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Anti-tumor Effect of Activated Canine B Cells with Interleukin-21 and Anti-B cell Receptor

인터루킨-21 및 항-B 세포 수용체를 이용하여
활성화된 개 B 세포의 항종양 효과

2023년 8월

서울대학교 대학원
수의학과 임상수의학(수의내과학) 전공
서 소 영

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이 논문을 수의학 석사 학위논문으로 제출함

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Abstract

Anti-tumor Effect of Activated Canine B Cells with Interleukin-21 and Anti-B cell Receptor

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Recently, novel studies on the pivotal role of B cells in tumor-microenvironment and anti-tumor immunity have been conducted. Additionally, Interleukin-21 (IL-21) and anti-B cell receptor (BCR) have been reported to stimulate B cells to secrete granzyme B, which is known to exhibit cytotoxic effects on tumor cells. However, the direct anti-tumor effect of B cells is not yet fully understood in the veterinary field. This study is the first attempt in veterinary medicine to identify the immediate effect of B cells on tumor suppression and the underlying mechanisms involved. In this study, canine B cells were isolated from peripheral blood and activated with IL-21 and anti-B cell receptor (BCR). The canine leukemia cell line GL-1 was co-cultured with B cells, and the anti-tumor effect was confirmed by assessing the changes in cell viability and apoptotic ratio.

When B cells were activated with IL-21 and anti-BCR, the secretion of

granzyme B and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) increased. Simultaneously, the viability of GL-1 cells decreased, and the apoptotic ratio increased, particularly when co-cultured with activated B cells.

The results demonstrated the direct anti-tumor effect of granzyme B-and TRAIL and its enhanced potential to inhibit tumor cells after activation with IL-21 and anti-BCR. This study may be a preliminary study dealing with immunomodulation in the canine tumor micro-environment, and further in vivo studies and clinical trials are expected.

Keywords: Canine, B cell, Anti-tumor effect, Interleukin 21, Anti-BCR, Granzyme B, TRAIL, Apoptosis

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1. Introduction

The immune system and alteration of the tumor's microenvironment are critical for the initiation and progression of cancer [1]. Genetic and epigenetic changes that sustain proliferative signals, escape growth suppressors, and trigger immunological responses cause cancer to develop over time [2]. The role of the immune system in cancer development has led to a breakthrough in immunotherapeutic strategies and has been validated for several types of cancer treatments, including cytokine therapies and anti-cancer vaccines [3].

Conventionally, only cytotoxic T cells and natural killer (NK) cells produce cytokines, upregulate inhibitory molecules, and directly kill tumor cells [3]. However, recently, the direct and indirect roles of B cells in anti-tumor responses have been appreciated [4-6]. B cells have diverse functions ranging from producing antibodies to presenting antigens [7]. In a tumor-specific microenvironment, B cells exhibit pro-tumoral effects by expressing cytokines such as interleukin 10 (IL-10), interleukin 35 (IL-35), and transforming growth factor-β (TGF-β) or inhibitory molecules such as Programmed Cell Death Protein 1 (PD-1) and Programmed Cell Death Ligand 1 (PD-L1) [8-10]. Conversely, B cells also suppress tumor progression by releasing tumor-specific antibodies and effector cytokines, presenting antigens to effector cells, and expressing cytotoxic molecules [11]. Tumor-infiltrating B cells are known to secrete antibodies that are against tumor-specific antigens and mediate tumor lysis by antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) [12]. Furthermore, B cells also promote T cell

responses by presenting antigens to helper T cells or directly activating cytotoxic T cells. Moreover, B cells directly kill tumor cells via granzyme B, perforin, and TRAIL [13].

Granzymes, serine proteases stored in the cytotoxic granules of immune cells, are released to eliminate infected transformed target cells. Granzymes are of numerous types, with granzymes A and B being the most abundant forms [14]. Granzyme B, which cleaves aspartate, activates the apoptotic pathway by proteolyzing multiple caspases or cleaving some key caspase substrates [15], also proceed directly to the nucleus and cleaves multiple nuclear substrates via an unknown pathway [16]. Several studies suggest that granzyme-secreting B cells function in early cell-mediated immune responses associated with inflammation and cancer [17, 18]. Granzyme-positive B cells infiltrate various solid tumors [19-21]. While the tumor-infiltrated B cells are associated with tumor progression, granzyme B-secreting B cells are known to contribute to the anti-tumor immune response [17].

TRAIL is a member of the tumor necrosis factor (TNF) family that triggers apoptosis by binding to TRAIL receptors [22]. TRAIL is expressed in the membrane or soluble form in numerous tissues and cells, but mainly on the surface of immune cells, where it acts as an inducer of apoptosis of target cells [23]. TRAIL binds to the Death Receptor 4/5 (DR4/5) to initiate the extrinsic pathway of cell death, which mainly involves the caspase pathway. The intrinsic pathway, which starts in the mitochondria, activates caspase proteins and the p53 pathway to initiate cell death [24]. TRAIL induces apoptosis in various cancer cells without affecting normal cells [25-27]. Cytotoxicity against tumor cells increases when B cells express high levels of TRAIL [28]. Thus, TRAIL has been considered a target for anti-tumor therapy

[29].

IL-21 and anti-BCR have been reported to play pivotal roles in determining B-cell activity. IL-21 proliferates B cells and promotes immunoglobulin production [30]. IL-21 also promotes the differentiation of B cells into long-lived and antibody-secreting plasma cells and stimulates B cells to express granzyme B in its active form [17]. A study showed that B cells activated properly with both IL-21 and anti-BCR increased granzyme B production and subsequently exerted a significant cytotoxic effect on tumor cells [31].

This study aimed to evaluate the anti-tumor effects of canine B cells activated with IL-21 and anti-BCR. Only a few studies have been done on the anti-cancer immunity of B cells. To our knowledge, this is the first research on canine B cells. We examined the interaction between canine leukemia cell line (GL-1) and canine B cells isolated from peripheral blood that was activated by IL-21 and anti-BCR. The ability of B cells to express granzyme B and TRAIL was evaluated, and their anti-tumor effect on GL-1 cells was assessed using cell viability and apoptosis assays.

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2. Materials and Methods

2.1. Canine leukemia B cell line and cell culture

This Prof. YukoGoto-Koshino (Tokyo University, Japan) provided the well-characterized canine leukemia B cell line GL-1. The GL-1 cell line was maintained in Roswell Park Memorial Institute (RPMI)-1640 (Welgene, Gyeong-San, Republic of Korea) medium supplemented with 10% fetal bovine serum (FBS, Gibco®, Paisley, Scotland), 1% solution of 10,000 units/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 µg/ml streptomycin (Sigma-Aldrich) (PS). The cell lines were cultured at 37 ˚C and 5% CO2. The media were added every other day, and cells were sub-cultured until their confluency reached 80%.

2.2. Isolation of canine B cells

The Canine Cluster of Differentiation 21 positive ($CD21^{+}$) B cells were isolated from canine peripheral blood mononuclear cells (cPBMCs). With the owners' permission, blood samples were collected from healthy dogs visiting the Seoul National University Veterinary Medical Teaching Hospital and participating in a blood donation program. This study was approved by the Institutional Animal Care and Use Committee of Seoul National University (protocol no. SNU-221102-1). Using the remaining white blood cell layer, Ficoll-Paque PREMIUM (Cytiva Life Sciences™, Marlborough, MA, USA) was used to isolate cPBMC, following the

manufacturer's instructions. The white blood cell layer was mixed with an equal amount of Dulbecco's phosphate-buffered saline (DPBS) solution (Welgene) and placed onto Ficoll-Paque PREMIUM according to the manufacturer's guidelines. After centrifugation at 480 ×g for 30 min at room temperature (RT) with a low brake, red blood cell lysis buffer (Sigma-Aldrich) was applied to the separated mononuclear cell layer at RT for 5 min. It was then washed with DPBS and centrifuged at 780 ×g for 10 min. CD21 antibody (dilution, 1:100; Invitrogen, Carlsbad, CA, USA) and anti-mouse immunoglobulin G microbeads (Miltenyi Biotec, Auburn, CA, USA) with MACS (Miltenyi Biotec) were utilized to isolate canine B cells according to the manufacturer's instructions. MACS separation buffer (Miltenyi Biotec) was used. The canine CD21 antibody was added to cPBMC and incubated at 4 ˚C for 1 h. $CD21^+$ B cells were collected by centrifuging at 780 ×g for 10 min. For magnetic labeling, 20 μl of anti-mouse IgG microbeads and 80 μl of MACS separation buffer were added to the $CD21^+$ B cells, which were incubated for 15 min. Magnetically labeled $CD21^+$ B cells were obtained after centrifugation at 780 ×g for 10 min. $CD21^+$ B cells were added to the LS column for isolation after washing it with 3 ml of MACS separation buffer. Finally, $CD21^+$ B cells were collected in a total of 6 ml of MACS separation buffer after an additional wash with 3 ml of MACS separation buffer.

2.3. B cell activation with anti-BCR and IL-21

The obtained $CD21^+$ B cells were stimulated with anti-BCR (Jackson ImmunoResearch Inc., West Grove, PA, USA) and IL-21 (Biotechne, MN, USA).

Cells were seeded at a density of 2×10^5 cells/ml in 6 well plates (SPL Life Science, Po-Cheon, Republic of Korea). Anti-BCR was added at a working concentration of 10 ug/ml, and IL-21 was added at a working concentration of 100 ng/ml to the activation group. The cells were incubated at 37 ˚C in 5% CO2 for 16 h for the activation.

2.4. B cell phenotyping analyses

After IL-21 and anti-BCR stimulation, B cells were fixed with ice-cold 70% alcohol overnight at 4 ℃. After fixation, B cells were washed thrice with ice-cold DPBS. They were then incubated for 30 min at 4 °C with antibodies against FITC-conjugated CD86 (1:100; BioLegend, San Diego, CA, USA) and phycoerythrin-conjugated CD80 (1:100; BioLegend). After three washes with ice-cold DPBS, flow cytometry was conducted within 1 h using FACS Aria II (BD Biosciences, Franklin Lakes, NJ, USA), and the results were analyzed using FlowJo v10.8.1 software (BD Biosciences).

2.5. Co-culture system

Approximately $1x10^6$ GL-1 cells were seeded in each well of 6 well plates (SPL). GL-1 cells were divided into three groups: negative control group without B cells, another group co-cultured with naïve B cells, and one group co-cultured with activated B cells. The 0.4-μm pore size inserts were placed in each well, and the insert was preconditioned with the media for an hour. Then, naïve and activated B

cells were seeded onto the insert at a density of 5×10^5 cells/well, at a ratio of 2:1 (GL-1:B cell). All groups of cells were incubated at 37 ºC in 5% CO2 for 24 h. The media of each insert was harvested for enzyme-linked immunosorbent assay (ELISA) of Granzyme B and TRAIL, and GL-1 cells were evaluated for cell viability and harvested for further apoptosis assays.

2.6. Enzyme-linked immunosorbent assay

The media obtained from naïve and activated B cells were centrifuged at 300 ×g for 5 min. The supernatant layer was separated and stored at - 20 ℃. The Canine Granzyme B ELISA kit (Mybiosource.com, CA, USA) and Canine Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) ELISA Kit (Reddot Biotech, TX, USA) were used. All ELISA procedures were performed according to manufacturer's instructions.

2.7. Cell viability assay

The Cell Counting Kit-8 (CCK-8) (Dong-in Biotech, Seoul, Republic of Korea) assay was used to confirm the anti-tumor effect of B cells. GL-1 cells were seeded at a density of 1 $x10^6$ cells/well in 6 well plates, and B cells were added at a ratio of 2:1 (GL-1:B cell). After incubation for 24 h, the CCK assay was performed according to the manufacturer’s instructions. Absorbance at 450 nm wavelength was determined using a spectrophotometer (Epoch Microplate Spectrophotometer; BioTek Instruments, Winooski, VT, USA).

2.8. Apoptosis analysis

Three groups of GL-1 cells, with or without B cell activation, were harvested and washed three times with cold DPBS. An Annexin V-FITC apoptosis detection kit (Enzo Life Science, Farmingdale, NY, USA) was used to detect apoptotic cells according to the manufacturer's protocol. The cells were resuspended in 1x binding buffer and stained with Annexin V-FITC and PI (dilution 1:20) for 15 min at RT in the dark. Flow cytometry was performed within 1 h using FACS Aria II (BD Biosciences), and the results were analyzed using FlowJo v10.8.1 software (BD Biosciences).

2.9. Statistical analysis

For statistical analyses, GraphPad Prism (version 9.3.1) software (GraphPad Software, San Diego, CA, USA) was used. Data were analyzed using the two-tailed unpaired Student's t-test and one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. The data are presented as mean value ± standard deviation. Statistical significance was set at P-value <0.05. All experiments were replicated at least three times.

3. Results

3.1. B cell phenotyping

In this study, cPBMC were obtained using Ficoll solution, and $CD21^+$ B cells were isolated using the MACS positive selection kit. In peripheral blood, CD21 is expressed in mature B cells and plays an important role in B cell activation and maturation. To confirm whether the isolated cells were genuine B cells, a flow cytometric assessment of CD80 and CD86 was performed [32, 33]. The results showed the expression of CD80 and CD86 on the surface of isolated cPBMC by flow cytometry; thus, the isolation of B cells from cPBMC was confirmed (Fig. 1a)[1].

3.2. B cell activation with IL-21 and anti-BCR

In accordance with previous studies, isolated human B cells were stimulated with IL-21 and anti-BCR to increase the anti-tumor effects in breast cancer cells [31]. After the activation of $CD21^+$ B cells with IL-21 and anti-BCR (Fig 2A), B cells were assessed by flow cytometry to determine whether the isolated and activated cells were genuine B cells. Both CD80 and CD86 were expressed in the stimulated cells, confirming that they were B cells (Fig. 1b)[2]. The experiments were replicated three times.

[1]In collaboration with Ga-Hyun Lim
[2]In collaboration with Ga-Hyun Lim

After activation, B cells and GL-1 cells were co-cultured using a transwell assay (Fig. 2b). The supernatant was acquired and stored to verify alterations in the levels of granzyme B and TRAIL secretion. ELISA analyzed the levels of secreted granzyme B and TRAIL. The results showed that granzyme B levels in $CD21^+$ B cells were 0.3360 ± 0.04088 pg/ml, whereas those in activated $CD21^+$ B cells were 0.4510 ± 0.09614 pg/ml. Therefore, we confirmed a statistically significant increase in granzyme B secretion in activated B cells (Fig. 3a, $p<0.01$)[3]. The level of TRAIL in $CD21^+$ B cells was 231.2 ± 1.261 pg/ml, and that in activated $CD21^+$ B cells was 708.3 ± 60.15 pg/ml. Notably, the secretion in activated B cells was higher than that of $CD21^+$ B cells (Fig. 3b, $p<0.0001$)[4].

3.3. Cytotoxic effect of activated B cell in canine leukemia B cell line

The cytotoxicity of activated B cells was confirmed by assessing the viability of the GL-1 cells. The Viability of naïve GL-1 cells without any activation, GL-1 cells co-cultured with $CD21^+$ B cells, and GL-1 cells co-cultured with $CD21^+$ B cells activated with IL-21 and anti-BCR were assessed using the CCK-8 assay. The results showed that the viability of GL-1 cells was significantly reduced when co-cultured with both $CD21^+$ B cells and activated $CD21^+$ B cells (Fig. 4)[5]. The viability of GL-1 cells co-cultured with $CD21^+$ B cells and activated $CD21^+$ B cells was 86.04 ± 5.683 % and 75.82 ± 7.967 %, respectively. Thus, a significant reduction

[3] In collaboration with Su-Min Park

[4] In collaboration with Su-Min Park

[5] In collaboration with Su-Min Park

in the viability of GL-1 cells was observed when co-cultured with B cells that were activated with IL-21 and anti-BCR (control vs. $CD21^+$ B cell, $p<0.05$; $CD21^+$ vs. Activated $CD21^+$ B cell, $p< 0.01$; control vs. Activated $CD21^+$ B cell $p<0.0001$).

3.4. Apoptosis effect of activated B cell in canine leukemia B cell line

Annexin V/PI staining and flow cytometry were performed to confirm the apoptotic effect of activated B cells on GL-1 cells. The results showed that apoptosis increased in GL-1 cells after co-culture with activated B cells (Fig. 5a, 5b)[6]. The ratio of annexin V positive cells represents the percentage of apoptotic cells. The apoptotic ratios for the negative control, GL-1 cells co-cultured with $CD21^+$ B cells were 2.793 ± 0.8211 % and 2.490 ± 0.3030 %, respectively. Compared with them, GL-1 cells co-cultured with B cells that were activated with IL-21 and anti-BCR showed an apoptotic ratio of 5.830 ± 1.059 %, which indicated a statistically significant increase in apoptotic ratio ($CD21^+$ vs. Activated $CD21^+$ B cell, $p< 0.01$; Control vs. Activated $CD21^+$ B cell, $p<0.001$). When these results and the anti-tumor mechanisms of granzyme B and TRAIL described previously are combined, the increase in the apoptotic ratio may be promoted by the cytotoxicity of granzyme B and TRAIL secreted by B cells.

[6] In collaboration with Ga-Hyun Lim

4. Discussion

Tumor-infiltrating B cells contribute to anti-tumor immunity through multiple mechanisms. The major roles of B cells in the tumor microenvironment are to present tumor-specific antigens to effector cells and secrete numerous cytokines to activate helper and cytotoxic T cells. Their novel roles in triggering the complement cascade, mediating phagocytosis of tumor cells, and inducing NK cells without antibody intervention have also been elucidated [32, 34]. In recent studies, the novel anti-tumor roles of B cells have been studied. These studies found that B cells directly contribute to tumor killing by secreting granzyme B and TRAIL [17, 31, 35]. Additionally, IL-21 and anti-BCR have also been reported to exert anti-tumor effects in leukemia and breast cancer cell lines by secreting granzyme B [6, 31, 36]. With the independence of the classical T-cell pathway, IL-21 stimulates B cells to produce and release granzyme B in its active form [35]. Therefore, we hypothesized that stimulation of canine B cells with IL-21 and anti-BCR resulted in enhanced granzyme B and TRAIL expression and suppressed tumor cell viability. Canine B cells were separated and stimulated with IL-21 and anti-BCR, and the anti-tumor effect of direct tumor-B cell contact was examined.

In this study, we isolated $CD21^+$ B cells from the peripheral blood of healthy dogs, stimulated them with IL-21 and anti-BCR, and subsequently confirmed their anti-tumor effects. Prior to this, the process of verifying whether the isolated cells were B cells was performed. CD80 and CD86 are proteins mainly expressed in antigen-presenting cells [16]. Several studies have demonstrated that tumor

infiltrating B cells express CD80 and CD86, which are co-stimulatory markers with the primary function of antigen presentation [12, 17]. Based on this information, the expression of CD80 and CD86 was confirmed by flow cytometric analysis of separated cells. Thus, we confirmed that the cells used in the next step were genuine B cells. Flow cytometry was conducted after stimulation with IL-21 or anti-BCR. CD80 and CD86 were also expressed in stimulated cells, confirming the cells used for the next step were B cells. In addition, the possibility of the isolated $CD21^+$ B cells to recognize tumor in the subsequent experiments was presented with the results.

In this study, we confirmed that granzyme B and TRAIL secretion increased in the B cell group activated with IL-21 and anti-BCR. IL-21 and anti-BCR have previously been shown to stimulate granzyme B synthesis in human B cells. IL-21 and anti-BCR are known to induce the differentiation of granzyme B-expressing cells via the STAT3 pathway without a CD40 signal [37, 38]. Anti-BCR has been reported to sensitize B cells to apoptosis induced by TRAIL [39], but its effect on TRAIL secretion has not been fully clarified. For the first time, this study confirmed an increase in soluble TRAIL secretion from B cells stimulated with IL-21 and anti-BCR. Based on these results and previous studies, IL-21 and anti-BCR are considered to increase the secretion of granzyme B and TRAIL in canine B cells, and further research on the pathway involved should be conducted.

Furthermore, compared with the control GL-1 group, both groups co-cultured with B cells showed decreased cell viability. Moreover, compared with the group co-cultured with $CD21^+$ B cells, viability was significantly reduced in the group co-cultured with stimulated $CD21^+$ B cells. To confirm that the cytotoxicity of GL-1 cells was induced by granzyme B and TRAIL, an apoptotic assay using flow

cytometry was performed. GL-1 cells co-cultured with B cells activated with IL-21 and anti-BCR showed a significant increase in apoptosis. This may provide supporting evidence that granzyme B and TRAIL produced by B cells contribute to the cytotoxicity of GL-1 cells via the apoptotic pathway.

Various mechanisms have been reported to induce tumor cell apoptosis by granzyme B and TRAIL. Granzyme B in cancer cells initiates proteolysis and activation of effector procaspase-3 and -7, permeabilizes the mitochondrial outer membrane, proteolyzes multiple housekeeping proteins, including certain membrane receptors that promote cell survival, and translocates into the nucleus to fragment multiple intranuclear proteins [40]. Previous studies reported that the soluble form of TRAIL promotes apoptosis in tumor cells via the extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is triggered by binding to the death receptor (DR) and induces apoptosis by activating effector caspases, whereas the intrinsic pathway is initiated by mitochondria to form an apoptosome and activate the effector caspase pathway [41]. Binding of TRAIL to its receptor recruits FAS-associated proteins with death domains and caspase 8 to develop a functioning death-inducing signaling complex; thus, induces apoptosis by releasing multiple mitochondrial proteins and forming a functional apoptosome. With the sequential activation of caspase 9 and caspase 3, cleavage of a large number of intracellular components results in apoptosis of the target cancer cells [15, 42]. In this study, stimulation with IL-21 and anti-BCR improved the anti-tumor effects of B cells. Combined with previous studies, we concluded that the anti-tumor effect is probably due to granzyme B and TRAIL secreted by activated B cells.

This study had several limitations. First, activated B cells have only been

applied to one type of canine cancer cell line, and a variety of malignant cell lines may be used in follow-up studies. Additionally, the anti-tumor effect was demonstrated by confirming an increase in apoptosis, whereas the exact pathway or molecule affected has not yet been determined. Therefore, further studies are required.

5. Conclusion

In conclusion, we activated canine B cells with IL-21 and anti-BCR and verified the alterations in the anti-tumor effects. The expression and secretion of granzyme B and TRAIL increased with activation, and when co-cultured with the canine leukemia cell line GL-1, cell viability decreased by inducing apoptosis.

To the best of our knowledge, this is a novel study that elucidated the anti-tumor effects of canine B cells isolated from peripheral blood. Contrary to conventional studies that mainly focused on cell therapy, we conducted research based on the immunomodulators, IL-21 and anti-BCR. In this study, we explored the potential of this novel immunotherapy for cancer treatment in veterinary medicine. Additional in vivo experiments and early clinical trials should be conducted to establish their clinical significance.

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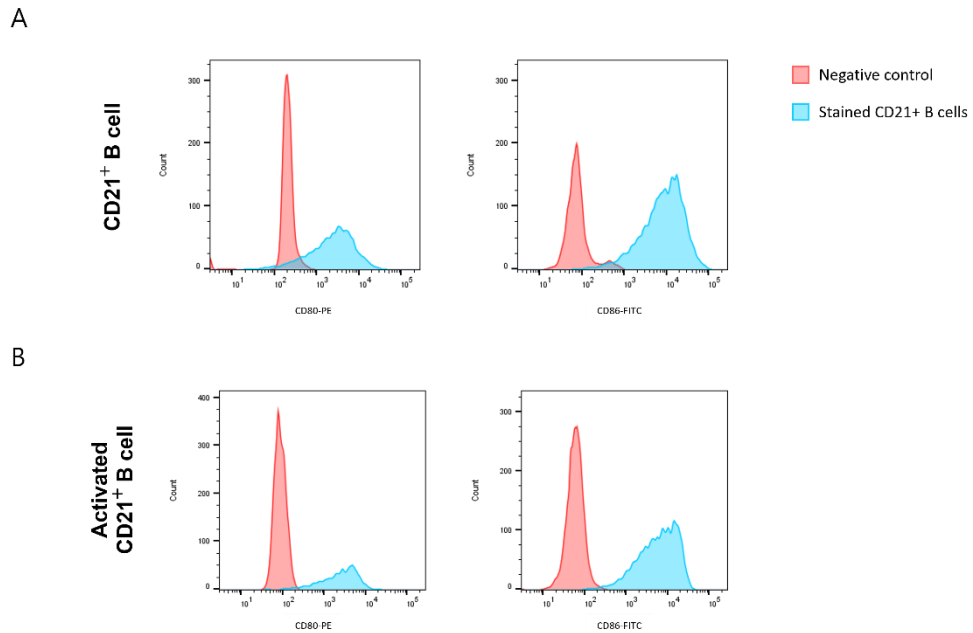

Figure 1. Expression of CD80 and CD86 was assessed by flow cytometry. (**A**) Representative flow cytometry plots of surface expression of CD80 and CD86 markers of canine B cells. Histograms represent the expression of CD80 and CD86 in the naïve $CD21^+$ B cells, **(B)** Surface expression of CD80 and CD86 markers of canine IL-21 and anti-BCR stimulated B cells. Histograms represent the expression of both CD80 and CD86 in the IL-21 and anti-BCR stimulated B cells. Experiments are performed in triplicates (n=3). Abbreviations: BCR, B cell receptor; CD, cluster of differentiation; IL, interleukin.

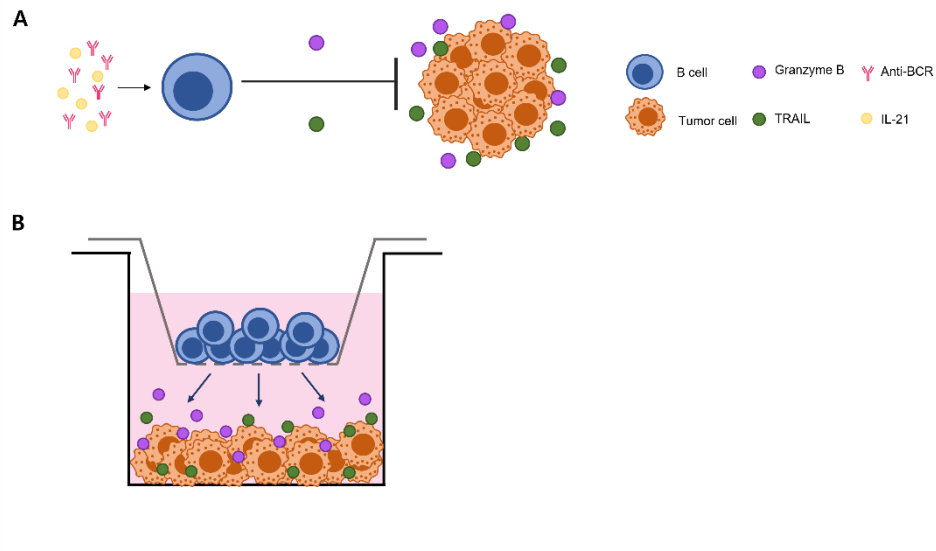

Figure 2. B cell activation with IL21 and anti-BCR. (A) The anti-tumor effect of B cells in the tumor micro-environment. B cells suppress tumor progression by secreting granzyme B and TRAIL, **(B)** Schematic representation of the two-dimensional co-culture with B cells and GL-1 cells using the Transwell assay. GL-1 cells were co-cultured with $CD21^{+}$ B cells and activated $CD21^{+}$ B cells. Abbreviations: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

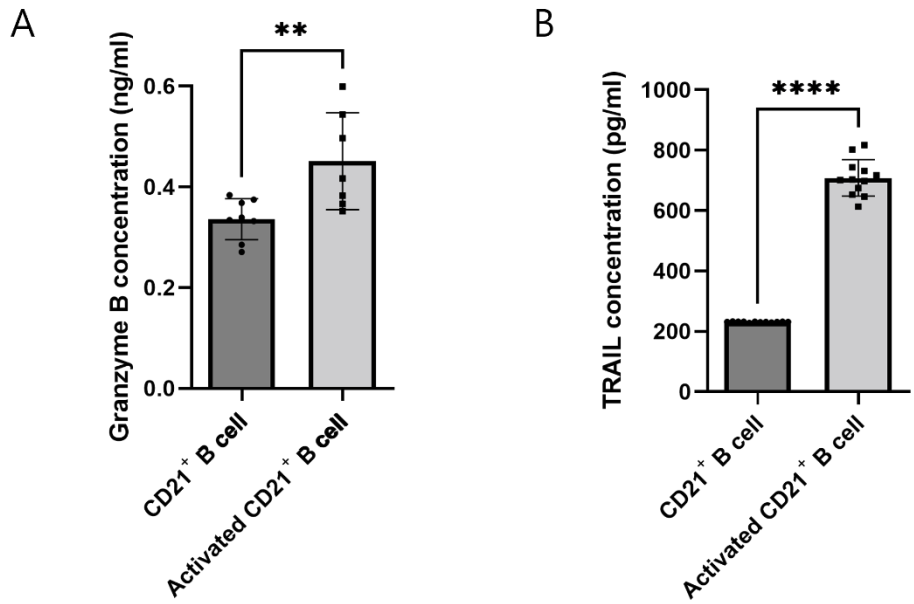

Figure 3. Expression of granzyme B and TRAIL using ELISA. (A) Compared to the $CD21^+$ B cell group, the activated B cell group displayed an increase in granzyme B level (n=7-8), **(B)** TRAIL level increased in the activated B cell group (n=12). Values are expressed as mean ± standard deviation. Statistical significance was determined using the two-tailed unpaired Student's t-test: **p<0.01, ****p<0.0001. Experiments are performed in triplicates. Abbreviations: ELISA, enzyme-linked immunosorbent assay; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

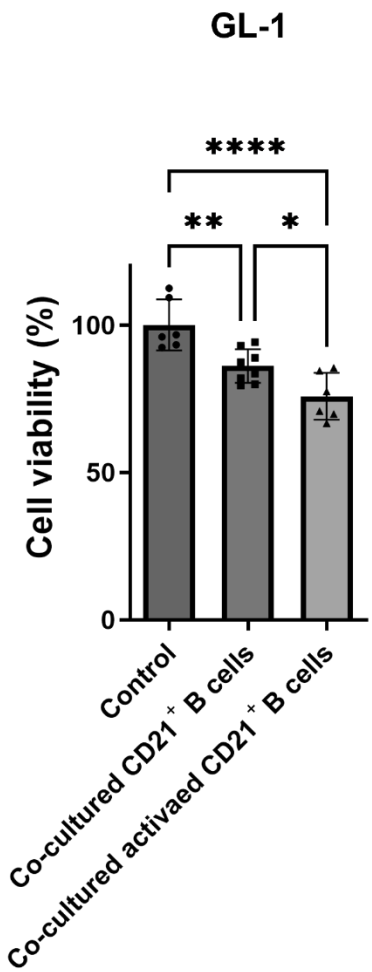

Figure 4. Cell viability assay of GL-1 cells. Cell viability of GL-1 cells that were co-cultured with B cells was assessed by CCK-8 analysis. The cell viability of GL-1 cells decreased when co-cultured with B cells. GL-1 cells co-cultured with the activated $CD21^+$ B cells showed more reduction in viability compared with the group co-cultured with $CD21^+$ B cells (n=6-8). Statistical significance was determined using the one-way analysis of variance (ANOVA) test followed by Tukey's multiple comparisons test: *$p<0.05$, **$p< 0.01$, ***$p<0.001$. Experiments are performed in triplicates. Abbreviations: CCK-8, cell counting kit-8.

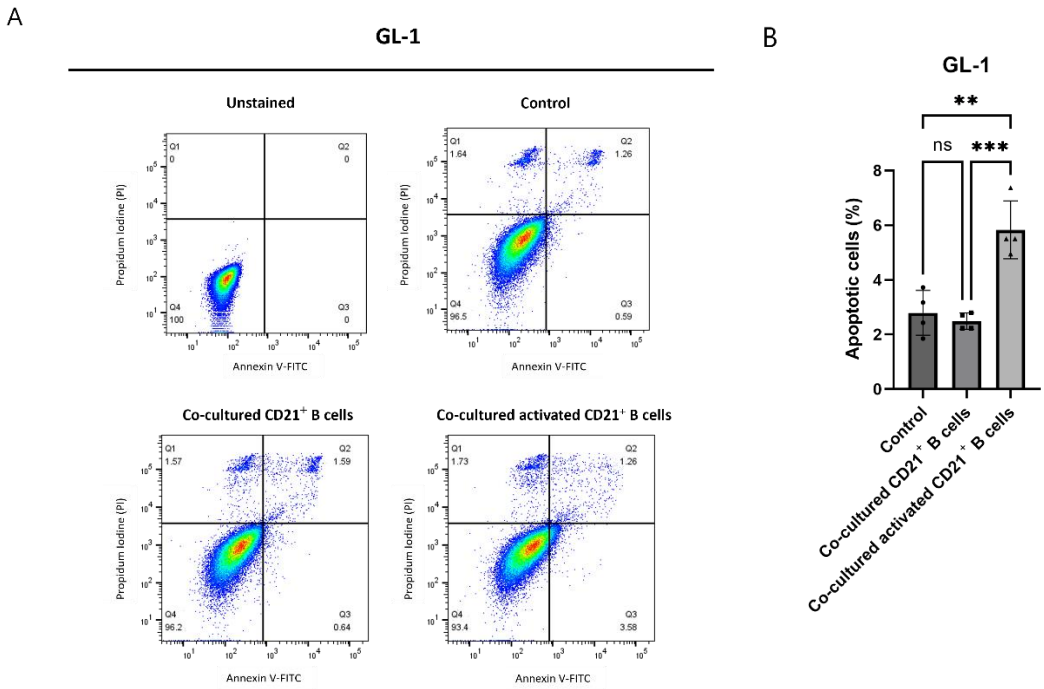

Figure 5. The apoptotic ratio was determined using flow cytometry. (A) Representative flow cytometry plots using Annexin V-FITC/PI staining for apoptosis. Apoptosis of GL-1 cells increased when co-culture with B cells activated with IL-21 and anti-BCR (n=4). The cells were gated based on unstained controls, **(B)** Quantification of data shown in B. Statistical significance was determined using the one-way analysis of variance (ANOVA) test followed by Tukey's multiple comparisons test: **p< 0.01, ***p<0.001; ns, not significant. Experiments are performed in triplicates.

7. 국문초록

인터루킨-21 및 항-B 세포 수용체를 이용하여 활성화된 개 B 세포의 항종양 효과

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최근 종양 미세환경 및 항종양 면역에서 B 세포의 주요한 역할에 대한 새로운 연구가 수행되고 있다. 또한 인터루킨-21(IL-21) 및 항-B 세포 수용체(BCR)는 B 세포를 자극하여 종양 세포에 세포독성 효과를 나타내는 그랜자임 B를 분비하는 것으로 보고되었다. 그러나, 수의학 분야에서 B 세포의 직접적인 항종양 효과에 대한 연구는 아직 부족한 상황이다. 본 연구는 종양 억제와 관련하여 B 세포의 직접적인 효과를 확인하는 수의학 분야의 새로운 연구이다. 본 연구에서 개 B 세포는 말

초 혈액으로부터 분리되었고 IL-21과 항 B 세포 수용체(BCR)로 활성화하였다. 개 백혈병 세포주 GL-1을 B 세포와 공동배양 하였으며, 세포 생존 여부 및 세포자살 비율의 변화를 평가하여 항종양 효과를 확인하였다.

IL-21과 항 BCR로 B 세포를 활성화시켰을 때, 그랜자임 B와 종양괴사인자 관련 세포자살 유도 수용체(TRAIL)의 분비가 유의적으로 증가했다. 동시에, GL-1 세포의 생존 능력은 유의미하게 감소했고, 특히 활성화된 B 세포와 함께 배양했을 때 세포자살 비율은 유의적으로 증가했다.

본 연구는 그랜자임 B와 TRAIL의 직접적인 항종양 효과와 IL-21 및 항 BCR로 활성화된 B 세포의 향상된 종양 억제 능력을 보여주었다. 이는 개에서 종양 미세 환경의 면역 조절을 다루는 초기 연구이며, 추가적인 생체 내 연구와 임상 시험으로 이어질 것으로 기대된다.

주요어: 개, B 세포, 항종양 효과, 인터루킨 21, 항-B 세포 수용체, 그랜자임 B, 세포자멸사

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