, 4). Thus, etanercept, a TNF-receptor-IgG Fc fusion protein and infliximab, an anti-TNF $\alpha$  monoclonal antibody, suppress the inflammation by inhibiting TNF $\alpha$ . Although several studies have disclosed the in vitro effects of these biological agents on the immune-competent cells (5, 6), their precise mechanisms of action in RA still remains unclear.

We have recently demonstrated that etanercept and infliximab suppressed the proliferation and interferon- $\gamma$  production of peripheral blood mononuclear cells (PBMC) stimulated with staphylococcal enterotoxin B (SEB) without direct effects on immobilized anti-CD3-stimulated CD4<sup>+</sup> T cells (7). It is thus suggested that etanercept and infliximab might suppress the activation of PBMC stimulated with SEB by inhibiting the function of monocytes (7).

The current studies were therefore undertaken to explore the effects of TNF inhibitors on human monocytes in detail. Special attention was paid to the capacities of TNF inhibitors to induce apoptosis of monocytes and to modulate the expression of costimulation molecules and cytokines.

## **Materials and methods**

### Monoclonal antibodies (mAbs)

A variety of mAbs were used in this study, including fluorescein isothiocyanate (FITC)-conjugated anti-CD80 (Immunotech, Marseille, France), FITC-conjugated anti-CD86 (Ancell, Bayport, MN), and FITC-conjugated control mouse IgG1 (Dako, Glostrup, Denmark).

### Cell preparation

PBMC were obtained from healthy adult volunteers with informed consent by centrifugation of heparinized venous blood over sodium diatrizoate-Ficoll gradients. Monocytes were prepared from PBMC using Monocyte Isolation Kit II (Miltenyi Biotec). Monocyte population obtained in this manner contained <0.1% CD3<sup>+</sup> cells, <0.1% CD19<sup>+</sup> cells, and >93% CD14<sup>+</sup> cells.

### Reagents

Infliximab and etanercept were purchased from Mitsubishi Tanabe Pharma Corporation (Tokyo, Japan) and Takeda Pharmaceutical Co. (Tokyo). Control human IgG1 was purified from serum of a patient with human IgG1 myeloma using DEAE-Sepharose column. Human IgG-F(ab')<sub>2</sub> (Gamma Venin® P) was purchased from Sanofi, Paris, France.

### Cell cultures

RPMI 1640 medium (Nikken, Kyoto, Japan) supplemented with penicillin G (100 U/ml)

(Life technologies, Grand Island, NY), streptomycin (100 µg/ml) (Life technologies), L-glutamine (0.3 mg/ml) (Sigma-Aldrich, St Louis, MO), and 10% fetal bovine serum (JRH Bio-Sciences, Lenexa, KS), were used for cultures. Purified monocytes ( $1 \times 10^6$  /well) were cultured in the presence of SEB (100 pg/ml) (Serva, Heidelberg, Germany) in each well of 24-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) with control IgG (10 µg/ml), or pharmacologically attainable concentrations of etanercept (10 µg/ml) or infliximab (10 µg/ml) (8, 9) for 24 or 48 hours.

#### Analysis of cell surface antigens by flow cytometry

The surface antigens on monocytes and the induction of apoptosis were analysed by flow cytometry. Briefly, after the incubation for 24 hours, the cells were washed once by phosphate buffered saline (PBS) containing 2% normal human serum and 0.1% sodium azide (staining buffer). The cells were then reacted in suspension by incubating for 30 minutes at 4°C with saturating concentrations of FITC-conjugated mAbs. After the cells were washed once with staining buffer and then twice with PBS, they were counterstained with phycoerythrin (PE)-conjugated Annexin V (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The cells were then analyzed using Cell Lab Quanta SC (Beckman Coulter, Miami, FL). To identify viable cells, the gating for the staining with Annexin V was used. In some experiments, the cells were stained with FITC-conjugated Annexin V (R&D Systems) and propidium iodide (PI), and were analysed by flow cytometry.

#### Measurement of IL-6

The concentrations of IL-6 in the culture supernatants were measured using Human IL-6



ELISA Development Kit (Peprotech, Rocky, Hill, NJ).

#### RNA isolation and real-time quantitative PCR

Total RNA was isolated from cultured cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's specifications. cDNA was prepared from 1 µg of total RNA using M-MLV reverse transcriptase (Takara Bio, Shiga, Japan) with random primers (Takara Bio), and was subjected to analysis with Real-time PCR using LightCycler 4.1 (Roche Diagnostics, Lewes, UK). Real-time PCR of IL-6 and β-actin was performed using SYBR Premix Ex Taq II (Takara Bio) with the following primers: sense, 5'-GGAGACTTGCCTGGTGAAAA-3' and antisense, 5'-GTCAGGGGTGGTTATTGCAT-3' for IL-6 (gene accession No. M14584); sense, 5'-TGGCACCCAGCACAATGAA-3' and antisense, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' for β-actin (gene accession No. NM001101). Amplification was performed according to the standard protocol recommended by the manufacturer. All results were calibrated to the copy number of β-actin obtained from the same cDNA samples.

#### Statistical analysis

Statistical significance was evaluated by Wilcoxon's signed rank test and paired t test where appropriate.

## Results

### Effects of TNF inhibitors on Annexin V expression of SEB-stimulated monocytes

Initial experiments examined the effects of TNF inhibitors on Annexin V expression of SEB-stimulated monocytes to explore their influences on the induction of apoptosis. As shown in Fig. 1, TNF inhibitors, etanercept and infliximab significantly increased the expression of Annexin V of monocytes compared with control IgG. It should be noted that these biological agents also increased the numbers of Annexin V-positive and PI-negative cells significantly. The results therefore indicate that TNF inhibitors promote apoptosis of SEB-stimulated monocytes. Moreover, the data confirm that human monocytes are direct targets of TNF inhibitors.

It was previously disclosed that etanercept and infliximab were able to induce apoptosis of TNF $\alpha$  expressing Jurkat T cells in the presence of human PBMCs, presumably through antibody dependent cellular cytotoxicity (ADCC), which requires the presence of Fc receptors (5). It is therefore possible that interactions with Fc receptors on monocytes might be required for the induction of apoptosis of monocytes by etanercept and infliximab. To explore this possibility, we next examined the influences of IgG and IgG-F(ab')<sub>2</sub> fragments on the capacities of these biological agents to induce apoptosis of SEB-stimulated monocytes. As shown in Fig. 2, addition of IgG, but not IgG-F(ab')<sub>2</sub>, almost completely reversed the capacities of not only etanercept, but infliximab, to induce apoptosis of SEB-stimulated monocytes ( $p < 0.05$ ). These results indicate that Fc receptors on monocytes are involved in the induction of apoptosis of SEB-stimulated monocytes by TNF inhibitors. Moreover, it is also suggested that the outside to inside signal via TNF $\alpha$  alone might not be sufficient for the induction of apoptosis of human

monocytes.

#### Effects of TNF inhibitors on the expression of CD80 and CD86 on SEB-stimulated monocytes

The next experiments examined the effects of each biological agent on the expression of costimulatory molecules on monocytes stimulated with SEB. Fig. 3A shows the typical histogram of the expression of CD80 and CD86 on Annexin V-negative monocytes from a normal healthy donor. Etanercept and infliximab markedly suppressed the expression of CD80 and CD86. Accordingly, both etanercept and infliximab significantly suppressed the expression of CD80 and CD86 on SEB-stimulated monocytes from 9 healthy individuals compared with control IgG (Fig. 3B). The results indicate that TNF inhibitors suppressed the expression of costimulation molecules on SEB-stimulated monocytes.

#### Effects of TNF inhibitors on the IL-6 production of SEB-stimulated monocytes

Recent studies have revealed that plasma concentration of IL-6 but not TNF $\alpha$  is correlated with the disease activity and radiographic progression in RA (10). Final experiments therefore examined the effects of TNF inhibitors on the production of IL-6 of monocytes stimulated with SEB. As shown in Fig. 4A, etanercept and infliximab decreased the concentrations of IL-6 in the culture supernatants of SEB-stimulated monocytes at pharmacologically attainable concentrations. Next, we examined the effects of these biological agents on the expression of mRNA for IL-6 in SEB-stimulated monocytes. As shown in Fig. 4B, etanercept and infliximab, suppressed the expression of mRNA for IL-6 in SEB-stimulated monocytes at their

pharmacologically attainable concentrations. The results indicate that etanercept and infliximab have direct effects on SEB-stimulated monocytes to suppress their expression of IL-6.

## Discussion

The current studies clearly demonstrate that etanercept and infliximab induce apoptosis of SEB-stimulated monocytes. Since addition of normal human IgG, but not IgG-F(ab')<sub>2</sub>, inhibited the induction of apoptosis by these biological agents, it is suggested that Fc receptors on monocytes are involved in the induction of apoptosis of SEB-stimulated monocytes by TNF inhibitors. Previous studies showed that ADCC is one of mechanisms of induction of apoptosis in target cells (11). Moreover, we examined the influences of IgG-Fc on the capacities of these biological agents to induce apoptosis of SEB-stimulated monocytes in 2 healthy individuals. Addition of IgG-Fc reversed the capacities of not only etanercept, but infliximab, to induce apoptosis of SEB-stimulated monocytes (data not shown). These results indicate that other IgG1 or IgGs should have the influences of inhibition of induction of apoptosis by these biological agents. Thus, it is strongly suggested that TNF inhibitors might induce apoptosis of human monocytes through ADCC. Of note, Mitoma et al demonstrated that infliximab and adalimumab, but not etanercept, induce apoptosis of Jurkat T cells expressing TNF $\alpha$  on the surface through outside to inside signals by membrane bound TNF $\alpha$  (5). Moreover, infliximab and adalimumab exerted much higher complement dependent cytotoxicity (CDC) to TNF $\alpha$ -expressing Jurkat T cells than etanercept (5). It should be emphasized, however, that ADCC activities to TNF $\alpha$ -expressing Jurkat T cells of etanercept were comparable to those of infliximab or adalimumab (5). Since addition of intact IgG almost completely abrogated the capacity of infliximab, etanercept to induce apoptosis of SEB activated monocytes in the present study, it is strongly suggested that induction of apoptosis of human monocytes by these biological compounds might be mediated through ADCC.

It was found that infliximab did not result in the increased apoptosis in the RA synovial tissue at 48 hours after initiation of treatment (12). However, 8 weeks of treatment with etanercept or with infliximab significantly increased apoptosis, accompanied by a significant decrease in the synovial macrophage population (13). Accordingly, etanercept and infliximab induced apoptosis of macrophages/monocytes in synovial fluid in vitro (13). It should be pointed out that SEB activates monocytes and dendritic cells through Toll-like receptor (TLR) 2 (14). On the other hand, previous studies demonstrated that the expression of TLR2 was up regulated in peripheral blood monocytes, synovial macrophages and synovial tissue of RA, thus playing an important role in the pathogenesis (15, 16). Since TNF inhibitors induced apoptosis of SEB-stimulated monocytes, it is possible that it also might induce apoptosis of monocytes in vivo in RA.

Since macrophages/monocytes derived from the bone marrow are precursors of type A synoviocytes (17, 18), the induction of apoptosis of peripheral blood monocytes might also lead to the reduction of synovial macrophages. Taken together, the results in our study further confirm that one of the important mechanisms of action of biological agents involves the induction of apoptosis of macrophages/monocytes in RA.

We have previously disclosed that infliximab as well as etanercept, but not tocilizumab, inhibited the proliferation and the interferon- $\gamma$  production of T cells, stimulated by SEB (7). Neither infliximab nor etanercept inhibited the T cell activation by immobilized anti-CD3 in the absence of monocytes. Since the activation of T cells by SEB requires the presence of antigen-presenting cells (19), the inhibition of SEB-stimulated T cells by

infliximab or etanercept is considered to be a result of inhibition of monocytes. Accordingly, infliximab as well as etanercept, suppressed the expression of HLA-DR on monocytes (7). Moreover, the data in the present study have shown that TNF inhibitors suppress the expression of CD80 and CD86 on SEB-stimulated monocytes. It is therefore likely that the suppression of SEB-mediated T cell activation by TNF inhibitors might be due to the inhibition of expression of HLA-DR as well as costimulation molecules, CD80 and CD86. The mechanisms of suppression of expression of these molecules by TNF inhibitors are currently unknown. Further studies to explore the influences of TNF inhibitors on the expression of mRNA for CD80, CD86 and HLA-DR and on the cleavages of these molecules from the membranes would be necessary to fully understand the mechanisms of action.

IL-6 has been found to play an important role in the pathogenesis of RA (20, 21). It has been shown that successful treatment with methotrexate (22) or with TNF inhibitors (23) results in the reduction of plasma IL-6. On the other hand, it has been also shown that infliximab markedly decreased the levels of IL-6 in the cerebrospinal fluid (CSF) from patients with chronic progressive neuro-Behcet's disease, who showed sustained elevation of CSF IL-6, on the next day of infusion (24). The results in the current studies demonstrate that TNF inhibitors suppressed the production and the expression of mRNA for IL-6 in SEB stimulated monocytes, confirming that TNF inhibitors inhibit the expression IL-6 in the sites of inflammation *in vivo*.

In summary, the results in the current studies have delineated the influences of TNF inhibitors on human monocytes. The common features of TNF inhibitors include the

induction of apoptosis and the inhibition of the expression of mRNA for IL-6. The differential capacities of TNF inhibitors on T cell activation appear to be a result of their differential capacities to inhibit the expression of HLA-DR and costimulation molecules. Further studies to delineate the precise mechanisms by which TNF inhibitors regulate the expression of HLA-DR, CD80 and CD86 would be important.

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### Figure legend

**Fig. 1 Effects of TNF inhibitors on Annexin V expression of SEB-stimulated monocytes.** Highly purified monocytes ( $1 \times 10^6$  /well) from 8 healthy individuals were cultured in the presence of SEB (100 pg/ml) in each well of 24-well flat-bottomed microtiter plates with etanercept (ETN) (10  $\mu$ g/ml), infliximab (IFX) (10  $\mu$ g/ml) or control IgG (10  $\mu$ g/ml) for 48 hours, after which the cells were stained with FITC-conjugated AnnexinV and propidium iodide (PI). The cells were then analyzed by flow cytometry. A. Representative dot plots for staining with FITC-conjugated Annexin V and PI. B, Percentages of total Annexin V-positive cells (upper) or PI-negative Annexin V-positive cells (lower) are shown. Statistical significance was evaluated by Wilcoxon's signed rank test.

**Fig. 2 Differential effects of human IgG and IgG F(ab')<sub>2</sub> fragments on the induction of apoptosis of SEB stimulated monocytes by TNF inhibitors.**

Highly purified monocytes ( $1 \times 10^6$  /well) from healthy individuals were cultured in the presence of SEB (100 pg/ml) in each well of 24-well flat-bottomed microtiter plates with etanercept (ETN) (10  $\mu$ g/ml), infliximab (IFX) (10  $\mu$ g/ml), or control IgG (10  $\mu$ g/ml) for 48 hours, without or with IgG (100  $\mu$ g/ml) or IgG F(ab')<sub>2</sub> fragments (100  $\mu$ g/ml). After which the cells were stained with PE-conjugated Annexin V. The cells were then analyzed by flow cytometry. Percentages of total Annexin V-positive cells (upper, n=7) or PI-negative Annexin V-positive cells (lower, n=2) are shown. Error bars indicate SD values of 7 (upper) or 2 (lower) different experiments. Statistical significance was evaluated by Wilcoxon's signed rank test.

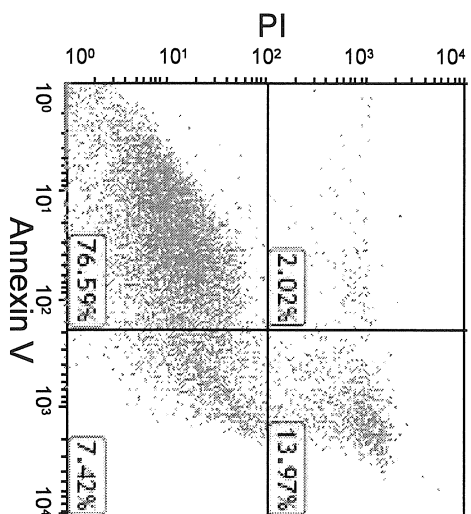
**Fig. 3 Effects of TNF inhibitors on the expression of CD80 and CD86 on SEB-stimulated monocytes.** Highly purified monocytes ( $1 \times 10^6$  /well) from 9 healthy

individuals were cultured in the presence of SEB (100 pg/ml) in each well of 24-well flat-bottomed microtiter plates with etanercept (ETN) (10 µg/ml), infliximab (IFX) (10 µg/ml), or control IgG (10 µg/ml) for 48 hours, after which the cells were stained with FITC-conjugated anti-CD80, anti-CD86, or control IgG1, followed by counterstaining with PE-conjugated Annexin V. The cells were then analyzed by flow cytometry. A. Representative histograms of the staining of various molecules on Annexin V-negative monocytes. The percents positive for specific mAb staining are indicated. Stainings with isotype-matched control mAb are indicated by shade. B. Percentages positive for each specific mAb staining of monocytes from 9 independent experiments are summarized. Statistical significance was evaluated by Wilcoxon's signed rank test.

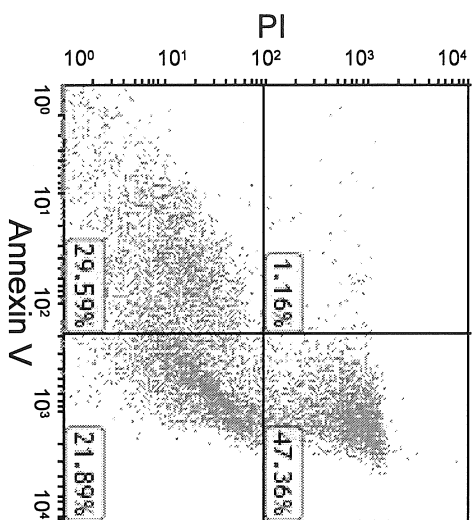
**Fig. 4 Effects of TNF inhibitors on the protein expression and mRNA expression for IL-6 of SEB-stimulated monocytes.** Highly purified monocytes ( $1 \times 10^6$  /well) from healthy individuals were cultured in the presence of SEB (100 pg/ml) in each well of 24-well flat-bottomed microtiter plates with etanercept (ETN) (10 µg/ml), infliximab (IFX) (10 µg/ml), or control IgG (10 µg/ml). A. After 48h of incubation, culture supernatants were collected and assayed for IL-6 by ELISA. Statistical significance was evaluated by Wilcoxon's signed rank test. B. After 24h of incubation, the cells were harvested and the expression of mRNA for IL-6 was examined by quantitative RT-PCR. Data are expressed as the ratio to copy numbers of  $\beta$ -actin mRNA. Statistical significance was evaluated by Wilcoxon's signed rank test.

A

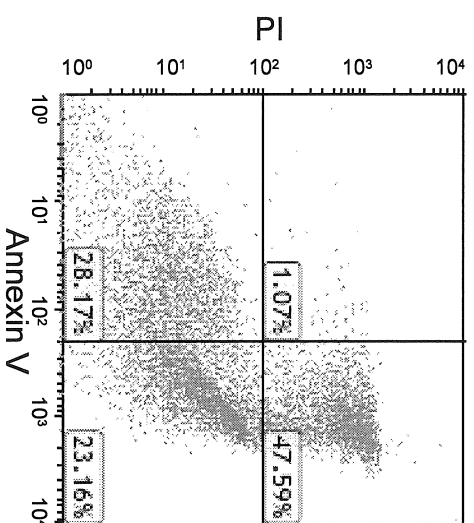
IgG



ETN



IFX



B

Figure 1

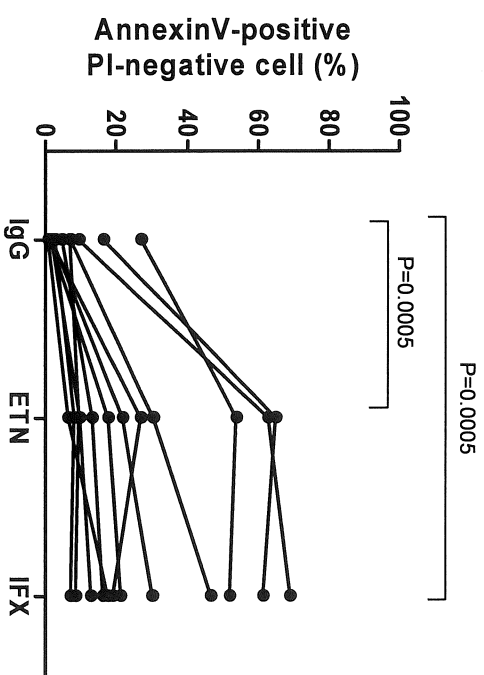
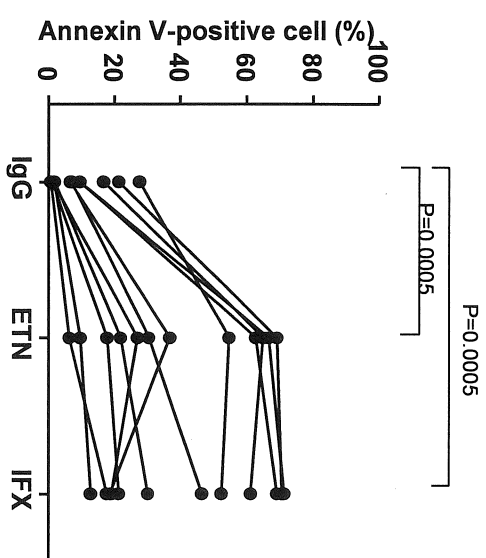
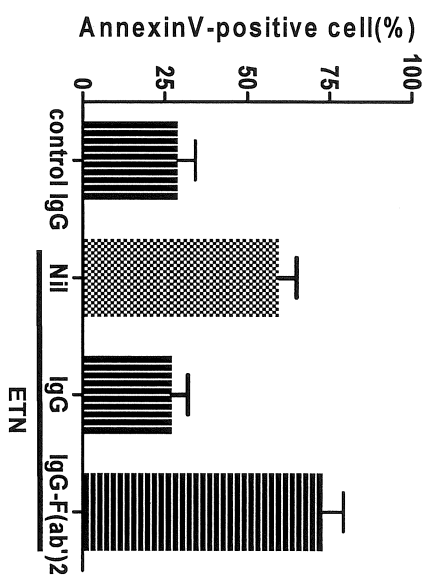


Figure 2

ETN



IFX

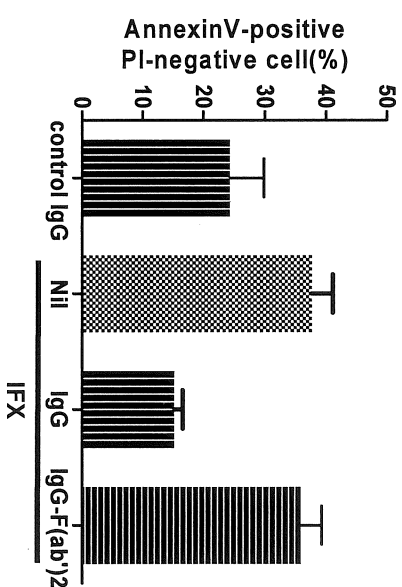
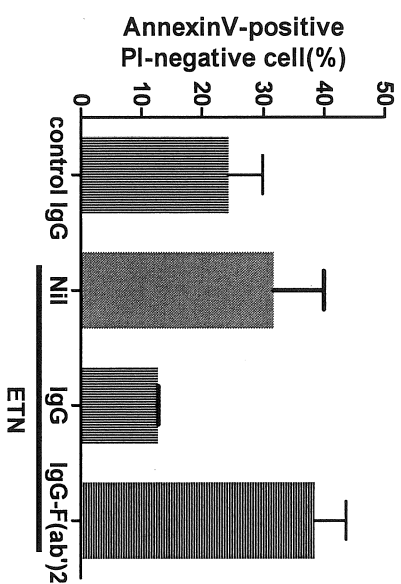
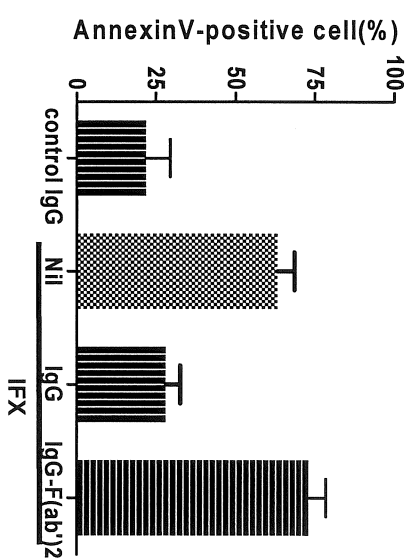
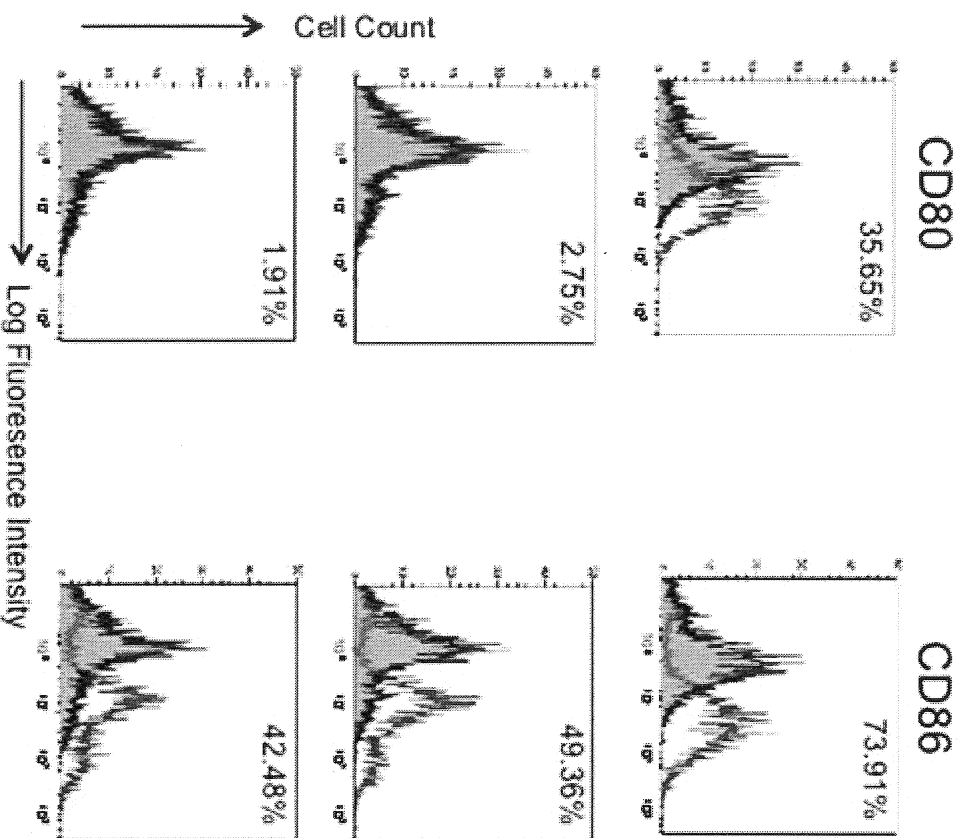




Figure 3

A



B

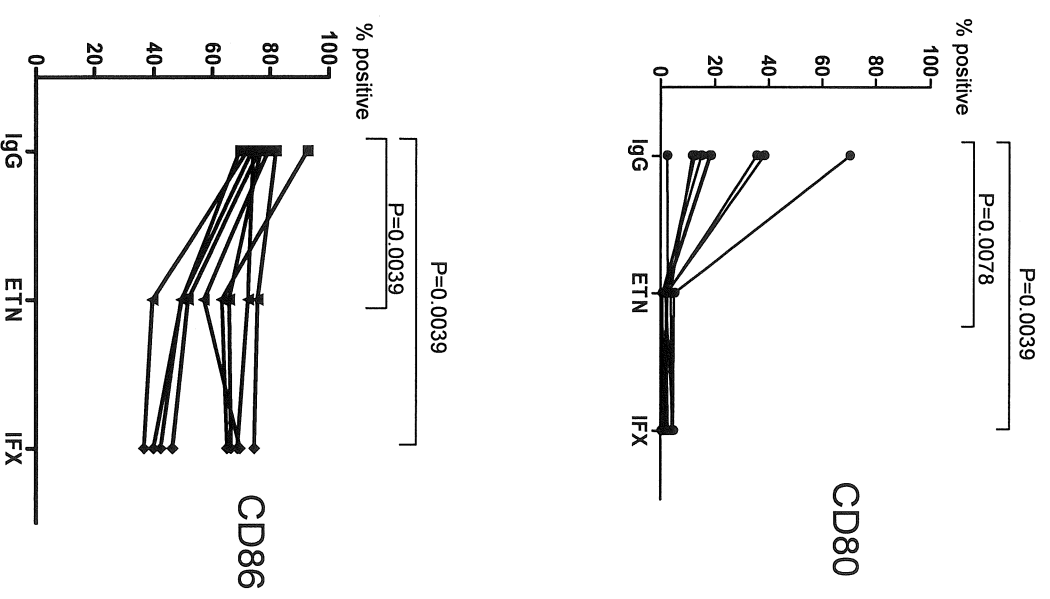
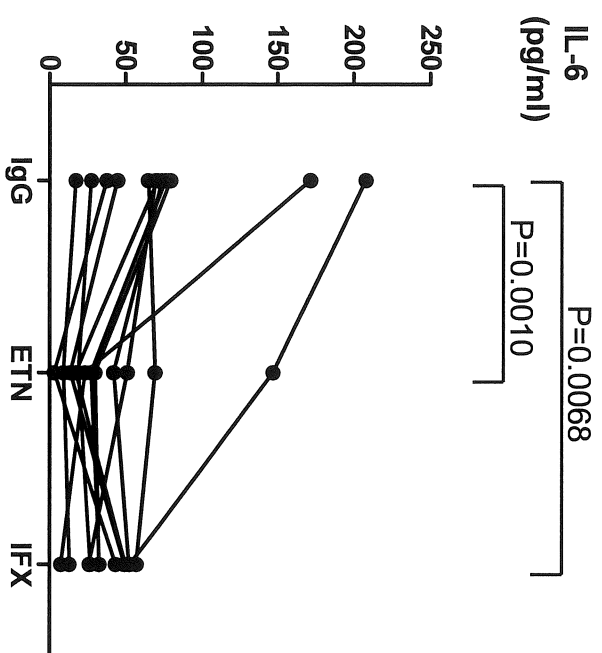


Figure 4

A



B

