PROGRESS REPORT
Division of Cancer Cell Biology

Daisuke Kitamura, M.D., D.M.S.
Members

Members (Kitamura's lab.)

Faculty members

Professor and Chairman
Daisuke Kitamura, M.D., D.M.S.

Assistant Professor
Mari Tenno, Ph.D.

Post-doctoral Researcher
Lalhaba Oinum, Ph.D.

Visiting Professor
Ryushin Mizuta, M.D., D.M.S.

Visiting Researcher
Yoshihito Nihei, M.D.
(Juntendo University)
Shogo Takatsuka, Ph.D.
(National Institute of Infectious Diseases)

Students

Graduate students
Hiroyuki Iwasaki
(Juntendo University Graduate School of Medicine)
Mizuki Higashiyama
Tsubasa Kobayashi
Miyuki Hata
Jianyi Zhang
Shu Takahashi
Qi Zhao

Undergraduate student
Yuko Emoto

Secretary
Yoshiko Hayashi

Shunsuke Kon, Ph.D.
Members

Faculty members

Junior Associate Professor
Shunsuke Kon, Ph.D.

Assistant Professor
Yuta Sakae, Ph.D.

Students

Graduate student
Kazuki Nakai
Hancheng Lin
Eilma Akter
Seo Hee Won
Kailai Xu

Undergraduate student
Olivia Marchia Kusuma

Division of Cancer Cell Biology

Chairman: Daisuke Kitamura, M.D., D.M.S.

Upon encountering antigens through the B-cell receptor (BCR), B cells are activated and take up antigens. If the antigen contains a protein ingredient, then B cells present the antigen-derived peptide on MHC class II to cognate helper T (Th) cells. In turn, Th cells stimulate B cells through the CD40-ligand (CD40L) and cytokines, such as IL-4 and IL-21, to facilitate their proliferation and switching of the BCR isotype from IgM/IgD to IgG, IgA, or IgE (class switching: CS). Some of these B cells then differentiate into short-lived plasma cells (PCs) and move to the extrafollicular region, whereas others proliferate further and form the germinal center (GC) in the B cell follicles. In GC, B cells undergo somatic hypermutation (SHM) of their immunoglobulin (Ig) V region genes to diversify their Ig repertoires. Among GC B cells, those expressing BCR (typically of IgG classes) that bind to the immunized antigen with high affinity are selected, and they differentiate into memory B cells (MBCs) or long-lived PCs (LLPCs), both contributing to long-lasting humoral immunological memory.

Despite the widely accepted dogma of T-cell-dependent (TD) immune responses, as summarized above, it has been revealed that GCs, SHM, and CS are not essential for generating MBCs. One representative finding is that IgG^+ MBCs without SHM can be generated in the absence of GC in mice devoid of Bcl6, a master regulator of GC B cell differentiation (Toyama et al. 2002, *Immunity* 17:329; Kaji et al. 2012, *J. Exp. Med*. 209:2079). In addition, IgM^+ MBCs have been appreciated to exist and to play a role in the humoral immunity (Dogan et al. 2009, *Nat. Immunol*. 10:1292, Pape et al. 2011, *Science* 331:1203). In contrast, GCs appear necessary for generating LLPC because mice deficient in IL-21/IL-21R signaling show premature contraction of GC and fewer LLPCs (Zotos et al. 2010, *J. Exp. Med*. 207:365; Rasheed et al. 2013, *J. Virol*. 87:7737).

GCs arise in secondary lymphoid tissues several days after immunization with protein-containing antigens, so-called TD antigens. GCs are mainly composed of intensely proliferating antigen-primed B cells (centroblasts), post-cycled B cells (centrocytes), follicular dendritic cells (FDCs) and follicular helper T (Tfh) cells. Tfh cells produce IL-21, which is critical for prolonged expansion of GC B cells, as mentioned above, and IL-4, which induces CS to IgG1 (in mice) or IgE. In addition, we previously reported that IL-4 induces epigenomic remodeling of the *Bcl6* locus by reprogramming the TCA cycle and the expression of Bcl6 (Haniuda et al. 2020, *Cell Rep*. 33:108333). It remains unknown which factors are necessary to induce the SHM of Ig genes in GC B cells. *In vitro* stimulation of B cells through CD40 or TLR4, together with IL-4 or IL-21, induces massive proliferation and CSR, but not SHM, although it induces the expression of activation-induced cytidine deaminase (AID), which is essential for SHM and CS recombination (CSR) of Ig genes. This suggests that some critical factors required for SHM are missing under these culture conditions. It is also unclear how a few high-affinity B cells are selected in the vast majority of other B cells in GCs, including those with considerable affinity for antigens, and how GC B cells differentiate into MBCs or LLPCs. Although the transcription factors Bcl-6 and Blimp1 are known to be necessary for B cells

to differentiate into GC B and PCs, respectively, little is known about the transcription factors that induce MBC differentiation, except for a few recent reports (Laidlaw et al. 2020, *Nat. Immunol*. 21:1082). The external stimuli that induce GC-B cell differentiation into MBCs or LLPCs remain elusive.

One reason for the difficulty in elucidating the mechanisms of GC B cell development could be the lack of appropriate *in vitro* systems that mimic the process of GC B cell development. To solve this problem, we previously established a B-cell culture system using an original feeder cell line expressing exogenous CD40L and BAFF (termed 40LB). When naive B cells are cultured sequentially with IL-4 and IL-21 on a 40LB feeder layer, B cells undergo massive expansion, express GC B cell markers such as GL7 and Fas, and undergo efficient CS either to IgG1 or IgE. We termed these cultured B cells "induced GC B (iGB) cells." This system enabled single B cell culture. Despite extensive proliferation, SHM of the V-region genes could not be detected in iGB cells. After primary culture with IL-4, iGB cells (iGB-4) can differentiate into memory-like B cells, termed "induced memory B (iMB) cells," after transfer into irradiated mice. When transferred with cognate antigen-primed Th cells to secondary recipient mice, antigen-specific iMB cells quickly responded to soluble cognate antigens to produce IgG1 antibodies, indicating the normal memory-recall function of iMB cells. In contrast, iGB cells after secondary culture with IL-21 (iGB-21) failed to develop into iMB cells in the recipients but instead differentiated into PCs in the bone marrow (Nojima et al. 2011, *Nat. Commun*. 2:465; Haniuda and Kitamura. 2019, *Bio-protocol* 9: e3163).

Thus, iGB cell culture appears to be a promising experimental system for the elucidation of several unsolved questions, including mechanisms for GC formation, generation of SHM and CSR, affinity selection, and B-lymphomagenesis. Indeed, many researchers worldwide have used this system and the results have been reported (Caganova et al. 2013, *J. Clin. Invest*. 123:5009; Wu et al. 2014, *PNAS* 111:e4638; Purwada et al. 2015, *Biomaterials* 63:24; Webb et al. 2016, *J. Immunol*. 196:207; Kuraoka et al. 2016, *Immunity* 44:542; Domeier et al. 2016, *J. Exp. Med*. 213:715; Kuraoka et al. 2017, *Cell Rep*. 18:1627; Lee et al. 2017, *J. Immunol*. 198:1066; Li et al. 2018, *Immunity* 48:530; Le Gallou et al. 2018, *J. Exp. Med*. 215:2035; Litzler et al. 2019, *Nat. Commun*. 10:22; Finney et al. 2019, *J. Immunol*. 203: 3268; Nojima et al. 2020, *J. Immunol*. 205:90; Wigton et al. 2021, *J. Exp. Med*. 218:e20201422; Fukushima et al. 2022, *Cell Rep*. 40:111373; Thomann et al. 2023, *PNAS*. 120 :e2300733120; Yada et al. 2024, *J. Exp. Med*. 221:e20222178; and many more).

Using this system, we have addressed the following questions: how is the generation of SHM regulated in GC B cells, how is the fate of GC B cells toward MBC subsets determined (Koike et al. 2019, *eLife* 8:e44245), how is the MBC recall response regulated (Fukao et al. 2014, *J. Immunol*. 193:635; Kodama et al. 2020, *Int. Immunol*. 32:385; Takatsuka et al. 2018, *Nat. Immunol*. 19:1025), and how are IgE-producing B cells restrained (Haniuda et al. 2016, *Nat. Immunol*. 17:1109)? Regarding the regulation of MBCs, we found that IL-9 autocrine signaling facilitated their proliferation and differentiation toward PCs, while suppressing their ICOS-L expression and differentiation toward GC B cells (Takatsuka et al. 2018). We also found that PC development from MBCs was suppressed by the cell-surface inhibitory receptor gp49B (also called Lilrb4), which is selectively expressed in MBCs and marginal zone B cells (Fukao et al. 2014). In addition, we previously reported that MBCs generated in the TD response are eliminated when their BCRs bind to specific antigens in the form of T-cell-independent type 2 (TI-2) antigens (Haniuda et al. 2011, *J. Immunol*. 186:5620). Thus, the recall response of MBCs appears to be strictly regulated, probably because MBCs are intrinsically hyper-responsive to

antigens owing to the properties of IgG BCR and the higher expression of MHC class II and co-stimulatory molecules, CD80 and CD86 (reviewed in Kitamura 2021, *Int. Immunol*. 33:791).

In addition, we established a system to culture mouse and human B cells for an unlimited length of time using iGB cells transduced with the Bach2 gene and attempted to establish an in vitro method to generate antibodies against tumor antigens as well as antibody-producing B cells using tumor-infiltrating B cells (Moutai et al. 2014, *PLoS One* 9:e92732; Wang et al. 2021, *PLoS One* 16:e0245608).

In 2020, Dr. Shunsuke Kon, a prominent scientist in cancer biology, started his laboratory in this division to study how normal, cancer, and immune cells interact with each other to maintain a normal cell society and break the norm into malignancy. On that occasion, we changed the name of this division from Division of Molecular Biology to Division of Cancer Cell Biology.

Mechanisms of B cell activation in T-cell-independent type 2 responses

The T-cell-independent type 2 (TI-2) response is crucial for protection against pathogens such, as Pneumococcus and Salmonella. For this response, marginal zone B cells in the spleen and B1 cells, innate-like B cells mainly residing in the peritoneal and pleural cavities, are responsible. When these B cells recognize TI-2 antigens, such as polysaccharides of bacterial capsules characterized by highly repetitive epitopes, strong crosslinking of BCR induces proliferation, differentiation into plasma cells, and the production of antibodies, such as IgM and IgG, without the help of T cells. BCR proximal signaling molecules such as Btk and BLNK, which are dispensable for the TD response, are necessary for the TI-2 response. However, the downstream signaling mechanisms remain unknown. We found that the proliferation, differentiation, and antibody production of B cells in the TI-2 response required intrinsic DNase1L3, an endonuclease known to digest genomic DNA in necrotic cells. DNase1L3 was selectively expressed in MZ B and B1 cells, in addition to macrophages, and it increased in amount and translocated into the nucleus upon stimulation with a model TI-2 antigen. However, the mechanism of its function in B-cell activation remains elusive (Kato et al. 2023, *Int. Immunol*. 35:275). We also found that protein kinase C (PKC) δ is activated in B cells by stimulation with a TI-2 antigen in vitro, and that B cell-intrinsic PKCδ is required for IgG production upon immunization with TI-2 antigens in vivo. In B cell-specific PKCδ-knockout (BKO) mice, serum IgG3 against commensal bacteria disappeared, and the mice were more susceptible to bacteremia caused by the disruption of the mucosal barrier upon administration of dextran sulfate. Mechanistically, we revealed that PKCδ is necessary for the induction of AID and class switching through upregulation of BATF expression (Fukao et al. 2021, *eLife* 10:e72116).

It has been reported that the TI-2 response is supported by dendritic cells or macrophages, but the underlining mechanism for this co-stimulation remains unknown. We identified IL-1α and IL-1β as efficient co-stimuli by *in vitro* screening of various cytokines for their ability to co-stimulate hydroxy-nitrophenyl acetyl (NP)-specific B cells with the TI-2 antigen NP-Ficoll to produce IgM and IgG3, the latter being the dominant switched Ig isotype in the TI-2 response. IL-1 strongly enhances B cell proliferation, AID expression, and CSR of the *IgG3* locus when added together with NP-Ficoll, but not when added alone (Fukao et al. 2021, *eLife* 10:e72116). We confirmed the role of IL-1 in the TI-2 response *in vivo* by immunizing mice deficient in IL1RAcP, a signaling component of IL-1R that binds to both IL-1α and IL-1β, with NP-Ficoll. Even before immunization, serum IgG3 levels were lower in IL1RAcP deficient mice compared to control mice, probably reflecting the contribution of IL-1

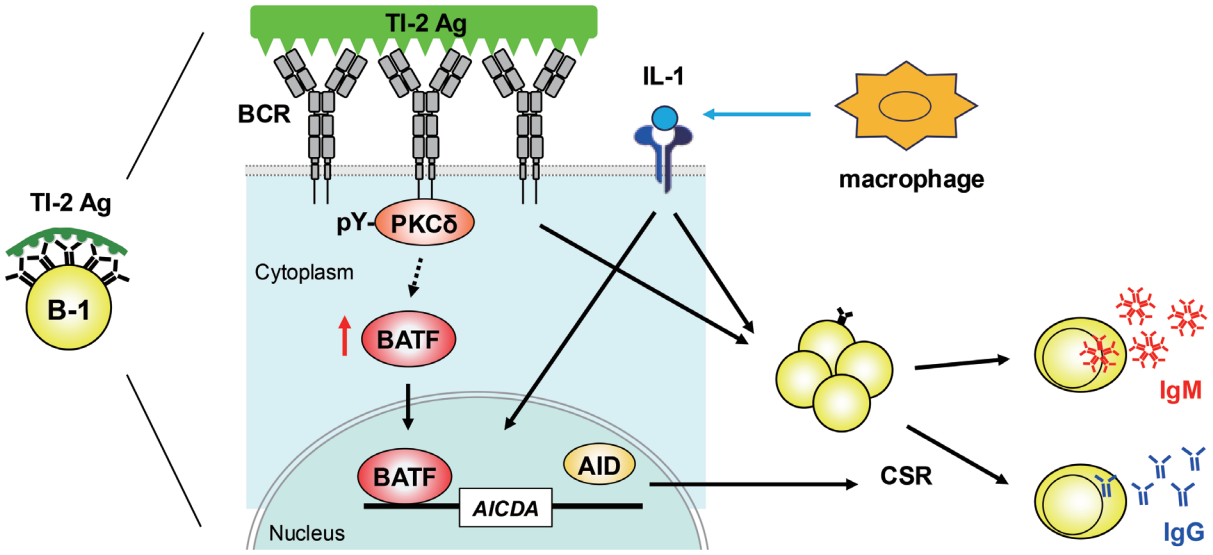

Figure 1. BCR and IL-1R signal crosstalk in B cell response to TI-2 antigen

to antibody production in response to commensal bacterial antigens at steady state. In addition, B1 cells from IL1RAcP-deficient mice did not respond to the immunized antigen NP-Ficoll when transferred into recipient mice carrying a transgenic non-NP-binding BCR, confirming a requirement of IL-1R on B1 cells. We also found that peritoneal macrophages produced IL-1α and IL-1β during the TI-2 response, and depletion of macrophages strongly suppressed antibody production. These data indicate that IL-1 produced by macrophages is a critical co-stimulant for B1 cells in the TI-2 response in the peritoneal cavity.

Collaborators:

Mari Tenno, Tang Xuyang, Takumi Umezu, Yuko Emoto, Saori Fukao, Kei Haniuda

Commensal bacteria-primed production of IgA autoantibodies against glomerular antigens in a mouse model of IgA nephropathy

IgA nephropathy (IgAN) is the most common type of primary glomerulonephritis, with approximately 40% of cases progressing to renal failure. IgAN is pathologically characterized by the deposition of IgA, IgG, and complement C3 in the glomerular mesangium, and proliferative changes in the mesangial cells and matrix. Glomerular IgA deposition has been ascribed to the abnormal glycosylation of IgA, namely galactose-deficient (Gd-) IgA1, which tends to form immune complexes (ICs) by self-aggregation or with autoantibodies against Gd-IgA1. However, such ICs would not only be deposited in the mesangial region but also at various locations in the glomeruli. Additionally, Gd-IgA1 can be found in healthy individuals and in patients with diseases other than IgAN. Therefore, Gd-IgA1 alone cannot explain the mesangium-specific IgA deposition in IgAN. Thus, the mechanisms underlying selective deposition and generation of pathogenic IgA in IgAN remain unclear.

To address these problems, we used an IgAN mouse model, “gddY” mice. These mice were generated by the selective intercrossing of mice in early onset groups of outbred ddY mice for more than 20 generations. The original ddY mice spontaneously developed IgAN but with a low incidence and highly variable timing of onset

among individuals. In contrast, essentially all gddY mice exhibit proteinuria and IgA deposition in the glomerular mesangium by 8 weeks of age, followed by glomerular injury, which is similar to that observed in human IgAN (Okazaki et al. 2012, *J. Am. Soc. Nephrol*. 23:1364). Recently, we found anti-mesangium IgA auto-Abs in the sera of gddY mice and human patients with IgAN and identified βII-spectrin as a target antigen in both gddY mice and IgAN patients. IgA^+ plasmablasts (PBs) accumulated in the kidneys of gddY mice and IgA Abs secreted by these PBs also bound to the mesangium and βII-spectrin. Although βII-spectrin is a cytoskeletal protein, it was expressed on the surface of mesangial cells and βII-spectrin-transfected HEK293T cells. A recombinant IgA antibody cloned from kidney PBs bound to the mesangial regions of the kidney in vivo after intravenous administration. From these data, we propose that the production of IgA autoantibodies against mesangial proteins, such as β2-spectrin, is the first trigger of the pathogenesis of IgAN, and therefore, IgAN is a tissue-specific autoimmune disease (Nihei et al. *Science Adv*. 2023).

Recently, we identified an additional self-antigen recognized by recombinant IgA auto-Abs (rAb#66 and others) derived from the kidney PBs of gddY mice. The protein isolated from mesangial lysates was CBX3, which is known to be located in the heterochromatin of the nucleus. However, CBX3 was detected on the surface of mesangial cells and recognized by IgA in the sera of gddY mice and IgAN patients. When BALB/c mice were immunized with CBX3, antigen-specific IgG and IgA antibodies were produced and deposited in the mesangium. Interestingly, after compound antibiotic treatment of gddY mice, IgA^+ PBs disappeared from the kidneys, glomerular IgA deposition was restrained, proteinuria was attenuated, and serum anti-CBX3 IgA disappeared, suggesting that commensal bacteria are involved in the production of IgA auto-Abs. In addition, feeding gddY mice an elemental diet caused a similar phenomenon. We found that gddY serum IgA and rAb#66 bound to oral, but not intestinal, commensal bacteria from gddY, but not BALB/c mice, and that rAb#66 binding to oral bacteria was competitively inhibited by the addition of CBX3 protein. We identified a bacterial strain that binds to rAb#66, which is previously unknown and tentatively termed "C42." Binding of rAb#66 to C42 was competed by CBX3, suggesting molecular mimicry between C42 and CBX3 epitopes. Immunization of BALB/c mice with C42 induced antibodies that bound to CBX3 and glomerular deposition of IgA in vivo. Finally, the proportion of C42 selectively decreased among the oral bacteria in gddY mice fed an elemental diet. Thus, our results indicate that particular strains of oral commensal bacteria can induce an immune response that leads to the production of anti-mesangial IgA auto-Abs in gddY mice. In addition, we detected CBX3-binding IgA^+ memory B cells in the periglandular and kidney lymph nodes (LNs), suggesting the initiation of the immune response around salivary glands, generating anti-mesengial memory B cells. Such memory B cells are probably recruited to kidney LNs and produce anti-mesengial IgA in response to mesangial antigens, perhaps drained from the kidney. These findings will facilitate an understanding of IgAN pathogenesis and therapeutic strategies for IgAN (Higashiyama et al. *Life Sci. Alliance* 2024).

Collaborators:

Mizuki Higashiyama, Kei Haniuda, Yoshihito Nihei[1], Hiroyuki Iwasaki[1], Riku Hisato, Qi Zhao, Saiko Kazuno[2], Mika Kikkawa[2], Yoshiki Miura[2],Yusuke Suzuki[1] ([1]Department of Nephrology, Faculty of Medicine, [2]Biomedical Research Core Facilities, Juntendo University)

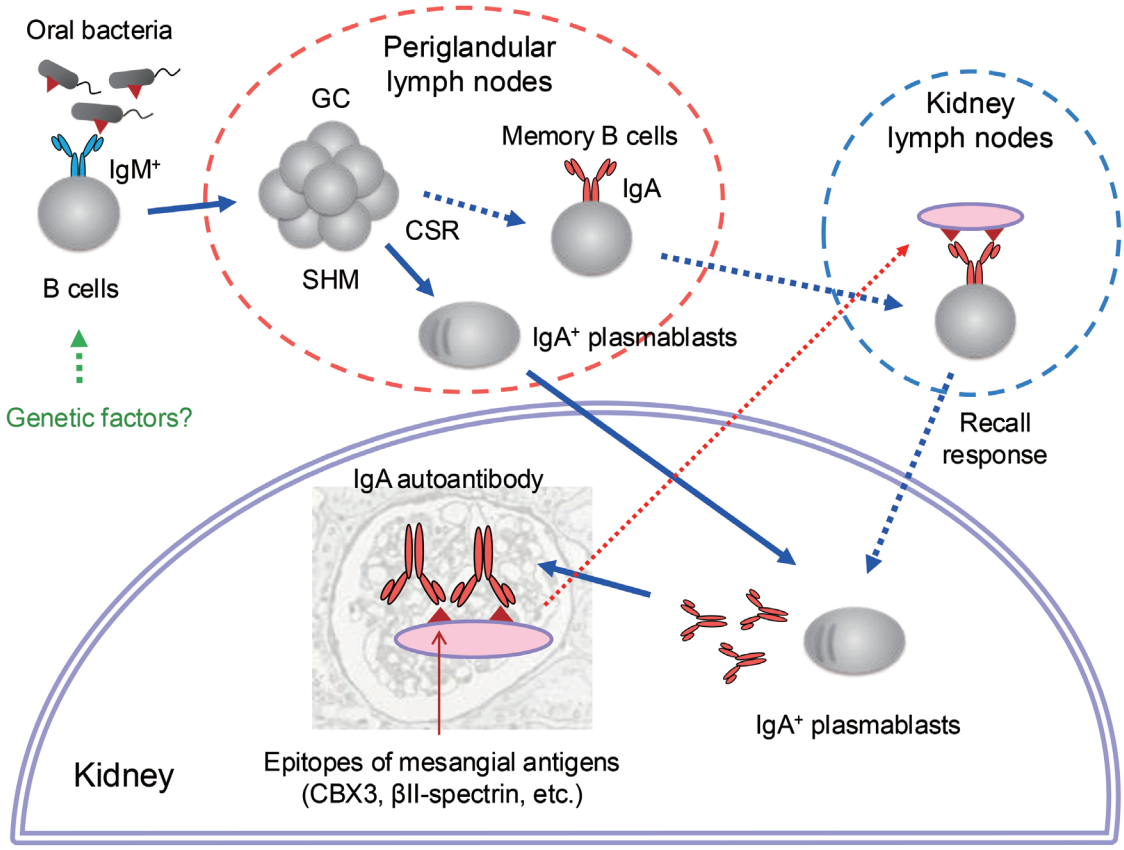

Figure 2. Oral commensal bacteria induce immune response producing anti-mesangial IgA through molecular mimicry to mesangial self-antigens in an IgA nephropathy model mouse

Commensal bacteria and the lung environment are responsible for natural IgE production in MyD88-deficient mice

IgE antibodies are common mediators of allergic responses, and are generally produced during type 2 immune responses to allergens. Allergen stimulation of IgE-bound FcεRI in mast cells or basophils induces production of chemical mediators and cytokines. In addition, IgE binding to FcεRI in the absence of allergens promotes the survival or proliferation of these cells and airway muscle cells. Thus, spontaneously produced natural IgE can increase an individual's susceptibility to allergic diseases. We previously reported that IgE^+ B cells are generated in GCs but immediately differentiate into short-lived PCs and die. This event is determined by the cell surface expression of the IgE class BCR and subsequent signaling through BLNK and CD19. For this reason, IgE^+ memory B cells and LLPCs are not generated, and blood IgE levels are normally maintained at very low levels (Haniuda et al. 2016, *Nat. Immunol*. 17:1109). However, nonspecific or natural IgE levels are often high in individuals who later develop allergic diseases.

Mice deficient in MyD88, a major TLR signaling molecule, have high serum levels of natural IgE, the mechanism of which remains unknown. We found that high serum IgE levels were maintained after weaning by memory B cells but not by LLPCs. IgE from PCs and sera of most *Myd88*$^{-/-}$ mice, but not of the *Myd88*$^{+/-}$ mice, recognized *Streptococcus azizii* (*S. azizii*), a commensal bacterium that is overrepresented in the lungs of *Myd88*$^{-/-}$ mice. $IgG1^+$ MBCs from the spleen also recognize *S. azizii*. Serum IgE

levels declined upon antibiotic administration and were boosted by challenge with *S. azizii* in *Myd88*$^{-/-}$ mice, indicating the contribution of *S. azizii*-specific IgG1^{+} MBCs to natural IgE production. Th2 cells, but not Th1, Th17, or Treg cells, were increased in the lungs of *Myd88*$^{-/-}$ mice and were activated upon addition of *S. azizii* to lung cells *ex vivo*. Finally, lung non-hematopoietic cells and CSF1 overproduced therefrom were responsible for natural IgE production in *Myd88*$^{-/-}$ mice. Indeed, conventional dendritic cells (cDC2) expressing the CSF1-receptor were increased in the lungs of *Myd88*$^{-/-}$ mice. Thus, CSF1 production in some lung cells is normally restrained by MyD88, which may be stimulated by commensal bacteria that interact with TLRs. When this signal is depleted, CSF1 is produced and stimulates cDC2 cells that initiate a Th2 response triggered by antigens derived from other commensal bacteria, such as *S. azizii*, which in turn generates memory B cells that continuously produce natural IgE through persistent recall responses (Amano et al. *J. Immunol*. 2023).

Collaborators:

Shunsuke Amano, Kei Haniuda, Saori Fukao, Hiroyasu Aoki*, Satoshi Ueha*, Jianyi Zhang, Lalhaba Oinum (*Division of Molecular Regulation of Inflammatory and Immune Diseases)

Publications

Daisuke Kitamura, M.D., D.M.S.

1. Higashiyama, M., Haniuda, K., Nihei, Y., Kazuno, S., Kikkawa, M., Miura, Y., Suzuki, Y., Kitamura, D. Oral bacteria induce IgA autoantibodies against a mesangial protein in IgA nephropathy model mice. ***Life Sci. Alliance*** 7:e202402588, 2024.
2. Yada, Y., Matsumoto, M., Inoue, T., Baba, A., Higuchi, R., Kawai, C., Yanagisawa, M., Kitamura, D., Ohga, S., Kurosaki, T., Baba, Y. STIM-mediated calcium influx regulates maintenance and selection of germinal center B cells. ***J. Exp. Med.*** 221:e20222178, 2024.
3. Thomann, A.S., McQuade, C.A., Pinjušić, K., Kolz, A., Schmitz, R., Kitamura, D., Wekerle, H., Peters, A. A B cell-driven EAE mouse model reveals the impact of B cell-derived cytokines on CNS autoimmunity. ***Proc. Natl. Acad. Sci. USA***. 120:e2300733120, 2023.
4. Horiuchi, S., Koike, T., Takebuchi, H., Hoshino, K., Sasaki, I., Fukuda-Ohta, Y., Kaisho, T., Kitamura, D. SpiB regulates the expression of B-cell-related genes and increases the longevity of memory B cells. ***Front. Immunol.*** 14:1250719, 2023.
5. Li, W., Nakano, H., Fan, W., Li, Y., Sil, P., Nakano, K., Zhao, F., Karmaus, P.W., Grimm, S.A., Shi, M., Xu, X., Mizuta, R., Kitamura, D., Wan, Y., Fessler, M.B., Cook, D.N., Shats, I., Li, X., Li, L. DNASE1L3 enhances antitumor immunity and suppresses tumor progression in colon cancer. ***JCI insight*** 8: e168161, 2023.
6. Nihei, Y., Higashiyama, M., Miyauchi, K., Haniuda, K., Suzuki, Y., Kubo, M., Kitamura, D.Subcutaneous immunization with zymosan generates mucosal IgA-eliciting memory and protects mice from heterologous influenza virus infection. ***Int. Immunol.*** 35: 377-389, 2023.
7. Kato, K., Haniuda, K., Fukao, S., Kitamura, D. B-cell-intrinsic DNase1L3 is essential for T-cell-independent type-II response in mice. ***Int. Immunol.*** 35:275-286, 2023.
8. Gokhale, S., Victor, E., Tsai, J., Spirollari, E., Matracz, B., Takatsuka, S., Jung, J., Kitamura, D., Xie, P. Upregulated expression of the IL-9 receptor on TRAF3-deficient B lymphocytes confers Ig isotype switching responsiveness to IL-9 in the presence of antigen receptor engagement and IL-4. ***J. Immunol.*** 210: 1059-1073, 2023.
9. Amano, S., Haniuda, K., Fukao, S., Aoki, H., Ueha, S., Kitamura, D. Commensal bacteria and the lung environment are responsible for

Th2-mediated memory yielding natural IgE in MyD88-deficient mice. ***J. Immunol.*** 210:959-972, 2023.

Division of Cancer Cell Biology

Shunsuke Kon, Ph.D.

Cancer is a leading cause of death in Japan, making the establishment of innovative and effective treatments urgently needed. Upon oncogenesis, a single epithelial cell undergoes an initial transformation due to gene mutations induced by various factors, such as pathogens, sunlight, exposure to carcinogens, and cell division. Subsequently, additional mutational burdens cause these transformed cells to become invasive malignant cells. It is estimated that thousands of transformed cells with oncogenic alterations are generated in the body every day. To eliminate these harmful transformed cells, which are detrimental to tissues, we employ tumor surveillance systems to fight against emerging transformed cells. Cell competition is an inherent property that living organisms prosses to fulfill this clearance function. When transformed cells appear within epithelial tissues, neighboring normal epithelial cells can recognize and eliminate them by pushing them into the apical lumen, effectively squeezing the transformed cells out of the epithelium. This biological phenomenon is referred to as 'apical extrusion' of transformed cells and represents one mechanism by which less fit transformed cells are eliminated through cell competition.

Our current primary focus is on understanding how cell competition is mechanically secured and how it becomes functionally disrupted at the onset of carcinogenesis. To investigate the functional alterations in cell competition during multi-sequential carcinogenesis, we utilize a cell competition mouse model that we previously established. We examine how the accumulation of genetic mutations affects the fate of transformed cells in a competitive environment. Additionally, we study the molecular mechanisms underlying the deregulation of cell competition caused by the accumulation of genetic insults. One of significant questions that need to be addressed in the field of cell competition is how normal epithelia cells recognize and eliminate neighboring transformed cells. To tackle this issue, we establish a novel platform in which normal cells adjacent to transformed cells are specifically labelled. Furthermore, we examine functional sex difference in cell competition using our mouse model. Beyond cell competition, we have expanded our research projects to unravel the principle underlying the invasion of cancer cells into lymphatic vessels. These studies will contribute to a comprehensive understanding of the physiological responses to the production of cancer cells.

Disruption of cell competition during multi-sequential carcinogenesis

We utilized familial adenomatous polyposis (FAP) in humans as a model to study the functional alteration of cell competition in multi-sequential carcinogenesis. During the development of FAP, the initial mutation of the APC gene induces neoplasia, and subsequent activation of the small GTPase Ras leads to carcinoma transition. To mimic the preexisting genetic insults of FAP in mice, we engineered the mice to sustain mosaic, somatic activation of Ras in the background of APC deficiency (APC^{min} mice). As a result, we found that a significantly higher number of RasV12-transformed cells diffusively invade the basal lamina in APC-

deficient mice compared to wild-type mice. In addition, APC^{min}/RasV12-transformed cells that have invaded the basal membrane penetrate through the basement membrane and expand in the stroma of mucosal epithelia, without accompanying any papillary adenomatous lesions in surrounding tissues, suggesting that cancer cells are directly generated from the normal mucous membrane through diffuse invasion of transformants (de novo carcinoma). Collectively, we found that Wnt activation induced by APC deficiency causes malfunction of cell competition and potentiates the diffuse invasion of transformed cells, resulting in de novo carcinoma in mice.

Collaborators:

Kazuki Nakai, Hancheng Lin

Molecular mechanism underlying diffuse invasion of transformed cells through malfunction of cell competition

This year, we have intensively explored the molecular basis of diffuse invasion of transformed cells caused by the malfunction of cell competition. To phenocopy the diffuse invasion of APC^{min}/RasV12 cells observed in mice in vitro, we had previously established Mardin-Darby Canine Kidney (MDCK) cells stably expressing an N-terminally deleted β-catenin mutant (β-catΔN), which is a non-degraded form of β-catenin (β-catΔN cells), and β-catΔN-expressing MDCK cells that express GFP-RasV12 in a tetracycline-dependent manner (β-catΔN/RasV12 cells). We then found that when β-catΔN/RasV12 cells are cultured alone, they remain within the epithelia. In contrast, when β-catΔN/RasV12 cells are co-cultured with β-catΔN cells at a ratio of 1:50, a substantial number of β-catΔN/RasV12 cells diffusively invade into the collagen matrix over time, highlighting the non-cell autonomous diffuse invasion of β-catΔN/RasV12 cells. Thus, we successfully generated an in vitro cell-culture system that recapitulates the salient feature of basal invasion of APC^{min}/RasV12 cells. Using this system, we conducted a comprehensive transcriptome analysis to search for molecules whose expression is changed in β-catΔN/RasV12 cells when co-cultured with β-catΔN cells. Consequently, we found that MMP21, a member of the matrix metalloproteinases (MMPs), is profoundly upregulated non-cell autonomously. Furthermore, quantitative PCR analysis revealed that MMP21 is by far the most abundant molecule among MMP isozymes. To characterize the proteolytic capabilities of MMP21, we produced a recombinant protein of the catalytic domain of MMP21 using E. coli and subjected it to incubation with several ECMs, which are principal constituents of connective tissues. Accordingly, the MMP21 catalytic domain extensively degrades collagen type I and collagen type IV, whereas digestion of fibronectin generates both small and large fragments. In contrast, laminin is resistant to hydrolysis, indicating that MMP21 is a competent Zn2+-dependent endoproteinase with unique specificity for collagen type I, collagen type IV, and fibronectin. We then knocked MMP21 down and found that the loss of MMP21 significantly decreases the frequency of non-cell autonomous diffuse invasion of β-catΔN/RasV12 cells, highlighting an active role of MMP21 in the non-cell autonomous basal invasion of transformants. Furthermore, GSEA analysis revealed that the NF-κB signal is activated in β-catΔN/RasV12 cells co-cultured with β-catΔN cells, and inhibition of the NF-κB pathway abrogates both non-cell autonomous MMP21 upregulation and basal invasion of β-catΔN/RasV12 cells. These results indicate that NF-κB signal positively regulates basal invasion of transformed cells through MMP21 upregulation (Nakai et al., Nature Communications, 2023; Figure 1).

Collaborators:

Kazuki Nakai, Hancheng Lin

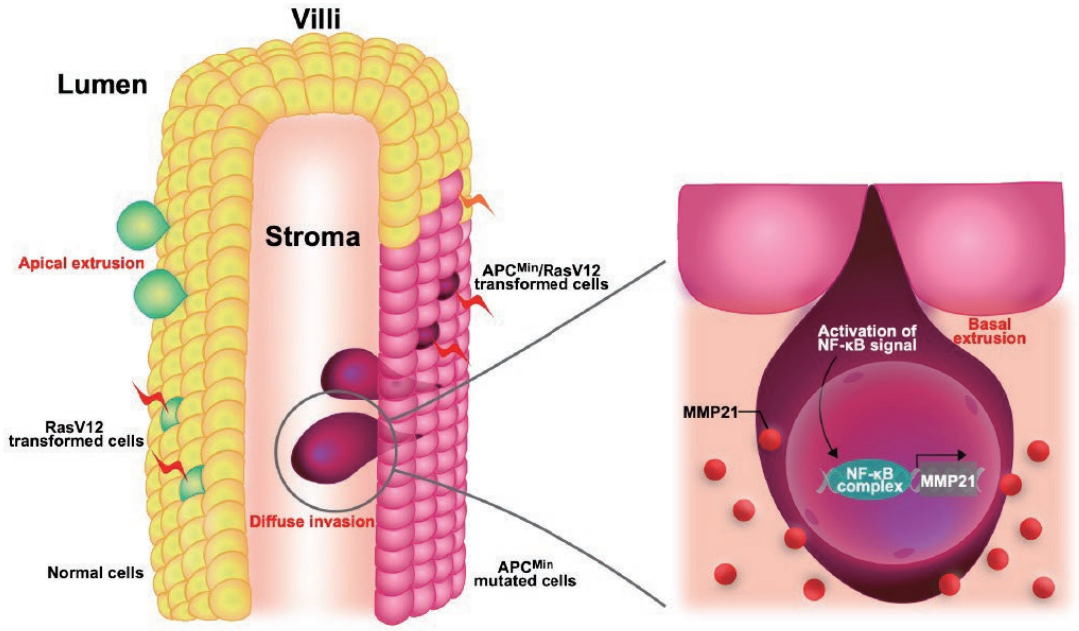

Figure 1. A schematic model for molecular mechanisms of the diffuse invasion of APC^{Min}/RasV12-transformed cells.

Identification of cell competition markers

Cellular events occurring during the extrusion of transformed cells have been well-studied, with perturbation in autophagic flux as one of the crucial modulators (Akter et al., Cell Rep., 2022). However, the molecular mechanisms underlying the elimination pressure exerted by neighboring normal cells remain incompletely understood. Moreover, the specific cell competition markers, which express in normal cells engaged in this process, have yet to be identified. A significant obstacle preventing us from addressing these issues has been the lack of methods to specifically label normal cells that are next to transformed cells. To overcome this challenge, we develop an in vitro culture system for specifically labelling and isolating normal cells adjacent to transformed cells by employing the secretory GPI anchored reconstitution-activated highlighting intercellular connections (sGRAPHIC) technique. This technique is based on the split GFP reconstitution system, in which soluble N-terminal GFP produced by doner cells binds to membrane anchoring C-terminal GFP expressed in targeted cells over a short range. Using this probe, we introduce either the membrane-associated N-terminal fragment of GFP gene (nGRAPHIC) or the secretory C-terminal fragment of GFP (cGRAPHIC) gene conjugated to Kusabira-Orange (KO2), as well as RasV12 gene into Madin-Darby canine kidney (MDCK) cells. Fluorescence emission from GFP occurs exclusively upon the binding of secreted cGRAPHIC fragments to nGRAPHIC fragments. Transcriptomic profiling of isolated GFP-positive normal cells was conducted to identify genes exhibiting upregulated expression patterns in the context of cell competition. Candidates identified through transcriptomic profiling were validated using quantitative polymerase chain reaction (qPCR), and their expression patterns *in vitro* were further investigated via fluorescence *in situ* hybridization (FISH). Subsequently, candidates displaying elevated expression levels *in vitro* were narrowed down to twelve genes, which were further validated through *in vivo* immunostaining. We plan to investigate the roles of these genes in cell competition, ultimately aiming to establish

the master regulators and markers of cell competition.

Collaborators:

Eilma Akter, Olivia Marchia Kusuma

Dissection of lymphovascular invasion by malignant cells

We have previously observed that APC^{min}/RasV12 cells often metastasize and preferentially invade lymphatic vessels rather than blood vessels. This prompted us to investigate the nature of lymphatic invasion by cancer cells, which has remained largely unexplored. This year, we develop a whole-mount staining method for the small intestine to visualize the 3D structure of vascular networks. Through this method, we discovered that Lyve-1-positive lymphatic vessels gradually disappear after the emergence of APC^{min}/RasV12 de novo cancer cells in stromal tissues. This conclusion is based on observations showing that the size, length, and width of lacteals, which comprise the lymphovascular system in the intestines, decrease over time in the presence of de novo tumors. To assess the function of lymphatic vessels, mice were administered with Oil Red O, which is absorbed in intestinal tissues through the lymphatic system. We found that lymphatic function is substantially disturbed in APC^{min}/RasV12-induced tumors. Notably, lymphatic endothelial cells close to cancer cells do not exhibit any signs of cell death, suggesting that APC^{min}/RasV12 de novo cancer cells induce regression of lymphatic vessels through a mechanism other than cell death induction. This led us to conduct single-cell RNA sequencing analysis, revealing profound changes in gene expression related to Endothelial-to-Mesenchymal Transition (EndMT) in tumor regions. Immunostaining with EndMT markers such as Transgelin or α-SMA shows that lymphatic endothelial cells surrounded by APC^{min}/RasV12 cells are positive for these markers. Furthermore, we found that the expression of Prox1, a master regulator of lymphatic endothelial lineage commitment, diminishes prior to the downregulation of Lyve-1 upon the formation of APC^{min}/RasV12-induced tumors. These results indicate that APC^{min}/RasV12 cancer cells induce the transdifferentiation of lymphatic endothelial cells into a certain type of mesenchymal cells,

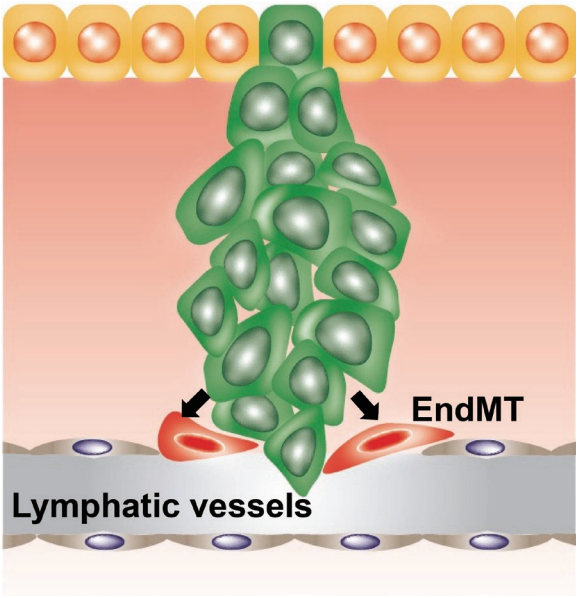

Figure 2. A schematic model for lymphovascular invasion of cancer cells.

leading to the disorganization of lymphatic vessels and potentiation of cancer cell invasion (Figure 2). We are now planning to establish an in vitro system to recapitulate EndMT induction caused by malignant cells and identify the key molecules that play a central role in this process in the coming year.

Collaborators:

Hancheng Lin, Kailai Xu

Sex difference in cell competition

We initially compared the elimination rates of RasV12-transformed cells by cell competition between adult males and females in the epithelial cell layers of the lung, pancreas, liver, and small intestine using a cell competition model mouse (*CK19*-CreERT2/ *DNMT1*-CAG-loxp-stop-loxp-RasV12-IRES-eGFP mouse). A sex difference was observed only in the pancreas, with females showing a higher elimination rate than males. However, in prepubertal (3-week-old) mice, no sex difference was observed in the pancreas, suggesting that the difference emerges during puberty. To explore the contribution of sex steroid hormones, which are implicated in various cases of functional sex differences, we surgically removed the gonads (testes or ovaries) from prepubertal and adult mice and quantified RasV12-transformed cell elimination rates in the pancreas 5 weeks later. Unexpectedly, sex differences in the elimination rates of RasV12-transformed cells persisted even after gonad removal, indicating that a sex steroid hormone-independent mechanism may be responsible for this phenomenon. This led us to focus on a molecule called UTX, an H3K27 demethylase located on the X chromosome that can escape X chromosome inactivation, as a potential factor in the mechanism underlying sex differences in pancreatic cell competition. Through fluorescent immunostaining, we quantified UTX levels in pancreatic ductal cells at prepubertal, pubertal, and adult stages, and found that UTX abundance was significantly higher in females than males at puberty. To examine the role of UTX, we inhibited its activity using the inhibitor GSK-J4 during the pubertal period (4–6 weeks of age). In the control group, the elimination rate of RasV12-transformed cells remained higher in females. In contrast, in the GSK-J4-treated group, the sex difference was abolished, and elimination rates were restored to those observed at prepubertal stages. These findings suggest that UTX-mediated H3K27 demethylation during puberty plays a crucial role in establishing sex differences for cell competition in pancreas. In upcoming year, we plan to use inhibitors and knockout mice to identify factors influencing UTX levels in males and females during puberty. Additionally, we will employ UTX CUT&Tag to uncover downstream target genes which are directly involved in cell competition.

Collaborators:

Yuta Sakae

Publication

1. Nakai, K., Lin, H., Yamano, S., Tanaka, S., Kitamoto, S., Saitoh, H., Sakuma, K., Kurauchi, J., Akter, E., Konno, M., Ishibashi, K., Kamata, R., Ohashi, A., Koseki, J., Takahashi, H., Yokoyama, H., Shiraki, Y., Enomoto, A., Abe, S., Hayakawa, Y., Ushiku, T., Mutoh, M., Fujita, Y. and **Kon, S**#. Wnt activation disturbs cell competition and causes diffuse invasion of transformed cells through NF-kB-MMP21 pathway. **Nature Communications**, 14, 7048. 2023 #corresponding author
2. Akter, E. and **Kon, S**#. Autophagic perturbation caused by reduced lysosomal activity positively regulates cell competition. **Autophagy** 19, 1874-1875. 2023 #corresponding author