1-O-001 Oral Sessions

Search of itch-producing substance(s) released from skin cells of atopic dermatitis model mice

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Atopic dermatitis (AD) is a highly pruritic, chronic skin disease. Some mediators other than histamine are thought to play a major role in itch in AD. We have recently established an AD mouse model characterized by severe itch. In the present study, we searched itch-producing substance (pruritogen) released from skin cells of the model mice. Hairless mice were fed a special diet deficient in unsaturated fatty acids and starch to induce skin barrier dysfunction, and then ointments containing a crude extract of house-dust mite were repeatedly applied to the skin of the mice (AD mice). In AD mice, robust scratching behavior was observed especially after application of the mite extract, which was not suppressed by a histamine H1 receptor antagonist olopatadine. To identify the pruritogen(s), skin cells isolated from the AD mice were incubated in the medium containing the mite extract, and the conditioned medium was intradermally injected into normal mice. Scratching response was clearly induced by the samples derived from mite extract-treated AD skin cells, which was not inhibited by olopatadine. On the other hand, the mite extract itself did not induce scratching response. In conclusion, we successfully collected pruritogenic substance(s) released from skin cells of the AD model mice.

Type 1 regulatory T (Tr1) cells increased by sublingual immunotherapy (SLIT) suppressed allergic inflammation

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Allergen-specific sublingual immunotherapy (SLIT) is clinically effective for allergic diseases such as Japanese cedar pollinosis, whereas mechanisms of the effectiveness have not been fully elucidated. The purpose of this study was to elucidate whether a subset of Treg cells, Tr1 cells play roles in the effectiveness of SLIT. SLIT treatment was started in Japanese cedar pollinosis patients in 2014 or 2015, and had been continued until May 2019. In May 2017 and May 2019, peripheral blood mononuclear cells (PBMCs) were collected from the patients, and analyzed by flow cytometer. Numbers of Tr1-like cells (IL-10-producing $Foxp3^-$ $CD4^+$ T cells) as well as $Foxp3^+$ Treg cells in PBMC collected in 2019 were significantly larger than those in 2017. Visual analogue scale score, a parameter of clinical effects for nasal symptoms in 2019 was significantly improved in comparison with that in 2017. In another experiment of mice, Tr1-like cells were induced in vitro by culture of splenocytes of ovalbumin (OVA)-sensitized mice with OVA and cytokines, and adoptively transferred to OVA-induced asthmatic mice. The adoptive transfer of Tr1-like cells significantly suppressed the development of airway hyperresponsiveness, and increases in IL-5 and eosinophils in the lung. In conclusion, Tr1 cells could play roles in clinical effectiveness of SLIT.

1-O-003 Oral Sessions

Thromboxane A_2 is involve in the skin inflammation in mice with atopy-like dermatitis

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Atopic dermatitis (AD) is chronic skin disease with sever pruritus and inflammation. Recently, we have reported that arachidonic acid metabolite thromboxane A_2 (TXA_2) is an itch mediator and is involved in itch-related responses in mice with AD-like dermatitis. However, it is still unknown whether TXA_2 is involved in the skin inflammation of atopic dermatitis. Therefore, we demonstrated the involvement of TXA_2 in the skin inflammation in AD. In this study, male NC/Nga mice with AD-like dermatitis (dermatitis mice), which were bred under conventional environmental, and male healthy NC/Nga mice (healthy mice), which were bred under specific pathogen free environmental, were used. TP thromboxane receptor antagonist was locally applied in the rostral back skin, which was shaved hair, once a day for 7 days. Repetitive application of TP receptor antagonist was inhibited epidermal hyperplasia, the increase of the number of inflammatory cells. Repetitive application of TP receptor agonist U-46619 in the skin induced epidermal hyperplasia and increased the number of inflammatory cells. In addition, TP receptor antagonist also inhibited several cytokinins (such as IL-5) and chemokinins (such as eotaxin). These results suggest that TXA_2 is involved in the skin inflammation of AD through the expression of cytokinins and chemokinins.

Establishment of a novel anti-CD20 monoclonal antibody C_{20}Mab-11 using the Cell-Based Immunization and Screening (CBIS) method for the detection of B cells in many applications

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Purpose: CD20 is one of B-lymphocyte antigens, and is known as an effective target for B cell lymphomas. Although anti-CD20 monoclonal antibody (mAb) drugs have brought significant survival benefits to B cell lymphoma patients, some patients have shown no clinical response in a second treatment of anti-CD20 mAbs; therefore, more effective treatment of B cell lymphomas should be developed. In this study, we aimed to develop useful anti-CD20 mAbs for treatment or research of B cell lymphoma.

Methods: For the anti-CD20 mAb development, we used Cell-Based Immunization and Screening (CBIS) method. CD20-overexpressed LN229 cells (LN229/CD20) were used for immunization. The screening of hybridomas was performed by flow cytometry (FCM) using CD20-overexpressed CHO cells.

Results: We used 8 Balb/c mice for the hybridoma development, and obtained 18 anti-CD20 mAbs. Of those clones, C_{20}Mab-11 (IgM, kappa) was shown to be useful for FCM and western blot (WB) for endogenous CD20-expressing cell lines. Furthermore, C_{20}Mab-11 strongly stained B cells of the lymph follicle via immunohistochemistry (IHC).

Conclusion: Using CBIS method, we successfully developed a sensitive and specific anti-CD20 mAb C_{20}Mab-11, which is useful for FCM, WB, and IHC.

Mechanism of action of pyrogallol on calcineurin-NFAT signaling

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Calcineurin-NFAT (CN/NFAT) signaling is one of the most well-known signaling pathways involving many biological functions. We have demonstrated that CN/NFAT signaling was responsible for the pathogenesis of allergic rhinitis and identified pyrogallol as an anti-allergic compound. Pyrogallol suppressed NFAT dephosphorylation and CN/NFAT signaling-mediated IL-9 gene up-regulation in RBL-2H3 cells. Pyrogallol improved toluene-2,4-diisocyanate (TDI)-induced nasal symptoms in TDI-sensitized allergy model rats. Here, we investigated the mechanism of action of pyrogallol for CN-NFAT signaling. Pyrogallol inhibited ionomycin-induced dephosphorylation and nuclear translocation of NFAT. Pull-down assay revealed that pyrogallol strengthened interaction between NFATc1 and calcineurin. Further studies demonstrated that calcineurin binding site 2 in NFATc1 was involved in pyrogallol's effect. Poly(U)-binding-splicing factor 60 (PUF60) was identified as NFATc2 binding protein using pyrogallol-immobilized affinity chromatography. Pyrogallol suppressed ionomycin-induced interaction of PUF60 with NFATc2. Knockout of *PUF60* gene suppressed ionomycin-induced IL-9 gene up-regulation in RBL-2H3 cells. These results suggest that pyrogallol suppressed CN/NFAT signaling through the inhibition of NFAT dephosphorylation by the isoform-dependent manner.

Phenotypic alteration of tumor infiltrating macrophage by PHD inhibitor lead to improve tumor microenvironment in vivo mouse model.

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Tumor tissue environment is generally exposed to low oxygen, low nutrition and high interstitial pressure condition. These milieus are caused by vascular hyper-permeability, irregular vascularization and immature vessels. We previously reported that prolyl hydroxylase inhibitor (PHDi) induced tumor blood vessel normalization and improved tumor microenvironment (TME) in tumor bearing mouse. In this study, we examined whether improvement of TME by PHDi elicit phenotypic alteration of tumor infiltrating immune cells, especially macrophage (Mf). Lewis lung carcinoma cells were transplanted subcutaneously. Mice were treated with PHDi intraperitoneally at day10 after tumor transplantation. Then tumor tissues were collected at day16 and analyzed immune cells by flowcytometry and immunofluorescence staining. Mf ratio in total leukocyte were significantly increased in PHDi treated tumor in both immunohistochemical and flowcytometric analysis. Lymphocyte ratio didn't change in PHDi treated tumor. we performed Mf transplanted analysis using sorted Mf from tumor tissue. Our experiments showed that some Mf population contribute to maintain tumor vessel normalization and tumor microenvironment improvement in PHDi treatment. In addition, PHDi treatment didn't induce tumor progression in LLC mouse model.

Angiotensin II stimulates proliferation and metastasis of murine TNBC 4T1 cells by affecting the tumor microenvironment

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Although it is known that tumor microenvironment affects tumor growth and metastasis, the effect of angiotensin II (Ang II) on tumor microenvironment has not been clarified. The aim of this study was, therefore, to examine the effect of Ang II on tumor microenvironment using a murine model of spontaneous cancer metastasis. Triple negative breast cancer 4T1-Luc cells, lacking Ang II type 1 receptor (AT1R) expression, were subcutaneously injected into mammary fat pad of BALB/c mice. After Ang II was administrated for 4 weeks using an osmotic pump, the primary tumor was weighed and analyzed for protein expressions. Metastasis to the lung was also evaluated by micro-CT and the measurement of luciferase activity. The weight of primary tumor and the number of lung colonies and their luciferase activity were significantly increased in the Ang II-treated mice. The expression levels of epithelial-to-mesenchymal transition markers (snail and vimentin) and cell proliferation markers (cyclin D1 and c-Myc) were also increased in primary tumors of the Ang II-treated mice. On the other hand, Ang II didn't alter the proliferation, migration, or infiltration of 4T1 cells *in vitro*. These results suggest that Ang II stimulates cancer growth and metastasis even in 4T1 cells lacking AT1R expression probably by affecting tumor microenvironment. Therefore, the Ang II signaling pathway could be an appropriate target for cancer therapy.

Potential metabolic changes mediated by cGAMP in astrocytes in contact with brain metastatic cancer

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In tumor brain metastasis, a gap junction was formed between astrocytes and cancer cells, and cGAMP (Cyclic 2'3'-GMP-AMP) was reported to be transmitted to astrocytes. It is known as a ligand for STING that involves innate immune response signaling, and that also can be a material for nucleic acids and amino acids. Thus, cGAMP transmission may alter the metabolic function of astrocytes, creating a favorable environment for tumor survival. In this study, after introducing cGAMP into the cell from the outside, the amount of cGAMP in the cell and the subsequent phenotypic changes were examined. Since cGAMP is hydrophilic, lipid nanoparticles (SS-cleavable and pH-Activated-like Material: ssPalm) were used as a carrier. The ssPalm-cGAMP complex was added to the cultured astrocytes. 12 ng of cGAMP was detected by CE-MS from astrocytes to which ssPalm-cGAMP complex equivalent to 8 μg of cGAMP was added. IFNβ mRNA expression and secretion into the culture supernatant, downstream of STING, were significantly increased by 15.3 and 1.7 times, respectively. Subsequently, the complex significantly increased the 2-deoxyglucose uptake by 1.1 times. For glutamate (Glu) metabolism, cGAMP was shown to increase the secretion of Glu into the supernatant while suppressing the conversion of intracellular Glu to Gln. These results suggest that cGAMP stimulates STING in astrocytes, moreover, promotes glucose utilization and activation of Glu metabolism. It should be further investigated that how this metabolic change affects the microenvironment formation of brain metastasis.

O-GlcNAcylation-mediated degradation of FBXL2 stabilizes FOXM1 oncogenic transcription factor to promote cancer progression

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O-GlcNAcylation is a dynamic and reversible post-translational modification of cytonuclear molecules and critical for intracellular signaling. The modification is regulated by only two enzymes, OGT and OGA, which add and remove a glucose metabolite, UDP-GlcNAc, respectively. Elevated *O*-GlcNAcylation is a hallmark of cancer and contributes to cancer malignancy. However, the molecular mechanism is not fully understood. Recently, we showed that FOXM1, which is a critical oncogenic transcription factor and wildly overexpressed in solid tumors, was elevated in a human cancer cell line by an OGA inhibitor, Thiamet G (TMG), inducing augmented *O*-GlcNAcylation. In this study, we identified FBXL2 E3 ligase as a new target of *O*-GlcNAcylation. The FOXM1 expression was increased accompanying with decreased its ubiquitination and degradation by TMG treatment. FBXL2 ubiquitinated FOXM1, and the ubiquitination of FOXM1 was reduced by TMG treatment. FBXL2 was ubiquitinated, which was promoted by TMG. Moreover, FBXL2 induction using the Tet-on system showed that FOXM1 expression and cell proliferation were reduced in NUGC-3 cells, and the reductions were attenuated by TMG. Taken together, we found that FOXM1 was stabilized by the *O*-GlcNAcylation-mediated degradation of FBXL2. These data suggest that elevated *O*-GlcNAcylation might contribute to cancer progression via the suppression of FBXL2-mediated degradation of FOXM1.

EP_4 receptor regulates cell migration and apoptosis in oral cancer

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【Background】The EP4 prostanoid receptors are one of the four receptor subtypes for Prostaglandin E2 (PGE2). EP4 plays an important role in cancer progression. Its inhibition is a potential strategy for cancer therapy. However, little information is available regarding cell apoptosis and cellular signaling pathway of EP4 in oral cancer. In current study, we examined that EP4 signal regulates cell apoptosis and chemotherapeutic resistance in oral cancer.

【Material and Method】Human-derived tongue squamous cell carcinoma cell lines, HSC-3 was used. Western blot analysis was performed to evaluate the proteins, which is associated with cell migration and apoptosis in cancer (E-cadherin, N-cadherin, claudin-1, ZEB1, ZO-1, galectin-3, fibronectin, Bcl-2, Bax). Cell apoptosis was evaluated by flowcytometry.

【Result】EP4 agonist (ONO-AE1-437) increased expression of galectin-3 in HSC-3 cells ($p<0.001$). EP4 agonist also increased claudin-1 expression. The other proteins were not changed by the EP4 agonist stimulation. Furthermore, EP4 agonist inhibited cisplatin-induced early apoptosis of oral cancer cells and decreased late apoptosis and necrosis.

【Conclusion】Activation of EP4 signal may increase the expression of galectin-3 and promote cell apoptosis, resulting in a chemotherapeutic resistance of oral cancer.

Astrocytes in the critical period regulate synapse-scaling microglia

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Glial cells play essential roles for the modulation of synaptic connections and healthy development of brain networks. They control the excitatory/inhibitory synaptic balance and assembles neural circuity by synapse-formation through synaptogenic factors or by synapse-elimination through phagocytosis. We have recently demonstrated that astrocytes form excitatory synapses in the adult injured brain, for which mGluR5 has an essential role. However, astrocytic mGluR5 is, in health brain, limitedly expressed in the early developmental stage (critical period). Thus, we surveyed how astrocytic mGluR5 in the critical period destines the subsequent synapse scaling using astrocyte-specific mGluR5 KO mice (cKO). Astrocytes in cKO were slightly reactive in the critical period. Unexpectedly, inhibitory synapses changed significantly more than excitatory synapses in cKO, and number of inhibitory synapses was significantly decreased in cKO throughout ages. This was mainly due to phagocytic microglia that frequently engulfed inhibitory synaptic elements in the critical period in cKO. Taken together, we conclude that astrocytic mGluR5 controls inhibitory synapses more significantly than excitatory synapses in the critical period, and its dysfunction results in decrease in inhibitory synapses throughout life, for which microglia has a pivotal role. We would like to emphasize that astrocytes in the critical period fate a lifelong inhibitory network by controlling microglia.

Melatonin receptor agonist ameliorates PTSD-like behaviors in *Fabp3*$^{-/-}$ mice.

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Fatty acid binding proteins (FABPs) are required for long-chain polyunsaturated fatty acids (LCPUFAs) intracellular trafficking, uptake, transport and metabolism. Whereas FABP3, FABP5 and FABP7 are expressed in the brain of mice and humans, FABP3 is predominantly expressed in the mature neurons. Previous clinical studies reported that supplementation of LCPUFAs relieves post-traumatic stress disorder (PTSD) symptoms. Here, we investigated relationship between PTSD-like symptoms and FABP3. FABP3 null (*Fabp3*$^{-/-}$) mice showed cognitive deficits, hyperlocomotion and impaired fear extinction as PTSD-like behaviors. We observed significantly reduction of calcium/calmodulin-dependent protein kinase II (CaMKII) autophosphorylation in the anterior cingulate cortex (ACC) of *Fabp3*$^{-/-}$ mice. By contrast, elevated CaMKII autophosphorylation and c-Fos expression levels were observed in the basolateral amygdala (BLA) after exposure to contextual fear conditions. Interestingly, Melatonin receptor (MTR) agonist ramelteon (1.0 mg/kg, p.o.) antagonized abnormal c-Fos expression and CaMKII autophosphorylation levels in the ACC and BLA, resulting in improvement of PTSD-like behaviors in *Fabp3*$^{-/-}$ mice. MTR antagonist luzindole (2.5 mg/kg, i.p.) inhibited the effect of ramelteon. Since melatonin receptors are few expressed in the amygdala, we suggest that ramelteon may restore decreased neuronal activity in ACC via MTR activation and in turn ameliorate aberrant BLA activity, thereby improving PTSD-like behaviors in *Fabp3*$^{-/-}$ mice.

Histamine neurons in the tuberomammillary nucleus modulate memory retrieval

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Brain histamine is produced mainly in the tuberomammillary nucleus (TMN) and is implicated in learning and memory as well as wakefulness and feeding. Previously, we demonstrated histamine H_3 receptor inverse agonists upregulate histamine release in the perirhinal cortex and promote the recall of forgotten object memories. However, since H_3 receptors are expressed in non-histaminergic neurons as well as histaminergic neurons, other neurotransmitter systems could be involved in the memory recovery. In this study, we examined whether chemogenetic manipulation of histamine neurons modulates memory retrieval in mice. We virally targeted hM3Dq, the excitatory DREADD receptor, to histamine neurons in the TMN of HDC-Cre mice. In a training session of the novel object recognition task, mice were placed in the field, in which two identical objects were positioned. One week later, they underwent a test session where one familiar and one novel object were presented. The pre-test injection of CNO to the mice receiving AAV-DIO-hM3Dq enhanced the discrimination between two objects as compared to controls. The activation of histamine neurons had no effect on anxiety-like behavior in the elevated plus maze test. These findings indicate that activation of histamine neurons enhances retrieval of a forgotten long-term object memory.

Role of glutamate release from melanin-concentrating hormone neurons in REM sleep regulation

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Neurons containing melanin-concentrating hormone (MCH) localized in the posterior lateral hypothalamus and have a crucial role in rapid eye movement sleep (REMs) regulation. As MCH neurons also contain a variety of other neurotransmitters such as glutamate. However, the specific neurotransmitter responsible for REMs regulation is not known. We hypothesized that glutamate, the primary fast-acting neurotransmitter in MCH neurons, is necessary for REMs regulation. To test this hypothesis, we generated mice deleted vesicular glutamate transporter (Vglut2; necessary for synaptic release of glutamate) specifically from MCH neurons by crossing MCH-Cre mice (expressing Cre recombinase only in MCH neurons) with Vglut2$^{flox/flox}$ mice (expressing LoxP-modified alleles in Vglut2). We then studied the amounts, architecture and diurnal variation of sleep-wake states in baseline conditions. Next, we activated the MCH neurons lacking glutamate release using chemogenetic methods and tested whether these MCH neurons still promoted REMs. Our results indicate that glutamate in MCH neurons contributes to normal diurnal variability of REMs by regulating the levels of REMs during the dark period, but MCH neurons can promote REMs even in the absence of glutamate.

HDAC3 inhibition ameliorates dystrophic axons and memory function via M2 microglia in a transgenic mouse model of Alzheimer's disease

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Amyloid β (Aβ) skews microglia to M1 phenotype and induces inflammation and neurodegeneration. On the other hand, another type of microglia, M2, shows anti-inflammatory and neurotrophic effects. We previously clarified that HDAC3 inhibition induced predominance of M2 microglia and axonal growth, and recovered locomotor function in spinal cord injured mice. Therefore, this study aimed to clarify that HDAC3 inhibition skewed to M2 microglia and restored memory function in Alzheimer's disease model mice. In cultured microglia, a treatment with an HDAC3 inhibitor, RGFP966, skewed to M2 microglia when treated 24 h after Aβ addition. Conditioned medium collected from RGFP966-treated microglia recovered Aβ-induced collapse of axonal growth cones. RGFP966 was intraperitoneally administered to 5XFAD mice, a transgenic model of Alzheimer's disease. RGFP966 decreased degenerated axons overlapping with Aβ plaques and improved novel object recognition memory. When microglia in the brain of 5XFAD mice were eliminated by intracerebroventricular administration of clophosome, the effects of RGFP966 were diminished. These results suggest that HDAC3 inhibition increased predominance of M2 microglia, recovered axonal degeneration, and ameliorated memory deficit in 5XFAD mice.