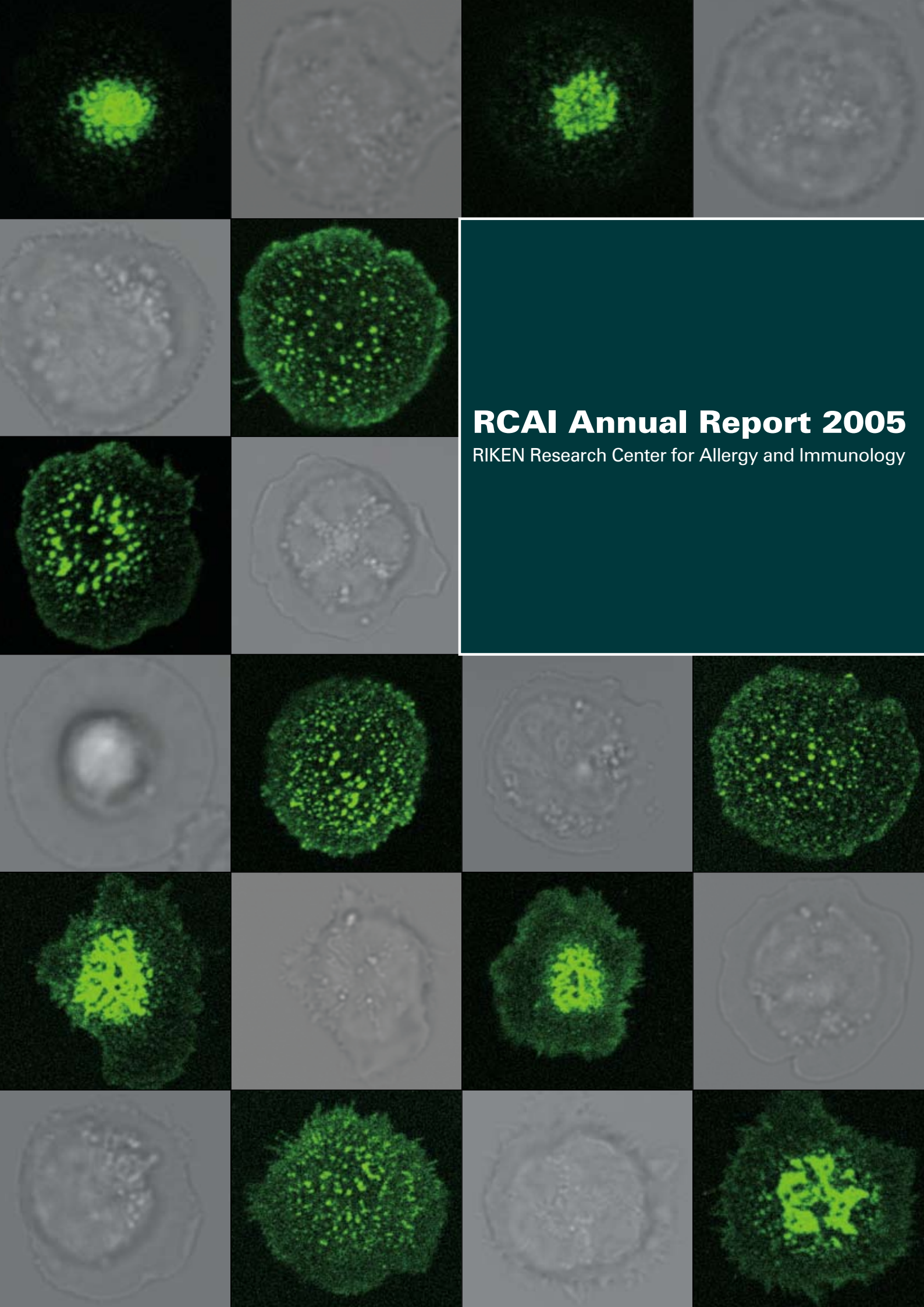

RCAI Annual Report 2005

RIKEN Research Center for Allergy and Immunology

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- Lab. for Immunogenetics **Hisahiro Yoshida M.D., Ph.D.**

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- Unit for Human Disease Model **Fumihiko Ishikawa M.D., Ph.D.**
- Unit for Allergy Transcriptome **Hirohisa Saito M.D., Ph.D.**

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- Unit for Cellular Immunotherapy **Shin-ichiro Fujii M.D., Ph.D.**
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Unit: Adjunct Research Program

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- Unit for Immune Homeostasis **Shohei Hori Ph.D.**

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- Unit for Lymphocyte Recognition **Facundo Damian Batista Ph.D.**
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- Unit for Thymic Environment **Willem van Ewijk Ph.D.**
- Unit for Thymocyte Differentiation **Wilfried Ellemeier Ph.D.**
- Unit for Immune Tolerance **Yun-Cai Liu Ph.D.**

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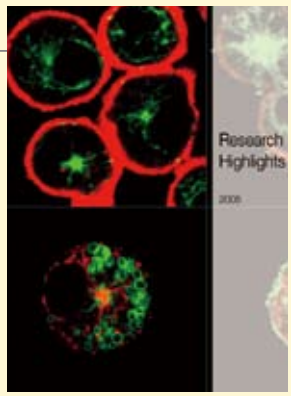

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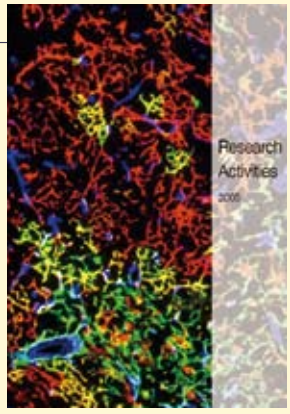

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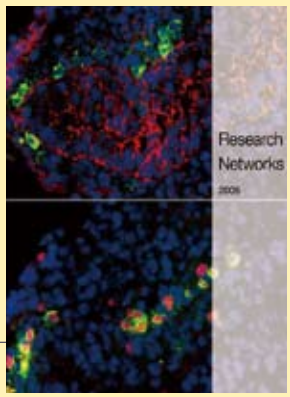

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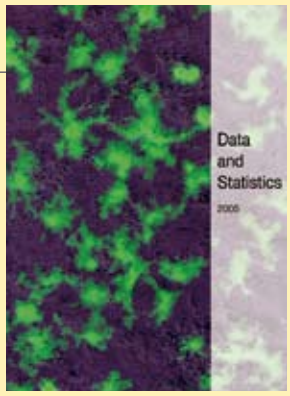

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Director's Report

This is the second annual report of the RIKEN Research Center for Allergy and Immunology (RCAI). This last year has been an important one for RCAI, albeit only its second year as a fully operational research center. During 2005, important directions for the future of the center have been set. For example, considerable effort has been placed on designing a translational research strategy for RCAI. But most of all, 2005 was a year noted for "promises kept, as it was referred to in a report by the RCAI Advisory Council.

"Promises Kept"

While I am flattered by the Council's tatement, as any director would be, it goes without saying that some qualification is in order. After all, the research center is barely two years old.

What were these promises? At least as concerns myself, my major goals were (1) to build-up, in a very short period of time, a unique place for the best minds in immunology to come together; (2) to create a research center geared toward the highest standards and exceptional productivity, yet one that also leaves sufficient breathing space for research efforts that are not simply safe bets, but that challenge established dogmas and ways of thinking in immunology today; (3) to create an environment where young investigators can thrive; (4) to develop a better interface between bench and bedside; and (5) to create a center for immunological research that is truly international.

I am extremely proud of the research published by RCAI scientists over the past year; it is a record of solid achievements, demonstrating that many of the decisions made in selecting principal investigators and in distributing the RCAI research budget were valid. Choosing the best among researchers with a long track record of success is easy, and the productivity of many established groups at the center has been outstanding. But some of the junior groups at the center have also demonstrated outstanding performance. Most of the junior groups have been very favorably reviewed and some have already made their mark with major publications in the world's best scientific journals. And, as you will see in the pages of this report, there are many more to come. In addition, many of the strategic efforts that we initiated over the past two years have already borne fruit. For example, the sophisticated microscopic techniques developed by Makio Tokunaga are used by an increasing number of scientists at the center, while our ENU screening effort has led to the development of two novel mouse models for atopic dermatitis, a result that is well aligned with our overall mission. Only a year ago we would not have dared to dream of such rapid progress.

Yet, as in any organization with a short history, there is much work that remains to be done. While we have created a framework to support translational and clinical research, this is only the beginning. Bringing scientists and clinicians together is particularly difficult in Japan. This is surprising given the fact that most Japanese immunologists have received medical training. Also, while we have been building an aggressive collaboration strategy, and are already working with many of the leading research organizations in the US and Europe, further efforts are required to fashion RCAI into a truly global research organization.

How have we done with respect to these five objectives? This report summarizes our efforts, and over the next few pages I will provide my own assessment of where RCAI stands today and what we should be aiming toward in the future.

An Environment for Science

For a director of any research organization, what matters most is perhaps less a list of great publications (of course we want that too) but that we have had the opportunity to contribute to an atmosphere that is conducive to research of the very highest quality. As the founding director of RCAI, I have been, and continue to be, responsible for building such an atmosphere. So, how have we done so far?

There are many dimensions to what could be considered the environment, from the neighborhood or the architecture of the building (and the way it organizes space and interaction), to the environment in an intellectual sense, or even culture of a research institute. One could argue that there are certainly better locations for a research center than a former industrial district. Yet the location within the RIKEN Yokohama Institute also offers large potential benefits. After all, there are a host of activities at the Yokohama campus that complement RCAIs efforts, from the large RIKEN mouse genomics efforts to research on structural genomics, SNPs, or even high-end computing applications for life sciences. After two years in Yokohama, we are only beginning to realize the potential synergies with the other RIKEN centers. And what about internal collaborations and the local culture? Here, I have to say, RCAI is still a young organization, but the number of both internal and external seminars has grown rapidly and I am very satisfied with the progress. We have also attempted to increase opportunities for internal discussion and, in thematic areas, such as allergy research, members of various laboratories discuss progress almost every week.

Much remains to be done to optimize the working environment for all staff at the center, from technician to group director. But managing a large research center is an ongoing learning process that is evolutionary in character, and there are no ready-made protocols that work simply.

Balancing Productivity and Breakthrough Developments

As a research center financed by the Japanese public, we constantly need to justify our work to numerous audiences. A standard way that the scientific community judges the success of any research institute is to count the number of its scientific publications in prestigious journals. Therefore having a strong publication record will be crucial for RCAIs long-term survival. Much of 2004 was spent settling in, breeding mice, and getting used to a new environment, and consequently 2005 was the first year for us in the new building. Given this, I am especially proud of the teams and groups that have produced many impressive results. This year, the more experienced research groups have done especially well.

Takashi Saito's group has been exceptionally productive with two intriguing new papers in *Nature Immunology* and a paper in *Science*. The two papers in *Nature Immunology* came from collaborative work with the single molecule immunoimaging unit, and provide new insights into the mechanistic and molecular basis of T cell signaling. These are the first papers to emerge from our efforts to bring single-molecule approaches to the field of immunology. One of the papers, co-authored with Mike L. Dustin at New York University, was funded by an RCAI International Research Collaboration Award.
Tomohiro Kurosaki's group has been equally productive. New papers in Immunity and *the Journal of Experimental Medicine* provide further details on the role of adapter proteins in B cell signaling. Toshio Hirano continues his work at the forefront of research on both cytokine signaling and early development, and a paper in *Immunity* showed that the IL-6-STAT-3 pathway controls MHC class II molecules in dendritic cells. In an imaginative paper published in *the Journal of Cell Biology*, Nishida et al. further dissect the process of mast cell degranulation.

In collaboration with Fukuoka University School of Medicine, my own group has shown that neutrophil production of IFN-γ triggered by Vα14 NKT cells is responsible for early graft loss in pancreatic islet transplants. In addition, we have shown that repeated administration of α-galactosylceramide prevents graft loss. In a second collaborative project, this time with the RIKEN Bioresource Center, we have shown that the success rate of the generation of cloned mice by direct nuclear transfer is dramatically increased when using NKT cells. Finally, our large ENU study, conducted by Hisahiro Yoshida, has already yielded results that were well beyond our initial expectations. Most importantly, we have been able to identify two novel mutants that show signs of a disease that mirror human atopic dermatitis.

For the younger groups I have put much less emphasis on publication. I feel that it is critical for younger investigators to have the time needed to identify and describe a major problem. This way they can become the investigators who develop the novel ideas and breakthroughs of the future. As we conducted the round of evaluation meetings during the second half of 2005, it was particularly pleasing to see that some of these pioneering groups were already publishing important new findings, and that most of the younger groups were well on track to contribute to the scientific literature soon.

Fostering Young Scientists

The majority of research teams and units at RCAI are led by relatively young scientists or, to be more precise, people who are managing a research group for the first time in their career. While their scientific careers may be young, most already have an M.D. or Ph.D. and so come with a wealth of experience. Given the time it takes to acquire both of these qualifications, many of the young leaders are aged 40 or more.

Many of RCAI's team and unit leaders have considerable experience at leading laboratories in immunology in Japan or overseas. They already have excellent publication track records and most

have published in top journals. And yet it is the first time for most of them to run their own laboratory and make their own decisions about who to hire, or how to allocate funding. At RCAI, these young investigators have ample resources, sufficient space, and highly productive and efficient central facilities on which to rely. If there is a limitation, it is time. Within 5 years they have to demonstrate their competence as leaders of their generation. In short, they are at the most important point of their scientific careers. While they are not under pressure to publish results, these young investigators need to develop their own scientific identity. They need to focus on a unique set of questions and problems, and establish a set of equally unique tools to tackle these questions.

Judging by the output of last year's round of annual evaluations, most of the younger scientists are well on their way, and already possess an identity that their colleagues recognize. The danger, if there is one, is that the center provides such a wealth of resources that some of the new groups have overstretched and tried to embark on too many projects. For these, I believe, the annual review exercise was an excellent opportunity to refocus activities. In some cases, the annual review also pointed to a somewhat new understanding of how scientific competition works. Understanding the rules of the game is crucial, especially for those who are determined to become leaders in their respective fields. This is especially important for small groups. For example, publishing a breakthrough finding in a leading journal is a great achievement, but it also causes a rapid increase in the number of laboratories working on that particular subject. The challenge is not only to publish, but also to stay ahead of competitors.

RCAI´s Translational Research Strategy

From its inception, RCAI has been a basic research center with a mission: to develop novel treatment strategies for allergic diseases. Our approach has been to step back from the often highly applied, and occasionally rather speculative, research in allergology and go back to the basics. One example has been to study the mechanisms of IgE regulation in mice and humans. Another has been to analyze the degranulation process in mast cells. On both subjects, I can report major new findings this year.

I should also mention the discovery, by Yasuyuki Ishii s group, of a surprising immunoregulatory effect. It appears that when an antigen is present, the effect that a ligand such as α-galacosylceramide is altered if it is delivered by a liposome. In Yasuyuki Ishii's approach, the administration of α-galacosylceramide through a liposome, together with antigen, can lead to the induction of an antigen-specific regulation of IgE.

Translational research is not about a simple application (or, for that matter, a translation) of new basic research findings to the clinic. New basic research findings in immunology are, in most cases, about the mouse immune system, not the human immune system. Translational research is, by definition, about making the work relevant to the human immune system. This is no small task.

Studying immunological processes in humans is far more difficult, demanding, time-consuming, and costly than work in murine models. It is also much less productive than research on the mouse system and many frequently-used techniques are simply not possible in humans. Humanized mouse models, in which immunodeficient mice are given a reconstituted human immune system, can bridge the gap by serving as a novel tool to study human immunity.

For RCAI, the development and study of humanized mouse models is now a major focus of the center's translational research efforts. While these models present extraordinary challenges, I believe that they have the potential to revolutionize research in immunology and to help us translate mice data into applications for humans. I am particularly excited that Dr. Ishikawa, a pioneer in the development of humanized mouse models, has recently joined RCAI as a Unit Leader. This work alone will also generate new areas for collaboration as the potential of these new models reaches well beyond the scope of RCAI. Already, we have begun several collaborative projects that use these models as tools for preclinical research on primary immunodeficiencies.

Global Networks

This brings me to my final topic: RCAI's position within the global networks of research in immunology today. Only two years ago, RCAI was largely unknown. In fact, when we moved into the new building in March 2004, I put a large empty map of the world on the wall of the lobby in front of the Director's office. Since then, we have marked the location of any important international collaboration on that board, and I must say that, within only two years, the board has filled impressively. There are, however, a number of important biases. While our international partners come from all over the world, most are from the United States, with a sizeable number also from Europe. The number of International Research Collaboration Awards has jumped from 5 at the end of 2004 to 10 at the end of 2005. Some of these collaborations are now bearing fruit and a number of papers are in preparation. But, while the center employs a few scientists or postdoctoral fellows from Asian countries, our links with emerging centers of biomedical research in Korea, Shanghai, and Singapore are still limited. In addition, the number of team leaders recruited from outside of Japan remains small and it is thus one of our most important goals to increase that number over the next few years.

In regard to global interlinkages, I need to mention two other issues. First, we held the 1st RCAI-JSI (Japanese Society for Immunology) Joint Symposium on Immunology in Yokohama in June 2005 and the RCAI Advisory Council was chaired by Max D. Cooper. When speakers start to redraft their presentations, shuffle around PowerPoint slides to include some of their latest raw data, you know that

a conference was a success. And, judging from the reactions we received, the RCAI-JSI Joint Symposium on Immunology certainly was a big success. I must give credit here to Toshio Hirano who, in his usual quiet manner, has done so much to make this happen. It was only appropriate that, halfway through the planning process of the event, he was selected to be President of the Japanese Society for Immunology, in addition to his role as RCAI deputy director.

Secondly, following the recommendations of the RCAI Advisory Boards inaugural meeting, all scientists at RCAI will need to undergo evaluation every year, and the evaluation panel draws much of its membership from outside the RCAI. The process we have chosen attempts not only to evaluate individuals, but also to act as one element of our program for mentoring the young groups. We have been able to recruit a number of prestigious people, notably Arthur Weiss, Alain Fischer, Max Cooper, Ruslan Medzhitov, Diane Mathis, Bernard Malissen, Paul Kincade, Antonio Coutinho, Ralph Steinman, Klaus Rajewsky, Tasuku Honjo, Masayuki Miyasaka, Nobuyuki Miyasaka, Hiromitsu Nakauchi, Takehiko Sasazuki, Kiyoshi Takatsu, Kazuo Sugamura, and Bill Paul. Consequently, 11 out of 18 members of the RCAI Advisory Council now come from outside of Japan. I am extremely grateful to this outstanding and immensely distinguished group of scientists and I am humbled that they have agreed to take upon themselves the burden of numerous trips to Japan.

After a year of annual reviews undertaken in small groups of 3-4 laboratories, both the scientists at the center and myself as a director have found the experience most worthwhile. The Advisory Council is therefore more than just a mechanism to ensure high quality research, but a strategic body of expertise that can assist me as director to manage the center. At least at RCAI, evaluation has nothing to do with the mundane rubberstamping of research that still can be seen at times elsewhere.

I am especially grateful to Dr. Max D. Cooper, one of the most amazing individuals I know. Dr. Cooper continues to amaze us all with his beautiful studies on the evolution of the adaptive immune system, and it is hard to overstate his contributions to immunology simply through the services he has performed for the community.

I started this brief summary of the year at RCAI with a reference to a comment in one of the reports by the RCAI Advisory Council. To me, promises kept simply means that we are on the right path. Science is an endless quest and this is, of course, what makes it so exciting!

Masaru Taniguchi, MD, PhD
Yokohama, March 2006

Prize Winners 2005


1


2

Four researchers at RCAI received prizes in 2005. Dr. Sidonia Fagarasan, Leader of the Laboratory for Mucosal Immunity, received the Minister of Education, Culture, Sports, Science and Technology (MEXT) Prize for Young Investigators, 2005 (Fig.1). This prize is awarded to young scientists (under 40 years of age) in recognition of creative and original research and outstanding ability to develop research projects in science. Dr. Fagarasan was awarded for her research on the mucosal immune system, focused particularly on immunoglobulin A production in the gut.

Dr. Toshio Hirano, Leader of the Laboratory for Cytokine Signaling, received Medical Award of The Japan Medical Association (Fig.2). The prize is awarded to members of the association whose work has greatly contributed to the field of basic medicine, social medicine or clinical medicine. Dr. Hirano received this award for his contribution to molecular biological and immunological studies on cytokines.

Not only the leaders but also young and active researchers at RCAI received prizes. Dr. Michio Hiroshima, a research scientist in Single Molecule Immunoimaging Research Unit, was awarded the Early Research in Biophysics Award 2005 for his poster presentation at The Biophysical Society of Japan (Fig.3). Dr. Kenichi Masuda, a research scientist in the Laboratory for Vaccine Design, received the Best Business Plan Award of the Kanagawa Science Park Venture Business School (Fig.4).

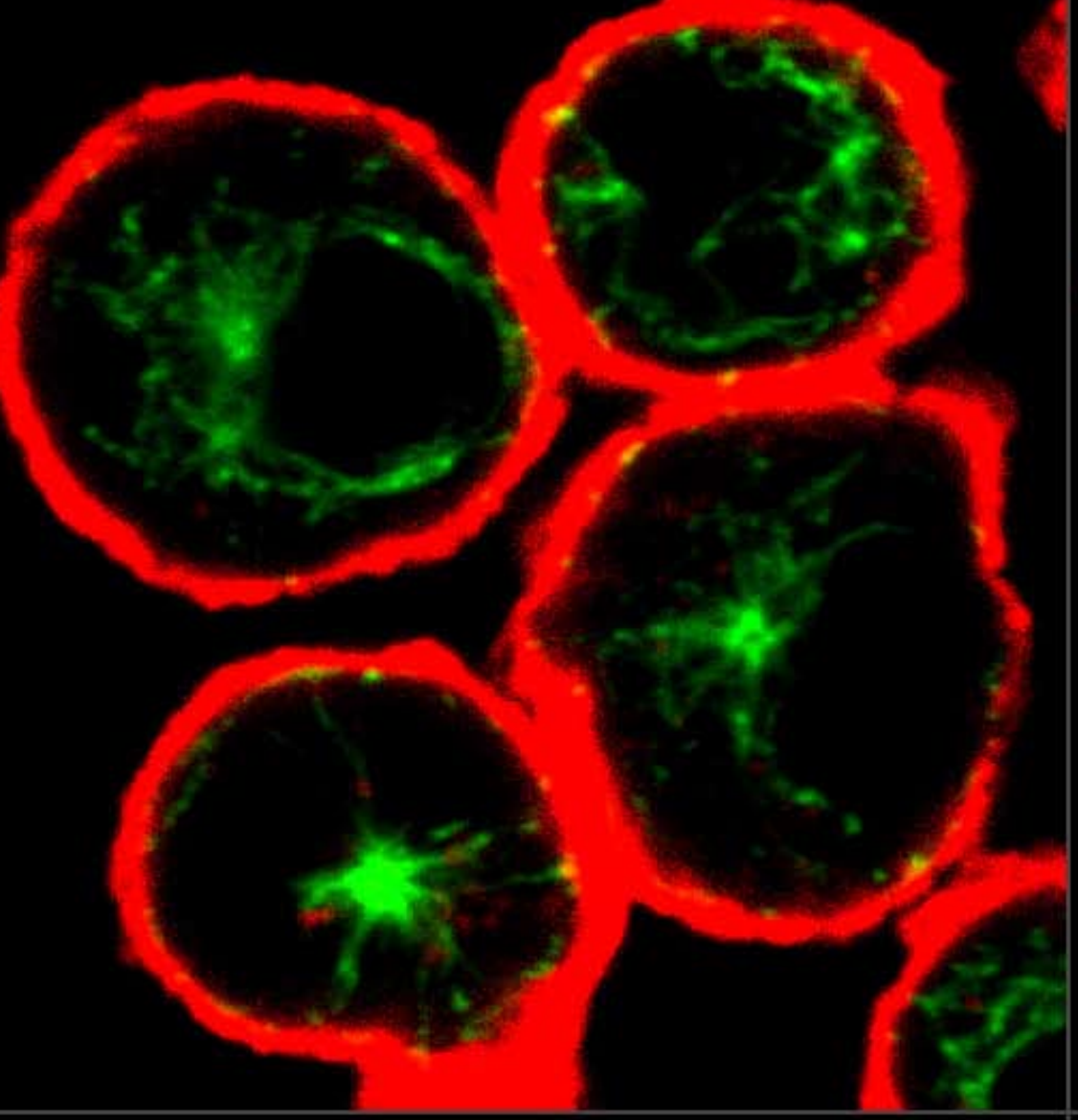

3

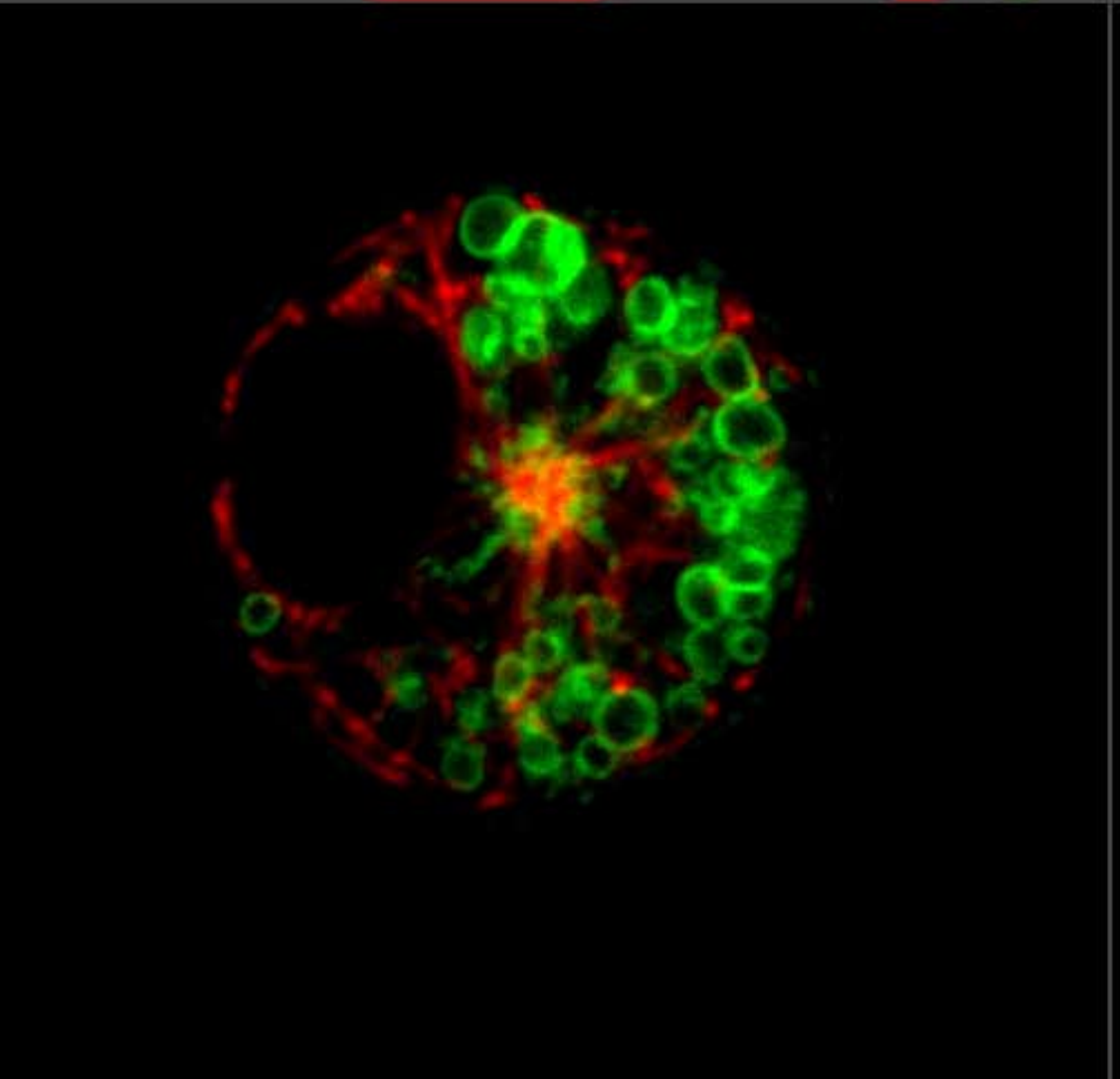

4

2005 Award for Excellent Papers of the Year

The RCAI Excellent Paper Award was established in 2004 to recognize exceptional publications of RCAI scientists. The award fund is supported by donations from Drs. Masaru Taniguchi and Dr. Toshio Hirano. This year, eight papers were selected, a reflection of the high quality of research being performed at RCAI.

Drs. Tadashi Yokosuka and Takashi Saito received the award for their paper that was published in *Nature Immunology*, concerning the use of the single-molecule microscope developed by Dr. Tokunaga (Single Molecule Immunoimaging Research Unit, RCAI) to demonstrate that TCR microclusters are the initiation sites of T cell activation. Drs. Keigo Nishida, Satoru Yamasaki and Toshio Hirano received the award for their paper published in *The Journal of Cell Biology*, in which they reported their identification of two separable steps in the process of mast cell degranulation. Drs. Sho Yamasaki and Takashi Saito received the award for their paper in *Nature Immunology*, in which they proposed that the spontaneous oligomerization of pre-TCR would make a pre-TCR ligand unnecessary. Other awardees were Drs. Kyoko Masuda and Hiroshi Kawamoto for their work on pre-thymic progenitors (*EMBO Journal*), Drs. Nobutaka Suzuki and Takashi Saito for demonstrating the role of IRAK-4 in both innate and acquired immune signaling (*Science*), Drs. Yuichi Aiba and Tomohiro Kurosaki for discovering BANK as a negative regulator of B cell responses (*Immunity*), Drs. Hisaaki Shinohara and Tomohiro Kurosaki for studies of the regulation of the BCR signaling pathway by PKCβ (*Journal of Experimental Medicine*), and Drs. Hidemitsu Kitamura and Toshio Hirano for defining the role of IL-6 in the maturation of dendritic cells (*Immunity*).





Research Highlights

2005

Development of Humanized Mice

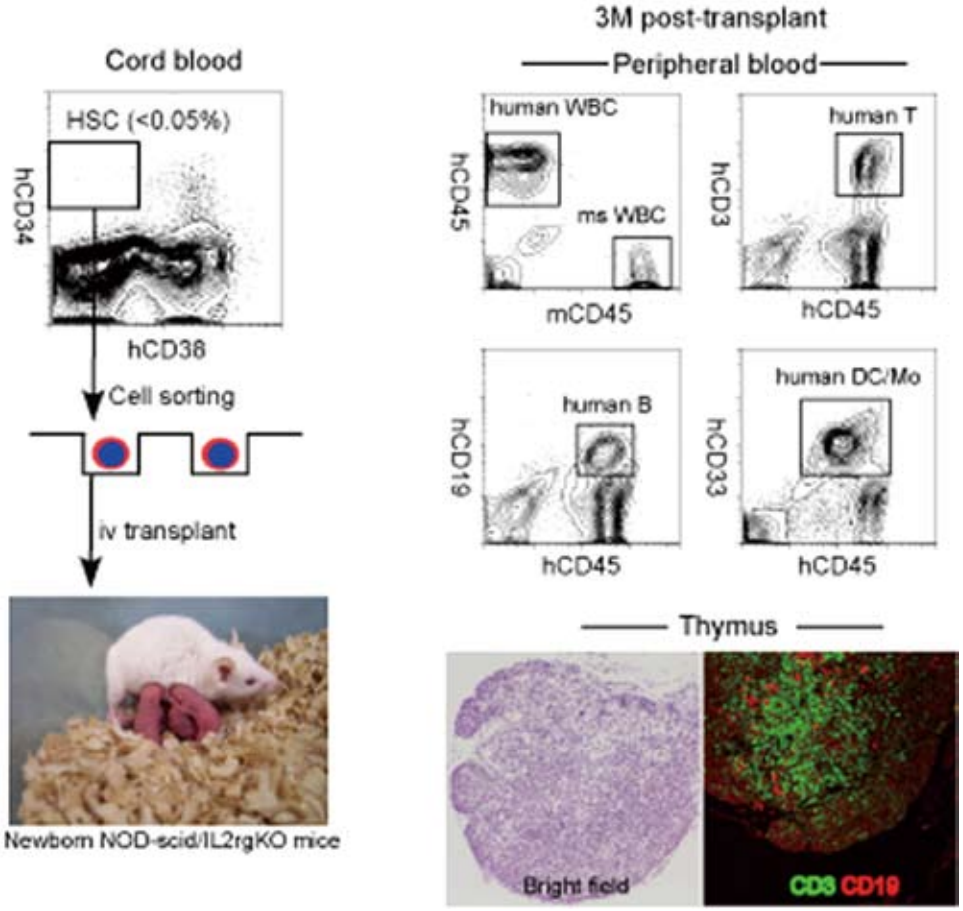


Figure: Development of human immunity in mice Human hematopoietic stem cells (Lin-CD34$^+$CD38$^-$) account for <0.05% of total cord blood mononuclear cells. These rare HSCs are purified by multi-color cell sorting, and transplanted into newborn NOD-scid/IL-2rγKO mice that are devoid of both acquired and innate immunity. At 3-month post-transplantation, more than 50% of leukocytes in the recipient mice are of human origin. Transplanted human HSCs give rise to all types of immune components in mice. The recipient thymus is highly repopulated by human T cells.

Our current understanding of stem cell biology has largely come from the knowledge of murine bone marrow-derived hematopoietic stem cells (HSCs). However, it is becoming apparent that the fundamental aspects of HSCs, such as their surface phenotype, are different between mice and humans. Such differences make it difficult to apply the knowledge of the murine stem cell systems to the study of the human system. All types of immune components differentiate from a HSC. Therefore, in order to study human immunity and to establish new immunotherapies, we aim to develop a model system which will enable us to dissect the human immune system in vivo by transplanting human HSCs into immunodeficient mice.

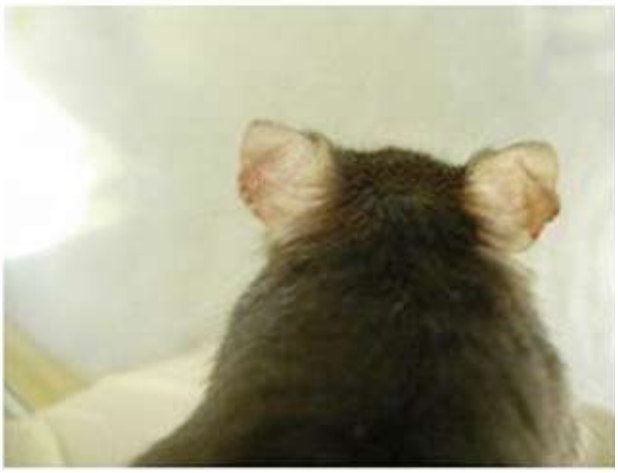
In the conventional adult scid repopulating assays using CB17-scids or even NOD-scids, there are a couple of problems, for example, impairment in T-cell commitment and maturation arrest in B-lineage cells without class-switching. We have taken two approaches to overcome these problems. First, in order to deplete innate immunity of the mice, null mutation of β2m or IL2Rγc was backcrossed onto the NOD-scid strain. Secondly, we transplanted human stem cells into neonatal mice, whose developing environment is more permissive and supportive for xenogeneic human cells, allowing them to engraft and expand their progeny.

When purified human cord blood hematopoietic stem cells (CD34$^+$CD38$^-$) were transplanted into newborn NOD/SCID/IL2γcnull mice, high levels of chimerism with human hematopoietic cells was achieved for over six months. In this model, human HSCs produced mature differentiated progenies including myelomonocytes, T and B cells, dendritic cells, erythrocytes and platelets. Human CD34$^+$ cells harvested from the bone marrow of the primary recipient mice could successfully reconstituted sublethally irradiated secondary recipients. Thus, the newborn NOD/SCID/IL2γcnull mouse system allows for the examination for self-renewal capacity in human HSCs.

Development of humanized mice enables us to analyze the histological examination of human B, T, and dendritic cells in the spleen or in the thymus that are difficult to obtain from human donors. More importantly, the humanized mouse system will be useful for studying various types of human diseases such as species-specific viral infection, hematological malignancies, and solid cancer. Evaluation of efficacy and safety of blood products, vaccines and drugs can be undertaken by using the humanized mouse system, and can be done more precisely and accurately than under the present systems. Thus, humanized mouse provides a unique system for studying human immunity and diseases in vivo; however, the present system still have practical limitations. We will have a plan to further develop the current mouse model by expressing human HLA class I and class II molecules and cytokines in mice to be trasnsplanted with human HSCs. This approach promotes more suitable conditions for appropriate expansion and maturation of human hematopoietic and immune system in the mouse.

ENU Project: Allergic Disease Model Mice

Early onset atopic dermatitis



Late onset atopic dermatitis

Figure: Appearance of atopic dermatitis model mice in ENU mutagenesis

Allergic disease such as asthma, rhinitis, and atopic dermatitis constitute an increasingly severe public health problem in Japan and other developing countries. Recent increase of those disease in developing countries indicate the environmental problems affect the disease onset, however, genetic disorder is also a important factor to induce allergic status.

In order to identify the genetic problems responsible for the onset of allergies, we have generated a pool of mutant mice by random chemical mutagenesis through ethilnitrosourea (ENU). ENU provides random point mutations on a genome, such that the offspring of male mice injected with ENU harbor various mutation on their genome. The incidence of random mutation is estimated to be 3,000 per a genome, it results in 100 coding regions are affected in a genome. To obtain the homozygote of those unknown mutation, we made the third generation by the second generation sib-mating. Up to now, we have screened approximately 6,000 mice from 60 pedigrees of mice to find mutants with phenotypes related to allergic status in autosomal recessive inheritance manner. In total, 46 mutant lines were found to have distinct phenotypes related to allergic disease; 25 mutant lines elevated the level of serum IgG1 or IgE, 10 lines had aberrant phenotypes for mast cells by their numbers, surface marker expression or degranulation, 3 lines had an eosinophil number increase.

Since these phenotypes are found among the animals kept under the clean, specific pathogen-free condition, we questioned whether they could be the disease models of human allergic diseases basically developed with environmental problems. In order to dissolve this question, we are now trying to re-screen the established mutant lines with allergen and adjuvant immunization, and found that tested mutant lines with elevated serum IgG1 and IgE responded quickly and intensively to the immunization experiment.

These results supported to start this immunization screen as a primary phenotype. With additional 8 pedigrees screens, we have found 6 new mutant lines showed aberrant serum IgE response to the immunization. Four of them had quick and intensive serum IgE elevation, and other 2 lines showed lowered or no response to allergen and adjuvant.

Interestingly, one of the mutants with high levels of serum IgE constantly scratched the skin of their face and ears from 3 months after birth, which led us to speculate that this behavior reflects atopic-dermatitis (AD)-like skin inflammation. Since we keep mice under specific pathogen-free conditions, this behavior must be induced by some genetic disorders held in this mutant genome. After close observation of the disease progress for a year, we have found the disease grade varied among the mutant animals. These results suggested that this AD-like disease may be induced by a combination of several mutations happened to be developed in a genome.

In collaboration with the Genome Science Center in RIKEN, now we can quickly map the mutant phenotype responsible loci. Now we are regionalizing the responsible loci of AD-like disease and IgE elevation in a genome. After regionalizing the responsible loci to a part of a chromosome, we can filter the candidate genes by a soft program PosMed established in RIKEN GSC. The infrastructure of RIKEN will accelerate the responsible mutation mapping. Identification and characterization of these mutations will shed light on the molecular mechanism of allergic disease development.

TCR-Microclusters Start and Maintain T cell Activation

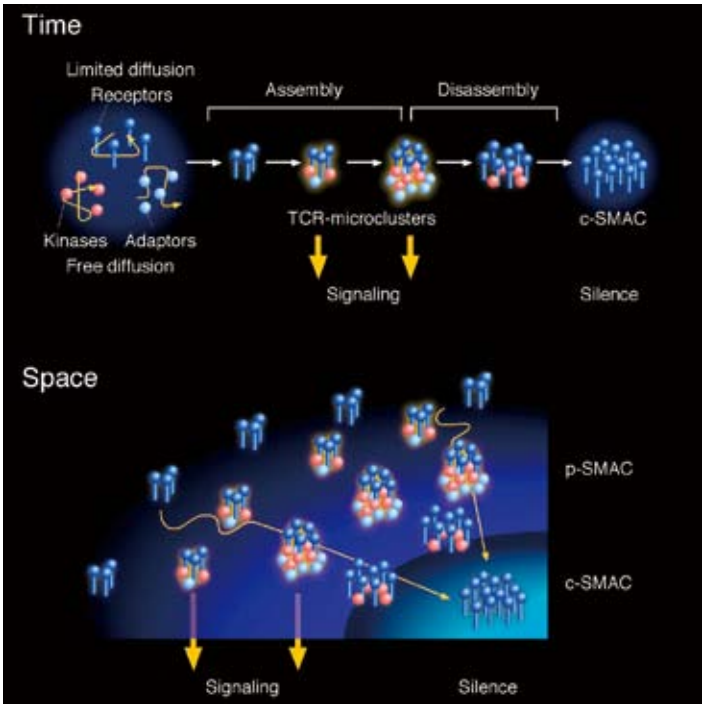


Figure: Spatio-temporal regulation of T cell activation through TCR-microclusters
(*Upper*) While receptors and kinases and adaptors make free movements during a quiescence status, TCR engagement with an antigen/MHC induces TCR assembly which recruits kinases and adaptors to form a signalsome, "TCR-microcluster (MC)". Tyrosine phosphorylation and Ca^{2+} influx are induced when a few TCR-MCs are formed, indicating that they are the start points for T cell activation. Consequently, TCR-MCs move to the center whereas kinases and adaptors dissociate from TCR-MCs.
(*Lower*) TCR-MCs are generated at the interface between a T cell and an antigen presenting cell or a planar membrane. Following the first attachment, a T cell generates new TCR-MCs at the spread leading edge. After the full expansion, the T cell begins to contract and TCR-MCs migrate to the central region of the interface, while kinases and adaptors dissociate from TCR-MCs. TCR-MCs finally accumulate at the center to form a c-SMAC. New TCR-MCs are continuously generated at the peripheral edge where TCR signal is sustained and lasts for hours.

When and how is T cell receptor (TCR) signaling generated? For many years, researchers in the T cell signaling field have analyzed the mechanisms using biochemical procedures, the black and white world. With the emergence of new imaging technologies in the last decade, the landmark for T cell signaling was discovered: the immunological synapse (IS). The IS is composed of TCRs and their downstream signaling molecules distributed in two concentric rings, and is formed at the interface between an antigen presenting cell (APC) and a T cell. After IS formation, T cells are activated and differentiate, consequently acquiring effector functions that control the fate of lymphocytes. Therefore, the IS is believed to be a site for antigen recognition and T cell activation.

Although the IS is a distinct structure for T cell signaling, there are some possibilities that dispute its real function. It is known that T cells are activated within a few minutes after TCR stimulation; however, approximately 5-10 minutes is needed for the complete formation of an IS. Therefore, the presence of a structure other than the IS for the initial activation is speculated.

Yokosuka et al. in the Laboratory for Cell Signaling found a novel site for T cell activation named "TCR-microcluster (MC)" using combined new technologies, namely, a supported planar membrane and total internal reflection microscopy (TIRFM). The planar membrane can act as an artificial APC due to the free-floating major histocompatibility complexes (MHCs) on it, and TIRFM can provide us nano-scale images required for single molecule analysis. A T cell that contacts the planar membrane spreads, continuously generating MCs at the contact interface. A MC is a signalsome that contains TCRs, kinases and adaptor molecules that act as TCR signaling inducers, and is formed prior to IS formation. One T cell contains a few hundred MCs at the initiation and one MC consists of 50 to 300 TCRs. Because early activation events such as tyrosine phosphorylation and the following $Ca2^{+}$ influx occur in parallel to the formation of several MCs, the MC is thought to be a site for antigen recognition and T cell activation.

Analysis from time zero to IS formation shows that MCs existing diffusively on a planar membrane begin migration to the central region of the interface, finally forming the so-called c-SMAC (central-supramolecular activation cluster), which is an aggregation of TCRs and is a feature of mature IS. Contrary to previous reports, kinases and adaptors dissociate from TCR-MCs before arrival at a c-SMAC and the c-SMAC has few signaling molecules except TCRs.

T cell activation for proliferation and cytokine secretion requires continuous signaling for several hours. Then, where and how is the sustained TCR signaling generated? Precise examination after c-SMAC formation revealed newly synthesized MCs on the leading edge of the interface. Furthermore, it was demonstrated that these MCs contained kinases and adaptors and maintained tyrosine phosphorylation. These results indicate that MCs continuously formed on the T cell-planar membrane interface are capable of generating T cell activation signals both at initiation and after mature IS formation.

Biochemical studies clarified the TCR signaling cascade and revealed the mechanisms of how T cells are activated and differentiated to show so many phenotypic variations. However, the analysis was conducted at the cell mass level. As each cell demonstrates a different reaction after a single stimulation, one molecule acts differentially in time and space within the same cell. The identified MCs made it possible to figure out the TCR signaling from the spatio-temporal point of view.

ORIGINAL RESEARCH PAPER

Yokosuka, T. et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. Nature Immunol 6, 1253-1262 (2005).

Identification of Common Regulator Between Innate and Acquired Immune Responses

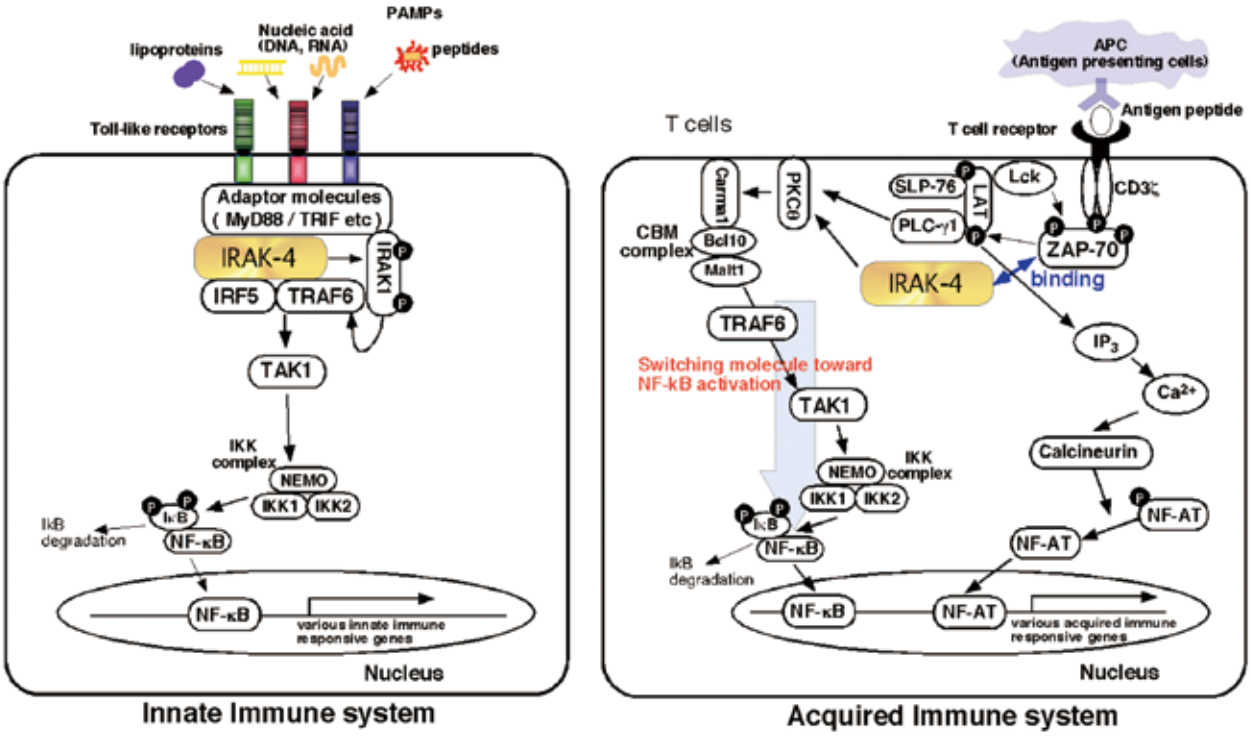


Figure: In innate immunity (*left*), upon PAMP (Pathogen-Associated Molecular Pattern) recognition, TLRs associate with adaptor proteins, which is followed by the recruitment of IRAK-4, IRAK-1, IRF5, and TRAF6 to TLRs. Finally, NF-κB is translocated into the nucleus where it can induce target gene expression. Thus, IRAK-4 is a critical signal transducer in innate responses. In acquired immunity (*right*), IRAK-4 constitutively associates with ZAP-70, and may be recruited to the TCR vicinity upon stimulation. IRAK-4 functions as a switch molecule toward NF-κB activation in TCR signaling. Since TLRs are expressed and function also in T cells, there may be crosstalk between TLR and TCR through IRAK-4 in T cells.

Our body is protected against external pathogens such as harmful microorganisms and viruses by the immune system. The immune system consists of 'innate' and 'adaptive' immunities. Innate immune responses rapidly detect invading pathogens and defend the body against them. Adaptive immune responses act later and ensure that the immune system 'remembers' invading pathogens, so that the body is equipped to respond more rapidly should the same pathogens be encountered again. Cells participating in both innate and adaptive immune responses express an array of receptors on their surface, which are able to 'sense' components of dangerous pathogens. Once triggered, each receptor activates a cascade of intracellular molecules that transmit stimulatory signals deep into the cell interior, ultimately resulting in the expression of defense genes.

Suzuki et al. in the Laboratory for Cell Signaling found that IRAK-4, an intracellular kinase essential for the transmission of signals in innate responses, also plays a crucial role in the transmission of signals of T cells that are in charge of adaptive immune responses (Figure). To gauge the role of IRAK-4 in T cell signalling, Suzuki et al. subjected IRAK-4-deficient T cells to a battery of immune function tests. In response to viral infection, mice generate cytotoxic T cells (CTLs) capable of eliminating infected cells and secrete cytokines. IRAK-4-deficient T cells exhibited defective development of CTLs and cytokine production. Because T cells in transplant recipients perceive donor tissue as 'foreign', graft rejection occurs. Compared to normal recipients, IRAK-4-deficient recipients showed delayed rejection of foreign skin grafts. By monitoring the activation status of T cell signalling molecules, they found that IRAK-4-deficient mice have a specific defect in NF-κB activation. T cell activation induces two representative transcription factors, NFAT and NF-κB. Detailed analyses showed that IRAK-4 mediates NF-κB activation by activating PKCθ through the association with ZAP-70 without alteration of the NFAT activation pathway. These findings suggest that the acquired immune system would utilize a useful innate immune molecule for signal transduction during evolution.

Previous studies identified the 'adaptive' immune roles of two proteins, RIP2 and TRAF6, which are established participants in 'innate' signalling cascades. These studies, together with the present study, illustrate that the distinction between molecules contributing to innate and adaptive immune responses may not be as sharp as previously thought. Innate and adaptive immune responses work in concert towards the common goal of defending the body against immune insults. In the same line of thinking, there must be much more evolutionarily conserved molecules between innate and acquired immune systems. These molecules are supposed to be potent key regulators for the development of immune-system-based drugs that are effective against infectious diseases, cancers, allergies, transplantation, and so on, because the inhibitors of these molecules should be effective against both innate and acquired immune systems.

ORIGINAL RESEARCH PAPER

Suzuki, N. et al. A critical role for the innate immune signaling molecule IRAK-4 in T cell activation. Science 311, 1927-1932 (2006).

Autonomous pre-TCR Signaling

Figure: Stereo diagrams of the three-dimensional structure model of the pTα-TCRβ (pre-TCR) complex
Functionally critical charged amino acids (D22, R24, R102 and R117) are represented by a ball and stick model. Acidic and basic residues are shown in blue and red, respectively.

T cells are generated within the thymus by selecting T cells that express right specificity and affinity of T cell receptor (TCR) through the depletion of T cells reactive to self molecules with high affinity and the selection of immunocompetent cells. A TCR repertoire is generated by sequential gene rearrangement from TCRβ and TCRα, and thymocytes expressing TCRαβ are subjected to selection. In immature thymocytes, a pre-TCR complex composed of TCRβ and an invariant pre-TCRα (pTα) is expressed, which transduces signals that regulate the allelic exclusion of the TCRβ gene and differentiation to express TCRα: a process called "β-selection." The pre-TCR is crucial for early T cell development and is proposed to function in a ligand-independent or autonomous manner. However, the molecular mechanism underlying such autonomous signaling remains elusive.

Yamasaki et al. in the Laboratory for Cell Signaling addressed this issue first by visualizing pre-TCR and TCRαβ using green fluorescence protein (GFP) fusion proteins of pTα and TCRα. When fluorescence-labeled pre-TCR and αβTCR were expressed in TCRα-deficient cells, the surface expression level of pre-TCR was much lower than that of TCRαβ, and a substantial fraction of pTα/GFP was observed in intracellular vesicles, suggesting that the pre-TCR is engaged autonomously.

To know the driving force of this engagement, they developed a novel system for the detection of subtle molecular interactions. Using the fusion protein of pTα and TCRα to the transmembrane and cytoplasmic domains of the human erythropoietin receptor (EPOR), the ability of autonomous oligomerization of the receptor was examined by analyzing the ligand-independent growth of BAF3 cells expressing pTα/EPOR and TCRα/EPOR on the basis of the finding that a self-dimerizing mutant of EPOR induced growth in a ligand-independent manner. Indeed, using this system, only BAF3 cells expressing pTα/EPOR showed IL-3-independent growth, indicating that the extracellular domain of pTα has the potential to form oligomers spontaneously. To elucidate the structural requirements for the oligomerization of pTα, several mutants were tested in this pTα/EPOR system and they found that four charged amino acids (R22, D24, R102 and R117) are critical for the self-oligomerizing property of pTα. Three-dimensional molecular modeling of the pTα-TCRβ dimer revealed that these amino acids are located on the molecular surface. Indeed, alteration of these residues eliminated the ability of pTα to support pre-TCR signaling *in vivo* when reconstituted into pTα-deficient mice.

Although it has been proposed that the 'cell-autonomous' localization of pre-TCR into lipid rafts is responsible for the ligand-independent signaling ability, they showed that the raft localization of pre-TCR was not sufficient for β-selection using raft-targeted CD3ε chimeras (LAT/CD3ε) in transgenic mice. In contrast, dimerized-CD3ε (hCD8/CD3ε) could bypass β-selection regardless of the raft localization. In addition, it has also been suggested that signaling thresholds are also altered during β-selection. They provided further evidence for the hypothesis that the low signaling threshold to the receptor may be a unique characteristic of $CD4^-CD8^-$ immature thymocytes.

Recently, charge-based autonomous interactions were also found in pre-BCR in immature B cells. Based on the salient similarities, charged-residue-mediated oligomerization via the surrogate invariant chains of the nascent antigen receptors in T cells and B cells seems to be a common but individually evolved strategy for cell-autonomous signaling that informs cells about the successful progress of the antigen receptor gene assembly.

ORIGINAL RESEARCH PAPER

Yamasaki, S. Ishikawa, E., Sakuma, M., Ogata, K., Sakata-Sogawa, K., Hiroshima, M., Wiest, D.L., Tokunaga, M. and Saito, T.: Mechanistic basis of pre-T cell receptor-mediated autonomous signaling critical for thymocyte development. Nat Immunol. 7: 67-75, 2006

Isolation of Prethymic T cell Progenitors

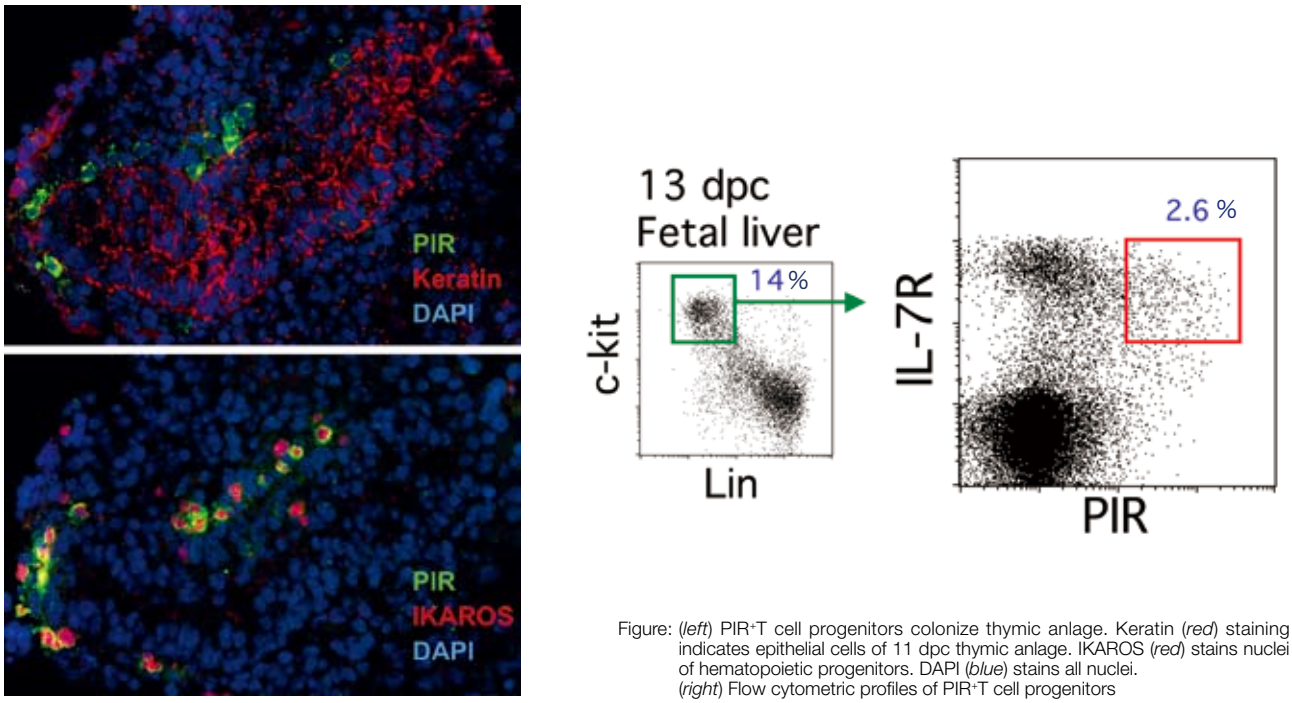


Figure: (*left*) PIR$^+$T cell progenitors colonize thymic anlage. Keratin (*red*) staining indicates epithelial cells of 11 dpc thymic anlage. IKAROS (*red*) stains nuclei of hematopoietic progenitors. DAPI (*blue*) stains all nuclei.
(*right*) Flow cytometric profiles of PIR$^+$T cell progenitors

Whether the restriction of progenitors to the T cell lineage takes place before they immigrate into the thymus has been a longstanding question in immunology as well as in developmental biology; however, it still remains controversial. In the present study, Masuda et al. succeeded in isolating prethymic T cell lineage-restricted progenitors in murine fetuses with the surface expression of paired immunoglobulin-like receptors (PIRs), and then disclosed the prethymic stage of T cells as a distinct stage from intrathymic stages. They further show genetic evidence that the production of PIR$^+$ progenitors is regulated differently from that of intrathymic progenitors, because PIR$^+$ prethymic progenitors do not depend on Hes1-mediated Notch signaling for their generation while intrathymic progenitors utterly do.

Previous studies have defined the prethymic T cell progenitors with well known differentiation markers such as Thy-1, NK1.1, and B220. It is unclear, however, whether these cells represent the major thymic immigrants, since these cells emerge at later stages in fetal liver or in fetal blood than the initial thymic colonization period, and do not correspond to the earliest thymic progenitors in terms of surface phenotypes. In the previous works, they used IL-7R as a marker for the enrichment of the prethymic T cell progenitors, and, using a clonal assay, they showed that the IL-7R$^+$ population of murine fetal liver contains a mixture of T and B cell progenitors but not common T/B progenitors. Based on these findings, we have proposed that T cell lineage commitment takes place at the prethymic stage in fetal liver. However, since B cell progenitors also express IL-7R, the exact identification or isolation of T cell progenitors can not be performed with the IL-7R as a marker. It was a prerequisite to find out a specific marker for prethymic T cell progenitors.

In this present study, they succeeded in defining the prethymic T cell developmental stage based on the expression of PIR. It is surprising that PIRs are expressed at progenitor stages specifically to the T cell lineage, since PIRs are known to be expressed on mature myeloid lineage cells, B cells and mast cells, but not on T cells. Of special interest is that PIR expression is immediately downregulated before thymic T cell progenitors express CD25 to enter the so-called DN2 stage, which has been considered as the first step in T cell lineage-specific differentiation.

By defining the prethymic stage with a specific surface marker, it became possible to examine whether Hes1, one of the target molecules of the Notch signal, which has been shown to be essential for early intrathymic T cell development, is required for prethymic T cell development. It is generally believed that Notch signaling controls the cell fate decision of uncommitted progenitors to the T cell lineage. Our present findings clearly demonstrated that Hes1-mediated Notch signaling is dispensable for the T cell lineage commitment.

The present findings not only provide a new surface marker for the prethymic stage of T cell development, but also define the prethymic stage as a novel, genetically separable stage from intrathymic stages. Thus, the present study disclosed the earliest phase of T cell production during ontogeny, which is one of the most essential events in the immune system. The definition of a novel stage of T cell development will be a guidepost for further studies in this field.

ORIGINAL RESEARCH PAPER

Masuda K, Kubagawa H, Ikawa T, Chen CC, Kakugawa K, Hattori M, Kageyama R, Cooper MD, Minato N, Katsura Y, Kawamoto H. Prethymic T-cell development defined by the expression of paired immunoglobulin-like receptors. EMBO J. 24, 4052-4060 (2005)

New Role for NKT Cells in Allograft Tolerance

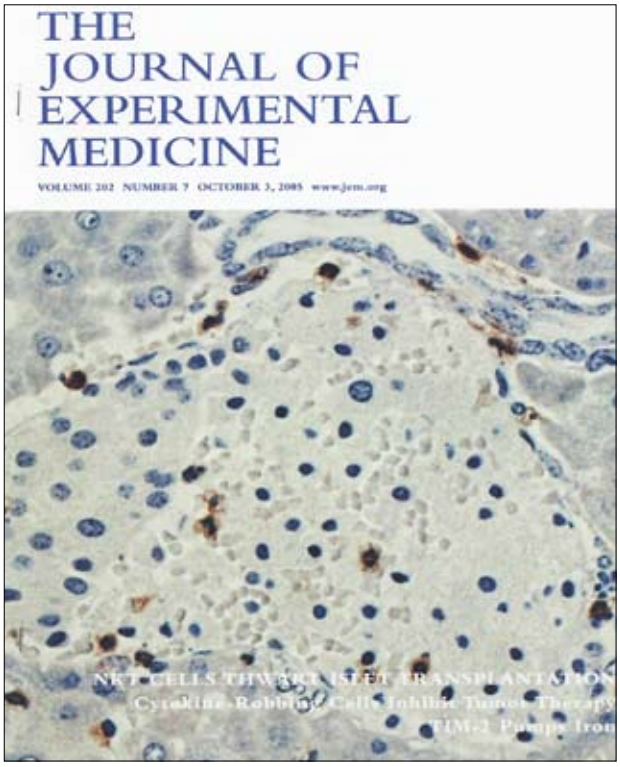


Figure: Interferonneutrophils (*brown*) infiltratingtransplanted pancreatic islet cells
NKT cells activate the neutrophils, and both cell types contribute to early islet cell rejection.

Although there has been significant excitement surrounding the transplantation of insulin-producing islets for the treatment of patients with type 1 diabetes, up to half of the patients that receive the transplants rapidly reject the donor islets and fail to achieve insulin independence. To try to explain this, Masaru Taniguchi and colleagues studied a mouse model of islet transplantation and now report that natural killer T (NKT) cells might be behind early graft loss.

NKT cells have an important role in immediate immune responses and, mainly through their ability to produce large amounts of interferon-γ (IFN-γ), function as a bridge between innate and adaptive immune responses. Because IFN-γ has been shown to be an important factor in the destruction of islet cells, the authors hypothesized that NKT cells might be involved in early islet graft failure.

Diabetes, as measured by hyperglycemia, was induced in C57BL/6 mice by intravenous injection of streptozocin. A transplant of 400 islets harvested from 2 syngeneic mice and injected into the liver of the diabetic mice was required to restore normal blood glucose levels; mice receiving 200 islets remained hyperglycemic and had few intact islets. But if the diabetic mice lacked NKT cells, a transplant of only 100 islets was sufficient to restore normal glucose levels, indicating that NKT cells may be responsible for the loss of the transplanted islets.

Tetramers of CD1d complexed with α-galactosylceramide (α-GalCer) were used to analyse NKT-cell numbers in mice that showed islet graft destruction. Although NKT-cell numbers seemed to decrease immediately after transplantation (consistent with activation-induced downregulation of their T-cell receptors), NKT cells were detected at high numbers 24 hours after transplantation. As a consequence of NKT-cell activation, neutrophils were induced to produce IFN-γ and were detected in the islet transplant, indicating that they might mediate destruction of the islets.

Based on the observation that a single dose of α-GalCer induces NKT-cell activation but repeated stimulation with α-GalCer inhibits NKT-cell activation, the authors tested whether graft loss could be prevented by repeated injection of α-GalCer. Diabetic mice that received 400 islets and were treated with a single injection of α-GalCer remained hyperglycemic and had increased IFN-γ production by neutrophils and NKT cells. By contrast, when diabetic mice were treated three times with α-GalCer, a transplant of only 200 islets was sufficient to restore normal blood glucose levels and reduce IFN-γ production by neutrophils. So, in vivo modulation of NKT-cell activation to prevent this collaboration between NKT cells and neutrophils might be a novel approach for improving efficiency of islet transplantation. (Written by Dr. Lucy Bird, *Nature Reviews Immunology*, Vol.1 5 p.830, 2005, Macmillan Magazines Ltd.)

ORIGINAL RESEARCH PAPER

Yasunami Y, Kojo S, Kitamura H, Toyofuku A, Satoh M, Nakano M, Nabeyama K, Nakamura Y, Matsuoka N, Ikeda S, Tanaka M, Ono J, Nagata N, Ohara O, Taniguchi M. Valpha14 NK T Cell-triggered IFN-gamma Production by Gr-1^{+}CD11b^{+} Cells Mediates Early Graft Loss of Syngeneic Transplanted Islets. J. Exp. Med. 202, 913-918 (2005).

Generation and Analysis of NKT Cell Cloned Mice

Figure: Mice cloned from NKT cells by direct nuclear transfer

The cloning of mammals by nuclear transfer remains inefficient. One fundamental question is whether clones have really been derived from differentiated cells rather than from stem cells sporadically present in donor-cell samples. To date, mature lymphocytes possessing genetic differentiation markers have been cloned to generate mice via a two-step nuclear transfer. In the first step, a donor cell nucleus that contains the animal's DNA is transferred into an egg that has had its nucleus removed. The embryo develops to the blastocyst stage, and the embryonic stem (ES) cell line is generated from the blastocyst. In the second step, the ES cells are injected into another blastocyst made up of tetraploid cells (cells having twice the DNA of normal cells), and placed into the uterus of a female mouse. Although this two-step procedure improves the cloning efficiency, certain problems remain: the ES cells have extra reprogramming time, and tetraploid cell line might contribute to most extra-embryonic tissues that adversely affect the components in cloned animals.

In collaboration with Dr. Ogura of the Bioresource Center at Tsukuba, Inoue et al. efficiently generated cloned mice with a single-step process. They used natural killer T (NKT) cells, a distinct subset of lymphocytes, as donor cells. The nuclei of NKT cells were transferred into eggs whose nuclei had been removed. The embryos were then cultured and placed into the uterus of a female mouse. The NKT cell-cloned embryos had a high developmental potential in vitro: Most (71%) developed to the morula/blastocyst stage, in marked contrast to embryos from peripheral blood T cells (12%). Furthermore, ES cell lines were efficiently established from these NKT cell blastocysts (4%) compared with clones from mature B and T lymphocytes (0.2%-0.3%). The transferred genomes from NKT cells generated embryonic as well as extra-embryonic tissues that play essential complementary roles in mammalian development. Approximately 1.5% of the embryos developed to term offspring. Although heavier than normal mice, the pups were healthy and fertile. The pups and their placentas possessed the rearranged TCR loci specific for NKT cells. The results provide the first direct evidence that fully differentiated cell nuclei carrying and expressing specific genetic markers can be reprogrammed within the oocyte cytoplasm to support full-term-embryo development.

These findings clearly indicate a high level of plasticity in the NKT cell genome. Thus, differentiation of the genome is not always a barrier to nuclear transfer cloning for either reproductive or therapeutic purposes, and we can now postulate that at least some mammals cloned to date have indeed been derived from differentiated donor cells. In addition, we found that cloned mice progeny possess increased number of NKT cells not only in the thymus but also in the periphery. We are now investigating the immunological phenotype and functions of NKT cells and the mechanisms of NKT cell differentiation in these mice.

ORIGINAL RESEARCH PAPER

Inoue K, Wakao H, Ogonuki N, Miki H, Seino K, Numbu-Wakao R, Noda S, Miyoshi H, Koseki H, Taniguchi M, Ogura A. Generation of Cloned Mice by Direct Nuclear Transfer from Natural Killer T Cells. Curr. Biol. 15, 1114-1118 (2005)

The Degranulation Two-step

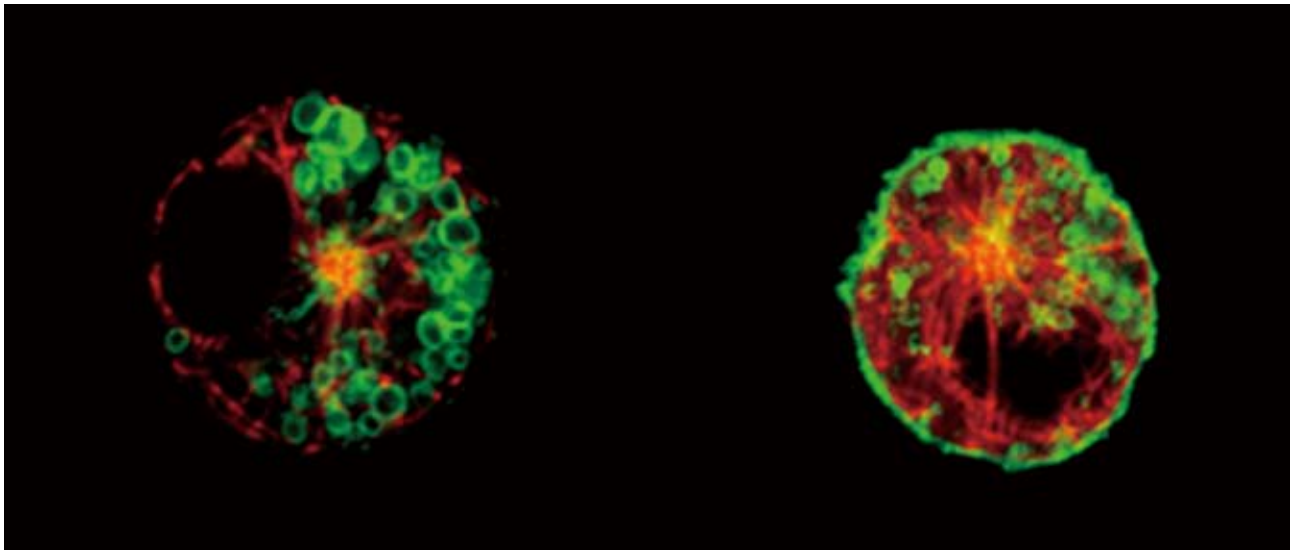


Figure: Granule translocation in mast cell
CD63-containing granules (*green*) move from cytoplasm (*left*) to the plasma membrane (*right*) and granules partially colocalize with microtubule (*red*) after stimulation (*right*).

Mast cells are granulated cells that play a pivotal role in allergy and inflammation. The granules contain inflammatory mediators such as histamine, proteases, lipid mediators, and cytokines. The activation of mast cells induces exocytosis and fusion of cytoplasmic granules with the plasma membrane, followed by the release of inflammatory mediators within minutes of stimulation. The aggregation of high-affinity IgE receptors (Fc ε RI) on mast cells which is induced by the antigen–IgE complexes is a potent stimulus for the release of inflammatory and allergic mediators from the cytoplasmic granules. However, the molecular mechanism of degranulation has not yet been established so far. It is still unclear how FcεRI-mediated signal transduction ultimately regulates the reorganization of the cytoskeleton and how these events lead to degranulation.

It is believed that a calcium-dependent event is sufficient for the Fc ε RI-induced degranulation in mast cells, since members of the SNARE (soluble NSF attachment protein receptor, where NSF is *N*-ethylmaleimide sensitive fusion protein) family were found to regulate granule-to-plasma or granule-to-granule membrane fusion in response to elevated cytosolic calcium concentrations. Nishida and Yamasaki et al. now show that in addition to the canonical calcium-dependent pathway, a calcium-independent and microtubule-dependent pathway is critically involved in degranulation. First, Fc ε RI stimulation triggers microtubule polymerization and granule translocation to the plasma membrane in a calcium-independent manner. Second, the granules fuse with the plasma membrane in a well-characterized calcium-dependent manner. Thus, the Fc ε RI-induced degranulation process could be dissected into two steps: a calcium-dependent process and a calcium-independent and microtubule-dependent one. Furthermore, using bone-marrow-derived mast cells (BMMCs) deficient in Fyn tyrosine kinase, they showed that Fyn, adaptor protein Gab2 and small G protein RhoA play a critical role in the calcium-independent, microtubule-dependent pathway. Lyn tyrosine kinase and the adaptor protein SLP-76, the essential molecules of the calcium-dependent pathway, were not involved in this calcium-independent and microtubule-dependent pathway. The signaling pathway plays a critical role in the calcium-independent, microtubule-dependent pathway.

Very recently, Toshio Hirano's group have shown that the zinc (Zn) chelator *N,N,N,N*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) is a novel anti-allergic agent. Nishida and Kabu et al. found that TPEN significantly inhibited the Fc ε RI-induced activation of BMMCs, which is indicated by β-hexosaminidase release, cytokine production, and leukotriene release (*J Immunol*. in press). Furthermore, in vivo allergic reactions such as passive cutaneous and systemic anaphylaxis were markedly decreased in TPEN-treated mice. Importantly, they clearly showed that TPEN inhibited Fc ε RI-induced granule translocation to the plasma membrane and confirmed that re-loading with Zn rescued the defect in Fc ε RI-induced granule translocation in Zn-depleted BMMCs. This suggested that the calcium-independent granule translocation process is totally dependent on Zn.

The above findings provide an insight to the molecular mechanisms of mast cell degranulation and might provide a relatively specific target for drugs aimed at blocking the release of chemical mediators from mast cells.

ORIGINAL RESEARCH PAPER

Nishida K, Yamasaki S, Ito Y, Kabu K, Hattori K, Tezuka T, Nishizumi H, Kitamura D, Goitsuka R, Geha RS, Yamamoto T, Yagi T, Hirano T. FcεRI-mediated Mast Cell Degranulation Requires Calcium-Independent Microtubule-Dependent Translocation of Granules to the Plasma Membrane. J. Cell Biol. 170, 115-126 (2005)

Role of IL-6/gp130 and Zinc Signaling in Dendritic Cell Function

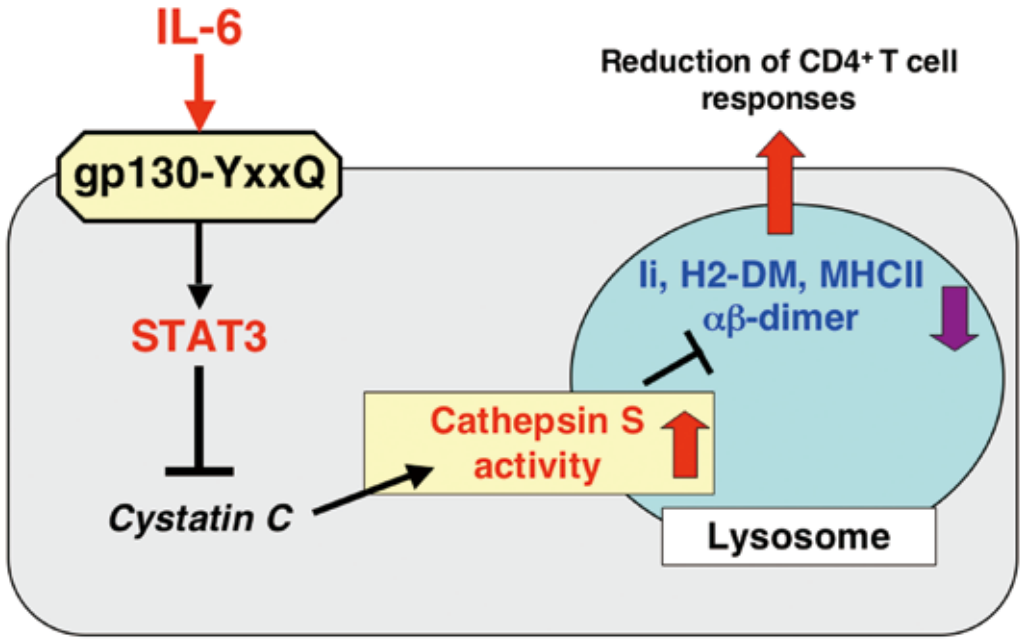


Figure: IL-6 STAT3 controls intracellular MHC class II αβ-dimer level through cathepsin S activity in dendritic cells, leading to reduction of CD4+ T cell response.

The regulation of major histocompatibility complex class II (MHCII) molecules in dendritic cells (DCs) is a critical factor in the host defense system and involves activation of the adaptive immune system. Antigen presentation through MHCII in DCs is critical for $CD4^+$ T cell-mediated immune responses. Toshio Hirano's group investigated the effect of IL-6/gp130 signaling on DC function related to antigen presentation. Previously, Park and colleagues reported that the IL-6-STAT3 pathway suppresses DC maturation, including the surface expression of MHCII and costimulators, and attenuates $CD4^+$ and $CD8^+$ T cell response *in vivo* and *in vitro*. Then, the group investigated how STAT3-mediated signaling inhibits the surface expression of MHCII in activated DCs. Now, Kitamura et al. provide evidence that IL-6-signal transducer and activator of transcription-3 (STAT3) signaling decreased intracellular MHCII αβ dimer, invariant chain (Ii), and MHC-like molecular chaperon (H2-DM) levels by enhancing cathepsin S activity in DCs, even before the activation. These mechanisms explain, at least in part, how STAT3-mediated signaling suppresses the LPS-mediated surface expression of MHCII in DCs. Importantly, their results indicate that a cathepsin S-mediated decrease in the MHCII αβ dimer level attenuated the subsequent $CD4^+$ T cell response.

According to these findings, regulation of endogenous protease activity in DCs by IL-6-dependent STAT3 activation is expected to become a powerful approach to control the DC-mediated immune response.

In a breakthrough discovery, Hirano's group published in *Nature* in 2004, Yamashita et al. identified the zinc transporter ZIP6 (also known as LIV1) as the target gene of the IL-6 cytokine signaling molecule STAT3 (Yamashita et al. *Nature*, 2004), and further demonstrated that ZIP6 plays an important role in the massive cell migration that takes place during the gastrulation process in the early development of the zebrafish embryo. Zinc is a trace element that is essential for the function of many enzymes and transcription factors. Zinc deficiency results in defects in innate and acquired immune responses. However, little is known how zinc controls immune cell function.

Very recently, Kitamura and his colleagues found that stimulation with the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS) altered the expression of zinc transporters in dendritic cells, thereby decreasing intracellular free zinc. Intracellular zinc depletion with the chelator upregulated surface MHCII level and $CD4^+$ T cell response, whereas zinc supplementation or overexpression of the gene encoding ZIP6, whose expression was reduced by LPS, inhibited LPS-induced upregulation of MHCII, costimulatory molecules, and $CD4^+$ T cell response (*Nature Immunology*, in press). These results strongly suggest that intracellular zinc homeostasis is closely related to DC-mediated immune responses. These findings establish a novel mechanism that links TLR to zinc signaling, indicating its crucial role in immune regulation.

ORIGINAL RESEARCH PAPER

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Negative Adaptor in B Cell Signaling

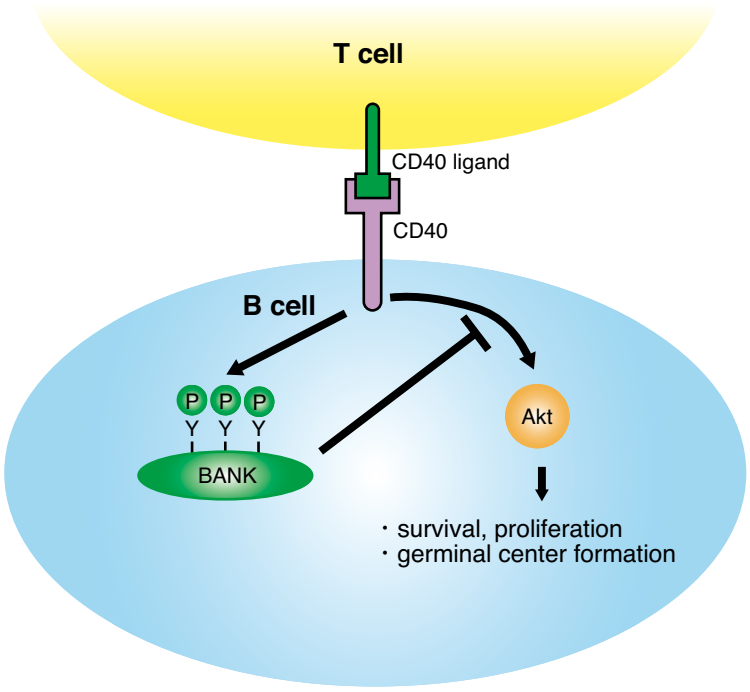

Figure: BANK functions as a negative regulator in CD40-mediated Akt activation, thereby preventing hyper-active B cell responses.

The fact that the immune system must respond to a bewildering number of challenges is reflected by its enormous diversity and plasticity. Take the example of B cells. B cells undergo negative selection in the bone marrow, generating humoral responses in the periphery and establishing tolerance and memory. Signaling through the B cell receptor (BCR) leads to a wide range of biological outcomes that are dependent on the developmental stage of the B cell as well as on the properties of the antigen and the surrounding tissues. It is clear that a simple "on-off" mode of signal transduction would not allow for such a diversity of responses. Rather, BCR signals must be precisely regulated in terms of their magnitude and duration. In addition to BCR, other cell surface receptors such as CD40 contribute to B cell responses. Transmembrane signaling through cell surface receptors should be further regulated or fine-tuned by an array of cytoplasmic signal transduction molecules.

As cytoplasmic signaling molecules, in addition to effector enzymes, adaptor proteins, which lack intrinsic enzymatic activity, have emerged to play an important role. In an earlier work, Dr. Kurosaki's group demonstrated that an adaptor molecule, BCAP, is essential for the development and differentiation of mature B cells as well as T cell independent antigen responses.

BANK, a recently purified adaptor molecule, shares overall structural features with BCAP; BANK and BCAP consist of an ankyrin-repeat-like region and a coiled-coil domain. Because of such structural similarity, BANK is thought to play a similar role to BCAP. However, in this new study, Aiba et al. now demonstrate that in contrast to the positive role of BCAP, BANK functions as a negative regulator in B cells. Indeed, BANK-deficient mice displayed enhanced germinal center formation and IgM production in response to T-dependent antigens. This hyper-phenotype is likely accounted for by hyper-CD40 signaling, because the hyper IgM production was blocked in CD40-BANK double knockout mice. Based on the findings that BCAP and BANK coexist in B cells and exhibit opposing roles, Kurosaki and colleagues propose the idea that changing the expression balance between BCAP and BANK may allow B cells to generate distinct responses under same stimulation conditions.

In addition to B cells, CD40 is expressed by a large variety of cell types including dendritic cells, monocytes, macrophages, mast cells, and endothelial cells. Mirroring such a broad distribution, CD40 is now thought to function as a general regulator of immune and inflammatory processes rather than a specific regulator of B cell mediated responses. Excess CD40 signaling in mouse models causes various complex anomalies leading to diseases including autoantibody production, chronic skin inflammation,and intestinal inflammation. These anomalies are manifested probably as a net outcome of multiple cell types. Thus, the identification of BANK as a B-cell-specific negative regulator of CD40 signaling could provide a tool to dissect such pleiotropic CD40 functions.

ORIGINAL RESEARCH PAPER

Aiba, Y., Yamazaki, T., Okada, T., Gotoh, K, Sanjo, H., Ogata, M. and Kurosaki, T. BANK negatively regulates Akt activation and subsequent B cell responses. Immunity 24, 259-268 (2006)

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Mechanism of NF-κB Signaling

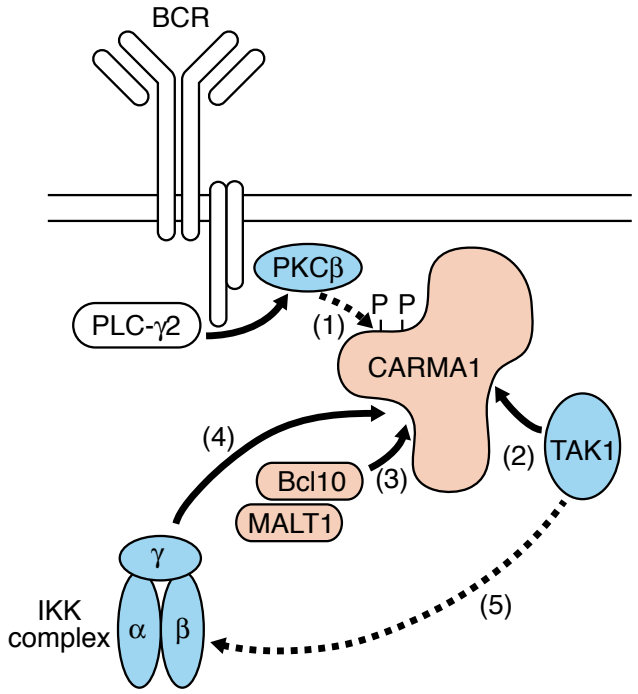


Figure: A model of BCR-mediated NF-κB activation. Stimulation of BCR leads to activation of proximal protein tyrosine kinases including Syk and Btk. Btk phosphorylates several tyrosine residues on PLC-γ2, and subsequently activates PKCβ. Activated PKCβ phosphorylates CARMA1 (1) directly or indirectly, which is able to recruit TAK1 to the phosphorylated CARMA1 (2). Meanwhile, the IKK complex, probably through the Bcl10/MALT1 complex, is recruited to the phosphorylated CARMA1 (3 and 4). These interactions (CARMA1-IKK and CARMA1-TAK1) contribute to the access of two key protein kinases, TAK1 and IKK, leading to activation of the IKK complex.

Inducible transcription factors regulate immediate and long-lived cellular responses necessary for organismal adaptation to environmental plasticity. One transcription factor that serves as a key responder to changes in the environment is nuclear factor κB (NF-κB). The biological system in which NF-κB plays the most important role is the immune system. For instance, NF-κB is critical for a variety of immunological stimuli such as B cell receptor in adaptive immunity or Toll-like receptors that sense and recognize intruding microorganisms in innate immunity. Therefore, the existence of signal-specific pathways for NF-κB activation is postulated.

In adaptive immunity, triggering of the B cell antigen receptor (BCR) leads to the initiation of multiple signaling pathways that regulate cellular proliferation and survival of immature and naive B lymphocytes, and the effector functions of mature B cells. Among them, the signaling pathway that leads to the activation of the transcription factors of NF-κB has a crucial role in these processes. The common feature of signals that induce NF-κB is the activation of an IκB kinase (IKK) complex consisting of two catalytic subunits, IKKα and IKKβ, and an essential regulatory subunit IKKγ. Indeed, IKK and subsequent NF-κB activation take place upon BCR engagement. In the BCR signaling context, protein kinase C (PKC) β is activated downstream of phospholipase C (PLC)-γ2, which in turn is important for IKK and subsequent NF-κB activation. In addition to PKCβ, adaptor proteins (CARMA1-Bcl10-MALT1) that participate in BCR-mediated IKK activation have been identified. However, it remains unclear whether PKCβ and the CARMA1-Bcl10-MALT1 complex connect and how, if any, these connections lead to IKK activation.

Shinohara et al. have demonstrated that the BCR-induced phosphorylation of CARMA1, mediated by PKCβ, contributes to bringing two key protein kinases, TGF-β-activated kinase 1 (TAK1) and IKK, into close proximity. As a consequence, TAK1 functions as an upstream kinase for IKK activation and subsequent NF-κB activation in the BCR signaling context. Therefore, this study reinforces the idea that adaptor proteins function as an interaction assembly point, thereby facilitating the interaction between binding partners.

TAK1 was reported to be a kinase that couples innate receptors, Toll-like receptors, to IKK activation. Therefore, the involvement of this kinase also in antigen-receptor–mediated IKK activation is somewhat surprising. Phosphorylation of a specific adaptor molecule, CARMA1, recruits two common NF-κB players, TAK1 and IKK, into the adaptive immune receptor signaling. Thus, the adaptive immune system in vertebrates may have evolved from the more primitive innate immune system by usurping the common key signaling components of the NF-κB pathway including TAK1 and IKK, using the CARMA1-Bcl10-MALT1 complex.

ORIGINAL RESEARCH PAPER

Shinohara, H, Yasuda, T, Aiba, Y, Sanjo, H, Hamadate, M, Watarai, H, Sakurai, H and Kurosaki, T. PKCβ Regulates BCR-Mediated IKK Activation by Facilitating the Interaction between TAK1 and CARMA1. J. Exp. Med. 202, 1423-1431 (2005)

DNA Polymerase for Mutations at C/G

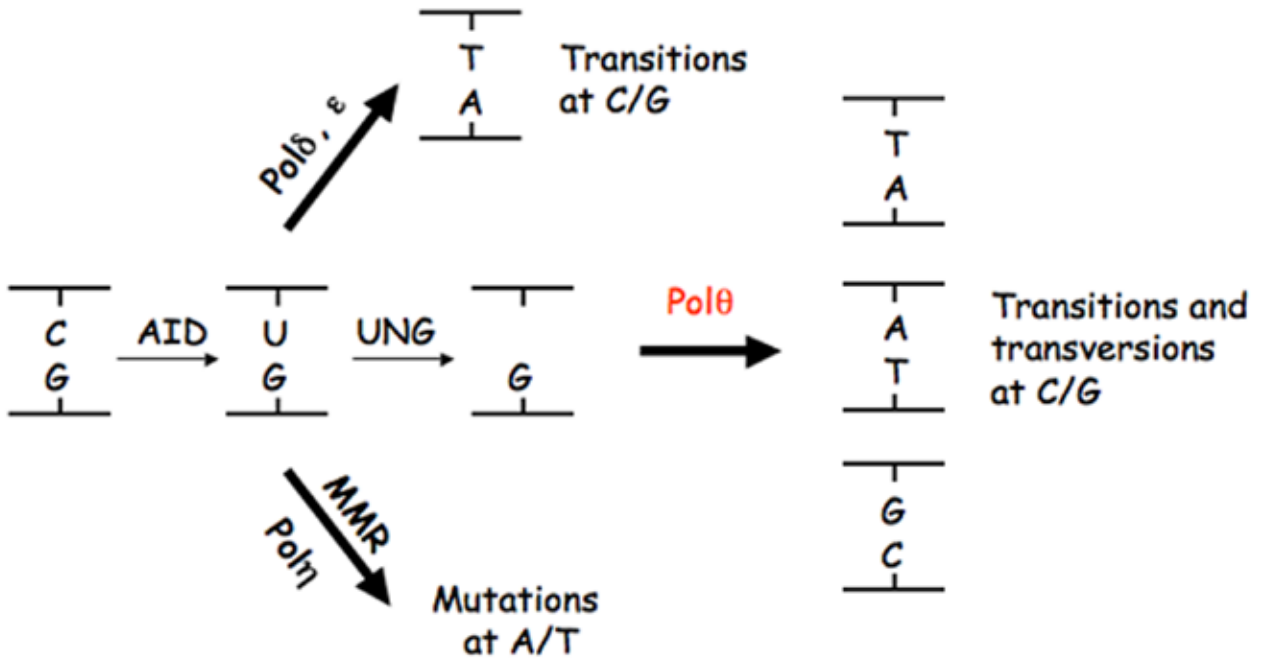


Figure: Role of Polθ in the SHM of Ig genes

Functional Ig genes are assembled in developing B cells by recombination-activating gene-mediated rearrangement of the germline V, D, and J gene segments. This process generates a primary repertoire of B cells expressing diversified surface immunoglobulins. Upon antigen stimulation and in the presence of T cell help, B cells undergo further diversification of their Ig genes, namely, somatic hypermutation (SHM) and class switch recombination (CSR), in the germinal centers (GCs) of secondary lymphoid organs such as spleen, lymph node, and Peyer's patches. Both SHM and CSR are initiated by a single enzyme, activation-induced cytidine deaminase, which catalyzes the deamination of C to U on DNA and/or possibly on an as-yet-hypothetical endonuclease mRNA. Although the mechanism of SHM is still not fully understood, it is thought that mutations are ultimately introduced by error-prone DNA polymerases during the DNA repair process.

Approximately 10 new low-fidelity DNA polymerases have been identified in the past several years. DNA polymerase η has been implicated in mutations at A/T but polymerases involved in C/G mutations have not been identified.

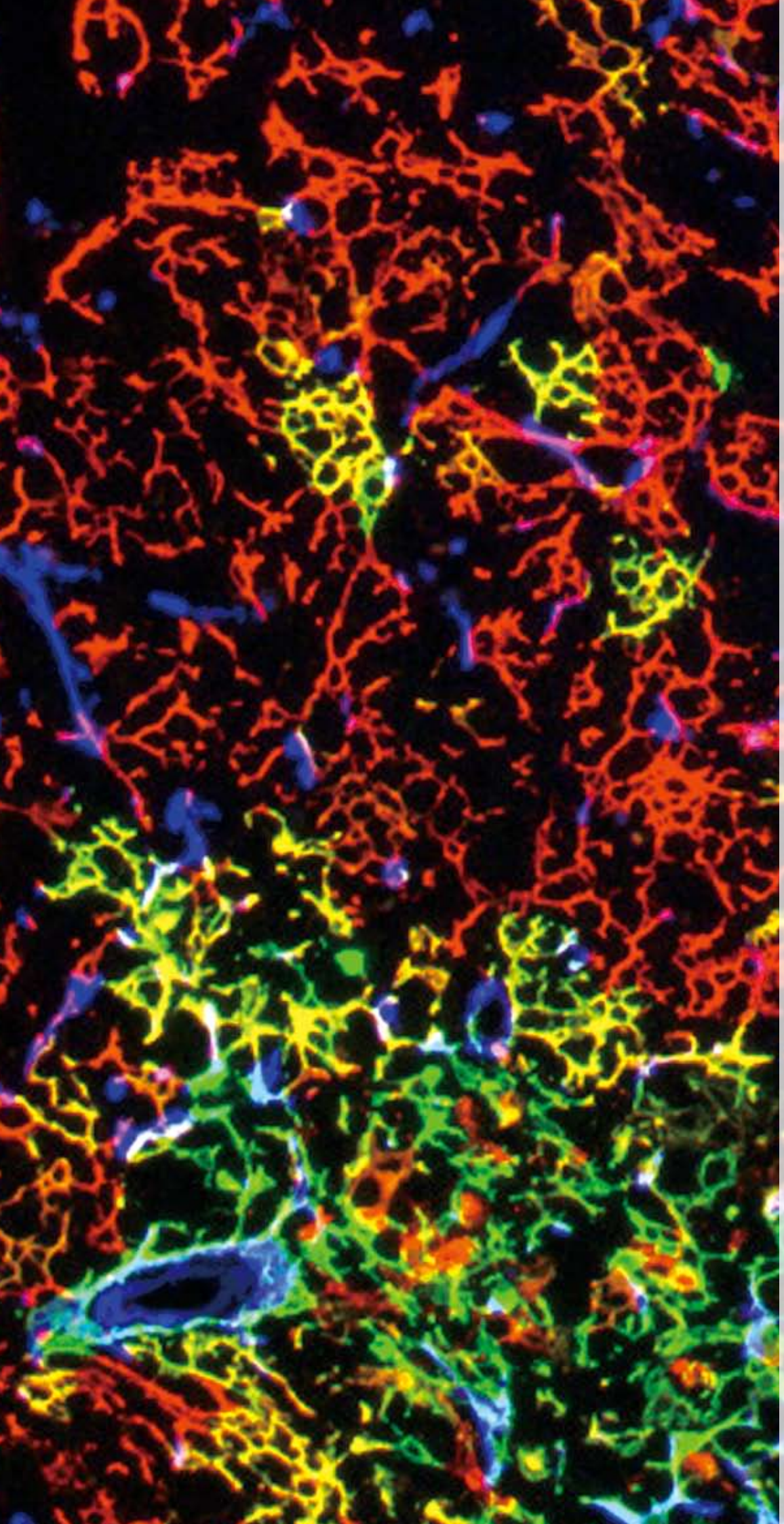
DNA polymerase (Polθ) exhibits a tissue-specific expression pattern. Unlike other low-fidelity DNA polymerases that are ubiquitously expressed, Dr. O-Wang's group found preferential expression of human and mouse *Polq*, the gene encoding Polθ, in lymphoid tissues. Most interestingly, abundant *Polq* transcripts were detected in GC B cells, the target cells of both SHM and CSR. The lymphoid-tissue-specific expression pattern of *Polq* in both human and mouse suggested that Polθ might have a specialized role in mammalian lymphocytes.

To investigate the polymerase function of Polθ in SHM, we have generated mutant mice expressing a DNA polymerase θ (Polθ) specifically devoid of polymerase activity. Compared to wild type mice, *Polq*-inactive mice exhibited reduced levels of serum IgM and IgG1. The mutant mice mounted relatively normal primary and secondary immune responses to a T-dependent antigen, but the production of high-affinity specific antibodies was partially impaired. Analysis of the mutation frequency in intronic sequences of the Ig J region revealed a slight reduction of the overall mutation frequency in *Polq*-inactive mice. Remarkably, however, only mutations at C/G were specifically reduced in *Polq*-inactive mice, whereas mutations at A/T were not affected, indicating an important, albeit not exclusive role of Polθ polymerase activity.

These results have led to the identification of the first DNA polymerase that is involved in the mutations at C/G, and suggest that multiple polymerases are required to generate all types of mutations. These findings, together with the recent observation that Polθ efficiently catalyzes the bypass of abasic sites, lead us to propose that Polθ introduces mutations at C/G by replicating over abasic sites generated via uracil-DNA glycosylase.

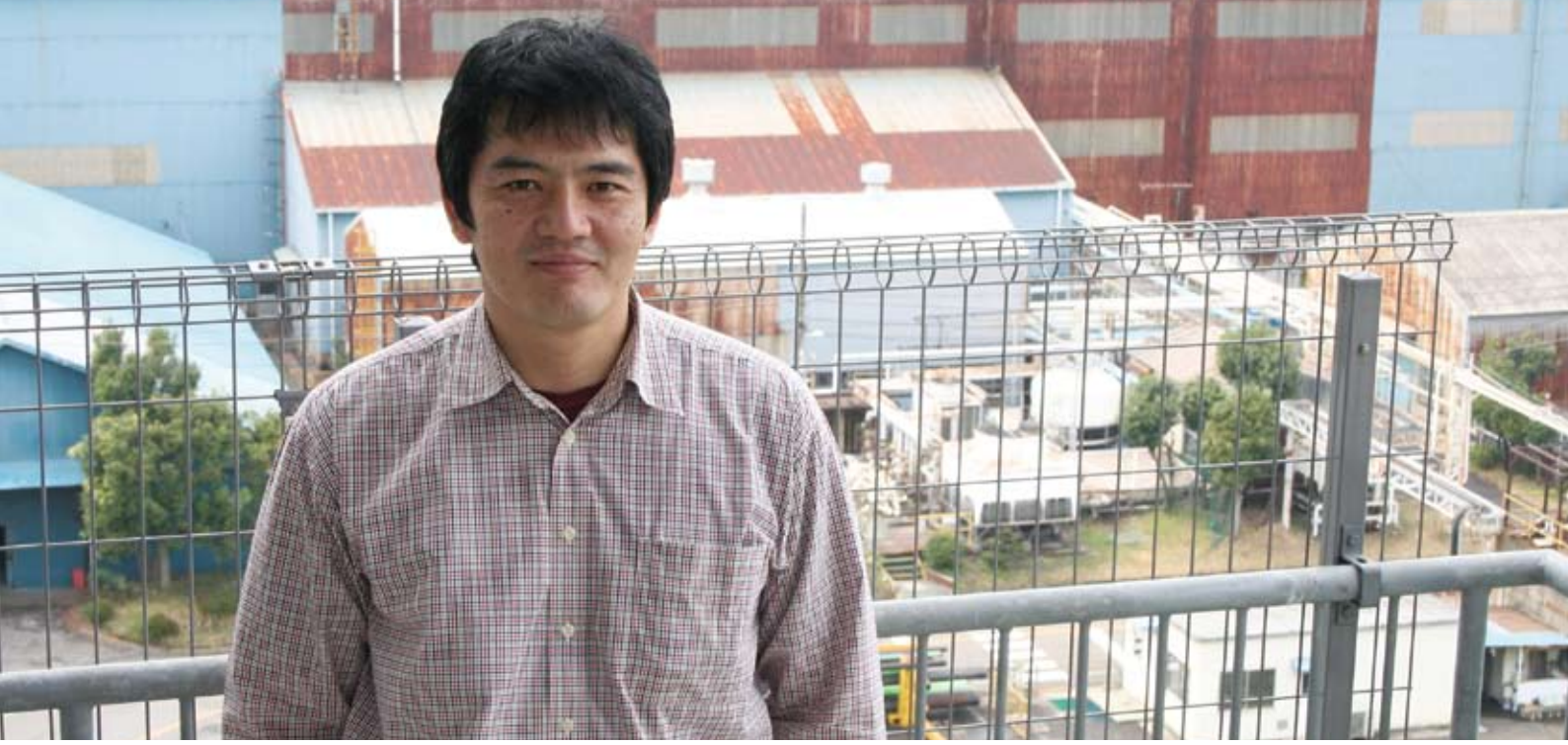
ORIGINAL RESEARCH PAPER

Masuda, K, Ouchida, R, Takeuchi, A, Saito, T, Koseki, H, Kawamura, K, Tagawa, M, Tokuhisa, T, Azuma, T and O-Wang, J. DNA Polymerase θ Contributes to the Generation of C/G Mutations during Somatic Hypermutation of Ig Genes. Proc. Natl. Acad. Sci. USA 102: 13986-13991 (2005)



Research Activities

2005



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The Developmental Genetics Research Group fulfills a double role within RCAI. A large portion of the manpower and financial resources of the group is devoted to the maintenance of a high-standard mouse facility at RCAI. Through the Animal Core Facility, the group is also responsible for the generation of knockout and transgenic animals for the various research laboratories at the center. At the same time, the laboratory is pursuing a research program to elucidate the molecular mechanisms underlying the epigenetic regulation mediated by Polycomb group (PcG) genes in development. PcG genes have been first isolated in Drosophila melanogaster as a group of genes required in the maintenance of segmental identity and have been shown to be structurally and functionally conserved in mammals. Genetic analyses of mammalian PcG proteins revealed their roles not only in the anterior-posterior specifications but also in cellular proliferation, differentiation, and senescence. Particularly, significant impacts of PcG functions on lymphocyte and lymphoid organ development have repeatedly been reported. PcG gene products form at least two different multimeric protein complexes; one mediates histone H3-K27 trimethylation, and another, H2A-K119 ubiquitinylation, on the chromatin. It is, however, unknown how PcG complexes mediate transcriptional repression and, consequently, exert their biological functions. To elucidate the molecular nature of PcG-mediated transcriptional regulation, we have been focusing our activity on the following issues: (1) association of PcG proteins to their targets, (2) molecular mechanisms underlying PcG-mediated repression, and (3) real-time imaging of PcG repressive complexes.

Major Results

Analysis of association of PcG proteins to their targets is undertaken in collaboration with Dr. Osamu Ohara (RCAI Immunogenomics Group). A main question addressed is how PcG multimeric complexes act on various target loci. Before addressing this issue, it is necessary to elucidate the target loci of PcG and related complexes. For this purpose, we have devised the chromatin immunoprecipitation (ChIP) technique using embryonic tissues, in which PcG proteins are in fact active. By combining this method with ChIP-on-Chip technique, we have systematically identified PcG target loci (Lee et al., 2006). Importantly, most of the PcG target genes turned out to be involved in development, patterning, differentiation, and cell growth. We further found a regulatory role of the Ring1B component for the complexes that eventually discriminated transcriptionally active and repressed domains (Fujimura et al., 2006).

Although PcG complexes have been shown to mediate transcriptional repression via its direct binding to the target and subsequent histone tail modifications, which transcriptional process is disrupted by PcG-binding and/or –mediated chromatin modifications has not yet been determined. We are looking for molecules that act after PcG binds to at the targets to repress transcription. In the last five years, we have endeavored to identify PcG binding proteins by yeast two-hybrid screening and purification of the complexes by tagging several PcG proteins. By generating mutant animals lacking respective PcG partners, we are currently conducting genetic analyses to elucidate the genetic epistasy. First, we have identified Phc2, a homolog of *Drosophila polyhomeotic*, as a constitutive component of the PcG complexes, which plays

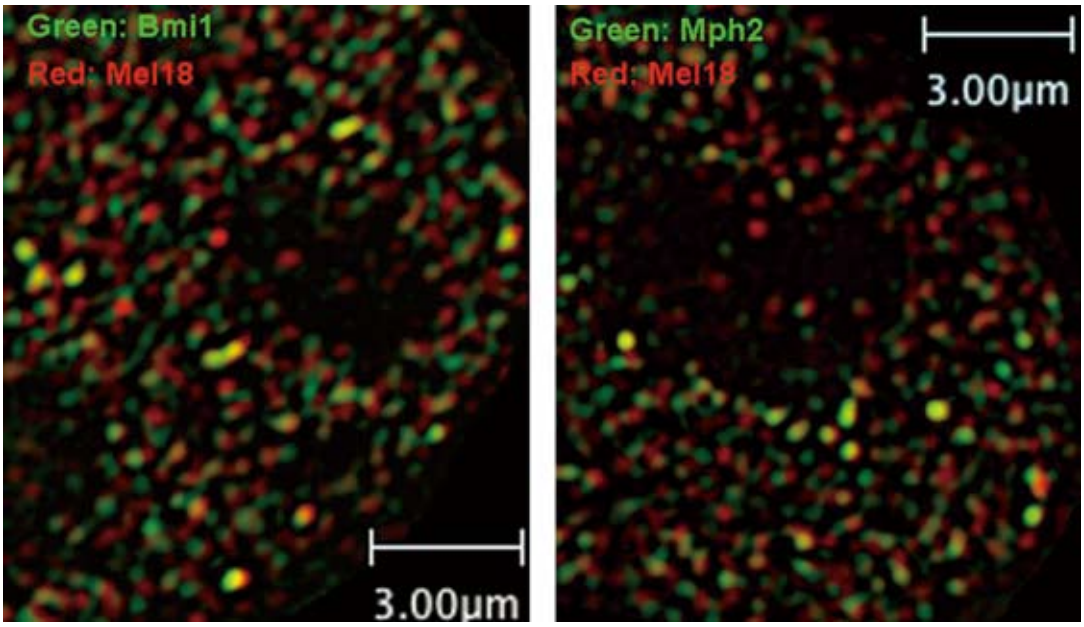

Figure: Deconvolved confocal images of PcG proteins by multicolor indirect immunofluorescence in MEFs. (*left panel*) Predominant colocalization of Mel18 and Bmi1 in large globular domains. (*right panel*) Predominant colocalization of Mel18 and Phc2 in large globular domains.

a role in oligomerizing PcG complexes in cooperation with its homolog Phc1 (Isono et al. 2005). Secondly, corepressors, homeoprotein-interacting kinases (HIPK1, -2, and -3), were identified as Phc2 binding proteins and their mutant animals exhibited homeotic transformations of the axis as well as PcG mutants in a gene dosage-dependent manner. Genetic analysis suggested epistatic roles of HIPKs for PcG complexes. It is likely that HIPKs could link PcG complexes with p300/CBP-containing HAT activity (Isono et al, 2006). Surprisingly, HIPKs have recently turned out to be a component of MOZ complexes, which are required for the maintenance of haematopoietic stem cells. By using HIPK1/2 double-knockout mice, HIPKs were shown to be required for the generation of haemangioblastic precursors from the lateral plate mesoderm (Kitabayashi et al., in press). Thirdly, we have recently copurified a ubiquitin-specific protease together with catalytically active PcG complexes. We are currently conducting genetic analysis to elucidate the role of this protein in PcG functions.

Another important issue is to elucidate the dynamics of PcG complexes in living cells. This work is undertaken in collaboration with Dr. Makio Tokunaga (RCAI, Single-molecule imaging Unit). For this purpose, we have been generating knock-in alleles for various PcG genes, in which PcG proteins tagged by fluorescent proteins are expressed from the respective endogenous loci. By combining such genetic resources and single-molecule imaging technique in living cells, we are currently describing the dynamics of PcG complexes quantitatively. We identified globular and filamentous domains formed by various PcG proteins in primary embryonic fibroblasts. Globular domains are mutually different in their size. Multicolor indirect immunofluorescence analyses revealed that larger globular domains are associated with various different PcG proteins and trimethylated H3-K27 (PcG proteins are self-associated through larger globular domains or PcG proteins are associated with different PcG proteins through each larger globular domains) whereas this association is excluded by acetylated or di- or trimethylated H3-K9. Our current results strongly suggest that large globular domains represent PcG-repressive domains. We, however, need further proof to justify this hypothesis.

Conclusions and perspectives

Based on the abovementioned three different approaches for analysis of PcG functions, we have recently found that the oligomerization of PcG core complexes could be a rate-limiting process to mediate the transcriptional repression and be regulated by both PcG-intrinsic and -extrinsic mechanisms. This intrinsic mechanism was clearly shown to involve PcG proteins that contain SPM domains such as Phc1, Phc2, Phc3 and Scmh1. These proteins appear to sense the degree of PcG oligomerization via interactions with other PcG components. In contrast, the extrinsic mechanism was suggested to involve the interactions of PcG complexes with various modified histone tails (M. Endoh, unpublished). Moreover, other groups have revealed the involvement of a dicer-dependent RNA machinery for this oligomerization process. It should be important to explore the molecular links that connect the intrinsic and extrinsic mechanisms.

Recent Publications

Lin L, Ozaki T, Takada Y, Kageyama H, Nakamura Y, Hata A, Zhang JH, Simonds WF, Nakagawara A, and Koseki H. topors, a p53 and topoisomerase I-binding RING finger protein, is a coactivator of p53 in growth suppression induced by DNA damage. **Oncogene** 24:3385-3396. (2005)

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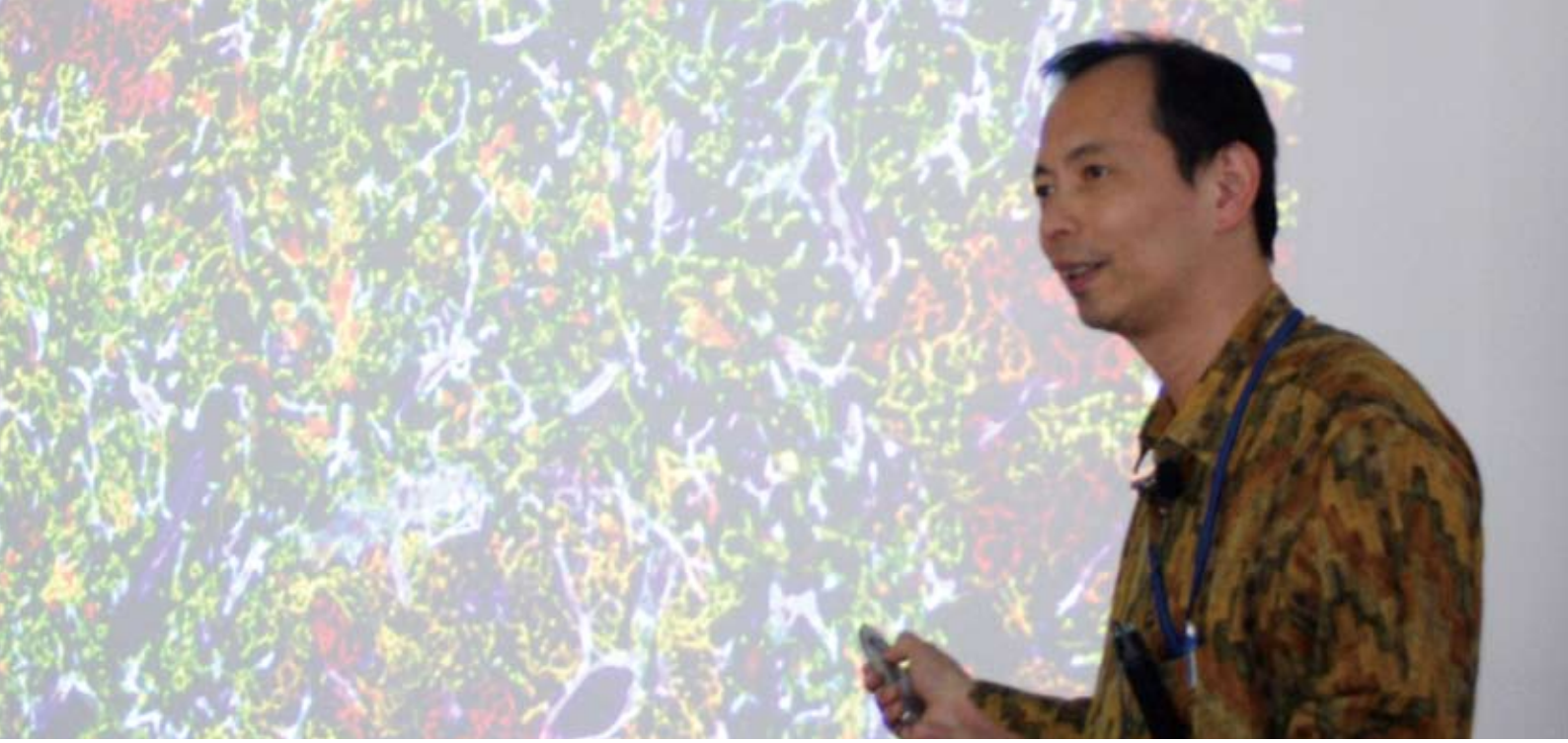

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In hematopoiesis, pluripotent hematopoietic stem cells (HSC) are sequentially restricted to give rise to a variety of lineage-committed progenitors. The major aim of the Laboratory for Lymphocyte Development is to elucidate the molecular mechanisms that regulate cell fate decisions in the process of lineage restriction from HSC to unipotent progenitors. We have previously established a clonal assay system to examine the developmental potential of individual progenitor cells toward T, B and myeloid cell lineages. This work has led to the fundamental redefinition of lymphoid progenitors and the ontogeny and phylogeny of T and B cell development.

Process of lineage commitment in hematopoiesis

The classic dichotomy model of hematopoiesis postulates that the first step of differentiation from HSC generates common myelo-erythroid and common lymphoid progenitors (CLP). Our previous studies in fetal mice, however, indicated that the first step of lineage restrictaion in HSC is the generation of myelo-lymphoid lineage progenitors and myelo-erythroid lineage progenitors. Most importantly, the myeloid potential is retained even after the segregation of myelo-lymphoid progenitor towards T and B cell lineages. Thus, each process of specification towards T, B and erythroid lineages appears to proceed accompanying the prototypical myeloid program. Recently, we call this model the "myeloid-based model" (Kawamoto H, *Trends Immunol*, in press). The CLP have, however, persisted in models of adult hematopoiesis, as several groups have provided experimental results supporting the presence of CLP in bone marrow. We are now studying whether the myeloid-based model is also applicable to adult hematopoiesis.

Prethymic stages of T cell development

We have previously reported that T cell lineage restricted progenitors are present in prethymic organs such as the aorta-gonad mesonephros (AGM) region, fetal liver, and fetal blood. However, it remains controversial whether the thymus-colonizing progenitors are committed to the T cell lineage. One problem is that the earliest intrathymic progenitors do not necessarily represent genuine thymic immigrants because their developmental potential should have been influenced by contact with the thymic microenvironment. We then examined the developmental potential of the ontogenically earliest thymic progenitors of day 11 murine fetus, which reside in the surrounding mesenchymal region and have not encountered thymic epithelial components. We examined the developmental potential of these cells, and the results provided direct evidence that the progenitors restricted to the T/NK/DC lineage selectively immigrate into the thymus. Very recently, we have found that the prethymic T cell progenitors express PIR (paired-immunoglobulin-like receptors) on their surface. This finding not only provides a tool for the isolation of prethymic T cell progenitors but also substantiates that the prethymic stage of T cell development is distinct from intrathymic stages. The isolated T cell progenitors will be a useful tool for gene therapy and regenerative medicine.

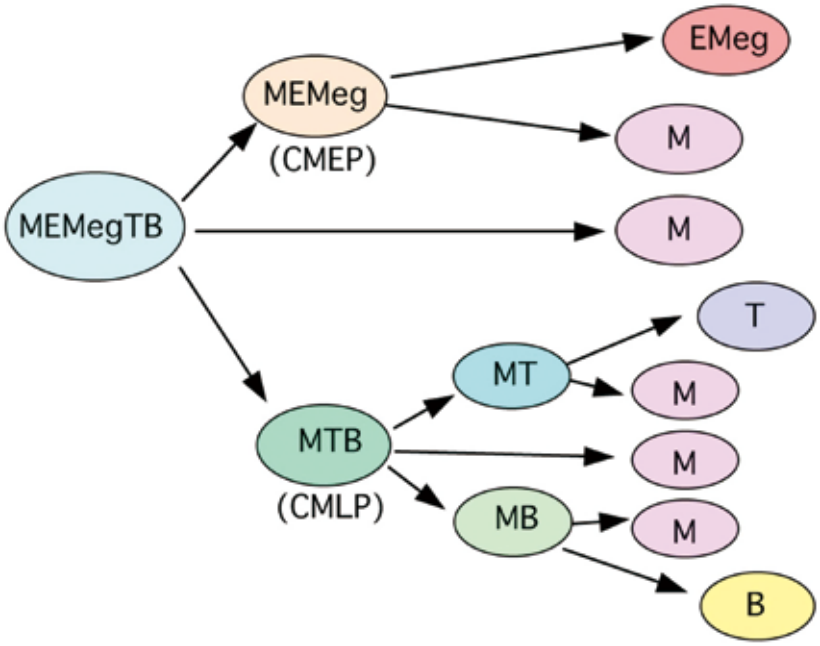

Figure: Myeloid-based model: This model proposes that the developmental programs towards T, B and erythroid lineages proceed independently on the basis of the prototypical myeloid program.

Besides the above two projects, we are interested in whether environmental factors instructively induce lineage commitment or selectively support autonomously committed progenitors in lymphopoiesis. To this end, it is important to establish an experimental system by which lineage commitment of progenitors can be monitored with real-time imaging. We have previously shown that the earliest T cell progenitors in the thymus retain the potential to generate NK cells and dendritic cells, and that the discontinuation of this tri-potentiality and clear T cell lineage committment happens before the initiation of TCRβ chain gene rearrangement. To establish an experimental system to directly visualize this differentiation step, we are using GFP transgenic mice in which the expression of GFP is controlled by the proximal promoter of T cell specific tyrosine kinase lck.

Research activities include the study of human lympho-hematopoiesis. Cord blood cells are used as the progenitor source, and the basic culture system is a modification of co-culture with murine stromal cells.

We are also interested in the development of thymic epithelial cells. Thymic epithelial cells support thymocyte development, while it is known that thymocytes in turn support the development of thymic epithelial cells. This mutual interaction is called thymic crosstalk. In collaboration with Professor van Ewijk, we are studying the molecular mechanisms of the regulation of thymic epithelial cell development by thymocytes, using the organ culture system. This project also includes studies on the progenitors of thymic epithelial cells.

The information and technology that will be acquired through these studies can be applied directly to regeneration therapy and gene therapy. We are trying to induce mature T cells from progenitors on monolayered stromal cells, which can be a source for immune cell therapy in the future.

Recent Publications

Masuda K, Kubagawa H, Ikawa T, Chen CC, Kakugawa K, Hattori M, Kageyama R, Cooper MD, Minato N, Katsura Y, Kawamoto H. Prethymic T-cell development defined by the expression of paired immunoglobulin-like receptors. EMBO J. 24, 4052-4060 (2005)

Lu M, Tayu R, Ikawa T, Masuda K, Matsumoto I, Mugishima H, Kawamoto H, Katsura Y. The earliest thymic progenitors in adults are restricted to T, NK and dendritic cell lineages and have a potential to form more diverse TCR-β chains than fetal progenitors. J. Immunol. 175, 5848-5956 (2005)

Masuda K, Itoi M, Amagai T, Minato N, Katsura Y, Kawamoto H. Thymic anlage is colonized by progenitors restricted to T, NK and dendritic cell lineages. J. Immunol. 174, 2525-2532 (2005)

Ikawa T, Kawamoto H, Wright LYT, Murre C. Long term cultured E2A-deficient hematopoietic progenitor cells are pluripotent. Immunity. 20, 349-360 (2004)

Ikawa T, Masuda K, Lu M, Minato N, Katsura Y, Kawamoto H. Identification of the earliest prethymic T cell progenitors in murine fetal blood. Blood. 103, 530-537 (2004)

Transcriptional Regulation Research Team

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Developmental programs in metazoans present the daunting challenge of spatiotemporal control of gene expression. The determination of the fate of progenitor cells differentiating into distinct lineage cells involves several steps. Following lineage specification, cell identity is established during a process known as lineage commitment. In fully differentiated cells, specific cellular function is regulated in part by the epigenetic maintenance of gene expression patterns. In our laboratory, we are trying to elucidate how progenitor cells sense external or intrinsic stimuli and switch on the genetic program for the regulation of lineage decision, and how the gene expression pattern established during the differentiation process is maintained by epigenetic mechanisms. We address these questions by studying CD4/CD8 gene regulation and the lineage choice of CD4$^+$CD8$^+$ DP thymocytes as a model system. We have shown that the Runx transcriptional factor complex is involved in CD4 gene silencing and cytotoxic T cell development. Further studies suggest that the target genes of the Runx complex are diverse and the Runx complex would be involved in the development of many lineages of hematopoietic cells. Therefore, our other goal is to understand the functions of the Runx transcriptional factor complex in immune system development and in regulating immune responses.

Lineage commitment during T lymphocyte development

Two major lineages of αβT lymphocytes, helper and cytotoxic T cells, differentiate from common progenitor CD4$^+$CD8$^+$ DP thymocytes. However, the molecular pathways that govern cell fate determination of CD4$^+$CD8$^+$ DP thymocytes remain poorly understood. Whereas cells expressing class II-restricted TCR differentiate into helper lineage and cease CD8 expression, cells expressing class I-restricted TCR differentiate into cytotoxic lineage and silence CD4 expression. Thus, the decisions of CD4$^+$CD8$^+$ DP thymocytes to differentiate into CD4$^-$CD8$^+$ cytotoxic or CD4$^+$CD8$^-$ helper lineage are correlated with the cessation of *Cd4* or *Cd8* gene expression, respectively. In this regard, it is likely that the mechanism regulating the expression of co-receptor genes would share a common regulatory pathway with the mechanism that regulates cell fate determination of CD4$^+$CD8$^+$ DP thymocytes. Lineage-specific expression of *Cd4* gene is controlled by an intronic silencer. We have identified the Runx transcriptional factor family as CD4 silencer binding proteins, and have shown that Runx3 plays a major role in the establishment of the cytotoxic lineage specific CD4 silencing. Interestingly, Runx3 is shown to bind to enhancers at the *Cd8* locus. We have shown that Runx3 deficiency resulted in partial loss of CD8$^+$ cytotoxic lineage T cells at the periphery, indicating the important roles of Runx3 in cytotoxic T cell differentiation. On the other hand, current genetic approaches have demonstrated that the expression of a functional Th-POK/cKrox transcriptional factor is essential and sufficient for helper T cell development. Therefore, it is very tempting to examine the relationship between Runx and Th-POK factors. Our laboratory has established mutant mouse strains for the conditional inactivation of *Runx1*, *Runx3* and *Cbf*β genes. By introducing three kinds of T cell specific Cre transgenes (*Lck-Cre*, *Cd4-Cre* and

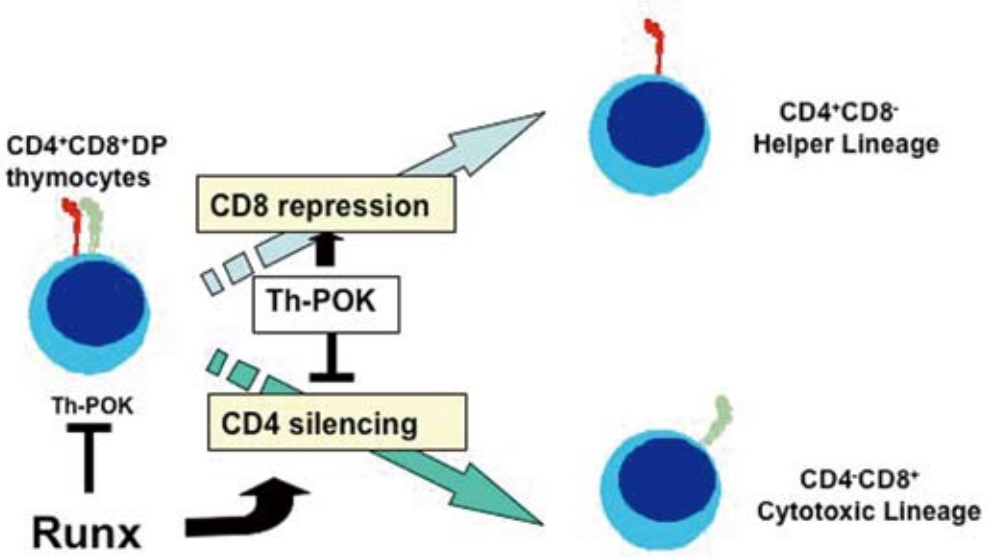

Figure: Possible transcriptional factor network in CD4/8 lineage choice

E8I-Cre) into these flox mutations for stage-specific inactivation of Runx genes, we are currently analyzing the roles of the Runx transcriptional factor family in the lineage decision of $CD4^+CD8^+$ DP thymocytes and T cell development.

Role of Runx transcriptional factors in development and regulation of immune system

The Runx complex has been shown to be involved in many developmental pathways in various species. However, its function in immune system regulation and development has not been well addressed mainly due to the lethal phenotype of germline null mutant mice. In addition to the conventional approach for the straight knockout of genes at germline, we recently applied the gene targeting technique to generate isoform-specific knockout and knock-in mutant mice of Runx family genes. For example, two isoform Cbfβ proteins, Cbfβ1 and Cbfβ2, are generated from the *Cbfβ* locus by alternative RNA splicing. We succeeded in generating mutant mice that specifically lack either Cbfβ1 or Cbfβ2 protein by targeting mutation into splicing donor signals at the *Cbfβ* gene. Since these mutations at the *Cbfβ* locus do not affect early mouse development, we are able to examine in vivo effect of the compromised function of the Runx complex. Interestingly, mice deficient for Cbfβ2 protein spontaneously developed asthma-like disease in lung, which is manifested by the proliferation of eosinophil-like cells and inflammatory bowel disease, indicating that the Runx complex plays important roles in immune system regulation. We are now extensively analyzing the pathogenesis of this disease model mouse by isolating pathogenic cells. Our preliminary characterization suggested that compromised Runx complex function induces Th2-dominant immune responses in an αβT cell intrinsic manner.

Another approach to dissecting the function of isoform Runx1 proteins led to the identification of the novel roles of the Runx complex in the organogenesis of lymphoid organs. In mutant mice in which the proximal promoter region at Runx1 gene is replaced with *Neo* gene, the formation of Peyer's patches was almost abrogated and a small number of peripheral lymph with ectopic location were observed. Thus, in addition to lymphocyte differentiation, the Runx complex also plays important roles in immune organ development.

In addition to conventional knockout approaches, we applied the gene targeting technique to generate the hypomorphic and flox mutant allele of Runx genes. The Runx mutant strains that have been generated, are being generated and will be generated, provide unique experimental tools to examine the in vivo function of the Runx transcriptional factor complex. We are trying to combine biochemical and molecular approaches with genetic studies to further understand the regulatory pathway of this complex. The identification of target genes, associated molecules and the post-translational modification of the Runx complex would provide answers to why the Runx complex exhibits multiple-biological functions. We believe that revealing the Runx-mediated regulatory pathway would shed light on the mechanism of lineage determination pathway that is shared by several developmental processes.

Recent Publications

Egawa T, Eberl G, Taniuchi I, Benlagha K, Geissmann F, Hennighausen L, Bendelac A, Littman D.R. Genetic evidence supporting selection of the Va14i NKT cell lineage from double positive thymocyte precursors. Immunity 22, 705-716 (2005)

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The long-term objective of our group is to determine the molecular mechanisms of T cell activation and homeostasis. In the past, our group focused primarily on the study of negative regulation of T cell Receptor (TCR) signaling through inhibitory adaptors and inhibitory co-stimulatory receptors. Currently, our group's activities encompass a broad variety of projects related to the basic mechanisms of T cell activation, differentiation, and functional regulation. TCR-mediated signaling is analyzed through dynamic molecular assembly, functional cloning, and related strategies that we hope will allow us to eventually assemble all components of the TCR signalsome. We are also increasingly employing new imaging tools to investigate the processes of recognition and activation in T cells at the single-cell and single-molecule level.

Real-time imaging of antigen-recognition and activation of T cells

Our group studies the function and formation of the immunological synapse as well as the dynamic movement of signaling molecules in the process of synapse formation and T cell activation at the single-cell level. In our studies, we use various advanced imaging techniques including single-molecule imaging with the Total Internal Reflection Fluorescence Microscopy (TIRF) approach. Specifically, we used a new single-molecule imaging technique developed by the RCAI Single Molecule Immunoimaging laboratory to visualize single fluorescence-tagged molecules in the dynamics of immunological synapse and lipid raft formation.

CD3ζ, ZAP-70 and SLP-76 are crucial molecules in the TCR signaling complex that function, respectively, as receptors, kinases, and adaptors. By tagging them with fluorescent molecules, we visualized the entire process of synapse formation and discovered that upon stimulation, microclusters containing TCR, kinases and adaptors were formed microclusters that provided the signal for initial activation.

The microclusters are continuously generated at the periphery of the interface and induce sustained activation signals even after the formation of the immunological synapse (see Research Highlights). Our results revealed that the microclusters are the sites for initial and sustained activation of T cells, and led us to re-analyze the function of the immunological synapse. At the same time, we are developing a number of knock-in mouse models to analyze dynamic assembly of the signalsome under physiological conditions. We also used single-molecule imaging techniques were also used to visualize the formation and translocation of lipid rafts on T cells.

Signal regulation in T cell development

Furthermore, advanced imaging techniques were used to investigate the formation of the pre-T cell receptors. Early T cell progenitors in the thymus express a functional pre-TCR composed of a TCR β-chain and an invariant TCR α-chain. T cells with successful TCR β rearrangement develop into $CD4^+CD8^+$ double-positive cells through pre-TCR signaling. This pre-TCR function is believed to be mediated in a ligand-independent, or autonomous fashion. While the pre-TCR is a crucial element in T cell development, very little is known about how pre-TCR signaling actually functions.

Our experiments suggest that autonomous pre-TCR signaling is caused by oligomerization of the pre-TCR α chain. Experiments using the CD8-CD3ε

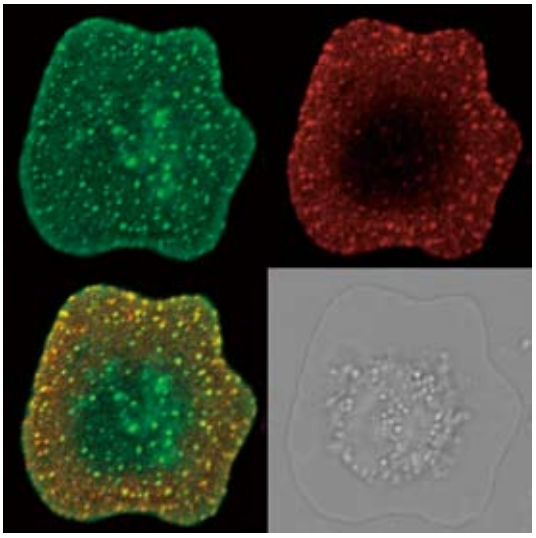

Figure 1: Tyrosine phosphorylated proteins (pY) (*red*) localized in CD3ζ microclusters (*green*) at the periphery of immunological synapse colocalization (yellow)

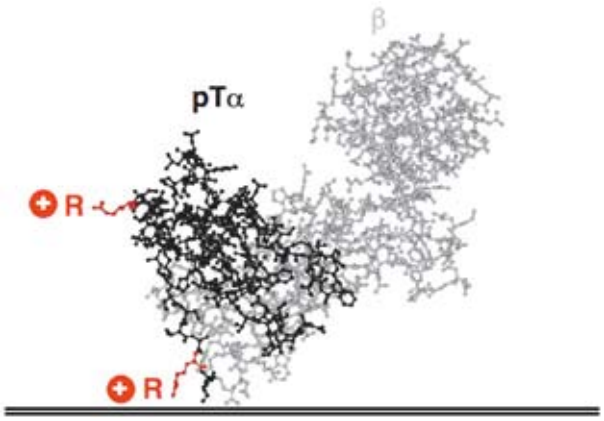

Figure 2: Three-dimensional structure of the pTα-TCRβ complex. Charged residues (R) critical for pre-TCR oligomerization are localized on the molecular surface.

chimeric molecule suggested that localization of the pre-TCR signaling complex on lipid rafts is not sufficient for beta-selection. Further, we determined the amino-acid residues on the extracellular domain of pre-TCR responsible for oligomerization, and showed that in a transgenic model lacking these residues pre-TCR signaling and beta-selection were abolished. Thus, our experiments suggest that the pre-TCR complex can signal through oligomerization of its alpha-chain, when induced by electrostatic interaction through charged amino-acid residues (see Research Highlights).

Co-regulation of NF-κB activation in innate and adaptive immunity

An intriguing new research direction within our group is the investigation of co-regulation of signaling in the adaptive and innate immune systems. The underlying hypothesis of this approach is that signaling molecules critical in activation pathways for the innate immune system may also play a role in the activation of adaptive immune system. In our research, we have mainly focused on signaling pathways to the NF-κB activation in T cells.

Most recently, we have demonstrated that IRAK-4, known as an important signaling molecule in innate responses through TLRs as well as the IL1 and IL18 receptors, is critical for T cell activation. Through *in vitro* T cell stimulation by antigens and antibodies, and *in vivo* analyses in DTH responses and skin graft rejection, we demonstrated that IRAK-4 is involved in early TCR signaling, particularly in the process of NF-κB activation by activating PKCθ (see Research Highlights). We expect that there are other molecules as common as IRAK-4 which are critical for both TLR and TCR signaling. In addition, given the implication of the same up-stream molecules in both TCR and TLR signaling, we hypothesize that direct links and active crosstalk between these two signaling paradigms exist. We hope to be able to prove this hypothesis through a detailed study of the function of TLRs on T cells, rather than dendritic cells.

Other results

We have also analyzed differential signal regulation of mast cell activation upon stimulation with IgE alone and IgE$^+$antigen. Using the CD8-FcRγ chimeric molecule, survival and degranulation of mast cells was induced by signal strength and duration. We demonstrated that IgE-mediated mast cell survival was induced by sustained Erk activation and subsequently secretion of autocrine IL-3.

We have cloned a pair of adhesion molecules, called CRTAM and Necl2 by subtraction and expression cloning. Heterophilic interaction of CRTAM-Necl2 induces cell-cell adhesion between CD8$^+$T cells and DC/epithelial cells. We established CRTAM-deficient mice and analyzed *in vivo* function of this interaction.

We had three important findings on the mechanisms of T cell activation this year. We first identified the TCR microcluster as the site for initial and sustained signals. In future, we will analyze the involvement of the downstream signal molecules and the function of the synapse. We unveiled the mechanism of pre-T cell activation by ligand-independent oligomerization of pre-TCR. We also found that T cell activation to lead NF-κB activation involved IRAK-4 and therefore IRAK4 is critical in both innate and acquired immunity.

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Yokosuka T, Sakata-Sogawa K, Kobayashi W, Hiroshima M, Hashimoto-Tane A, Tokunaga M, Dustin ML, Saito T. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap 70 and SLP-76. Nat Immunol. 6, 1253-1262 (2005)

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Suzuki N, Suzuki S, Millar DG, Unno M, Hara H, Calzascia T, Yamasaki S, Yokosuka T, Chen N-J, Elford AR, Suzuki J-I, Takeuchi A, Mirtsos C, Bouchard D, Ohashi P, S. Yeh W-C, Saito T. A critical role for the innate immune signaling molecule IRAK-4 in T cell activation. Science 311, 1927-1932 (2006)

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The normal function of the immune system is dependent on the ability of B cells to develop an appropriate repertoire of B cell antigen receptors (BCRs) and to respond to a wide range of foreign antigens, resulting in the generation of functional mature effector B cells. This process is accomplished by B cell signaling through the BCR, which leads a wide range of biological outputs that depend on the developmental stage of the B cell and the properties of the antigen. In addition to the BCR, other cell-surface receptors such as CD40 contribute to B cell response by functioning as co-receptors, by supporting survival and proliferation, or by interacting with other types of cells including T cells and stromal cells. Our aim is to understand the molecular basis of signaling complexes that contribute to cell fate decisions such as differentiation, growth, or apoptosis of B cells. We are particularly interested in adaptor molecules that are commonly defined as proteins possessing protein-protein or protein-lipid interaction domains but not intrinsic enzymatic activity. Our analysis demonstrated that adaptor molecules direct the appropriate subcellular localization of effector enzymes and regulate their activity by inducing conformational changes, both of which contribute to the spatiotemporal precision of B cell signaling events.

Negative regulation of CD40 signaling by adaptor molecule BANK

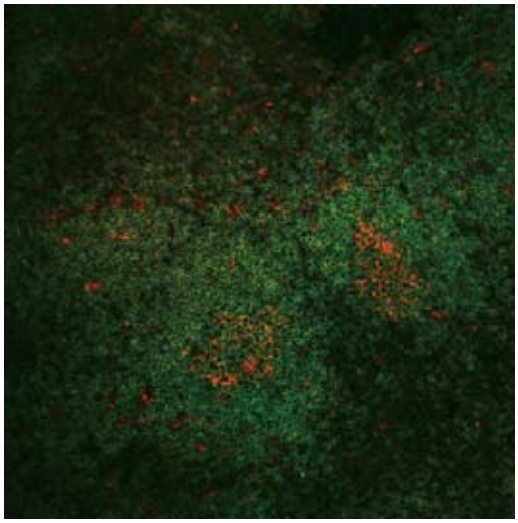
BANK, a recently purified adaptor molecule, shares overall structural features with its relatives BCAP and Dof; these molecules consist of an ankyrin-repeat-like region and a coiled-coil domain. Functionally, BCAP plays a positive role in B cell development and activation; for instance, in mice deficient in BCAP, the number of B lymphocytes is reduced and T cell-independent immune response is perturbed. In contrast, BANK-deficient mice display enhanced germinal center formation and IgM production in response to T cell–dependent antigens. This hyper-phenotype is likely accounted for by hyper-CD40 signaling, because hyper-IgM production is blocked in CD40-BANK double knockout mice.

These findings demonstrate that BANK functions as a negative adaptor in CD40-mediated signaling, thereby preventing hyperactive B cell response to T-cell-dependent antigens. Considering that BCAP and BANK coexist in B cells and exert opposing roles despite their similar structures, a promising idea has emerged that changing the expression balance between such positive and negative adaptors would allow B cells to generate graded responses under the same stimulation conditions.

Regulation of NF-κB pathway by CARMA1

The importance of IκB kinase (IKK)-induced NF-κB activation in B cell development as well as B cell activation is well recognized. Upon BCR engagement, protein kinase C (PKC)β is activated downstream of PLC-γ2, which in turn is important for IKK and subsequent NF-κB activation. In addition to PKCβ, adaptor proteins (CARMA1-Bcl10-MALT1) that participate in BCR-mediated IKK activation have been identified. However, it remains unclear whether PKCβ and the

WT mouse



BANK-dificient mouse

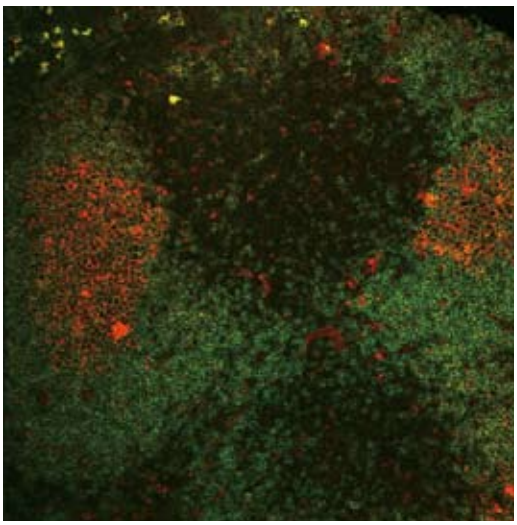


Figure : Enhanced germinal center formation in BANK-deficient mice. Increased numbers of germinal center B cells (red) in the spleen from BANK-deficient mice.

CARMA1-Bcl-10-MALT1 complex connect and how, if any, their connection leads to IKK activation. It was demonstrated that the BCR-induced phosphorylation of CARMA1, mediated by PKCβ, contributes to bringing two key protein kinases, TAK1 and IKK, to close proximity. As a consequence, TAK1 functions as an upstream kinase for IKK activation and subsequent NF-κB activation in the BCR signaling context.

We have already clarified that adaptor molecules (BLNK, BCAP, and BANK) regulate PLC-γ2 activation in distinct manners. BLNK brings two key enzymes, Btk and PLC-γ2, in close proximity to each other wherein Btk is able to phosphorylate PLC-γ2, thereby increasing its enzymatic activity. On the other hand, BCAP and BANK regulate PI3K positively and negatively, respectively, which in turn, modulates PLC-γ2 activation. Based on these backgrounds, we are focusing our efforts on how PLC-γ2 modulates B cell response. As mentioned above, PKCβ acts as a key enzyme downstream of PLC-γ2 in IKK activation. Hence, by using imaging and genetic analysis, we are now studying the interactions among CARMA1, TAK1, and PKCβ and the functional importance of these interactions. As another important target of PLC-γ2, we have already identified RasGRP3, one of the Ras guanine nucleotide exchange factors (GEFs). PLC-γ2 regulates RasGRP3 membrane localization and subsequent Ras activation in the BCR signaling context. To address the biological function of RasGRP3, we have established RasGRP3 knockout mice in a B-cell-specific manner and conducted intensive examinations.

To further understand the action mechanisms of adaptor molecules, two approaches are inevitable; 1) identifying binding partners and clarifying the functional consequences of these partners by current genetic and biochemical techniques; 2) looking at dynamic movement of adaptors/binding partners by using imaging techniques. By combining these technologies, we are focusing upon action mechanisms of three adaptor molecules (BCAP, BANK, and CARMA1). Particularly, in regard to imaging techniques, we are now trying to develop this system as a collaboration of Dr. F. Batista (UK).

Recent Publications

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Shinohara, H., Yasuda, T., Aiba, Y., Sanjo, H., Hamadate, M., Watarai, H., Sakurai, H. and **Kurosaki, T.** PKCβ regulates BCR-mediated IKK activation by facilitating the interaction between TAK1 and CARMA1. J. Exp. Med. 202, 1423-1431 (2005)

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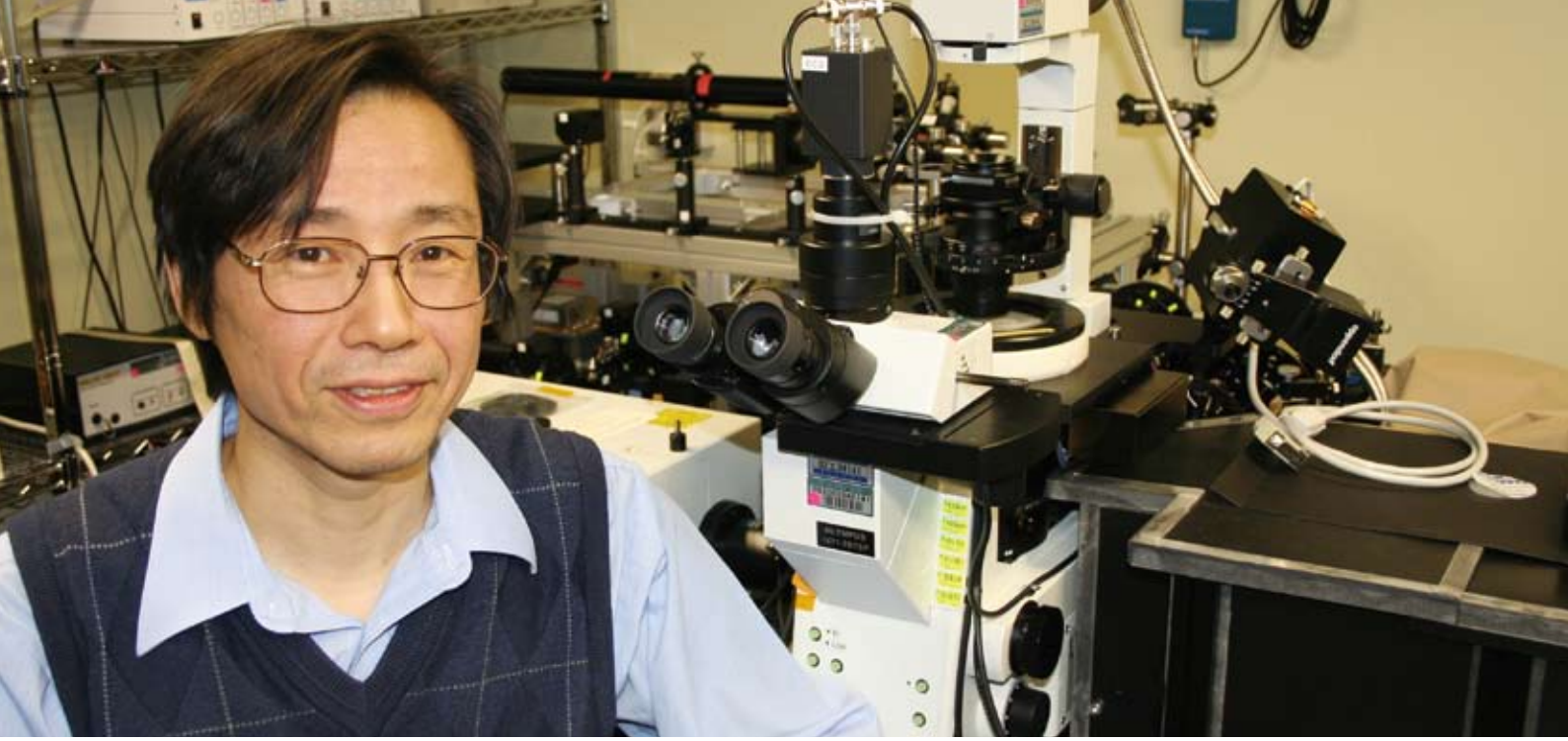

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Based on a novel approach to single molecule imaging, our team has pioneered the application of single-molecule studies to immunology. The goal of single-molecule immunoimaging is to visualize immunological responses and signaling processes at the single-molecule level and within single cells. To this end, a new type of fluorescence microscopy, termed "Highly Inclined and Laminated Optical Sheet" (HILO) microscopy, was developed. A microscope system was installed at RCAI and various collaborations with different groups at the center have been initiated. Our group has focused on the development of novel types of fluorescence microscopy for use in single cell/single molecule studies. In addition, we are developing analysis software tools for imaging and quantitative analysis. Presently, more than eight external research collaborations including international collaborations are ongoing.

HILO Microscopy

We have been involved in the development of total internal reflection fluorescence (TIRF) microscopy, a light-microscopic technique that uses evanescent light to illuminate single molecules. TIRF has become a widespread technique for single-molecule imaging at surfaces, but cannot be used for single-cell imaging due to the very limited depth of evanescent light. HILO is built upon the TIRF approach but enables the imaging of single molecules within living cells. Most importantly, HILO visualizes single molecules not only on cell surfaces but also inside living cells. Further, it has been shown that single molecule imaging and quantification of molecular interactions are useful new tools to elucidate the molecular mechanism of cellular functions. HILO microscopy can be used for observations within living cells that are sensitive to the illuminated light, and for time-lapse observation over long periods since only weak illumination is required. Using HILO, the dynamic movements of large numbers of single molecules inside a cell can be traced. Thus, HILO is also useful for quantitative studies of the distribution, dynamic movement, or interaction of large numbers of molecules. Whereas a single-color HILO was developed initially, a new multi-color system has been installed to observe intermolecular interactions in greater detail.

Visualization of Initiation of T Cell Activation

Although the immunological synapse has been thought to mediate antigen recognition and activation of T cells, the sites for initiating and sustaining T cell receptor (TCR) signals remain elusive. We have developed the "time 0 method" and used it to clearly visualize the initiation of T cell activation from the moment of the activation. Using molecular imaging, we have demonstrated that T cell activation is initiated and sustained in TCR-containing microclusters generated at the initial contact areas and at the periphery of the mature immunological synapse. Microclusters containing TCR, kinase ZAP-70 and adaptor SLP-76 are continuously generated at the periphery. Whereas microclusters migrate toward the central region, SLP-76 disappears completely and ZAP-70 is mostly lost before the microclusters join the TCR-rich central

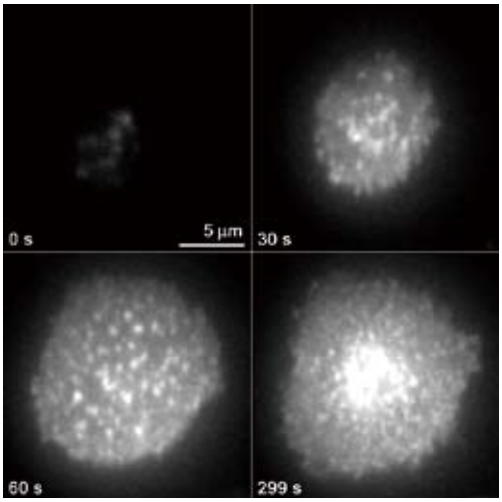

Figure 1: Single-cell molecular imaging: Microclustering of T cell receptor by signaling activation.

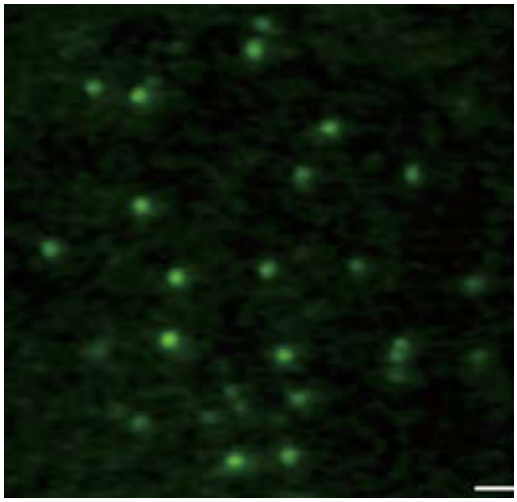

Figure 2: Single molecula imaging of importin βduring nuclear import. Bar, 1 µm.

region. These results suggest that TCR microclusters initiate and sustain TCR signals.

Application to Nuclear Pore Complex

Nuclear transport is a highly dynamic process occurring in the nuclear pore complex (NPC). Using novel microscopy, we have clearly visualized single fluorescent molecules inside cells during nuclear import. Molecular interactions with the assembled NPC were quantified by single molecule analysis. The retention times, the number of associated molecules, the dissociation constant, and the stoichiometry of import were all determined. Simulation based on a model with two types of multi-binding sites using these parameters well explained the molecular kinetics in cells. The combination of single molecule quantification and modeling opens new approaches for the development of molecular system biology.

Other Ongoing Projects

In addition to the above, a number of other internal and external collaborations are ongoing. For example, together with Takashi Saito's group, we are studying single molecule imaging of lipid rafts. Visualization of Vav protein function is ongoing in cooperation with Dr. Swat Wojciech at the University of Washington. Further, in cooperation with Haruhiko Koseki, we are attempting to visualize chromatin modifier proteins and nuclear dynamics. Studies on STAT4 signaling molecule and the zinc transporter protein ZnT3 are likewise ongoing.

Recent Publications

Yokosuka T., Sakata-Sogawa K., Kobayashi, W., Hiroshima, M., Hashimoto-Tane, A., Tokunaga M., Dustin, M. L., Saito, T. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nature Immunol.* **6**, 1253-1262 (2005).

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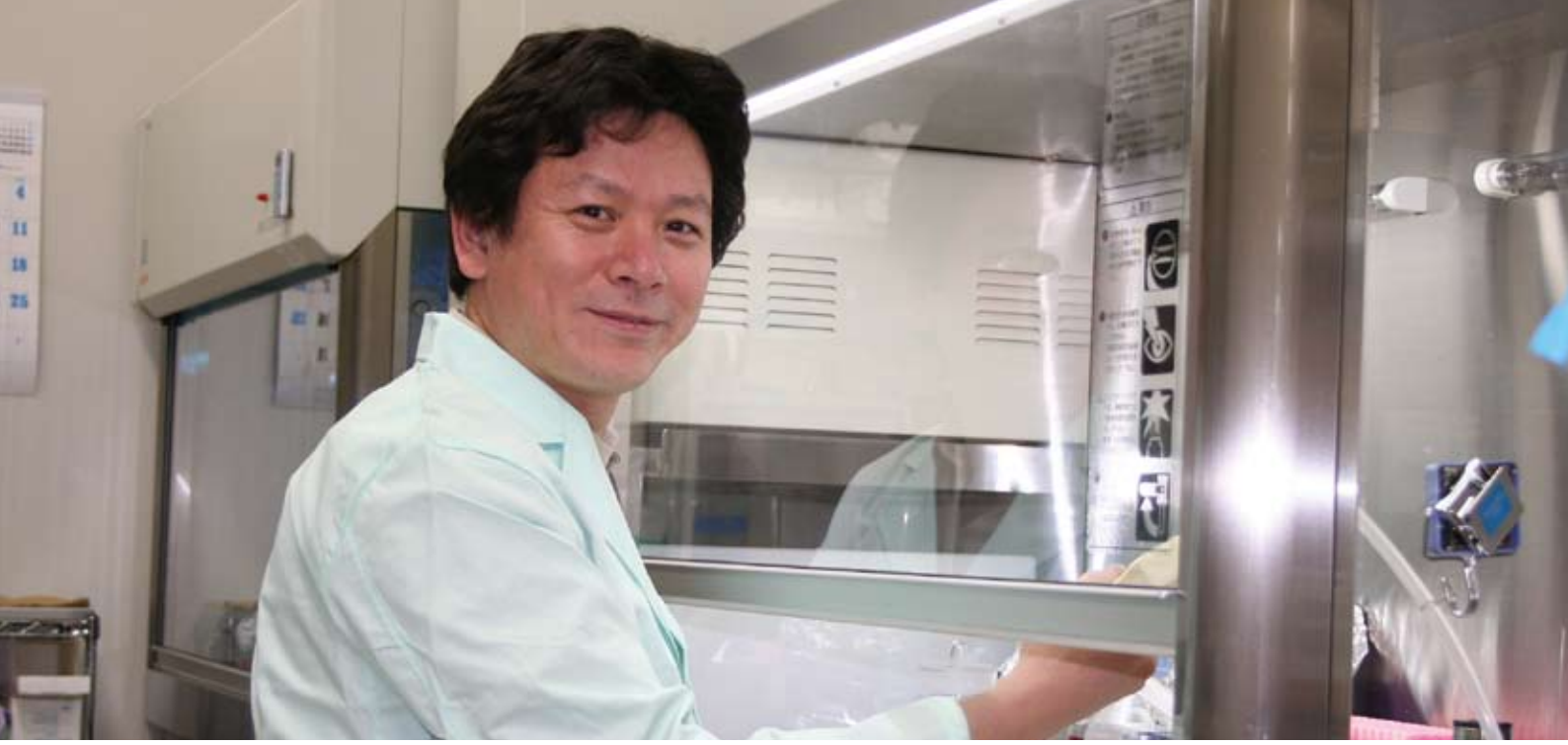

Antigen Receptor Diversity Research Team

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Germinal center (GC) B lymphocytes are a unique cell population that arises during a T cell-dependent antibody response. In GC B cells, the immunoglobulin (Ig) genes undergo two dynamic genetic alterations: first, somatic hypermutation (SHM), which is focused on the variable-region exons and can alter the antibody affinity for the antigen, and second, class switch recombination (CSR), which changes the constant region of the antibody to acquire different effector functions. Both SHM and CSR are initiated by a single enzyme, activation-induced cytidine deaminase (AID), which catalyzes the deamination of C to U; however,the precise mechanism of SHM still remains elusive. Our focus is on the study of genetic alterations in germinal center (GC) B cells and, in particular, on the role of various low-fidelity DNA polymerases. These polymerases are unique as they can replicate DNA past unrepaired lesions and have been implicated in both mutagenesis and mutation avoidance. We believe that the study of these polymerases will yield new insights into the molecular machinery of SHM and CSR. Further, we are investigating the roles of these polymerases in the maintenance of genome stability and in the development of lymphoid malignancies.

Role of the low-fidelity DNA polymerases in the somatic hypermutation of Ig genes

As many as 10 low-fidelity DNA polymerases have recently been identified. At least four of these polymerases, Polθ, Polη, Polζ and Rev1, have been shown to participate in SHM of Ig genes. Polθ is a ~300-kDa family A polymerase with a unique structure, having a helicase domain in its N-terminal portion and a polymerase domain in its C-terminus. We have previously shown that inactivation of its polymerase domain resulted in a specific reduction of C/G mutations of Ig genes. To further understand the role of Polθ in Ig gene SHM, we have also generated mice that completely lack *Polq* expression (*Polq*-null). *Polq*-null mice showed a reduction of both C/G and A/T mutations and there was a significant increase of G to C transversions. These results suggest that Polθ is involved in not only C/G but also A/T mutations of Ig genes.

Polη has been shown to play a dominant role in the generation of A/T mutations. To investigate how Polθ and Polη differentially participate in the generation of A/T mutations, we have analyzed the frequency and patterns of Ig gene mutations in mice deficient for both Polθ and Polη and compared them with the results from mice singly deficient for either enzyme. We found that Polθ and Polη function in the same genetic pathway and cooperate to generate mutations at A/T. One possibility is that Polη may insert a wrong nucleotide opposite A or T, which is then extended by Polθ. We are now performing biochemical experiments using recombinant Polη and Polθ to verify this hypothesis.

Additionally, we have generated *Polz* and *Rev1* knockin mice, which express a catalytically inactive Polζ and Rev1, respectively. We will analyze the Ig gene SHM in these knockin mice and will also cross them with *Polq*-null and *Polh*-KO mice. We believe that analyses of mice deficient for two or more of these enzymes will provide important insights toward

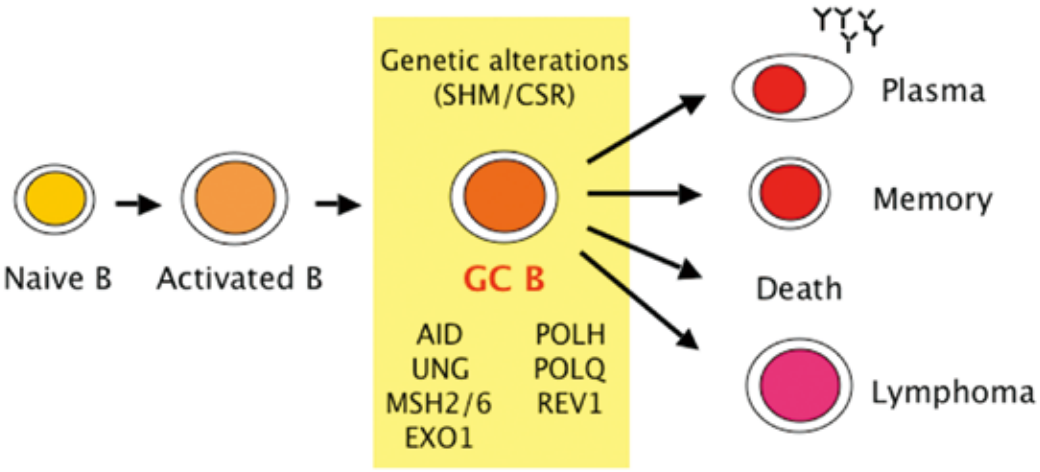

Figure: Genetic alterations are crucial for the maturation of Germinal Center B cells—but can also cause cancer.

understanding how these polymerases collaborate and compete to generate different types of point mutations during SHM of Ig genes.

Why do GC B cells have increased mutations at A/T?

Using lacZ-transgenic mice in which the genome mutation frequency and patterns can be easily detected with high sensitivity, we have examined the mutations in naïve and germinal center B cells. We found that GC B cells have a dramatic increase in mutations that occur at A/T. This is particularly interesting since overexpression of AID in fibroblasts only induces mutations at C/G. It thus appears that GC B cells provide an environment that allows efficient induction of A/T mutations. A number of experiments are now in progress to examine whether certain DNA polymerases might play a role in the induction of A/T mutations. Clarifying the molecular mechanism of the A/T mutations has significant implications in understanding how mutations are targeted to each nucleotide and how GC B cells acquired the ability to generate all the types of mutations necessary for the unfettered affinity maturation of antibodies.

Analysis of Clast6/Laptm5, a novel lysosomal protein involved in the regulation of T cell activation

Clast6/Laptm5 is a lysosomal protein that contains five membrane-spanning segments and a proline-rich domain at its C-terminus. Clast6 is specifically expressed in lymphoid tissues and its expression is rapidly and transiently down-regulated in both B and T cells by various activation signals. To explore its physiological role in the immune system, we have generated Clast6 knockout (Clast6-KO) mice by homologous recombination in embryonic stem cells. B and T cell development and maturation were normal in Clast6-KO mice although there was a moderate increase in the total cellularity in the bone marrow. Clast6-KO B cells showed no apparent abnormalities in terms of their proliferative responsiveness to various B cell activation signals. However, in comparison with WT T cells, Clast6 KO T cells exhibited elevated proliferation and cytokine production in response to CD3 stimulation. Both CD4 and CD8 T cells of Clast6 KO mice were hypersensitive to CD3-mediated proliferative signals. To explore the mechanism of the T cell hyperresponsiveness in the absence of Clast6, we examined the expression of molecules known to be involved in the signal transduction through the T cell antigen receptor (TCR). We consistently observed increased protein levels of the CD3ζ chain of the TCR complex in Clast6-KO T cells as compared with WT T cells, suggesting that Clast6 might directly or indirectly regulate CD3ζ expression. Co-transfection of Clast6 and CD3ζ into fibroblasts resulted in a dramatic reduction of the CD3ζ protein level, compared with CD3ζ alone transfectants, without affecting its transcript level. Moreover, overexpression of Clast6 in a T cell hybridoma decreased the level of the endogenous CD3ζ chain. These results collectively suggest that Clast6/laptm5 mediates the degradation of the CD3ζ chain and thereby negatively regulates T cell activation.

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Immune Surveillance Research Unit

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This laboratory is partially funded through the Immune Surveillance Project, by a special grant in priority areas funded by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for a total of four years, starting in 2003. This project, which is coordinated by our laboratory, funds 66 research groups all over Japan.

Immune surveillance project

The goal of the Immune Surveillance Project is to apply the knowledge on the immune system, which has been accumulated over the recent years, to the study of immune surveillance. The projects are focusing on the following three major subjects: (1) the spatial and temporal regulation of immune responses and immunosurveillance, (2) the constitution of immune memory and approaches to regulating long-term immune memory, (3) immune surveillance against cancer cells, pathogens or other dangers, and the development of novel methods to restore, reinforce, and/or regulate immune surveillance. We have been working on the establishment of a novel strategy to restore and reinforce the immunological ability in order to conquer severe infection and cancer.

Artificial lymph nodes

We have previously demonstrated that artificially generated lymph-node-like tissues (artificial lymph nodes), which were prepared by the transplantation of stromal-cell-embedded biocompatible scaffolds into the renal subcapsular space in mice, possess a well-organized tissue structure similar to that of secondary lymphoid organs. Artificial lymph nodes contain T cells, B cells and dendritic cells, the same as natural lymph nodes with definite separation of T cell and B cell areas accompanying the formation of B cell follicles. In addition, the existence of germinal centers is confirmed, in which there are many plasma cells and vigorously multiplying B cells. The artificial lymph-node-like tissue was transplanted into naïve normal mice as well as into severe combined immunodeficiency (SCID) mice. The artificial lymph nodes support extremely strong antigen-specific antibody formation in SCID mice. Large numbers of antigen-specific antibody-forming cells were detected not only in the artificial lymph nodes but also in the spleen and BM in SCID mice carrying transplanted artificial lymph nodes. In particular, when artificial lymph nodes were transplanted into SCID mice, the cells in artificial lymph nodes migrated to the empty spaces in immunological tissues, where they explosively and clonally expanded upon antigen stimulation. As a result, large numbers of antigen-specific antibody-forming cells appeared in the spleen and BM of SCID mice. It is thought that the movement of cells from artificial lymph nodes to the spleen in SCID mice is induced by sphingosine 1-phosphate (S1P) signaling. Furthermore, the structure of artificial lymph nodes remained stable for a long time after transplantation. Surprisingly, memory-type $CD4^+$ T cells are abundant in the artificial lymph nodes.

This novel simplified system of lymphoid tissue construction will facilitate the analyses of the cell-cell interactions required for the development of secondary lymphoid organs and the efficient induction of adaptive secondary immune responses, and may have possible applications in the treatment of

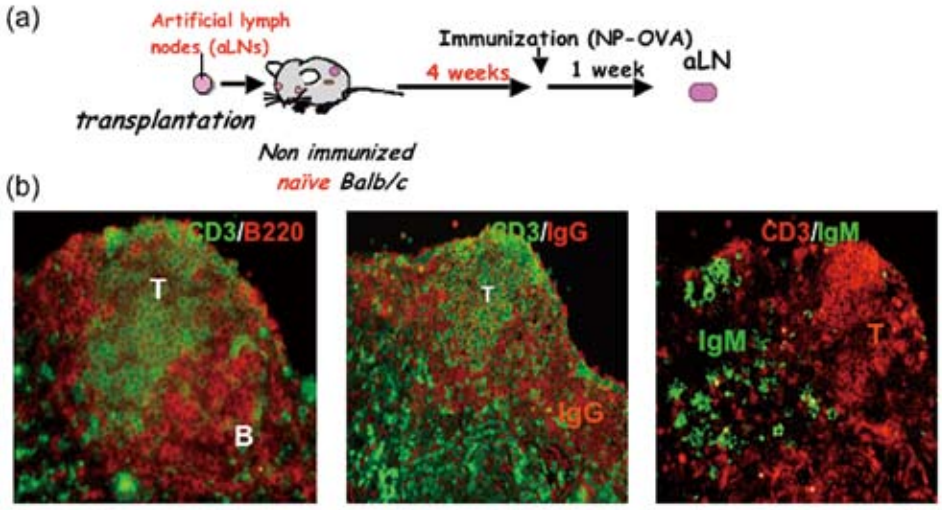

Figure: (a) A strong antigen-specific secondary IgG but weak IgM response is induced in artificial lymph nodes even 4 weeks after transplantation into naïve recipient.
(b) Artificial lymph nodes (28 days after transplantation)

immune deficiency, severe infection and cancer in the near future.

Humanized mouse models

Thanks to the tremendous effort of Dr. Fumihiko Ishikawa, we have been able to report the successful engraftment of human hematopoietic cell lineages and the generation of functional human T and B cells, as well as DCs, and NK and NKT cells, by the injection of human spinal- or cord-blood-derived hematopoietic stem cells into a NOD/SCID/IL2 γ chain^{null} newborn mouse. Humanized mouse models have enormous potential, both as innovative tools to study the human immune system and as model systems in preclinical research as a substitute for the human body. The humanized mouse model, in conjunction with the reconstitution techniques that we are developing in our laboratory, is a powerful new approach to studying human immune tissues. We are currently trying to develop a human-type artificial lymph node by transplanting stromal-cell-embedded biocompatible scaffolds into the renal subcapsular space in humanized mice.

Regulatory roles of histamine receptor signaling in immune responses

We have been investigating the role of histamine H1 and H2 receptor (H1R and H2R)-mediated signals in the regulation of immune responses. In the present work, we examined the role of H1R in allergic inflammation in vivo using a mouse asthma model. Allergen-stimulated splenic CD4$^+$ T cells from H1R-deficient mice exhibited enhanced Th2 cytokine production, as we previously reported. However, allergen-challenged H1R-deficient mice exhibited a diminished lung Th2 cytokine mRNA level, airway inflammation, goblet cell metaplasia, and airway hyper-responsiveness (AHR). We found that H1-receptor-deficient CD4$^+$ Th2 cells failed either to migrate to local regions of the lung or to confer airway inflammation or AHR. Our work clearly established that histamine and H1R play a part in promoting the migration of Th2 cells into sites of allergen exposure (Bryce P.J. et al. *J. Clin. Invest*. in press).

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Ishikawa F., Yasukawa M., Lyons B., Yoshida S., Miyamoto T., Yoshimoto G., Watanabe T., Akashi K., Shultz L. D., Harada M. Development of Functional Human Blood and Immune Systems in NOD/SCID/IL2 Receptor γ chain^{null} Mice. **Blood 106, 1565-1573 (2005)**

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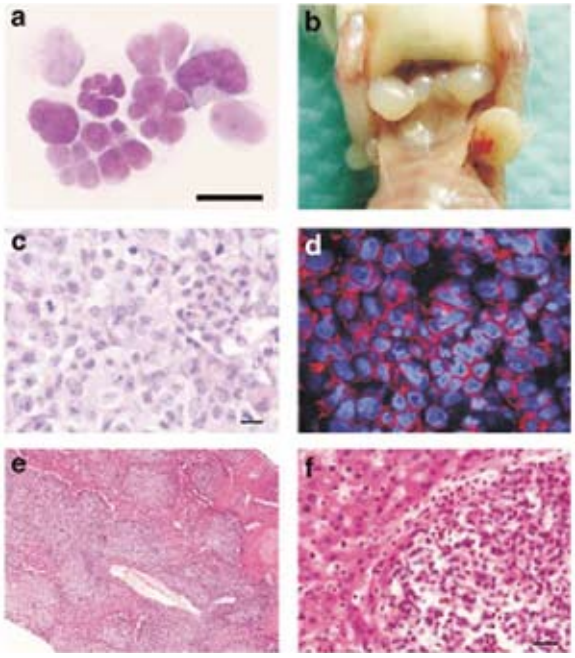
To develop suitable *in vivo* models of human normal hematopoietic and immune systems as well as of diseases involving these systems, various immunosuppressed mouse strains were constructed in the past, including CB17/SCID, NOD/SCID and NOD/SCID/β2m^{null} mice, to name a few. While these strains have allowed human immunohematopoietic system development to some extent when transplanted with human hematopoietic stem and progenitor cells, their short life spans and age-dependent leakiness of murine humoral immunity have presented obstacles for the long-term evaluation of human hematopoiesis *in vivo*. To overcome these problems, we have recently created a novel immunodeficient strain with improved long-term xenogeneic engraftment, NOD.Cg-*Prkdc*^{scid}*Il2rg*$^{tm1Wjl/J}$ (NOD/SCID/IL2rγ^{null}), carrying a complete null mutation of the common γ chain. As this strain has a life expectancy of >90 weeks, it enables the assessment of reconstitution and immune system development capacity of human hematopoietic stem and progenitor cells. Using this strain, we created humanized mouse with full representation of human hematopoietic and immune systems, in contrast with existing models that have limited human myeloid/lymphoid development capacity. Using this model, we aim to address such issues as human thymic immune cell development and the identification of human lymphoid progenitors, and to develop an *in vivo* model of immunotherapy using a full complement of human immune cells. In addition, we aim to create *in vivo* models of human diseases such as hematologic malignancies with the long-term goal of developing novel therapeutic modalities for such diseases.

Development of normal human immunohematopoietic system in NOD/SCID/IL2rγ^{null} mice

In the past, the full development of the human immunohematopoietic system in an *in vivo* mouse model was hindered by the lack of suitable animal models. For instance, NOD/SCID mice support human B cell differentiation from transplanted human hematopoietic stem cells (HSCs) but not T cells or dendritic cells. In contrast, using the novel newborn NOD/SCID/IL2rγ^{null} HSC transplantation model, we developed humanized mouse with full representation of the human immunohematopoietic system. When purified human cord blood CD34^{+} stem/progenitor cells or CD34^{+}CD38^{-} stem cells were transplanted into newborn NOD/SCID/IL2rγ^{null} mice, more than 50% human hematopoietic chimerism was achieved for over six months. In this model, human HSCs produced mature differentiated progeny including myelomonocytes, T and B cells, dendritic cells, erythrocytes and platelets. Differentiation occurred via known intermediates such as common lymphoid and common myeloid progenitors, recapitulating normal human hematopoiesis. Functionally, human B cells underwent normal class switching and produced antigen-specific immunoglobulins upon immunization using ovalbumin. In addition, IgA-secreting human B cells were found in the murine intestinal mucosa. Furthermore, human T cells exhibited the HLA-dependent cytotoxic function. These findings suggest that the NOD/SCID/IL2rγ^{null} newborn transplant model is an excellent model to study the human immunohematopoietic system *in vivo*. Currently, we are conducting experiments to



Figures: (a) Cytospin specimen of blood derived from a recipient mouse showed multi-lobulated "lower-like cells".
(b) Multiple lymphadenopathy was seen in recipients.
(c) Pleomorphic and mitotic changes of cells were identified in the recipient lymph nodes.
(d) Human CD4 staining of lymph node cells of a recipient (red). Nuclei of cells were stained with DAPI (blue).
(e) and (f) Hematoxylin-eosin staining of the liver showed nodular infiltration of ATL cells at low (e) and high
(f) magnification.

further analyze human immune cell subsets and their function in the mouse model.

Development of *in vivo* model of adult T-cell leukemia (ATL)

ATL is caused by the clonal expansion of $CD4^+$ cells infected with human T-cell leukemia virus type I (HTLV-I). Clinically, ATL is classified into four subtypes: acute, smoldering chronic, and lymphoma. While the smoldering type is initially indolent, the majority progress to acute-type ATL over time. Since there is no cure for acute-type ATL, it is crucial to develop an animal model to study its pathogenesis and to develop effective therapy. However, the establishment of engraftment of primary acute-type ATL cells in previous animal models such as CB17-scid mice or NOD/SCID mice has not been achieved so far. Using the newborn NOD/ SCID/ β2-microglobulin^{null} (NOD/ SCID/ $\beta2m^{null}$) transplantation model, it was possible to successfully engraft primary acute-type ATL cells in the peripheral blood and the lymph nodes of recipients at high efficiency. Engrafted ATL cells were $hCD4^+hCD25^+$ and showed identical pattern of HTLV-I integration to the patient's ATL cells. Transplanted ATL cells showed infiltration into the recipient liver, forming nodular lesions that recapitulate the clinical features of the patient. In contrast, multiple clones of primary smoldering-type ATL cells were engrafted in the NOD/ SCID/$\beta2m^{null}$ mice. Upon secondary transplantation, a single clone became predominant, suggesting that a clone with dominant proliferative capacity is being selected competitively in this xenotransplant model.

The NOD/SCID/IL2rγ^{null} mouse model is an excellent *in vivo* model to study normal or abnormal human hematopoietic and immune systems. It is superior to existing models as it supports high levels of human cell engraftment and full differentiation of immune components. Using this model, it is possible to recreate *in vivo* normal human hematopoietic and immune development by reconstituting normal human bone marrow or umbilical cord blood derived hematopoietic stem and progenitor cells. It is also possible to recapitulate *in vivo* the pathogenesis of various diseases affecting the hematopoietic and immune systems by transplanting primary patient hematopoietic stem and progenitor cells. We are also developing next-generation immunosuppressed mice with an even greater capacity for human hematopoietic and immune reconstitution.

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The mucosal epithelium that lines the inner surfaces of the body, especially within the intestine, is exposed to various macromolecules and microorganisms whose efficient uptake is crucial to maintaining normal immune regulation. Epithelial cells that cover the gut-associated lymphoid tissue (GALT), such as Peyer's patches (PPs) and isolated lymphoid follicles, are both histologically and biochemically distinct from normal absorptive epithelial cells of the villi and are termed follicle-associated epithelium (FAE). FAE contains a specialized subset of epithelial cells, M cells, that play an important role in immune surveillance by delivering ingested macromolecules and microorganisms to the underlying lymphoid cells via transcytosis. Despite their significance, studies of M cells remain in their infancy, mainly because the low frequency of M cells and the lack of specific surface markers make it difficult to purify the M cells required for molecular/biochemical analyses. Accordingly, one of the primary aims of our laboratory is to understand the mechanisms that underlie the differentiation and function of FAE and M cells. Our research team is also investigating the recognition of commensal microbiota by intestinal epithelium and its influence on the mucosal and systemic immune system.

Identification of markers for FAE and M cells

We have devised a method to dissect the epithelial layer from mesenchymes and have thereby collected M-cell-enriched FAE and intestinal epithelial cells (IECs) of the villi from the small intestines of mice. RNA was then extracted from the sampled tissue and amplified. Using Affymetrix high-density oligonucleotide microarrays, we analyzed the gene expression profiles of the FAE and M cells to characterize their cellular phenotypes. The microarray data revealed that among approximately 14,000 genes, 409 were expressed in the FAE at more than twice the level observed in the IECs. These included genes that are involved in membrane traffic, host defense, and transcriptional regulation as well as uncharacterized genes. Subsequent real-time RT-PCR and *in situ* hybridization analyses identified three molecules–ubiquitin D (Ub-D), tumor necrosis factor receptor superfamily 12a (TNFRsf12a), and transmembrane 4 superfamily 4 (Tm4sf4)–that were predominantly distributed throughout FAE but rarely expressed in IECs. In contrast, transcripts of secretory granule neuroendocrine protein 1 (Sgne-1) were scattered in the FAE and co-localized with the signal of *Ulex europaeus* agglutinin-1 (UEA-1); this is relatively specific for M cells in the FAE in mice, although UEA-1 also reacts with goblet cells in villi and Paneth cells in the crypt of the small intestine. This finding clearly suggests the M-cell-specific expression of Sgne-1 in the gut (Figure). Such a unique pattern of gene expression distinguishes FAE and M cells from IECs, and may reflect their cellular phenotype(s) associated with specific functional features. We are also currently performing proteome analysis of M cells and FAE, as well as microarray analysis of human biopsy samples obtained by endoscopic examination. This project is being undertaken in collaboration with the RCAI Allergy Transcriptome Research Unit, the Immunogenomics Research Group, the Developmental Genetics Research Group, and Dr. Mitsutoshi Iimura of Tokyo Women's Medical University.

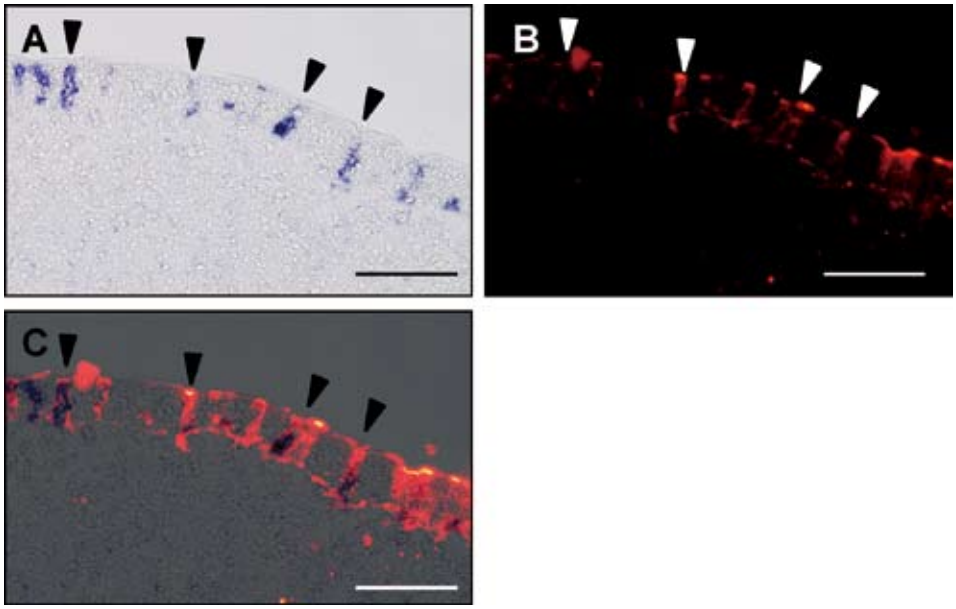

Figure: M-cell-specific expression of Sgne-1. The section following in situ hybridization staining (A) was further labeled with an M-cell marker, UEA-1 (B). The merged image (C) reveals that Sgne-1 expression is restricted in UEA-1+ M cells (indicated by arrowheads). Scale bar: 50 µm.

The role of chemokines in the crosstalk between FAE and GALT

The spatial distribution of immune cells in PPs is most likely controlled by chemokine-driven processes. For example, CCL20 is constitutively expressed by the FAE in both mice and humans. Similarly, murine CCL9 and its potential human counterpart CCL23 are selectively produced by FAE but not IECs. The recently identified CXCL16 has dual functions as a transmembrane adhesion molecule and a soluble chemokine. In our research, we found that CXCL16 mRNA and protein were expressed constitutively on the FAE that cover PPs, isolated lymphoid follicles, and cecal patches, but only minimally on IECs within the murine gastrointestinal tract. CXCL16 was also expressed on the FAE of human ileal lymphoid follicles. The CXCL16 receptor CXCR6/Bonzo was constitutively expressed on subpopulations of $CD4^+$ and $CD8^+$ T cells isolated from PPs. The expression of CXCR6/Bonzo on the PP T cells was upregulated after stimulation with anti-CD3 and anti-CD28 mAbs, suggesting that CXCR6 is expressed on the activated/memory PP T cells. The activated PP T cells showed chemotactic migration in response to the soluble N-terminal chemokine domain of CXCL16. Furthermore, the activated PP T cells selectively adhered to those cells expressing CXCL16.

To determine the physiological role of CXCL16 in GALT, we first carefully analyzed the distribution of T cells in PPs. We found that T cells are localized in the interfollicular T-cell region (IFR) and to a lesser extent in the subepithelial dome (SED) and the germinal center of lymphoid follicles. Accordingly, the majority of the adoptive transferred activated T cells migrated into the SED and the IFR; however, the neutralization of CXCL16 by anti-CXCL16 antibody injection specifically reduced the migration of the adoptive transferred activated T cells into the SED of PPs. These data suggest that CXCL16 expressed on the FAE plays an important role in the recruitment and retention of activated T cells in the SED, and should therefore be at least partially responsible for lymphocyte compartmentalization in GALT.

In our research, we have identified several M-cell-specific genes in addition to Sgne-1, and we are in the process of determining the precise characteristics of these gene products, including the generation of transgenic and/or knockout mice. We are also attempting to clone the gene for the as-yet unidentified IgA receptor expressed on the M-cell luminal surface. For chemokines expressed by the FAE/M cells, we obtained knockout mice for CXCL16, CCL9, and CCR6 (the receptor for CCL20), and are now analyzing the effect of the absence of these proteins on the differentiation and function of the FAE, M cells, and PPs.

We are also interested in host–commensal microbiota interaction. The gut is colonized by some 100 billion commensal bacteria as intestinal microbiota that together with the host cells themselves comprise the host–microbiota symbiotic ecosystem. It is believed that intestinal microbiota is closely related to various lifestyle-related diseases and intestinal infections, and certain intestinal bacteria such as lactobacilli and bifidobacteria are thought to be beneficial against such disease states. Accordingly, consuming these bacteria as probiotics, or functional food, is recommended as a strategy for promoting health and/or preventing disease, although scientific evidence of any benefit in this regard is largely lacking. We are currently addressing this lack of data by using gnotobiotic mice as a model system.

Recent publications

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Mitsunari T, Nakatsu F, Shioda N, Love PE, Grinberg A, Bonifacino JS, Ohno H. Clathrin Adaptor AP-2 is Essential for Early Embryonal Development. Mol. Cell. Biol. 25, 9318-9323 (2005)

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One site having perhaps the most complex and dynamic mutualistic eukaryotic-prokaryotic relationship is the gastrointestinal tract. The gut is thought to harbor 500-1000 microbial species that provide metabolic traits including the ability to break down otherwise undigestible food components, as well as natural defense against colonization by pathogens. Indeed, the recognition of commensal bacterial products through toll-like receptors (TLRs) plays a critical role in epithelial homeostasis by inducing the secretion of protective factors that strengthen epithelial resistance to pathogens. Furthermore, the interaction of commensal bacteria with specialized cells located in intestinal crypts, called Paneth cells, leads to the secretion of antimicrobial products that contribute not only to the enforcement of the epithelium and the surveillance of stem cell niche, but also to systemic response to infection. Perhaps the most important role played by the gut microbiota is in "educating" the immune system. The continuous dialog between immune cells and bacteria ensures the fitness of the immune system, which is translated into a state of hypo-responsiveness against some environmental antigens (such as dietary antigens and commensals) and paradoxically, a state of readiness that allows for efficient and prompt response against other antigens such as pathogens.

One conspicuous response of the immune system following microbial gut colonization is the production of IgA by gut-associated lymphoid tissues (GALTs).

In humans, at least 80% of plasma cells are located in the gut lamina propria, and together they produce more IgA (40-60 mg/kg/day) than any other immunoglobulin isotypes combined.

All current projects in our laboratory are aimed at improving our understanding of the molecular and cellular foundations for gut IgA production, as well as the role of commensal bacteria in the regulation of mucosal and systemic immune responses.

Recently, we have provided an answer to one long-standing question in mucosal immunity, namely, how IgA plasma cells are produced in the absence of organized follicular structures in the gut.

Using alymphoplasia *aly/aly* mice that cannot develop any follicular structures because of impaired LTβR signaling on stromal cells, we carried out a series of reconstitution experiments aimed at restoring the IgA compartment in gut, and demonstrated the existence of two independent pathways for the recruitment of naïve and gut primed IgM^+ B cells to the gut LP.

We found that a functional NIK on LP stromal cells is critical for the homing of naïve but not gut-primed B cells to the LP of the small intestine. The transfer of normal BM cells into *aly/aly* mice failed to rescue gut IgA (Figure a) in spite of the complete recovery of IgA^+ B cells and plasma cells in spleen. In contrast, $B220^+IgM^+$ B cells isolated from PP or naïve B cells that had been allowed to experience an NIK-sufficient gut environment could migrate to the LP and generate IgA plasma cells in *aly/aly* mice (Figure b). Thus, it appears that the gut environment imprints naïve B cells with gut-seeking properties. The results together indicate that the role of organized structures in the gut is not only in the induction of mucosal IgA B cell development, but also in the reprogramming of the homing properties of B cells.

One signature of gut-experienced B cells is

the modulation of the expression and activation of mucosal integrin α4β7. In support of this notion, only B cells that express high levels of integrin α4β7 after experiencing the gut environment could migrate to the *aly/aly* intestine.

Unlike gut-experienced B cells, naïve B cell recruitment to the LP requires gut-specific stromal cells with a functional NIK. This is based on the observation that co-injection of BM with LP stromal cells but not of BM alone can induce the migration of BM B cells to the *aly/aly* gut (Figure c). Thus, LP stromal cells can recruit naïve B cells to the gut through an independent pathway that involves the activation of NF-κB by NIK. This novel, and probably a more primitive pathway, would explain the surprising observation that ROR $gt^{-/-}$ mice and $Id2^{-/-}$ mice that completely lack follicular organization in the gut have normal B cells and IgA plasma cells in their LP.

Regulation of mucosal immune response by gut stromal cells

In order to understand the fundamental basis of mucosal immune response, we need to characterize all the elements involved including not only non-static immune cells but also their structural backbone of immobile, stromal cells. Because every immune cell acquires different properties in different environment conditions, an understanding of the mucosal environment would certainly help elucidate the regional characteristics of the immune cells in the gut. Our studies are based on the following assumptions:a. Stromal cells would regulate the migration and location/distribution of immune cells, as a structural backbone within both organized and diffuse lymphoid tissues (i.e., gut lamina propria);b. The continuous interactions of immune cells with stromal cells would reciprocally influence their survival, homeostatic expansion and phenotypic differentiation;c. The unique characteristics of stromal cells located in strategic defensive sites would profoundly influence the functional capacities of local cells, leading to "functional imprinting" for that particular environment. This would ensure prompt and efficient local or regional immune defense response without the necessity to implicate the whole immune system.

Our objectives are to characterize at both cellular and molecular levels the stromal cell compartment in adult gut and to understand the molecular mechanisms of immune-stromal cell crosstalk and their functional implications.

Understanding commensal host-bacterial relationships in the gut, and their role in educating the immune system

The interactions between host and commensal microbiota are highly complex and heterogeneous and clearly have a wide-ranging impact on host immunity and physiology. However, the molecular mechanisms underlying the mutualistic interactions have remained largely unknown because we understand so little about the complex consortia of bacteria that live in (or) adjacent to the mucus layer overlying the intestinal epithelial cells. We are investigating the mutualism between commensal bacteria and the host immune system through the following experimental setups.

Firstly, we are studying the bacterial community in the intestine of normal mice or genetically manipulated mice without IgA or B cells, using molecular approaches that allow us to qualitatively and quantitatively evaluate bacteria present in different intestinal niches.

Secondly, using mice strains with genetic lesions affecting both innate and adaptive immunities, such as Toll-like-receptor-deficient, T-cell-deficient, AID-knockout mice, we are trying to dissect molecular foundations that lead to positive or negative signals for the induction and regulation of gut immune response.

Participation of innate-like peritoneal B1 cells in gut immune response

Innate-like B1 cells residing mainly in the peritoneal cavity were found to generate large amounts of antibodies outside their niche (i.e., IgM in the spleen or IgA in the small intestine). These antibodies play a critical role in the early phase of infection, well before the adaptive immune response is established. However, in spite of their importance, the molecular signals required for B1 cell mobilization are totally unknown. We wish to elucidate the molecular aspects for the recruitment of B1 cells from the peritoneum or peritoneum-associated tissues and their participation in mucosal and systemic immune responses.

To create a solid foundation with impact on both future vaccine strategies and therapeutic approaches to immune dysregulation, it is our aim to characterize and functionally understand all partners involved in immune dialog at mucosal surfaces, including their continuous interactions. Ongoing projects in our laboratory are focused on three important players: bacteria, immune cells and gut stromal cells. The elucidation of bacteria-stromal cell-immune cell interactions in normal steady-state conditions will help us understand the pathophysiologic mechanisms in immune dysregulation, such as allergy and inflammation.

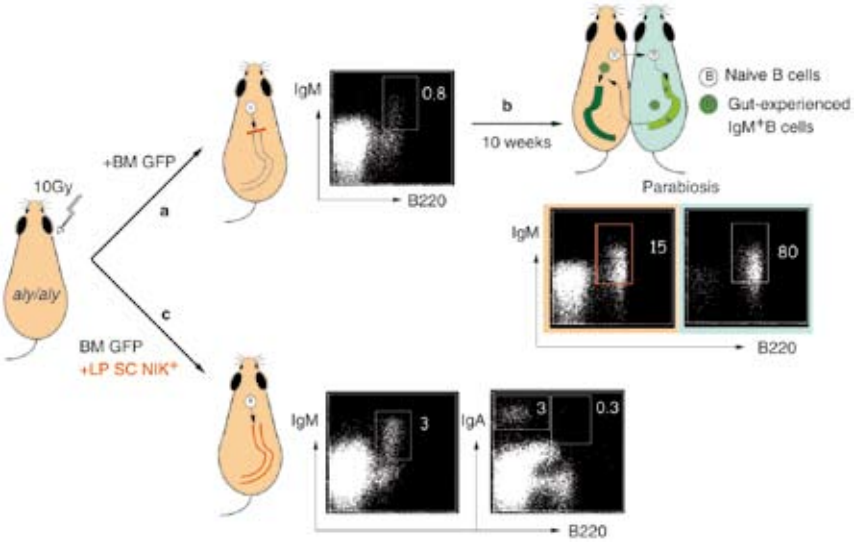

Figure: Migration of IgM+ B cells to gut lamina propria of *aly/aly* mice (a) Naïve IgM+ B cells derived from BM of GFP Tg mice and injected into lethally irradiated *aly/aly* mice failed to reconstitute IgM+ in the LP, (b) but migrated into RAG2-/- intestine, after parabiosis. The cells that experienced RAG2-/- gut efficiently migrated to LP of *aly/aly* parabiotic partner. (c) Naïve IgM+ BM-derived B cells co-injected with NIK-sufficient LP stromal cells also migrate to LP of *aly/aly* mice, generating *in situ* B220-IgA+ plasma cells.

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Fagarasan S, Honjo T Regulation of IgA synthesis at mucosal surfaces. Curr Opin Immunol. 2004 Jun;16(3):277-83.

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Host defense in mammals consists of innate and adaptive immunities. Innate immunity functions as a pathogen sensor and is involved in eradicating pathogens. Furthermore, innate immunity contributes to the establishment of adaptive immunity. Dendritic cells (DCs) are antigen-presenting cells that are critically involved in the sequence of these immune responses. DCs sense various pathogen-derived molecular components and exert their immunostimulatory functions by producing inflammatory cytokines or upregulating the expression of costimulatory molecules. These components are termed immune adjuvants on the basis of their DC-activating abilities. Immune adjuvants are recognized by various types of pattern-recognition receptors, including Toll-like receptors (TLRs). The identification of new types of immune adjuvants and the characterization of the mechanism by which they activate DCs will likely contribute to the development of novel immunoregulatory maneuvers. The aim of our present research is to clarify how DCs are activated via pattern-recognition receptors and to obtain critical information required to effectively manipulate the immune response. Various immune adjuvants such as TLR ligands and gene-targeting mice are important tools to achieve this aim.

Mechanism by which TLRs show pleiotropic effects on DCs

TLRs are able to recognize a variety of immune adjuvants. These adjuvants can be categorized into lipids, proteins, and nucleic acids according to their molecular characteristics. TLRs are able to transduce common immunostimulatory signals, but each TLR is also able to exhibit its own function via its molecular mechanism. In our research, we have been clarifying how TLR adapters are critically involved in TLR signaling, but much remains unknown. We are attempting to clarify how TLRs exert their pleiotropic functions, especially on DCs.

A cytoplasmic adapter, MyD88, is required for the signaling of all TLRs except TLR3. This has been verified by an analysis on MyD88-deficient mice that are refractory to TLR2, TLR5, TLR7, and TLR9 signaling. Another adapter, TIR domain Containing adaptor inducing IFN-β (TRIF), is critical for the MyD88-independent pathway downstream of TLR3 and TLR4, which are receptors for double-stranded RNA (dsRNA) and lipopolysaccharides (LPSs), respectively. TRIF is involved in the activation of interferon regulatory factor (IRF)-3 and the translocation to the nucleus. As a result, IFN-β gene expression is induced. TLR3 and TLR4 signaling can activate IFN-β gene expression in MyD88-deficient cells. This IFN-β gene induction was severely impaired in mutant mice lacking two IκB kinase (IKK) family members: IKKε/ι and TANK-binding kinase (TBK)1. Importantly, such mutant mice also lacked Type I IFN production after viral infection. Compared with double knockout mice, single knockout mice lacking IKKε/ι or TBK1 showed mild phenotypes, although TBK1 mutants exhibited more severe phenotypes than IKKε/ι mutants. These results clearly demonstrate that IKKε/ι and TBK1 cooperatively function as critical kinases for IRF-3 activation and IFN-gene induction in both TLR3/4 and the cytosolic viral recognition pathway.

Notably, nucleic acid adjuvants are characterized by their ability to induce Type I IFNs, especially IFN-α.

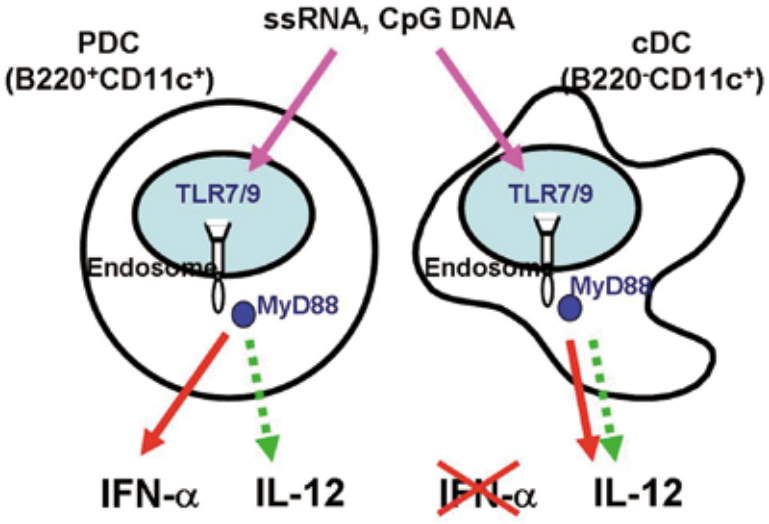

Figure: TLR7 and TLR9 signaling can activate a DC subset, PDC, to produce Type I IFN via a cytoplasmic adapter, MyD88.

Single-stranded RNA (ssRNA) and CpG DNA function as the ligands for TLR7 and TLR9, respectively. Although TLR7 and TLR9 signaling can induce the production of Type I IFNs, the induction depends on a DC subset termed plasmacytoid dendritic cells (PDCs). PDCs express only TLR7 and TLR9 among TLRs, and are also known as Type I IFN producing cells during viral infection.

There are two types of CpG DNA: D/A- and K/B-type CpG DNA. These two types are structurally and functionally different. K/B-type CpG DNA is able to activate B cells and induce DCs to produce inflammatory cytokines. These effects are weaker in D/A-type CpG DNA, although D/A-type CpG DNA shows much stronger effects in inducing Type I IFNs. In our research, we have characterized the effects of these two types of CpG DNA in various DC subsets and found that all of these functions depend on TLR9. TLR9 signaling depends on MyD88, and indeed this adapter was critically required for induction of both inflammatory cytokines and Type I IFNs.

Downstream of MyD88, the TLR9 signaling pathway is bifurcated into two pathways for inflammatory cytokine and Type I IFN induction. We recently identified IKKα as a critical kinase that is selectively involved in the Type I IFN induction pathway.

We have characterized the cellular and molecular mechanisms by which immune adjuvants activate DCs. Compared with lipid or protein immune adjuvants, nucleic acid adjuvants show several distinct characteristics. In particular, nucleic acids are peculiar in their ability to induce Type I IFNs. This ability is important not only because it can provoke antiviral defense but also because it is involved in the pathogenesis of certain autoimmune diseases such as systemic lupus erythematosus (SLE). PDC is a critical DC subset in this context, but other DC subsets are also able to produce Type I IFNs in response to nucleic acid adjuvants. Clarification of DC subset function is likely to be critical in characterizing the pleiotropic function of immune adjuvants, including TLR ligands. Furthermore, analyzing the effects of various adjuvants on DC subsets should also contribute to the identification or development of novel types of vaccines.

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Dendritic cells (DCs), the most potent antigen (Ag)-presenting cells (APCs), are characterized by their dendritic morphology and unique phenotype, and consist of heterogeneous subsets with myeloid or lymphoid lineage as well as differing with maturities in both lymphoid and peripheral tissues. Immature DCs (iDCs) sense the presence of invading pathogens via various pattern-recognition receptors (PRRs) and process the pathogens intracellularly in inflammatory tissues, developing into mature DCs (mDCs) with the upregulation of major histocompatibility complex (MHC) and costimulatory molecules in inflammatory microenvironments. Subsequently, mDCs migrate into secondary lymphoid tissues where they present the processed Ags to naïve T cells to effectively generate type 1 helper T (Th1) cells and Th2 cells, depending on their lineage and activation signals. Furthermore, mDCs produce various sets of cytokines that activate innate immune cells, thereby playing a crucial role in linking innate and adaptive immunities. On the other hand, accumulating evidence suggests that iDCs are involved in the induction of peripheral tolerance under steady-state conditions in vivo. In addition, the modification of iDCs with certain immunosuppressive molecules generates tolerogenic DCs that not only reduce T cell stimulatory capacity but also induce anergic T cells and regulatory T (T_R) cells. We have established human and murine tolerogenic DCs having greater capacity to induce anergic T cells and T_R cells than the previously known tolerogenic DCs in vivo and in vitro, and designated them as regulatory DCs (DC_{reg}).

Our goals are as follows: (1) to clarify the molecular mechanisms underlying the T cell regulatory function of DC_{reg}; (2) to develop immunotherapeutic approaches with DC_{reg} for immunopathogenic diseases; and (3) to characterize specific DC subsets involved in immune regulation. The major observations of two main projects in our laboratory are discussed below.

Regulatory dendritic cells act as regulators of acute lethal systemic inflammatory response

Bacterial infection triggers host inflammation through the activation of immune cells, leading to elimination of bacteria. However, the regulatory mechanisms of host inflammatory response remain unknown. We observed that DC_{reg}, a subset of potent tolerogenic DCs in mice, control systemic inflammatory response. Unlike normal DCs that produce proinflammatory cytokines in response to bacterial lipopolysaccharide (LPS), DC_{reg} produce less proinflammatory cytokines and instead preferentially produce IL-10, and these events involve the expression of IκBNS and Bcl-3 as well as the cyclic AMP (cAMP)-mediated activation of protein kinase A (PKA). In addition, murine DC_{reg} suppress LPS-induced production of proinflammatory cytokines in macrophages. Furthermore, DC_{reg} protect mice against lethality induced by experimental endotoxemia and bacterial peritonitis. The inhibitory effect of DC_{reg} against inflammatory responses involves the production of IL-10. On the other hand, $CD11c^{low}CD45RB^{high}$ DCs, the naturally existing tolerogenic DC subset producing IL-10, also suppress LPS-induced host inflammatory response. Thus, subsets of tolerogenic DCs act as potential regulators of host inflammatory

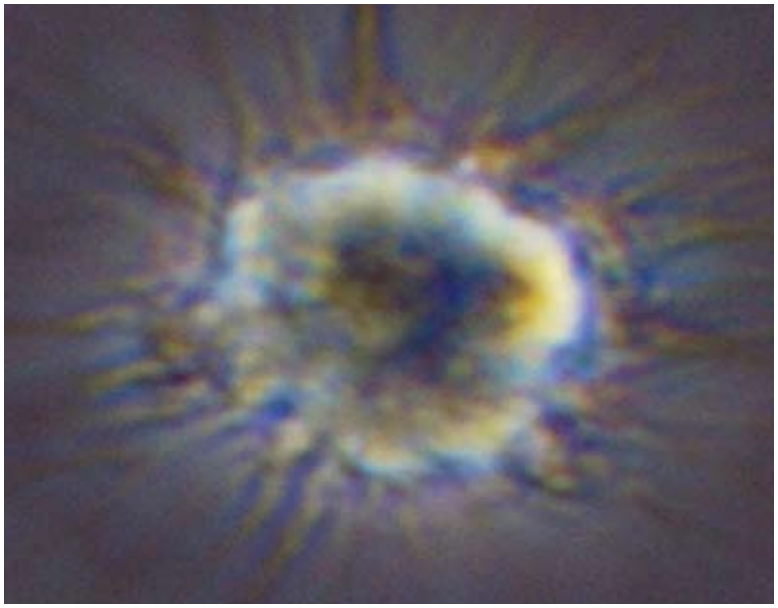

Figure: Tolerance induction through dendritic cells: A promising immunotherapeutic tool

response, and may have preventive and therapeutic potential for the treatment of systemic as well as local inflammatory diseases.

We are continuing our studies of the functions of human and murine DC_{reg}. As novel immunotherapeutic approaches that employ DC_{reg} against immune diseases show much promise, we are developing immunotherapeutic approaches using DC_{reg} for allergic and autoimmune diseases, and are now in various stages of research and preclinical evaluation. Finally, we are focusing on the molecular basis underlying the T cell regulatory function of DC_{reg}, and are attempting to identify functional molecules that specifically express on DC_{reg}.

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Recently, we have discovered a novel E3 ubiquitin ligase family that consists of viral E3 ubiquitin ligases (E3s) and their mammalian homologues. These novel E3s are membrane-bound molecules that share the secondary structure and catalytic domain for E3 activity. All family members have two transmembrane regions at the center and a RING-CH domain at the amino terminus. Overexpression of these novel E3s has been shown to reduce the surface expression of various membrane proteins through ubiquitination of target molecules. Initial examples of viral E3s were identified in Kaposi's sarcoma-associated herpesvirus (KSHV) and murine γ-herpesvirus 68 (MHV-68) and have been designated as Modulator of Immune Recognition (MIR) 1, 2 and mK3, respectively. MIR 1, 2 and mK3 are able to downregulate MHC class I expression, and mK3 is required to establish an effective latent viral infection in vivo. The first characterized mammalian homologue to MIR 1, 2 and mK3 is c-MIR1. Overexpression of c-MIR1 downregulates B7-2, a costimulatory molecule important for antigen presentation. Subsequently, several mammalian molecules related of c-MIR1 have been characterized and named as MIR family. However, the precise physiological function of MIR family members remains unknown. Therefore, we intended to elucidate the physiological functions of MIR family members by generating genetically modified mice.

Major observations

Kaposi's sarcoma-associated herpesvirus (KSHV) encodes two proteins, modulator of immune recognition 1 and 2 (MIR1 and MIR2), which are involved in the evasion of host immunity. MIR1 and 2 have been shown to function as an E3 ubiquitin ligase for immune recognition-related molecules (e.g., MHC class I, B7-2 and ICAM-1) through the RINGv domain. We showed that the human genome also encodes a novel RINGv domain-containing protein that functions as an E3 ubiquitin ligase and whose putative substrate is the B7-2 costimulatory molecule. This novel E3 ubiquitin ligase was designated as c-MIR (cellular-MIR) based on its functional and structural similarity to MIR1 and 2. Forced expression of c-MIR induced specific downregulation of B7-2 surface expression through ubiquitination, rapid endocytosis and lysosomal degradation of the target molecule. This specific targeting was dependent on the binding of c-MIR to B7-2. Replacing the RINGv domain of MIR1 with the corresponding domain of c-MIR did not alter MIR1 function.

Thus, we identified a novel E3 ubiquitin ligase (E3), designated as c-MIR, which targets B7-2 to lysosomal degradation and downregulates the B7-2 surface expression through ubiquitination of its cytoplasmic tail. B7-2 is well known as a costimulatory molecule for antigen presentation, suggesting that the manipulation of c-MIR expression modulates immune responses *in vivo*. To examine this hypothesis, we generated genetically modified mice in which c-MIR1 was expressed under an invariant chain promoter. Dendritic cells derived from genetically engineered mice showed low ability to present antigens. In addition, these mice showed resistance to the onset of experimental autoimmune encephalomyelitis and an impaired development of CD4 T cells in the thymus and the periphery. These findings led us to conclude

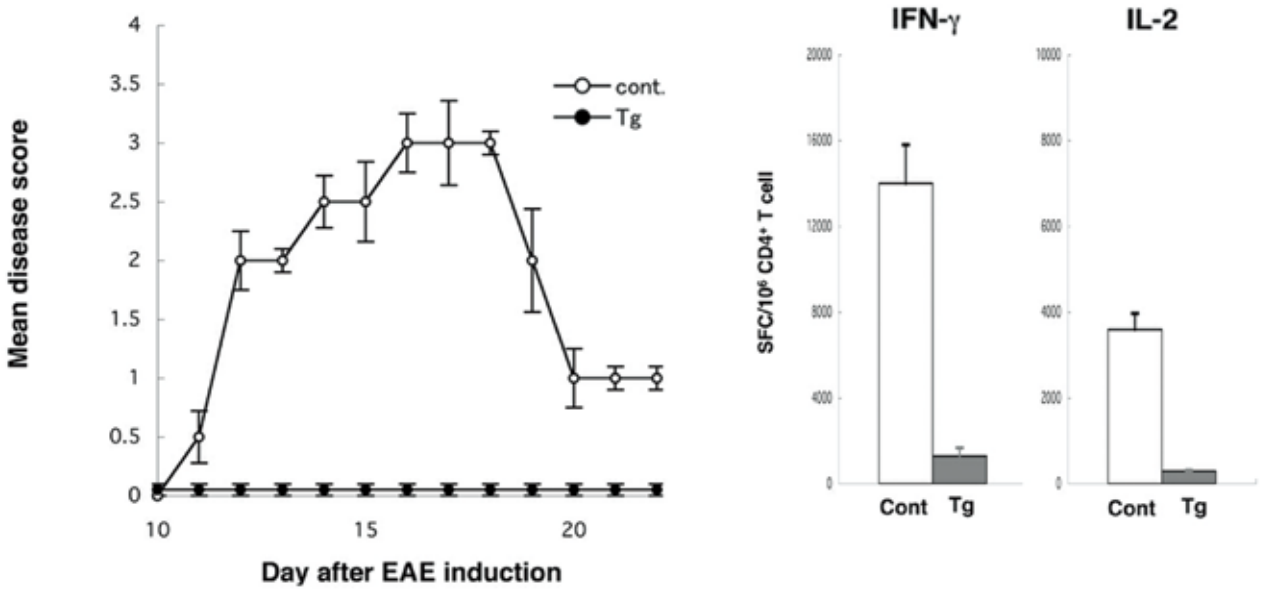

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Figure : Prevention of the onset of experimental autoimmune encephalomyelitis (EAE) by c-MIR1.The mice were immunized with 200 μg of MOG peptide in CFA. The mice were scored on a scale of 0-5. Data represent mean clinical score of each group plotted against time (n=6 for each group). Data are mean ± SEM. Data shown are representative of three independent experiments. The same number of splenic CD4 T cells was obtained from each type of mice 10 days after second injection of pertussis toxin, and were cultured with irradiated splenocytes obtained from control littermates in the presence of MOG_{35-55} in 96-well ELISPOT plates. IFN-γ– or IL-2-producing CD4 T cells were detected by ELISPOT. The numbers of cells secreting either IL-2 or IFN-γ among 1 x 10^6 CD4 T cells are presented. Tg: c-MIR1 transgenic mice; Cont: control littermates

that MHC class II (MHC II) is an additional target for c-MIR1. Indeed, forced expression of c-MIR1 in several B cell lines downregulated the surface expression of MHC II, and downregulation was found to depend on the presence of a single lysine residue in the cytoplasmic tail of the I-A β chain. In a reconstitution system using 293T cells, we found that the lysine residue at position 225 in the I-A β chain was ubiquitinated by c-MIR1.

Conclusion with perspectives

Based on our recent observations, we concluded that forcibly expressed c-MIR functions as a potent immune modulator *in vivo* and *in vitro*, and that MHC II and B7-2 are targets of c-MIR. To our knowledge, c-MIR is the first example of an E3 that is capable of targeting MHC II. Hence, further analysis of c-MIR might provide new insight into the molecular mechanism of antigen presentation. In addition, the discovery of c-MIR, a novel E3 ubiquitin ligase, highlights the possibility that viral immune regulatory proteins originated in the host genome and presents unique functions of RINGv domain-containing proteins in mammals.

At present, the physiological role of c-MIR is still unknown. Since another research group reported that immature DCs degrade MHC II-peptide complexes much faster than mature DCs, and we found that c-MIR is moderately expressed in splenic macrophages and splenic DCs, which are physiological APCs *in vivo*, we hypothesize that c-MIR might function as a modulator of MHC II expression in immature DCs. To examine this hypothesis, we are presently generating c-MIR-deficient mice.

Given the striking effect of c-MIR overexpression on the immune system, manipulation of the function and/or expression of c-MIR might be used as a strategy for artificial immune modulation *in vivo*. At present, there are several projects that attempt to identify and produce the novel subsets of DCs that induce immunological tolerance. These DCs, which have been designated as tolerogenic DCs, or regulatory DCs (DCreg), show a very limited capability of antigen presentation. In this connection, bone marrow-derived DCs (BMDCs) in c-MIR transgenic mice (c-MIR Tg) did not present several antigens to T cells efficiently, suggesting that APCs forcibly expressing c-MIR might function as tolerogenic DCs. Preliminary experiments grouping our lab showed that allogenic T cells stimulated with c-MIR Tg-derived mature BMDCs were not able to proliferate efficiently upon stimulation when compared to control littermate mice-derived BMDCs, while T cell responsiveness was improved by adding an excess amount of IL-2. Therefore, we are now investigating possible applications of c-MIR for immune tolerance induction.

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Apoptotic cell death is a critical and an evolutionarily conserved process for the elimination of unnecessary cells. After cells undergo apoptosis, apoptotic cell corpses are rapidly recognized and phagocytosed by professional phagocytes such as macrophages and dendritic cells. The rapid removal of apoptotic cells by phagocytes prevents the release of potentially toxic or immunogenic materials from dying cells. In mammals, the recognition and engulfment of apoptotic cells by phagocytes is an intricate process, and a number of molecules on phagocytes are involved in this phenomenon. We previously showed that the milk fat globule-EGF-factor 8 (MFG-E8), a bridging molecule between apoptotic cells and phagocytes, is expressed in tingible body macrophages located in the germinal center (GC) of spleen and lymph nodes. MFG-E8-deficient mice showed failure of apoptotic cell clearance in GC, and spontaneously developed autoantibody production. We also found that the intravenous injection of an MFG-E8 mutant protein, D89E protein, induced the production of autoantibodies in mice by inhibiting apoptotic cell clearance. These results indicate that the complete removal of apoptotic cells by phagocytes plays a critical role in the maintenance of self-tolerance. The Laboratory for Innate Cellular Immunity is investigating the molecular mechanisms for the recognition and phagocytosis of dying cells by phagocytes, and the pathological relevance of impaired phagocytosis to inflammatory disorders including autoimmune diseases.

Tolerance induction by apoptotic cell clearance

Experimental autoimmune encephalomyelitis (EAE) is characterized by neurological impairment such as progressive paralysis that first affects the tail and hind limbs. EAE is a model of human multiple sclerosis, and is induced in experimental animals by injecting them with central nervous system (CNS) tissue homogenates or purified components of the myelin sheath with adjuvants. The immunization results in the activation of myelin antigen-specific, autoreactive Th1 cells that induce inflammation and demyelination in the CNS. Therefore, the induction of antigen-specific T-cell tolerance could be a therapeutic approach to suppress the onset and progression of EAE. We examined whether the delivery of MOG epitope via apoptotic cells induced tolerance in MOG-specific T cells and subsequently prevented EAE. To this end, we induced apoptosis in transformants expressing MOG fragments by treatment with Fas ligand, and injected these apoptotic cells intravenously into C57BL/6J mice. Thereafter, the mice were immunized with MOG in complete Freund's adjuvant to induce EAE. The prior injection of apoptotic cells expressing an MOG fragment prevented the development of EAE, while the injection of apoptotic parent cells had no effect. We also examined whether the administration of apoptotic cells expressing an MOG fragment after MOG immunization had any effects on EAE progression. Injection of apoptotic cells either 8 days or 3 days before MOG immunization prevented the progression of EAE. On the other hand, when apoptotic cells were injected 8 days after MOG immunization, the severity of EAE was not different from that of mice without

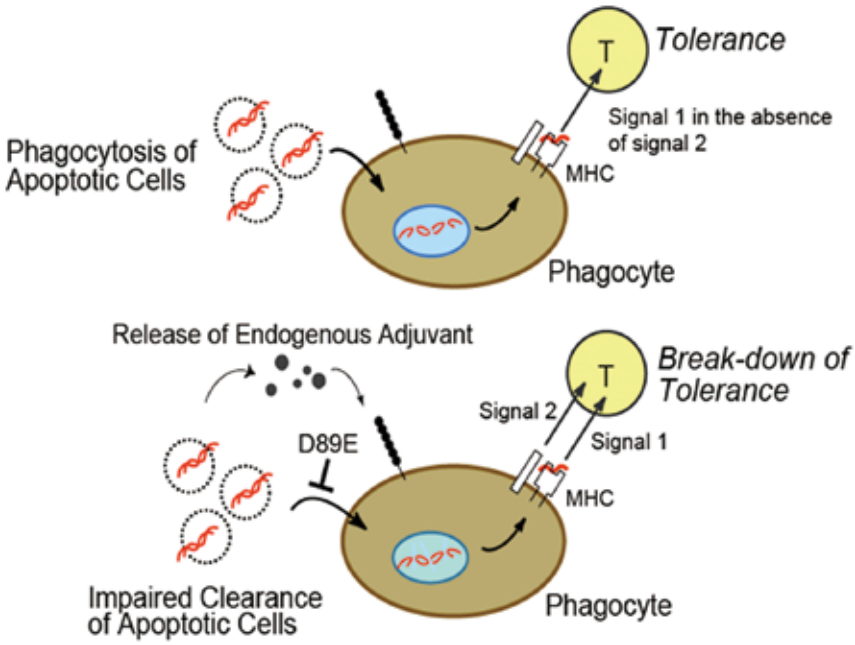

Figure: Phagocytosis of apoptotic cells induces tolerance to antigens in apoptotic cells.

any pretreatment, indicating that pretreatment with apoptotic cells was required for the inhibition of EAE progression. The suppressive effects of apoptotic cells expressing an MOG fragment were largely, but not completely, canceled when the mice were injected with apoptotic cells pretreated with an MFG-E8 mutant protein, D89E, which exerts an inhibitory effect on apoptotic cell engulfment by masking phosphatidylserine (PS). This result suggested that the PS-dependent engulfment of apoptotic cells is required, at least in part, for the inhibition of EAE. We are now analyzing the mechanisms of the tolerance induction in association with apoptotic cell clearance.

Mouse model of acute respiratory distress syndrome

To study the effects of loss of a specific cell type in animal models, several approaches have been attempted for the inducible ablation of a specific cell type. One such method involves the establishment of a transgene of the human diphtheria toxin receptor (DTR) into mice, named TRECK (Toxin-REceptor-mediated Cell Knockout). Human HB-EGF acts as a DTR, while mouse HB-EGF possesses negligible affinity to diphtheria toxin (DT). Thus, mouse cells are far more resistant to DT than human cells. When the human HB-EGF gene is transduced into mice under the control of a cell-specific promoter, the target cells are transiently depleted by DT administration *in vivo*. This technique allows us to achieve efficient inducible ablation of a specific cell type by administration of DT. To establish inducible macrophage-depletion mice, we generated LysM-DTR mice in which human DTR was expressed under the control of the lysozyme M gene, which is specifically expressed in myelomonocytic cells such as macrophages and monocytes.

Our initial goal was to induce the ablation of various macrophages *in vivo*. In fact, various macrophages were depleted by DT administration in LysM-DTR mice. However, when a large amount of DT was injected into transgenic mice, all mice died within 96 h. Histological analysis of the lungs revealed that the alveolar walls became thick, and the alveolar spaces were diminished. Moreover, red blood cells were notably found in the interstitial mesenchyme, indicating severe congestion in the lungs. In immunohistochemical analysis, alveolar epithelial type II cells as well as alveolar macrophages underwent apoptosis on DT administration. Consistent with the damage of alveolar epithelial type II cells, the production of surfactant proteins was greatly reduced in DT-treated LysM-DTR mice. These results indicated that the mice suffered from acute respiratory failure in association with the lack of surfactant proteins on DT administration. We could also generate conditional alveolar epithelial type II cell ablation with or without ablation of alveolar macrophages by means of bone marrow transplantation. This transgenic mouse serves as a novel animal model to study the pathophysiology of acute respiratory distress syndrome due to the dysfunction of alveolar epithelial type II cells.

We are trying to reveal the cellular and molecular mechanisms of the tolerance induction to cell-associated antigens.

Recent Publications

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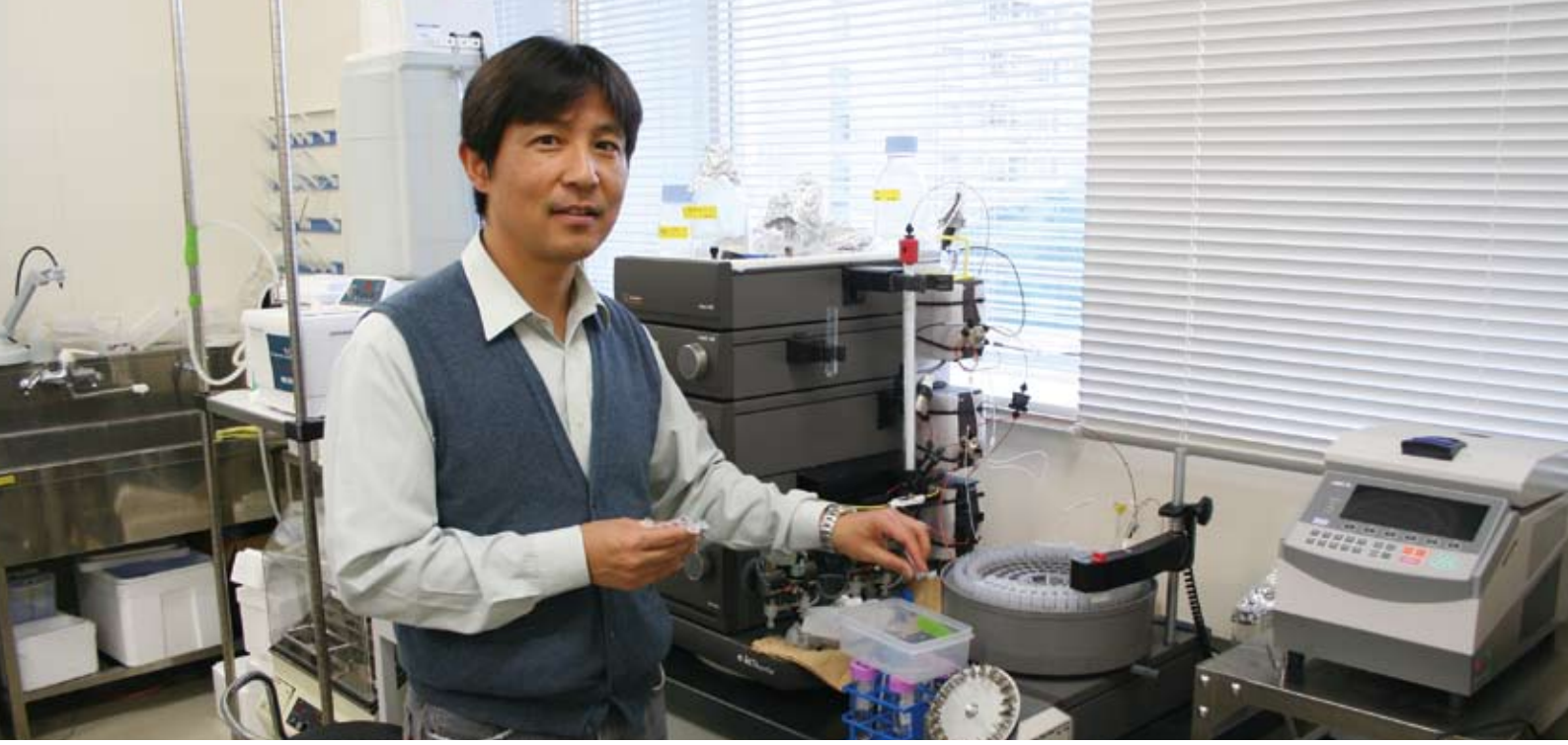

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Unfolded or denatured proteins in cells are rapidly degraded, otherwise, they may cause cell death through apoptosis. The destruction machinery for those proteins is linked to the ubiquitin-proteasome pathway. Unfolded or damaged proteins are first recognized by molecular chaperones such as hsp90 and hsc70. These chaperones may simultaneously recruit E3 ubiquitin ligase to polyubiquitinylate those proteins, resulting in the delivery of the proteins to the proteasome. This chaperone-assisted quality control system for cellular proteins, in the context of immunity, is the main source of the antigenic peptides presented by MHC class I molecules. It depends on the structure of the 20S proteasome and its regulatory particles, such as PA28 and PA700 (19S cap) how peptide fragments produced from the whole protein. The degradation patterns of cellular proteins determine the peptide repertoires presented by MHC class I molecules. The precise regulatory mechanism by which the proteasome produces peptides, however, is not well understood. Our laboratory focuses on the regulatory mechanism, particularly with respect to PA28 and the molecular chaperone, hsp90, that regulate the processing of cellular proteins and peptides.

HSPs in antigen processing

MHC class I ligands are mainly produced by the proteasome. The proteasome activator, PA28, comprising a heterohexamer of α and β chains, is thus PA28$\alpha_3\beta_3$ and is constitutively expressed in almost all cells. *In vivo* processing of $TRP2_{181\text{-}188}$, a murine melanoma antigen, is completely abolished in PA28$\alpha^{-/-}\beta^{-/-}$ cells, while the processing of the $OVA_{257\text{-}264}$ epitope remains intact in the mutant. This finding implies that hsp90 substitutes for PA28, resulting in the normal processing of the $OVA_{257\text{-}264}$ epitope in PA28$\alpha^{-/-}\beta^{-/-}$ cells. However, the underlying mechanism had been largely unknown.

We established a method that enables us to isolate 26S-type proteasomes (RCR, RC and hybrid proteasomes) as well as PA28-assisted proteasomes from a small amount of any kind of cellular extract. Applying this method, we found that Hsp90α pulled-down (or reconstituted) three distinct proteasomes from cell extracts *in vitro*: hsp90α-RC (singly capped 26S proteasome), hsp90α/Hop/hsc70/hsp40-20S, and hsp90α, the carboxyl-terminus of the hsc70 interacting protein (CHIP)-20S. Of these, the first two produce, *in vitro*, epitopes that can sensitize the epitope-specific cytotoxic T lymphocytes (CTLs). CHIP is known to interact with hsp90 and hsc70 through its tetratricopeptide repeat (TPR) motif to ubiquitinylate the proteins that are to be digested by the 26S proteasome. By CTL sensitization assay, we found that hsp90α-RC (one of the 26S proteasomes) and hsp90α /Hop/hsc70/hsp40-20S could process the *Plasmodium yoelii* $CSP_{281\text{-}289}$ T cell epitope, but PA28-assisted proteasome could not.

In order to further analyze the processing mechanism, we used several T cell epitopes spanning both N- and C-terminal flanking regions and established the ESI-based LC/MS-mediated peptide digestion assay with the 26S proteasome and the PA28-assisted proteasome. We found that *Plasmodium yoelii* $CSP_{281\text{-}289}$ was processed by the 26S proteasome but destroyed within the epitope sequence by PA28-20S. In contrast, the $TRP2_{181\text{-}188}$ epitope was processed by PA28-20S

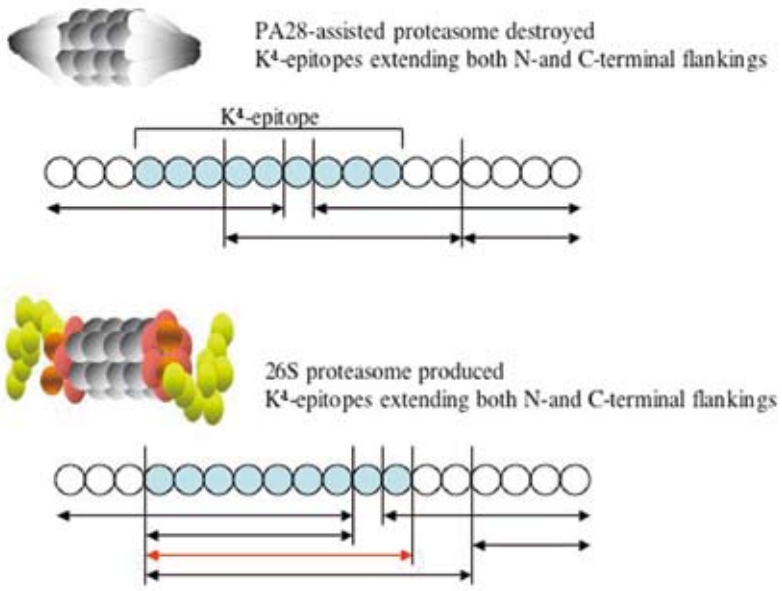

Figure: Regulatory particles of the proteasome-controlled production of MHC class I ligands.

but destroyed by the 26S proteasome. Our results indicate that the regulatory particles of proteasomes such as PA28 and 19S cap (PA700) not only deliver substrates into the 20S but also affect the digestion pattern, resulting in the production or destruction of the T cell epitopes. We suggest that hsp90α stimulated the assembly of the 26S proteasome with which antigen processing was augmented.

PA28 in antigen processing

IFN-γ stimulation increases the likelihood of football- and hybrid-shaped proteasomes. The former is a complex in which PA28 is attached to both ends of the central 20S proteasome while the latter comprises the 20S proteasome flanked by PA28 on one side and PA700 (that is, 19S Cap) on the other and functions as an ATP-dependent protease, similar to the 26S proteasomes that have PA700 at both sides. The physiological role of PA28 is still largely unknown. The 20S proteasome consists of α and β rings stacked as αββα. The α-ring, through which substrates enter, is usually closed, therefore, a regulatory particle such as PA28 is essential for opening the α-ring. The effect of PA28 is thus called the gate-opening effect. PA700 is also implicated to be a chaperone complex that passes through substrates into the core 20S. On the basis of that perspective, both PA28 and PA700 may open the α-ring of the 20S, which allows peptides to be loaded into the 20S. It is not known whether this gate-opening is the only task of these regulatory particles. We speculated that PA28 not only opened the gate but also influenced the pattern of peptide digestion by the proteasome, resulting in the production of a set of peptides different from those produced by the 26S proteasome. In fact, the *Plasmodium yoelii* $CSP_{281-289}$ T cell epitope was processed by the 26S proteasome but destroyed by PA28-20S, indicating different digestion patterns for these two proteasome complexes. To gain more insight into the role of PA28, we carried out LC/MS-based peptide digestion assay using many synthetic peptides harboring mouse MHC class I K^b and K^d ligands and the PA28-20S proteasome. We found that many of the K^b ligands were processed by PA28-20S, whereas K^d ligands within the epitopes were digested and destroyed. Furthermore, we found that knock-down of both PA28α and β by RNAi significantly augmented K^d and D^d expressions but reduced that of L^d on the same cell surface, indicating that the effect of PA28 on antigen processing depends on MHC polymorphism.

Antigen processing, as described above, is regulated by the ubiquitin–proteasome system. The peptide repertoire produced by the proteasome is determined by the kind of regulatory particle, such as PA28 and PA700, and the structure of the 20S proteasome itself. Further analysis of the structure of the proteasome and its digestion pattern of peptides harboring MHC class I ligands will help to elucidate the processing mechanism. The structure of the proteasomes is generally determined by the cell lineage, tissue or organ, but intriguingly, they are also regulated by cytokines such as interferon-γ (IFNγ). For example, IFNγ induces PA28-assisted immunoproteasome, which in turn suggests that the analysis of the relationship between cytokines and the structure of the proteasome may provide a further understanding of antigen processing, and hence, of the adaptive immune system.

Recent Publications

Honma K., Udono H., Kohno T., Yamamoto K., Ogawa A., Takemori T., Kumatori A., Suzuki S., Matsuyama T., Yui K. Interferon Regulatory Factor-4 Negatively Regulates the Production of Proinflammatory Cytokines by Macrophages in Response to LPS. Proc. Natl. Acad. Sci. USA. 102, 16001-16006 (2005)

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Type 1 diabetes mellitus is an autoimmune disease caused by a destruction of pancreatic beta cells by autoreactive T cells. In both human and animal models of type I diabetes, class II major histocompatibility (MHC) antigens are the most important genetic factor for susceptibility to the disease. Furthermore, various environmental factors such as infection and active immunization with model antigens are known to influence disease development both positively and negatively. The goals of our research group are to understand the disease process and to develop means to modulate this process. The disease process is shown in the Figure, and our projects are (1) to identify the antigen(s) responsible for the initiation of the disease, (2) to elucidate biochemical and molecular mechanisms by which certain MHC genotypes confer susceptibility to type I diabetes, (3) to establish an effective method to regulate disease development in vivo, and (4) to understand the final effector mechanisms by which pancreatic beta cells are destroyed. All experiments are carried out using the NOD mouse model of type I diabetes, but it is our expectation that the results obtained using this mouse model will be relevant to the development of new strategies to treat human type I diabetes.

Screening of diabetes antigens

The search for diabetes antigens has haunted the world of immunology for many years. Although a number of antigens have been shown to play a role in the pathogenesis of type I diabetes in NOD mice, no antigen has been demonstrated to stimulate pathogenic T cells that cause diabetes *in vivo*. We use a saturation screening approach to identify pancreatic beta cell antigen that stimulates diabetogenic T cells. Using whole genome analysis, we have identified about 100 beta cell-specific cDNAs. Proteins are produced from these cDNAs using a cell-free expression system and screened for their capacity to stimulate diabetogenic T cells *in vitro*. We also use retroviral-mediated expression of these cDNAs in an antigen- presenting cell. Antigen-presenting cells expressing beta cell antigens will also be tested with diabetogenic T cells. These approaches will cover all possible antigens expressed in pancreatic beta cells and are likely to succeed in identifying diabetogenic antigen(s) in the NOD mouse.

We are attempting to extend this method to the identification of antigen(s) in human Type I diabetes.

Class II MHC and presentation of diabetogenic antigen

I-Ag7 in mouse and DQ8/2 in human are the most important genetic factors for the susceptibility to type I diabetes. These MHC molecules share a non-Asp residue at position 57 of the beta chain. Also, these MHC molecules are known to bind all peptides with weak affinity. We hypothesized that i) poor peptide-MHC interaction generates an unstable complex. ii) This unstable complex exhibits multiple transitional conformations, and iii) T cells recognize this transitional conformation to form a stable trimolecular TCR/MHC/peptide for activation. To test this hypothesis, we are using diffracted X-ray tracking (DXT) to visualize the movement of the peptide in the MHC groove.

Regulatory T cells in diabetes development

We have shown that regulatory T cells play an impor-

tant role in the regulation of diabetes development in NOD mice. The following conditions favor the generation of regulatory T cells leading to the complete suppression of diabetes development: I) MHC class II heterozygosity, II) lack of interferon gamma signaling, and III) exposure of T cells to soluble but not to the cell-bound form of the antigen. These results indicate that insufficient activation of T cells preferentially induces regulatory functions. Currently, we plan to generate regulatory T cells from naïve T cells with specificity for beta cell antigen. These T cells will be tested for the regulation of autoimmune diseases as well as inhibition of transplanted pancreatic islet rejection.

Regulation of T cell function by cytokines

We have shown that IL-2 and IL-15 that share all of the known signaling components of the receptor exhibit very different functions on T cells. IL-2 preferentially induces effector CD8 T cells and maintains regulatory CD4 T cells. IL-15 plays an important role in maintaining memory CD8 T cells with no effect on CD4 T cell function. We plan to examine the mechanisms by which these two cytokines differentially regulate the fate of antigen-specific T cells in vivo. Currently we are attempting to carry out structural analysis of the interaction between IL-2/IL-15 and their receptors. Furthermore, a single-molecule microscope will be used to investigate possible kinetics differences between IL-2 and IL-15 for the activation of the key signaling molecule STAT-5.

Other projects in our laboratory

Role of TcR alpha chain secondary rearrangement for the formation and maintenance of T cell repertoire: Using a TcR alpha chain Knock-In mouse and single J alpha chain mice, we demonstrate that the secondary rearrangement of the TcR alpha chain plays a critical role during thymocyte selection and for the formation of peripheral T cell repertoire. In the following studies, we found that interaction between foreign antigen-specific T cells and endogenous antigens leads to the deletion of the T cells in the periphery in an age-dependent manner. We plan to identify the gene(s) and antigen(s) that interact with the T cells and modify the mature T cell pool in aged mice. Understanding the mechanisms by which endogenous antigens modify the T cell repertoire in an age-dependent fashion may provide insight to the age-dependent change/decline in immune function in both mouse and man (Huang et al. *Proc Natl Acad Sci USA* ,2005).

Gads, a Grb2-like adaptor protein, plays a critical role in thymocyte development. We demonstrate that mast cells from Gads-deficient (-/-) mice have selective functional defects. Bone marrow-derived mast cells (BMMCs) from Gads$^{-/-}$ mice failed to respond to crosslinking the FcεRI in Ca^{2+} mobilization, degranulation and cytokine production. This is likely due to the lack of Gads-mediated association of SLP-76 and LAT. In contrast, activation of ERK, JNK and p38 MAPK by the same stimulation was intact in the absence of Gads. Stimulation of mast cells through a Toll-like receptor to produce cytokines was not impaired in Gads$^{-/-}$ mast cells. In agreement with these *in vitro* results, mice lacking the Gads gene showed greatly impaired passive cutaneous anaphylaxis, while no difference was observed in mast cell-dependent resistance to acute peritoneal bacterial infection between Gads$^{+/-}$ and Gads$^{-/-}$ mice. Moreover, mature T and B cells from Gads$^{-/-}$ mice showed no apparent functional impairment. Thus, the inhibition of Gads *in vivo* would suppress IgE-mediated allergic reaction with no adverse effect on both innate and acquired immune responses, and Gads could be an ideal target for the control of allergic responses (To be submitted).

Cytomegalovirus (CMV) infection is one of the most common complications in immunocompromised individuals, such as organ and bone marrow transplant patients. Both innate and adaptive immune responses are required for defense against CMV infection. In murine CMV (MCMV) infection, strains harboring the MCMV-specific NK cell activation receptor, Ly49H, are resistant. In contrast, MCMV infection of mice lacking the Ly49H gene causes early mortality due to uncontrolled viral replication. In this context, we discover the successful protection of mice against lethal MCMV infection using gene-transferred polyclonal CD8 T-cells. CD8 T-cells expressing a chimeric receptor comprising Ly49H extracellular and CD3ζ cytoplasmic domains are capable of killing target cells expressing the MCMV protein, m157. The chimeric receptor-expressing CD8 T cells protect mice in vivo from lethality in the acute phase of MCMV infection, leading to the establishment of long-term protection. These data provide proof-of-principle evidence that a novel strategy of harnessing CD8 cytolytic function through a TCR-independent yet pathogen-specific receptor can result in effective protection of hosts against pathogens (submitted).

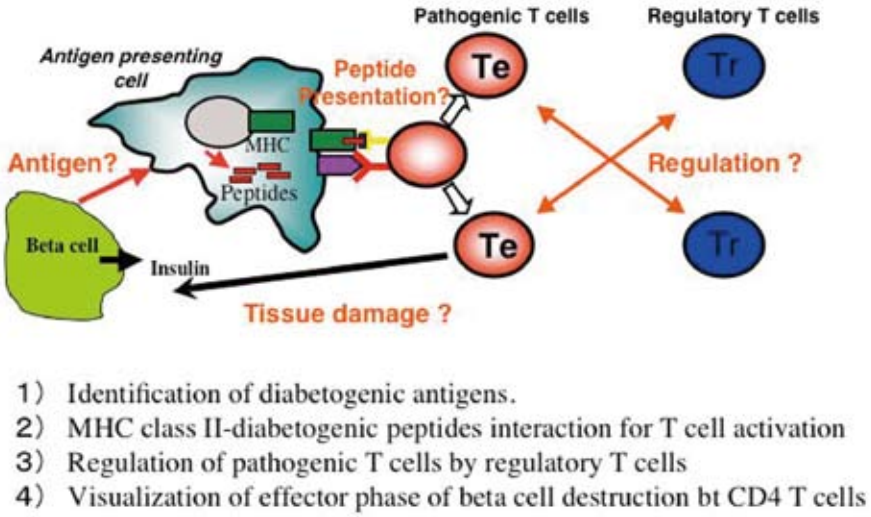

Figure: Pathogenesis and control of Type I diabetes

Recent Publications

Huang CY, Sleckman BP, Kanagawa O. Revision of T cell receptor α chain genes is required for normal T lymphocyte development. Proc Natl Acad Sci U S A. 2005,102:14356-61.

Aya K, Alhawagri M, Hagen-Stapleton A, Kitaura H, Kanagawa O, Novack DV NF-κB-inducing kinase controls lymphocyte and osteoclast activities in inflammatory arthritis. J Clin Invest. 2005, 115:1848-54.

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Suri A, Shimizu J, Katz JD, Sakaguchi S, Unanue ER, Kanagawa O. Regulation of autoimmune diabetes by non-islet-specific T cells - a role for the glucocorticoid-induced TNF receptor. Eur J Immunol. 2004, 34: 447-54.

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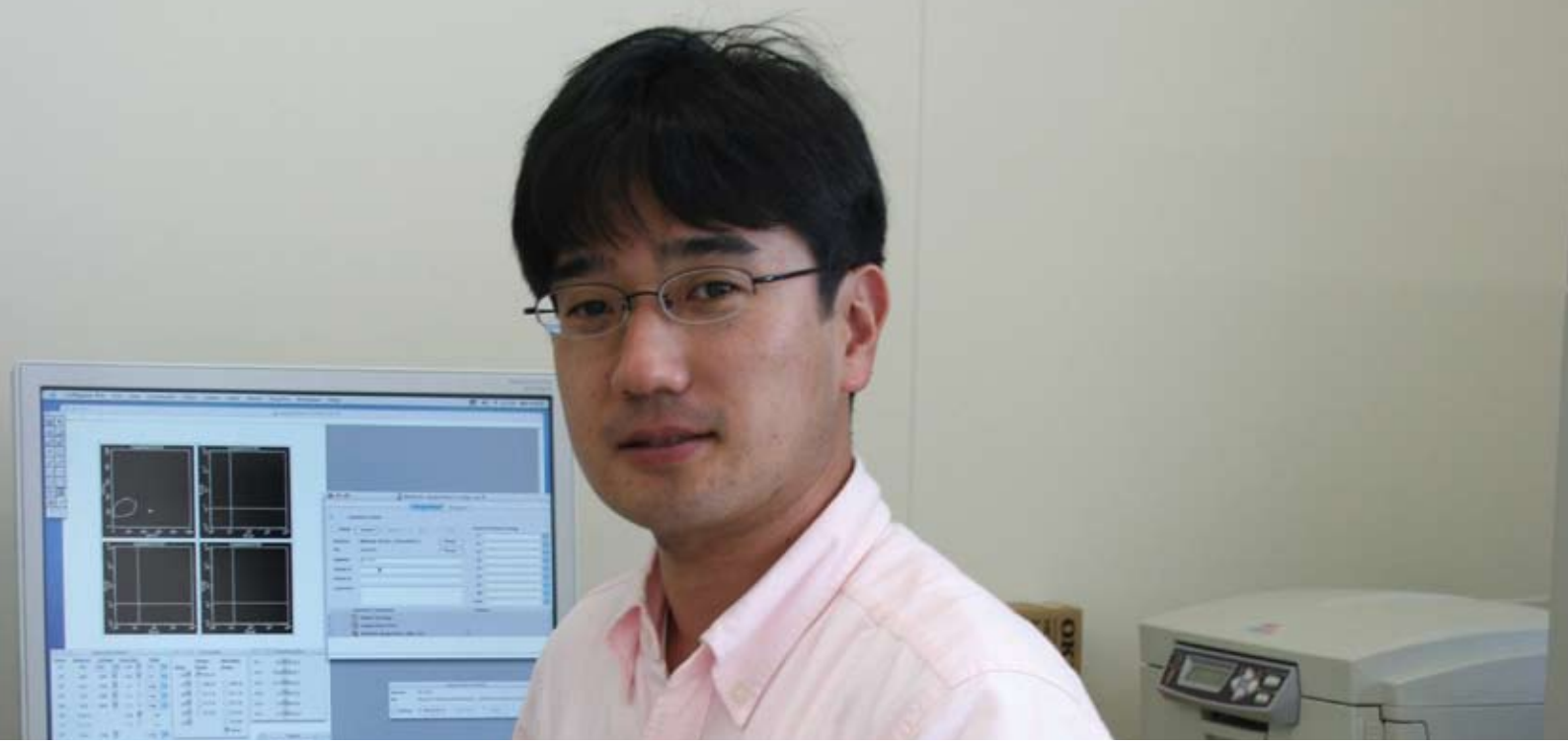

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A small subpopulation of T lymphocytes, known as regulatory T cells (T_{reg}), plays a central role in preventing pathological immune responses such as autoimmunity, inflammation and allergy, and thus ensures dominant tolerance to self- and innocuous environmental antigens. This has been best illustrated by our recent finding that the development and function of T_{reg} is controlled by the transcription factor Foxp3, a genetic deficiency of which leads to the development of a fatal autoimmune, inflammatory and allergic disease in a natural mouse mutant strain called scurfy and in human patients with the IPEX syndrome.

The identification of Foxp3 as a "master" regulator of T_{reg} development and function has provided a key to a number of outstanding unresolved questions concerning their role in tolerance and immune regulation and the physiology of these cells, which include their origins, the molecular/cellular mechanisms controlling their development and function, and their antigen specificity. Resolving these issues is the goal of this laboratory.

Etiology of Immune Dysregulation in Foxp3-mutant Mice

Since *scurfy* or Foxp3-deficient mice fail to generate T_{reg} cells, it is now believed that this defective T_{reg} generation is the primary cause of the immune dysregulation in Foxp3-mutant mice and humans. It has also been proposed, however, that Foxp3-deficient mice develop immune pathology due to a defect in non-hematopoietic tissues, particularly the thymic epithelium, the tissue essential for T cell development and repertoire selection. This hypothesis is supported by the findings that lethally irradiated wild-type host mice reconstituted with *scurfy* donor bone marrow cells fail to transmit the fatal immune pathology and that Foxp3 has recently been reported to be expressed in the thymic epithelium as well. Aiming at resolving this apparent discrepancy, we repeated this radiation chimera experiment and found that such chimeric mice still harbored a control number of host-derived $Foxp3^+$ T_{reg} cells. These host-derived $Foxp3^+$ T_{reg} cells are responsible for the disease protection since antibody-mediated or genetic ablation of host T cells resulted in the development of a fatal immune pathology. Furthermore, our results have clearly excluded the proposed hypothesis that Foxp3 inactivation in non-hematopoietic tissues is required for the disease development in Foxp3-deficient mice. Collectively, the results of our analyses enforce the notion that Foxp3-deficient mice develop a fatal immune pathology due indeed to defective T_{reg} development, and therefore establish that $Foxp3^+$ T_{reg} cells play an indispensable role in dominant tolerance.

Furthermore, our studies of the bone marrow chimeras have also demonstrated that, in the absence of thymic generation, the peripheral $Foxp3^+$ T_{reg} pool is fully restored and maintained from a small number of radioresistant host cells through "homeostatic" expansion. This illustrates that the number of $Foxp3^+$ T_{reg} cells is tightly controlled and thus they occupy a specific "niche" in the peripheral lymphoid organs. The molecular and cellular mechanisms underlying T_{reg} homeostasis are currently under investigation.

We are also developing a "knock-in" mouse model in which either Thy1.1 or the human CD52 reporter is knocked into the Foxp3 locus. These mice will be a valuable tool not only to track Foxp3 expression

in vivo but also to establish the functions of Foxp3$^+$ T_{reg} in tolerance and immune regulation in normal, non-lymphopenic animals, because these mice will allow us to eliminate Foxp3$^+$ T_{reg} cells from the body by injecting depleting monoclonal antibodies against these surface antigens.

Impacts of IPEX Mutations on T_{reg} development and Function

Having established that Foxp3-deficient mice develop immune pathology due to defective T_{reg} development, we then addressed whether and how Foxp3 mutations that had been identified in IPEX patients affect T_{reg} development and function. To this end, we generated Foxp3 mutants carrying IPEX mutations and retrovirally transduced them into conventional T cells. Our analyses revealed that all the mutations we examined were amorphic or hypomorphic in that T cells expressing these mutants failed to exert full suppressive activity. Most of the mutations also affected the expression of T_{reg} phenotypic markers, suggesting defective T_{reg} development in patients carrying these mutations. Serendipitously, T cells transduced with one of the mutations normally expressed T_{reg} phenotypic markers despite their impaired suppressive activity, suggesting that the patients carrying this particular mutation develop IPEX syndrome due to impaired T_{reg} effector function rather than defective T_{reg} development. This observation prompted us to compare the gene expression profiles of T cells transduced with wild-type Foxp3 versus this mutant by DNA microarray analyses. Our results demonstrated that these two groups of T cells were strikingly similar in terms of gene expression, except for a small subset of Foxp3 target genes affected by this mutation. Since the expression of these genes is affected in a manner strongly associated with the loss of suppressive activity, we hypothesize that these genes may be important for T_{reg} effector function. We are currently undertaking genetic studies to test this hypothesis.

One of the central unresolved questions regarding T_{reg} cell biology is the molecular mechanisms by which they suppress immune responses. Specifically, despite extensive worldwide studies at a number of laboratories, the molecular identity that mediates T_{reg} suppressor effector function still remains unknown. Our analyses of IPEX mutations appear to have provided a promising clue to resolving this question. By comparing gene expression profiles of T cells transduced with wild-type and mutant Foxp3, we have identified a small number of Foxp3 target genes which are affected by the mutation and are therefore strongly associated with T_{reg} suppressor effector functions. The identification of T_{reg} effector molecules will be particularly important in manipulating T_{reg} functions for the treatment of autoimmunity, inflammation, allergy, chronic infection and cancer.

This laboratory has been partially funded by the PRESTO program of the Japan Science and Technology Agency (JST).

Recent Publications

Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic Maintenance of Natural Foxp3$^+$CD25$^+$CD4$^+$ Regulatory T Cells by IL-2 and Induction of Autoimmune Disease by IL-2 Neutralization. J. Exp. Med. *201, 723-735 (2005)*

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Our focus is to control autoimmune diseases including rheumatoid arthritis (RA) and polymyositis (PM). RA afflicts 0.5-1% of the Japanese population. The main pathology of the affected synovial tissues consists of inflammation and hypergrowth of synovial fibroblasts. Current clinilal and experimental approaches to regulate RA or its animal models mainly focus on the control of underlying immune abnormalities, but such treatments are often ineffective in controlling cartilage and bone destruction. One approach we have been pursuing is the suppression of rheumatoid inflammation by controlling the synovial cell cycle. Also, we succeeded in developing a new model of polymyositis, another autoimmune disease, for which we do not have specific treatment in clinical settings.

Cell cycle regulation therapy for treatment of RA

RA is characterized by inflammation of joint cartilage followed by hyperplasia of synovial tissues. We have shown that the transfer of cyclin-dependent kinase inhibitor (CDKI) genes such as $p16^{INK4a}$ and $p21^{clp1}$ into synovial tissues in RA animal models ameliorated the symptoms of RA. The treatment suppressed not only the inflammation but also the proliferation of synovial tissues; however, it remains obscure how CDKI associated with cell cycle control exerts an anti-inflammatory effect. It could be an indirect effect of cell cycle inhibition because it inhibits platform formation of inhibition. Alternatively, it could be due to the effect of CDKI per se and/or inhibition of CDK activity.

In order to clarify the underlying mechanisms, we studied how $p21^{clp1}$ gene transfer treatment affects gene expression in synovial fibroblasts. We found that the expression levels of various cytokines (IL-6, IL-8, MCP-1 and MIP1α) and proteolytic enzymes (cathepsins B and K, and MMP-1 and -3) were decreased after the gene transfer treatment. Then, we studied the inhibitory mechanisms on the gene expression. We discovered that the transferred gene, $p21^{clp1}$, bound to and inhibited JNK MAP kinase, an intracellular signaling molecule, and reduced the activities of NFκB and AP-1, which are essential molecules for the inflammatory-related gene expression.

These effects were not observed when $p16^{INK4a}$ gene was transferred. However, $p16^{INK4a}$ gene transfer decreased the expression of such inflammatory mediators as MCP-1 and MMP-3. In addition, we found that CDK4/6 specific inhibitors suppressed $p16^{INK4a}$ and decreased the expression levels of MCP-1 and MMP-3. CDK4 and 6 cannot be functionally distinguished *in vivo* and *in vitro*. When CDK4 activity was artificially up-regulated by induction of CDK4 and cyclin D1 gens, the expression levels of these inflammatory mediators were also enhanced in synovial fibroblasts and osteoclasts. Our results suggest that CDK4/6 regulates the expression of inflammatory mediators and induces the destruction of cartilages and bones. The close relationships between the regulation of cell cycle and the expression levels of inflammatory effectors indicate the possibility that the regulation of CDK4/6 activity may accompany suppression of hyperplasia and inflammation in RA. (*Arthritis Rheum*. in press)

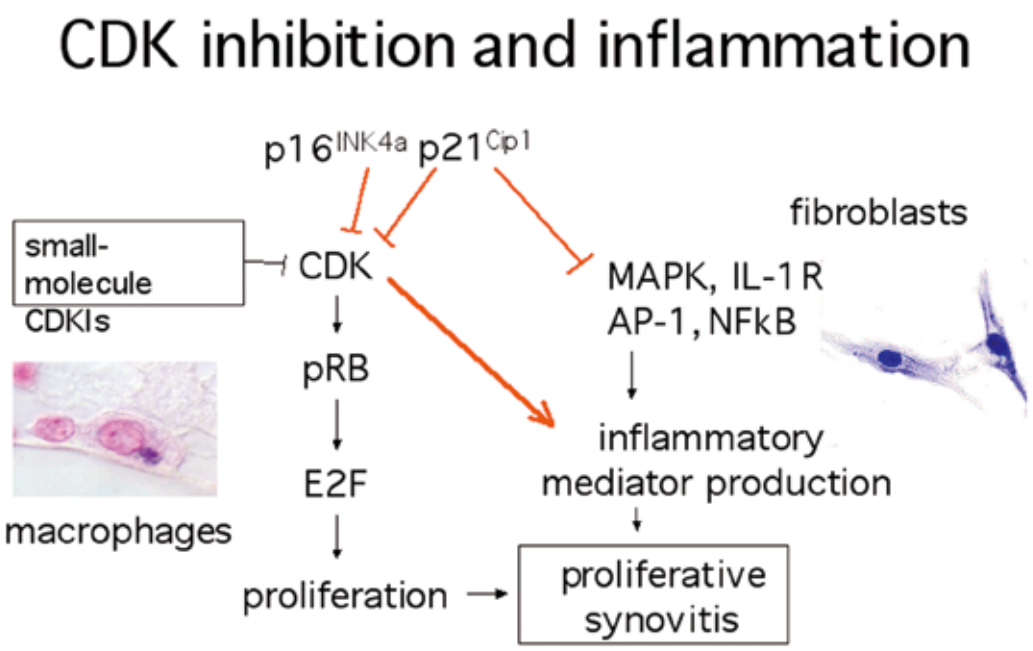

Figure: CDK inhibition and inflammation

CDK4/6 inhibitors have been developed by pharmaceutical companies for the treatment of cancer. However, clinical trials have shown that their efficacy against cancer is rather low although they only have mild side effects. When administered systemically, patients showed decreases in the number of white blood cells and diarrhea, but no obvious organic failures.

Thus, we administered a CDK4/6 inhibitor to a mouse model of RA. Joint inflammation was suppressed by the administration of a low dose compared with that for cancer. Antibody production was not affected by the CDK4/6 administration.

We expect that the CDK4/6 inhibitor will serve as a novel drug for RA as it will not affect the function of lymphocytes during treatment. Based on these results, we have applied for patents and plan to proceed to translational research.

Establishment of animal model for polymyositis

Polymyositis (PM) is one of the major systemic autoimmune diseases and can affect skeletal muscles of the whole body. We have succeeded in establishing a new model of PM in C57BL/6 mice. Unlike previous models, this model can be easily applied to studies with gene knockout mice, and can be used to investigate the immunopathology of PM. The pathology of the new animal model closely mimics human PM. In the analysis of gene knockout mice, we found that humoral autoimmunity is not necessary for the development of myositis. We will use this model to search for new therapeutic approaches that specifically address the pathology of PM.

Our goal is to develop novel therapies for autoimmune diseases based on the immunological research we are performing. We are proceeding with research to develop novel therapies for RA, and using the newly developed PM model, pursuing target molecules for the treatment of PM.

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CD1d-restricted natural killer T (NKT) cells, which express both a single invariant Vα14 antigen receptor (and are therefore also known as Vα14$^+$ NKT or Vα14i NKT cells) and NK receptors, such as NK1.1, occupy a unique intermediary position between innate and acquired immunity. Furthermore, because of their ability to quickly release large amount of cytokines, such as interferon (IFN)-γ and IL-4, and their apparent self-reactivity, it has been demonstrated that NKT cells play an important role in the regulation of various autoimmune disease development, such as rheumatoid arthritis, SLE, type I diabetes, systemic sclerosis and mediates the maintenance of tolerance. allergic diseases such as asthma. NKT cells also mediate protective immune responses against pathogenic infections or tumor. Our group studies the regulation of NKT cell-mediated immune functions and responses, both beneficial and harmful, which we believe will contribute to the development of new strategies for human immune therapy.

Cellular and molecular mechanisms of NKT cell-mediated regulation of immune responses

NKT cells are known to be able to regulate various immune responses. Satoshi Kojo et al. have investigated the cellular and molecular mechanisms of the NKT cell-mediated immune regulation. So far, we have found the mechanisms for the generation of regulatory dendritic cells (DCs) by stimulation of NKT cells *in vivo*. NKT cell functions or cytokine profiles can be altered by additional mechanisms, including the modulation of TCR signaling. A single stimulation with α-galactosylceramide (α-GalCer), a specific ligand for NKT cells, resulted in the production of high levels of IFN- by the NKT cells. This α-GalCer-induced IFN-γ production is the basis for the adjuvant effect of NKT cell activation on NK and CD8 T$^+$ cells, and contributes to protective immune responses such as tumor rejection. In contrast, repeated stimulation with α-GalCer caused NKT cells to produce more IL-10 and less (if any) IFN-γ, thus favoring the development of regulatory NKT cells. These regulatory NKT cells educate naive DCs to acquire properties of regulatory DCs in IL-10-dependent fashion, marked by non-matured phenotypes and increased IL-10 but reduced IL-12 production. The unique cytokine profile in these DCs appears to be regulated by extracellular signal-regulated kinase-1/2 and IκBNS. These DCs also showed an ability to suppress the development of experimental allergic encephalomyelitis (Kojo S, *J. Immunol.* 2005) and transplantation tolerance (Jiang X, *J. Immunol.* 2005) by generating IL-10-producing regulatory CD4$^+$ T cells in vivo. These studies suggest that the TCR signal intensity influences the function of NKT cells, in a manner similar to the effect of TCR avility on positive and negative selection of developing T cells in the thymus.

Plasma membrane-focused proteomics of dendritic cells and identification of a novel plasmacytoid dendritic cell-specific surface receptor, PDC-TREM

The differential expression of surface molecules on dendritic cells (DCs) reflects their functional differences as immature and mature subsets. It is dif-

Figure: Structural features of PDC-TREM

ficult, however, to characterize differences in surface expression by standard proteomic approaches, due mainly to the hydrophobic nature and low abundance of the individual proteins in question. Watarai et al. established a method for obtaining high yield plasmalemma preparations, in which surface molecules are enriched over two hundred-fold by coating cells with beads conjugated with antibodies against a cell-type-specific cell surface molecule, followed by nitrogen cavitated disruption, magnetic separation and density gradient ultracentrifugation. We identified and quantified 339 MoDC transmembrane proteins, including 33 previously uncharacterized molecules. Whereas 106 proteins were selectively expressed in immature cells or down-regulated after maturation, 191 proteins were selectively expressed in mature cells or up-regulated after maturation. We identified a new molecule among those previously uncharacterized, PDC-TREM, which is selectively expressed in activated plasmacytoid dendritic cells (also known as natural type I interferon producing cells) and responsible for robust production of type I interferon.

NKT cell-triggered IFN-γ production by Gr-1$^+$CD11b$^+$ cells mediates early graft loss of syngeneic transplanted Islets

See Research Highlights section

Generation and analysis of NKT cell cloned mice

See Research Highlights section

Translation research: phase I/IIa clinical trials for lung cancer patients using α-GalCer/DC therapy

See Translational Research Section

In the next two years, we will focus on two projects: first, the identification of precursor cells for NKT cells and their selection mechanisms by using NKT cell cloned mice *in vivo* and also ES cells derived from NKT cell cloned mice *in vitro*, and second, the roles of PDC-TREM expressed on activated PDCs in the early events of innate and acquired immune responses in the protection of pathogens. In addition to these basic projects, we will continue to do the phase IIa clinical trials of α-GalCer-pulsed DC therapy on lung cancer patients in collaboration with Chiba University.

Recent Publications

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Despite their increasing prevalence in developed countries, the molecular mechanisms behind autoimmune diseases and allergy remain poorly understood. The eventual goal of the Cytokine Signaling Research Group is to contribute to the elucidation of the molecular and immunological mechanisms of autoimmune diseases and allergy from the viewpoint of signal transduction within the immune system. More than two decades ago, we were involved in the cloning of "soluble factors" that were later to become interleukin 6 (IL-6), and are continuously exploring molecular processes related to IL-6 and the IL-6 receptor, focusing on STAT3 (Signal Transducer and Activator of Transcription 3), various members of the Gab family of proteins and, most recently, the largely unknown universe of signaling through zinc transporter proteins, to reveal the role of zinc in immune functions.

Role of gp130 and zinc signaling in the immune system

The effect of IL-6/gp130 signaling on dendritic cell (DC) functions related to antigen presentation. Previously, we reported the immunosuppressive effect of IL-6 through the down-regulation of MHC class II molecules on DCs, in vivo and in vitro. Further, we were able to clarify the detailed molecular mechanisms of this effect and to find that IL-6/gp130 signaling activates the lysosomal enzyme cathepsin S through STAT3 and causes a reduction of the MHC class II αβ-dimer level in DCs and subsequently $CD4^+$T cell activation (Kitamura et al., *Immunity*, 2005).

In a breakthrough discovery published in *Nature* in 2004, we identified the zinc (Zn) transporter Zip6 (also known as Liv1) as the target gene of the IL-6 cytokine signaling molecule STAT3 and further demonstrated that Zip6 plays an important role in the cell migration that takes place during the gastrulation process in the early development of the zebrafish embryo. Zn is a trace element that is essential for the function of many enzymes and transcription factors. Zn deficiency results in defects in innate and acquired immune responses. However, little is known about the mechanism(s) by which Zn affects immune cell function. We found that stimulation with the toll-like receptor (TLR) 4 agonist lipopolysaccharide (LPS) altered the expression of Zn transporters in DCs, thereby decreasing intracellular free Zn. A Zn chelator mimicked the effects of LPS, whereas Zn supplementation or overexpression of the gene encoding Zip6, a Zn transporter whose expression was reduced by LPS, inhibited LPS-induced upregulation of MHC class II and costimulatory molecules. These results have led to the establishment of a link between TLR signaling and Zn homeostasis, and strongly suggest that intracellular Zn is closely related to DC maturation as well as to the regulation of MHC class II molecules through endocytosis and trafficking (Kitamura et al., *Nature Immunology*, in press). In order to further elucidate the intracellular role of the Zn in DC maturation, we are currently trying to identify the target molecule of Zn in signaling cascades related to DC maturation.

Molecular mechanisms of mast cell degranulation

The immunological and molecular mechanisms of

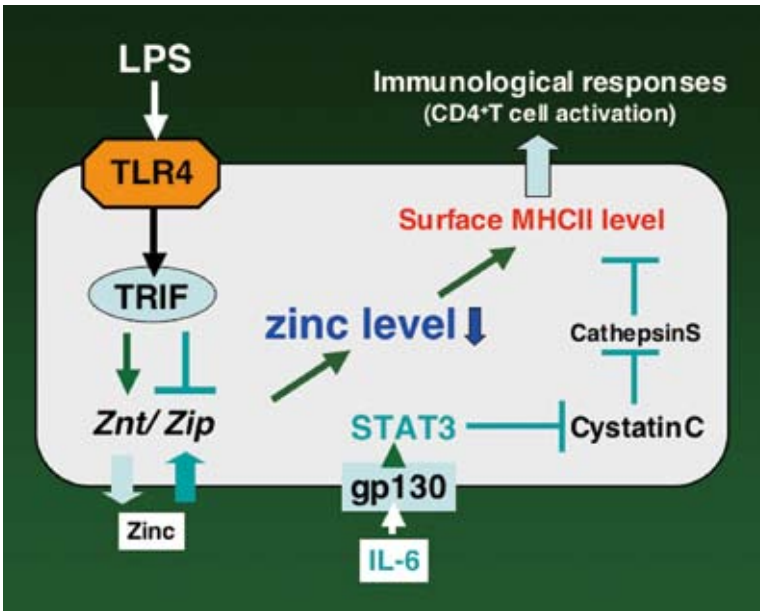

Figure: TLR-mediated zinc signaling controls DC maturation

mast cells in allergy, inflammation, and autoimmune diseases were investigated. Most importantly, the degranulation process of mast cells was dissected. First, Fc ε RI stimulation triggers microtubule polymerization and granule translocation to the plasma membrane in a calcium-independent manner. Second, the granules fuse with the plasma membrane in a well-characterized calcium-dependent manner. Furthermore, it was shown that not Lyn/SLP-76 but the Fyn/Gab2/RhoA signaling pathway plays a critical role in the calcium-independent microtubule-dependent pathway (Nishida and Yamasaki et al., *J Cell Biol*. 2005). Our present research concerns mainly the clarification of the molecular mechanism of calcium-independent, microtubule-dependent granule translocation. Very recently, it was found that Zn is required for Fc ε RI induced granule translocation to the plasma membrane, suggesting that the Zn chelator is a potential new antiallergic agent. Zn is also required for cytokine production in mast cells. In addition, it was shown that zinc deficiency in mast cell prevents the translocation of protein kinase C (PKC) as well as downstream events such as the phosphorylation and nuclear translocation of NF-κB and the production of such cytokines as IL-6 and TNF α (Kabu et al., J. Immunol. in press). Based on these findings, we are now searching for Zn-associated molecules that regulate granule translocation and cytokine production in mast cells. Furthermore, we are trying to identify tubulin-associated molecules involved in granule translocation and to establish knock-in mice expressing a mutant Gab2 molecule in order to further dissect Fc ε RI mediated signaling and to better clarify the in vivo function of mast cells in allergy, inflammation and autoimmune diseases.

Our group has been studying these signaling molecules using a broad variety of tools employed in molecular biology, cell biology, immunology, or development. When it turned out that STAT3 has an important function in development, we expanded our activities into developmental biology. When Zn transporter proteins were found being involved in signaling processes orchestrated by STAT3, we set up an entirely new research program to study the biological function of zinc.

Presently, the research group at RCAI is focusing on signaling processes relevant to the immune system; in particular, it is investigating the molecular mechanisms of IL-6/IL-6 family cytokine-mediated immune responses in health and disease, as well as trying to elucidate the molecular mechanisms of mast cell function, including degranulation and cytokine production related to allergy, autoimmune diseases, and inflammation. A second research group based at Osaka University is focusing on the more basic aspects of IL-6-related signaling molecules and their functional roles in relation to development, oncogenesis, and immunity.

Recent Publications

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T cells play a central role in the promotion of the effector and regulatory functions in the immunological surveillance system, and aberrations of these functions result in various immunological disorders. T cells produce various types of cytokines in the immune response, which are critical factors in the transmission of information from the receptor to the nucleus as well as in the generation of communication networks among cells. Thus, the cytokines secreted by T cells directly promote the effector and regulatory functions of T cells.

The main scientific goal of our laboratory is to understand the molecular basis of epigenetical regulation of effector cytokine locus and signal transduction cascades through cytokine signaling that regulates effector cytokine expression during T cell differentiation. We are interested in how the expression of *Il4* and *Il13* cytokine genes is independently controlled among various cell lineages, including lymphoid and myriad lineages. Research activities are focused on the biochemical mechanisms of cytokine pathways and the lineage-specific transcriptional regulation in Th2 cytokine gene expression.

Regulation of cis-acting elements of *Il4* locus among different lineages producing IL-4 and IL-13

In order to understand epigenetic changes in chromatin structure at the T helper (Th2) locus correlate with IL-4 and IL-13 expressions, we established a series of reporter transgenic mice and deletion mutant mice of cis-acting elements from mouse genome. We have recently demonstrated that conserved noncoding sequence-2 (CNS-2), an essential enhancer at the downstream of the *Il4* locus, is constitutively acting in NKT cells as well as in a subset of $CD44^{hi}$ memory phenotype $CD4^{+}$ T cells. Furthermore, we observed that Th2 differentiation was selectively impaired in T-cell-specific, RBP-J-deficient mice that lack Notch signal. T-cell-specific, RBP-J-deficient mice showed abrogation of the CNS-2 enhancer activity and initial IL-4 expression in NKT and memory phenotype $CD4^{+}$ T cells, and CNS-2 enhancer-deficient mice resulted in a loss of initial IL-4 expression in NKT and memory phenotype CD4 T cells and Th2 differentiation under neutral condition. Therefore, the major role of the Notch signal is to regulate CNS-2 enhancer activity in NKT and memory phenotype CD4 T cells. This result also shows a functional significance of memory phenotype T cells for facilitating Th2-mediated allergic responses. Our GFP transgenic system distinguishes IL-4-producing and IL-17 producing memory phenotype CD4T cells based on the cytokine production profile. These two memory cells play an important role in determining the direction of the immune response as immune regulators.

Role of suppressor of cytokine signaling (SOCS) in immune regulation

The cytokine environment at the site of initial antigen stimulation determines the direction of helper T cell differentiation into either Th1 or Th2 cells. The SOCS3 and SOCS5 proteins are implicated in this process of controlling the balance between Th1 and Th2 cells through the inhibition of IL-12 and IL-4 signaling pathways, respectively. The Th2 environment accelerates the number of CD4 T cells expressing

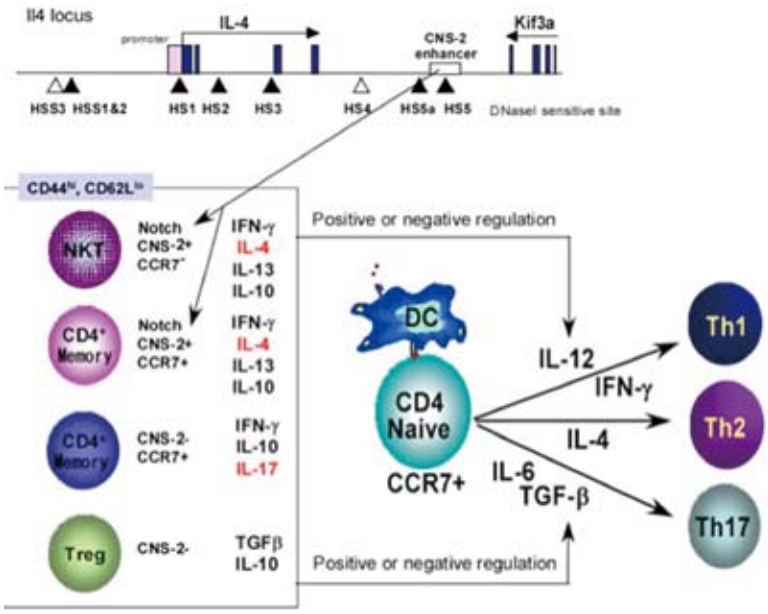

Figure: Regulatory elements on Il4 locus and role of CD44high memory phenotype T cells in helper T cell differentiation CNS-2 located downstream of the Il4 locus functions as an enhancer to regulate IL-4 production in NKT cells and memory phenotype CD4+ T cells through the Notch signaling pathway. CD44high memory phenotype T cells, NKT cells and Treg cells play a significant role in regulating the direction of helper T cell differentiation.

SOCS3, particularly in the allergic inflammatory site, subsequently enhancing the incidence of allergic diseases, including allergic conjunctivitis and atopic asthma. Therefore, the modification of SOCS3 and SOCS5 functions and their expressions could be a beneficial target for therapeutic intervention in many aspects of allergic diseases.

The suppressors of cytokine signaling (SOCS) are a family of proteins that negatively regulate cytokine signaling. SOCS1 is a powerful negative regulator capable of inhibiting a wide range of cytokines through binding to all family members of the Jak tyrosine kinases via its SH2 domain. In order to understand how the abrogation of cytokine signaling by SOCS1 affects the development and maintenance of naive and memory CD4 T cells, we investigated the generation of these cells in SOCS1 Tg mice. Cell surface phenotype analysis confirmed that most peripheral CD4 T cells in SOCS1 Tg mice had a memory phenotype even in a DO11.10 TCR transgenic or *cd28*-deficient background. Adoptive transfer of $CD44^{low}$ CD4 thymocytes from SOCS1 Tg mice again resulted in the development of $CD44^{high}$ CD4 T cells. Our results suggest that IL-7-mediated STAT5 activation is essential for long-term survival of naive CD4 cells after export from the thymus, but that another SOCS1-sensitive cytokine is critical for short-term naive T cell survival.

Th2 cytokines, such as IL-4 and IL-13, are expressed in distinct lineages of cells that develop in different tissue environments. Several cell types have been reported to secrete IL-4 and IL-13, including CD4 T cells, Th2 cells, NKT cells, mast cells, basophils, and eosinophils, although considerable research emphasis has been placed on understanding the molecular basis of *Il4* gene regulation in CD4 T cells. However, the mechanisms of Th2 cytokine gene regulation among the distinct lineages are unclear. Our reporter transgenic mice and deletion mutant mice of cis-acting elements on Il4 and Il13 locus would be beneficial tools for answering the question of whether Th2 cytokine genes are distinctively regulated in different lineages of cells. Our GFP reporter transgenic system indicated that distinct enhancer elements regulated the transcription of the *Il4* gene in distinct lineages of cells, and therefore this system would be a useful tool for visualizing the behavior of Th2 cells, memory T cells, NKT cells, mast cells, and basophils in various inflammatory sites and lymph nodes.

We have demonstrated that the SOCS family of proteins is implicated in many aspects of acquired immunity by controling the balance between Th1 and Th2 cells. We recently demonstrate that modification of the Th1 response by SOCS5 expression in T cells augments innate immunity during septic peritonitis induced by cecal ligation and puncture. In vitro bactericidal activities of macrophages and neutrophils were markedly augmented in SOCS5 transgenic mice. These findings strengthen the evidences that intervention of SOCS5 expression in T cells affects innate immunity and provide us new insight to investigate crosstalk between innate and acquired immunity.

Recent Publications

Tanigaki, K., Tsuji, M., Han, H., Yamamoto, N., Tsukada, J., Inoue, H., **Kubo, M.**, Honjo, T.: Regulation of αβ/γδ T cell lineage commitment and peripheral T cell response by Notch/RBP-J signaling. ***Immunity*** 20, 611-622, 2004

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Mast cells evoke immediate-type allergic reaction and chronic inflammation through the release of a variety of mediators and cytokines upon allergen challenge. In addition, mast cells were recently found to be involved in innate immunity, angiogenesis or tissue remodeling as a consequence of chronic inflammation. Mast cells are relatively indifferent to glucocorticoid treatment, the first-line therapy for asthma, whereas T cell activation is sensitive to it. Moreover, currently available mast cell stabilizing anti-allergic drugs such as cromolyn are less effective against human mast cells than against rodent mast cells. Therefore, targeting mast cell activation is an important strategy for the development of drugs to treat intractable allergic diseases. Yet, if we want to avoid the suppression of mast cell function, we must try as much as possible to understand these cells in a most comprehensive fashion.

This laboratory has considerable experience in culturing human mast cells. The immediate goals of our laboratory are (1) to analyze the genome-wide expression profiles of various types of human mast cells (organ origin, resting vs. activated, mast cells vs. other leukocytes and human vs. mouse, etc.) by standard oligonucleotide microarrays; (2) to identify and characterize important genes for mast cell activation; and (3) to elucidate yet unknown mast cell function.

Amphiregulin plays a crucial role in steroid-resistant asthma pathogenesis

Inhaled glucocorticoid (steroid) is widely recognized as the first-line therapy for bronchial asthma. Thus, there is no need to develop new drugs that function similarly to the inhaled steroid. What we need is to focus on pathological issues that cannot be resolved with state-of-the-art therapies such as inhaled steroid. Only 16 genes were found from approximately 23,000 genes when they were selected on the basis of the following conditions: (1) they were upregulated by IgE-mediated stimulation, (2) they were not suppressed by glucocorticoid pretreatment, and (3) they were preferentially expressed by mast cells compared with other cell types including 11 different leukocyte types and fibroblasts. Amphiregulin was further selected in this study since it was recently found to be involved in tissue remodeling. Then, we found through histological sectioning that amphiregulin was expressed almost exclusively on mast cells from 40 asthmatic subjects but not on those from six healthy control subjects (Figure). Furthermore, amphiregulin increased the expression levels of mucin genes in airway epithelial cells, which are related to airway tissue remodeling. Thus, after exposure to allergens, human mast cells may induce sputum overproduction via the release of amphiregulin, suggesting that amphiregulin is a new therapeutic target to treat overproduction of sputum in asthma. We did not use a mouse model because amphiregulin was weakly expressed in mouse mast cells.

Identification of mast cell subtype-specific genes

We also identified the genes selectively expressed by mast cells, eosinophils and basophils that are crucial to allergic reactions and inflammation. These mast cell-, basophil- and/or eosinophil-specific genes could

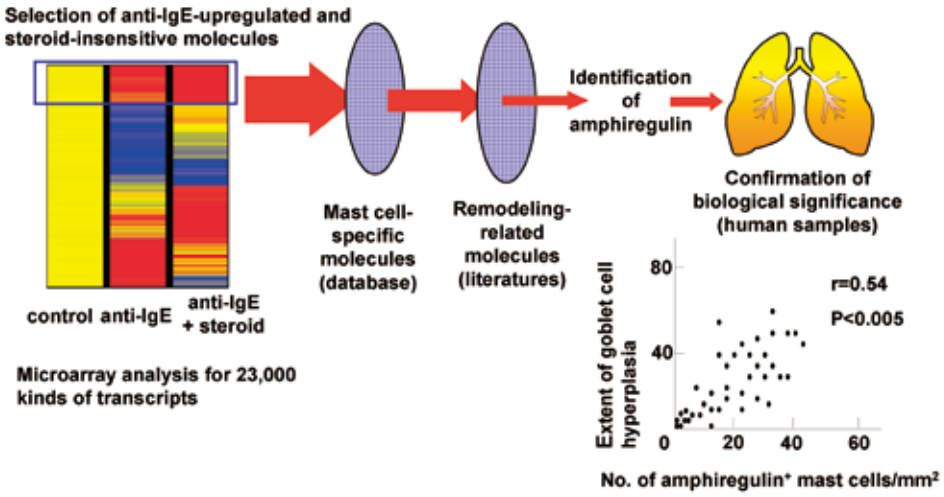

Figure: Identification of Molecule Responsible for Airway Remodeling (Typical Research Strategy of Allergy Transcriptome Unit)
From approximately 23,000 genes, we selected 16 genes that were upregulated by IgE-mediated stimulation, insensitive to glucocorticoid pretreatment, and preferentially expressed by mast cells compared with other cell types. Amphiregulin was further selected since it is involved in tissue remodeling. Then, we found through histological sectioning that amphiregulin was exclusively expressed on mast cells from asthmatic patients.

be potential therapeutic targets for allergic diseases. However, the functions of mast cells including their drug sensitivity somewhat differ among mast cell subtypes depending on the tissue where mast cells dwell. This year, we identified lung-type mast-cell-specific genes and skin-type mast-cell-specific genes (2006, in press). The gene for chymase, one of the mast-cell-unique granular proteases *(CMA1)*, and some related genes were markedly upregulated in skin-type mast cells and down-regulated in lung-derived mast cells. Among those *CMA1*-related genes, *HEY1*, a transcription factor, was the most upregulated in skin-type mast cells and its expression pattern was most closely related to the *CMA1* gene. C5a is a potent secretagogue for mast cells and granulocytes, and plays an important role in pathogenesis of allergies or other inflammatory diseases. In the present study, C5a receptor expression was upregulated on skin-type mast cells but not on lung-type mast cells, as has been reported. No such non-immunological secretagogues have been found on lung-type mast cells. In the present genomic study, we found that the platelet-activating factor (PAF) receptor was highly expressed on lung mast cells but not on skin mast cells. From this observation, we are now investigating whether PAF can induce lung mast cell activation. Our results are expected to highlight the new role of mast cells in asthma pathogenesis (unpublished data).

Systemic role of human mast cells in asthma pathogenesis

We will investigate whether amphiregulin could serve as a diagnostic marker in childhood asthma to predict the progress of airway tissue remodeling. So far, we have found that amphiregulin levels are markedly increased in sputum from asthmatic children during exacerbation. After 10 days, however, the amphiregulin levels returned to normal. However, we also realized that the levels of amphiregulin decreased slowly in some patients. As our future plan, we will investigate patient profiles prospectively according the levels of amphiregulin during asthma attacks. We have also found that human mast cells produce abundant levels of thymic stromal lymphopoietin (TSLP) in response to IgE-dependent stimulation, suggesting that mast cells may play a crucial role in producing Th2 memory cells when they are antigen-presented by dendritic cells. In this regard, we will further examine the pathological role of various mast-cell-specific molecules that we have identified with microarrays using patient's samples.

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Our group deals with tumor immunology, focusing on the link between innate and adaptive immunities through the interaction between dendritic cells (DCs) and NKT cells. In addition to the direct anti-tumor response of activated NKT cells, we found an adjuvant effect of α-galactosylceramide (α-GalCer) in mice upon intravenous administration of antigen plus α-GalCer. We are studying the processes of activation of adaptive immunity, i.e., the mechanism of antitumor T cell immunity mediated by augmented innate immunity, and have conducted investigations of adequate tumor antigen delivery systems. Our goal is to develop novel therapies for cancers employing a strategy to establish adaptive antitumor immunity by augmented innate immunity. Since it is often difficult to evaluate NKT cell function in patients with malignancies due to the reduced numbers of NKT cells, we have attempted to develop an approach to evaluate the functions of NKT cells and antigen presenting cells (APCs) in cancer patients to understand the pathologic status of cancer diseases.

Study of the relationship between augmentation of NKT cells and induction of adaptive immunity using a new ligand, α-C-GalCer

α-GalCer is a prototype compound used for studies of the presentation of glycolipids on CD1d molecules to NKT lymphocytes. A single intravenous dose of glycolipid triggers the production of several cytokines over the course of one day, the short-lived activation of NKT and NK cells, and a prolonged adaptive T cell immune response if certain antigens are given together with α-GalCer. Recently, CD1d-binding glycolipid analogs including sphingolipids and sulfatide variants have been synthesized. These molecules induce the response of NKT cells with the secretion of IFN-γ and IL-4 in human and mouse. One of CD1d-binding glycolipid analogs, α-C-GalCer, is known to be much more active than α-GalCer in inducing resistance to coadministered tumor cells and malaria parasites. In this study, we determined if the more active glycolipid α-C-GalCer is a more potent inducer of DC function in various forms of innate and adaptive resistance.

We found that a new analogue, α-C-GalCer, potently induces these innate and adaptive immune responses in mice. α-C-GalCer triggers IL-12 and IFN-γ production more effectively than α-GalCer, whereas it minimally elicits IL-4 and TNF-α release into the serum. α-C-GalCer also better mobilizes NKT and NK cells to resist B16 melanoma than α-GalCer. In addition, we found that DCs loaded with α-C-GalCer induce NKT cell response in vivo with greater magnitude and longer kinetics than DCs loaded with α-GalCer. Such an effect could be attributed to the stable binding of α-C-GalCer to DCs compared with α-GalCer. When glycolipid is targeted to DCs in spleen together with antigens in dying cells, such as irradiated tumor cells, α-C-CalCer is active as an adjuvant for T-cell immunity at as low dose as 20 ng/mouse, where it is also able to upregulate the required CD40L costimulatory molecule on NKT cells. Therefore, α-C-GalCer represents a glycolipid that binds more stably to DCs and acts as a more effective link between innate and adaptive immunities *in vivo*. (*Proc Natl Acad Sci USA* in press)

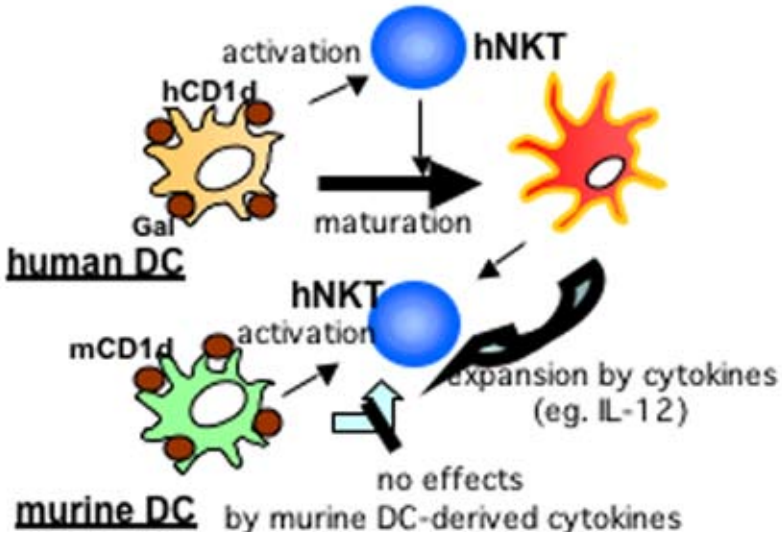

Figure: Depicting the interaction between human or murine DCs and human NKT cells.

When human NKT cells (hNKT) cells are cultured with a-GalCer-loaded autologous DCs, hNKT cells mature human DCs. Subsequently, hNKT cells are synergistically activated with human IL-12 and costimulatory molecules on DCs as well as a-GalCer. In contrast, hNKT cells can be activated by a-GalCer-loaded murine DCs in a ligand specific manner. By using the phenomena, we established

Evaluation of function of human invariant NKT cells from cancer patients

NKT cells play a role in the immunological regulation of certain diseases, and their frequency and/or function may be related to disease prognosis. However, it is often difficult to evaluate NKT cell function in patients with malignancies due to the reduced numbers of NKT cells as well as the dysfunction of APCs used as stimulators. To detect NKT cell function independent of human APCs, we focused on a homogenous population of murine DCs. NKT cells recognize glycolipids presented by the nonpolymorphic MHC class I-like molecule CD1d, which is related to β2-microglobulin-associated transmembrane proteins. The high sequence homology between human and murine CD1d (60.4% for the α1 domain and 62.4% for the α2 domain) allows human Vα24$^+$ NKT cells to respond to murine CD1d. Our goal is to find an approach to evaluate the function of human NKT cells by comparing human and murine DCs.

We found that NKT cell function could not be evaluated by conventional ELISPOT assays, confirming the impaired function of APCs in patients with chronic myelogenous leukemia (CML) in the chronic phase. To overcome this problem, we have established a sensitive assay using murine DCs to evaluate the function of small numbers of human NKT (hNKT) cells in the absence of autologous APCs. We found that CML patients in the chronic phase and under medication with imatinib showed complete cytogenetic response (CCR) and had hNKT cells capable of producing IFN-γ. In contrast, hNKT cells appeared to be nonfunctional in patients partially responsive (PR) to imatinib treatment, who did not produce IFN-γ upon stimulation. The results indicate the possibility of developing effective immunotherapy through the generation of functionally potent hNKT cells in *ex vivo* by α-GalCer-pulsed DCs. (*Journal of Immunology* in press)

When hNKT cells are cultured with α-GalCer-loaded autologous DCs, hNKT cells mature human DCs. Subsequently, hNKT cells are synergistically activated with human IL-12 and costimulatory molecules on DCs as well as α-GalCer. In contrast, hNKT cells can be activated by α-GalCer-loaded murine DCs in a ligand-specific manner. Using this phenomenon, we established an assay system for the detection of low NKT cells in an autologous APC independent manner.

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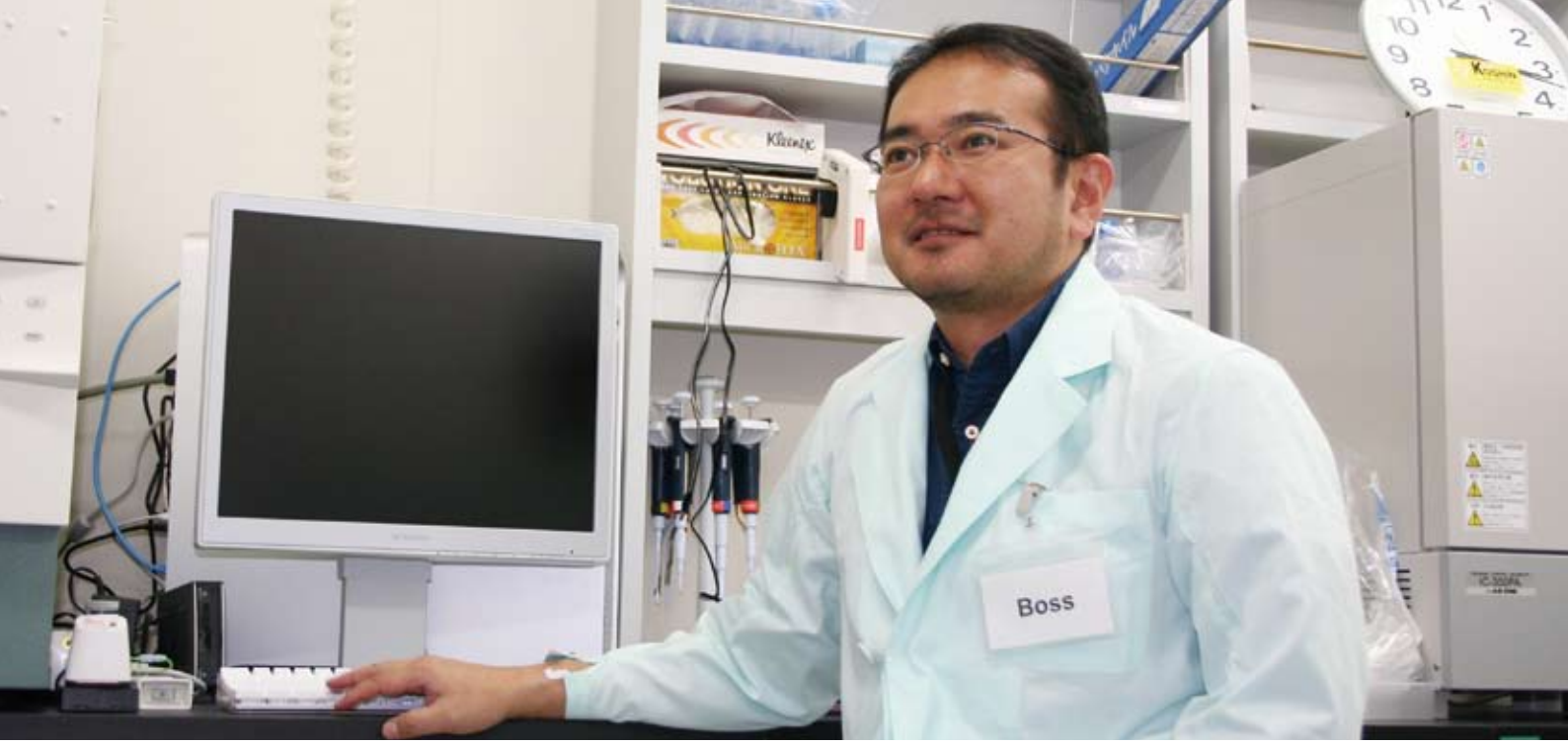

Clinical Allergy Research Unit

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Our aim of the Clinical Allergy Unit is to clarify the immunological and molecular mechanisms underlying the prevention, or suppression, of allergic responses and is to establish innovative technologies for the manufacturing of allergy vaccines against Japanese cedar pollinosis. Our focus is therefore on he modulation of IgE production, a potentially powerful, yet not well explored approach to mitigate allergic diseases. Further, within RCAI, part of our mission is to promote and initiate translational and clinical research in the field of allergy and to develop a network of collaborations with clinical research centers and industry. Recently, we have successfully generated liposome vaccine that effectively induces immunoregulatory cells and suppresses both primary and secondary IgE antibody responses.

Background for the development of liposome vaccine

IIt is generally accepted that polarization of immune response towards the Th2 type is the most important prerequisite for IgE antibody formation and the development of atopic disorders. In this context, it is important to know which regulatory elements can down-modulate the dominant Th2 response. In an experimental model of anterior-chamber-associated immune deviation (ACAID), NKT cells were shown to be involved in the development of systemic immune tolerance. In particular, NKT cell-derived IL-10 appears to play a critical role in the development of antigen-specific regulatory T (Treg) cells and immune tolerance. In addition, splenic dendritic cells (DCs) acquire the properties of regulatory DCs in an IL-10-dependent fashion after repeated injections of α-galactosylceramide (α-GalCer). These findings collectively suggest the possibility that stimulation of NKT cells with α-GalCer at the time of (or prior to) antigen presentation to naïve T cells may facilitate the differentiation of Treg, rather than Th cells specific for the antigen.

Mechanisms of IgE suppression mediated by liposome allergy vaccine

In view of the findings in the ACAID system, we speculated that stimulation of NKT cells in the vicinity of antigen presentation to T cells might facilitate the generation of regulatory DCs, and this was followed by the development of antigen-specific Treg cells in vivo. We therefore prepared a liposome that contained α-GalCer in the lipid bilayer and encapsulated OVA protein. Since the formulation of an antigen in a liposome enhances antigen-uptake by antigen presenting cells (APCs), we expected that α-GalCer and OVA in the liposome would be efficiently presented by APCs to NKT cells and antigen-specific T cells, respectively.

α-GalCer-OVA-liposome was injected into groups of mice prior to immunization with OVA. Antigen specific primary IgE antibody responses were completely suppressed by pretreatment with α-GalCer-OVA-liposome. The suppressive mechanism is due to the generation of IL-10-producing regulatory DCs after coculture with liposome-activated NKT cells. We further confirmed that the regulatory DCs was increased by treatment with α-GalCer-liposome. Such an effect was hardly detected in NKT cell-deficient mice, suggesting that NKT cells play a critical role in the generation

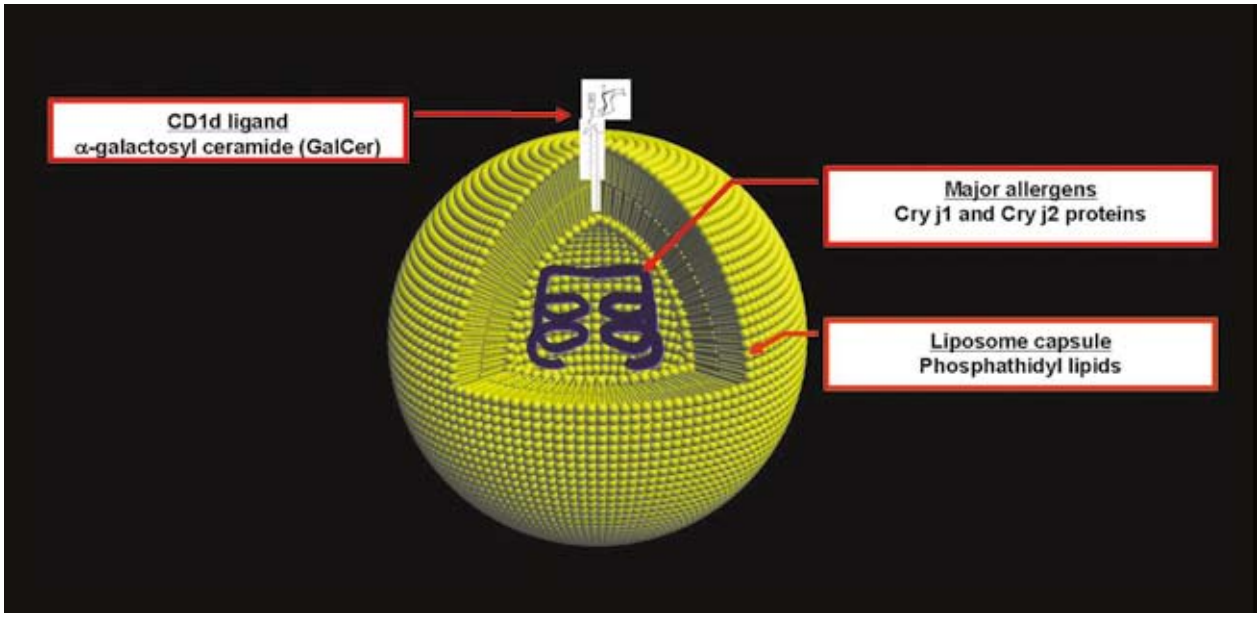

Figure: αGalCer-Cryj1/2-liposome vaccine against Japanese cedar pollinosis

of regulatory DCs. Furthermore, the regulatory DCs generated by the interaction with liposome-activated NKT cells induced regulatory CD4 T cells suppressing IgE responses in an antigen specific fashion.

Future plan

An appropriate animal model for preclinical examination is required to develop allergen-specific immunotherapy against Japanese cedar pollinosis. Since no allergic clinical signs are developed in mice, other animal models to test the efficacy of liposome allergy vaccine developed in our laboratory. Because dogs are known to show clinical signs of allergy to Japanese cedar pollen, probably owing to type I hypersensitivity to Japanese cedar pollen antigen, we expect that this animal model could be useful for preclinical studies of the efficacy and safety of α-GalCer-liposome manipulation. In fact, experimental sensitization to Japanese cedar pollen was induced in beagles. In addition, it has been reported that a dog model sensitized to Japanese cedar pollen antigen can be used to investigate the in vivo efficacy of immunotherapy.

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Japanese cedar pollinosis has become a common allergic disease in Japan. As an allergen-specific treatment, allergen immunotherapy is an attractive therapeutic tool to shift immune response against allergens from Th2 to Th1 skewed response. However, current approach has not widely been used in clinics because of its low therapeutic efficacy and the risk of side effects. Therefore, there has been an emerging need to develop an allergen immunotherapy with a high efficacy and low side effects. It has been observed that CpG oligodeoxynucleotides stimulate the Th1 skewed immune response. Accordingly, the conjugation of CpG with allergens induces Th1 responses in an allergen-specific manner, resulting in inhibition of IgE antibody response to allergens. In order to develop an allergen-specific immunotherapy against Japanese cedar pollinosis, CpG conjugation with the major allergens of Japanese cedar pollen can be the most powerful candidate. Our final research goal is to establish CpG-conjugated Cry j 1 immunotherapy as a novel treatment for Japanese cedar pollinosis.

Major allergens of Japanese cedar pollinosis as a target of immunotherapy

In order to develop a widely used immunotherapy, it is necessary to investigate the major allergens of Japanese cedar pollen. We have previously identified Cry j 1 and Cry j 2 as the main allergens in Japanese cedar pollinosis; however, it has been speculated that a third major allergen of Japanese cedar pollen exists. In analogy to the recently identified mountain cedar allergen Jun a 3, we have characterized the third major allergen of Japanese cedar pollen, Cry j 3, by two-dimensional western blotting using serum from patients with Japanese cedar pollinosis. It was found that approximately 27% of patients suffering from Japanese cedar pollinosis had IgE specific to Cry j 3, as determined by ELISA. This may be useful to cover the patients with Japanese cedar pollen, who can be treated with allergen-specific immunotherapy. Because we are trying to develop a monoclonal antibody against Cry j 3, in the future, the assay system for Cry j 3 as well as for Cry j 1 and Cry j 2 may be useful in identifying patients who could respond effectively to a novel immunotherapy.

CpG adjuvant vaccine and its clinical efficacy

Immunostimulatory sequences of oligodeoxynucleotides, CpG motif in bacterial DNA, are one of the most attractive tools for developing an immunotherapy against allergic diseases because it can stimulate Th1-skewed responses to allergens. In order to utilize such an effective nature of the CpG in suppressing allergy, our goal is to develop a vaccine for Japanese cedar pollinosis using animal models of Japanese cedar pollinosis which manifest clinical signs upon exposure to Japanese cedar pollen. We first used CpG-conjugated Cry j 1 as a novel immunotherapy for Japanese cedar pollinosis. In mice sensitized to Cry j 1 with alum, CpG-Cry j 1 suppressed the increase in IgE specific to Cry j 1 even after boosting with Cry j 1, but induced a high serum concentration of IgG2a specific to Cry j 1. The serum concentration balance of IgE and IgG2a specific to Cry j 1 was strengthened by Cry j 1 boosting. It was found that serum from

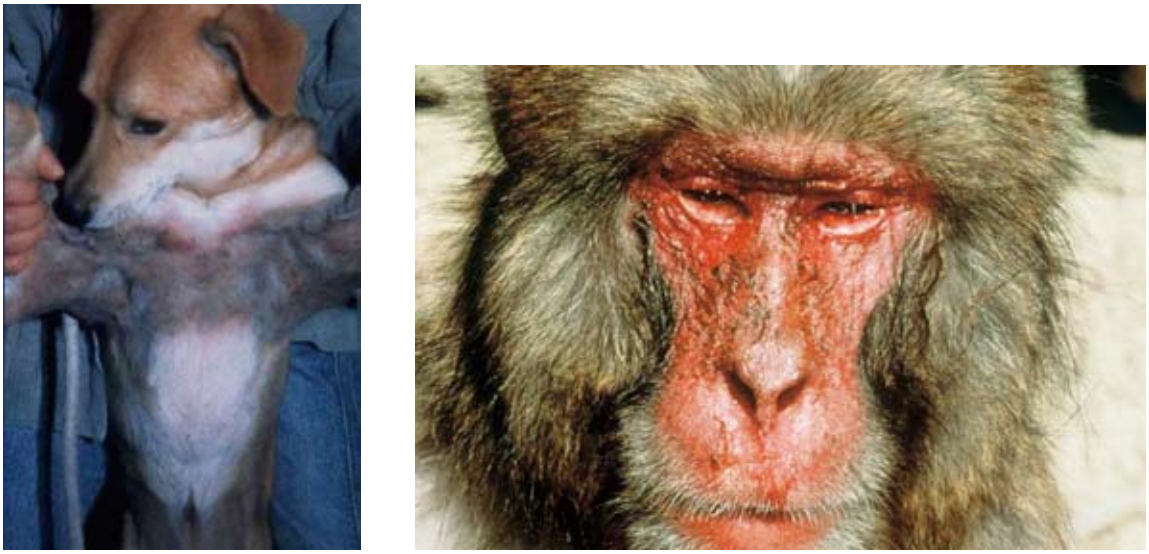

Figure: Atopic dermatitis in dogs (*left*) and cedar pollinosis in monkeys (*right*)

mice treated with CpG-Cry j 1 suppressed Cry j 1-specific degranulation of bone marrow-derived cultured mouse mast cells, compared with the degranulation using serum from mice treated with mutant CpG-Cry j 1. These indicated that CpG-Cry j 1 might suppress the increase in IgE specific to Cry j 1 after exposure to Japanese cedar pollen, resulting in the suppression of type I hypersensitivity to Cry j 1.

In order to establish the clinical efficacy of CpG-Cry j 1, an appropriate animal model must be developed. The animals showing clinical signs of allergy are monkeys and dogs. As a first step towards developing the animal model suitable for investigating allergic reaction to Japanese cedar pollen, dogs were selected and examined as a possible animal model. A dog colony has been found to be spontaneously increasing serum IgE concentration against food antigens and showing clinical manifestations of allergy such as pruritic skin and diarrhea after food intake. It was also found that these dogs were sensitized to house dust mite (HDM) antigen when they were exposed to the antigen via the skin with increase of serum IgE specific to HDM. The dogs also show skin lesions at the site where the antigen is exposed. In addition, purified B cells from PBMCs in a healthy dog proliferated against *in vitro* stimulation using CpG-ODN 1018 in a manner similar to human B cells. We are planning to analyze the allergic reaction to Cry j 1 in the high-IgE dog colony, which will be used for a future research on the clinical efficacy of CpG-ODN conjugated with Cry j 1.

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One of the aims of our research group is to function as a gateway to genomics for immunologists. Consequently, our research activities are divided into: (1) Central support activities, (2) Strategic and collaborative research activities, and (3) Exploratory research activities, according to the research frameworks and aims of our staff. Although the research goals for (1) and (2) vary widely depending on the goals of the projects with which we are involved, our mission is always to provide the best expertise in transcriptomics, proteomics, and bioinformatics for immunologists. Our research activities in terms of (3) are focused on the development of new technologies and approaches in the field of Immunogenomics. While the outcomes of this research might not always be directly relevant to immunology at the proof-of-principle stage, the ultimate goal is to apply these newly developed technologies to address important immunological issues in collaboration with other members of RCAI.

As described above, we have made a considerable effort in terms of central support activities and strategic/collaborative research activities; however, as the outcomes of these research activities are largely descriptive and/or wide-ranging, only the main topics that are targeted in our exploratory research are described below.

Construction of an Immunogenomics Reference Database

This project involves collaborative research between different research groups within RCAI. The goal of the project is to construct an informational platform that enables immunologists to fully exploit "Omics" data. To generate the Immunogenomics reference database, we have undertaken many mRNA profiling experiments (>100 experiments and growing) and 2D-gel-based proteomic analyses of various kinds of immune cells at "reference" states. Quantitative proteome data based on 2D-gel electrophoresis were obtained using a method developed within our group (Kimura et al., *Proteomics*, in press). All the transcriptomic and proteomic data obtained in this way have been compiled in the database and are thereby easily retrieved on demand. We have also developed several bioinformatics microarray analysis tools that effectively mine the data of the RCAI database (manuscript in preparation). Although there are several reports that describe the collection of transcriptome data related to immune cells, the unsurpassed features of our database, named RefDIC (Reference genomic Database for Immune Cells), include (1) the capability to cross-reference from transcriptomic data to proteomic data and vice versa, and (2) functions for data sharing. The latter feature is particularly important for Immunogenomics because data sharing in the research community is considered to play a key role in the "Omics" approach. The database will be opened to the public in mid-late 2006 to increase the profile of RCAI in the research community. The homepage of the RefDIC Internet site is shown in Fig. 1.

Development of Microscopic Platform for Real-Time Monitoring of Biomolecular Interactions

This study is being conducted in collaboration with Dr. Harada and her colleagues at the Tokyo Metropolitan Institute of Medical Science. The motivation behind the project is derived from a number of observations that have emerged from the RCAI: (1) the rate-limiting step of transcriptomic and proteomic analyses is usually

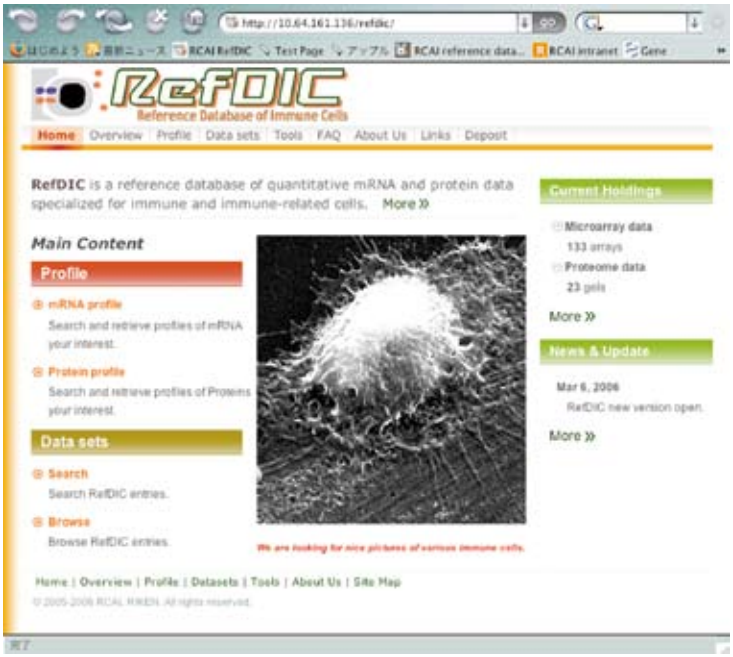

Figure: Homepage of the RefDIC Internet site.

the preparation of sufficient numbers of immune cells for the analyses; and (2) the degree of homogeneity of immune cells to be analyzed varies widely depending on experimental conditions. These two problems are general and serious concerns in functional genomics. A logical solution to these problems is to dramatically miniaturize the analysis platform to enable the monitoring of biomolecular interactions even within an extract from a single cell. A further problem to be addressed in this study is how to monitor unstable interactions, i.e., interactions with a high dissociation rate constant. If we are able to solve these problems, the resultant analysis platforms will be used not only for mRNA/protein profiling but also for real-time monitoring of biomolecular interactions in general and serve as the technological basis of single-cell biology. To this end, we have demonstrated that a newly developed microscopic platform enables us to monitor binding reactions in picolitter or less in real time by combining ultrahigh-density microarraying technology and total internal reflection fluorescence microscopy. As a demonstration of the power of this platform, we demonstrated that real-time monitoring of the interaction of NFκB p50 and DNA enabled us to detect even unstable interactions. Although this platform is only at the proof-of-principle stage, the results obtained to date have encouraged us to develop microfluidics-based transcriptome/proteome analysis platforms as an extension of this platform for immunology.

In the field of genomics, new technologies are continuously emerging. Thus, it is necessary for our research group to keep abreast of these new technologies according to the needs of immunologists and make the technologies accessible to immunologists within RCAI. We are also expected to pioneer new approaches in transcriptome and proteome analysis to address important immunological problems. To this end, we have made efforts to develop new applications of genomic technologies in the field of immunology. For example, we are currently seeking to open the way to comprehensively analyzing post-transcriptional events in immune cells. This research is expected to fill the gap that exists between transcriptomic and proteomic data. In addition, our reference Immunogenomics database will be extended to include additional data that enable us to consider immune systems on a molecular basis. While the development of new analysis platforms for single-cell biochemistry is an extremely challenging and keenly competitive field of research, we have pursued this avenue because biochemical analyses at the single-cell level will inevitably provide us with novel and indispensable information for a comprehensive understanding of the immune system. Although our exploratory research is apparently divergent in scope, the various outcomes are expected to collectively serve as the basis of Immunogenomics.

To successfully achieve the aims of our research group, it is important to maintain a balance between central support activities, strategic/collaborative research activity, and exploratory research activity. Thus, research activities must be carefully managed to maintain a satisfactory balance of these multipronged activities in accordance with the demands of immunologists within RCAI. Interactive collaborations with other research groups within RCAI have provided us with many sound lessons in this respect and will surely contribute to establishing the research field of Immunogenomics.

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The main activity of our team is focused on screening a large ENU mutant panel and is undertaken in cooperation with the RIKEN Genomic Sciences Center. An important goal of this project for RCAI is the development of novel mouse models for various immunological disorders, notably, allergic and autoimmune disorders. Since the ENU mutagenesis introduces approximately 3,000 point mutations on a genome, we can expect 100 coding region mutations in one pedigree. In parallel, we are screening those mutations under environmental bias, using a variety of approaches, including the immunization of mutants with various allergic antigens and adjuvants to identify the modifier genes.

ENU Mutant Panel Study

In order to identify the genetic basis for immune disorders, we are screening a pool of mutant mice generated by random chemical mutagenesis through ethylnitrosourea (ENU) administration. ENU induces a random single base pair change in genomic DNA at approximately 3,000 sites throughout the whole genome and approximately 100 sites in the coding regions of proteins per first-generation (G1) mutant mouse. It is expected that screening approximately 300 G1 pedigrees will be sufficient to establish a mutant mouse library with mutations in all gene products. The following prescription of ENU for C57BL/6 strain, dominant mutant analysis was started in August 2003 and recessive mutant analysis in December 2003 in the GSC research building as a pilot study. In this process, we screened 102 dominant inheritances from 16 pedigrees and around 400 recessive inheritances from 58 mouse pedigrees. After moving into the RCAI research building, we screened, within the first year, approximately 8,000 recessive inheritances from 60 mouse pedigrees. For basic phenotypic screening, we examined the levels of immunoglobulins, cytokines, and auto-antibodies, as well as PBC surface marker expression, in peripheral blood. Using tissue sections from animals at 16 weeks of age, we clarified the mutant phenotype by pathological, cytological and molecular biological examinations of affected tissues, lymphoid organs and blood cells. Collaborating teams examined the dissected samples by their own methods. All screening data are updated on the internal database system in the RCAI web server, and we define the mutant phenotype at meetings with collaborating teams held every month. All the attendants discuss the selection of candidate mutant lines.

A phenotype identified as a mutant candidate line is reexamined in a later development stage, and if there is the same phenotype in both sexes, we start mating them for phenotype inheritance tests. If only single-gender mutant animals are obtained, the phenotype is disregarded but is noted as an interesting one. The responsible mutation loci are mapped by backcrossing mutant individuals with the C3H/HeJ strain for gene detection by SNP analysis. After approximate mapping, candidate gene sequences are checked against public databases by means of the PosMed research system established by RIKEN GSC.

Allergic Disease Modifying Mutant Mouse Model

By ENU recessive mutant screening, we identified and established a mutant reminiscent of typical hu-

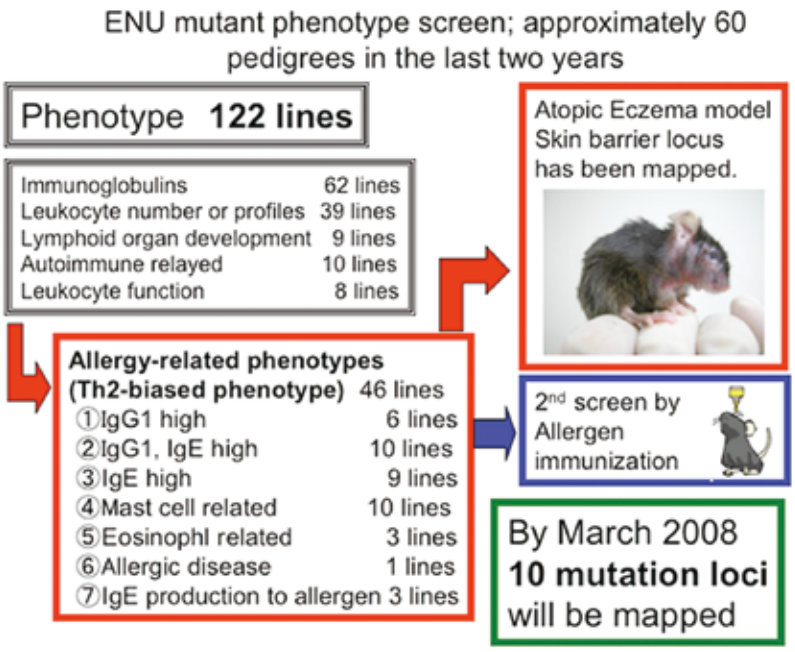


Figure: Summary of two years of ENU mutant screening. In total, approximately 6,000 mutant mice from 50 genomes have been screened and 120 phenotypes have been identified as mutant lines. The mouse shown presents a mutant phenotype reminiscent of human atopic dermatitis.

man allergic disease. The serum IgG1 or IgE level of these mice gradually increased during growth. The ear skin became thicker and red, and the mice started to scratch the ear skin or face continuously. Pathologically, the epidermal layer was hypertrophic and many lymphocytes were found in the dermis of the lesion. Mast cell numbers increased in the same region, and many of them bcame enlarged and emitted metachromatic particles. All these symptoms or findings seemed compatible with the criteria for human atopic dermatitis (AD). Therefore, we have started to study this mutant line intensively. The disease grade or onset timing of atopic dermatitis in the mutant mice varies within a litter, and we could distinguish them into several groups. Since ENU induces multiple mutations in a pedigree, it is plausible that a combination of a few mutations affects the clinical symptoms. In order to identify every responsible gene for those phenotypes, we are collaborating with the Phenome Informatic Team and Mouse Mutation Resource Exploration Team in GSC, RIKEN. These teams can quickly detect the responsible region roughly to 5cM from 30 mutant DNA. Subsequently, we can focus on the candidate genes by means of database analysis using our own software program.

By ENU recessive mutant screening, we have identified and established more than 120 mutant lines of immune or blood disease models. In line with our expectation, more than 40 lines showed phenotypes related to allergic diseases. For example, more than ten lines exhibited elevated serum IgE levels, and some of them showed aberrant responses against the induction of artificial allergic disease. We still require more than a year to confirm the phenotype inheritance of all these mutants. We are now backcrossing some of them to the C3H/HeJ strain in order to map the responsible loci. As of August 2006, we have mapped three independent mutant-responsible loci to distinct regions, and two of them have been clarified to be point mutations of known genes. The third mutant we are focusing on is the atopic dermatitis model described above. After mapping to 10cM in a chromosome, we have chosen a candidate gene using the PosMed system and are preparing to sequence it. We plan to map ten more responsible loci for allergic disease model mutation within a year.

Recent Publications

Yuhki M, Yamada M, Kawano M, Iwasato T, Itohara S, Yoshida H, Ogawa M, Mishina Y. BMP Receptor IA Signaling Is Necessary for Hair Follicle Cycling and Hair Shaft Differentiation in Mice. Development 131, 1825-1833 (2004)

Weston JA, Yoshida H, Robinson V, Nishikawa S, Fraser ST, Nishikawa S. Neural Crest and the Origin of Ectomesenchyme: Neural Fold Heterogeneity Suggests an Alternative Hypothesis. Dev. Dyn. 229, 118-130 (2004)

Hirano M, Yamamoto A, Yoshimura N, Tokunaga T, Motohashi T, Ishizaki K, Yoshida H, Okazaki K, Yamazaki H, Hayashi S, Kunisada T. Generation of Structures Formed by Lens and Retinal Cells Differentiating from Embryonic Stem Cells. Dev. Dyn. 228, 664-671 (2003)

Iiyama R, Kanai T, Uraushihara K, Ishikura T, Makita S, Totsuka T, Yamazaki M, Nakamura T, Miyata T, Yoshida H, Takeuchi O, Hoshino K, Takeda K, Ishikawa H, Akira S, Watanabe M. Normal Development of the Gut-Associated Lymphoid Tissue Except Peyer's Patch in MyD88-Deficient Mice. Scand. J. Immunol. 58, 620-627 (2003)

Nishikawa S-I, Honda K, Vieira P, Yoshida H. Organogenesis of Peripheral Lymphoid Organs. Immunological Reviews 195, 72-80 (2003)

Central Facilities

In April, 2006, RCAI's Advisory Council Board reported that the services provided by the Center's "central facilities" had been both timely and of the highest quality. The central facilities are the resource and competency pools created by RCAI to provide all researchers in the Center with access to the most advanced equipment and technology. The Advisory Council mentioned that access to these outstanding core facilities had empowered smaller research groups by speeding up the progress of their internationally competitive research projects.

The central facilities provide all needs for immunological, genetic, and animal experiments: (1) support for various immunological and cell-biological techniques, such as confocal microscopy, FACS, cell sorting and production of monoclonal antibodies; (2) advanced research approaches in genomics, proteomics, and bioinformatics, such as DNA sequencing, microarrays, protein arrays and mass spectroscopy; (3) an animal facility capable of breeding as many as 68,000 mice and supporting the generation of transgenic/knock-out/cloned mice and the preparation of frozen embryos for preservation (Table).

Some of the equipment is maintained under collaboration with related companies such as Becton-Dickinson for FACS and Leica for confocal microscopes. In addition, proteomics facility collaborates with Shimadz, and monoclonal antibodies facility collaborates with MBL for technological development.

Most of the central facilities laboratories are managed by related group leaders (Table). Basically, each central facility lab. is divided into subdivisions which are supervised by responsible research scientists or highly experienced technical staff, Dr. Akiko Furuno (Confocal microscopy), Ms. Hanae Fujimoto (FACS), Mr. Shinji Seki (Monoclonal Ab.), Dr. Hiroshi Kitamura (Genome/ microarray analysis), Mr. Atsushi Hijikata (Bioinformatics), Dr. Yayoi Kimura (Two-dimensional gel electrophoresis), Mr. Takanori Hasegawa (Animal facility) and Mr. Yasuaki Murahashi (IT infrastructure).

Table: RCAI central facilities

Facility	Available instruments	Functions
Confocal microscopy Managed by Dr. Osami Kanagawa	Inverted (HiVIS+405)	Multicolor staining (up to 8 colors): colocalization/organellar localization
	Inverted (VIS+UV)	FRET (Fluorescence Resonance Energy Transfer): real-time protein-protein interaction
	Upright (VIS+UV)	FLAP (Fluorescence Recovery after Photobleaching): dynamic movement of molecules among organelles
		Time lapse: molecular movement/ cell-cell interaction
	Inverted (VIS+MP)	Multi-photon (MP): thicker samples (tissue, whole mount staining)
	Intravital (VIS+MP) (Fig.1)	Visualization of immune cells in living mice
	TIRFM	Single Molecule Microscopy
FACS and cell sorting Managed by Dr. Takashi Saito	Vantage Diva (Laser;488nm/Dye/UV,Analysis;max 8 color, Sort;4way/plate, Mode;normal/turbo)	Cell Sorter (Fig. 2)
	Vantage SE (Laser;488nm/He-Ne/UV,Analysis;max 6 color, Sort;2way/plate, Mode;normal/turbo)	
	Vantage SE (Laser;488nm/He-Ne/Kr,Analysis;max 6 color, Sort;2way/plate, Mode;normal/turbo)	
	Vantage SE (Laser;488nm/Dye,Analysis;max 6 color, Sort;2way/plate, Mode;normal/turbo)	
	Aria (Laser;488nm/He-Ne/405nm,Analysis;max 9 color, Sort;4way/plate, Mode;20psi/35psi/75psi)	
	Calibur HTS (Laser; 488nm/633nm, Analysis; max 4-color)(3)	Cell analysis
	Calibur normal (Laser; 488nm/633nm, Analysis; max 4-color)(3)	
	LSR (Laser; 488nm/633nm, Analysis; max 6-color)	
Monoclonal Ab Managed by Dr. Takashi Saito		Production of monoclonal antibody
DNA analysis Managed by Dr. Osamu Ohara	MFX9600 (Toyobo)(2)	Plasmid extraction robot
	Biomek FX (Beckman Coulter)(1)	Laboratory automation workstations
	Biomek 2000 (Beckman Coulter)(1)	
	My cycler (Bio Rad)(1)	Thermal cyclers
	ABI9600 (Applied Biosystems)(2)	
	ABI 3100 (Applied Biosystems)(4)	DNA sequencers
	RISA384 (Shimadzu Biotech)(1)	
Transcriptome analysis Managed by Dr. Osamu Ohara	GeneChip analyzing system (Affymetrix)(1)	Microarray analyzing system (Fig. 3)
	FLA8000 (Fuji Film)(1)	High-resolution array scanner
	iCycler iQ real time PCR detection system (Bio Rad)(4)	Real-time PCR analyzers
	ABI7000 (Applied Biosystems)(1)	
	Genespring workstation (Silicon Genetics)(1)	Data mining software
Proteome analysis Managed by Dr. Osamu Ohara	ProExpress (Perkin Elmer)(1)	High-resolution scanner
	Progenesis workstation (Nonlinear Dynamics)(1)	Proteome data mining software
	Xcise (ProteomeSystems/Shimadzu Biotech)(1)	Integrated gel-excision processor
	AKTAexplorer 10S system (Amersham Biosciences)(1)	Liquid chromatography
	Agilent1100 system (Agilent)(1)	
	Esquire3000plus (Bruker Daltonics)(1)	Mass spectrometry (Fig. 4)
	Axima-CFR (Kratos/Shimadzu Biotech)(1)	
	BIACORE3000 (BIACORE)	SPR (Surface Plasmon Resonance) detection system
Animal facility Managed by Dr. Haruhiko Koseki	Maximum Number of Mice: 69,000	Maintenance of SPF area
	Maximum Number of Cages: 13,800	Production of immunocompromised mouse strains
	13 SPF rooms	Generation of genetically-engineered mice (transgenics and targeted mutagenesis)
	5 isolation rooms	Generation for ENU-treated G3 offsprings for phenotype-driven screening
	Embryonic transfer room (Fig. 5)	Cloning of lymphocytes by nuclear transfer
	Micro injection and ES cell culture equipments	Generation of ES cells from investigator supplied targeting constructs
		Cryopreservation of mouse embryos
Radioisotope facility Managed by Dr. Jiang O-Wang		Control for the safe use of radioisotopes

Translational Research

In the present political/funding climate in Japan and RIKEN it is crucial for RCAI to have the ability to fund translational and clinical research that is taking place outside the center in order to ensure the adequate clinical development of research at RCAI in collaborations with external partners. Models for such collaborations exist and have been reviewed favorably by the Advisory Council. A program has been created to provide incentives for scientists in basic research to expand their research towards disease-oriented studies. Furthermore, RCAI has established research groups inside the center that are responsible for preclinical research in allergy and cell therapy. These groups provide linkages to clinical research facilities.

We currently have two projects: clinical trials of immune cell therapy for cancer patients, and a proof-of-principle study on the development of a liposome vaccine for cedar pollen allergy (pollinosis).

Phase I/IIa Clinical Trials for Lung Cancer Patients using αGalCer/DC Therapy

In collaboration with Chiba University (Prof. Toshinori Nakayama, Prof. Takehiko Fujisawa, and Associate Prof. Shin-ichiro Motohashi), we have carried out phase I/IIa clinical trials on lung cancer patients with intravenous injections of α GalCer-pulsed dendritic dells (DCs). Eleven patients were enrolled in the phase I, and 20 cases are ongoing in the phase IIa studies receiving α GalCer-pulsed DCs. No severe adverse events were observed during the phase I study.

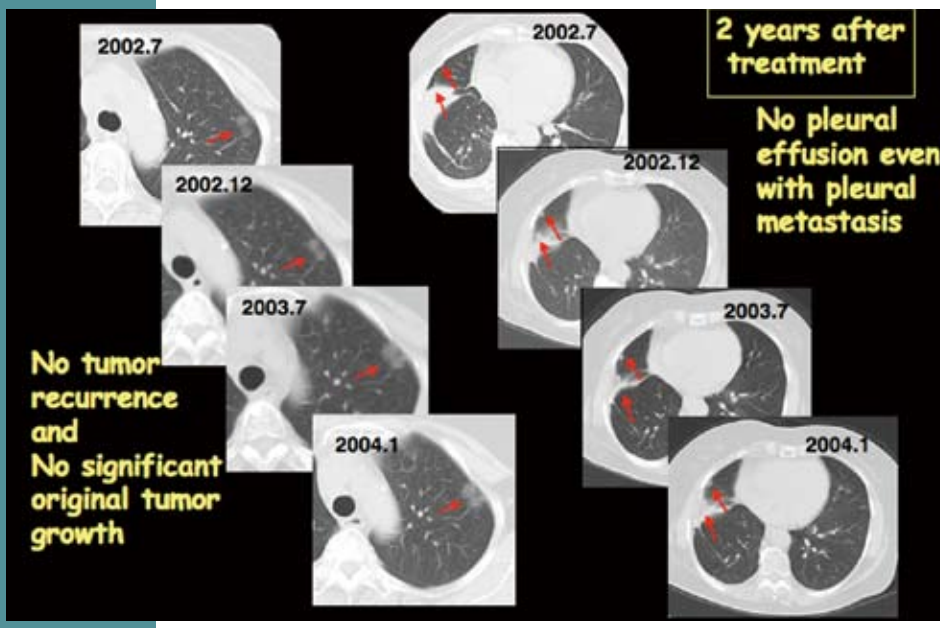


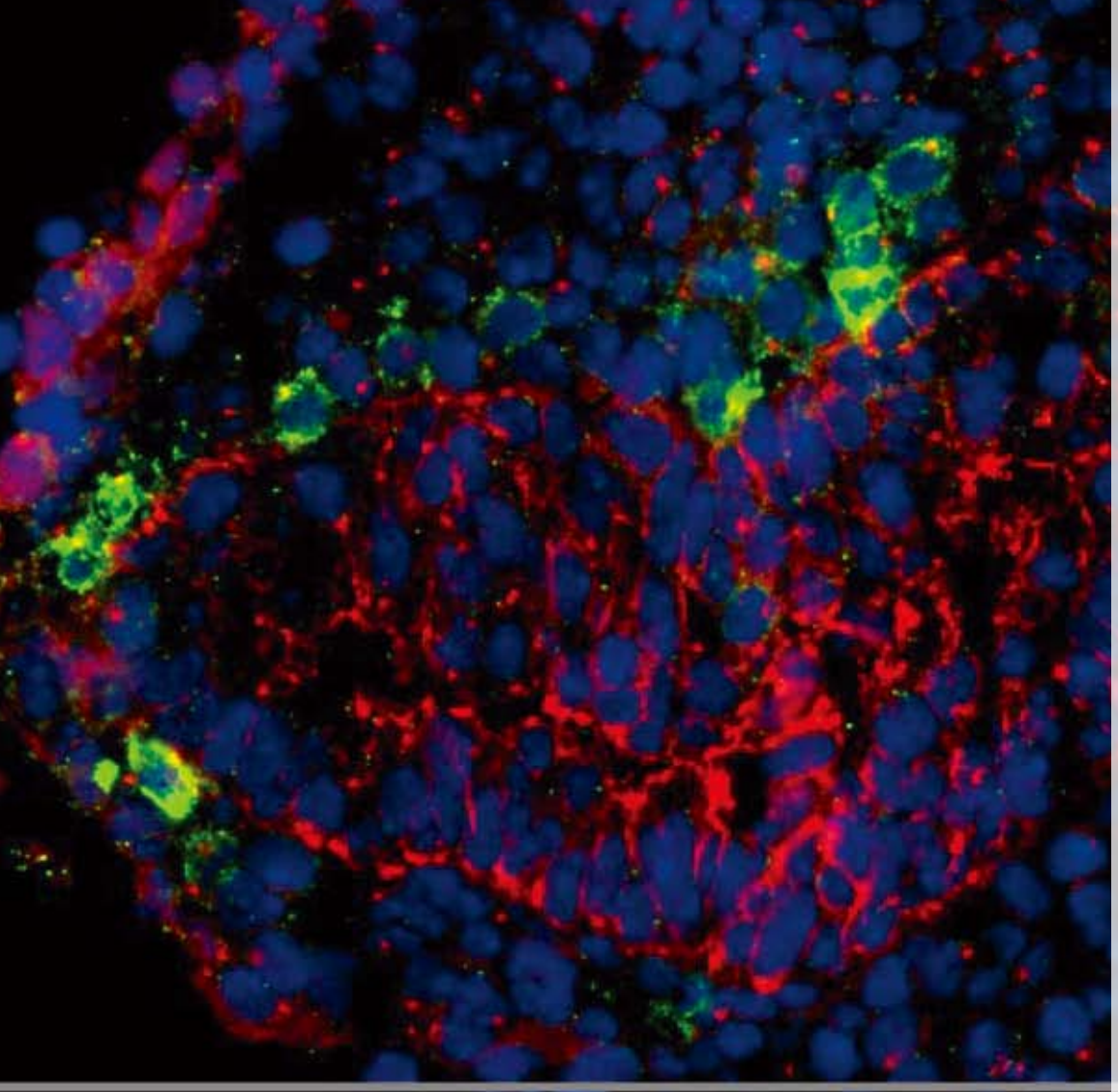
Figure: CT scan of a lung cancer patient

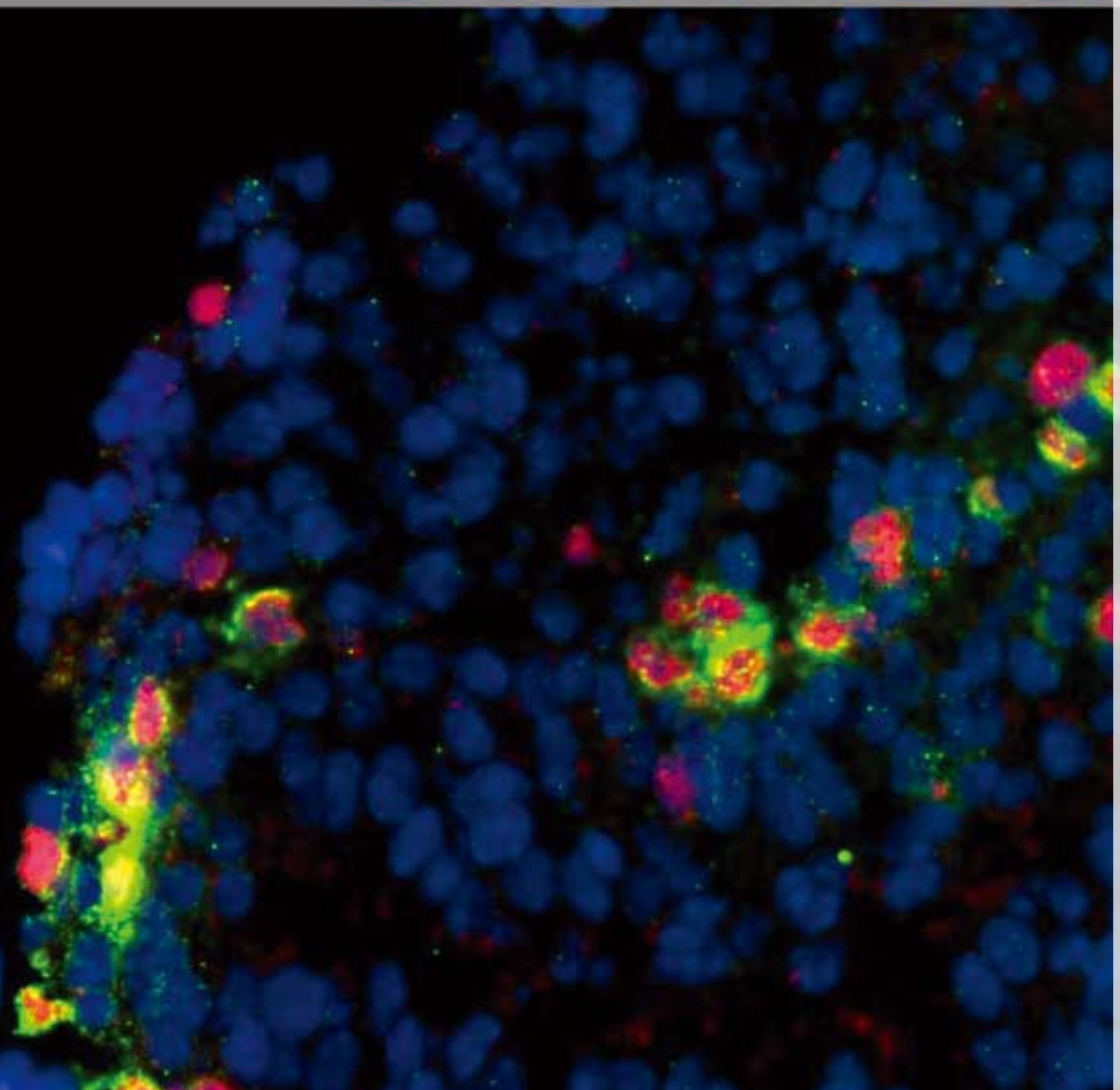
In phase IIa, a dramatic increase in peripheral blood human NKT cells was observed in >50% cases, and significant IFN-γ responses were seen in >90% cases. No patient was found to meet the criteria for either a partial or complete tumor regression, while in several cases the tumor size remained unchanged without pleural effusion for more than 2 years with good QOL. Since we have not completed the phase IIa studies, median survival time cannot be calculated. However, the tentative median survival time by α GalCer/DC therapy (based on the 12 cases who have finished the protocols) is >19 months (as of March 31, 2006), which is better than the median survival time with conventional therapies (7.8 months). Thus, in these clinical trials, α GalCer-pulsed DC therapy was well tolerated, and has been carried out safely even in patients with advanced diseases.

Study on the Development of a Liposome Vaccine for Cedar Pollinosis

The Research Unit for Clinical Allergy aims to clarify the immunological and molecular mechanisms underlying allergic responses and to apply this knowledge to the development of immunotherapy for patients suffering from allergies. The demand for such a therapy is quite strong in Japan, with an estimated 30% of the population suffering from allergies to the Japanese cedar pollen.

Considering the previous report that NKT cells generate tolerogenic DCs, followed by the induction of antigen-specific regulatory T cells suppressing antibody responses in an antigen specific fashion, a liposome vaccine, which contained α GalCer in the lipid bilayer and encapsulated antigen, was developed. Once the α GalCer-antigen-liposome was injected intraperitoneally into mice prior to the immunization with alum-absorbed antigen, the primary, secondary and ongoing responses of IgE, IgG1 and IgG2a antibodies were significantly suppressed. Furthermore, the enhancement of IL-10 production by α GalCer-liposome caused expansion of regulatory DCs in vivo. These effects were hardly detected in the NKT cell-deficient mice, suggesting that NKT cells play a critical role in the expansion of the regulatory DCs. Upon stimulation in vitro with antigen, in the presence of regulatory DCs from the mice treated with α GalCer-liposome, $CD4^+$ T cells became regulatory. This suggested that regulatory DCs could induce regulatory T cells. Thus, the regulatory cascade initiated by α GalCer-OVA-liposome suppresses IgE responses against allergens.





Research Networks

2005

Research Collaborations

In order to study problems in immunology, researchers must often apply different lines of thought and various approaches to tackle problems. Therefore it is very important to maintain an environment where people can carry out discussions freely, because such discussions often lead to collaboration. As shown in the left figure, 63% of the on-going projects at RCAI are collaborative projects in which two or more laboratories are involved. When the collaborative projects were analyzed, it became clear that internal collaborations within the Center (projects for which RCAI laboratories collaborate) and domestic collaborations (collaborations of RCAI laboratories and external organizations in Japan) make up one-third each, indicating that domestic collaborations are extensively carried out. The number of international collaborations was also very high (24%). Among the 22 international collaborations, a half of them were supported by RCAI's International Research Collaboration Award, indicating that the Award effectively contributes to the promotion of international collaboration. In comparison, collaborations with other centers of RIKEN were relatively low (12.2%), reflecting the difficulty of interdisciplinary projects. However, by taking full advantage of the RIKEN Yokohama Campus, where RCAI and three other biological centers (Genomic Sciences Center, SNP Research Center and Plant Science Center) are located, the number of interdisciplinary projects is expected to grow in the near future.

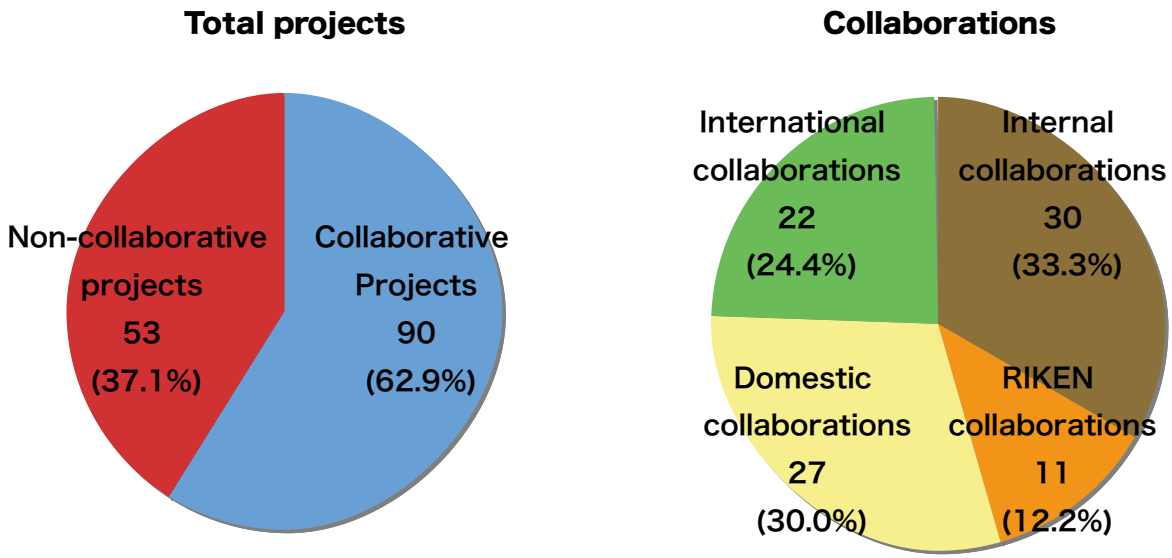


Figure: Collaborative projects at RCAI

International Programs

Table 1: Awardees of RCAI International Collaboration Award Program

Year	Awardee	Title of Research	Host Lab.
2004-	**Michael Dustin** New York University School of Medicine	Analysis of dynamism and function of limmunological synapse using planar membrane and knock-in T cells	**Takashi Saito** Laboratory of Cell Signaling,
2004-	**Willem van Ewijk** Leiden University Medical Center	Regulatory role of lymphoid progenitors during development of thymic microenvironments	**Hiroshi Kawamoto** Laboratory for Lymphocyte Development
2004-	**Miguel Vidal** Centro de Investigaciones Biologicas, CSIC	Genomic and functional analysis of the role of the Polycomb Ring1 genes in B-cell development	**Haruhiko Koseki** Laboratory for Developmental Genetics
2004- 2006. Mar.	**Steven F. Ziegler** Benaroya Research Institute at Virginia Mason Medical Center	Role of NKT cells in TSLP-Mmediated Aallergic linflammation	**Masaru Taniguchi** Laboratory for Immune Regulation
2004-	**Peter D. Burrows** University of Alabama at Birmingham	Expression and function of FcRY-α novel Fc receptor-related gene expressed in B cells	**Jiyang O-Wang** Laboratory for Antigen Receptor Diversity
2005-	**Wilfried Ellemeier** Institute of Immunology, Medical University Vienna **Hilde Cheroute** La Jolla Institute for Allergy and Immunology	Study of T cell differentiation mediated by regulated expression of CD8 genes	**Ichiro Taniuchi** Laboratory for Transcriptional Regulation
2005-	**Mark Bix** University of Washington, Seattle, Washington	Understanding genetic regulation of interleukin 4 production by a CD4(+) T cell-intrinsic mechanism.	**Masato Kubo** Laboratory for Signal Network
2005-	**Yun-Cai Liu** La Jolla Institute for Allergy and Immunology	Gene-array analysis and proteomics of Th2 tolerance	**Yasuyuki Ishii** Research Unit for Clinical Allergy
2005-	**Kenneth M. Murphy** Howard Hughes Medical Institute Washington University School of Medicine	Visualization of STAT protein in the cytokine mediated signaling at a single molecular level.	**Osami Kanagawa** Laboratory for Autoimmune Regulation
2005-	**Facundo Damian Batista** Cancer Research UK London	Role of signaling molecules in B cell synapse formation and its maintenance	**Tomohiro Kurosaki** Laboratory for Lymphocyte Differentiation

RCAI has established a unique supporting program for collaborations with groups outside of Japan. The International Collaborative Research Awards Program provides 15,000,000 JPY/year to selected collaborative research projects. It allows foreign scientists to set up a semi-independent International Research Unit within the laboratory of their collaboration partner at the Center. Currently, 10 programs are under way, including the five started in 2005 (Table 1).

The programs have already resulted in successful discoveries: Drs. Takashi Saito and Michael Dustin successfully observed the initiation process of T cell activation (*Nature Immunology*, 2005 and *Immunity*, 2006, See Research Highlights), and Drs. Haruhiko Koseki and Miguel Vidal discovered the function of the Polycomb gene in lymphocyte development (*Dev. Cell*, 2004 and *Development,* 2006). Drs. Hiroshi Kawamoto and Willem van Ewijk found a novel population of progenitor cells (*Development*, 2006 in press). In addition to these scientific accomplishments, the program promotes international exchange of people and stimulates their creativity for new research areas in immunology.

The Center's Short-Term Invited Lectureship Program invites internationally recognized investigators to give lectures and discuss ongoing research with young RCAI investigators (Table 2).

In addition to the above programs, the Center carries out joint seminars and the exchange of young scientists in collaborative programs with foreign institutes. RCAI signed a collaboration agreement with Max Planck Institute of Immunology and Max Planck Institute for Infection Biology in 2005, and a joint workshop is planned for 2006. The first joint workshop with the La Jolla Institute of Allergy and Immunology was held in February 2006 in San Diego, CA.

Table 2: Short-term Lectureship Program 2005

Month	Lecturer	
Apr	Mark Bix	Univ of Washington
Apr	Jean-Laurent Casanova	Univ of Paris, Necker Medical School
Apr	Jocelyne Demengeot	Instituto Gulbenkian de Ciencia
May	Casey T. Weaver	Univ Alabama at Birmingham
May	Marc Bonneville	INSERM
Aug	Stefan Kaufmann	Max Planck Institute for Infection Biology
Oct	Hilde Cheroutre	La Jolla Institute

2005 RCAI-JSI International Symposium on Immunology

Mechanisms of Immune Responses in Health and Diseases

The first International Symposium on Immunology organized jointly by RCAI and the Japanese Society for Immunology (JSI) was held from June 17 to 19 at the Pacifico Yokohama. The first symposium in a series of annual conferences was entitled "Mechanisms of Immune Responses in Health and Diseases". The intention of this first conferences was to keep the agenda open and bring together some of the leading and most productive scientists in immunology today, both abroad and in Japan.

In an environment that is characterized by an overload of increasingly specialized events, conferences, seminars, and workshops as well as an increasing number of commercial events and venues, to organize a successful international conference that covers some of the major trends in immunology today and that caters to a broader audience is no small task. Our answer to this problem was to put a strong accent on quality. Thus, the goal of this first international symposium was, first and foremost, to bring together a distinguished group of immunobiologists and immunologists, selected by the quality and originality of their recent research, rather than the thematic orientation of their work. Also, rather than focus on a narrowly defined topic, we intentionally kept the thematic agenda of the symposium as open as possible, in order to let new ideas emerge and flourish.

This formula is not without its risks. Few branches of science are as specialized as immunology. In a field in which literally tens of thousands of scientists publish an hundrets of highly specialized research papers every day, launching a conference series that is explicitly aimed at bringing the many loose ends together into a broader perspective is a daring undertaking. And yet, this formula had proved a considerable success at an earlier event organized by RCAI and Osaka University in 2003. It was the highly positive response to this first international conference that had encouraged RCAI to launch an annual series of events, together with the Japanese Society for Immunology (JSI).

With 553 attendees, this first symposium—like the earlier meeting in Osaka—can certainly count as a important success. And a success it was both for the invited speakers and the audience. A simple sign of success for any conference is when speakers start to re-arrange their presentations in last minute, adding unpublished data or now findings that they had perhaps initially reserved for later occasions.

Still, while this first event had put an emphasis on topics with a system-wide relevance, future events will be more narrowly focused on a given disease paradigm or research approach of relevance to RCAI. But, the ambition and the approach will remain the same: To bring together some of the most creative minds in immunology today and to provide a venue to test new ideas and interconnections that cut across the narrowly defined specialist niches that dominate scientific research today.


1


02


03


04

Session I: Recognition in the immune system

Ralph M. Steinman, The Rockefeller University, USA
Kenneth L. Rock, University of Massachusetts Medical School, USA
Kayo Inaba, Kyoto University, Japan
Mark M. Davis, HHMI and Stanford University School of Medicine, USA
Takashi Saito, RIKEN RCAI, Japan
Arthur Weiss, HHMI and University of California, San Francisco, USA

Session II: Development of the immune system

Michel C. Nussenzweig, The Rockefeller University, USA
Mark S. Schlissel, University of California, Berkeley, USA
Tasuku Honjo, Kyoto University, Japan
Nobuo Sakaguchi, Kumamoto University, Japan
Tomohiro Kurosaki, RIKEN RCAI, Japan
Klaus Rajewsky, Harvard Medical School, USA
Meinrad Busslinger, Research Institute of Molecular Pathology, Austria

Session III: Immune diseases and immunomodulation

Hiroshi Kiyono, The University of Tokyo, Japan
Jeffrey V. Ravetch, The Rockefeller University, USA
Shigekazu Nagata, Osaka University, Japan
Diane J. Mathis, Harvard Medical School, USA
Nora Sarvetnick, The Scripps Research Institute, USA
Toshio Hirano, Osaka University and RIKEN RCAI, Japan
Tadamitsu Kishimoto, Osaka University, Japan

Session IV: Regulation and homeostasis of immune responses

Kouji Matsushima, The University of Tokyo, Japan
Rafi Ahmed, Emory School of Medicine, USA
Takeshi Tokuhisa, Chiba University, Japan
Shimon Sakaguchi, Kyoto University, Japan
Michael J. Bevan, HHMI and University of Washington, USA
Anjana Rao, Harvard Medical School, USA

Session V: Triangular immunological interaction of the host, pathogen, and tumor

Shizuo Akira, Osaka University, Japan
William R. Heath, The Walter and Eliza Hall Institute of Medical Research, Australia
Shigeo Koyasu, Keio University, Japan
Tadatsugu Taniguchi, The University of Tokyo, Japan
Lewis L. Lanier, University of California, San Francisco, USA
Masaru Taniguchi, RIKEN RCAI, Japan

What guarantees success in a large international conference is not only a distinguished group of speakers [01]; the two other necessary ingredients are a highly dedicated staff and a receptive audience [02]. One of the speakers at the conference, Klaus Rajewsky, now at Harvard University, [03] met many of his former students from Japan at the event. The small-talk at any scientific conference is as important—if not more important—as the presentations at the podium: Signaling experts Arthur Weiss (UCSF) and Tomohiro Kurosaki (RCAI) during a coffee break [04]. T cell development specialist Ichiro Tainuchi (RCAI) listens attentively to Meinrad Büsslinger, (IMP, Vienna) who presented his most recent findings on the role of the Pax5 gene in B cell development [05]. RCAI director Masaru Taniguchi and Ralph Steinman (Rockefeller University) at the symposium dinner [06]. The RCAI Symposium, like the earlier conference in Osaka, was largely the brainchild of RCAI deputy director Toshio Hirano [07] who oversaw the planning of the event and who is already hard at work on the agenda for the coming years.


05


06


07

Events 2005

RCAI Workshop on Epigenetic Regulation During Immune System Development
April 15, 2005

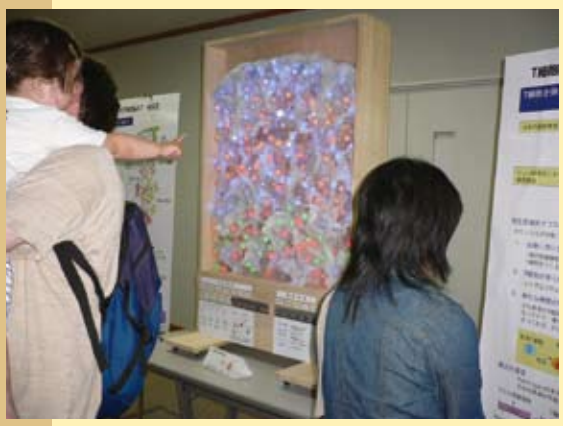
The Second RCAI Workshop, entitled "Epigenetic Regulation during Immune System Development", was organized by Drs. Ichiro Taniuchi and Masato Kubo and attended by 125 people. The focus of this workshop was on understanding the epigenetical program that regulates tissue- and stage–specific gene expression during immune system development. Since many genes have been well characterized in their spatiotemporal expression pattern and for their roles in lymphocyte development, the immune system could provide us with several beneficial points in the study of chromatin-based gene expression. This workshop was organized to encourage the exchange of information and to promote collaborations between groups inside and outside of RIKEN.

2005 RCAI-JSI International Symposium on Immunology
June 17-19, 2005

This international symposium was held under the joint auspices of RCAI and the Japanese Society for Immunology. More than 550 people from various countries attended and contributed to building an international foundation. (See International Symposium in the previous page).

RIKEN Yokohama Open Campus
June 25, 2005

The Yokohama campus of RIKEN, where RCAI is located, is open to the public once a year. This year, as many as 1,663 people visited the campus. At RCAI, researchers from more than 18 laboratories explained their field of research to the visitors. The display which gathered the most attention was the hand-made model of the thymus. Dr. Hiroshi Kawamoto and his colleagues in the Laboratory for Lymphocyte Development lit the model with colorful lights and explained to the people how the immune cells develop in the thymus. Dr. Masahiro Sakaguchi, leader of Vaccine design Research Team, gave a special lecture on "Allergy for Japanese Cedar Pollen". The visitors, from kindergarteners to retired folk, avidly asked questions to the researchers and shared their common interests in science.



RCAI Retreat
August 29-30, 2005

One hundred sixty-three researchers at RCAI gathered for an overnight event that took place at Japan Aerobics Center in Chiba prefecture. The invited lecturers, Drs. Willem van Ewijk and Steven Ziegler, and four selected researchers from RCAI, Drs. Bob Meek, Takashi Tanaka, Hiroshi Wakao and Ryouji Yagi also gave speeches. All the researchers presented their posters during the poster session, and principle investigators reviewed and selected posters for the Best Posters Awards 2005.

RCAI Workshop on Pathogen Recognition and Adaptive Immunity Regulation by Dendritic Cells
October 21, 2005

Drs. Tsuneyasu Kaisho and Katsuaki Sato organized the third RCAI Workshop entitled "Pathogen recognition and adaptive immunity regulation by dendritic cells". Several speakers active in research were invited and they presented their excellent recent work, prompting discussions on how innate immunity senses pathogens and regulates adaptive immunity.

Super Science High School
January 14, 2006

The Ministry of Education, Culture, Sports, Science and Technology designates high schools that focus specifically on scientific education as "Super Science High Schools". There are currently 82 Super Science Highs Schools in Japan. RCAI held a special workshop for students from five of these Super Science High schools. After lectures on "Aids Virus and Immunity" and "Immunological Organs" by Drs. Tomohiro Kurosaki and Hisahiro Yoshida, the students were divided into groups for hands-on practice of the dissection and histological staining of immunological organs.

Symposium on Pollen Allergy
January 17, 2006

The Symposium on Pollen Allergy was held under the joint auspices of the Ministry of Education, Culture, Sports, Science and Technology. Two hundred eighty-two people from the general public attended the symposium, through which RCAI's research activities were introduced to a wide range of people. In the first session, Drs. Masaru Taniguchi and Emiko Noguchi (Tsukuba Univ.) presented a general overview of cedar pollen allergy in Japan. Then, Drs. Toshinori Nakayama (Chiba Univ.), Shizuo Akira (Osaka Univ.), Toshiyuki Matsuoka (Kyoto Univ.), and Toshio Hirano (RCAI) presented the basic mechanisms of allergy and the potential targets in the regulation of allergic reactions. In the final session, Drs. Yasuyuki Ishii and Eyal Raz (UCSD) presented the novel therapeutic approaches they developed.

LIAI-RCAI Joint Workshop
February 2, 2006

The La Jolla Institute for Allergy and Immunology (LIAI) and RCAI are unique research organizations focused on basic and applied research in immunology and allergy. In order to enhance the mutual research collaboration, they agreed to hold a series of workshops. The first joint workshop with the La Jolla Institute for Allergy and Immunology was held at the La Jolla Institute in San Diego, CA. Six members from RCAI, Drs. Masaru Taniguchi, Takashi Saito, Sidonia Fagarasan, Tsuneyasu Kaisho, Shohei Hori and Keigo Nishida, presented their recent research results at the workshop and exchanged information. The next workshop will be held at RCAI in early 2007.

Graduate Student Program and Tutorial System

RCAI accepts graduate school students through the adjunct professorship at various universities. This year, RCAI and Osaka University Graduate School of Medicine have agreed to set up a cooperative program in the Division of Preventive and Environmental Medicine. Drs. Osami Kanagawa (Leader, Lab. for Autoimmune Regulation) and Tsuneyasu Kaisho (Leader, Lab. for Host Defense) were newly appointed as adjunct professors, and Dr. Toshiyuki Fukada (senior research scientist, Lab. for Cytokine Signaling) became an adjunct associate professor of the graduate school. In addition to this new program with Osaka University, Dr. Hiroshi Ohno has been an adjunct professor at Yokohama City University; Drs. Takashi Saito, Tomohiro Kurosaki have been appointed as adjunct professors, and Dr. Sidonia Fagarasan has been made adjunct associate professor at Tokyo Medical and Dental University.

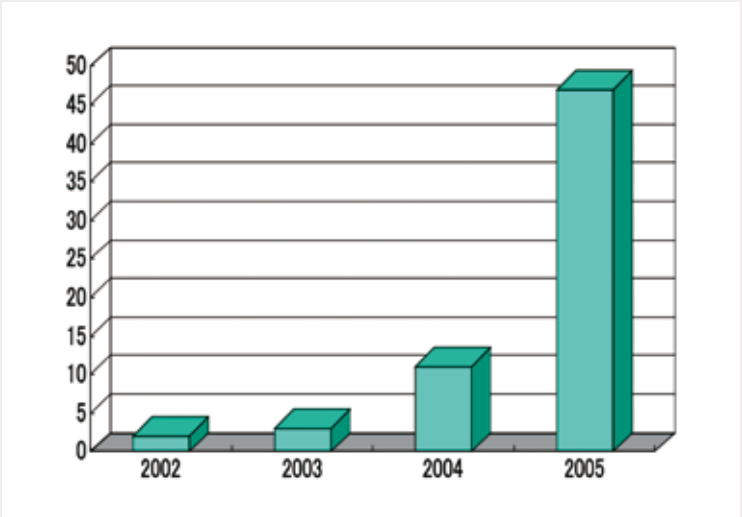
RCAI had been accepting students from schools including these graduate schools, and the number of students at RCAI reached 48 in 2005. This year, RCAI started a tutorial system for these students. Each student has two tutors, and every tutor meets the students regularly so that he/she can consult with the tutor on any subject at any time.

Support for Patent Application

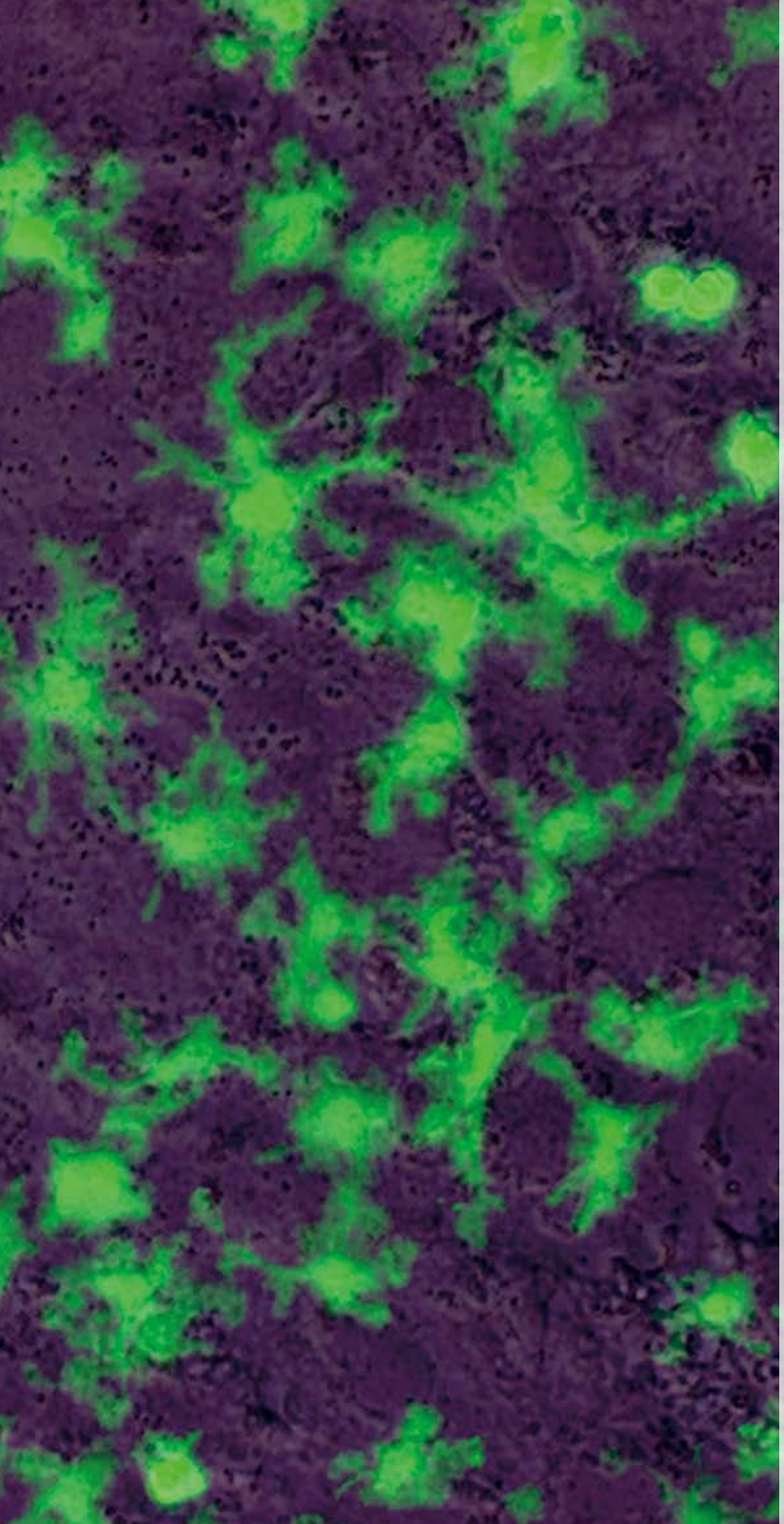
RCAI regards the development of new technology in immunological studies as one of its missions. In order to support such innovations, RCAI has developed the following unique support system for patent application.

A Japanese patent law firm visits RCAI twice a year to conduct hearings on the candidate inventions for patent applications. This law firm prepares the necessary documents on the inventions that are suitable for application as patents. RCAI submits the reports to the Center for Intellectual Property Strategies of RIKEN.

The number of patent applications from RCAI increased dramatically in 2005, as shown in the graph. Forty-seven patent applications were submitted this year, suggesting that many research discoveries have high potential for becoming patented, whereas most of them would have been neglected had there not been the support by the experts.



Graph: Number of patent applications



Data and Statistics

2005

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Publication Table

Jan.-Dec. 2005

Journal Title	2005
Advances in Immunology	1
Blood	6
Cancer Research	1
Current Biology	1
Genes & Development	2
Immunity	2
International Immunology	4
Journal of Biological Chemistry	2
Journal of Cell Biology	2
Journal of Clinical Investigation	1
Journal of Experimental Medicine	9
Journal of Immunology	19
Molecular and Cellular Biology	3
Nature Immunology	1
Nature Medicine	1
Science	1
PNAS	7
Other Journals/ Books	68
Total	131

Invited Lectures & Seminars

RCAI Seminers 2005				
4-Apr	**Naohiro Inohara**	University of Michigan Medical School	USA	Nod protein-mediated signaling and related diseases
5-Apr	**Michael L. Dustin**	Skirballl Institute of Biomolecular Medicine New York University	USA	Location of sustained TCR signaling in the immunological synapse
6-Apr	**Takeshi Egawa**	Skirball Institute of Biomolecular Medicine, New York University	USA	Genetic Evidences for V-alpha14i NKT Cell Development & Transcription Factor Requirements
11-Apr	**Ethan Shevach**	National Institute of Allergy and Infectious Diseases, NIH	USA	Activation and deactivation of regulatory T cell function
19-Apr	**Yongwon Choi**	University of Pennsylvania School of Medicine	USA	TRAF6 and Autoimmunity
20-Apr	**Jean-Laurent Casanova**	Necker-Enfants Malades Medical School	France	The human model: a genetic dissection of immunity to infection in natural conditions
26-Apr	**Jocelyne Demengeot**	Instituto Gulbenkian de Ciencia, Oeiras	Portugal	Immune regulation at the frontier of innate and adaptive immunity
9-May	**Casey T. Weaver**	University of Alabama at Birmingham	USA	A new lineage of effector CD4 T cells
10-May	**Casey T. Weaver**	University of Alabama at Birmingham	USA	Insights into regulatory T cell function from dynamic imaging of DC-T cell interactions
11-May	**Marc Bonneville**	University of Nantes	France	Structural and functional basis of tumor and infected cell recognition by a major human gdT cell subset
12-May	**Hidehiro Kishimoto**	Research Institute for Biological Sciences, Tokyo University of Science	Japan	The thymus and central tolerance
30-May	**Steven Ziegler**	Benaroya Research Institute at Virginia Mason	USA	TSLP and Allergic Inflammation
2-Jun	**Tamotsu Yoshimori**	National Institute of Genetics	Japan	Autophagy unveiled: molecular machinery and role in diseases
27-Jun	**Kunihiro Ohta**	RIKEN Wako Institute	Japan	Acceleration of gene conversion of Ig gene locus in DT40 cells and its application to antibody production
1-Jul	**Hajime Takashima, Tohru Yamazaki**	Takashima International Patent Office	Japan	Applying patents: Focus on academic patents
6-Jul	**Hiromi Kubagawa**	The University of Alabama at Birmingham	USA	Structure and function of Fc receptor for IgA and IgM
28-Jul	**Akiko Iwasaki**	Yale University School of Medicine	USA	Innate control of antiviral immunity
22-Aug	**Stefan H.E. Kaufmann**	Max-Planck-Institute for Infection Biology	Germany	How the immune system deals with proteins and lipids of the most successful pathogen, Mycobacterium tuberculosis
26-Aug	**Werne Mueller**	German Research Center for Biotechnology in Braunschweig	Germany	Which cell type has to produce IL-10 in innate and adaptive immune responses?

8-Sep	**Wen Chen Yeh**	University of Tronto	Canada	Inflammatory signaling in immune responses and cancers
22-Sep	**Wilfried Ellmeier**	Institute of Immunology, Medical University of Vienna	Austria	Epigenetic and transcriptional regulation of gene expression during T cell development
5-Oct	**Hilde Cheroutre**	La Jolla Institute for Allergy and Immunology	USA	A role for the counteracting receptor, CD8aa, in the differentiation of CD8ab+ memory T cells
27-Oct	**Facundo D.Batista**	Cancer Reseach UK, London Research Institute	UK	B cell antigen recognition and the immunologcal synapse
31-Oct	**Brenard Malissen**	Center d'Immunologie de Mareseille-Luminy INSERM-CNRS-University of Mediterranee	France	Dynamics and function of langerhans cells in vivo
1-Nov	**Paul W. Kincade**	Oklahoma Medical Research Foundation	USA	A new paradigm for how the innate immune system can be replensished during infections
7-Nov	**Amnon Altman**	La Jolla Insutitute for Allergy and Immunology	USA	Regulation of T cell activation and anergy by reversible protein palmitoyation
7-Nov	**Daniel A Peterson**	Barnes-Jewish-Hospital, St Louis	USA	Specificty in the Mucosal Immune Response to a Model Gut Symbiont
10-Nov	**Fumihiko Ishikawa**	Kyushu University Graduate school of Medical Sciences	Japan	Studying Human Hematopoietic and Immune Systems in Mice
16-Dec	**Caetano Reis e Sousa**	Cancer Research UK,London Research Institute	UK	Innate pathways in dendritic cell activation
20-Dec	**Takehiro Kawano**	Samuel Lunenfeld Research Institute Mount Sinai Hospital	Japan	Regulator of matrix Proteinases orchestrates organshape fprmation in C.elegans
16-Jan	**Oliver Lantz**	Curie Insstitute	France	Mucosal associated invariant T(MAIT) cell: a new evolutionarily conserved T cell subset
18-Jan	**Eyal Raz**	University of California, San Diego	USA	Diverse role of TLR signaling in gut homeostasis
15-Feb	**Shinya Kimura**	Kyoto University	Japan	Block the BCR-ABL signaling
16-Feb	**Shigeaki Nonoyama**	Department of Pediatrics National Defense Medical College	Japan	Identification and functional analysis of the genes responsible for human primary immunodeficiency diseases
16-Feb	**Martin F. Kagnoff**	University of California, San Diego	USA	Epithelial cells: Lessons for homeland defense
1-Mar	**Neil A.Mabbott**	Institute for Animal Health, Edinburgh	UK	Prions and the immune system
14-Mar	**Diane Mathis**	Joslin Diabetes Center Harvard medical School	USA	Aire control of Immunological tolerance

University Graduation Record

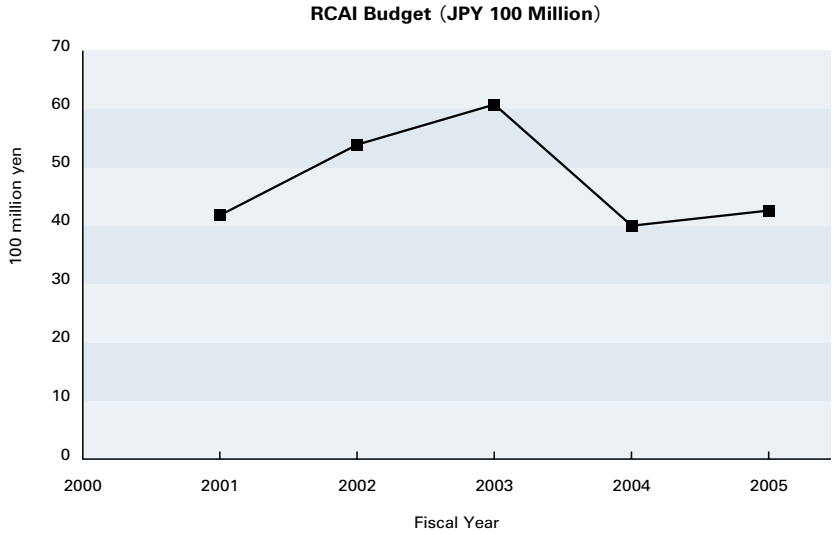
List of Universities

The table indicates the universities where RCAI researchers took their Ph.D. degrees.

* The following includes Director, Deputy Director, Principle Investigators (incl. International Research Units), Senior Scientists, Scientists and Coordinaters. The following does not include Research Associates, Technical Staff or Assistants.

Graduate School	Number of Graduates Working at RCAI*
The University of Tokyo	**19**
Kyoto University	**15**
Osaka University	**11**
Chiba University	**8**
Hokkaido University	**6**
Kyushu University	**5**
Okayama University	**3**
Tokyo Medical and Dental University	**3**
Tokyo Institute of Technology	**3**
Kumamoto University	**2**
Saitama University	**2**
Kobe University	**2**
Tokyo University of Science	**2**
Tohoku University	**2**
Harvard University	**2**
Johns Hopkins University	**2**
Ghent State University	**1**
Iuliu Hatieganu University	**1**
University of Southampton	**1**
Yokohama City University	**1**
Gifu Pharmaceutical University	**1**
Kurume University	**1**
Keio University	**1**
Juntendo University	**1**
Osaka Prefecture University	**1**
University of Tsukuba	**1**
Sichuan University	**1**
Nagasaki University	**1**
Tokyo Dental College	**1**
Tokyo Metropolitan University	**1**
Toyama Medical and Pharmaceutical	**1**
University of Alabama at Microbiology Birmingham	**1**
Erasmus University of Rotterdam	**1**
Universidad Complutense	**1**
MIT	**1**
Gunma University	**1**
University of Vienna	**1**
International School of Advance Studies	**1**

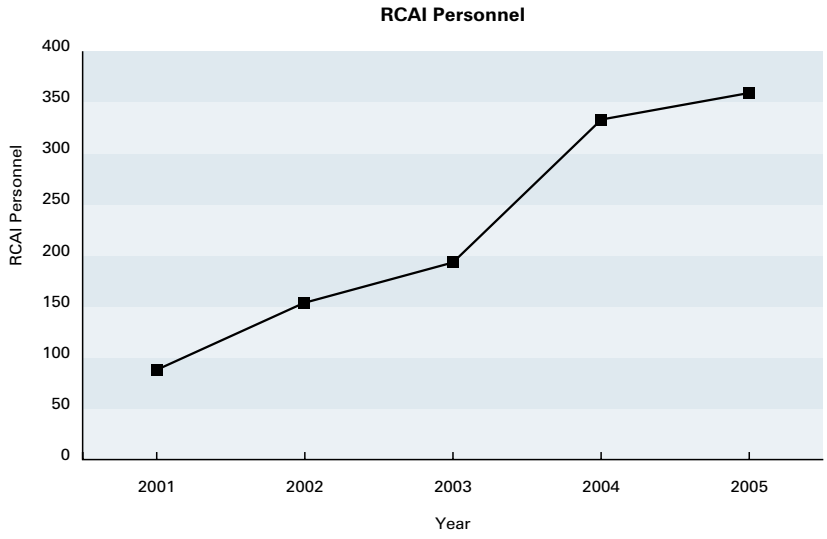
Budget and Personnel



RCAI Budget 2001-2005 (JPY 100 Million)

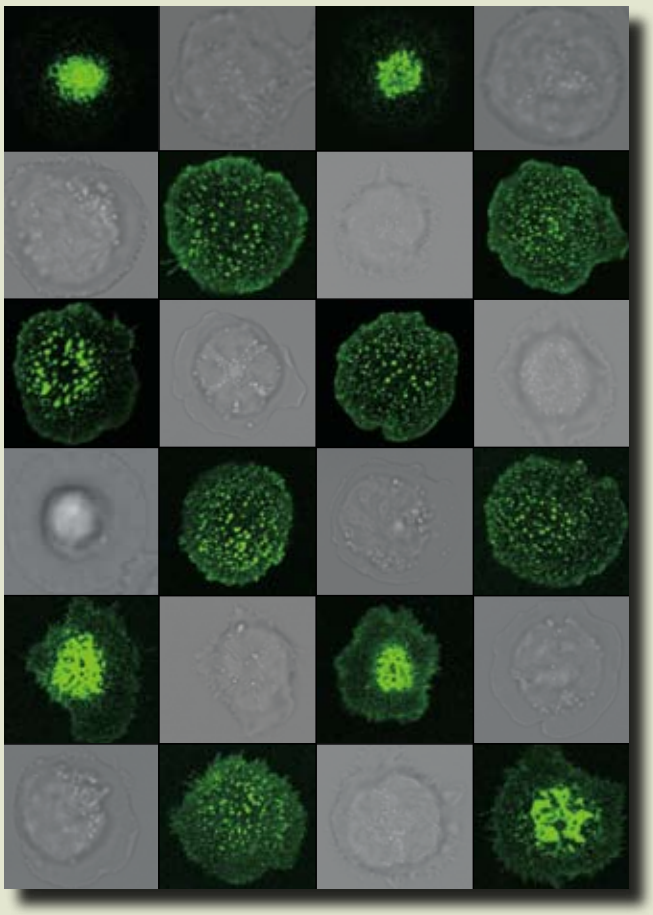
Note:Budgets for 2001-2003 include construction expenses for RCAI facility

	RCAI Budget JPY 100 Million
2001	41.74
2002	54.23
2003	60.48
2004	40.1
2005	42.92



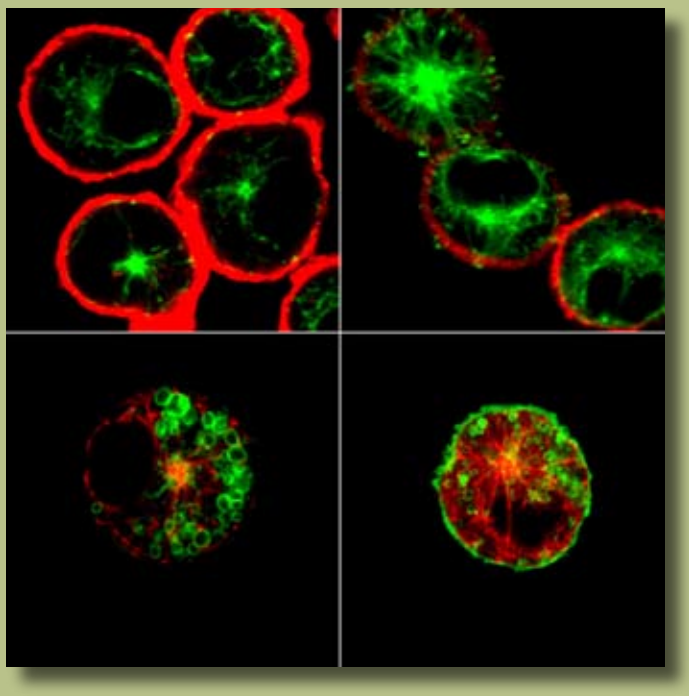
	RCAI Personnel
2001	84
2002	156
2003	191
2004	328
2005	363

RCAI Staff Composition (as of March 31, 2005)	number of employment
Director	1
Special Advisor	1
Principle Investigators	27
Coordinater	1
Senior Scientists	8
Scientists	67
Senior Techical Staff	2
Technical Staff	3
Technician	101
Graduate Students	48
Research Associates	2
Visiting Scientist	26
Assistant	26
Part-time Staff	32
Temporary Employment	18
Total	363



Cover:

Generation and dynamic movement of T cell receptor microclusters during the course of immunological synapse formation. A novel imaging technique to visualize single fluorescence-tagged molecule in lymphocytes has been developed by Single Molecule Immunoimaging Research Unit. Using this technique, Cell Signaling Research Group monitored the movement of signaling molecules during T cell activation and identified microclusters responsible for initial and sustained T cell activation (See Research Highlights). The images were singled out at various time points. T cell receptor-microclusters were visualized by CD3ζ−EGFP fusion proteins (*green*) , and black and white photos are the transparent images counterpart to green fluorescent ones. (Images by the courtesy of Cell Signaling Research Group)



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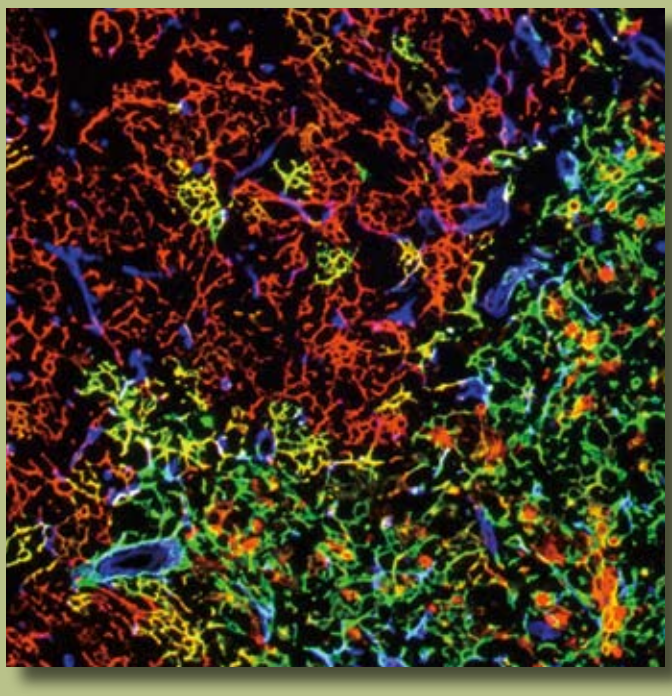

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1. **Front page of Research Highlights section:**

   Degranulation of mast cells involves FcεRI-induced calcium-independent microtubule-dependent granule translocation, reported by Nishida and Yamasaki et al. in JCB. Upper panel shows antigen stimulation triggers microtubule polymerization (*green*) and cortical F-actin ring disassembly (*red*). Lower panel shows antigen stimulation-induced granule translocation to the plasma membrane and partial co-localization of granules (*green*) with microtubule (*red*) (See Research Highlights). (Image courtesy of Cytokine Signaling Research Group)

2. **Front page of Research Activities section:**

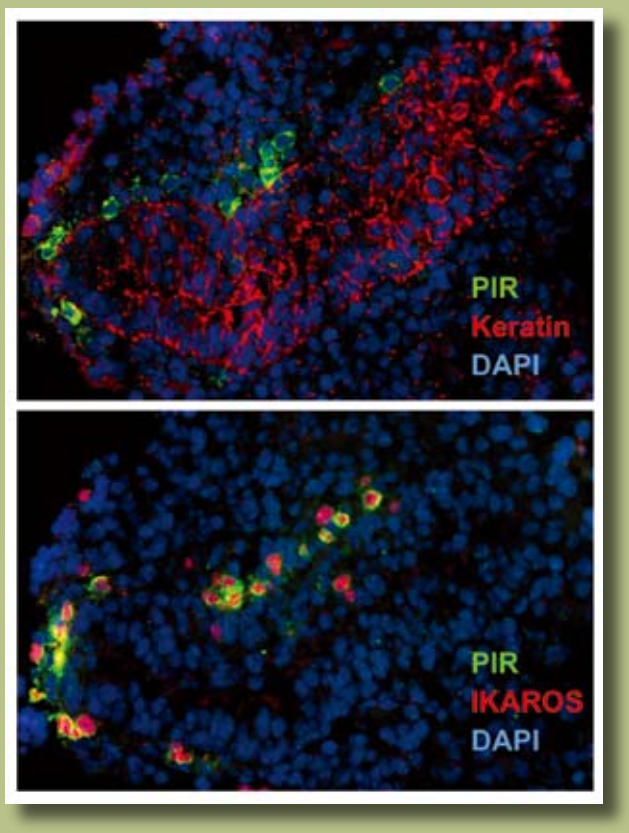
   An immunohistochemically stained section of adult thymus. Thymic cortical epithelial cells (*red*), thymic medually epithelial cells (*green*) and mesenchymal cells (blue) are seen. (Image courtesy of Lymphocyte Development Research Team)

3. **Front page of Research Networks section:**

   Pre-thymic T cell progenitor cells migrate into thymus. Paired Immunoglobulin-like Receptors (PIR) (*green*) are expressed on the surface of these migrating progenitors (See Research Highlights). (Image courtesy of Lymphocyte Development Research Team)

4. **Front page of Data and Statistics section:**

   Dendritic cells (*green*) generated from a intrathymic T cell progenitor on a monolayered stromal cells. (Image courtesy of Lymphocyte Development Research Team)



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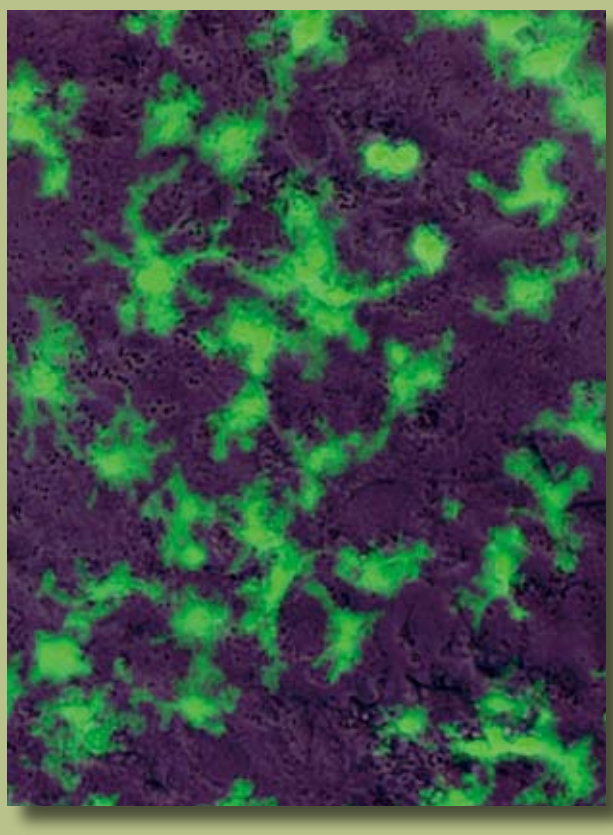

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