

LANDMARK

Selection, memory and selective memories: T cells, B cells and Sir Mac 1968

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In 1968, Sir MacFarlane Burnet published two papers, one in *Nature*,¹ the other in the *Lancet*² that, in an evolutionary and infectious disease context, respectively, addressed the then very recent Miller and Mitchell observations on cellular interactions in antibody formation. The system we were using was the 19S (immunoglobulin M) haemolysin antibody response to sheep erythrocytes (SRBC) in CBA mice measured in the also recently developed (and revolutionary) Jerne plaque-forming cell (PFC) assay. Much use was made of the two T-cell-deficiency models pioneered since 1961 by Jaq Miller, namely, the neonatally thymectomized mouse and the adult-thymectomized, irradiated, bone marrow-reconstituted mouse. SRBCs were the 'workhorse' antigen at the time, well suited to the Jerne PFC assay.

We had shown that thymus-derived, antigen-reactive cells (probably comprising the bulk of Gowan's recirculating pool of long-lived small lymphocytes) responded to SRBC not by production of antibody but by facilitating its production by a separate lineage of bone marrow-derived precursors of PFC. This was virtually the start of studies on *immunoregulation* at a cellular level.

The cells were termed T and B cells by Roitt *et al.*³ (We are indebted to Ivan Roitt and colleagues for introducing these 'reader-friendly' terms but, as I will indicate below, Sir Mac almost did it more than a year earlier. Why Jaq and I missed this opportunity to come up with the terms T and B cells is a source of some anguish. We laboured with thymus-derived antigen-reactive cells and bone marrow-derived antibody-forming cell precursors.)

Sir Mac^{1,2} postulated that both T and B cells have antigenic specificity in the Miller and Mitchell system and that B-cell production of antibody is influenced by nonspecific pharmacologically active substances (for which read *cytokines*) elaborated by responding T cells. In passing, Sir Mac gave us a bit of a serve for not thinking about this possibility rather than even hinting at the ridiculous notion of instruction of passive B cells by clonally individuated T cells through information transfer (e.g. V region mRNA). This, of course, led to some experiments specifically designed to prove that the great man's Clonal Selection Theory was only half right in that it pertained only to T cells (the thymus being GOD, the generator of diversity) and that the decimated instructionists may yet have their day (perhaps half day) in the sun. At the very least, a clear demonstration of such a mechanism, and a major alteration to the best hypothesis yet devised to account for adaptive immunity, memory, tolerance and immune surveillance, would probably not be a career-limiting discovery for a young PhD student! Also, it would not hurt positioning of the thymus as the key organ and orchestrator in immunology. In retrospect, the principal reason we were able to entertain (and entertainment was really what it was) the entirely heretical information transfer idea was that it actually took quite some time to demonstrate tolerance and memory (that is specificity) in *B cells*, tolerance (\pm memory) in T cells being demonstrated very early.

T CELL–B CELL INTERACTION

The central question of my PhD thesis⁴ that commenced in 1966 with Jaq Miller and Gus Nossal as my supervisors was: 'Does the peripheral lymphocyte population contain a large number of thymus-derived cells and do

these cells respond to antigen by producing a progeny of antibody-forming cells?' In this quote, the 'peripheral lymphocyte population' referred to was essentially Jim Gowans⁵ recirculating pool of thoracic duct small lymphocytes known to contain 'immunologically competent cells' or 'antigen-reactive cells' involved in expression of *both* cell-mediated immunity and antibody production on transfer to recipients. Using intra-thymic labelling, Gus Nossal, Irv Weissman and others produced evidence for migration of cells from the thymus, though the extent of such was difficult to determine. Don Metcalf's experiments commencing in the 1950s were strongly suggesting a humoral influence of the thymus on immune reactivity with little or no actual cell migration.⁶ Jaq's position on all this was clear: bone marrow-derived cells seeded the thymus where they underwent proliferation and differentiation followed by export to peripheral lymphoid organs and the recirculating pool. They were postulated to be short lived unless they encountered antigen. Despite work in chickens on the bursa of Fabricius, there was some expectation that these thymus migrants may produce antibodies. In his view, thymic humoral influences were more likely operative within the thymus, even though some experiments with thymus in diffusion chambers (performed in collaboration with David Osoba just prior to Jaq's arrival back in Australia in 1966) suggested that factors may sometimes act at a distance. My thesis work actually started as something of a Miller vs Metcalf exercise: in the friendliest of atmospheres of course!

At the time, two groups had demonstrated an interaction between thymus and bone marrow cell types in antibody production. In a complex experimental system in which thymectomized irradiated adult mice received bone marrow and thymus from donors that,

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genetically, were slightly different, Davies *et al.*^{7–9} obtained clear evidence for synergism. However, they were not able to identify the cell type(s) producing antibody: thus ‘...it may be that thymus-derived cells can produce antibody, but only in the presence of cells of bone marrow origin. Equally, cells of bone marrow origin.... may be the cells whose immunological potential is enhanced by association with cells of thymic origin. These are not problems which the present analysis can resolve.’⁹

In a much simpler system, Claman *et al.*^{10–12} demonstrated clear synergism between thymus and bone marrow cell suspensions in antibody production in irradiated mice. They were not able to determine which cell type contained the precursors of antibody-forming cells but suggested that bone marrow provides ‘effector’ cells and the thymus the ‘auxiliary’ cells. Radovich *et al.*¹³ in a similar system provided data, which they believed favoured the alternative hypothesis. Responses in these systems were well below what could be expected in an intact mouse and this had some influence on their immediate impact and integration into thinking on the inductive events of antibody production.

Jaq and I came upon cellular collaboration in antibody production through our studies on the extent of the cellular defect following neonatal thymectomy (alternatively, adult thymectomy, lethal irradiation plus bone marrow cell reconstitution) and the search for a direct contribution of cells from the thymus to the antigen-reactive cells of the recirculating pool of lymphocytes. Probably, the most compelling data on cellular interaction (plus the identity of antibody-forming cells) were obtained in adult thymectomized CBA mice, heavily irradiated and protected with syngeneic bone marrow and injected *some weeks later* with (CBA×C57Bl/6) F₁ thoracic duct cells together with SRBC challenge. PFC responses in the spleens were of a magnitude expected of *normal* mice (an important point according to some)—that is around 50–100 000 19S PFC per spleen. Using aliquots of spleen cells containing the PFC, anti-CBA serum eliminated the PFC, whereas anti-C57Bl/6 serum had no such effect.¹⁴ In a syngeneic system using chromosomally marked CBA/T6T6 bone marrow cells and CBA thoracic duct cells, Nossal *et al.*¹⁵ demonstrated with chromosomal spreads of single antibody-forming cells that the PFC carried the marker of the bone marrow donor. In reconstituted neonatally thymectomized mice, PFCs were of host origin using either chromosomal or immunogenetic markers.^{15,16}

With good data in hand we could speculate (In my first overseas seminar presentation in Mill Hill in 1969, Av Mitchison in the discussion period said that the data I had presented was some of the best he had seen and the hypothesis was brilliant; however, he saw no connection whatsoever between the two! Something for a young student to contemplate. Also, at that time and reflecting early scepticism on cell interactions in antibody production, Bede Morris of the John Curtin School famously stated that B and T were the first and last letters of (expletive deleted!)—that assuredly wasn’t ‘Burnet.’) and I will discuss these speculations in the context of interaction with Sir Mac, whom we honour on this occasion.

T CELL–B CELL–SIR MAC INTERACTION

In 1967, Sir Mac communicated our paper to *Proc Natl Acad Sci USA* with initial data demonstrating that T cells react with antigen, yet are not the precursors of antibody-secreting cells.¹⁷ In the discussion section of that *Proc Natl Acad Sci USA* paper, we posed the question ‘...do (thymus-derived cells) act as *specific* recognition agents capable of transferring to essentially *passive* bone marrow-derived cells, the *instructions* required for the synthesis of specific antibody molecules?’ (my emphases).

Clearly, this was too much for Sir Mac! In the two 1968 papers referred to above, he proposed one hypothesis only to account for this cellular interaction—nonspecific signals pass between specifically reactive T and B cells. (In passing, it is fascinating to note the contexts in which his comments about the new data on T cell–B cell interaction in antibody production were made (though it is of no real surprise bearing in mind Sir Mac’s life work).

The *Nature* paper came at it from an evolutionary perspective—the title being ‘Evolution of the immune process in vertebrates’; the *Lancet* paper was from the perspective of the pathogenesis of measles virus. To make sense of the quotes to follow, it must be remembered that the late 1960s was the height of the search for the mammalian equivalent of the bursa of Fabricius in birds. The mammalian central lymphoid organ for antibody-based immunity if it existed at all (c.f. the thymus for cell-mediated immunity) was thought to be located somewhere in the gut-associated lymphoid tissue (GALT).

Sir Mac wrote: ‘...in most or all immunological situations where antigenic stimulation is taking place we have in the relevant lymphoid tissue.... progenitor immunocytes (lymphocytes) of thymic origin (PI-T), and

progenitor immunocytes of GALT origin (PI-G)...’ ‘...one can envisage the first step as stimulation by antigen (of the PI-T cells)... to develop to pyroninophil blasts and to liberate pharmacological agents which can influence and activate adjacent cells. One of these effects is presumed to be on any PI-G cells of potential reactivity with the antigen concerned. Only when they are so stimulated do they become susceptible to react with antigen (especially with antigen on dendritic phagocytic cells) by taking on plasmablast morphology and initiating clones of antibody-producing cells.’¹ In the *Lancet* paper,² he used the abbreviations T-D immunocytes and G-D immunocytes (that is getting close to Ivan Roitt’s splendid 1969 designations of T and B cells) and wrote.... ‘my interpretation of this as a non-specific pharmacological stimulation is not mentioned by Mitchell and Miller, but seems best to fit the facts’. (In the *Nature* paper, he was probably also enforcing the subliminal serve when he said that... ‘simple interpretations are always less subtle than biological realities but they can sometimes be helpful.’)

Clearly, this was too much for Jaq! These papers and speculation (and Sir Mac’s ability to get things published so quickly) drove us to prove him wrong (though not too strenuously). In the event, Sir Mac was pretty much on the money. There are a couple of observations to be made about Sir Mac’s insight now, 40 years later. ‘Nonspecific pharmacological agents’ would immediately connote histamine and the other small molecules of immediate-hypersensitivity reactions. At that time, Sir Mac had been speculating that mast cells may be thymus-derived cells following observations of large number of mast cells in the thymus, if I recall in hybrid NZB mice. There was also John David’s newly discovered factor (actually an ‘activity’), macrophage migration inhibition factor in T-cell-dependent delayed-type hypersensitivity reactions and Don Metcalf’s thymus-derived ‘lymphocytosis-stimulating factor’ of around 10 years standing (also an ‘activity’).

At the time, the word ‘cytokine’ was some years away. (There was a word sometimes used in this regard, this being ‘chalone’ (again, my memory is being tested.)) Why did Sir Mac speculate that ‘the B cell’ had to be activated by ‘the T-cell cytokine’ to *become* antigen reactive rather than being antigen reactive *ab initio*. In other words, the emphasis was on ‘conditioning’ and recruitment of the B cell by T-cell factors rather than B-cell proliferation and differentiation to antibody-secreting plasma cells after antigenic stimulation.

Without knowing of course, there may be two reasons. I suspect his thinking at the time was dominated by the fact that so many antibody responses to so many antigens (wrongly as it turns out) were considered to be T-cell independent and therefore a T-cell influence on *some* B cells pre-antigen recognition was more likely than any obligatory T-cell influence on all B cells after antigen triggering.

The second reason for invoking a T-cell influence on B-cell recruitment may be more interesting—he was avoiding a need to give credence or emphasis to Don Metcalf's notion that thymus humoral factors act in concert with antigen to accelerate the proliferative and differentiative response of antigen-reactive cells.¹⁸ Just on this point, Jaq Miller was emphasizing the alternate idea that any thymic humoral factor (from thymus epithelial tissue) was involved intra-thymically in maturation of antigen-reactive cells from some precursor cell population.¹⁹ Also of interest is that Sir Mac incorporated a role for antigen located in follicles and germinal centres to account for the contemporary work of Gus Nossal, Gordon Ada and colleagues at The Walter & Eliza Hall Institute.

Why did Jaq and I pursue the notion for some time that T cells were clonally individuated and that B cells may receive their instructions from T cells? Of course, we also proposed other mechanisms and were not wedded to heresy. The range of possibilities we canvassed to account for T cell–B cell cooperation was as follows,^{4,17,20} all of which had precedents or at least were not entirely fanciful:

Nonspecific influence

- (1) Trophocytic function—T cells are a source of feeder nucleosides or other nutrients and precursor substances for proliferating B cells;
- (2) Phagocytic function—T cells transform to macrophage-like cells responsible for antigen processing for B-cell recognition;
- (3) Pharmacological—T cells liberate factors that promote recruitment, differentiation or proliferation of B cells and
- (4) Absorption of carrier molecules—T cells absorb 19S antibody and through subsequent binding to carrier determinants 'orient' haptenic determinants of antigens for B cells.²¹ This hypothesis derived from the phenomenon known as the 'carrier effect' of Mitchison²² and the data of Henry and Jerne²³ on immunoenhancing effects of 19S antibodies.

Specific influence

- (5) Information transfer (see below)
- (6) Antigen recognition and 'focussing'—the carrier effect (see below).

At the time of our initial observations (that is 1967–1968), there was no lack of speculation/demonstration that 'inducer factors' were a feature of some immune responses with candidates being 'informational low MW RNA', RNA-antigen ('superantigen') complexes, DNA, episomal genetic elements, 'viral antibodies', etc. (I recall as a post-doc at Stanford a wonderful exchange at a 1970 meeting in California between Dan Campbell and Frank Dixon, the latter being unable to repeat Dan's experiments on immune effects of nucleic acid preparations. After a fairly robust if not heated discussion, admirably typical of our US colleagues and quite enlightening for me, Frank finally said, 'Well Dan, my DNA is obviously different from your DNA, a fact for which I am sure we are both eternally grateful.') Moreover, we were emboldened by the fact that there was absolutely no demonstration at the time of either memory or tolerance in B cells, only tolerance (\pm memory) in T cells. We designed an experiment to reinforce our wild speculation and to discount Sir Mac's idea.

We induced tolerance in donor mice using the SRBC plus cyclophosphamide regime of Dietrich and Dukor.²⁴ As a source of tolerant T cells, we took thoracic duct lymphocytes from these mice and injected them into neonatally thymectomized mice together with SRBC and horse erythrocytes (HRBC) (horse and sheep erythrocytes having been shown by Alastair Cunningham to be non-cross reactive). We reasoned that, if Sir Mac was right, the HRBC should stimulate (non-tolerant) HRBC-reactive T cells to liberate their 'nonspecific pharmacological factors/cytokines' that would promote anti-SRBC antibody by (non-tolerant) B cells in the host.

All controls 'behaved' perfectly. The anti-SRBC PFC response of reconstituted thymectomized recipients of non-tolerant thoracic duct lymphocytes challenged with SRBC+HRBC was around 60 000 PFC per spleen. The anti-SRBC PFC response of recipients of SRBC-tolerant thoracic duct lymphocytes challenged with both erythrocyte types was 3–5 \times lower and, importantly, there was no significant difference in recipients of tolerant T cells whether challenged with SRBC alone or SRBC+HRBC. However, looking back at the data, it could be said that they were somewhat inconclusive.^{4,20,25}

The death knell came upon all this speculation on information transfer with clear

demonstrations of tolerance in T and B cells (e.g. Chiller *et al.*²⁶) and memory in both (e.g. Mitchell *et al.*²⁷ using allotypic immunoglobulin markers and anti-theta (Thy1) sera). Moreover, all the phenomena described as the 'carrier effect' by Av Mitchison and Martin Raff could be accommodated readily by T-cell carrier specificity and B-cell hapten specificity with a 'second signal' delivered by T cells: except the small matter raised by Bretscher and Cohn²⁸ of two rare cells with specificity finding each other, an issue rendered somewhat irrelevant by mobility of T cells, anatomical facilitation of interaction and clonal expansion.

(As a postscript, Jaq has reminded me that, with regard to information transfer, we did receive some encouragement from none other than Nobel Laureate, Jacques Monod:²⁵

'To speculate, let us say that there are Ig genes with a constant region (C) gene somewhere and a number of variable region (V) genes elsewhere. The first assumption is that there are cells which differentiate in such a way that the V region in them tends to be excluded out of the chromosome into an episome. This is nothing very extraordinary: we know of cases of the sort in bacteria. I would further add that this is true of lymphocytes in particular, but it is more or less true depending on which class of lymphocytes you are looking at. The properties of thymus cells would be such that in these cells this particular genetic region will exist mostly in the episomal form. So we have an interpretation of what your interaction means: it is a transfer of information, but information concerning only the V region. Therefore this is not contradicted by the fact that the allotype comes from the host... . The bone marrow cell may be one where differentiation is such that the episome tends to be inserted back very quickly into the system.'

REFLECTIONS

This Symposium and Special Issue of *Immunol Cell Biol* are a celebration of the genius of Sir MacFarlane Burnet, who influenced a multitude of fields and people and whose Clonal Selection Theory of Antibody Diversity has stood the harshest test—that of time. On many occasions, Gus Nossal has spoken of his mentor as the personification of 'science is primarily about ideas'. Sir Mac certainly did not lack ideas, rarely enunciated in discussion after a seminar or in a group (at least when I knew him as an older man—that is Sir Mac!), but rather in the tranquillity of his study or office with reference material all around; very much like my first mentor, primary PhD supervisor and good friend, Jaq Miller.

I have often recounted an anecdote about my four mentors in the late 1960s at The Walter & Eliza Hall Institute—Jaq Miller, Gus Nossal, Don Metcalf and Noel Warner. With a new discovery in hand and taking that discovery to Jaq, he would outline three repeat experiments which must be performed before we said anything; to Don, he would provide three alternative interpretations of the data than the one I was promulgating; to Noel, he would give me three references that were highly relevant to my discovery such as his encyclopaedic knowledge of the literature; and to Gus, he would leave me in no doubt after 3 min that I should craft my Nobel Prize acceptance speech right now, such was his enthusiasm. The nice thing about this story is that I can transpose most of the names with most of the responses without doing any injustices or taking any liberty with the truth!

What would Sir Mac have said if he had been a close mentor—rather than being across the road in the Department of Microbiology of The University of Melbourne keeping well out of the way of his successor, Gus, and enabling the latter to effect the dictum, ‘New Director, New Directions’ unencumbered. He would have had no immediate response. Subsequently, he would have urged me to put the observation/discovery in an evolutionary context, a self—non-self discrimination context as appropriate or a host—pathogen infectious disease context. (In a later era for me as an immunoparasitologist, Burnet’s writings on the evolution of host—parasite relationships from an immunological perspective were our starting point (in 1975)—a solid conceptual biological framework on which to tackle the immunology of this relationship looking for the Achilles’ heel to exploit in terms of vaccines. We were only partially successful²⁹ but this was more our naivety unlikely to be worthy of Sir Mac’s prescience. However, like T cell—B cell interactions, our studies on host—parasite interactions at The Walter & Eliza Hall Institute yielded a wealth of new information and were a source of great enjoyment because of a brilliant team, different personalities, huge challenges, good funding and unstinting support from the Director.)

On the issue of specificity in T and B cells and the broad concept of cytokine influence of one on the other—and where we had some minor jousting—Sir Mac was right of course. But so was Jaq and so was Don. Sir Mac got it wrong regarding T-cell effects on B-cell pre-antigen stimulation of the B cell; Don Metcalf was right in that *one* humoral ‘thymic influence’ is at the level of post-antigen stimulation of the B cell; Jaq Miller was right in

that this particular thymic influence is mediated by exported T cells. Both T and B cells react to antigen and *short-range* T-cell cytokines (in keeping with the hapten-carrier phenomenon), with no antigenic specificity, influence B-cell behaviour post-antigen stimulation at several levels.

My own pet hypothesis before I left the field in the mid-1970s was that T-cell lymphokines activate macrophages to remove antigen and reduce its tolerogenicity for high-affinity B cells and that the actual ‘second signal’ (that is the triggering event for B cells) follows from protease release by T cells that, acting at close range, deaggregate antigen-induced B-cell receptor clustering.^{30,31} No wonder one took up the simple problem of host—parasite relationships and parasite vaccine development!

As indicated above, demonstrations of cell—cell interactions in antibody formation and the clear identification of the precursor of antibody-secreting cells (that is the B cell), virtually initiated the field of immunoregulation at the cellular level. Soon after, cellular immunology went through a period that, in my view, was unfortunate. Without any idea of how cells communicated with each other—it would take molecular genetics and good protein chemistry/structural biology at a later date to pin down molecular events—we all came up with some elaborate schemes. I recall many diagrams with arrows going in all directions and all sorts of T cells imagined—helpers, suppressors, contrasuppressors, helpers of helpers, etc etc. Any antibody response that went up or down was ascribable to a raft of interacting cell types with the focus only on immune *induction*. The cartoons of molecular events and signals between cells had something of a ‘spaghetti and meatballs’ appearance. I would suggest that this was not the ‘high table’ of cellular immunology and, happily, a better menu came later.

CONCLUDING COMMENT

This account will seem rather quaint in the context of modern immunology and to someone entering the field in 2007. My only objective is to paint a picture of the prevailing circumstances and state of knowledge (pre-molecular biology) to account for a literature that, 40 years hence, still makes for some fascinating reading. One thing is for sure—only commit to writing that which you will still be proud of decades later because someone is likely to revisit it all as I have just done. In passing and also on a lighter note, I have additionally tried to give some hint of several interpersonal dynamics involving Sir Mac in

the late 1960s: of course, in the great sweep of history these are trivial and irrelevant but, at the time, quite important in being highly motivational!

I hope these reflections while honouring the memory of Sir Mac (and the 50th anniversary of the Clonal Selection Theory) will also resonate with current and prospective practitioners determined to prove through their own creativity and inventiveness that the golden era of immunology was not way back then but is just around the corner. In any event, I hope like myself in 1966, those starting their scientific career in 2007 will be beneficiaries of the influence of giants such as Sir Mac, Jaq Miller, Gus Nossal, Don Metcalf *et al.* One could be so fortunate even as I have moved, and continue to move, some distance from the field though following it with fascination—one never forgets one’s first love!

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