Functional Analysis of Fibroblastic Reticular Cells Derived from Mouse Lymph Node via Bidirectional Crosstalk with T Cells

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Fibroblastic reticular cells (FRCs) form the structural backbone of the T zone provide a guidance path for immigrating T cells in the lymph node (LN). FRCs may contribute directly to developing T-cell biology in the LN and allow analyses of fundamental aspects of FRC biology related to T cells. FRCs inhibited T-cell apoptosis, and FRC culture supernatants strongly induced the expression of Bcl-xL in T cells against doxorubicin. Coculture of FRC and T cells resulted in rearrangements of the actin cytoskeleton, as well as global changes in the morphology of the FRCs. In addition, when cocultured, the T cells adhered to the FRC monolayer, and the membrane intercellular adhesion molecule (ICAM)-1 was slightly increased by day-dependent manner. In contrast, the expression of soluble ICAM-1 was dramatically increased in a day-dependent manner. Several chemokines, such as CCL5, CXCL1, CXCL5, CXCL16, CCL8, CXCL13, and ICAM-1, and MMPs were expressed in FRCs sensed by tumor necrosis factor (TNF) families. Nuclear factor kappa B (NFκB)-RelA of the NFκB canonical pathway was translocated into FRC nuclear by TNFα. In contrast, p52 proteolyzed from p100, a counterpart of RelB of the noncanonical NFκB pathway, accumulated in the peripheral FRC nucleus by agonistic anti-LTβR antibody. In summary, we propose a model in which FRCs engage in bidirectional crosstalk to increase the efficiency of T-cell biology. This cooperative feedback loop may help to maintain tissue integrity and function during immune responses.

Key words : Agonistic anti-LTβR antibody, FRC, NFκB, T cell, TNFα

Introduction

Immune system is composed of primary lymphoid organs, secondary lymphoid organs (SLO), immune cells, and immune regulation factors. SLO, such as lymph nodes (LN), Peyer's patches and spleen, are critical for efficient start of adaptive immunity. The LN structure provides meeting places where lymphocytes coming from the blood encounter toxic pathogens and their products brought from infected region. LN is partitioned into cortical, paracortical and medullary areas [13, 18]. Moreover, LN contained hematopoietic immune cells and non-hematopoietic stromal cell. The cortical region contains follicles, which include B cells and follicular dendritic cells (FDC) [13], and the paracortical region, which includes T cells, interdigitating cells, dendritic

cells, macrophages, fibroblastic reticular cells (FRC). Additionally, non-hematopoietic stromal cells, such as endothelial cells, marginal reticular cell, integrin α7⁺ pericyte and undefined stroma provide a structural framework for the LN through functional signals like chemokines and cytokines [13, 18]. So far, stromal cells of SLOs have long been recognized as constructor and maintenance players of SLO tertiary architecture environments in each SLO partition [1, 6, 11]. FRC is thought to be highly specialized to the development of the lymphoid microenvironment. FRC secretes and ensheathes an extracellular matrix (ECM)-based reticular or conduit network within the T-cell zones of LNs [14, 17, 23, 24]. Within the LN, naive T cells are activated in the FRC-rich paracortical region. Accordingly, others were interested in understanding how the presence of FRCs might affect the progression of the T-cell response [16]. In vitro studies have demonstrated that ECM secretion of FRC is dramatically enhanced by the presence of T cells [24]. It suggests FRC may contribute directly to developing T-cell biology in LN. However, our understanding of these fibroblast subsets is still very limited, and much progress must have been made in defining various functions of FRC related to T cell

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immunity. Thus, it allows for us to investigate fundamental aspects of FRC biology between FRC and T cells.

Materials and Methods

Cell culture

FRC was established as described previously [14]. FRC was maintained in 10% fetal calf serum - dulbecco's modified eagle's medium (DMEM) and CD4⁺ EL4 T lymphoma was cultured in roswell park memorial institute medium (RPMI) 1640 supplement with 10% FCS supplemented with streptomycin and penicillin.

Antibody

The antibodies used for immunostaining or Western blot were as follows: as primary reagents, in biotin anti - intercellular adhesion molecule (ICAM)-1 antibody (Immunotech, Monrovia, CA, USA), rabbit anti-Bcl-xL antibody, rabbit anti-RelA (p65) antibody, monoclonal anti-p52 antibody (Santa Cruz Biotechnology, Dallas, Texas, USA), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody, as secondary reagents, fluorescein isothiocyanate (FITC) anti-mouse IgG antibody, FITC anti - rabbit IgG antibody (Jackson Immuno Research Laboratories, West Grove, PA, USA), horse radish peroxidase (HRP)-anti-rabbit IgG antibody, HRP-streptavidin (Molecular Probes, Eugene, OR, USA). Polyclonal goat agonistic anti-lymphotoxin- β receptor (LT β R) antibody against mouse LT β R extracellular domain and control IgG for cell stimulation were purchased from R&D Systems (Minneapolis, MN, USA).

T cell viability cocultured with FRC

Co-cultures were initiated by seeding FRCs (1×10^5 cells/dish) onto 6 ϕ cell culture dish for 12 h and cocultured with 8 μ g/ml Dox-treated CD4⁺ EL4 T cells (1×10^6 cells/dish) in RPMI1640 medium supplement with 10% FCS for 24 h. T cells were harvested after 24 h by pipetting the medium that contained the cells to rule out the presence of FRC, and then centrifuging at 1,500 rpm for 3 min. Following removal of the supernatants (sup), fresh medium was added to the T cell pellets, and then the suspended cell medium was transferred into 96-well plate. Cell viability was determined using CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI, US). Cells were placed in 96-well plastic culture plates, and then 100 μ l of 0.5 mg/ml tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyph-

enyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution was added to each well and incubated for 4 h at 37°C [2, 4, 22]. Formazan absorbance was read at 490 nm using a plate reader.

Western blot analysis

Cell lysate was extracted in 1% Nonidet P-40, 120 mM NaCl, 50 mM Tris (pH 8.0), 1 mM ethylenediaminetetraacetic acid, 0.1 mM phenylmethylsulfonylfluoride, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin. For preparing culture sup of FRC, FRC (5×10^6 cells) in 6 ϕ dish were cultured for 24 h. Then, culture sup were collected, filtered and stored until experiments. T cells (5×10^6 cells) were pretreated with FRC sup (100 ng/ml) and incubated for 1 h, and followed by further incubation for 24 h in the presence or absence of FRC sup and doxorubicin (Dox) (100 μ g/ml) (Sigma Chemical, St. Louis, MO, USA) for the expression degree of Bcl-xL. To investigate ICAM-1 expression in FRCs, FRC (5×10^6 cells) cocultured with T cells (5×10^6 cells) was grown on 6 ϕ dish plate for 1day (D), 2D, and 3D. After coculturing, T cells were removed out by pipetting the medium that contained the T cells to rule out the presence of FRC monolayer. The expression degree of membrane bound ICAM-1 (mICAM-1) and soluble ICAM-1 (sICAM-1) was detected by utilizing biotin-anti-ICAM-1 antibody and streptavidin-HRP. GAPDH was used to demonstrate equal loading of lysates. Protein concentration was determined by BCA method. After the samples were boiled, equal amounts of total lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were soaked in a blocking solution (5% skim milk and 0.2% Tween 20-PBS) for 1 h, and then incubated with polyclonal anti-LT β R antibody for 1 h. After being washed with Tween 20-PBS, membranes were incubated with HRP-conjugated anti-rabbit antibodies for 1 h. Specific bands were visualized by an ECL method (ECL⁺ Amersham Biosciences, Piscataway, NJ, USA).

RNA isolation

Total RNA was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA were extracted with chloroform (Sigma Chemical, St. Louis, MO, USA) and followed by ethanol precipitation. RNA was quantified using NanoDrop®ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). And RNA quality was assessed using Agilent RNA Nano 6000 LabChip kits

and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Microarray analysis

control and test RNAs, the synthesis of target cRNA probes and hybridization were performed using Agilent's Low RNA Input Linear Amplification kit (Agilent Technology, Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, each 1 µg total RNA and T7 promoter primer mix and incubated at 65°C for 10 min. cDNA master mix was prepared and added to the reaction mixer. The samples were incubated at 40°C for 2 h and then the RT and dsDNA synthesis was terminated by incubating at 65°C for 15 min. The transcription master mix was prepared as the manufacturer's protocol (4X Transcription buffer, 0.1 M DTT, NTP mix, 50% PEG, RNase-Out, Inorganic pyrophosphatase, T7-RNA polymerase, and Cyanine 3/5-CTP). Transcription of dsDNA was performed by adding the transcription master mix to the dsDNA reaction samples and incubating at 40°C for 2 h. After purification, labeling, fragmentation and assembling onto Agilent's Mouse Oligo Microarray (44K) of cRNA the arrays hybridized at 65°C for 17 h using Agilent Hybridization oven (Agilent Technology, Santa Clara, CA, USA). The hybridized microarrays were washed as the manufacturer's washing protocol (Agilent Technology, Santa Clara, CA, USA). The hybridized images were scanned using Agilent's DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technology, Santa Clara, CA, USA). All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3 (Agilent Technology, Santa Clara, CA, USA). The averages of normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control channel intensity.

Fluorescence staining

FRC monolayers on chamber slides were cocultured with CD4+EL4 T cells for 24 h. Cells were stained with rhodamine phalloidin (5 µg/ml) (Cell Signaling Technology, Danvers, MA, USA) for 45 min to observe changes in F-actin distribution after fixation and washing, and examined by Zeiss photomicroscope equipped for fluorescence microscopy. Nuclear was stained with 4'-6-Diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA). FRC monolayers grown on chamber slides were treated with tumor necrosis factor α (TNFα) (100 ng/ml), and agonistic anti-LTβR anti-

body (1 µg/ml) for 6 h in FRC. Cells were stained with anti-p65 antibody for canonical pathway and anti-p52 antibody for noncanonical pathway after fixation, permeabilization, block and washing. Immune fluorescence images of the nuclear translocation of nuclear factor kappa B (NFκB)-p65 and NFκB-p52 in response to 100 ng/ml TNFα and 1 µg/ml agonistic anti-LTβR antibody stimulation were examined by Zeiss photomicroscope equipped for fluorescence microscopy.

Results

FRC affected T cell survival against drug cytotoxicity

FDC affects B cell biology via the promotion of germinal center B cell survival by providing survival soluble factors including cytokines, and chemokines via tissue fluid [29]. Based on this phenomenon, we hypothesized that these FRC also have a regulatory effect on T cell biological behavior. Thus, *in vitro* co-culture of T cells in the presence of FRC monolayer made onto cell culture dish to investigate the influence of FRC in involvement with T cell biological actions was performed. First of all, we analyzed the survival or proliferation of T cells as one of T cell biological markers via MTS assay in this culture system with anti-cancer agent, Dox. The presence of Dox in this culture did not disturb the FRC viability pattern (Fig. 1). In situation of T cell survival with or without Dox, Dox affected less T cell survival during the 24 h of T cell culture (Fig. 1). Additional T cell culture in the presence or in the absence of FRC monolayer resulted in further T cellular proliferation (Fig. 1). In contrast, as shown in Fig. 1, the addition of Dox in FRC+T cell co-culture resulted in the decrease of T cell survival. Collectively, FRC affected T cell biology in terms of T cell survival, especially.

B cell lymphoma (Bcl)-xL was involved in survival of T cell against Dox

To understand the phenomenon underlying T cell survival enhanced in the presence or absence of FRC monolayer against Dox (Fig. 1), we thought that FRC secrete soluble survival factors into cell culture sup similar to the secretion of FRC into tissue fluid *in vivo*. Thus, it is possible to contain soluble survival factors in FRC culture sup. The enhancement of T cell survival was regulated by the expression of pro-survival Bcl-2 family protein, one of cell survival factors [19]. Thus, FRC culture sup was prepared as description in

Fig. 1. Effect of co-cultured FRC on drug cytotoxicity in T cells. Confluent FRC monolayer was made onto 6 ϕ dish and cocultured with 100 μ g/ml Dox-treated CD4⁺ EL4 T cells (1×10^6 cells/dish) in RPMI1640 medium supplement with 10% FCS for 24 h. After incubation, the unbound or bound T cells were removed by washing with PBS, and then, culture sup including the FRCs were collected and cell viability was measured by MTS assay. The data shown represent three independent experiments and are represented as the mean $\pm$ S.D.

material and method to investigate the effect of FRC sup about T cell survival with the involvement of the cell survival protein Bcl-xL. As shown in Fig. 2, FRC sup strongly induced increased expression of Bcl-xL in comparison with T cell as a negative control. Dox+FRC sup induced an increase in Bcl-xL expression, but only Dox-treated T cell decreased expression of Bcl-xL (Fig. 2). It suggests that FRC culture media contain soluble factors derived from FRC in influencing Bcl-xL expression.

Morphological alteration of FRC monolayer co-cultured with T cells

To directly probe the cell biological change such as FRC shape, T cell adhesion against FRC monolayer, FRC was co-cultured with T cells and observed under fluorescence microscope with rhodamine phalloidin staining. As shown in Fig. 3, this co-culture resulted in large-scale structural rearrangements in the actin cytoskeleton as well as global changes of cell shape. In addition, several T cells were attached to FRC (Fig. 3). Moreover, actin stress fibers were disrupted in co-cultured FRC and F-actin was diminished in FRC co-cultured with T cell (Fig. 3). This finding suggests that FRC physically interact with T cell and is involved in T cell biology.

Fig. 2. Effect of FRC sup on Bcl-xL expression in T cells. FRC (5×10^6 cells) was grown on 6 ϕ dish plate for 24 h. Culture sup of the FRCs were collected, filtrated, and stored until experiments. And protein concentration of sup was measured by BCA method. T cells (5×10^6 cells) were pretreated with FRC sup (100 ng/ml) and incubated for 1 h, and followed by further incubation for 24 h in the presence or absence of FRC sup and Dox (100 μ g/ml). The expression degree of Bcl-xL was detected by Western blot. GAPDH was used to demonstrate equal loading of lysates.

Expression pattern of adhesion molecular ICAM-1 in FRC co-cultured with T cell

ICAM-1 plays an important role in firm arrest to the cell-cell interaction via suitable ligands/ICAM-1. Activated leukocytes are adhered firmly, initiate transendothelial migration as well as the binding between ICAM-1 and lymphocyte function-associated antigen (LFA)-1 facilitates close contact between the antigen presenting cell and T cells for the immunological synapse formation [9, 25]. Moreover, ligation of LFA-1 and ICAM-1 is important in a variety of cellular events including Ag-specific T cell activation, T cell migration and antigen scanning in LN [12]. sICAM-1 serves as a counter-receptor or competitor for the LFA-1 for the cell physiological regulation [31]. As shown Fig 3, when FRC and T cell were co-cultured, T cell was adhered to FRC monolayer. Thus, we investigated the expression of ICAM-1 in FRC cocultured with T cell. FRC expressed mICAM-1 and sICAM-1. mICAM-1 slightly increased by day-dependent manner, however, sICAM-1 drastically increased by day-dependent manner (Fig. 4). These findings suggest that FRC has the ability to provide cooperative interaction and cell biological function to T cell.

Gene alteration in FRC by agonistic anti-LT β R antibody and TNF α

shown in Fig. 3 and 4, morphological change, stress fiber

Fig. 3. Alteration of cell shape and stress fibers in FRC using a fluorescence microscopy. FRC monolayers (arrow) on chamber slides were cocultured with CD4⁺ EL4 T cells (arrow head) for 24 h. Cells were stained with rodamine phalloidin for changes in F-actin distribution after fixation and washing, and examined by Zeiss photomicroscope equipped for fluorescence microscopy. Nuclear was stained with DAPI.

alteration, and increased sICAM-1 production were detected in FRC co-cultured with T cell. Accumulation of lymphocytes induces alteration of FRC such that they produce chemokines for further lymphocyte attraction via the secretion of TNF α (or related cytokines) and likely also via direct contacts mediated by adhesion molecules and LT α 1 β 2 [14]. TNF family cytokines including the membrane heterotrimeric form of LT α 1 β 2 are important for lymphoid tissue archi-

tecture and organogenesis [20]. Thus, it allows for us to examine the roles of these mediators in the gene alteration of FRC. We compared the panel of transcripts whose expression was altered in TNF α -treated and agonistic anti-LT β R antibody-treated FRC compared to normal FRC. Our results demonstrate that TNF α in FRC ($p < 0.05$) affected the expression of 723 identified transcripts regulated minimally by greater than or equal to a 2-fold change to total probe # 39,429. Of the identified regulated genes, 902 were down-regulated. In addition, agonistic anti-LT β R antibody affected the expression of 391 transcripts regulated minimally by greater than or equal to a 2-fold change to total probe # 39,429 and 496 genes were down-regulated. Chemokines lead to leukocyte homing and are implicated actively in immune reaction. Metallo matrix proteinase (MMP) functions the remodel of ECM and digests membrane bound chemokines or adhesion molecules to facilitate the function of T lymphocyte in LN. Thus, we analyzed the expression pattern of these factors in gene chip results. Of the regulated genes, the following was known to affect leukocyte homing and T cell biological function (Table 1). Soluble factors described in table 1, 2 were related to T cell response. These results indicate that FRC was tightly connected with T cell rather than B cell.

Fig. 4. Effect of T cell coculture on ICAM-1 expression in FRCs. FRC (5×10^6 cells) was grown on 6 ϕ dish plate for indicated time. T cells (5×10^6 cells) were coculture with FRC. After coculture, T cells were removed out by washing with PBS. Cell was lysed with RIPA buffer and protein concentration of FRC lysate was measured by BCA method. The expression degree of mICAM-1 and sICAM-1 was detected by Western blot. GAPDH was used to demonstrate equal loading of lysates.

Table 1. Chemokine and cytokine transcripts modulated in FRCs by TNF α exposure

Systematic	GeneSymbol	Normalization*	Regulation*	Remark
A_51_P184484	Mmp13	93.943954	UP	T cell mediated tissue remodelling
A_51_P255699	Mmp3	51.54251	UP	Extracellular matrix remodeling involved in CD4+ T lymphocyte migration
A_52_P638459	Ccl5	28.396307	UP	Directional movement induction of activated CXCR3 and CCR5 bearing T cells
A_51_P363187	Cxcl1	24.750534	UP	Effector CD8 T cell recruitment
A_51_P120830	Mmp10	20.856514	UP	Degradation of various components of the extracellular matrix; Activation of ICAM-1/LFA-1-mediated intercellular contact
A_55_P1990032	Cxcl5	16.097998	UP	Neutrophil attractant
A_52_P99888	Cxcl16	14.880662	UP	Transmembrane chemokine; CD8+ T cell attraction and adhesion in LN
A_55_P2016462	Cxcl10	8.553003	UP	Chemotaxis of CXCR3+ Th1 and Tc1
A_51_P464703	Ccl8	7.2886925	UP	CD4(+) T lymphocytes homing
A_52_P232813	Cxcl3	5.551653	UP	Inhibition of T cell proliferation
A_52_P613241	Icam1	5.525103	UP	Cell adhesion and chemotaxis of T cell
A_55_P1961451	Adamts7	5.5199876	UP	Extracellular matrix (ECM)-degrading proteinases in VSMC migration
A_51_P231320	Mmp8	5.5022626	UP	Collagenases, macrophage chemoattractant, Inflammation
A_52_P507214	Mmp9	4.4453382	UP	Up-regulated antigen-specific T-cell responses
A_52_P52618	Csf2rb	4.142702	UP	Regulation of Th2 immunity
A_51_P331752	Ccl11	3.4064958	UP	Regulation of Th2 environment
A_51_P286737	Ccl2	2.0796976	UP	monocyte chemoattractant protein
A_55_P2068723	Ccl27a	0.3715961	DOWN	inflammatory T cell response; Chemoattractant for Th-2 type T-cells
A_51_P196925	Cx3cl1	0.3694497	DOWN	Integrin-independent leukocyte adhesion
A_51_P114462	Ccl17	0.2876406	DOWN	Th2-attracting chemokines

*Normalization was the ratio between TNF α treated sample value vs control value.

*Regulation was decided by normalized values (*i.e.*, values above 2 fold) as UP regulation and (values below 0.5 fold) as DOWN regulation.

Activation of NF κ B pathway in TNF α -treated and agonistic anti-LT β R antibody-treated FRC

mediators such as chemokines and MMPs modulate immune system through activation of the transcription factor NF κ B pathway [3, 27]. Furthermore, we investigated the expression of several chemokines and MMPs in FRC treated with TNF α and agonistic anti-LT β R antibody (Table 1, 2). TNF α elicits triggers signal cascade via its receptors (TNFR1 and TNFR2) toward RelA (p65)-p50 complex (p105) (canonical pathway). In contrast, LT α 1 β 2 activates a distinct signaling pathway toward RelB - p52 (p100) complex (noncanonical pathway) via LT β R [5, 30]. Thus, NF κ B activation mechanism was investigated via immune-staining in FRC treated with TNF α and agonistic anti-LT β R antibody. In resting cells, NF κ B was located in the cytoplasm, how-

ever, NF κ B was activated and translocated into nuclear when TNF α was treated in FRC (Fig. 5A). In contrast, strikingly, we observed that p52 proteolyzed from p100, counter-partner of RelB of non-canonical NF κ B pathway, was accumulated in peripheral nucleus when agonistic anti-LT β R antibody was treated in FRC (Fig. 5B). The fact that FRC could transcribe chemokines and MMPs despite their inability to translocate p52 suggested that other NF κ B family members may regulate LT α 1 β 2-LT β R signaling pathway.

Discussion

The three dimensional architecture of LN is thought to optimize antigen correction and presentation to naive circulating lymphocytes and allow for clonal selection and rapid

Table 2. Chemokine and cytokine transcripts modulated in FRCs by agonistic anti LT β R antibody exposure

Systematic	GeneSymbol	Normalization [†]	Regulation*	Remark
A_52_P99888	Cxcl16	14.948073	UP	T cell migration
A_52_P507214	Mmp9	9.049398	UP	Up-regulated antigen-specific T-cell responses
A_51_P120830	Mmp10	8.330407	UP	Degradation of various components of the extracellular matrix; Activation of ICAM-1/LFA-1-mediated intercellular contact
A_51_P255699	Mmp3	5.987079	UP	Extracellular matrix remodeling involved in CD4 ⁺ T lymphocyte migration
A_51_P331752	Ccl11	5.610748	UP	Angiogenesis, Migration of activated T cell
A_52_P52618	Csf2rb	5.2937036	UP	Regulation of Th2 immunity
A_51_P464703	Ccl8	4.5239396	UP	Inflammation, Development of DC
A_52_P638459	Ccl5	4.4313483	UP	T cell activation
A_55_P2016462	Cxcl10	3.1940744	UP	Chronic inflammation, monocyte, T cell, NK cell chemotaxis
A_51_P363187	Cxcl1	2.6172688	UP	Neutrophil migration, T cell migration
A_55_P1990032	Cxcl5	2.500141	UP	Hematopoietic stem cell migration, Leukocyte recruitment, Neutrophil homeostasis

[†]Normalization was the ratio between TNF α treated sample value vs control value.

*Regulation was decided by normalized values (*i.e.*, values above 2 fold) as UP regulation.

Fig. 5. Involvement of canonical and noncanonical pathway of NF κ B in FRC. FRC monolayers on chamber slides were cultured. Cells were stained with anti-p65 antibody for canonical pathway (A) and anti-p52 antibody for noncanonical pathway (B) after fixation, permeabilization, block and washing. Immune fluorescence images of the nuclear translocation of NF κ B-p65 and NF κ B-p52 in response to 100 ng/ml TNF α and 1 μ g/ml agonistic anti-LT β R antibody stimulation for 6 h in FRC were examined by examined by Zeiss photomicroscope equipped for fluorescence microscopy.

cell proliferation of antigen-specific T and cognated B lymphocytes, as well as their differentiation into effector cells. To make these processes more efficient LN are zoned into structurally distinct microparts: The B zone (follicle or outer cortex), T zone (paracortex), medulla zone and entry and exit zones. In this study, we investigated the directional cell biological involvement between FRC and T cell for the cross-talk, each other. FRC resides in T zones and constitutively secrete the chemokines to attract and retain T cells [21, 28]. So far, main role of FRC has been addressed to make network, which acts as physical migration road during T cell migration [13, 21]. While FRC form the structural backbone of the T cell rich zones in LN, but also actively influ-

ence the T cell biological situation. FRC are the major source of constitutive IL-7 expression in LN and thereby regulate the fitness, survival and homeostasis of naive recirculating T cells [7, 10]. As shown in Fig. 1, FRC co-cultured with T cells inhibited T cell apoptosis against Dox. Thus, the effect of T cell anti-apoptosis against Dox may be related to action of IL-7 secreted from FRC. We demonstrated the expression of *Bcl-xL* was significantly increased in T cells treated with FRC sup (Fig. 2). The expression of *Bcl-2* family genes is important for the survival of T cells [19]. Recently, T cells in LN were significantly decreased in signal transducer and activator of transcription 3 (Stat3)-deficient mice [15]. Expression of the cell survival genes *Bcl-2* and *BclxL* was

markedly decreased in the T cell - specific Stat3-deleted mice [15]. Stat3, as a key regulator of Bcl-2 family genes, promotes the proliferation of activated T cells and regulates the lifespan of T lymphocytes. Stat 3 regulates T cell homeostasis and survival by maintaining the required naïve T-cell population in LN [15]. It suggests that Stat3 helps to maintain the T-cell pool in the resting condition by promoting the expression of *Bcl-2* family genes. Therefore, cell survival factors such as survivin, Stat3 and cleaved caspase-3 protein levels must be checked by further study. Overnight incubation of FRC with T cells led to a morphological change and disruption of stress fiber. It seems likely that unknown factors produced by T cells are sensed by FRC leading to alteration induction. The signals exchanged between FRC and T cells leading to alteration induction is unclear. However, within the first 20 h upon TCR triggering, T cells produce a first wave of TNF α cytokines. It seems likely that TNF α produced by T cells are sensed by FRC leading to change induction. We detected that FRC cocultured with T cell expressed mICAM-1 and sICAM-1 day-dependent manner (Fig. 4). For the firm and physical attachment for the interaction between T cells and other cells, the contact between T cells and other cells is strengthened by additional interactions between the integrin LFA-1 on the T cell surface and the ICAM-1 on other cell surface [26]. sICAM-1 is constitutively expressed or is inducible on the cell surface of different cell lines [31]. sICAM-1 binding to LFA is capable of inhibiting lymphocyte attachment to endothelial cells [31]. These findings suggest the concept that FRC has the ability to provide cooperative response for the cell biological role. Detailed functions of mICAM-1 and sICAM-1 between FRC and T cell remain to be elucidated. Members of TNF family cytokines including the membrane heterotrimeric form of LT α 1 β 2 of T cell are important for lymphoid tissue architecture and organogenesis [14]. Thus, we examined the roles of these mediators in the FRC gene alteration. Stimulation of FRCs with TNF α and commercially well-known agonistic anti-LT β R antibody raised against the extracellular part of LT β R mimic receptor are able to induce downstream signals [14]. Many cytokines and MMPs were produced by FRC in table 1, 2, and the FRC-derived these signals are thought to stimulate T cell behavior onto FRC monolayer. TNF α uses its receptors with TNFR1 and TNFR2 toward RelA-p50 (NF κ B-canonical pathway). In contrast, LT α 1 β 2 triggers a distinct signaling pathway toward RelB-p52 (p100) (NF κ B-noncanonical pathway) via LT β R [21, 28]. NF κ B has multiple roles in the im-

mune system, both in the development, differentiation, and survival regulation of its effector cells, as well as in the dynamic regulation of local and systemic immune activity. TNF α was able to trigger the nuclear translocation of RelA, however, agonistic anti-LT β R antibody was able to trigger the peripheral nuclear translocation of p52, RelB counterpart (Fig. 5). Five mammalian NF κ B genes give rise to five transcription factor proteins, RelA, cRel, RelB, p50, and p52. Five NF κ B polypeptides can form 15 transcription factors through homo- and hetero-dimerization [8]. In a given cell, a subset of dimers may be present, depending on the cell stage, and conditioning by additional cues in case of agonistic anti-LT β R antibody. In this research we discussed our findings which suggest that FRC in T zone are not simple scaffolding cells, but actively influence T cell biology.

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초록 : T세포와 양방향 작용을 통한 마우스 림프절로부터 분리된 fibroblastic reticular cell의 기능적 분석

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Fibroblastic reticular cells (FRC)는 림프절 T세포 지역에 구조적 골격 형성을 하며 유입 T 세포의 안내길을 제공한다. FRC는 림프절에서 T세포 생물학 발달에 기여한다. 따라서, 이것이 FRC와 T세포 사이에서 FRC의 세포생물학적 근본 기능을 알아보게 하였다. FRC 배양 상등액은 T세포 사멸을 저해하였다. FRC 상등액은 doxorubicin에 대하여 T세포에 Bcl-xL의 발현을 증가시켰다. FRC와 T세포의 공배양은 FRC의 액틴 골격의 변화와 형태적 변화를 유도하였다. 또한, FRC와 T세포의 공배양은 T 세포가 FRC 단일층에 부착하는 결과를 유도하였고 막결합형 intercellular adhesion molecule (ICAM)-1 단백질의 발현은 약간 증가한 반면 용해성 ICAM-1 (sICAM-1) 발현은 시간 의존적으로 드라마틱하게 증가하였다. FRC는 T세포에 의해 분비되는 tumor necrosis factor (TNF) 패밀리에 의해 CCL5, CXCL1, CXCL5, CXCL16, CCL8, CXCL13와 같은 케모카인들과 ICAM-1 그리고 MMPs의 발현량을 증가시켰다. TNF α 가 FRC에 처리 되었을때 NF κ B canonical pathway의 RelA는 핵으로 전좌 되었지만, agonistic anti-LT β R antibody로 처리된 FRC에서 non-canonical NF κ B pathway의 RelB의 카운터 파트너인 p100의 분해산물 p52는 핵주변부로 축적되었다. 결론적으로 FRC는 FRC와 T세포 양방향 협력을 통해 T세포 생물학적 기능을 증진한다. 이러한 상호협력 관계는 면역반응 동안 조직의 통합성과 기능을 유지하는데 도움을 줄 것으로 사료된다.