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A bird's eye view on human B cells

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## Summary

We show in this review that there is a continuum between the chicken B cell system classified as the first GALT model described and the human B cell system. We propose that humans have conserved for one B-cell subpopulation, the marginal zone B-cell subset in charge of T-independent responses, the strategies of diversification used by GALT species to generate their pre-immune repertoire.

When the chicken model of B cell development was described at the molecular level, it provided a different version of the GOD's (generation of diversity) solution, introducing gut-associated lymphoid tissues (GALT) as the site where the all pre-immune B cell repertoire is produced by post-rearrangement diversification processes<sup>1</sup>.

At that time the GOD's enigma seemed more or less solved by the mouse model which had showed that Ig diversity was generated by the « roulette of rearrangement »: a few hundred V genes recombining to a small number of D and/or J elements in a random fashion, allowing the generation throughout life in the bone marrow of an infinite number of B cells, each of them carrying theoretically a different Ig receptor<sup>2</sup>.

In the chicken several steps appeared strikingly different<sup>3</sup>:

- 1) Rearrangement is used to produce a quasi-monoclonal population of B cells, each of them having rearranged a unique functional  $V_H$ - $V_L$  pair.
- 2) Ig gene rearrangement occurs during a short period of time during embryonic development and targets a few million progenitors. A few hundred thousands B cell progenitors having performed a productive H-L rearrangement localize in the epithelium of the bursa of Fabricius and proliferate within bursal follicles (10,000 follicles with 1-10 founder cells). A few days before hatching and several weeks after, bursal B cells start to migrate to the periphery, generating the B cell compartment of the animal.
- 3) Diversity is generated on this  $V_H$ - $V_L$  pair by ongoing gene conversion involving upstream pseudogenes, starting during embryonic development and thereafter during the first months of life in the bursa, a primary lymphoid organ that involutes and disappears around 6 months after hatching.

GALT models of B cell development were further discovered in mammalian species with the molecular description of the sheep and rabbit B cell immune system (table 1). The search for a « mammalian bursa» had been the goal of the pioneers who described the chicken immune system. Their first choice was the rabbit (rightfully, see below), having shown that removal at birth of appendix and other GALT (Sacculus rotundus and Peyer's patches) reduced considerably the B cell response in this species<sup>4</sup>. However at the same time, it was shown that, in the mouse and human models, transfer of bone marrow precursors could restore the B cell lineage of the receiver<sup>5,6</sup>. The description of an ontogenetic process leading to the generation of B cells in the mouse bone marrow consolidated further the model and the idea of a mammalian bursa-equivalent was abandoned<sup>7</sup>.

### Bursa-equivalent primary organs in mammals

In readdressing the question of the existence of a mammalian bursa, we were motivated by the work of Reynolds and Morris who showed that Peyer's patches were of two sorts in sheep: the ileal Peyer's patches, which morphologically and functionally behaved like a primary lymphoid organ, and the jejunal Peyer's patches, which on the contrary possessed all the properties of secondary lymphoid organs<sup>8</sup>.

The study of the light chain locus during development indeed showed that the sheep used its ileal Peyer's patches to generate its B cell pre-immune repertoire with similarities and differences with the chicken model<sup>9,10</sup>.

1) Rearrangement occurs in sheep during a short window of development during fetal life, mostly in the fetal liver and in the spleen. The light chain lambda locus (the major light chain isotype) contains around 100 genes among which approximately half are functional, but 2  $V\lambda$  genes contribute 50% of the rearrangements. A few million cells having performed a productive rearrangement reach the ileal epithelium during fetal life and induce the formation of 100,000 follicles in which they proliferate for several months after birth.

2) During proliferation in ileal Peyer's patch follicles, all B cells diversify their Ig receptor by somatic hypermutation, the very process used by most species during affinity maturation for the response to T-dependent (TD) antigens.

3) Similarly to what occurs in the chicken, Ig diversification starts in the antigen-free environment of the embryo and goes on for several months after birth as an antigen- and T-independent process. Diversified B cells migrate to the periphery and install the B cell compartment for the whole life of the animal. Ileal Peyer's patches involute and completely disappear after one year while the jejunal Peyer's patches remain throughout life.

The rabbit model was shown by the group of K. Knight to be another variation on the GALT theme, confirming that this mode of B cell development had been conserved in the mammalian kingdom. At the genomic level, the heavy chain locus was very « mouse-like », as it includes a large family of functional  $V_H$  genes, but all  $V_H$  genes, as in the chicken, belong to a single  $V_H$  family ( $V_{H3}$ ). Instead of rearranging at the same frequency any one of these genes, the most proximal  $V_{H1}$  gene is rearranged in 80% of its B cells. This  $V_H$  gene is thereafter diversified by a « chicken-like » ongoing gene conversion process involving upstream functional or non-functional  $V_H$  genes while the CDR3 region is diversified by a « sheep-like » hypermutation mechanism<sup>11</sup>. As in the chicken, rearrangement occurs essentially during early development while diversification by gene

conversion occurs mainly in appendix follicles<sup>12</sup>. Contrarily to the chicken and to the sheep, B cell proliferation and diversification only start after birth and the GALT remain once the B cell compartment is installed after 2-3 months, shifting their activities from that of a primary organ of diversification to that of a secondary lymphoid organ with clear germinal centers involved in T-dependent immune responses.

As for the lymphoid organs in which these events take place, we have proposed, on the basis of experiments performed on germ free sheep and on ileal loops realized on fetal sheep, that gut bacterial constituents essentially provide the B cells present in follicles with a proliferative signal rather than with an antigenic stimuli<sup>10</sup>. A similar proposition was recently formulated for the rabbit model in which it was shown that it is the presence of these bacterial constituents in the gut that permits the initiation of this proliferative phase in the GALT, most probably again due to their mitotic enhancement capacity<sup>13,14</sup>. It is therefore tempting to associate at the evolutionary level these post-rearrangement diversification processes requiring many cycles of proliferation and selection with an organ in which this proliferation can be naturally maintained. Others GALT models have since been described, in cattle, pigs, dogs, and it is highly probable that a large number of mammalian species uses this strategy to generate their B cell repertoire<sup>14</sup>. Another surprising property of the GALT species is that once their B cell compartment is installed at the periphery at the end of development, there is no more *de novo* production of new B cells, implying that this peripheral compartment is sufficient to protect the animal for its life time. How is this peripheral compartment maintained is not known at the moment. It must be composed of memory and naive B cells but whether the latter ones can self-renew or are maintained by a pool of progenitors with stem cell properties is an open question. Human and mice would have thus evolved this luxury to continually produce B cells with the possibility that any new antigen being encountered (Mel Cohn's moon dust<sup>15</sup>) may be taken care of by any one of these newly produced VDJ recombinations.

At the evolutionary level, whether diversity is stored in V pseudogenes or functional genes and whether the mechanism of diversification of the pre-immune repertoire is ongoing rearrangement or ongoing gene conversion and hypermutation does not make much difference. In such sense the chicken and the rabbit pseudoV genes are indeed functional donors of diversity for an acceptor V gene. Nevertheless there is a great difference between an ongoing rearrangement process involving mainly a variability at the CDR3 level and the variability generated on the three CDRs by gene conversion or hypermutation. Surprisingly enough, hypermutation appears to be a fully efficient mechanism to generate a pre-immune repertoire. This comes from two different processes:

first, a co-evolution of the mutation mechanism and of the target V gene sequence, by which mutation hotspots leading to replacement mutations are specifically clustered in CDRs, thus allowing a strong concentration and a high R/S ratio of CDR mutations<sup>10,16</sup>. Second, a constant selection for cells with a functional BCR at the surface that can provide the appropriate tonic signal, ensuring the counter-selection of deleterious mutations in framework regions (and a low R/S ratio of framework mutations). Both of these features result in a mutation selection pattern very similar to the one observed during antigen-selected immune responses<sup>10</sup>.

### **A T-independent diversification pathway in humans**

In mice and humans, germline antibodies display a rather low affinity for their specific antigens and show some polyspecificity. It has been proposed that this loose specificity could be explained by the flexibility of their combining site. According to this model, affinity maturation and selection, after encounter with its specific protein antigen, should allow for the fixation of this antibody-combining site to its ligand, thus suppressing its flexibility and polyspecificity<sup>17</sup>. The most obvious explanation for this strategy would be to preserve the pre-immune repertoire from the holes of negative selection. Included in this scheme is the fact that immune responses to non-protein antigens will only raise germ-line antibodies responses.

In the GALT models all types of affinities will emerge from the primary repertoire and in fact most de novo produced B cells will die in the GALT and only around 5% will migrate to the periphery. Immune responses to proteins antigens will still be improved by an additional diversification step in germinal centers by hypermutation and/or gene conversion. Immune responses to T-independent antigens on the other hand will theoretically generate high affinity responses with mutated antibodies. In human the T-independent responses represent a paradox since they are by definition against non-protein antigens and therefore germinal center-independent. Strikingly, antibodies raised by T-independent vaccines such as bacterial capsular polysaccharides are usually mutated and, to explain this paradox, it is proposed the rather unlikely scenario that these bacterial polysaccharides in each vaccine preparation may be coated with bacterial proteins that would drive them into a T-dependent hypermutation process in germinal centers<sup>18</sup>. Another explanation could be that some specialized B cells may in human mutate their Ig receptor after stimulation by T-independent antigens. A third explanation which we favor and which does not exclude the latter one is that the B cells in charge of T-I responses in humans already mutate their Ig receptor during their postnatal development, as it occurs

in rabbit, before their immune response to external antigens. In this last scheme, the TI-antigens would select for B cell clones with the best affinity in the splenic marginal zone and induce a second round of hypermutation after stimulation.

We have come to this proposition because of the following observations<sup>19,20</sup>:

- 1) Hyper-IgM patients with either CD40L or CD40 deficiency, which do not have germinal centers, possess a population of circulating IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> B cells with a mutated Ig receptor, in the complete absence of CD27<sup>+</sup> IgM-only and switched memory B cells. In both type of patients the frequency of Ig mutations observed in these cells is very similar to the one observed in controls although the number of IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> blood cells is in general lower in the patients as compared to normal children. The absence of CD40 or CD40L may alter B cell development by hampering the network of cytokines interactions involving dendritic cells, activated T cells and natural killer cells. Nevertheless the fact that these patients never display any CD27<sup>+</sup> IgM-only B cells indicate that the IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> B cells present are not produced in some cryptic germinal center structures, but rather belong to a separate population. Conversely, in AID-deficient patients in which germinal centers are formed but B cells are unable to activate hypermutation and isotype switching, there is an expansion of the circulating IgM-only CD27<sup>+</sup> cells, which can amount to 10% of their total B cells whereas such cells are extremely minor in normal individuals.
- 2) Phenotypic analysis and gene expression profiling indicate that blood IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> cells are circulating marginal zone B cells.
- 3) After vaccination with a non-conjugated pneumococcal vaccine, blood and splenic IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> B cells are specifically mobilized in T-independent responses. This response is taken care of by mutated antibodies. Upon expansion of these cells in the splenic marginal zone during the week following vaccination, the hypermutation process seems to be reactivated.
- 4) Most strikingly, the blood IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> B cell population was well expanded and mutated in children below two years, an age at which toddlers do not respond to T-independent antigens, thus strongly suggesting that this diversification results at this stage from a developmental program and not from an immune response.

Our conclusion from these preliminary investigations was, as proposed in our first report<sup>19</sup>, that this so-called memory IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> B cell population, which represent 10-30% of B cells in blood and spleen, belonged to a different pathway of differentiation and was linked to the response against TI-antigens. A similar conclusion was reached by Carsetti and her colleagues in a recent report, based on their study on the responding



capacity of normal individuals and asplenic or splenectomized patients to T-independent pneumococcal antigens<sup>21</sup>.

What can be concluded from this first series of results? We would like to propose that during early ontogeny blood IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> B cells develop and mutate their Ig receptor before 2 years of age, at which stage they become functionally competent against TI antigens, this competence being probably caused by the differentiation of the appropriate accessory cells in the splenic marginal zone microenvironment (fig. 1).

But how is this population produced and diversified throughout life? It may either be maintained in the periphery after its initial production and thus constitute like mouse B1 cells a separate self-renewing B cell lineage with a large repertoire of specificities and affinities<sup>22,23</sup>. Alternatively and more likely, it may be continuously produced, sharing up to a certain stage a common pathway of differentiation with conventional B cells. At the transitional bifurcation step, it could be driven by self or bacterial antigens and by specific interactions with neighboring cells to its final marginal zone B cell phenotype<sup>24-26</sup>. As shown for GALT species, these ligands, whether internal or provided by commensal bacteria, would only induce the proliferation and Ig diversification of these B cells and would not trigger any effector function, i.e. the production of antibodies. This pre-diversification step, rather than in B cell follicles along the gut, could take place in the splenic marginal zone, or in the marginal zone-like regions of tonsils, lymph nodes, and Peyer's patches. Asplenic patients indeed harbor IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> B cells with normally mutated genes but their frequency appears somewhat reduced in the adults, suggesting that such marginal zone-like sites, if they can compensate for the diversification function of the spleen, are not able to support the production and/or survival of these cells to the same extent.

There are some apparent differences among GALT species that may confuse the picture. In chickens and sheep, diversification of the pre-immune repertoire starts in the sterile environment of the embryo and then goes on for several months under the proliferative stimulation of the gut bacterial constituents. In rabbits, diversification of the B cell repertoire requires the gut constituents since it starts only after birth, but here again their role is essentially to drive B cell proliferation. In humans, as it occurs in rabbits, there is little expansion and diversification of the IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> B cells at birth, and by 2 years of age, the frequency of mutations carried by these cells is more or less equivalent to the one found in adults, suggesting that each new wave of cells undergoes the same diversification process. What drives pre-diversification of marginal zone B cells in humans and how does it occur is one of the next burning question.

B cells thus appear to possess the intrinsic property to diversify their Ig receptor outside the strict engagement of their B-cell receptor. Strikingly, this has been shown recently to occur in the mouse under specific experimental conditions: one of these settings corresponds to a situation of strong germline repertoire restriction, leading to pre-immune diversification by hypermutation<sup>27</sup>; the second one to a constitutive B cell signaling, turning on hypermutation in the context of a microbial-driven, non BCR-mediated, stimulation<sup>28</sup>.

In conclusion, this model of differentiation for human marginal zone B cells that includes a pre-diversification step provides an explanation for the surprising fact that T-independent responses in humans are taken care by mutated antibodies. It also shows that humans may have conserved for one arm of their B cell system, in its normal physiological function, the strategies used by GALT species to diversify their pre-immune repertoire.

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### Figure legend

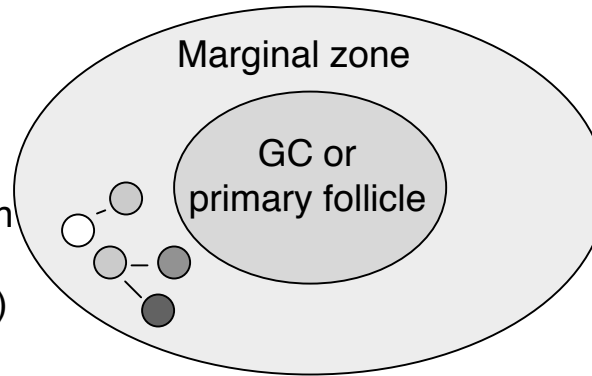
Figure 1. A proposed scheme for the diversification of human marginal zone B cells. Marginal zone is schematized in light gray, surrounding a primary follicle or a germinal center (GC). B cells within this environment undergo repertoire diversification, driven by antigenic stimuli in the absence of antigenic response before two years (the scale of grays represents an increasing V gene diversity). As the marginal zone environment matures, B cells become able to respond to T-independent antigens by differentiating into antibody-secreting cells while possibly triggering another round of diversification by hypermutation. It is also proposed that newly produced B cells are constantly driven to diversify outside of an immune response by antigenic stimuli, in a process similar to the one occurring in young children.

Table 1. Characteristics of B cell diversification in GALT

	<b>Chicken</b>	<b>Sheep</b>	<b>Rabbit</b>
Tissue involved in pre-immune repertoire formation	Bursa of Fabricius	Ileal Peyer's patches	Appendix
Molecular mechanism involved in the formation of the pre-immune repertoire	Gene conversion	Somatic mutation	Gene conversion and somatic mutation
Development before birth	Yes	Yes	No
Involution at adult stage	Yes	Yes	No
Function	Primary lymphoid organ	Primary lymphoid organ	Primary, then secondary lymphoid organ
Role of exogenous antigens after birth	Stimulation of proliferation	Stimulation of proliferation (no effect on diversification observed in germfree sheep or in sterile ileal segments)	Stimulation of proliferation (necessary for development after birth))

**Repertoire diversification  
(0-2 years)**

bacterial or self antigen-driven  
diversification  
(immature microenvironment)



**Repertoire diversification and immune response  
(after 2 years)**

bacterial or self antigen-driven  
diversification

T-independent  
antigen-specific response  
(mature microenvironment,  
MΦ, DC, NK-T)

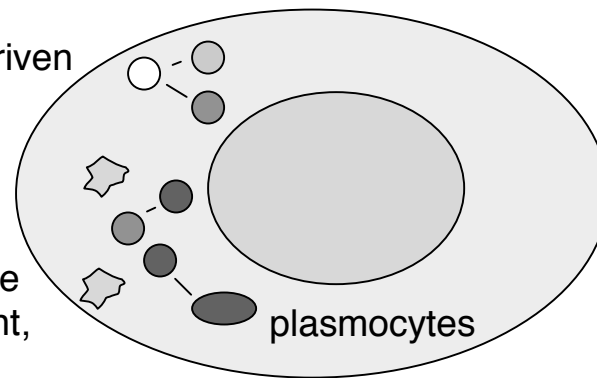


Figure 1