ORIGINS OF B CELL LINEAGES: ASPECTS OF THE DIFFERENCE BETWEEN B-1 AND CONVENTIONAL B CELLS IN M54 μ TRANSGENIC MICE

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For many years, all of the available evidence on lymphocyte differentiation was consistent with the idea that all lymphocytes and myeloid cells in mouse and man are derived from a single, pluripotent stem cell population that arises during fetal life and persists unchanged thereafter as a self-replenishing population, resident principally in the bone marrow in adults. Recent studies, however, demonstrate that the regenerative potential of the hematopoietic stem cell population in adult mice is more restricted than in fetal animals. In essence, these findings (reviewed in 1,2) show that certain B and T cell

H. Nariuchi *et al.* ed: Molecular Basis of Immune Responses. Academic Press(1993)

subpopulations are actually separate lineages that derive from independent progenitors.

We see these data as evidence that the evolution of the immune system brought a series of stem cells into existence that sequentially give rise to lymphocytes that are similar to their predecessors but have either lost or gained functional capabilities. Since the evolutionary success of the latest "layer" in such a system depends on its ability to introduce a selective advantage, each new layer can be expected to increase the sophistication of the immune system with respect to its ability to efficiently protect the animal against challenges within its environment. Thus, while B-1 cells tend to create a first line of defense by producing low affinity, broad-specificity antibodies that react with ubiquitous micro-organisms, conventional B cells produce high affinity antibodies that specifically react with particular pathogens. Similarly, the repertoire of γδ T cells is considerably more restricted than the repertoire of αβ T cells.

This concept of an evolutionarily layered immune system presents a framework within which the B cells that we have defined here can be organized and related to the T cell lineages defined in other recent studies. Thus, it offers a potentially productive route for unraveling the complexities of an immune system that has evolved to provide a multiplicity of functions concerned with protecting against attacks from without and from within.

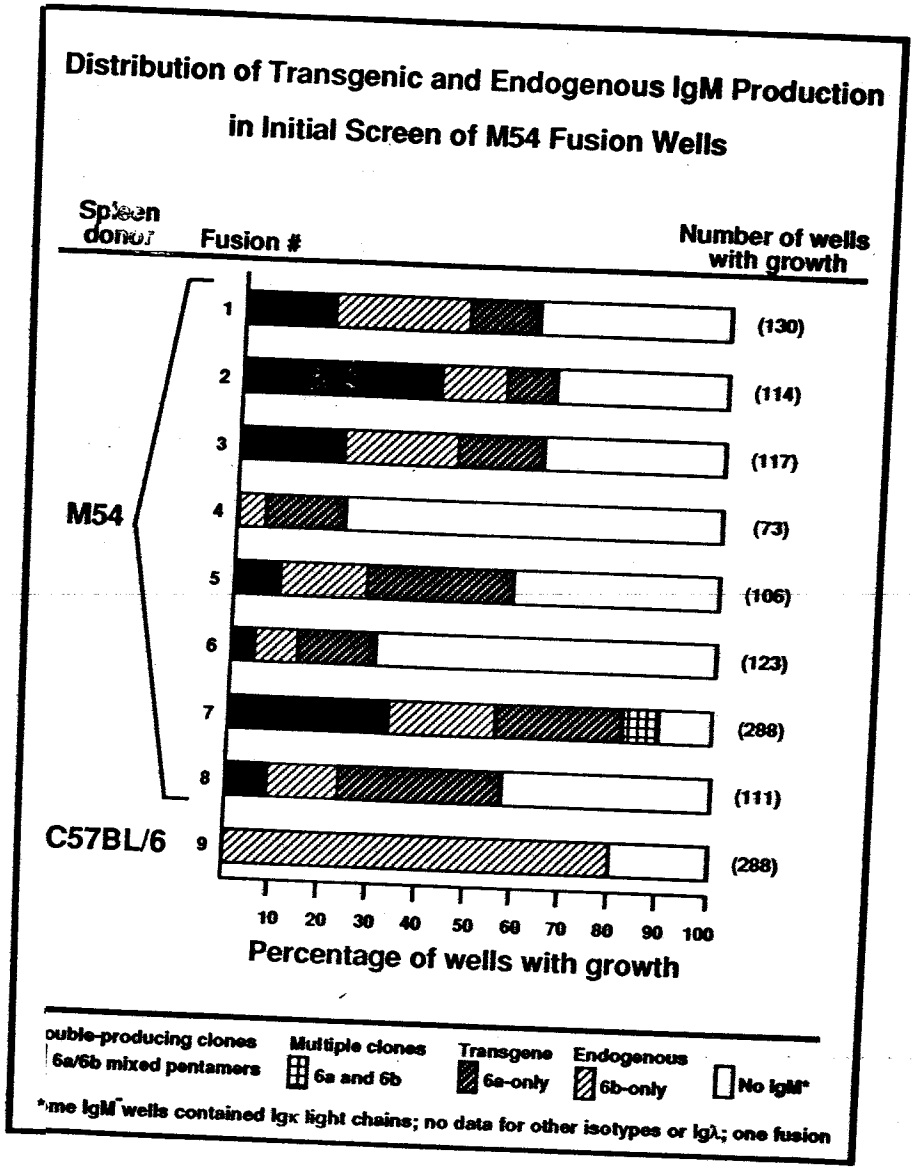
The studies that led us to this formulation have by and large centered on defining the characteristics and the origins of the B-1 and conventional B cell lineages. We have recently reviewed this work in detail (1,2); therefore, we will use the space allotted here to discuss our most recent findings, which focus on the differences between B-1 and conventional B cell development and Ig rearrangements in the M54 transgenic mouse line, which carries the 17.2.25 immunoglobulin μ heavy-chain gene.

M54 transgenic mice were initially produced to study the mechanisms involved in allelic exclusion. Later, when they were found to rearrange and express endogenous μ chains in addition to expressing the transgene-encoded IgM, Weaver, Baltimore, Imanishi-Kari and colleagues (3) used them to produce a series of hybridomas that would allow exploration of the influence of the transgenic IgM on the generation of the immune repertoire.

Our M54 studies, begun after this work appeared, demonstrated that B cell development is severely impaired in M54 mice and that many of the B cells and plasma cells that express endogenous IgM in these mice are "double-producers" that simultaneously produce transgenic and endogenous μ molecules (summarized below) (4,5). Consistent with this, we showed that "mixed" IgM pentamers containing both endogenous and transgenic μ heavy chains are readily detectable in M54 sera. Since Weaver *et al.* (3) failed to recover double-producing hybridomas, the evidence we presented demonstrating a high frequency of double-producers in M54 mice was taken as support for the highly publicized challenge raised to those studies (6).

Transgenic and endogenous μ heavy chains are in/on the same cells in M54 mice

B Cells	Double-expressing cells that carry both endogenous and transgenic μ are detectable by FACS analysis.
Plasma Cells	Plasma cells containing both endogenous and transgenic μ are detectable in spleen; plasma cells containing transgenic μ and endogenous IgG or IgA are also detectable in spleen or gut associated lymphoid tissue. These double-expressing plasma cells are visible either in cyto-spin or histologic sections.
Serum Ig	Hybrid Ig molecules are detectable in serum by ELISA.
Hybridomas	Fusions with M54 spleen cells yield many double-expressing hybridomas that have mRNA for transgenic and endogenous μ chains and secrete mixed IgM pentamers containing both heavy chains. These hybridomas are *highly unstable* and rapidly lose the ability to produce one or the other μ chain.



Our recent studies, which primarily focus on the disgulation of Ig rearrangement in M54 mice, resolve the apparent contradiction between our findings and Weaver *et al.* We have now shown that although double-producing hybridomas can readily be generated from M54 mice, they are are extremely unstable and rapidly yield variants producing either transgenic or endogenous μ. Thus although we detected double-producers in the majority of the initial hybridoma supernatants, the *stable* cloned lines we obtained were, as in Weaver et al (3), almost all single producers. In fact, a fair number of the wells that contained double-producers when tested initially lost the ability to produce one of the heavy chains when tested subsequently (prior to subcloning).

Double-producing hybridomas were identified in the initial screen by a two step procedure in which wells that contained both transgenic and endogenous IgM heavy chains were re-tested to determine whether they contained "mixed pentamers" in an ELISA designed to specifically detect such molecules. In all fusions but one (#7), wells that scored positive for the two types of IgM always contained mixed molecules. In fusion #7, plating efficiency was very high. Therefore, all wells contained at least one clone and many wells contained several clones. Thus, in this fusion, wells were detected that contained "pure" endogenous *and* "pure" transgenic IgM pentamers (produced by independent clones) but did not contain the mixed pentamers required to give a positive signal in the assay designed to detect these molecules.

To further verify the double-producing wells, and to remove any doubt that the ELISA and FACS-staining assays that we used for these studies independently detect transgenic and endogenous μ chains, we did southern and northern gel analyses that demonstrated that double-producers had mRNA and genomic DNA for each of the μ chains they produced whereas single producers derived from these doubles had only the single message and genomic sequence corresponding to the μ they produced.

The demonstration that double producing hybridomas are consistently readily obtained from M54 mice but very difficult to maintain is sufficient to explain the discrepancy between our finding of double-expressing B cells in M54 mice and Weaver et al's failure to detect such hybridomas in their studies. The issue of whether the failure to have an assay that would detect mixed pentamers corrupted the data presented by these investigators thus becomes moot. There is little likelihood that any of the hybridomas that they characterized were producing anything

other than the Ig molecules that were recognized with the assays used.

We have not extensively investigated the other findings reported by Weaver *et al.* (3); however, our data are consistent with essentially all of these findings. For example, the initial studies by Weaver *et al.* (3) and extended by Iacomini *et al.* (7,8) demonstrated that hybridomas recovered from M54 mice disproportionally express V_H genes from the proximal (V_HQ52 and V_H81X) V_H families. We found a similar skewing in that 4/6 hybridomas that we characterized for V_H gene family expression also expressed V_H genes from these families. This skewing is consistent with the idea that the V_H in M54 mice is specially selected, perhaps by a mechanism influence by the presence of transgenic IgM.

Weaver et al presented anti-idiotype data suggesting the skewing might reflect idiotype network interactions that resulted in the production of endogenous idiotypes that mimic idiotype(s) expressed by the transgene. However, although this possibility is intriguing, we chose not to investigate the *in situ* M54 idiotype repertoire for three reasons. First, conventional rabbit and guinea pig anti-idiotype antibodies such as those used by Weaver et al are not easily produced and vary sufficiently from lot to lot to make their use highly problematical, particularly when trying to repeat earlier work. Secondly, the V_H genes that are over-represented in M54 animals are known to code for Ig molecules that frequently cross react with a variety of antigens, including other Ig molecules, and thus could mimic idiotype mimicry. Finally, these kinds of studies are difficult with sera or cells from intact M54 mice (rather than with hybridomas) since M54 mice have sizable numbers of double-producing cells that carry or secrete mixed pentamers that would indeed confuse anti-idiotype studies.

The completion of the hybridoma work and the the validation of our earlier demonstration of double-producing B cells in M54 mice cleared the way for our most recent work, which demonstrates that the μ transgene differentially affects Ig rearrangements in the two B cell lineages (conventional and B-1). In essence, we have now obtained cell transfer and FACS phenotyping data that show that the expression of endogenous μ (in the presence or absence of transgenic μ) in M54 mice is essentially restricted to cells of the B-1 lineage. Few (if any) conventional B cells express endogenous μ in these mice. This restriction is particularly evident in recipients of M54 bone marrow, where there are essentially no cells producing the endogenous Ig encoded by the M54 IgH chromosome. These findings are not universal with μ transgenic mice; however, similar results have been obtained with the SP6 (9) line and some others.

The μ heavy chain transgene interferes with B cell development in M54 mice

	In situ	Developing in recipients* of M54 bone marrow
Conventional B cells	Pre-B and B cells depleted Some B cells mature Almost all express transgene μ Very few express endogenous μ	D-J rearrangement impaired Some B cell reconstituted All express transgene μ None express endogenous μ
B-1 cells	>95% express endogenous μ Many also express transgene μ VH gene repertoire is skewed	No reconstitution

*Lethally irradiated recipients reconstituted with M54 bone marrow.

Further examination of the IgH rearrangements in M54 conventional B cells (sorted from the bone marrow recipients) suggests that the failure to express a functionally rearranged, endogenous VDJ is due to expression (of the membrane form) of the transgene at a very early stage of development, essentially at or before the D_H-J_H rearrangement occurs. This initial IgH rearrangement normally occurs on both chromosomes in all splenic B cells. However, we find that it occurs on only 60 percent of the IgH chromosomes in (FACS-sorted) M54 conventional B cells. Forty percent of these chromosomes still have D_H and J_H in the germline configuration, even though the cells successfully

rearranged a functional light chain which is expressed as IgM molecules in association with the transgene-encoded μ chain.

The bulk analysis methods used thus far in these studies cannot distinguish between random interference with D_H-J_H rearrangements and the selective prevention of both D_H-J_H rearrangements in 40 percent of the cells. However, in either case, the data demonstrate that transgene expression in M54 mice can block IgH rearrangement at a very early stage, a stage in fact that pre-dates commitment to expression of one of the two IgH chromosomes in the cell (allelic exclusion).

These findings suggest that there may be important developmental differences between B-1 and conventional B cells. The premature expression of membrane μ in conventional B cells appears to selectively block IgH rearrangement in conventional B cells and, in a small number of cells, to allow development to maturity without expression of a functional IgH rearrangement. B-1 cells apparently ignore this signal since the majority of the cells in the M54 B-1 population express endogenous μ heavy chains. We do not as yet know whether B-1 cells can rearrange and express a μ chain even though their D_H-J_H rearrangment is partially blocked (on one IgH chromosome) or whether B-1 cells develop normally and complete both D_H-J_H rearrangements before starting the next phase of the rearrangement process (to V_H-D_H-J_H); however, in either case, the premature expression of the transgenic μ chain appears to have a considerably different impact on the development of conventional and B-1 cells.

The differences between B-1 and conventional B cells probably reflect relatively large differences in the times at which these B cell lineages evolved (1,2, 10,11). B-1 cells appear to be quite "primitive" in nature and to behave in many ways like avian B cells. Conventional B cells, in contrast, appear to be evolutionary late-comers that made their appearance in mammals and became the predominant B cell lineage in most mammalian species. Thus, it is not surprising that a variety of conditions (e.g., temporally inappropriate expression of rearranged μ heavy chain) that do not interfere with the development of the more "primitive" B-1 lineage may be able to block development of the evolutionarily more advanced conventional B cells. Recognition of these kinds of differences is difficult; however, it is crucial to studies of B cell development and to an understanding of the roles of the B cell lineages in the immune system.

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Discussion

Q. Tada: We have been learning from you for several years about the nature of Ly-1 B cells; lineage difference, gene expression, and the development. But still we have not been informed of your opinion on the regulatory role of the Ly-1 B cells. Several years ago we suggested that the Ly-1 B cells may have a regulatory role in the production of certain idiotypes. Tell us what you think about their regulatory roles.

A. Leonore Herzenberg: Kyoko Hayakawa demonstrated the existence of the Ly-1 B cell and some relationship to regulation of allotype expression while in Dr. Tada's laboratory. In following that we found the Ly-1 B (B-1) lineage. When we treated the B-1 cell with antibody to allotypes early in development, we depleted the entire B-1 population. When we treat it with an anti-IgM allotype reagent allotype in allotype heterozygotes, we deplete half the B-1 population. A feedback regulation prevents those cells from coming back, thus permanently depleting the B-1 cells that were initially depleted by the treatment antibody. Conventional B cells recover to normal levels. We believe that that is the key to the allotype suppression. The implication is that that is also the key to idiotype regulations as Rajewsky and Eichman talked about when they were treating neonates. In the absence of the depleted B-1 cells, we induced a suppressor cell. I want very much to go back to working on that problem because I think these cells are key to the regulation of immune responses.