PROGRESS REPORT
Division of Cell Fate Regulation

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Members

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- Ryo Goitsuka, Ph.D., D.V.M.

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- Naoko Nakano, Ph.D.

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Graduate student
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Division of Cell Fate Regulation

Chairman: Ryo Goitsuka, Ph.D., D.V.M.

Infancy and childhood are critical periods for the development and maturation of adaptive immunity. During this time, the immune system establishes protective immunity to pathogens and tolerance to innocuous microbial and environmental antigens. The tissue-resident immune cells, such as macrophages and innate lymphoid cells, which are derived from both embryonic and postnatal precursors, show differences in distribution as well as in functions. Their tissue-specific signatures appear to be acquired during this narrow perinatal window of time, thus implicating any defects in these processes in the risk for immune-associated diseases. As a key perinatal organ that connects fetal liver to bone marrow hematopoiesis, the spleen acts as a transient site both for hematopoiesis and lymphopoiesis, as well as for destruction of fetal-type erythrocytes. Fetal-type B cells, called B-1 cells, have unique autoreactive B cell antigen receptor specificities and first appear in the neonatal spleen. Later, they move into the peritoneal and pleural cavities, where they are clonally maintained as long-lived B cells by self-renewal. Their physiological functions remain mysterious, but they serve as a source of IL-10-producing regulatory B cells under inflammatory conditions as well as precursors of plasma cells that produce natural antibodies. To understand the function of fetal-derived lymphoid cells in adulthood in detail, we have recently established an experimental system that enables genetic manipulation of fetal lymphoid cells in adulthood, thus being ready to expand our knowledge on early life immunity that might affect susceptibility and severity of immune-mediated disorders.

B cells of early-life origin defined by RAG2-based lymphoid cell tracking under native hematopoietic conditions

During the perinatal period, the immune system sets the threshold to select either response or tolerance to environmental antigens, which leads to the potential to provide a lifetime of protection and health. B-1a B cells have been demonstrated to develop during this perinatal time window, showing a unique and restricted BCR repertoire, and these cells play a major role in natural Ab secretion and immune regulation. In the current study, we developed a highly efficient temporally controllable RAG2-based lymphoid lineage cell labeling and tracking system and applied this system to understand the biological properties and contribution of B-1a cells generated at distinct developmental periods to the adult B-1a compartments. This approach revealed that B-1a cells with a history of RAG2 expression during the embryonic and neonatal periods dominate the adult B-1a compartment, including those in the bone marrow (BM), peritoneal cavity, and spleen. Moreover, the BCR repertoire of B-1a cells with a history of RAG2 expression during the embryonic period was restricted, becoming gradually more diverse during the neonatal period and then heterogeneous at the adult stage. Furthermore, more than half of plasmablasts/plasma cells in the adult BM had embryonic and neonatal RAG2 expression histories. Moreover, BCR analysis revealed a high relatedness between BM plasmablasts/plasma cells and B-1a cells derived from embryonic and neonatal periods, suggesting that these cell types have a common origin. Together, these findings define, under

native hematopoietic conditions, the importance in adulthood of B-1a cells generated during the perinatal period.

Collaborators:

Fujisaki K., Okazaki S., Ogawa S., Takeda M., Sugihara E., Imai K., Mizuno S., Takahashi S.

The J chain acts as a critical regulator for intestinal IgA$^+$ plasma cell differentiation

The J chain is essential for IgA dimer formation and poly-Ig receptor binding. However, it shows no homology to any known protein structure, and there are no family molecules, the function of the J chain is incompletely understood, regardless of its immunological importance. We thus sought to examine the functions of the J chain in intestinal IgA-secreting plasma cells with the use of the reporter allele, in which a splice acceptor, EGFP-2A-CreERT2 cassette, and a poly-A sequence are knocked into the first intron of the J chain gene. In the pups harboring homozygous reporter alleles that lack the intact J chain protein (JKO), EGFP$^+$ IgA$^+$ cells were detected only at the base of the intestinal lamina propria, but absent in the villi, where EGFP$^+$ IgA$^+$ cells were abundant in heterozygous pups before weaning. RNA-seq analyses revealed that JKO EGFP$^+$ IgA$^+$ cells lack expression of CXCR5, a ligand of which CXCL13 is selectively expressed at the tip of the villi, suggesting the inability to migrate to the tip of the intestinal villi. To understand the life of cells expressing the J chain before weaning, newborn pups simultaneously having the Rosa26-tdTomato allele were treated with tamoxifen within 24 hours after the birth, and reporter expression in EGFP$^+$ IgA$^+$ plasma cells was analyzed at 10 weeks after the birth. In the intestinal lamina propria, approximately 20-30% of IgA$^+$ plasma cells were tdTomato$^+$ EGFP$^+$, whereas tdTomato$^+$ plasma cells expressing either IgA or IgM were almost absent in other tissues, including the bone marrow, spleen, Peyer's patches, and mesenteric lymph nodes. These tdTomato$^+$ EGFP$^+$ IgA$^+$ plasma cells disappeared by the loss of the J chain. Gene expression profiles differed between tdTomato$^+$ EGFP$^+$ and tdTomato$^-$ EGFP$^+$ cell populations, implying the distinct nature of intestinal IgA$^+$ plasma cells before and after weaning. Of note, IgA$^+$ plasma cells derived from before weaning express IL-18 and P2rx1, the former involving intestinal immune tolerance and the latter functioning as a sensor for extracellular ATP potentially provided by intestinal epithelial cells. Taken together, these findings indicate that intestinal IgA$^+$ plasma cells developed before weaning require the J chain to reach the niche of the villi, which leads to the persistent residence at the tip of the villi throughout life, and that these cells maintain the immune tolerance in the intestinal microenvironment.

Collaborators:

Fujisaki K., Okazaki S., Ogawa S., Kon, S.

The orphan G protein-coupled receptor 141 in myeloid cells is an inflammatory suppressor

G protein-coupled receptors (GPCRs) regulate many cellular processes in response to various stimuli, including light, hormones, neurotransmitters, and odorants, some of which play critical roles in innate and adaptive immune responses. However, the physiological functions of many GPCRs and their involvement in autoimmune diseases of the central nervous system remain unclear. Here, we demonstrate that GPR141, an orphan GPCR belonging to the class A receptor family, suppresses immune responses. High GPR141 messenger RNA levels were expressed in myeloid-lineage cells, including neutrophils (CD11b$^+$ Gr1$^+$), monocytes (CD11b$^+$ Gr1$^-$Ly6C$^+$ and CD11b$^+$ Gr1$^-$Ly6C$^-$), macrophages (F4/80$^+$), and dendritic cells (CD11c$^+$). Gpr141-/-

mice, which we independently generated, displayed almost no abnormalities in myeloid cell differentiation and compartmentalization in the spleen and bone marrow under steady-state conditions. However, Gpr141 deficiency exacerbated disease conditions of experimental autoimmune encephalomyelitis, an autoimmune disease model for multiple sclerosis, with increased inflammation in the spinal cord. $Gpr141^{-/-}$ mice showed increased $CD11b^{+}$ $Gr1^{+}$ neutrophils, $CD11b^{+}$ Gr1- monocytes, $CD11c^{+}$ dendritic cells, and $CD4^{+}$ T cell infiltration into the experimental autoimmune encephalomyelitis-induced spinal cord compared with littermate control mice. Lymphocytes enriched from $Gpr141^{-/-}$ mice immunized with myelin oligodendrocyte glycoprotein 35-55 produced high amounts of interferon-γ, interleukin-17A, and interleukin-6 compared with those from wild-type mice. Moreover, $CD11c^{+}$ dendritic cells (DCs) purified from $Gpr141^{-/-}$ mice increased cytokine production of myelin oligodendrocyte glycoprotein 35-55-specific T cells. These findings suggest that GPR141 functions as a negative regulator of immune responses by controlling the functions of monocytes and dendritic cells and that targeting GPR141 may be a possible therapeutic intervention for modulating chronic inflammatory diseases.

Collaborators:

Sawabe A., Okazaki S., Nakamura A., Kaifu T.

Publications

Fujisaki K., Okazaki S., Ogawa S., Takeda M., Sugihara E., Imai K., Mizuno S., Takahashi S., and Goitsuka R.: B cells of early-life origin defined by RAG2-based lymphoid cell tracking under native hematopoietic conditions. ***J. Immunol.***, 213(3):296-305. (2024)

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Oda, A., Furukawa, S., Kitabatake, M., Ouji-Sageshima, N., Sonobe, S., Horiuchi, K., Nakajima, Y., Ogiwara, K., Goitsuka, R., Shima, M., Ito, T., Nogami, K.: The spleen is the major site for the development and expansion of inhibitor producing-cells in hemophilia A mice upon FVIII infusion developing high-titer inhibitor. ***Thromb. Res.***, doi: 10.1016/j.thromres.2023.03.003. (2023)

Kugii, Y., Kuroki, Y., Hara, Y., Goitsuka, R. and Nakano, N.: Lack of HVEM signals in thymocytes impairs conventional CD8 T cell selection and promotes memory-like CD8 T cell development. ***J. Immunol.***, 210, 1482-1493. (2023)

Division of Cell Fate Regulation

Masayuki Sakurai, Ph.D.

***Research Interest**: Deciphering Molecular Mechanisms of Nucleobase Modification:*

In the gene expression process, which is the "central dogma of life" that transcribes necessary genes from DNA to RNA and produces proteins as functional expression entities, appropriate gene expression is maintained by various regulatory mechanisms at each step. One of these regulatory mechanisms is the modification of the chemical structure of the four bases [A, G, C, T(U)] of DNA and RNA, which are the genetic information itself. Our research focuses on the elucidation of biological phenomena that ultimately contribute to changes in the chemical structures of bases at the molecular level.

Research Topics

We are currently focusing on the modification of adenosine (A), called inosine (I, Ino), by a deamination reaction. In the Watson-Crick type base pairing, which is essential in the central dogma, A forms an [A:T(U)] pair with thymine (T) or uridine (U), and guanosine (G) forms a [G:C] pair with cytidine (C). However, after deamination of A, Ino forms a base pair with C as well as G. As a result, base pairing is changed from A:T(U) to Ino:C, which is equivalent to editing from A to G in the genetic information and is called the A-to-I editing mechanism (Fig.1a). This mechanism was discovered in metazoans, and is carried out by the enzyme adenosine deaminase acting on dsRNAs (ADAR), which uses intracellular double-stranded RNA as a substrate. ADARs have functions that are closely related to the gene sequence of transcripts, splicing, protein-binding structures, translation efficiency, and innate immunity to foreign RNAs. Because of their name and history, ADARs are thought to act specifically on double-stranded RNAs. Recently, however, scientists, including our research group, have discovered that ADAR can edit RNA and DNA strands A-to-I RNA and A-to-I DNA using RNA:DNA hybrid strands as substrates. This finding suggests that an active A-to-G base-editing mechanism that results from A-to-I DNA editing in genomic DNA is intrinsic to mammals.

We are working to elucidate the regulatory mechanisms of gene expression that are modified and optimized by RNA-driven editing of DNA and the base chemical structure of RNA, which we view as nucleic acid editing biology. With the addition of this concept, we are developing a new field of research using RNA, which has been overlooked in the central dogma of life, as a mediator of linkage. In particular, we are investigating the molecular mechanism and biological significance of adenosine deamination editing of DNA (A-to-I DNA editing) using the DNA:RNA strand as a substrate, and the mechanism of cellular oncogenesis and disease onset caused by the disruption. As the main research item, we will establish a technique for labeling, purifying, and identifying inosine sites in RNA and DNA strands, which has been impossible until now [discovery through the development of new technology]. Next, we will elucidate the mechanisms by which DNA:RNA strand formation and inosinylation regulate gene expression and genome dynamics [Elucidation of Functions and Mechanisms], and apply our

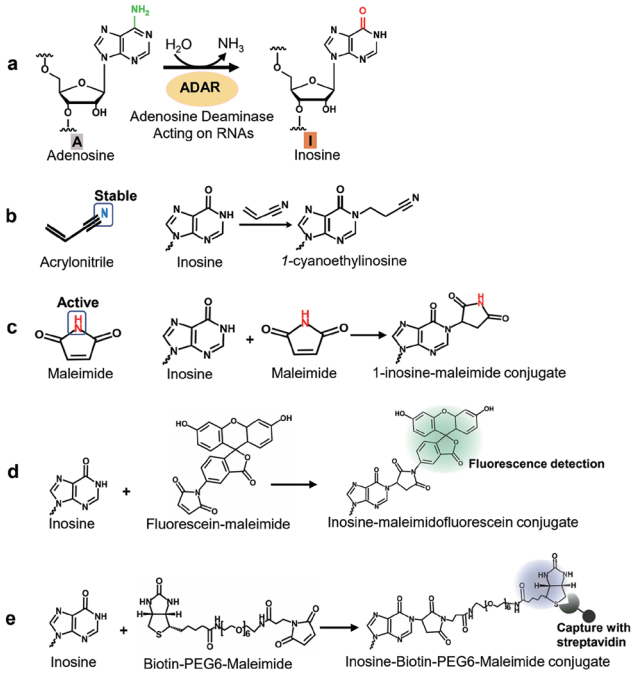

Fig1. A-to-I editing and invented chemical reactions for inosine.

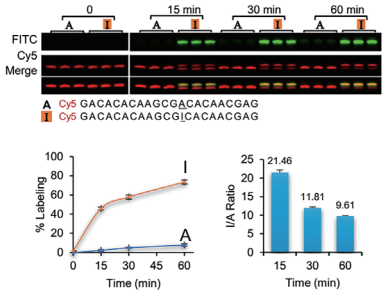

Fig2. Inosine specific fluoro-labeling.

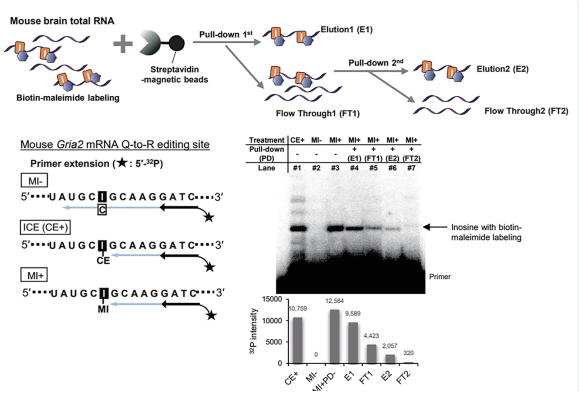

Fig3. Inosine specific molecular purification.

findings to medical and genetic engineering applications by developing artificial methods for the site-specific introduction and inhibition of A-to-I RNA and DNA editing [Applicative utilization of the mechanism].

Establishment of labeling and purification techniques for inosine modifications

Because Ino forms a base pair with C as well as G after editing, Ino on DNA functions as a G:C base pair after the replication phase or as a transcriptional template strand. Therefore, A-to-I DNA editing can be regarded as an active intracellular genome A>G (guanosine) mutagenesis mechanism. The reasons why active A-to-I DNA editing sites have not been identified so far are as follows: 1) The cause of A>G mutations in DNA is thought to be a simple accidental and spontaneous chemical change of adenosine (base part: adenine, A) to inosine (base part: hypoxanthine, H); 2) Ino is replaced by G in PCR amplification, and it is technically impossible to distinguish Ino from G; 3) the formation and function of the substrate RNA:DNA is unclear; and 4) it is extremely difficult to detect inosine on DNA, where only two molecules from homologous chromosomes exist per region, by sequence analysis methods that do not involve any kind of enrichment. To solve these difficulties, we developed a detection and identification method based on the chemical properties of inosine.

Sakurai and collaborators have already developed a technique (ICE method) to prove that it is Ino, not G, even through PCR reaction by using inosine-specific cyanoethyl group addition reaction(Fig.1b). This method has an accuracy of 97% and can be applied to next-generation sequencing. However, the sensitivity (depth) of

inosine detection depends on the expression level of each transcript, as in general transcriptome analysis. As the goal of this study was to identify inosine in genomic DNA or regulatory noncoding RNAs with low expression levels, the optimal solution was considered to be the enrichment and purification of inosine-containing nucleic acids. However, the cyanoethylation described above makes it difficult to label and purify inosine with additional fluorescence or tags because the additional functional groups are very stable and do not react.

Therefore, in this study, we developed a fluorescent or functional tag labeling technique specifically for inosine, which is applicable to both RNA and DNA(Fig.1c-e). We have established the conditions for inosine-specific fluorescent labeling (Fig.2) and other functional groups in nucleic acids, and succeeded in purifying nucleic acid molecules containing inosine by affinity purification in a functional group-dependent manner(Fig.3). This technique is called inosine-chemical labeling and affinity molecular purification (ICLAMP). We applied this technique to patents and published our research results based on this method (FEBS Letters 2024). In parallel, we have identified inosine sites in minor RNA and DNA in cultured human cells and mouse tissues using a method applied to next-generation sequencing and are currently analyzing and optimizing the data.

Collaborators:

Yuxi Yang (Sakurai Lab), Koki Nakayama, Shunpei Okada (Alumni member), Takeshi Wada, Kazuki Sato (Tokyo University of Science, Faculty of Pharmaceutical Sciences), Michiaki Hamada, Chao Zeng (Waseda University), Hiroki Ueda (The University of Tokyo)

Double-stranded RNA and DNA: RNA strand dynamics and significance of A-to-I editing

Focusing on the binding and targeting of ADAR proteins to double-stranded RNA and DNA:RNA hybrid strands and their dynamics in cells, the project aims to elucidate the A-to-I editing mechanism by ADARs and their interactions with nucleic acid-binding cofactor proteins. We are currently working to propose and establish the concept of a gene network of nucleic acid base modifications, which is a nucleic acid regulatory system for genome dynamics, transcription, RNA processing, and translation by enzymatic chemical editing of nucleic acids. We are currently investigating the significance of the A-to-I DNA editing ability and binding of ADARs to the DNA:RNA strand for life, and are focusing on a structure called R-loop as a candidate site of DNA:RNA strand formation where A-to-I DNA editing can occur. Normally, the newly synthesized RNA strand is immediately dissociated from the template DNA by transcription; however, in some sequences, the RNA strand is paired with the template DNA and remains to form a stable DNA:RNA strand. This structure is called the R-loop and is assumed to be involved in destabilizing factors of the genome structure and transcriptional regulation.

Phenotypic analysis of ADAR suppression in cultured cells revealed increased phosphorylation of γH2AX, RPA32, and DNA-PKCs, indicating increased DNA damage and activation of the repair systems. In HeLa cells, the localization of the R-loop in the nucleus was consistent with that of ADAR, and an increase in the amount of the R-loop was observed when ADAR expression was suppressed. In addition, an increase in the amount of R-loop was observed when ADAR expression was suppressed. We are currently investigating the molecular mechanism of mitosis-specific mitotic arrest observed upon ADAR suppression. We found that genes involved in the Spindle Checkpoint and genes

regulating mitotic entry form a complex with ADAR in the M-phase. We also found that protein phosphate modification was involved in the regulatory process. Next, to investigate the dynamics of ADAR in the M-phase, DNA-IP and RNA-IP by ADAR were analyzed by next-generation sequencing. We identified characteristic binding targets of ADARs using these analyses.

On the other hand, transcriptome analysis during ADAR repression identified transcription factor E as the gene with the highest transcriptional update. Furthermore, a comprehensive analysis using R-loop-specific antibodies revealed R-loop formation in the transcription initiation region of transcription factor E. Furthermore, it has been reported that transcription initiation of transcription factor E involves a neighboring noncoding RNA. We hypothesize that ADAR is involved in RNA:DNA strand-bound R-loop regulation in the transcription initiation region of the transcription factor E gene and are currently testing this hypothesis. We are also attempting to identify the R-loop region of the same type. We are also preparing recombinant ADARs and reconstructing them in vitro to elucidate the molecular mechanisms of their interaction with ADARs.

Collaborators:

Eito Ichihashi, Mai Kubota, Yuki Minato, Ryotaro Yanoshita, Michiaki Hamada (Waseda University), Chao Zeng (Waseda University), Nobuyoshi Akimitsu (The University of Tokyo)

Development of artificial A-to-I editing of target DNA/RNA sites for use in drug discovery

We are developing a technology to artificially inosinylate (synonymous with A>G substitution) target DNA sites (or RNA sites) by introducing synthetic guide RNA from outside the cell, utilizing the A-to-I DNA editing activity of ADAR and its knowledge, and applying it to genetic engineering and medical science as a genomic DNA base-editing method. We aimed to apply this technology to genetic engineering and medical science. To enable real-time measurement of editing efficiency in cells, we constructed an embedded reporter system in the genome of cultured human cells that expresses green fluorescent protein (GFP) when editing occurs. The success of artificial A-to-I editing depends on achieving high editing efficiency and ensuring specificity (avoiding off-targets). This depends on the subcellular localization of the introduced guide RNA, specificity of the complementary strand (efficiency of complementary strand formation with the target substrate), resistance to nuclease degradation (intracellular stability), binding affinity to ADAR (double-stranded nucleic acid A-type helix structure), and efficiency of deamination by ADAR (A-locusing at the enzyme activity center). We designed and used a guide nucleic acid chain with a double-stranded structure and a single-stranded guide sequence as the basic form and obtained GFP-positive cells in 1-10% of the cell population. These cells were isolated and purified, and inosinylation was verified.

Collaborators:

Yuxi Yang, Koki Takemura, Terutaka Kubota

Publication

1. Yang, Y., Nakayama, K., Okada, S., Sato, K., Wada, T., Sakaguchi, Y., Murayama, A., Suzuki, T., Sakurai, M. "ICLAMP: A Novel Technique to Explore Adenosine Deamination via Inosine Chemical Labeling and Affinity Molecular Purification. " *FEBS Lett*.598:1080-1093 [DOI:10.1002/1873-3468.14854 PMID: 38523059.] (2024)
2. Ichihashi, E., Kubota, M., Shiromoto, Y., Sakurai, M. "ADAR1 RNA editing enzyme regulates telomeric R-loop formation." Methods Mol. Biol. (2024, in press).