PROGRESS REPORT

Division of Cancer Cell Biology

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Members (Kitamura's lab.)

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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. It is known that GC is not essential for generating MBCs (Toyama et al. 2002, *Immunity* 17:329; Kaji et al. 2012, *J. Exp. Med*. 209:2079), but necessary for generating LLPC (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, 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, suggesting missing factor(s) required for SHM. It is also unclear 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. Our in-vitro-induced germinal center B cell (iGB) culture system may be useful to solve these questions (Nojima et al. 2011, *Nat. Commun*. 2:465; Haniuda and Kitamura. 2019, *Bio-protocol* 9: e3163). Indeed, iGB culture system has been utilized for the studies of B cells all over the world including ourselves. (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 the generation of SHM is regulated in GC B cells, how the fate of GC B cells toward MBC subsets is determined (Koike et al. 2019, *eLife* 8:e44245), how the MBC recall response is 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 IgE-producing B cells are 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).

Recently, we have more focused on the mechanisms of B-cell activation during T-cell-independent type 2 (TI-2) response, well-known antibody-producing immune response to particular antigens, which does not require T-cell help nor the pathogen-associated molecular patterns (PAMPs) such as LPS, components of TI-1 antigens, as detailed below.

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

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

The TI-2 response is crucial for protection against pathogens, such as Pneumococcus and Salmonella. Marginal zone B cells in the spleen and B1 cells, innate-like B cells mainly residing in the peritoneal and pleural cavities, are major players in this response. 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 underlying this process remain unknown. We recently reported that the proliferation, differentiation, and antibody production of splenic and peritoneal 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 its expression increased and translocated into the nucleus upon stimulation with a model TI-2 antigen [hydroxy-nitrophenyl acetyl (NP)-Ficoll]. Early BCR signaling upon TI-2 stimulation, including phosphorylation of Syk, BLNK, Erk, and NF-κB p65, was intact in DNase1L3-deficient B cells, and NF-κB was necessary for the upregulation of *DNase1L3* mRNA upon the same stimulation. Gene expression analysis of activated MZ B cells early after TI-2 immunization revealed that IRF4 and its target genes were significantly downregulated in DNase1L3-deficient B cells. In addition, the proliferation and plasma cell differentiation of DNase1L3-deficient B cells after TI-2 immunization were restored by IRF4 transduction. Thus, DNase1L3 appears to mediate B cell activation by BCR stimulation with the TI-2 antigen through the upregulation of IRF4 (Kato et al. 2023, *Int. Immunol*. 35:275). We also reported 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 dextran sulfate administration. Mechanistically, we revealed that PKCδ is necessary for the induction of AID and class switching through the 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; however, the underlying mechanism of this co-stimulation is 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 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 than in control mice, probably reflecting the contribution of IL-1 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 the 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 that macrophage depletion 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. However, peritoneal macrophages produced IL-1 during TI-2 stimulation in vitro only when B cells coexisted, suggesting that antigen-activated B cells promote IL-1 production by macrophages. We further found that stimulation of B cells with TI-2 antigen, IL-1, or both synergistically induced tyrosine phosphorylation of STAT3, with a lower level in DNase1L3-deficient B cells than in wild-type B cells. In mice with STAT3-deficient B cells, the TI-2 immune response was severely dampened. Finally, transduction of the constitutively active form of STAT3 restored the proliferation and plasma cell differentiation of DNase1L3-deficient B cells after stimulation with the TI-2 antigen and IL-1. As STAT3 has been reported to bind to *IRF4* gene loci, STAT3 may function downstream of DNase1L3 and requires DNase1L3 for its sufficient activation. However, the mechanism underlying the effect of DNase1L3 remains unclear.

Collaborators:

Mari Tenno, Yuko Emoto, Miyuki Hata[1], Kei Kato[1], Saori Fukao[1], Kei Haniuda[1] (former members of Kitamura lab.)

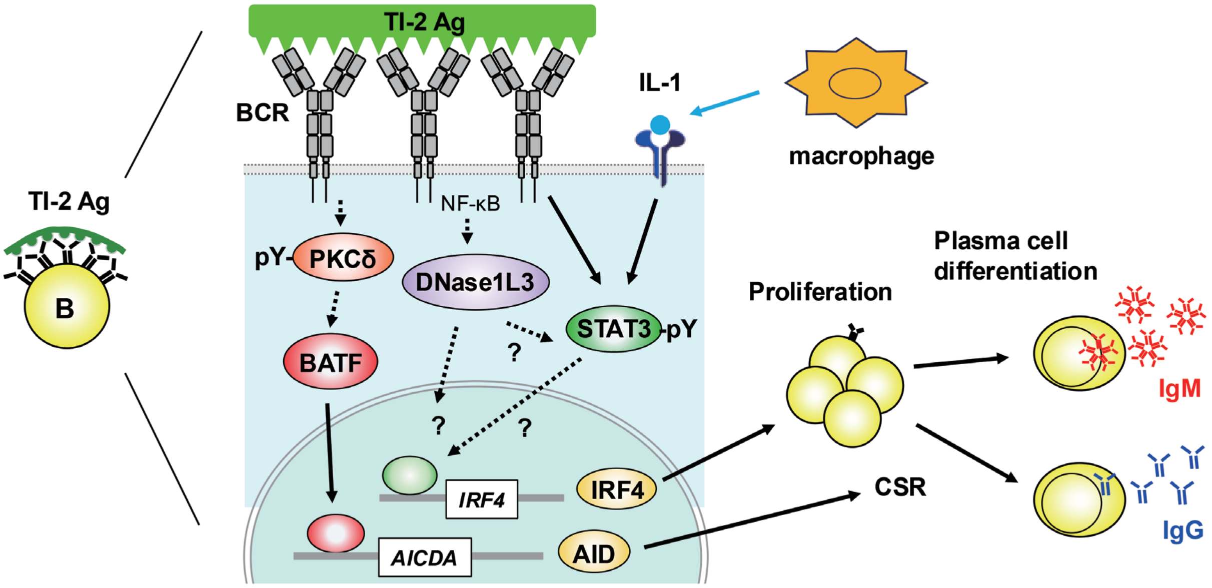

Figure 1. Molecular mechanisms of B cell activation in the TI-2 response

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 patients with renal diseases other than IgAN. Therefore, Gd-IgA1 alone cannot explain mesangium-specific IgA deposition in IgAN. Thus, the mechanisms underlying the selective deposition and generation of pathogenic IgA in IgAN remain unclear.

To address these problems, we have been studying by using gddY mice, a faithful IgAN mouse model. These mice were generated by the selective intercrossing of mice in the 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, resembling that observed in human IgAN (Okazaki et al. 2012, *J. Am. Soc. Nephrol.* 23:1364). Using this disease model, we reported the presence of 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 found 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, following intravenous administration. Based on these data, we proposed that the production of IgA autoantibodies against mesangial proteins, such as β2-spectrin, is the first trigger of IgAN pathogenesis; therefore, IgAN is a tissue-specific autoimmune disease (Nihei et al. *Science Adv.* 2023).

Subsequently, 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 self-antigen isolated from mesangial lysates was CBX3, a known heterochromatin protein. CBX3 was detected on the surface of mesangial cells and recognized by IgA in the sera of gddY mice and IgAN patients. Upon immunization with recombinant CBX3, BALB/c mice produced CBX3-specific IgG and IgA antibodies that were deposited in the mesangium. IgA$^+$ PBs accumulation in the kidneys, glomerular IgA deposition, proteinuria, and serum anti-CBX3 IgA production in gddY mice appeared to be dependent on commensal bacteria. GddY mouse serum IgA and rAb#66 bound to oral but not intestinal commensal bacteria from gddY mice, but not from BALB/c mice, and 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.” The binding of rAb#66 to C42 was competed by CBX3, suggesting molecular mimicry between the C42 and CBX3 epitopes. Immunization of BALB/c mice with C42 induced antibodies that bound to CBX3 and resulted in glomerular deposition of IgA in vivo. These 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 an immune response around the salivary glands, generating anti-mesengial memory B cells. These memory B cells are likely recruited to the kidney LNs and produce anti-mesangial IgA in response to mesangial antigens, which may be drained from the kidney. These findings will facilitate an

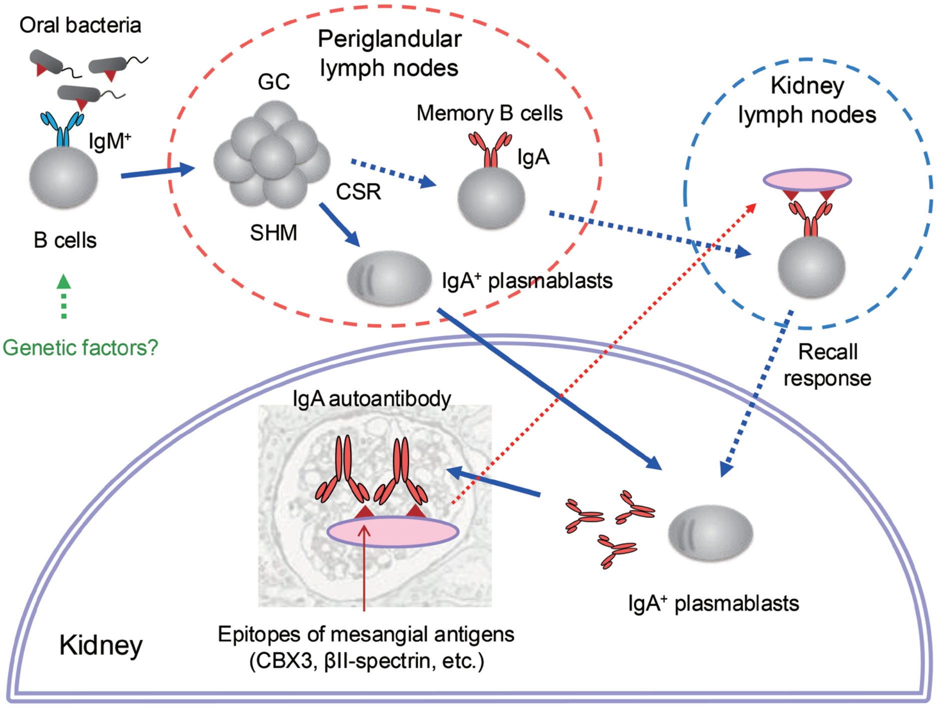

Figure 2. Oral commensal bacteria induce immune response producing anti-mesangial IgA through molecular mimicry to mesangial self-antigens in an IgA nephropathy model mouse

understanding of IgAN pathogenesis and therapeutic strategies for IgAN (Higashiyama et al. *Life Sci. Alliance* 2024). We are currently characterizing IgA autoantibodies recognizing other mesangial self-antigens in gddY mice and IgAN patients.

Collaborators:

Mizuki Higashiyama[1], Kei Haniuda[1], Yoshihito Nihei[2], Hiroyuki Iwasaki[2], Riku Hisato[1], Qi Zhao[1], Saiko Kazuno[3], Mika Kikkawa[3], Yoshiki Miura[3],Yusuke Suzuki[2] ([1]former members of Kitamura lab.; [2]Department of Nephrology, Faculty of Medicine, [3]Biomedical Research Core Facilities, Juntendo University)

Effects of airway allergic reactions on ciliary movement and mucus clearance in mouse trachea

The cilia present in the mammalian tracheal epithelium play a central role in mucus clearance by generating unidirectional mucus flow through the coordinated asymmetric movement of propulsive and recovery strokes. Mucus clearance in the tracheal epithelium is a vital biological defense mechanism against pathogenic invasion of the respiratory tract. The immune response associated with airway allergy is known to cause goblet cell hyperplasia, excessive mucus production, and eosinophil infiltration in the tracheal epithelium, mainly through the action of type 2 cytokines. However, its effects on tracheal ciliary movement remain poorly understood. We investigated this using an ovalbumin (OVA)-induced airway allergy model in mice to elucidate the changes in tracheal epithelial ciliary motility and mucus transport capacity during airway allergy, aiming to clarify the causal relationship between the immune response and impaired ciliary transport. Upon secondary challenge with intranasal OVA administration, we observed inflammatory cell infiltration into the epithelium and mucin accumulation on the epithelial surface using tissue histology. Additionally, the number of CD45-positive cells in the trachea increased, along with elevated mRNA expression of IL-4 and IL-13 in the trachea. Serum IgE levels were also elevated, confirming the induction of an allergic response.

The longitudinally bisected trachea of these mice was placed in a dish, and the tracheal epithelium was observed under an epifluorescence microscope. Fluid flow in the tracheal lumen, evaluated by the migration speed of fluorescent microbeads, was greatly reduced in allergy-induced mice. Ciliary motion was evaluated as the movement trajectories of fluorescent beads attached to ciliary tips using a three-dimensional position detection microscope, which revealed that its velocity, amplitude, and frequency were reduced in allergy-induced mice, and the asymmetry between effective and recovery stroke velocities was lost.

Based on these results, it is considered that the allergic reaction causes a reduction in the amplitude of ciliary movement, in which the waveform becomes nearly symmetrical, leading to a reduction in liquid flow generation capacity. We are currently working to elucidate the molecular mechanisms underlying the decline in ciliary function caused by allergic reactions.

Collaborators:

Shu Takahashi, Mari Tenno, Tomoko Masaike[1] ([1]Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science)

Involvement of autoantibodies in streptozotocin-induced type 1 diabetes mouse model

Type 1 diabetes (T1D) is an autoimmune disease primarily characterized by T cell-mediated destruction of pancreatic β-cells. While the role of T cells in T1D pathogenesis has been extensively studied, the contribution of B cells to this process remains unclear. In this study, we aimed to elucidate the involvement of B cells in T1D using a streptozotocin (STZ)-induced diabetic mouse model. Our results demonstrated the production of anti-insulin antibodies following low-dose STZ treatment and revealed that repertoire-sufficient B cells and plasma cells are essential for the development of T1D in this model. Furthermore, antigen-specific autoantibodies are necessary for disease onset, and depletion of the microbiota by antibiotics prevents T1D development. These findings suggest that B cells primed with commensal bacteria may be activated by self-antigens derived from STZ-damaged pancreatic β-cells, subsequently producing autoantibodies that induce pancreatic inflammation. This study highlights the previously underappreciated roles of B cells and microbiota in T1D pathogenesis, offering novel insights into potential therapeutic targets.

Collaborators:

Kenta Ueda, Mari Tenno, Chen Luo[1] ([1]former member of Kitamura lab.)

Publications

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

1. He M, D’Aulerio R, Pinho LG, Doukoumopoulos E, Yong T, Vieira RC, Oliveira MMS, Galvani RG, Sedimbi S, Seitz C, Kuznetsov NV, Zuriaga MA, Kitamura D, Fuster JJ, Cotta-de-Almeida V, Ström L, Degn SE, Westerberg LS. AID facilitates TET2 demethylation of *Irf4* for plasma cell fate in germinal center B cells. ***BioRxiv,*** 2025 doi: https://doi.org/10.1101/2025.05.02.651162 (manuscript under review).

2. Nihei Y, Kitamura D. Pathogenesis of IgA nephropathy as a tissue-specific autoimmune disease. ***Int. Immunol.*** 37: 75-81, 2024.

3. 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.

Division of Cancer Cell Biology

Shunsuke Kon, Ph.D.

Cancer remains a leading cause of death in Japan, underscoring the urgent need to establish innovative and effective treatments. Oncogenesis begins when a single epithelial cell undergoes an initial transformation caused by gene mutations induced by various factors such as pathogens, ultraviolet, radiation, carcinogen exposure, or errors during cell division. Subsequent accumulation of additional mutations drives these transformed cells to become invasive malignant cells. It is estimated that thousands of transformed cells with oncogenic alterations arise in the human body every day. To eliminate these potentially harmful cells, which are detrimental to tissues, the body employs tumor surveillance systems that act against emerging transformed cells. Cell competition is an intrinsic property of living organisms that 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, thereby expelling the transformed cells from the epithelium. This biological phenomenon, known as 'apical extrusion' of transformed cells, represents one mechanism through which less fit cells are removed by cell competition.

Our current primary focus is to understand how cell competition is mechanically secured and how it becomes functionally disrupted at the onset of carcinogenesis. A key unresolved question in the field is how normal epithelial cells recognize and eliminate their transformed neighbors. To address this, we have developed a novel platform that enables specific labeling of normal cells adjacent to transformed cells. In addition, we are exploring sex-specific difference in cell competition using our mouse model. Beyond the scope of cell competition, by utilizing the *de novo* cancer mouse model that we previously established (Nakai et al., Nat. Commun., 2023), our research has expanded to uncover the principles governing cancer cell invasion into lymphatic vessels and relationship between cancer malignancy and cellular senescence. Collectively, these studies aim to provide a comprehensive understanding of the physiological responses that occur the emergence of cancer cells.

Identification of cell competition markers

Cellular events occurring during the extrusion of transformed cells have been well-studied, with perturbations in autophagic flux identified as one of the key modulators (Akter et al., Cell Rep., 2022). However, the molecular mechanisms underlying the elimination pressure exerted by neighboring normal cells remain incompletely understood. Moreover, specific markers that are expressed in normal cells engaged in this process have yet to be identified. A major obstacle in addressing these issues has been the lack of methods to specifically label normal cells that are adjacent to transformed cells. To overcome this challenge, we developed an *in vitro* culture system that enables selective labelling and isolation of normal cells neighboring transformed cells by employing the secretory GPI-anchored reconstitution-activated highlighting intercellular connections (sGRAPHIC) technique. This approach is based on a split-GFP

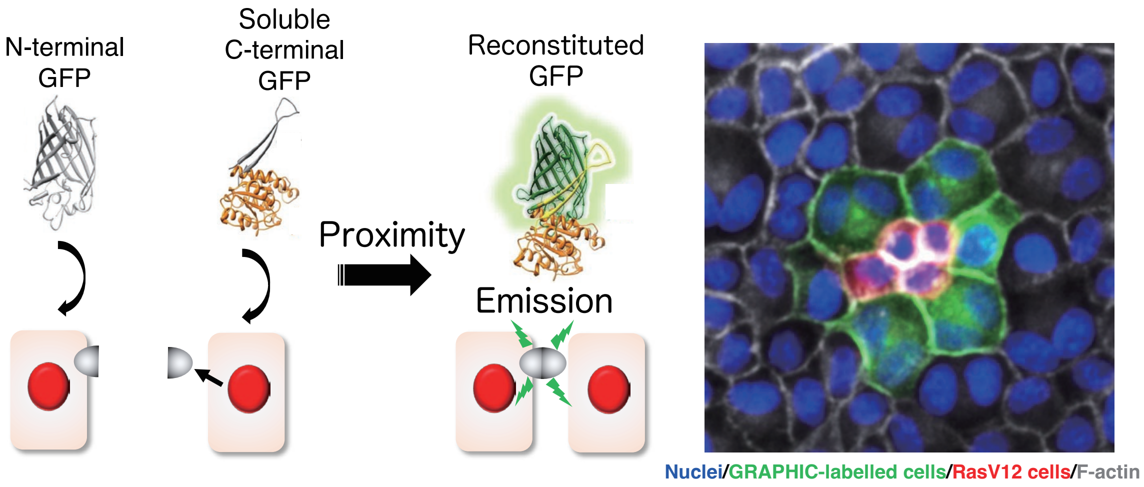

Figure 1. A schematic view of sGRAPHIC and an image showing GFP-labelled normal cells neighboring RasV12-transformed cells.

reconstitution system, in which soluble C-terminal GFP produced by doner cells binds over a short range to membrane-anchored N-terminal GFP expressed in targeted cells. Using this probe, we introduced into Madin-Darby canine kidney (MDCK) cells either the membrane-associated N-terminal fragment of GFP (nGRAPHIC) or the secretory C-terminal fragment of GFP (cGRAPHIC) conjugated to Kusabira-Orange (KO2), along with the oncogenic *RasV12* gene. We found that normal cells adjacent to *RasV12*-expressing cells were efficiently labeled with GFP (Figure 1). Subsequently, transcriptomic profiling of the isolated GFP-positive normal cells was conducted to identify genes whose expression was upregulated in the context of cell competition. Candidate genes identified through transcriptomic profiling were validated using quantitative polymerase chain reaction (qPCR), and their expression patterns *in vitro* were further examined by fluorescence *in situ* hybridization (FISH) and immunofluorescence. From these analyses, twelve genes showing elevated expression levels *in vitro* were selected for further validation *in vivo* using a cell competition mouse model. We plan to investigate the roles of these genes in cell competition, ultimately aiming to establish the master regulators and definitive markers of cell competition.

Collaborators:

Eilma Akter, Olivia Marchia Kusuma, Kengo Matsubaww

Dissection of lymphovascular invasion by cancer cells

we previously 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 greater number of RasV12-transformed cells diffusively invade the basal lamina in APC-deficient mice compared with wild-type mice. In addition, APC^{min}/RasV12-transformed cells that have invaded the basal membrane penetrate through the basement membrane and expand within the stromal compartment of the mucosal epithelium, without forming any papillary adenomatous lesions in the surrounding tissues. These findings suggest that cancer cells arise directly from the normal mucosal membrane through diffuse invasion of transformants; a process indicative of *de novo* carcinoma formation (Nakai et al., Nature Commun., 2023).

Using this mouse model, we observed that APC^{min}/RasV12 cells frequently metastasize and preferentially invade lymphatic vessels rather than blood vessels. This prompted us to investigate the mechanisms underlying lymphatic invasion by cancer cells, a process that has remained largely unexplored. This year, we developed a whole-mount staining method for the small intestine to visualize the three-dimensional architecture of vascular networks. Using this technique, we discovered that Lyve-1-positive lymphatic vessels gradually disappear following the emergence of APC^{min}/RasV12 *de novo* cancer cells in stromal tissues. This conclusion is supported by quantitative analyses showing that the size, length, and width of lacteals, which comprise the central lymphatic vessels of intestinal villi, progressively decrease over time in the presence of *de novo* tumors. To assess lymphatic function, we administered Oil Red O, a lipid dye that is absorbed into intestinal tissues through the lymphatic system. We found that lymphatic transport is markedly impaired in APC^{min}/RasV12-induced tumors. Notably, lymphatic endothelial cells close to cancer cells did not exhibit any signs of cell death, suggesting that regression of lymphatic vessels is mediated by a mechanism other than cell death induction. This led us to perform single-cell RNA sequencing, which revealed profound alterations in gene expression associated with Endothelial-to-Mesenchymal Transition (EndMT) in tumor regions. Immunostaining with EndMT markers such as Transgelin and α-SMA demonstrated that lymphatic endothelial cells surrounded by APC^{min}/RasV12 cells were positive for these markers. Furthermore, we observed that the expression of Prox1, a master regulator of lymphatic endothelial cell lineage commitment, diminishes prior to the downregulation of Lyve-1 upon formation of APC^{min}/RasV12-induced tumors. Collectively, these findings indicate that APC^{min}/RasV12 cancer cells induce transdifferentiation of lymphatic endothelial cells into mesenchymal-like cells, leading to disorganization of lymphatic vessels and enhanced cancer cell invasion (Figure 2). We are now planning to establish an *in vitro* system to recapitulate cancer cell-induced EndMT and to identify key molecular mediators that drive this process in the coming year.

Collaborators:

Hancheng Lin, Kailai Xu, Kosuke Terao, Yukari Yamazaki

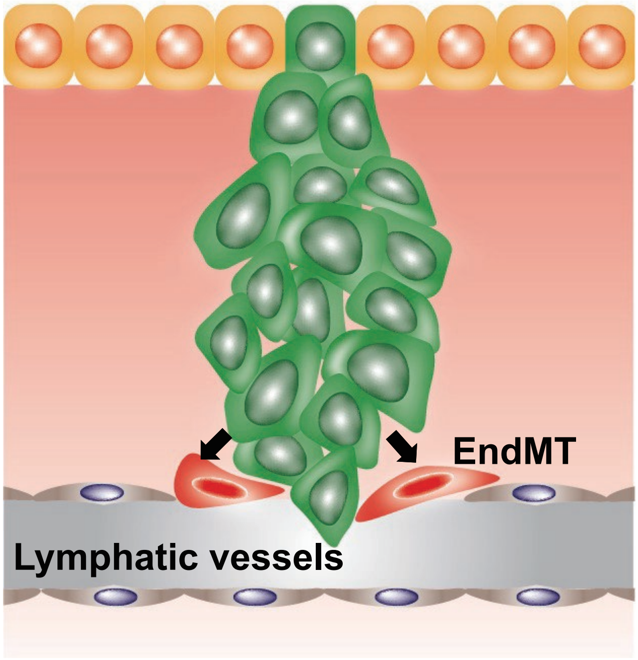

Figure 2. A schematic model for lymphovascular invasion of cancer cells.

Sex difference in cell competition

Previous studies using a mouse model of cell competition revealed sex differences in pancreatic cell competition, with females exhibiting a higher rate of RasV12-transformed cell elimination than males. Furthermore, these sex differences in cell competition in the pancreas were established during puberty; however, sex steroid hormones were not involved in their establishment. We therefore focused on UTX as a potential factor contributing to development of sex differences in pancreatic cell competition. UTX is an H3K27 demethylase located on the X chromosome that can escape X-chromosome inactivation. Using immunofluorescence staining, we quantified abundance of UTX in the nuclei of pancreatic ductal epithelial cells and found that females showed higher UTX level than males only during puberty. To examine the functional relevance of this finding, we inhibited the enzymatic activity of UTX using an inhibitor (GSK-J4) during puberty (4-6 weeks of age). As previously noted, females exhibited a higher rate of RasV12-transformed cell elimination in the pancreas of the than males, whereas no sex difference was observed in the GSK-J4-treated group. In contrast, adult mice treated with GSK-J4 for one week still displayed a sex difference in the rate of elimination of RasV12-transformed cells comparable to the control group. These results suggest that epigenetic changes mediated by UTX-mediated H3K27 demethylation during puberty contribute to the establishment of sex difference in pancreatic cell competition. We next focused on leptin as a candidate factor responsible for inducing UTX expression in pubertal females. Leptin is known as a key upstream regulator of puberty onset, although its effects on non-neuronal organs are largely unknown. Immunofluorescence staining revealed expression of leptin receptors, pSTAT3 and pSTAT5, in pancreatic ductal epithelial cells. The results showed that leptin receptors and pSTAT3 were expressed in pancreatic ductal epithelial cells. Moreover, a STAT3-binding sequence was identified approximately 12 kbp upstream of the UTX transcription start site, suggesting that leptin-STAT3 signaling may induce UTX expression. To test this hypothesis, we used a short-term culture system of pancreatic tissue fragments and found that leptin treatment indeed induced UTX expression in the female pancreas. We next sought to perform CUT&Tag analysis using isolated pancreatic ductal epithelial cells to identify downstream tragets regulated by UTX. However, attempts to isolate these cells from mouse pancreases by FACS resulted in contamination with acinar and endocrine cells, as confirmed by RT-qPCR, making pure isolation infeasible. To overcome this limitation, we plan to utilize Sox9-EGFP KI mice, which have been successfully used for the isolation of pancreatic ductal epithelial cells, and attempt cell isolation next year. Furthermore, to determine whether the increased UTX expression in female pancreatic ductal epithelial cells during puberty results from escape from X-chromosome inactivation, we introduced KI mice carrying S-Tag and FLAG-Tag sequences inserted at the N-terminus of the endogenous UTX locus. Using heterozygous females, we will perform fluorescent immunostaining for these tags to determine whether UTX specifically escapes X-chromosome inactivation in pancreatic ductal epithelial cells during puberty.

Collaborators:

Yuta Sakae

Relationship between cancer malignancy and cellular senescence

Cellular senescence play a double sword in cancer development. It has been initially conceived that senescence plays an tumor-suppressive role by inhibiting aggressive proliferation of cancer cells. In contrast, recent studies have suggested that senescence-induced cancer cells potentiate tumor growth through secreting Senescence-associated secretory phenotype (SASP) factors, which sustain to maintain stable tumor microenvironment. Thus, it remains debatable how senescence is involved in cancer development. This year, we initially found that cancer cells produced in APC^{min}/RasV12 mice (see details in above) gradually invaded in depth from mucosa to muscle layer, suggesting that they gain malignancy in time dependency. Interestingly, highly invasive malignant cells exhibit non-proliferative status, as the number of Ki67-positive cells get decreased when they invade into submucosa and muscle layer. This brought us to explore whether senescence is induced in malignant cancer cells by staining p21 molecule, which is a surrogate marker for senescence. As a result, the more cancer cells invade in depth, the more cancer cells are positive for p21. Collectively, these results imply that senescence is induced in cancer cells to acquire invasive phenotype. To explore this hypothesis in vitro, we set up the platform, using boyden-chamber, where we can evaluate invasion using cancer cell lines established from APC^{min}/RasV12 mice (named as LMPDC cells). We then found that LMPDC cells that invade into lower chamber show lower ki-67 positive fraction and higher p21-positive fraction compared to those of cells maintained in upper chamber, recapitulating the mice data. We are now planning to find molecules that serve to link between cancer malignancy and cellular senescence by utilizing scRNA-seq data.

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