



Serendipities of Acquired Immunity

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INTRODUCTION

For a long time, biology was perceived as the lesser of the natural sciences because, unlike physics, deductive reasoning could not be used to solve biological problems. Biology has been full of mystery since I started my career in biological sciences almost half a century ago. Although the basic principles of biology stem from the rules of physics, biological systems have such an extraordinary, layered complexity derived from a tremendous number of parameters, beautifully and magically intertwined and controlled to achieve what we call “life”. Paradoxically, we start with a rather limited number (about 20,000) of coding region genes. However, many transcripts can be generated from a single coding gene locus, indicating that a single gene can produce many proteins. Furthermore, there are a much higher number of non-coding transcripts that may affect the expression of the coding genes. DNA, RNA and proteins can be chemically modified by methylation, phosphorylation and acetylation. In addition, at least 20,000 metabolites circulate in our blood. These can also be sensed by cells, interacting with various proteins and influencing gene expression, thus generating enormously complicated regulatory mechanisms to achieve homeostasis. The origin of metabolites can be traced not only to the biochemistry of our own cells but also to the diverse communities of

microbes inhabiting every surface of the body. If we imagine roughly 10^{13} order of our own cells, each expressing different proteins and containing different metabolites, in constant dialog with 10^{14} order of microbial cells, also in different metabolic states, the complexity of our biological system exceeds by far the physical and chemical complexity of the universe. The task of a medical scientist is deciphering this overwhelming complexity in order to understand the healthy state and diseases. We have yet to get a clear vision of exactly how gene expression is regulated. To make a long story short, the two major questions that we need to answer in biology are, what the mechanisms are for diverse yet strictly specific systems, and how these systems, are regulated. Biological regulation is robust, with many layers of redundancy as a safeguard. While we still do not have a thorough understanding of single-celled organisms like *E. coli* or yeast, multicellular organisms like human beings have far more complex regulatory systems with multiple layers of functional redundancy as well as tremendous specificities.

The immune system is perhaps one of the best-studied integrative regulatory systems in mammals. A large number of immune cells in different activation stages play specific roles, either through direct cell interactions or communication via soluble molecules like cytokines and their receptors. Although immunology is interesting and fascinating for a large audience, the immunologists' language is often misunderstood by other biologists, probably because of their use of scientific jargon. The best example is the cluster of differentiation (CD) annotation of lymphocyte surface molecules, which is necessary but at the same time very limiting and confusing. Even more difficult for non-aficionados is to understand sophisticated hypotheses and conceptual frameworks of the regulatory mechanisms of the immune system, including its innate and acquired immunity branches. The acquired immunity emerged during the late stage of our evolution. Therefore, this branch of the immune system utilizes all pre-existing regulatory systems employed by other systems, such as the nervous and endocrine systems. As such, the immune system is an ideal biological system to study the enormous specificity and highly sophisticated regulatory networks acquired by the biological systems during evolution.

When I started working on the immune system after my training in biochemistry and molecular biology, I often felt uneasy because immunologists were concerned mostly with functional aspects of the immune system rather than the molecules responsible. To give just one example, many immunologists used the term "antibody" for serum containing hundreds of proteins! Nevertheless, I witnessed a drastic change in the field with the advent of molecular biological techniques that could be easily applied to address immunological questions. I was very fortunate to begin

my career at a time when molecular biology facilitated the elucidation of molecular mechanisms for the immune functions using their valuable assay systems. The most important conceptual questions in immunology were: a) how does the acquired immune system generate the enormous diversity required to distinguish myriads of microorganisms and substances foreign to our body and b) how does this enormously diverse recognition system discriminate between self *versus* non-self? Basically, these major immunological questions also represent the core questions in biology: how organisms can have enormous numbers of reactions with highly specific yet robust regulations.

IMMUNOGLOBULIN DIVERSITY

Karl Landsteiner was astonished by the observations that almost all synthesized organic compounds bound to proteins could induce generation of specific antibodies (Ab) or immunoglobulins (Ig) by B cells in animals. This brought the question of how many Ig genes might exist there to generate such a diverse repertoire of Ab. Around 1970, the Ig structure had been elucidated by the efforts of many scientists, including Rodney Porter and Gerald Edelman, by finding that a typical Ab like IgG, consists of two identical light (L) and two heavy (H) chains linked by disulfide bonds (Edelman, 1959; Porter, 1959). Frank Putnam (Titani *et al.*, 1965) and two of Edelman's colleagues, Norbert Hilschman and Lyman Craig (Hilschmann *et al.*, 1965), discovered that the human L chain has two distinct regions, the N-terminal variable (V) and the C-terminal constant (C) region. The next obvious question was related to the number of V and C genes. How many V genes or C genes are there? Do we have the same number of V and C genes, or perhaps a large number of V genes and a small number of C genes? Two major hypotheses were put forward to explain the enormous Ab diversity. The 'germline hypothesis' claimed that there might be as many as one thousand genes responsible for the Ab diversity phenomenon. Another hypothesis proposed by Sir Frank Macfarlane Burnet, called 'somatic hypermutation theory', claimed that somatic mutations could be responsible for the generation of enormous diversity of the lymphocyte repertoire starting from a rather limited number of inherited genes. This debate caught the attention of not only immunologists but also biologists in general.

After I obtained my Ph. D. in Osamu Hayaishi's laboratory, I went to the United States of America to carry out my postdoctoral training (Fig. 1). In 1972, I listened to a lecture by Donald Brown from the Department of Embryology of the Carnegie Institution of Washington in Baltimore (Fig. 2). He proposed a very brave idea in support of the germline hypothesis, which postulates that animals have a large number of V and C genes.



Figure 1. Osamu Hayaishi and myself around 1996.

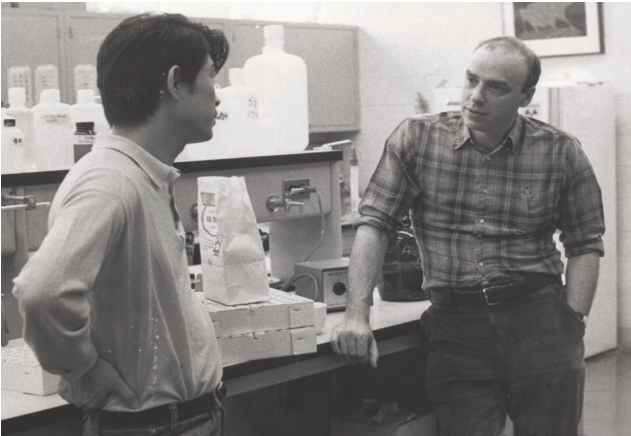


Figure 2. Donald Brown at Carnegie Institution in Baltimore, 1971.

Assuming the V and C sequences to be reiterated more than a hundred times, the major problem of the germline hypothesis was finding a mechanism for keeping many copies of the C gene unchanged while many copies of V genes varied. The evolutionary selection pressure on each copy of the C gene would be very weak if there were many copies of the same gene. Don proposed that the frequent homologous recombination could repair genetic mutations and maintain a large number of copies of the Ig C genes. The idea came from his own study on the structure of the ribosomal RNA or the 5S RNA genes, both of which consist of the spacer and RNA coding regions which correspond to alternating V and C region sequences, respectively (Brown *et al.*, 1968). A set of the spacer and coding regions is tandemly repeated almost a thousand times in the ribosomal RNA gene loci, which are often located close to the telomere of the

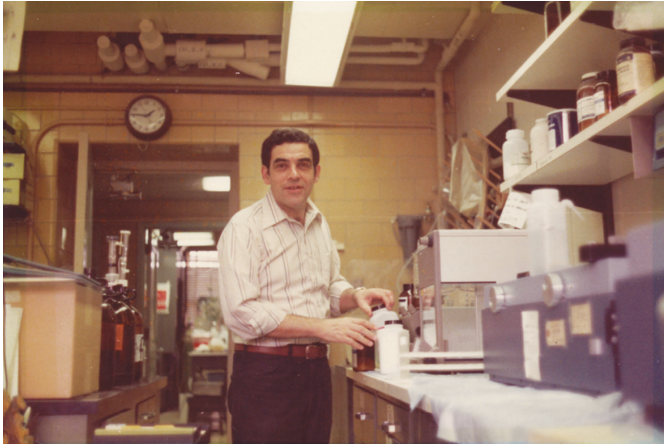


Figure 3. Philip Leder at NIH, 1973.

chromosome where the homologous recombination frequency is expected to be high.

That seminar struck me like a lightning bolt. I knew that the question was very important, but only then did I realize that emerging molecular biological techniques could be used to find an answer. I was fortunate that Philip Leder at NIH accepted me into his laboratory to work on this question (Fig. 3). Our results indicated that the number of copies of the IgL (C_{κ} or C_{λ}) chain gene is low, one or a few at most, ironically disproving Don's germline hypothesis (Honjo *et al.*, 1974). The results also suggested that some gene alterations, either somatic mutations, recombination events or both are required to generate diverse Ab from a small number of C genes. Indeed, soon afterwards Susumu Tonegawa discovered that recombination of a small number of variable (V), diversity (D) and joining (J) segments generates a repertoire of millions of unique receptor genes during lymphocyte differentiation (Brack *et al.*, 1978).

CLASS SWITCH RECOMBINATION AND AID

Upon returning to Japan in 1974 to the University of Tokyo, I was thinking long and hard about what fundamental problem I could tackle while setting up my own small laboratory. I finally decided to work on the IgH chain genes to address the specific question related to molecular mechanism of Ig isotype or class switching, thus avoiding direct competition for the mechanism of Ab repertoire generation on which Phil, Susumu and many others were working. It was known that antigen stimulation of mature B cells induces isotype class switching, a process that replaces the H chain C (C_H) region gene without changing antigen-binding specificity of the V region gene. The C_H region determines the Ig classes such as IgM,

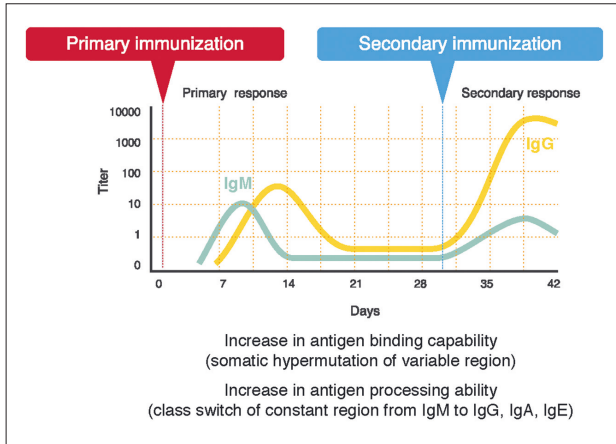


Figure 4. Antibody memory generation by vaccine (antigen) administration.

IgG, IgE and IgA. Another genetic alteration associated with antigenic stimulation of B cells is somatic hypermutation, a process known to facilitate generation of high-affinity Ab. These two phenomena represent the basis of immunological antibody memory and are critical to successful vaccination (Fig. 4).

We were very fortunate to find a collaborator, Masao Ono, who established the method for immunoprecipitating the Ig protein-mRNA complexes in polysomes, from which we could purify H chain messenger RNA rather quickly (Ono *et al.*, 1977). Using the cDNA from this purified mRNA, Tohru Kataoka and I were surprised to find the copy number of the C_H gene differed among DNA obtained from various myelomas producing different classes of Ig. By comparing the copy number of C_γ genes in DNAs of more than 10 myelomas expressing different C_H genes, I realized that a simple deletion between the expressed V_H gene and the C_H gene to be expressed could explain this phenomenon if the C_H gene were aligned in a specific order. We proposed the allelic deletion model of the Ig C_H locus (Honjo *et al.*, 1978).

In 1997, I isolated the first important C_H gene, $C_{\gamma 1}$ from purified $C_{\gamma 1}$ mRNA while on three-month stay in Phil's lab. Once $C_{\gamma 1}$ cDNA was cloned, it did not take long to clone other C_H genes and to convincingly prove that DNA recombination occurs by the isolation of actively transcribed Ig H chain genes (Kataoka *et al.*, 1980). This discovery was made amid incredible competition, as in addition to us, four other groups reached the same conclusion almost simultaneously (Cory, 1981; Davis *et al.*, 1980; Maki *et al.*, 1980; Rabbitts *et al.*, 1981). Finally, four years after we first conceived the allelic deletion, we were able to elucidate how all C_H genes are aligned on the mouse chromosome (Shimizu *et al.*, 1982).

We then proved that the deleted DNA segments are looped out from the chromosome (Iwasato *et al.*, 1990), a useful finding allowing us later to measure ongoing class switch recombination (CSR). Importantly, CSR takes place in the intergenic regions in the C_H locus, characterized by highly repetitive sequences. By studying how the Ig H chain locus is arranged in the mouse chromosome, we saw that each C_H region gene is preceded by a region (a few to 10 Kb) of highly repetitive DNA which we named the switch (S) region (Kataoka *et al.*, 1981) (Fig. 5). Biologically, CSR must be very efficient because it takes place immediately after antigen stimulation. The structure we revealed fulfills this requirement because CSR can take place anywhere within the large S regions, unlike the site-specificity of V(D)J recombination.

Retrospectively, these studies were milestones in understanding immune diversity, yet I was determined to identify the enzyme(s) responsible for this efficient type of recombination. For this purpose, it was essential to set up a good cellular system to measure class switching *in vitro*. It was fortunate that Eva Severinson proposed collaboration for isolation of as-yet-unknown soluble factor that induces class switching. Using a factor-secreting T cell line that she developed, we isolated cDNA encoding interleukin 4 (IL-4) and IL-5, which turned out to be critical cytokines to not only class switching of B cells but also other important aspects of lymphocyte differentiation (Azuma *et al.*, 1986; Noma *et al.*, 1986).

Another important tool that helped elucidating enzymatic mechanisms of CSR was the B1 cell tumor-derived line CH12, obtained from Warren Strobel at NIH in the early '90s. Although the original cell line had a very high background and low switching efficiency, we repeatedly recloned the CH12 cells and finally obtained the CH12F3 line. This subclone contained

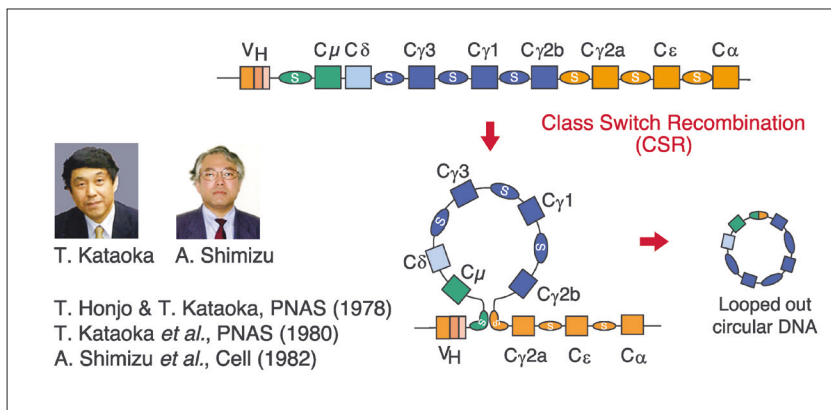


Figure 5. Class switch recombination takes place by deletion of a large DNA segment.

less than 1 percent IgA positive cells under non-stimulated condition but switched to more than 50 percent IgA positive cells after stimulation with IL-4, TGF- β 1 and CD40 ligand (Nakamura *et al.*, 1996). The system was used by Masamichi Muramatsu to carry out subtractive hybridization between nonstimulated and stimulated cells, which led to identification of the 23c9 cDNA clone, specifically expressed by activated CH12F3 cells, and also uniquely detected in germinal center B cells but not in other cells. Its sequence revealed the strongest homology with the RNA editing enzyme APOBEC1, and had weak cytidine deaminase activity *in vitro*. We named it Activation-Induced cytidine Deaminase (AID) (Muramatsu *et al.*, 1999). AID-deficient mice completely lost not only class switching but also SHM, a finding which was unexpected as these two genetic alterations were thought to be mediated by different enzymes (Muramatsu *et al.*, 2000) (Fig. 6). AID was thus shown to be the enzyme I had dreamed of finding for so long. Using the human AID primers, we designed, and our collaborators Anne Durandy and Allan Fisher identified, that every one of their Hyper-IgM syndrome type 2 patients had mutations in the AID gene (Revy *et al.*, 2000). The phenotypes of AID deficiency in human and mouse were remarkably similar. Thus, we obtained clear evidence that AID is the enzyme that engraves antigen memory in the antibody genes, which serves as the mechanistic basis of vaccination.

However, the molecular mechanism of AID is still not fully resolved. Although we have accumulated evidence supporting the idea that RNA is directly edited by AID (Begum *et al.*, 2015), most scientists in this field

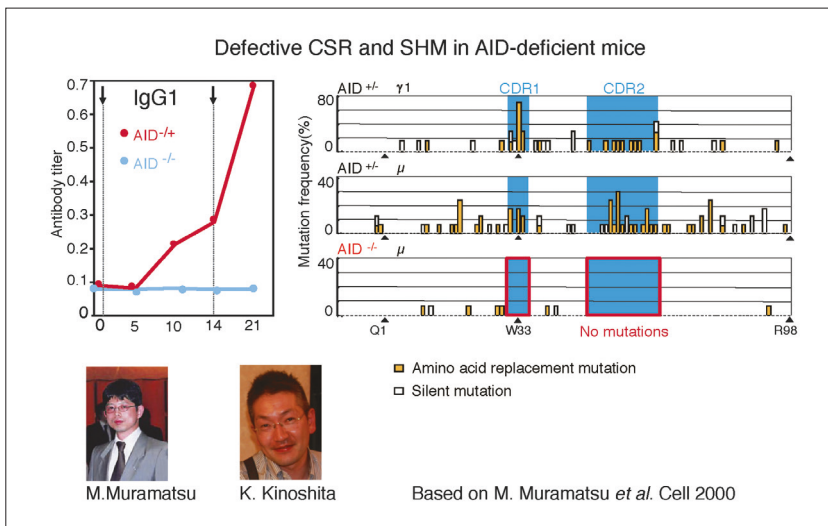


Figure 6. AID-deficient mice lost both CSR and SHM.

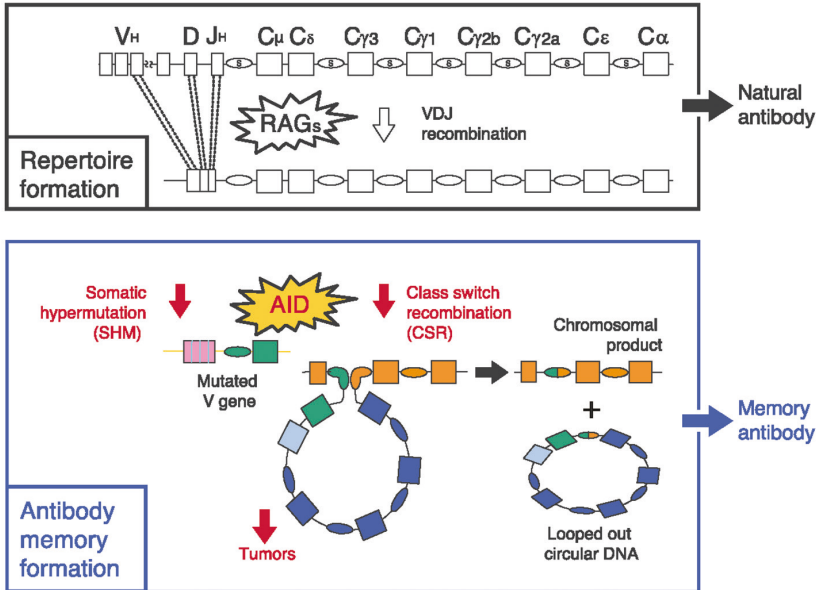


Figure 7. AID engraves Ab memory in the genome for effective vaccination.

believe that AID edits the DNA directly (Meng *et al.*, 2015). Nonetheless, it is now clear that B cell DNA rearrangements occur in two stages. Developing B cells in the bone marrow first generate the V region repertoire by V(D)J recombination. When B cells encounter antigens in the peripheral lymphoid tissues, they are activated to express the AID enzyme that induces SHM and CSR (Fig. 7). Occasionally, however, AID expression can cause aberrant chromosomal translocation between Ig and oncogenes like *c-myc*, generating leukemias like B cell lymphoma. The idea that immune activation could lead to tumorigenesis was totally unexpected by many immunologists at the time.

DISCOVERY OF PD-1 AND ITS APPLICATION

The great mystery that comes with our immense immune diversity is how to avoid immune activity against oneself. The phenomenon, known as immune tolerance, was theoretically proposed by Sir Frank Macfarlane Burnet and demonstrated by Sir Peter Brian Medawar, for which they shared the 1960 Nobel Prize in Physiology or Medicine. Sir Frank also proposed that tumor cells may express non-self antigens which the immune system would recognize and eliminate by the mechanism called immune surveillance (Burnet, 1957). Tumor cells, however, can grow when they induce the immune system into an inactive state i.e. immune

tolerance by reasons unknown even today. Such ideas and observations initiated what would become the field of cancer immunotherapy.

Many scientists tested various approaches to cure cancers; for example, some identified tumor-specific antigens and injected them as vaccines into patients. Others infused patients with their own T cells after expanding the cells in the laboratory with IL-2. Injections with immune-stimulating cytokines such as interferon- γ , IL-2 or IL-12 were also extensively pursued. Unfortunately, these trials did not provide any clear clinical benefits. In retrospect, all these trials were analogous to pressing an accelerator of the immune system under the condition of strong braking, because the immune system of cancer patients was in a state of immune tolerance. Indeed, until the mid-1990s, none of the molecules responsible for this negative immune regulation had been identified.

The new era of the immune regulation came when Pierre Golstein discovered the CTLA-4 molecule and when subsequent studies by Jim Allison, Jeffrey Bluestone, Craig Thomson, Tak Mak and Arlene Sharpe's groups revealed that CTLA-4 is a negative regulator involved in preventing the initial activation of the immune system (Brunet *et al.*, 1987; Krummel *et al.*, 1995; Tivol *et al.*, 1995; Walunas *et al.*, 1994; Waterhouse *et al.*, 1995). Indeed, CTLA-4 regulates the immune activation in an on-off manner in pair with CD28, in a manner equivalent to the parking brake (Fig. 8). Independently, we found another negative regulator called PD-1 that regulates the immune reaction in a rheostatic manner in pair with the accelerator ICOS. PD-1 was fortuitously isolated by Yasumasa Ishida, who had worked intensely on isolating the proteins involved in T cell selection in thymus, in order to answer the major question of how immune cells establish self-tolerance during development. He stimulated T cell lines derived from the thymus followed by cDNA subtraction to compare the

Action	Drive	Stop	Action mode
Parking [Activation]	Ignition [CD28]	Parking brake [CTLA4]	ON/OFF [Drastic]
Driving [Attack]	Accelerator [ICOS]	Brake [PD-1]	~ 100 km/h [Mild]

Figure 8. Brakes and accelerators control immune reactions like those in a car.

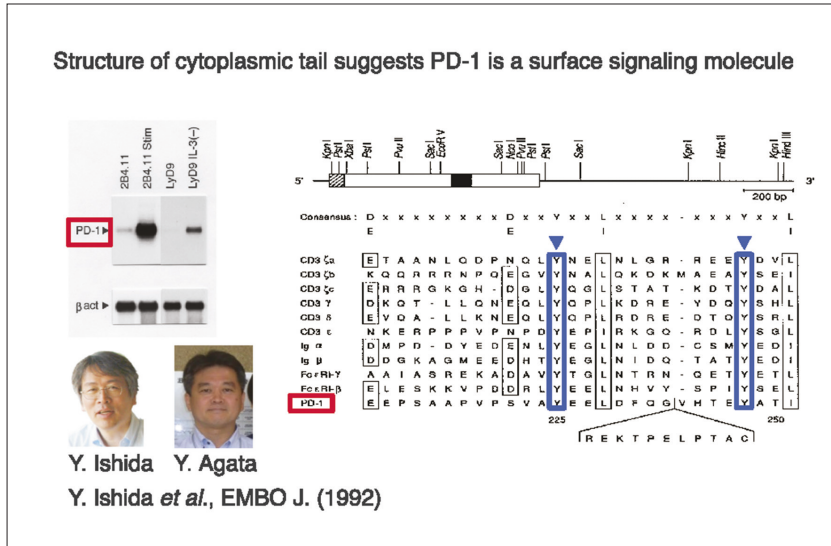


Figure 9. Discovery of PD-1 (programmed death-1) cDNA and its structure.

genes expressed by either growing or dying cells. He isolated four independent clones, which were surprisingly derived from a single mRNA. The cDNA sequence revealed a very interesting molecule we named programmed cell death 1 (PD-1) (Ishida *et al.*, 1992). PD-1 is a cell surface receptor with a cytoplasmic domain containing a pair of tyrosines, which is conserved among then known activation-signal transducing receptors. However, PD-1 is distinct from the rest by the double length between the two tyrosine residues (Fig. 9). We subsequently found that PD-1 expression is strongly induced in T cells and B cells upon stimulation (Agata *et al.*, 1996). We easily excluded the possibility that PD-1 is involved in cell death by the absence of PD-1 expression during the normal cell apoptosis induced by dexamethazone.

Although Hiroyuki Nishimura generated the PD-1 knockout (KO) mice in 1994, it took us a long time to understand the function of PD-1 *in vivo*, as deleting the gene in mice of a mixed genetic background seemed to confer no ill effects. The first hint of the negative regulatory function of PD-1 was obtained when he found that B cells from PD-1 deficient mice had stronger responses to antigen stimulation *in vitro* (Nishimura *et al.*, 1998). When crossing the PD-1 KO mice with autoimmune-prone *lpr/lpr* mice, PD-1 deficiency accelerated the development of severe nephritis and arthritis around five months after birth. Eventually, we also found that when using the common laboratory C57BL/6 strain, PD-1 KO mice also developed autoimmune nephritis and arthritis but only by around 14

months (Nishimura *et al.*, 1999). Surprisingly, on the BALB/c background, the PD-1 deficiency induced dilated cardiomyopathy, suggesting that while PD-1 is important for fending off the initiation of autoimmune disease, the specific organs targeted might be affected by other genes (Nishimura *et al.*, 2001). To confirm this idea, we examined consequences of the lack of PD-1 inhibitory function by crossing PD-1 KO mice with other autoimmune-prone mice commonly used by immunologists. Taku Okazaki found that PD-1 deficiency on the NOD background causes exacerbation of diabetes, while on the MRL background the absence of PD-1 causes severe myocarditis (Wang *et al.*, 2005; Wang *et al.*, 2010) (Fig. 10). Regarding the signaling characteristics of PD-1, Taku was able to elucidate the molecular mechanism for its immune brake function. He showed that the engagement of PD-1 induces phosphorylation of the conserved tyrosine residues, which recruits SHP2 phosphatase and dephosphorylates signaling molecules phosphorylated by antigen receptor engagement, resulting in down-modulation of activation signals (Okazaki *et al.*, 2001). All the above results provided strong evidence that PD-1 is the second new negative regulator of the immune system. However, in contrast to CTLA-4 deficiency, which develops severe autoimmunity leading to death by four to five weeks after birth, PD-1 deficiency results in autoimmunity developing more slowly and with milder phenotypes. Such results

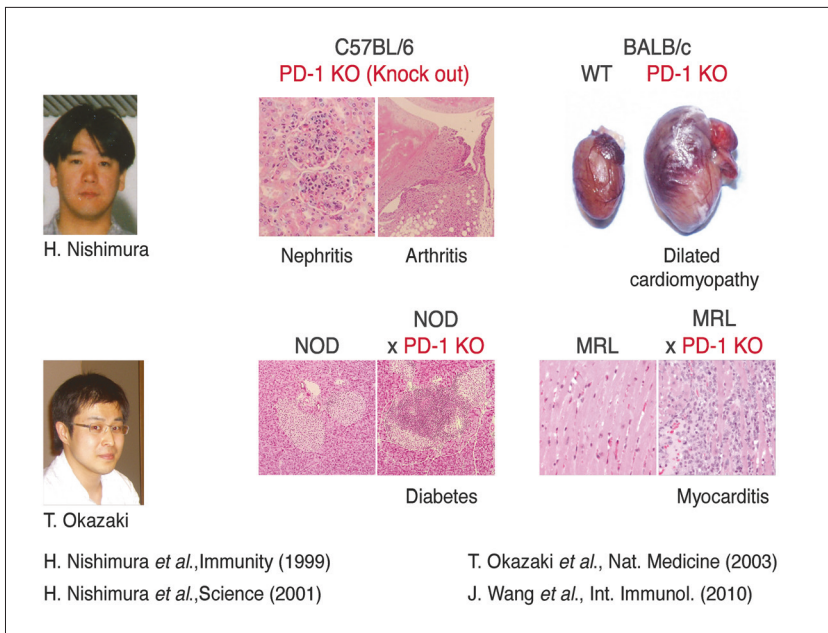


Figure 10. PD-1 is a negative regulator.

convinced us that PD-1 modulation might be a very good approach for the treatment of various immune-related diseases, including cancer.

Meantime, I wanted to isolate the ligand for PD-1 and collaborated with Steve Clark at the Genetic Institute (GI) in Boston. Unexpectedly, Gordon Freeman from the Dana-Farber Cancer Institute chose many cDNAs encoding B7 family member proteins from a database list, and one of the clones, 292, was shown to bind our PD-1-expressing cell line by the GI group. We confirmed their results and further demonstrated that 292 protein binding to the PD-1 receptor inhibits T cell proliferation. The identification of PD-1 ligand, which we named PD-L1, was reported in 2000 (Freeman *et al.* 2000).

Before the dawn of the new millennium, we started to test the effect of PD-1 deficiency on tumor cell growth. Yoshiko Iwai performed experiments comparing the growth of myelomas expressing PD-L1 between mice with or without PD-1 by the middle of 2000. She found that PD-1 deficiency suppressed the growth of PD-L1 expressing tumor cells (Fig. 11). The results convinced me that blocking PD-1 with an antibody would be effective to suppress the tumor growth. We urgently needed a PD-1 blocking Ab to confirm that this strategy could be used for cancer therapy. Our long-term collaborator, Nagahiro Minato, and his colleagues had already begun generating good Ab against PD-1 and PD-L1. Our laboratories embarked on an exciting and fruitful collaborative effort, and Yoshimasa Tanaka from Minato's laboratory showed that the PD-L1 Ab

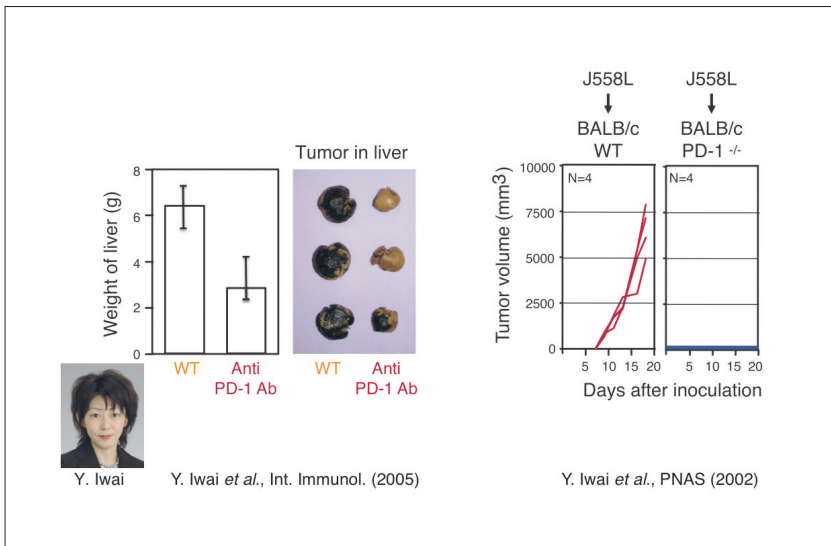


Figure 11. Suppression of B16 melanoma metastasis by PD-1 Ab and inhibition of tumorigenesis of myeloma (J558L) in PD-1^{-/-} mice.

they had generated could efficiently suppress tumor growth in a variety of mouse models (Iwai *et al.*, 2002) (Fig. 12). Yoshiko further confirmed that PD-1 blockade by either genetic manipulation or PD-1 Ab treatment clearly suppressed the metastasis of B16 melanoma cells from spleen to liver (Iwai *et al.*, 2005) (Fig. 11). These findings showed that PD-1 blockade by Ab against either PD-1 or PD-L1 is a powerful tool for treatment of cancers, preventing and delaying the growth and spread of tumor cells (Fig. 13).

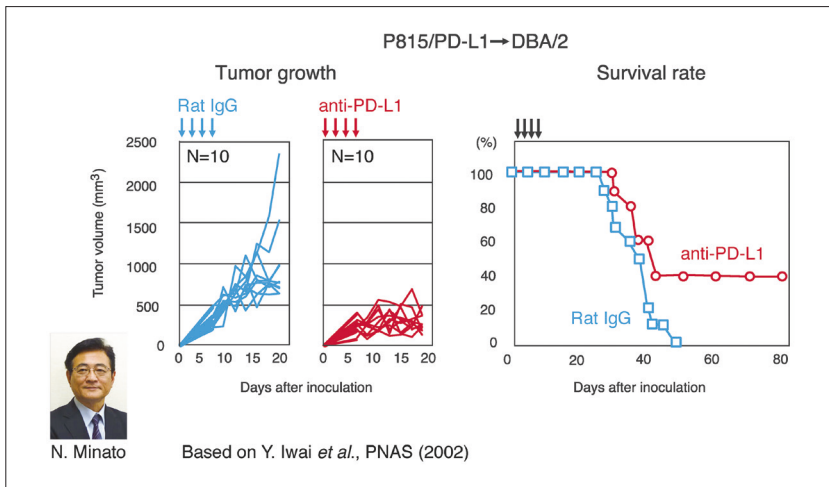


Figure 12. Growth inhibition of PD-L1 expressing mastocytoma (P815/PD-L1) and extension of animal survival by PD-L1 Ab.

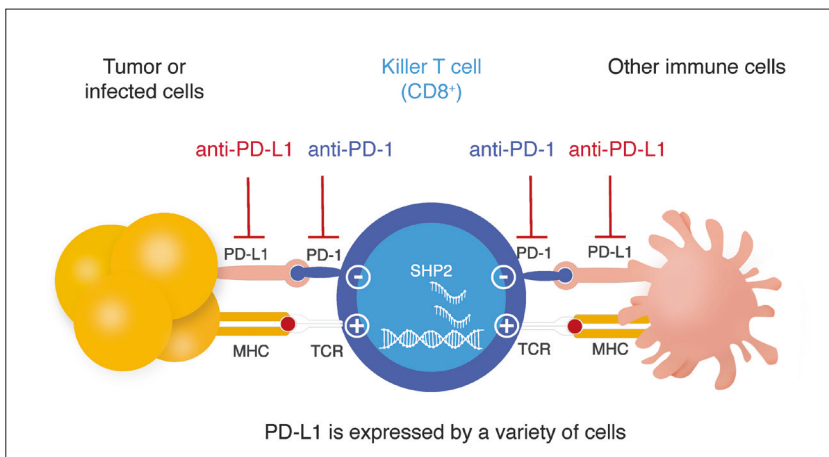


Figure 13. Schematic representation of tumor killing using Ab against either PD-1 or PD-L1.

CANCER IMMUNOTHERAPY BY PD-1 BLOCKADE

In order to apply this treatment to humans, we needed to file a patent application and obtain help from the pharmaceutical industry. After much struggle and many illusions and disillusion – which I describe in my biography – Medarex, a small venture capital already working with Jim Allison on a CTLA-4 monoclonal Ab, began to generate blocking PD-1 Abs in collaboration with Ono Pharmaceutical Co., Ltd. The human anti-PD-1 Ab named Nivolumab was an IgG4 with a mutation at S228P to reduce Ab-dependent cell-mediated cytotoxicity and approved as an investigation new drug by the FDA on August 1, 2006. Clinical trials were initiated immediately in the United States and two years later in Japan. The first Phase I clinical trial was comprehensively summarized by Suzanne Topalian and her colleagues (Topalian *et al.*, 2012). The results of this study surprised the scientific immunology community and many clinicians, because about 20 to 30 percent of terminal-stage patients with very aggressive forms of cancers such as non-small cell lung cancer, melanoma or renal cancer showed complete or partial responses. Perhaps even more surprising was the finding that 20 out of 31 responders of melanoma patients continued to respond for more than one year, some even after stopping the treatment (Fig. 14). Such durable responses had not been experienced with any other chemotherapy regimens. We also started

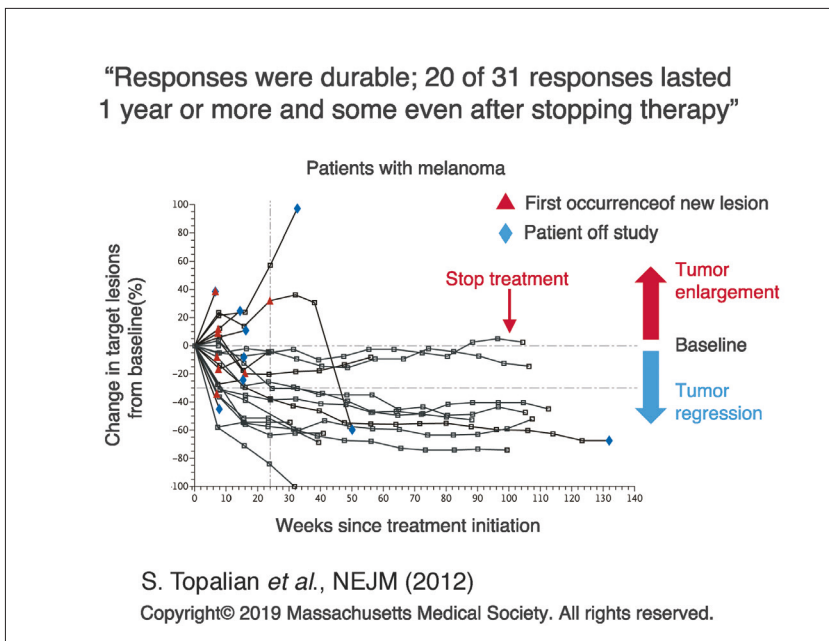


Figure 14. Durable response to PD-1 blockade.

a Phase II trial on platinum-resistant ovarian cancer patients in collaboration with the Gynecology Department of Kyoto University Hospital. Although the study was rather small, the results were promising. We were astonished to find that in about 40 percent of patients, responded or completely stopped the tumor growth (Hamanishi *et al.*, 2015) (Fig. 15). Two patients who showed a complete response after one year of PD-1 blockade are still healthy almost five years after stopping the treatment (Fig. 16).

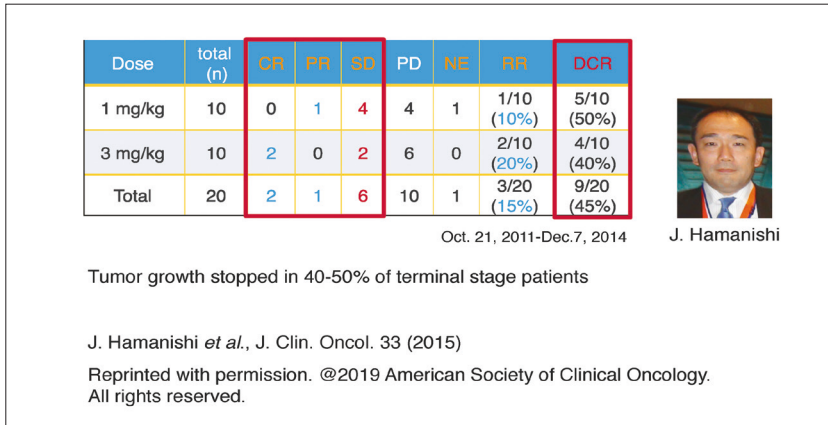


Figure 15. Phase II trial of anti-PD-1 Ab in patients with platinum-resistant ovarian cancer.

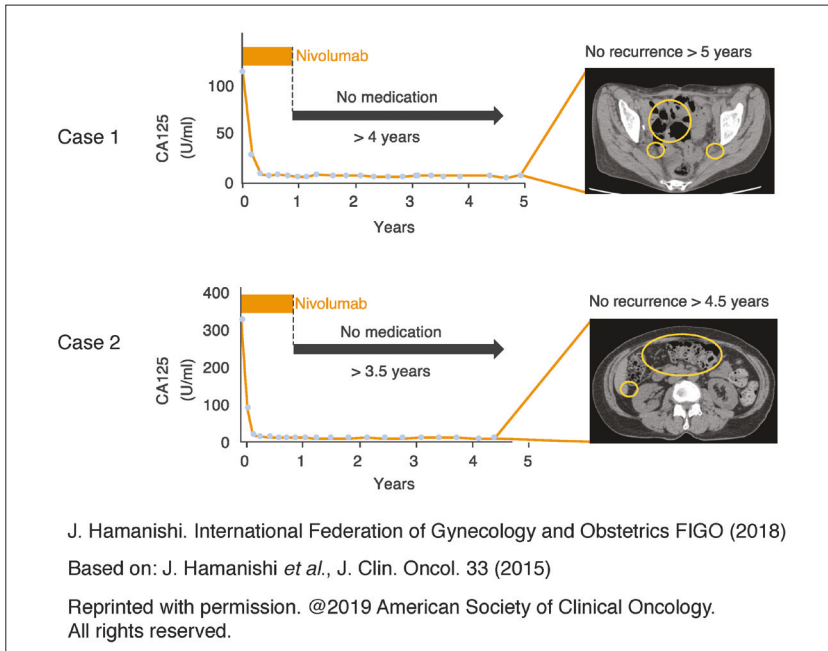


Figure 16. Durable complete responses of ovarian cancer patients to Nivolumab.

Numerous clinical trials carried out around the world during the last six years were recently summarized (Gong *et al.*, 2018). I was most impressed by a randomized study on 418 melanoma patients, on whom the effect of either Nivolumab or chemotherapy (Dacarbazine) was compared. The outcome was quite impressive. Almost 70 percent of the Nivolumab group survived after a year and a half, compared with less than 20 percent of the Dacarbazine group. At this point the study was stopped as it was deemed unethical to continue Dacarbazine treatment in the face of such clear clinical benefits from Nivolumab (Robert *et al.*, 2015) (Fig. 17). This large-scale study was carried out by an international team of clinicians in the European Union, Canada and Australia. Another striking clinical study has been reported for Hodgkin lymphoma cases. Without exception, all of the 23 Hodgkin lymphoma patients with various treatment histories showed either complete responses (four cases), partial responses (16 cases) or stable diseases (three cases) (Ansell *et al.*, 2015). Thanks to the enormous effort of scientists, clinical doctors, patients, bioinformaticians and other researchers in these studies and many more, by 2018 more than a dozen tumor types have been approved for the treatment by PD-1 blockade therapy. Most recently, the FDA approved this treatment to all microsatellite instability-high (MSI-H) cancers, regardless of the origin of their tumors. I view this as a very wise decision by the regulator, as it lifts the financial burden from drug companies to run expensive clinical trials for the many potential tumor types that might benefit from PD-1 treatment, greatly speeding up access to this new drug for many additional patients. Clearly, PD-1 blockade treatment has brought a paradigm shift in cancer therapy due to its efficacy over a wide range of tumors; durability of its effects in those who respond; and importantly, there are few adverse effects compared with previous treatments.

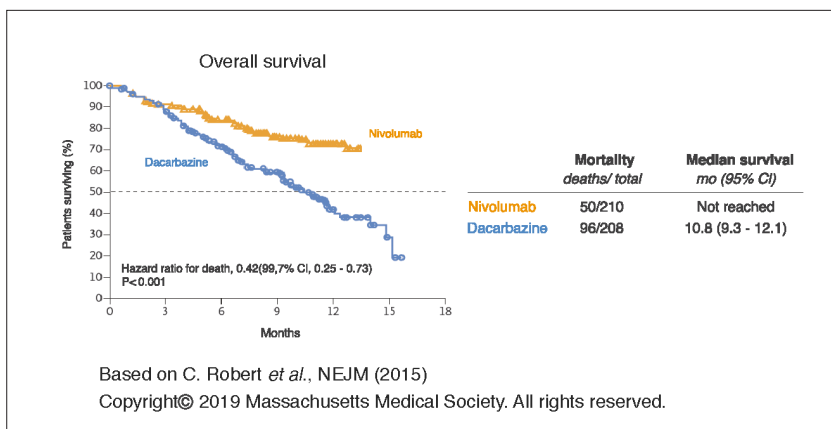


Figure 17. Randomized study on untreated melanoma patients with Nivolumab and Dacarbazine (chemotherapy).

But how can we explain the success of checkpoint immunotherapy such as PD-1 Ab treatment? Extensive DNA sequencing of a variety of tumors revealed enormous accumulation of mutations in the DNA of almost all cancer cells. Mutation frequencies in coding regions can reach the levels of one thousand to 10 thousand-fold higher than those of normal cells, which generally have few, if any, mutations (Alexandrov *et al.*, 2013; Martincorena *et al.*, 2015). Melanomas have the highest mutation frequency, followed by lung cancers. In fact, almost all types of tumors have 100 times more mutations compared with normal cells. Summarizing the results of many studies as follows: Firstly, cells become cancerous by accumulating a large number of mutations, changing their appearance from normal cells. These changes can be detected by our immune system's ability to home in on 'foreignness', meaning cancer immunotherapy is potentially effective for all types of cancers; secondly, this large increase in mutations also makes it difficult to identify which among them can be targeted by chemotherapy drugs; thirdly, frequent mutations continue to generate tumor cells that are resistant to chemotherapy, whereas the T cells with enormous immune repertoire can recognize and attack not only original but also mutated tumor cells.

Nevertheless, there are many hurdles ahead before declaring immunotherapy's complete victory over cancer. There are several obvious issues emerging from the clinical trials. First of all, the efficacy of this treatment is still limited. For example, for melanoma, the success rate of PD-1 blockade therapy as the first line of treatment reached about 70 percent at best (Robert *et al.*, 2015). In most other cases, especially when used as the second or the third line of treatment, the efficacy rate was 20–30 percent. Thus, we must urgently address two problems. One is how we can predictively distinguish responders from non-responders to PD-1 blockade. The other is how we can significantly improve the efficacy of the PD-1 immunotherapy.

Predictive markers could be related to tumor characteristics. For example, a high mutation rate of tumor cells is one good indicator for responsiveness to PD-1 blockade therapy as mentioned above. PD-L1 expression by tumors was also considered but unfortunately was not efficiently predictive of responder status (Colwell, 2015). The other factors influencing the response rate of cancer immunotherapy are related to the individual's immune capacity, which is at present very difficult to predict. Since there are hundreds of genes involved in the immune responses, and multiple layers of regulation, it is not easy to predict an individual's immune power, for example, by looking at their genetic polymorphisms. Environmental factors, such as the host microbial communities in the gut, also seem likely to play an important role in modulating immune responses and perhaps tumor features as well. Thus, in the near future, we will strive to

understand the best combinatorial factors predictive for a positive outcome of immunotherapy.

Needless to say, improving the immunotherapy response rate would help a great many additional cancer patients, and we are working towards this in two different ways in our current research. Firstly, we are testing ways to increase the number of cytotoxic T cells (CTL) that infiltrate and kill tumors. We recently showed that the first important steps in CTL activation and proliferation occur in tumor-draining lymph nodes. Once they are activated and decorated with responsive receptors, CTLs migrate to the tumor site in response to a gradient of chemokines secreted from the tumor site (Chamoto *et al.*, 2017). We showed that tumor growth could no longer be suppressed by PD-L1 Ab when draining lymph nodes were surgically removed in tumor-bearing mice that were otherwise responsive to PD-1 blockade. This is because activation of CTL in draining lymph nodes is prerequisite for upregulation of CXCR3, a receptor responding to several chemokines, such as CXCL9, CXCL10 and CXCL11. Interestingly, CXCL9 is actually produced by tumor tissues in response to interferon- γ secreted by pre-existing tumor, infiltrating CTLs upon stimulation with PD-1 blocking Ab. Thus, lymph nodes appear to be critical to initiate the activation and to deploy the CTLs to the tumor sites, in turn further facilitating the recruitment of more CTLs from lymph nodes in what appears to be a positive feedback loop. These results suggest that surgeons should avoid removing healthy lymph nodes during dissection of tumors in order to safeguard protective immune responses.

Another approach to strengthening PD-1 blockade which is being pursued by many scientists and pharmaceutical companies around the world is combination therapy. Amazingly, in the United States alone, more than one thousand clinical trials testing PD-1 blockade in combination therapy are currently ongoing (Tang *et al.*, 2018). The most commonly used combination partners for PD-1 blockade are CTLA-4 Ab, followed by chemotherapy, radiotherapy, anti-VEGF agents, and chemoradiotherapy. It is important to remember that neither chemo- nor radiotherapy could show appropriate effects on tumor growth if the immune system is defective in animal models (Apetoh *et al.*, 2007; Takeshima *et al.*, 2010).

Our own laboratory took a totally different approach. We and others found that T cell activation is accompanied by a burst of activity by the cells' mitochondria, the energy-producing organ of the cell (Chamoto *et al.*, 2017; Fox *et al.*, 2005). This energy is probably needed to support the cell division that quickly follows antigenic stimulation. CTLs divide almost every 8 hours, requiring a large amount of building blocks and energy to produce the proteins and nucleic acids required to build a daughter cell several times per day. We therefore searched for chemicals that can boost mitochondrial activity in active T cells. PGC-1 α is a key

transcriptional cofactor boosting fatty-acid oxidation and oxidative phosphorylation, which are crucial to mitochondrial activation (Chamoto *et al.*, 2017; Ventura-Clapier *et al.*, 2008). In my lab, Kenji Chamoto screened for chemicals which influenced PGC1- α activity in the context of PD-1 blockade therapy. He found that Bezafibrate, already approved as a drug for hyperlipidemia, synergizes with anti-PD-L1 Ab for suppression of tumor growth (Chamoto *et al.*, 2017) (Fig. 18). Subsequently, we found that Bezafibrate has two important activities for increasing the number of tumor-specific CTLs at the tumor site (Chowdhury *et al.*, 2018). By activating PGC-1 α and PPAR signaling, Bezafibrate facilitates rapid proliferation of effector CTLs. Also, enhanced PPAR signaling by Bezafibrate appears to prevent cell death, and to facilitate long-term survival of the activated CTLs. We are now carrying out the Phase I clinical trial in Japan, combining PD-1 blockade with Bezafibrate. I hope the large number of trials currently underway will soon identify many other strategies to improve the efficacy of PD-1 blockade.

FUTURE PROSPECTS FOR PD-1 BLOCKADE CANCER THERAPY

In parallel with improvement strategies for successful therapy, I see the need to push forward the basic fundamental understanding of the whole body. In other words, we need to invest more effort into understanding physiology, a challenging task as I have already pointed out. We found that PD-1 deficiency manifests different types of organ-specific autoimmunity in different laboratory mouse strains. As expected, some of the

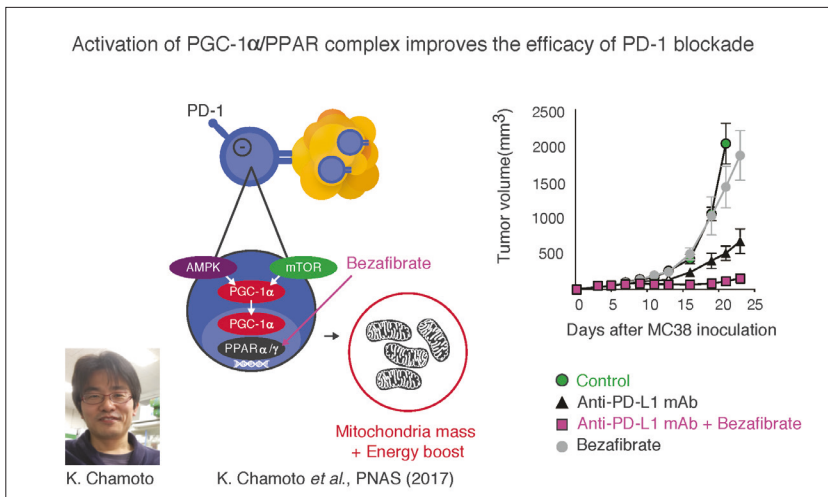


Figure 18. Improvement of PD-1 blockade therapy by Bezafibrate.

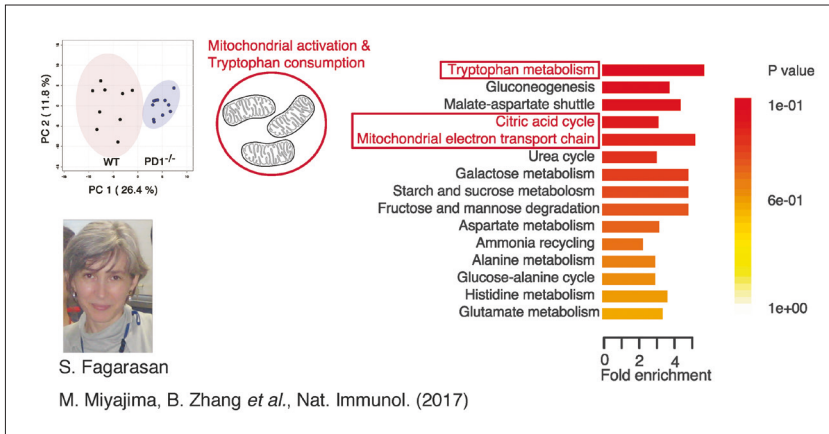


Figure 19. Hyperimmune activity can be read in the blood biochemistry of PD-1 KO mice.

patients undergoing PD-1 blockade therapy develop similar autoimmune features. Some adverse effects of PD-1 blockade are partly due to the fact that T cells proliferate vigorously after stimulation to generate functional progeny, requiring a huge amount of energy and metabolites to build new nucleic acids and proteins. Indeed, Sidonia Fagarasan's group recently showed that activation of the immune system limits the systemic availability of amino acids, hindering the synthesis of neurotransmitters in the brain (Miyajima *et al.*, 2017) (Fig. 19). Serum depletion of tryptophan and tyrosine, for example, reduces the amounts of serotonin and dopamine in the brain and causes behavioral changes like enhanced fear responses and anxiety. Such systemic biochemical changes were observed in PD-1 deficient mice as well as in normal mice under strong antigenic stimulation.

Furthermore, the harmful hyperimmune activation in PD-1 deficient mice is caused not only by the absence of this inhibitory receptor but also by dysregulated microbial communities in the gut (Kawamoto *et al.*, 2012). Indeed, Sidonia's group showed that in addition to AID, PD-1 is an essential molecule required to generate and select proper IgAs responsible for maintenance of a healthy microbial ecosystem in the gut (Fig. 20). The absence of PD-1 causes a skewing of balance between T and B cells in the germinal centers, with expansion of germinal center T cells perturbing the normal process of selection and maturation of IgA repertoire needed to maintain gut symbiosis.

Sidonia's amazing research in this area extended our understanding of PD-1 functions and grew from her own findings on hyperimmune activation caused by microbiota dysbiosis in AID-deficient mice (Fagarasan *et al.*, 2002; Suzuki *et al.*, 2004). She is the first person who showed that skewed microbiota in the gut can activate the immune system throughout

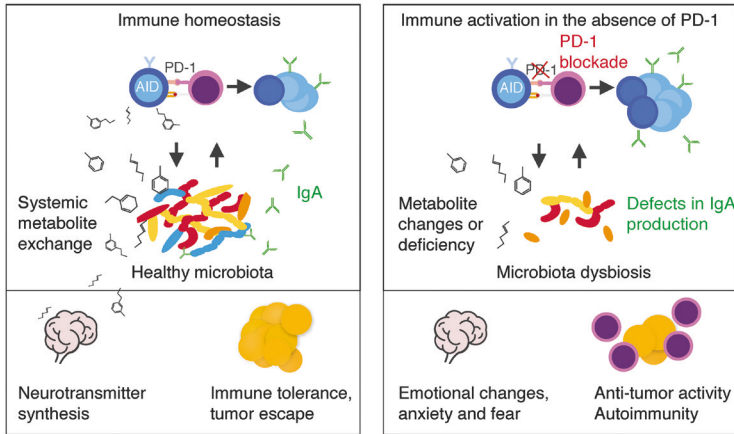


Figure 20. AID and PD-1 cooperate in germinal centers for IgA selection to maintain microbiome and body homeostasis.

the body in the AID-deficiency mouse model. Most strikingly, in the absence of IgA, expansion of certain species of anaerobic bacteria caused enlarged nodular structures along the whole intestinal tract, as well as hypertrophy of all lymphoid tissues. How fortunate I am to have discovered the two critical molecules, AID and PD-1, which by a certain amount of chance became the focus of my research, and collaborate in such a fascinating way to control not only the immune system but also the ecosystem of the gut which is so important for whole-body balance.

Based on these studies, it was perhaps not surprising to find that PD-1 blockade therapy can be influenced by the bacterial communities in the gut (Sivan *et al.*, 2015). We also put into test this by evaluating the anti-tumor activity of AID-deficient mice and found that tumor growth is dramatically inhibited in AID-deficient mice even without PD-1 Ab treatment (M. Akrami and R. Menzies, unpublished data). Sidonia's group further confirmed that the hyperimmune activity against tumor growth in AID-deficient mice depends on microbiota, because AID-deficient mice raised in germ-free conditions lost strong anti-tumor activity (M. Miyajima and S. Fagarasan, unpublished data). Thus, PD-1 is critical for regulation of CTLs against tumors but AID as well as PD-1 is important for boosting the immune potential against tumors through microbiota regulation, strongly indicating that gut microbiota is critical to the anti-tumor activity of individual immunity (Fig. 20).

In summary, I feel that despite enormous recent progress, our knowledge of the immune system and its interactions with other systems is still very limited. We are just at the beginning of an exploration that will lead us to a better understanding of how our bodies function. Even a single

molecule, PD-1, has so many unanticipated effects on aspects of health and disease, from tipping the balance of the immune system towards autoimmunity, to regulating bacterial communities, to major changes in the biochemistry of the immune, circulatory and nervous systems. Achieving successful immunotherapy implies learning more not only about improving cancer outcomes, but also understanding all the effects of the treatment on the rest of the body.

In 2016 Andy Coghlan published an article entitled “Closing in on cancer” in *New Scientist magazine*. Regarding the PD-1 blockade therapy, he quoted Dan Chen from Genentech saying “We are at the point where we have discovered the cancer equivalent to penicillin.” They considered PD-1 blockade therapy to be comparable to the discovery of penicillin for infectious diseases. “Although penicillin itself couldn’t cure all infections, it gave rise to a whole generation of antibiotics that changed medicine forever, consigning most previously fatal infections to history.” (Coghlan, 2016). I really hope this dream about the future of cancer and immunotherapy will come true. At this stage in 2018, however, first line treatment by PD-1 blockade or in combination with CTLA-4 blockade is limited to melanoma, lung cancer and renal cancer only. In the near future, I hope many more types of cancer may be treated by immunotherapy as the first-line therapy. I hope that by fine-tuning a therapeutic balance between tumor growth and immune surveillance using PD-1 blockade, this devastating disease may become instead of a death sentence, a chronic and manageable condition. It might sometimes be preferable to merely control or slow tumor growth, especially in more fragile, elderly patients, making cancer a disease we live with, rather than die from. Looking back, vaccination and antibiotic discoveries were two great leaps in human health-care which greatly reduced infectious diseases. WHO has even declared the human smallpox virus to be completely eradicated. I truly hope that within this century the next leap forward will be controlling cancer by immunotherapy and its improved protocols, which might include microbiome manipulation. We are still at a very early stage in our understanding of the microbiome, metabolome, and all the other possible factors affecting immunity and physiology, including the aging process. However, as we have seen very rapid development of cancer immunotherapy during last 10 years, I believe the future of cancer treatment is promising and really hope that the 21st century will be remembered as the century in which cancer was conquered.

Neither infectious disease control nor cancer immunotherapy would have been possible without acquired immunity, which developed at a very early stage of the vertebrate evolution. Acquired immunity originated in jawless fish, in which AID orthologues with a unique enzymatic ability were responsible for generating the enormously diverse molecules for



Figure 21. Current colleagues and long-term collaborators.

both the T and B lineage equivalents (Guo *et al.*, 2009). Later, in jawed fish, a transposon containing ancestral RAG-1 and RAG-2 ‘jumped’ into a primordial immunoglobulin-like gene. The transposon insertion generated a split gene, an ancestor of the V(D)J segments as well as recombinase enzymes. RAGs replaced the function of AID in the repertoire generation, while AID kept its function for antigen-induced antibody memory including SHM and CSR. All these drastic genetic mechanisms were selected during evolution for the advantage they conferred in the fight against infectious pathogens. As a result, humans now benefit from the sophisticated acquired immune system in the fight against cancer. Cancer cells accumulate mutations and develop into non-self-cells expressing neoantigens that are easily recognized by the acquired immune system. This is obviously an extra bonus from acquired immunity that evolution did not anticipate. In that sense, we humans are very lucky to enjoy this unexpected gift from evolution.

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