**Focal adhesion kinase (FAK) mediates human leukocyte histocompatibility antigen (HLA) class II-induced signaling in gingival fibroblasts**

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**Short title:** HLA II-induced signals in gingival fibroblasts.

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**Key words:** gingival fibroblast; human leukocyte antigen-II; focal adhesion kinase; luteolin

**Abstract**

*Background*: The role of human leukocyte histocompatibility antigen (HLA) class II molecules on non-antigen presenting cells has been a matter of controversy. We previously reported HLA-II molecules on human gingival fibroblasts (GF) do not present antigens, but transduce signals into the cells by making complex with antigenic peptide-T-cell receptor or by stimulating cell surface HLA-DR molecules with anti-HLA-DR antibody (L243) which mimics the formation of HLA-II –antigenic peptide-T-cell receptor complex, resulting in the expression of several cytokines.

*Objective*: The aim of this study was to detect HLA-II-associated molecules mediating HLA-II-induced signals into the cells.

*Materials and Methods*: Antibody-based protein-microarray analysis was performed to detect activated signaling molecules in GF stimulated *via* HLA-II molecules. Then, we examined if these molecules structurally associate with HLA-II, and actually transduce signals into the cells.

*Results*: Stimulation of HLA-II on GF by L243 resulted in enhanced phosphorylation of focal adhesion kinase (FAK). FAK was co-immunoprecipitated with HLA-DR by L243. Stimulation of GF with L243 induced phosphorylation of FAK. Luteolin, putative FAK inhibitor, suppressed phosphorylation of FAK, and dose dependently inhibited HLA-II-induced cytokine production.

*Conclusion*: FAK is structurally associated with HLA-DR, and mediates HLA-II-induced signals in GF.

## Introduction

The physiological role of human leukocyte histocompatibility antigen (HLA) class II molecules on non-antigen presenting cells such as fibroblasts has not been fully understood yet, although these cells are known to express HLA class II molecules on their cell surfaces, especially highly upon stimulation with interferon-γ (IFN-γ). For example, gingival fibroblasts in inflamed periodontal tissues have been suggested to express HLA-II molecules on their cell surface (1). We previously reported that ligation of HLA-DR molecules on cultured human gingival fibroblasts with anti-HLA-DR monoclonal antibodies (L243) or with antigenic peptide/T cell receptor complex resulted in a secretion of several cytokine/chemokines such as regulated upon activation, normal T expressed and secreted (RANTES), monocyte chemoattractant protein 1 (MCP-1) and interleukin-6 (IL-6), and suggested that cytokines thus secreted could further deteriorate the clinical course of inflammatory periodontal diseases (2). Additionally, we recently reported that ligation of HLA-DR molecules with anti-DR antibodies on IFN-γ-stimulated fibroblasts resulted in the activation of intracellular signaling molecule c-jun N-terminal kinase (JNK), one of the mitogen activated protein (MAP) kinases, and JNK activity is at least involved in the RANTES production in such cells, as specific inhibitor for JNK inhibited HLA-II-induced RANTES expression in these cells (3). However, so far, it is still unclear as to what molecules structurally associate with HLA-DR molecules in non-antigen presenting cells, and, if any, whether such molecules transduce signals into the cells. Because it is known that intracellular domain of HLA-II molecules is quite short in length, and this domain even lacks the amino acid residues potentially receiving phosphorylation which transduce signals into the cells, some other molecules which structurally associate with HLA-class II

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molecules are actually suggested to mediate HLA-II-induced signals (4). Therefore, identification and characterization of HLA-class II-associated molecules potentially transducing HLA-II-induced signals into the cells in non-antigen presenting cells would greatly enhance our understanding the overall physiological role of HLA-II molecules expressed on non-antigen presenting cells. Especially, it is important to identify such molecules in specific cells types such as gingival fibroblasts which are easily exposed to chronic inflammatory conditions, as HLA-II molecules expressed on these cells under inflammatory conditions may greatly alter the clinical course of inflammatory periodontal diseases.

In this study, therefore, to try to identify associate molecules with HLA-DR and putative molecules mediating HLA-DR-induced signals in gingival fibroblasts, we first utilized antibody-based protein microarray technique. We then tried to see if such molecules structurally associate with HLA-DR molecules, and actively mediate HLA-II-induced signaling into the cells.

## Materials and methods

### Reagents

Mouse anti-HLA-DR monoclonal antibodies (L243: Leinco Technologies Inc., Ballwin, MO, USA) were used for ligation with HLA-DR molecules. Isotype matched control mouse IgG2a were obtained from Pharmingen (San Diego, CA, USA). IFN-γ was obtained from Genzyme (Cambridge, MA, USA). As putative inhibitors for focal adhesion kinase (FAK), luteolin and quercetin were used. Luteolin was purchased from Extrasynthese (Genay, France), while quercetin was from Nacalai

Tesque (Kyoto, Japan).

**Cells and cell culture**

Human gingival fibroblasts were isolated from healthy volunteers' gingival tissues during the extraction of impacted third molar. Gingival tissues were plated in a 35mm cell culture dish pre-coated with type I collagen and fibronectin. Outgrown cells from tissue explants were expanded and maintained as described previously (2). Briefly, the cells were cultured in a medium composed of Dulbecco's modified Eagle medium supplemented with 10 % fetal bovine serum, 10 mg/ml gentamicin, 0.1 mM non-essential amino acids, vitamins, 2 mM L-glutamine. When the cells reached confluent, the cells were passaged with a split ratio of 1:4. The cells were used between passage 5 and 7 for all experiments.

**Antibody microarray analyses**

To detect putative signaling molecules in fibroblasts stimulated with L243, activated molecules were detected by antibody-based protein-microarray technique (Panorama TM Ab. Microarray, SIGMA, St. Louis, MO). The cells were first cultured till sub-confluency, and the medium was then changed to serum-free medium and the cells were cultured for 24h for serum starvation, followed by 48h culture with or without 500 U/ml of IFN-γ in serum-free medium. Then, the cells were stimulated either with L243 (1 μg/ml) or control IgG. At the indicated time period, the cells were lysed with cell lysis buffer included in the microarray kit and the soluble fractions were obtained The cellular

proteins were labeled either with fluorescent dye, Cy3 or Cy5, and the labeled proteins were

subjected to microarray analyses according to the manufacture's instructions. The fluorescent

intensity of each reaction was measured by using GenePix 4000B Microarray scanner (Amersham

Biosciences, Buckinghamshire, England), and the data was quantified by using Array Vision

software (Amersham Biosciences).

**Immunoprecipitation**

To isolate putative associate molecules with HLA-DR, IFN-γ-treated fibroblasts for 48h were first

lysed with 1% CHAPS buffer containing 150 mM NaCl, 20 mM Tris-HCl, 1mM NaF and 5 mM

EDTA, and the soluble fraction was obtained by centrifugation. Then, the lysates were incubated

with L243, and L243-protein complex was immunoprecipitated with Protein A Magnetic Beads

(New England Biolabs, MA, USA) according to the manufacture's instruction. The complex was

then subjected to SDS-PAGE. In some experiments, the cells were first stimulated either with L243

or control IgG for indicated time period and lysed, followed by the immunoprecipitation with

Protein A Magnetic beads.

**Western immunoblotting**

Detection of tyrosine phosphorylated FAK, and FAK proteins were performed by western

immunoblotting. Briefly, protein samples obtained by immunoprecipitation described above were

separated by SDS-PAGE, and blotted onto PVDF membrane. To detect FAK, rabbit anti human FAK

polyclonal antibody (SIGMA, Saint Louis, MO) was used, while to detect phospho-FAK, rabbit anti phospho-FAK polyclonal antibody (pY397: SIGMA) was used as a primary antibody, respectively. Horseradish peroxidase conjugated anti-rabbit IgG (Amersham Biosciences) was used as a secondary antibody. Immuno-reactive proteins were detected by enhanced chemiluminescence method (ECL: Amersham Biosciences).

In some experiments, the cells were cultured with or without indicated concentration of luteolin or quercetin, putative FAK inhibitors, for 48h, and the cells were lysed with cell lysis buffer (1% CHAPS, 2mM $Na_3VO_4$, 1mM NaF, 5mM EDTA, proteinase inhibitor mixture [Roche Diagnostics GmbH, Mannheim, Germany] in 20mM Tris-150mM NaCl buffer, pH 7.6) at each indicated time point. Protein concentration was measured by protein assay (Bio-Rad, Hercules, CA). 10 μg of protein samples were separated by SDS-PAGE, and blotted onto the membrane. Phospho-FAK and FAK were detected as described. To see the effects of luteolin on the phosphorylation of ERK, p38, and JNK MAP kinases, and c-jun, same samples were subjected to SDS-PAGE, followed by the western immunoblotting with rabbit anti-phospho ERK, ERK, phospho-p38, p38, phospho-JNK polyclonal antibody, anti-JNK polyclonal antibody, anti-phospho c-jun polyclonal antibody and anti-c-jun polyclonal antibody (all from Cell Signaling, Berverly, MA, USA), as luteolin has been suggested to influence the phosphorylation of these proteins in other cell types. In these experiments, the membranes were first probed with the antibodies against each native protein, and then re-probed with the antibodies recognizing phosphorylated form of each protein after stripping bound antibodies with stripping buffer (2% SDS, 100mM mercaptoethanol in 62.5mM Tris-HCl buffer, pH 6.7).

**Cytokine assay**

RANTES, MCP-1, and IL-6 concentration in culture supernatants of the cells stimulated *via* HLA-DR molecules with or without pre-incubation of the cells with indicated concentration of luteolin for 48h was measured by using commercial immunoassay kit (human RANTES, MCP-1, and IL-6 ELISA kit, Endogen Inc., Woburn, MA, USA) following 16h of cell culture.

**Statistical analyses**

Statistical analyses comparing the cytokine productivity between the cells stimulated with L243 in the presence or absence of luteolin was performed by using student T-test.

## Results

**Ligation of HLA-DR molecules on fibroblasts with L243 results in the activation of several signaling molecules**

Ligation of HLA-DR molecules with L243 resulted in the enhanced phosphorylation of several molecules including FAK, Raf, and JNK (Figure 1). About two fold higher phosphorylation was observed in each kinase as compared with the cells stimulated with control IgG. As for FAK, all three antibodies recognizing distinct phosphorylation sites (pY397, pS910, and pY577) reacted strongly with the proteins obtained from L243-stimulated cells, suggesting that FAK is highly

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activated by L243 stimulation.

**HLA-DR molecules on fibroblasts directly associate with FAK**

As FAK usually associates with intracellular domain of integrins, we next tried to see if HLA-DR molecule on fibroblasts directly associates with FAK. Cellular proteins co-immunoprecipitated with either L243 or control IgG were separated by SDS-PAGE, and FAK was detected by western immunoblotting. As in figure 2, FAK was clearly observed in a fraction immunoprecipitated with L243 obtained from IFN-γ-stimulated cells. A weak band was also observed in a fraction immunoprecipitated with L243 obtained from the cells without prior IFN-γ stimulation. In contrast, no corresponding band was detected in a fraction immunoprecipitated with control IgG. Thus, the result indicated that HLA-DR molecule on fibroblasts directly associated with FAK.

**Ligation of HLA-DR molecules on fibroblasts with L243 results in the phosphorylation of FAK**

To see FAK is actually activated in fibroblasts upon stimulation with L243, IFN-γ-treated cells were first stimulated with L243 or with control IgG, and then, the cells were lysed, followed by the immunoprecipitation. As in figure 3, enhanced phosphorylation of FAK was actually observed in 10 min following L243 stimulation, while the level of phosphorylation was weak in control IgG-stimulated cells. The amounts of total cellular FAK proteins did not change regardless the stimulation with L243 (Figure 3, lower panel). Thus, the result indicated that HLA-DR-associated FAK is activated in fibroblasts upon stimulation with L243.

**Luteolin, but not quercetin, suppresses phosphorylation of FAK, while it does not influence the phosphorylation of ERK, p38 and JNK mitogen activated protein (MAP) kinases and c-jun**

We next wondered if any reagent suppressed phosphorylation of FAK. As certain flavonoids have been reported to inhibit FAK activity in different cell types, we tried to see the effects of flavonoids as luteolin and quercetin on the phosphorylation of FAK. As these reagents were suggested to suppress activities of other signaling molecules in other cell types, we also tested the effects of these reagents on the activities of reported other kinases. Luteolin, but not quercetin suppressed the phosphorylation of FAK (pY397) in a dose dependent manner (Figure 4A). However, luteolin did not affect the phosphorylation of ERK, p38 and JNK MAP kinase and c-jun (Figure 4B). In some kinases such as p38, JNK and c-jun, slightly enhanced phosphorylation was observed. Additionally, 100 μM luteolin resulted in the loss of both ERK and p-ERK. Cell viability was not influenced by the experimental concentration of luteolin, as judged by the trypan blue dye exclusion assay. Based on these results, we utilized luteolin for subsequent experiments.

**Luteolin inhibits the production of MCP-1, IL-6 and RANTES in fibroblasts stimulated with L243**

Finally, we tried to see the effects of luteolin on L243-stimulated MCP-1, IL-6, and RANTES production in fibroblasts. We treated the cells with different concentration of luteolin for 48h and then stimulated the cells either with L243 or control IgG. 16hr after stimulation, the culture

supernatants were collected and the amounts of RANTES, MCP-1, and IL-6 were measured. The

results were shown in figure 5. Luteolin suppressed L243-induced production of RANTES, MCP-1,

and IL-6 in a dose dependent manner.


## Discussion


The present study can be summarized as follows; 1) HLA-DR molecules directly associate

with FAK in fibroblasts, 2) FAK can mediates HLA-DR-induced signals into the cells, 3) certain

flavonoid such as luteolin suppresses FAK phosphorylation, and 4) luteolin suppressed

HLA-DR-induced cytokine and chemokine production from fibroblasts. Therefore, FAK structurally

associates with HLA-II molecules and actually mediates HLA-II-induced signaling which leads to

the secretion of cytokines.

Although it is well-accepted that FAK signals play an important role in cell migration,

proliferation and differentiation, no reports are available regarding the selective inhibitors for FAK.

In this study, thus, we searched the literature to find possible inhibitors for FAK, and chose quercetin

and luteolin, as these two reagents were the only reported inhibitors for FAK (5). The results

indicated that quercetin did not influence the phosphorylation status of FAK in fibroblasts, while

luteolin inhibited the phosphorylation of FAK in a dose dependent manner. However, luteolin has

been suggested to affect other signaling molecules such as ERK, p38 MAP kinase and JNK activity

in murine macrophage cell line, RAW 264, human cultured mast cells, vascular sooth muscle cells,

Hep G2 cells, colon epithelial cells, and HUVEC cells (6-11). Additionally, luteolin has recently

been reported to suppress the phosphorylation of c-jun without affecting MAP kinase activity,

thereby suppressing the transcription factor, activator protein (AP)-1, activity in basophilic cell line, KU812 (12). Therefore, we tested the possibility that luteolin might also influence the phosphorylation of reported signaling molecules such as MAP kinases and c-jun in gingival fibroblasts. However, no apparent inhibition on the phosphorylation of ERK, p38, JNK, and c-jun were observed. Additionally, luteolin even enhanced the phosphorylation of some signaling molecules such as p38, p-JNK, and c-jun. We thus speculate that the effects of luteolin could differ from cell type to cell type.

In our previously study, we reported that ligation of HLA-DR molecules with L243 resulted in the phosphorylation of JNK-2 in gingival fibroblasts, and inhibition of JNK activity with specific JNK inhibitor dose-dependently suppressed RANTES production (3). However, in our present study, luteolin did not influence the phosphorylation status of JNK, although it inhibited RANTES production in fibroblasts. We therefore speculate that JNK is one of the downstream signaling molecules of HLA-DR-induced FAK-mediated signals in fibroblasts. In fact, JNK was previously reported to be one of the downstream signaling molecules of FAK (13, 14). Additionally, enhanced phosphorylation of Raf was observed in fibroblasts when stimulated with L243 by protein microarray analyses, which was also reported to locates downstream of FAK signals (15). Therefore, HLA-II-induced signals leading to RANTES production may be mediated through FAK, followed by JNK, while such signals leading to IL-6 and MCP-1 production could also be mediated through FAK, but not followed by JNK. In this case, other downstream signaling molecules of FAK might mediate such signals. HLA-II-mediated signaling pathways in gingival fibroblasts revealed so far including our current results are summarized in figure 6.

It is generally accepted that FAK plays an important role in cell migration. Therefore, we

tested the possibility that L243 may promote cell migration for fibroblasts. However, we did not observe apparent chemotactic responses of the cells against L243 as judged by chemotaxis assay (data not shown). Recently, however, ligation of HLA-DR on malignant melanoma cells by L243 has been reported to increase the phosphorylation of FAK, and proliferative activity of such cells was greatly impaired when the HLA-DR molecules were stimulated with L243 (16). Based on this observation and our current study, it is possible that HLA-DR molecules in both fibroblasts and epithelial cells associate with FAK. Also, HLA molecules belong to the immunoglobulin superfamily and some family members such as intercellular adhesion molecule-1 and CD146 have recently been reported to associate with FAK (17, 18). Interestingly, HLA-class I molecule also appeared to associate with FAK in endothelial cells (19, 20). Taken together, it is not surprising that HLA-II molecules on gingival fibroblasts structurally associate with FAK.

In this study, we found that luteolin suppressed FAK phosphorylation and inhibited HLA-II-induced cytokine production. This may partially account for the reported anti-oxidant and anti-inflammatory property of luteolin (21). As this reagent is one of the flavonoids contained in certain natural plants, this could be useful for clinically reducing unfavorable inflammatory responses leading to further tissue destruction in case of chronic periodontal disease and/or some autoimmune diseases such as rheumatoid arthritis.

In conclusion, this study demonstrated that HLA class II molecules on gingival fibroblasts associate with FAK in non-professional antigen presenting, and non-malignant normal cells such as gingival fibroblasts. Additionally, FAK appeared to transduce HLA-II-induced signals into the cells leading to the production of cytokines such as IL-6, MCP-1, and RANTES.

## Acknowledgments

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**Figure Legends**

**Figure 1. Ligation of HLA-DR molecule on fibroblasts with L243 induces enhanced phosphorylation of FAK, JNK, and Raf-1.**

The cells were stimulated either with L243 or control IgG. The cell lysates were obtained at indicated time period, labeled with fluorescent dye, and were subjected to protein microarray analyses as described in "Materials and methods" Data are expressed as fold increase of fluorescent intensity of test samples against that of control samples obtained from the cells stimulated with isotype-matched control IgG.

**Figure 2. FAK directly associates with HLA-DR molecules on fibroblasts.**

INF-γ-treated or -untreated fibroblasts were lysed, and the cell lysates were immunoprecipated with L243, followed by the western immunoblotting with anti-phospho FAK antibody (pY397). As control, IFN-γ-treated cells were similarly lysed, and the lysates were immunoprecipitatade with control IgG. FAK was highly detected in a fraction immunoprecipitated with L243 obtained from the cells stimulated with IFN-γ. Weak reactivity was also observed in a fraction immunoprecipitated with L243 obtained from IFN-γ-untreated cells.

**Figure 3. FAK is phosphorylated upon stimulation with L243.**

IFN-γ-treated cells were first stimulated either with L243 or control IgG for indicated time period. The cells were lysed, and the lysates were immunoprecipitated. 10min following stimulation with L243, larger amounts of phosphorylated form of FAK were observed. Lower panel indicates the total

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FAK proteins detected from total cell lysates in each sample.


**Figure 4. Luteolin, but not quercetin inhibits FAK phosphorylation (A), and the effects of luteolin on the phosphorylation of ERK, p38, JNK, MAP kinases, and c-jun (B).**

The effects of luteolin and quercetin on the phosphorylation of FAK were examined (A). Luteolin suppressed the phosphorylation of FAK in a dose dependent manner. The effects of luteolin on the phosphorylation of MAP kinases and c-jun were examined (B). The membrane was first probed with the antibody against ERK, p38, JNK, and c-jun, respectively. Then, the same membrane was re-probed with the antibody reacting with the phosphorylated form of each molecule following stripping. Luteolin did not suppress the phosphorylation of ERK, p38, JNK MAP kinases and c-jun.


**Figure 5. Luteolin suppresses HLA-DR-induced cytokine production in fibroblasts.**

The cells were first treated with or without indicated concentration of luteolin for 48h, and then stimulated either with L243 or control IgG. 16h following stimulation, culture supernatants were collected, and the concentration of MCP-1, IL-6, and RANTES was measured. Luteolin dose dependently suppressed MCP-1, IL-6, and RANTES production in fibroblasts stimulated with L243. Data are expressed as % production of each cytokine from the cells stimulated with L243 in the presence of luteolin against that from the cells cultured without luteolin. *p<0.05, and **<0.01 by student T-test.


**Figure 6. Schematic presentation of the HLA-II-mediated signals in gingival fibroblasts.**

Formation of HLA-II-antigenic peptide-T-cell receptor complex between fibroblasts and CD4^{+}

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T-cells activates FAK signaling pathways. FAK directly or indirectly activates JNK, resulting in the production of RANTES, while at the same time FAK phosphorylatiuon results in the production of MCP-1 and IL-6 *via* signaling molecules other than JNK.
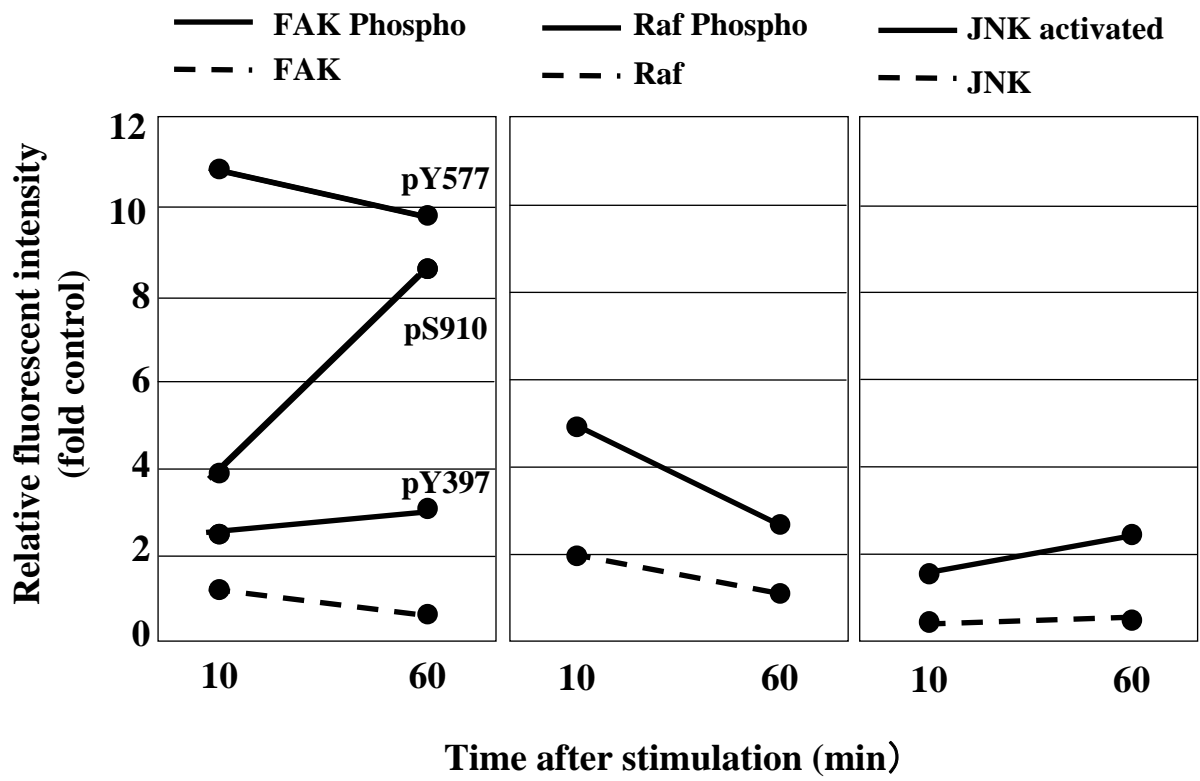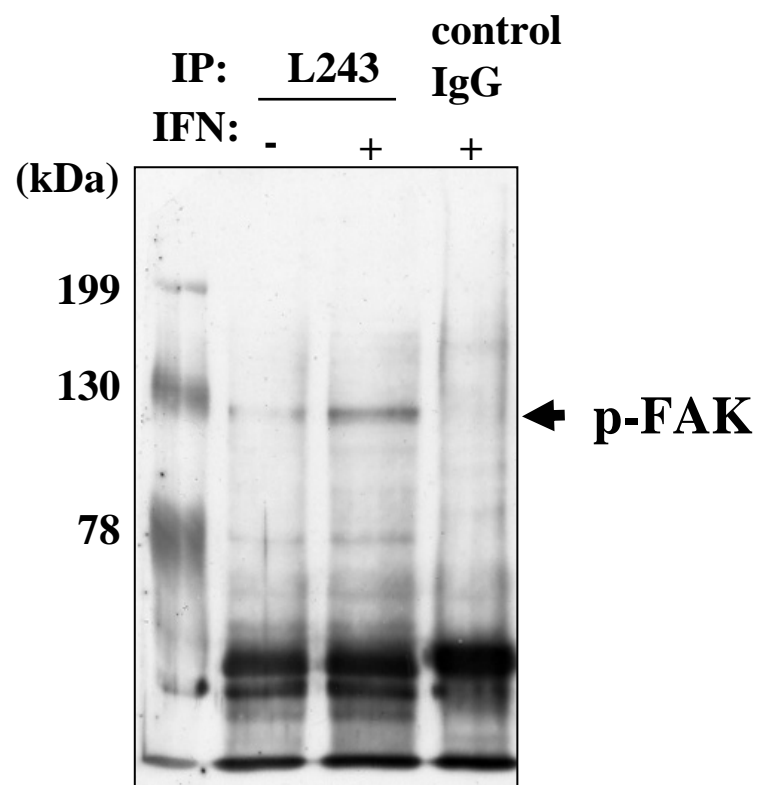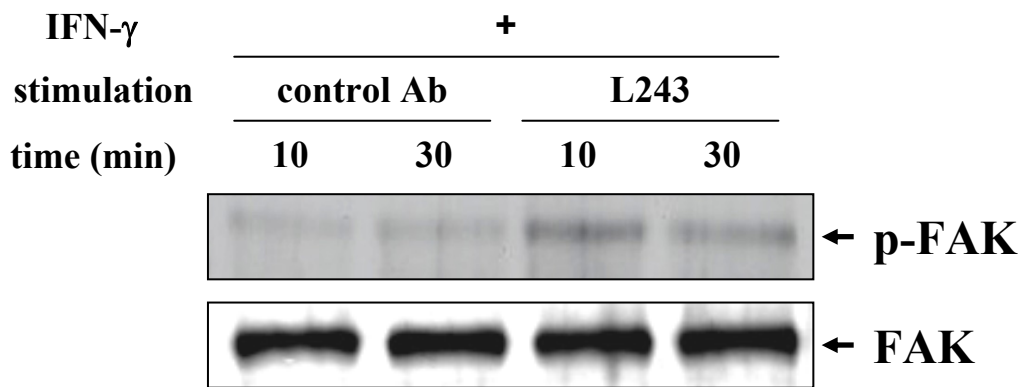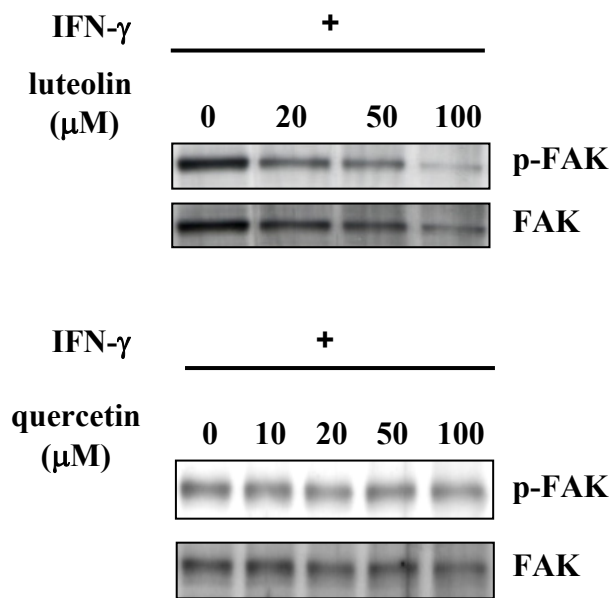
**Figure 1**

Figure 2

**IFN-γ**  +

| stimulation | control Ab | | L243 | |
|---|---|---|---|---|
| **time (min)** | **10** | **30** | **10** | **30** |

← **p-FAK**

← **FAK**

**Figure 3**

**(A)**

| IFN-γ | | + | | |
|---|---|---|---|---|
| luteolin (μM) | 0 | 20 | 50 | 100 |

p-FAK

FAK

| IFN-γ | | + | | | |
|---|---|---|---|---|---|
| quercetin (μM) | 0 | 10 | 20 | 50 | 100 |

p-FAK

FAK

**(B)**

| IFN-γ | | + | | | |
|---|---|---|---|---|---|
| luteolin (μM) | 0 | 10 | 20 | 50 | 100 |

p-ERK

ERK

p-p38

p38

p-JNK

JNK

p-c-Jun

c-Jun

**Figure 4**

Figure 5

CD4+ T-helper cells

T-cell receptor

HLA-II molecule

Antigenic peptide

FAK P

HGF

JNK P

? P

RANTES

MCP-1
IL-6

Figure 6