

From Fleas to T Lymphocytes and Beyond: How did I Become an Immunologist, and What Has Resulted from It?

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Abstract

A narrative from a co-author of studies explaining the cellular mechanisms of self/nonself discrimination by the immune system, set against the backdrop of significant advancements in immunology during the last quarter of the 20th century. This review focuses on demonstrating the functional diversity of T cells by identifying their two major subsets: regulatory CD4 and cytotoxic CD8. It also examines the first T-cell receptor (TCR) transgenic mice, providing definitive evidence for both positive and negative selection of immature thymocytes as mechanisms for generating a self-tolerant and MHC-restricted TCR repertoire. The origins of these experiments have been discussed.

Keywords

Self/nonself discrimination • Central tolerance • Positive selection • MHC-restriction

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Motto:

*It's not enough to know the word by sight. One must
Know how it issued from the furrowed dust:
How it bred there, how it grew and swelled—
Not how it rings, but how, resounding, belled;
Not how it sounds, but with what rounding frame
It there matured and came to term and name,
Then burst into expression. The poet's duty—
In the word's growth in time to find its beauty.*
/Julian Tuwim/- translated from Polish by Jakub Ziguras

By substituting “poet” for “immunologist” and “word” for “lymphocyte,” one gains a poetic description of the motivation that has driven me and many others to study the development, differentiation, and function of these cells.

My scientific career began when I identified, “by sight,” two major types of thymus-derived lymphocytes called T cells and concluded by observing how they “burst into expression,” i.e., how they are born and selected in the thymus. This research led to a better understanding of the cellular mechanisms that enable the immune system to distinguish between normal “healthy” cells and “good” commensal microflora—which are protected—and cancerous or infected cells, as well as pathogenic microorganisms and foreign cells that are combated.

This fundamental problem of self/nonself discrimination, a long-standing immunological puzzle, has captivated and inspired generations of researchers. Over 100 years ago, Ehrlich (1900) recognized this issue and coined the term “*horror autotoxicus*” to illustrate the severe consequences that could arise if the immune system turns against the tissues of its organism. Burnet’s clonal selection theory (Burnet 1957) marked the first attempt to explain the cellular basis of the immune system’s enigmatic ability. It initiated a new era in immunological research by establishing a key objective for future studies. I have actively participated in a long struggle to verify the proposed cellular mechanisms responsible for producing defensive lymphocytes that do not self-destruct, and I would like to share my journey.

Like my whole life, my scientific career was a series of serendipitous events. The following individuals played significant roles: Sergiusz Riabinin, Czesław Radzikowski, Lloyd Old, Hiroshi Shiku, and Harald von Boehmer, along with molecules such as the $\alpha\beta$ T-cell receptor (TCR), major histocompatibility complex molecules class I and class II (MHC-I, MHC-II), and T-cell co-receptors (CD4 and CD8).

As a young man, I did not dream, like many scientists, of exploring the world’s mysteries. For a long time, I was uncertain about what I wanted to do. However, from early childhood, I knew I wanted the freedom to do what I wanted, when I wanted. This desire came from observing my uncle, Sergiusz Riabinin, a zoologist, who helped me understand that, as shown by Fleming’s example, important discoveries can come from accidental observations by careful observers motivated solely by curiosity, rather than by preconceived ideas that can weaken perceptiveness.

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My uncle spent his time watching birds from early spring until late autumn. During the winter, he sat at home writing his observations. I liked this lifestyle and decided to study biology to become an ornithologist. Soon, however, it became clear that watching birds would not lead me, like James Watson (Watson 1962), to a great discovery, so I switched to studying insects. Unfortunately, they were not *Drosophila*, but fleas, which also turned out to be a mistake. With this strange specialty, it was difficult to find a decent job, which was quite frustrating.

One day in 1967, while walking past an oncological institute in my hometown of Gliwice, I thought it would be intriguing to study tumor cells. However, my rudimentary knowledge of cell and molecular biology posed a challenge. Although it still leaves much to be desired, good fortune has accompanied me since then. My friend knew people at this institute and introduced me to a courageous man, Dr. Czesław Radzikowski. After a brief interview, despite my lack of qualifications, he offered me an assistant position. He asked Barbara Sikora (Eng. Tit)—my future wife and greatest discovery, more than compensating for my failure as an ornithologist—to teach me cell culture methods. This was the first time I saw a living cell under the microscope, and I became fascinated. It was a cell line of murine leukemia. My task was to adapt this line to grow *in vitro* (Kisielow 1970) and define its immunogenic and antigenic properties by testing antibodies from immunized, genetically compatible, and incompatible mice. The activity and specificity of the antibodies were measured by the now obsolete trypan blue exclusion method. At that time, it was the only known and widely used method to study cell surface antigens, which involved estimating the proportion of cells killed by the antibodies by counting under the microscope the cells stained blue.

While I was counting blue cells under the microscope, Jacques Miller in Australia (Miller and Mitchell 1967) and Max Cooper in the USA (Cooper et al. 1965) discovered that lymphocytes from different origins mediate immune responses: thymus-dependent T cells and bone marrow-derived, antibody-producing B cells. I was unaware of these findings because access to Western journals in Poland was limited at the time, and hardly anyone at the institute knew about them. Fortunately, in the Soviet camp, Poland was considered the happiest barrack—as it was called—and people were allowed to accept fellowships from selected Western foundations.

In 1972, I was fortunate to receive a 1-year WHO fellowship to work with Dr. Lloyd Old at the Sloan-Kettering Institute (SKI) in New York. At that time, the clonal selection theory was widely accepted, confirming the basic principle that a single lymphocyte secretes antibodies of only one specificity. However, a fundamental question rooted in clonal selection theory, which set the research direction for the coming decades, remained unresolved. This concerned the problem of self-tolerance, which is the absence of immune reactions

against normal, “healthy” self-body components, despite the immune system’s capacity to generate lymphocytes and antibodies specific to all kinds of natural and artificial molecules. The possible solution to this conundrum was suggested in 1953 by the experiments conducted by Peter Medawar and colleagues (Billingham et al. 1953), which demonstrated that transplantation tolerance in adults could be induced by the neonatal injection of foreign donor cells, hinting at a potential resolution. This led Joshua Lederberg (Lederberg 1959) to postulate that the lack of auto-reactivity may be due to negative selection, i.e., clonal deletion of self-reactive lymphocytes at early developmental stages.

Alongside the big question of self/nonself discrimination, many smaller yet equally fundamental questions remained unanswered, and these needed to be resolved before any progress could be made in addressing this problem.

1. What is the role of the thymus in lymphocyte development?

After Jacques Miller demonstrated that the thymus is necessary for lymphocyte development (Miller 1961), the fate and function of the major population of small, cortisone-sensitive thymocytes—accounting for 90% of lymphoid cells in this organ—remained unclear. It was uncertain whether they represented an intermediate, immature stage in T-cell development, negatively selected “useless” or auto-aggressive lymphocytes, or what also seemed possible: the source of necessary hormones.

2. What is the physiological function of class I and class II MHC antigens?

For a long time, the only known function of MHC molecules was to induce graft rejection of foreign donors, which was unlikely to reflect their true physiological role.

3. How is the seemingly endless diversity of antigen-specific receptors generated?

At that time, the knowledge suggested that our genomes literally could not contain enough genes to account for the vast diversity of these molecules in each individual.

4. Are antigen-specific receptors on B and T cells of the same or different nature?

This was one of the most sensitive and elusive questions in immunology during the next decade, and some respected immunologists mistakenly believed it was an antibody molecule.

5. Are T lymphocytes functionally homogeneous or heterogeneous?

It was known that they could kill all types of cells and help B cells produce antibodies, but the important question regarding the identity of T cells performing these functions remained unanswered. Could the same cell kill or help, depending on the circumstances, or were these activities carried out by highly specialized cells

from different lineages? Until the late '60s, even authorities like James Gowans, who discovered the immune function of small lymphocytes (Gowans et al. 1962), considered the possibility that they represented a single variety of cells. As recalled by Jacques Miller (Miller and Sadelain 2015), the conviction that lymphocytes are uniform was so strong that, for some time after the discovery, B and T cells existed for many only as the first and last letters of the word "BullshiT."

During my stay at Dr. Old's laboratory, groundbreaking discoveries that opened the way to answer many of the above questions, made in the laboratories of future Nobel Prize winners, were emerging like mushrooms after the rain.

- Zinkernagel and Doherty (1974) explained the physiological function of MHC molecules by discovering the phenomenon of MHC restriction in T-cell recognition of antigens. They demonstrated that T cells can kill target cells only if the antigen is presented in the context of class I MHC molecules from the host in which the T cells have developed, thereby identifying MHC molecules as the ligands for cytotoxic T cells.
- David et al. (1973) and Cullen et al. (1974) identified class II MHC molecules as the products of immune response genes.
- Erb and Feldman (1975) demonstrated that class II MHC molecules restrict the antigen recognition of T cells, thereby facilitating B cells' production of antibodies.
- Köhler and Milstein (1975) discovered how to produce monoclonal antibodies.
- Hozumi and Tonegawa (1976) discovered the molecular mechanism that generates a diverse range of antibodies and antigen-specific receptors, involving DNA rearrangement.
- Jaenisch and Mintz (1974) demonstrated that foreign DNA could integrate into the DNA of early mouse embryos, creating the first transgenic mammal.
- I, along with Hiroshi Shiku and John Hirst (Kisielow et al. 1975; Kisielow 2014; and see below), demonstrated that cytotoxic and helper regulatory functions are mediated by different T cells. Although of lesser significance than all the above, it was considered crucial: "Identification of different lines of T lymphocytes that may perform different function is a crucial step in our understanding of immunological responses, and hence is of relevance to cancer no less than to several other fields of medicine"—Edward A. Boyse in the letter to WHO dated August 7, 1973, asking for prolongation of my stipend. "There is no doubt that identification of the two major T cell subsets was highly significant to further immunology work"—Jacques F.A.P. Miller in a letter from August 28, 2020.

In Dr. Old's laboratory, the primary focus was on identifying tumor-specific antigens and understanding tumor-specific immune responses. However, during our first conversation, Dr. Old suggested a different project aimed at understanding the molecular mechanism of cell-mediated killing. He proposed that I join Hiroshi Shiku, who, like me, had just arrived at his laboratory and used a novel *in vitro* method to study cytotoxicity against non-lymphoid adherent target cells. Dr. Old suggested trying to find whether antisera against various cell surface antigens present on lymphocytes identified by him and Dr. Boyse could inhibit killing.

Dr. Boyse provided me with a panel of antisera that reacted with lymphocyte surfaces, including mouse antisera against Ly1 (later renamed CD5) and Ly2 (renamed CD8) molecules (Boyse et al. 1968). His lab focused on the role of thymic hormones in lymphopoiesis, and no functional studies of lymphocytes were on the agenda. While determining the optimal dilution of antisera for our experiments, I noticed that the proportions of cortisone-resistant thymocytes and lymphocytes from lymph nodes and the spleen, lysed by CD5 and CD8 antisera, differed by a few percent. Despite this, my colleagues were skeptical about the value of these results due to the questionable precision of the trypan blue exclusion test. However, when our initial attempts to block the killing of target cells failed, I realized that my observation, suggesting that CD5 and CD8 molecules are not equally expressed on all T cells, could be validated if the elimination of cells with antisera against different CD molecules produced varying effects on the ability of surviving cells to perform various functions. The results we obtained with Hiroshi Shiku (Kisielow et al. 1975, and Shiku et al. 1975) unequivocally showed that T lymphocytes are functionally heterogeneous: T helpers could be distinguished and separated from T killers based on their different phenotypes: CD5⁺CD8⁻ and CD5⁻CD8⁺, respectively. Our enthusiasm was high, but mine was the highest because I had all the results in my notebook. However, my enthusiasm did not last long because the next day, I was told that I should not worry about being a co-author on the paper, but I "couldn't play captain on this boat." I vehemently protested and continued to conduct my experiments at night with the moral support of Peter Beverley. Dr. Old, who in the meantime became a vice president of SKI, was inaccessible, and I could not ask him for help.

In my subsequent clandestine experiments, I examined the impact of eliminating T cells from non-immunized mice using CD5 and CD8 antisera on their ability to induce graft versus host reactions, which involved both initiating and implementing the immune response (Kisielow et al. 1975). The results strongly suggested that T cells characterized by different CD5/8 phenotypes represent distinct lineages developing independently of antigenic stimulation, and may cooperate during cell-mediated responses. In parallel, I analyzed the

Ly phenotype of thymocytes and gathered evidence suggesting the existence of four populations of T lymphocytes in the thymus: a major CD5⁺8⁺ (double-positive [DP]) population and three minor populations: CD5⁺8⁻, CD5⁻8⁺ (single-positive [SP]), and CD5⁻8⁻ (double-negative [DN]). Five years later, the CD4 molecule (Reinherz et al. 1979; Dialynas et al. 1983), a functional counterpart of the CD8 molecule, on helper T cells, replaced CD5 as a specific marker of regulatory T cells. Since then, the subsets originally identified by the CD5 and CD8 molecules have been referred to as CD4⁺8⁺, CD4⁺8⁻, CD4⁻8⁺, and CD4⁻8⁻. The developmental relationship between thymocyte subpopulations remained unclear for many years, but at least we had the tools to investigate this issue. Strong indications that SP thymocytes may develop from DP were suggested by observations (Ceredig et al. 1983; Kisielow et al. 1984) that they appear before SP during ontogeny. However, there was no direct evidence that they contained precursors of SP thymocytes. Conversely, existing evidence suggested that DP thymocytes are “infertile” and never leave the thymus but die *in situ* (Shortman and Jackson 1974).

I handed the first draft of a paper describing our findings to Dr. Boyse before my return to Poland in 1974. After a month without hearing from Dr. Boyse, I asked Peter Beverley to help me finish the manuscript, which we sent to him, informing him that if he did not respond, we would submit the paper on our own. In response, the paper, which included several names unrelated to our discovery, was sent to *Nature* for publication and appeared in January 1975 (Kisielow et al. 1975). After my departure, Dr. Boyse discontinued the supply of antisera to others and began collaborating with Harvey Cantor, who replicated our experiments and provided further evidence that T cells identified by different CD5/CD8 phenotypes develop in mice without intentional immunization and can cooperate. Their two influential publications (Cantor and Boyse 1975a, b), which did not openly cite our experiments, overshadowed our earlier paper in *Nature*. What happened next resembled a dam breaking. The number of publications that followed, using CD5 (and later CD4) and CD8 markers to explore T cell heterogeneity, development, selection, specificity, function, mechanisms of antigen recognition, and interactions with other immune cells, grew exponentially, rarely referencing our original paper that described the discovery. In the autumn of 1975, a *Nature* report signed by Peter Medawar and Elisabeth Simpson (Medawar and Simpson 1975) pointed out the importance of our findings but did not refer to our paper. When I asked the Editors about the reason for the omission, I learned it had been accidentally removed from the manuscript during revision.

It soon became evident that we had uncovered only the tips of two icebergs because later studies showed that subsets identified by CD8 and particularly by CD4, which eliminated the use of CD5, turned out to be exceedingly heterogeneous.

The most important finding with fundamental significance for understanding the peripheral mechanism of self-tolerance was the identification of “suppressor” T cells within the population of CD4 T cells (Sakaguchi 2004), termed Treg, them from CD4 helper T cells. At the turn of the ‘70s, the application of monoclonal antibodies revolutionized the field. CD4 and CD8 T-cell lineages were identified in other vertebrates, including humans, and many new functionally important lymphocyte-specific molecules were discovered.

Back in Poland, equipped with small aliquots of CD5 and CD8 antisera, I sought to investigate the developmental potential of DP and the functional potential of SP thymocyte subpopulations. Rudolf Jaenisch generously provided me with his Moloney leukemia virus transgenic mice, which we aimed to use for tracking T-cell development. However, the poor conditions of the inadequately equipped laboratory rendered my efforts futile. At that time, studying T-cell development in TCR transgenic mice was unthinkable because the TCR itself, along with its genes, was unknown.

Spoiled by my success in the USA and frustrated by the lack of opportunities for conducting reasonable science in Poland, I decided to quit and start a new life as a gardener, but I failed. Suddenly, a fortunate coincidence (good fortune cannot be avoided!) made it possible for me to continue studying T-cell development at the Basel Institute for Immunology in Switzerland.

The discoveries made in the ‘70s (David et al. 1973; Cullen et al. 1974; Jaenisch and Mintz 1974; Zinkernagel and Doherty 1974; Erb and Feldman 1975; Kisielow et al. 1975; Köhler and Milstein 1975; Hozumi and Tonegawa 1976), including ours, equipped the researchers with new knowledge and tools to fill many gaps that blocked progress toward solving the self/nonself discrimination puzzle. The way was opened to identify the function of key molecules participating in antigen recognition by T cells, such as the TCR, class I and class II MHC, CD4, and CD8. Elucidating their role in the thymic education of T cells was crucial for understanding how a self-tolerant, self-MHC-restricted repertoire is selected.

Initially, it was thought that a negative selection of self-reacting T cells would be sufficient to ensure that the repertoire of mature T cells remained functional and non-autoreactive. It was speculated that non-self-reacting, MHC-restricted T lymphocytes could spontaneously follow their intrinsic developmental program, in which self-MHC restriction was evolutionarily imprinted. The controversial idea of positive selection emerged later when Bevan (1977) and Zinkernagel (1978) demonstrated that the MHC restriction mechanism of T cell antigen recognition is imposed in the thymus, suggesting an active process of positive selection of thymocytes by MHC antigens through an unknown mechanism.

Harald von Boehmer, whom I first met in 1980 at the Immunology Congress in Paris, recognized my interest in how CD4 and CD8 T cells are generated in the thymus and

invited me to his laboratory. At that time, it became clear that studying the development, selection, and fate of T cells required a better understanding of activation mechanisms and the identification of the surface molecules involved in this process.

Identifying *TCR* genes (Hedrick et al. 1984; Yanagi et al. 1984) and proteins (Haskins et al. 1983; McIntyre and Allison 1983) in the mid-80s opened new horizons. Other parallel findings that critically contributed to the elucidation of T-cell antigen recognition and activation demonstrated that:

- TCR ligand consists of a complex formed by MHC molecules binding and presenting peptides to T cells (Allen et al. 1984; Bjorkman et al. 1987).
- The MHC/peptide complex is bound by a single TCR having “double” specificity: one for MHC and another for peptide (Dembic et al. 1986).
- Class I MHC molecules bind peptides resulting from intracellular digestion of endogenous proteins (Nuchtern et al. 1989), while class II binds peptides of degraded exogenously derived proteins (Lamb et al. 1991).
- CD4 and CD8 molecules bind to class II (Cammarota et al. 1992) and class I (Salter et al. 1990) MHC molecules, respectively, and function as TCR co-receptors during T-cell antigen recognition and activation.
- The above discoveries identified the quintet of key cell surface molecules: TCR, MHC-I, MHC-II, CD4, and CD8 molecules, which control the activation of T cells.

Alongside these discoveries, the methods for creating transgenic and gene-knockout mice were refined and shared, opening up the possibility of clarifying the role of these molecules in generating the self-tolerant, MHC-restricted repertoire of mature T cells.

The concept of using mice that express only transgenic TCR on all T lymphocytes to validate the old postulate that the deletion of immature self-reactive lymphocytes is a central mechanism of self-tolerance likely originated from several minds. The primary challenge was to select the appropriate receptor, which is specific for self-antigen and mirrors the natural physiological receptor-ligand interaction.

Harald von Boehmer and Werner Haas had a brilliant idea that allowed us “to catch two magpies by the tail” because it provided definitive evidence indicating that both negative and positive selection of immature CD4⁺8⁺ thymocytes are responsible for shaping the self-tolerant, self-MHC restricted repertoire of T cell receptors. The idea was to use one of Harald’s cytotoxic CD8 T cell clones specific for male antigen (HY) and restricted by class I MHC molecules as a donor of transgenic *TCR* genes. The hope was that by comparing the development of HY-specific transgenic T cells in male and female mice, we could track their fate in the presence and absence of self-antigen in the system, reflecting the natural

process as closely as possible at that time. The clarity of the results surpassed all expectations.

Michael Steinmetz, Yas Uematsu, and Horst Bluthman at BII and Anton Berns with Paul Krimpenfort in the Netherlands had the necessary expertise and, after successfully conquering many hurdles, obtained the mice.

When I arrived, the first mice were born. Harald was on sabbatical in the USA, and we had to communicate by mail. Since none of the molecular biologists knew how to track T-cell development, I had the privilege of doing it. The priority was to test the clonal deletion hypothesis of immunologic tolerance by checking whether HY-TCR-bearing T cells were present in males. To my amazement, the results appeared to fulfill the predictions. Males had very few T cells and a rudimentary thymus (Kisielow et al. 1988a). It was incredible because hardly anyone expected to see such a spectacular effect. Although more control experiments were needed, our joy was immense. Harald envied me for being the first to witness it.

In males, nearly all thymocytes were deleted at the DP CD4⁺8⁺ stage. This indicated that the recognition of self-antigens by immature lymphocytes leads to their death, representing the central mechanism of self-tolerance (Kisielow et al. 1988a). However, one could still argue that the death of DP thymocytes, which are cortisone-sensitive, was a nonspecific effect of stress. To provide direct evidence that the deletion results from the binding of cognate peptide by DP thymocytes, my students, Wojtek Swat and Leszek Ignatowicz, and I performed an *in vitro* experiment in which female DP thymocytes were mixed with male antigen-presenting cells, demonstrating that it leads to HY antigen-specific death by apoptosis (Swat et al. 1991).

Antigen-specific deletion of DP thymocytes and the observation that peripheral organs contained only a few T cells strongly suggested that DP thymocytes contain T-cell precursors and that this is the critical stage at which decisions about the fate of developing T cells are taken.

The full potential of the HY transgenic mice model was revealed through analyzing T-cell development in transgenic females (Kisielow et al. 1988b; Teh et al. 1988). This analysis confirmed the existence of a positive selection mechanism suggested by Bevan’s (Bevan 1977) and Zinkernagel’s (Zinkernagel 1978) experiments, clarifying the cellular mechanisms involved. Clear evidence showed that DP thymocytes survive and develop into mature T cells only when they bind to self-MHC molecules presenting non-cognate peptides. Moreover, it is not a “single hit” phenomenon; the survival of SP mature T cells requires continuous interaction between TCR and MHC molecules: class I for CD8 (Kisielow and Miazek 1995) and class II for CD4 T cells, as demonstrated by others (Kaye et al. 1989). In females, DP thymocytes were not deleted, and the ratio of CD4 to CD8 SP T cells observed in normal mice was reversed, favoring CD8 cells, all of which expressed class I MHC (Db) restricted HY-specific

transgenic receptors. I realized I was witnessing CD8 T cells “*in statu nascendi*.” Seeing the birth of the CD8 T-cell, which we discovered 13 years earlier, was, for me, an especially joyful moment, crowning my scientific career.

Synopsis

From studies using HY-TCR transgenic mice, the following picture of T-cell selection emerged:

The TCR repertoire of mature T cells is shaped in the thymus by two opposing processes: negative and positive selection of CD4⁺8⁺ thymocytes. Their fate mainly depends on the degree of complementarity between TCR and MHC molecules presenting self-peptides. Cells expressing “useless” TCR, i.e., having no complementarity to the self MHC/peptide complex, die by spontaneous apoptosis, i.e., genetically programmed cell death. This phenomenon is

referred to as “death by neglect.” Cells expressing TCR that are fully complementary to both MHC and the presented cognate peptide and bind the complex with high affinity are also negatively selected and die by antigen-induced apoptosis *via* TCR signaling. This process is called “clonal deletion” or “central tolerance.” Cells carrying a TCR that matches a particular MHC allele, not bound to the cognate peptide, are “saved,” i.e., positively selected and protected from death by neglect; their further development depends on the class of MHC molecule. Recognition of class I molecules that cross-link TCR with CD8 molecules directs differentiation toward the CD8 lineage. In contrast, recognition of class II molecules that cross-link TCR with CD4 molecules directs differentiation toward CD4 lineages. Binding antigens by mature T lymphocytes enhances the signal generated by contact with MHC alone and stimulates them to fulfill their functions.

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