Selection of natural autoreactive B cells

R.R. Hardy, K. Hayakawa

Fox Chase Cancer Center, Philadelphia, PA, USA.

Richard R. Hardy, PhD
Kyoko Hayakawa, MD, PhD

Please address correspondence to:
Richard R. Hardy, PhD,
Fox Chase Cancer Center,
333 Cottman Ave.,
Philadelphia, PA 19111, USA.
E-mail: richard.hardy@fccc.edu

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ABSTRACT

Natural antibodies produced by CD5+ B1 B cells include anti-thymocyte autoantibody (ATA). Transgenic mice bearing the Ig-μ heavy chain of a prototypic ATA, V_H3609Vκ21c, demonstrated a critical requirement for self-antigen in the accumulation of ATA B cells and production of high levels of serum ATA. Further work with ATA-μκ transgenic mice revealed that, while development of most B cells were blocked at an immature stage in spleen, some mature ATA B cells were present. ATA-μκ transgenic mice with varying levels of Thy-1 autoantigen showed a clear relationship between BCR crosslinking and B cell fate, with low levels generating marginal zone ATA B cells and complete antigen absence allowing maturation to follicular ATA B cells. Finally, different fates of developing ATA B cells encountering high levels self-antigen may be accounted for by variations in the response of newly formed B cells arising from foetal and adult development.

Introduction

The discovery of a B cell subpopulation expressing the pan-T cell surface glycoprotein CD5, in the autoimmune-prone mouse strain NZB, led naturally to an investigation of the antibodies produced by these cells (1). This analysis found that certain natural autoantibodies were produced by CD5+ B cells (also termed B1 B cells), based on experiments altering their numbers, either by cell sorting or in mutant mouse strains such as Xid and MeV (2, 3). Here we describe the selection of B cells bearing a B cell antigen receptor associated with the CD5+ B1 B cell subset: antibody binding a determinant on thymocytes, termed anti-thymocyte autoantibody or ATA. This specificity is germline-encoded and typical of a class of IgM autoantibodies that are abundant in sera of certain autoimmune mouse strains, such as NZB, but that are also present in the serum of normal animals.

A natural autoantibody: V_H3609Vk21c anti-Thy1/CD90 ATA

In our screen of hybridomas made from sorted CD5+ B1 B cells we isolated a group of hybridomas that secreted IgM capable of binding to thymocytes, as revealed by staining and flow cytometry analysis (4). The binding of several of these could be blocked by pre-incubation of thymocytes with an anti-Thy-1 monoclonal antibody. Staining analysis of mutant cell lines that had lost Thy-1 expression, including a line where Thy-1 had been re-introduced by transfection, demonstrated a clear association of this determinant with Thy-1. Based on observed periodate sensitivity of thymocyte staining and the finding that some Thy-1+ cells, particularly peripheral CD8+ T cells and many CD4+ T cells, were not labelled by these antibodies, we concluded that the ATA determinant is a specific glycosylation present on certain Thy-1 molecules. Half of these hybridomas, including the group that stained thymocytes most intensely, contained a V_H3609 rearranged immunoglobulin (Ig) heavy chain. The prototypic anti-thymocyte/Thy-1 autoantibody (ATA), clone 1-6C10 (from hybridoma SM6C10), paired this heavy chain with an Ig light chain from the Vκ21 family, Vκ21c. Further analysis of the antigen epitope recognised by 6C10 ATA identified it as a tissue- and differentiation stage-specific N-glycosylated determinant on Thy-1/CD90, as we could immunoprecipitate a fraction of the Thy-1 peptides from a thymocyte lysate, shown by 2-D gel analysis and Western blotting (5). The epitope is species-specific, as the ATA antibody does not bind to Thy-1 in rat. Thy-1 glycoforms expressed on neural tissue and on haematopoietic stem cells in mouse are also not detected by this antibody.

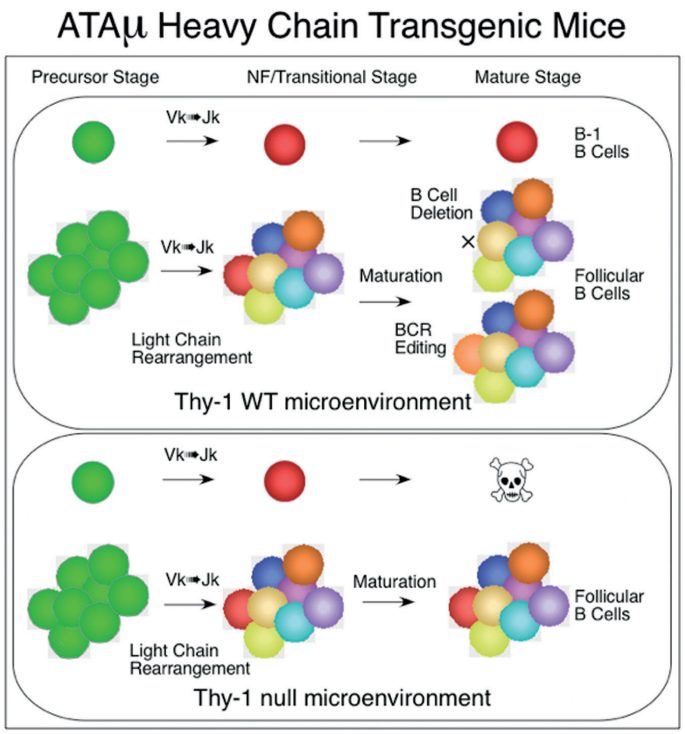

Fig. 1. ATA μ heavy chain transgenic (Tg) model system reveals a key role for antigen in selection of ATA$^+$ B cells. In a Thy-1$^+$ microenvironment, some B cells that pair the Vκ21c light chain with the ATA-μ Tg heavy chain (ATA BCR; red) are selected into the B1 B cell pool and consequently are responsible for serum ATA IgM. Most bone marrow derived B cells that express the ATA BCR are either deleted or undergo receptor editing to a different specificity; cells without Vκ21c light chain are not autoreactive and become follicular B cells. In a Thy-1$^-$ microenvironment, ATA B1 B cells do not accumulate and ATA$^+$ B cells can become follicular B cells.

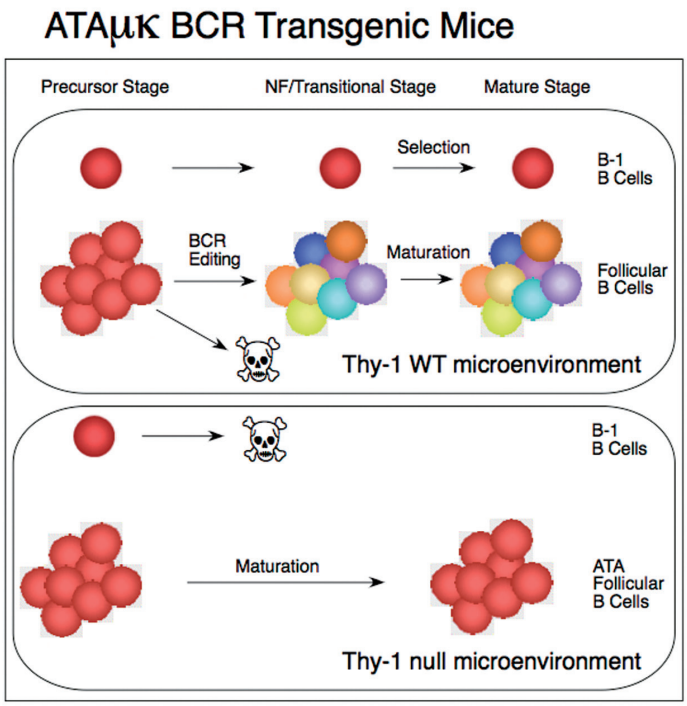

Fig. 2. ATA heavy/light chain transgenic model system generates different B cell fates, depending on the presence of Thy-1. In a Thy-1$^+$ microenvironment, developing ATA B cells are arrested at a short-lived immature stage in spleen. Nevertheless there is abundant ATA IgM in serum, derived from differentiation of B1 type B cells during foetal/neonatal development. In contrast, in a Thy-1$^-$ microenvironment, ATA B cells do not enter the B1 B cell pool and serum ATA levels are low. ATA B cell maturation is efficient in bone marrow, generating a follicular B cell pool, with most cells expressing the ATA BCR.

ATA-μ transgenic mice: evidence for B cell positive selection

The observation that the 6C10 ATA epitope was critically dependent on Thy-1 suggested a novel approach to studying the development of natural autoreactive B cells: for the first time, it was possible to eliminate the natural autoantigen, by analyzing the development of ATA B cells in mutant animals that lacked Thy-1, mice generated by gene targeting (6). The normal frequency of B cells with a V_H3609/6C10 rearrangement is low, so we made Ig heavy chain transgenic mice bearing this specific VDJ-μ and then first studied development of B cells in these mice on a Thy-1 wild-type background (7). We found that ATA-μ transgenic mice had a high titer of serum ATA, easily demonstrable by its capacity to bind to thymocytes, as revealed by fluorescence staining. By generating monoclonal antibodies specific for the ATA Ig heavy and light chain V_H segments we were able to show that most B cells in these mice expressed the transgenic

Ig heavy chain, paired with a diverse set of light chains. Only a small fraction of B cells paired the ATA heavy chain with a Vκ21c light chain to generate the thymocyte-binding specificity. Such cells were readily detectable in the peritoneal cavity, where they had a $CD5^+$ B1a cell phenotype.

In contrast with these results, analysis of the ATA-μ transgene on a Thy-1^- background revealed a completely different picture: serum ATA titer was close to background and the $CD5^+$ B1 B cell population in peritoneal cavity predominantly consisted of cells with endogenous Ig heavy chain expression, with few of the ATA-Tg^+ cells found in Thy-1 wild-type animals. We could restore the production of serum ATA and accumulation of ATA B1 B cells by providing Thy-1 antigen, performing a co-transfer of haematopoietic stem cells from ATATg^+Thy1^- and Tg^-Thy1^+ mice into immunodeficient SCID recipients. Thus, both the accumulation of ATA $CD5^+$ B cells and elevated level of serum ATA were dependent on the presence of the autoantigen, Thy-1, indicating a requirement for antigen in the selection of these ATA $CD5^+$ B1 B cells. In the absence of ATA antigen (*i.e.* in Thy-1^- mice) the $CD5^+$ B cell pool that developed was evidently selected on the basis of alternate self-antigens, resulting in a set of cells that had undergone rearrangement of endogenous heavy chains and so expressed different BCRs. We reported this as the first example of positive selection for B cells, as diagrammed in Figure 1.

ATA-μκ transgenic mice: evidence for B cell negative selection

To understand more about this selection process, we cloned the prototype Vκ21c light chain from the ATA hybridoma and produced transgenic mice expressing both 6C10 V_H3609 heavy chain (the ATA-μ) and Vκ21c kappa light chain, referred to as ATA-μκ transgenic mice (8). We examined B cell development in these transgenic mice, both on wildtype (Thy-1^+) and Thy-1^- backgrounds. Unlike ATA-μ transgenic B cells which pair the ATA heavy chain with a diverse set of Ig light chains, in these animals all newly formed B cells in bone marrow express the ATA specificity, allowing careful assessment of the consequences of this self-reactive B cell antigen receptor (BCR) on bone marrow B cell development. We found that ATA B cells in a Thy-1^+ environment became arrested at an immature stage in spleen, shortly after exiting the bone marrow. By examining several independently generated ATA-μκ transgenic lines, we found two outcomes: in some lines the arrested cells died; in other lines the arrested cells revised their BCR, pairing the ATA transgenic heavy chain with a non-transgenic light chain produced by rearrangement of the normal (endogenous) kappa locus, thereby eliminating the ATA specificity (Fig. 2). Arrested B cells in spleen showed an immature $CD93^+$ phenotype and had a high turnover (*i.e.* were short-lived) as assessed by BrdU incorporation, typical of immature B cells. These cells showed another similarity to immature B cells, failing to proliferate when exposed to antigen. Thus these cells were similar to self-reactive B cells in "classical" B cell tolerance models of negative selection, such as with anti-HEL/HEL, anti-MHC class I, and anti-dsDNA (9-12).

As expected, this maturation arrest did not occur on a Thy-1 null background, where instead cells progressed to a typical follicular (Fo) stage B cell (Fig. 2). These mature Fo B cells responded robustly to antigen exposure. Interestingly, even with the obvious negative impact of antigen on ATA B cell maturation, we nevertheless still detected high levels of ATA IgM in the serum of Thy-1 wildtype mice, and this level decreased in the absence of Thy-1, similar to results with ATA-μ transgenic mice. In order to determine whether this ATA production derived from B1 B cells or instead from maturation-arrested B cells in spleen, we transferred either spleen "arrested" ATA B cells or peritoneal cavity B1 B cells from ATA-μκ transgenic mice into B cell deficient J_H null mice and monitored the production of serum ATA. Although serum ATA was readily detectable in the recipients of peritoneal cavity B cells, none was detected in recipient mice that received spleen maturation-arrested B cells. We concluded that most bone marrow developing B cells reach spleen, become inactivated, and then either die or revise their BCR specificity away from ATA. In contrast a minor fraction of cells in peritoneal cavity (and spleen) is responsible for ATA production, with some cells showing a B1 B cell phenotype.

Thus, we found both positive and negative impacts of self-antigen in these ATA-μκ transgenic mice, indicating that at least some developing B cells with this specificity could be selected by interaction with antigen to become antibody-forming cells (Fig. 3). The reason for ATA B cells adopting these two different cell fates, either negative selection or positive selection, with development to B1 B cells and some differentiation to antibody-forming cells may be due to differences in selection during foetal and adult life, as described below. Furthermore, the B cells initially generated in neonatal animals are critically dependent on strong signalling through the BCR for their survival and respond positively to self-antigen exposure, whereas, once such a population becomes established, with attendant production of antigen-antibody complexes, the B cells developing afterward experience excessive BCR signalling in conjunction with non-BCR signals and so respond negatively.

Altering ATA BCR crosslinking: marginal zone B cell development

The experiments described above analysed two distinct ATA BCR signalling states, one with little or no crosslinking in the absence of Thy-1 antigen and one with strong BCR signalling in the presence of Thy-1 (on a wildtype background). We extended this work to investigate the consequences of intermediate signalling states by providing different levels of Thy1 antigen to developing ATA BCR B cells (13). We cloned and expressed Thy1 as a transgene in T cells under control of the Lck proximal promoter, selecting mouse lines with different expression levels. Sub-physiologic levels of antigen could be obtained with transgenic lines that showed low Thy-1 on a Thy-1^- background. Above-physiologic levels could be obtained using a high Thy-1 transgenic

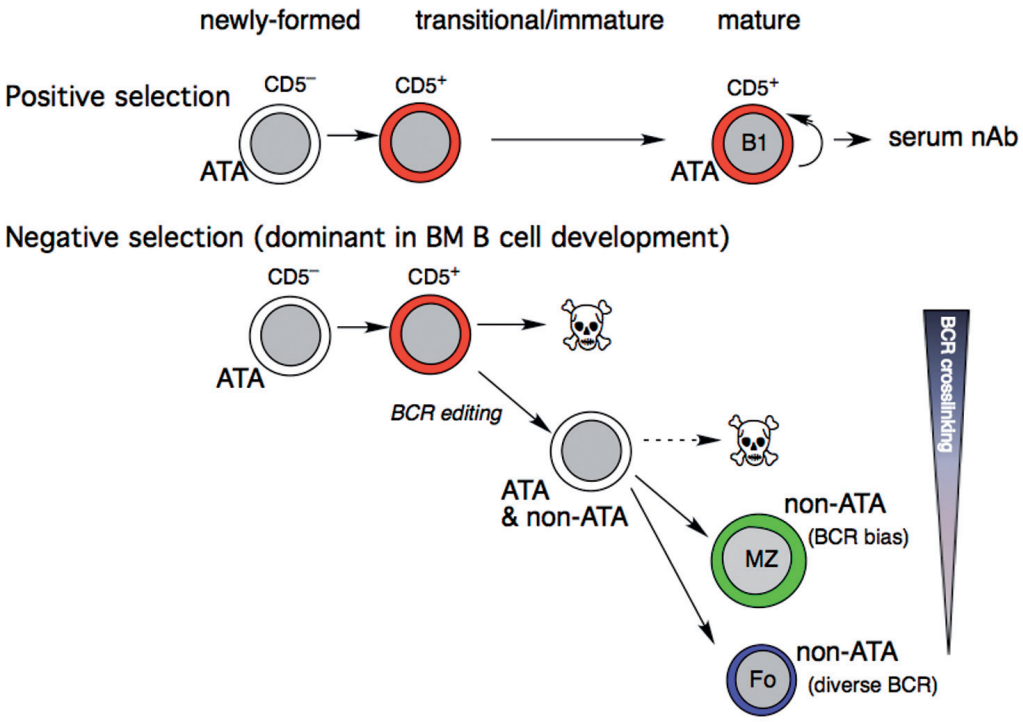

Fig. 3. Fates of ATA B cells undergoing positive and negative selection. Newly-formed ATA B cells generated during foetal development up-regulate CD5 and persist in adult animals after encountering Thy-1 self-antigen and some will secrete the ATA natural antibody. In adult mice, most ATA B cells encountering Thy-1 undergo developmental arrest at an immature stage and either die or edit the ATA BCR, replacing the transgenic Ig light chain with a different light chain.

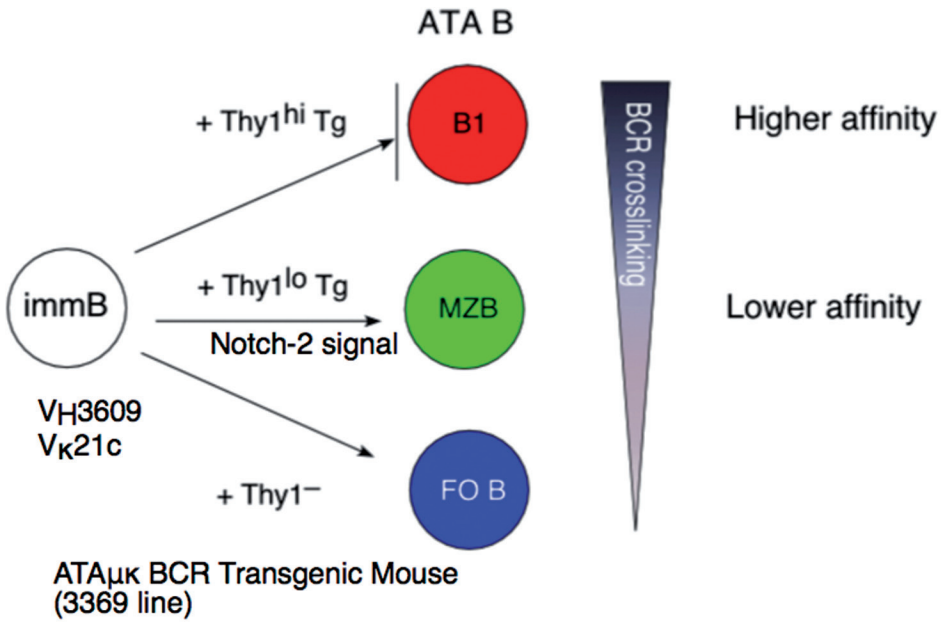

Fig. 4. BCR crosslinking intensity and B cell fate. Developing ATA B cells that encounter different levels of Thy-1 self-antigen may: 1) experience maturation arrest and die or else become B1 B cells with high Thy-1 levels; 2) enter the marginal zone B cell pool if appropriately signaled by Notch-2 and low levels of Thy-1; or 3) mature to a follicular B cell pool when the antigen is absent in Thy-1⁻ mice.

line on a Thy-1 wildtype background. Extremely low levels of antigen exposure could be accomplished by simply using Rag-1⁻ mice, where T cell development is blocked at a very early stage due to the failure of T cell receptor rearrangement, decreasing the number of Thy-1⁺ cells by several orders of magnitude. It is important to note that Thy-1 is not an integral membrane protein, but instead is glycophosphatidylinositol (GPI) linked, and so can be shed into serum. Considering the relative lack of T cells in bone marrow, it is likely that developing B cells encounter shed Thy-1, and so altered levels of Thy-1 expression result in different amounts of shed antigen in serum.

We had already determined in prior work that ATA BCR B cells developed to the default follicular (Fo) B cell fate in the absence of self antigen and so the most striking finding in this series of experiments was the decreased production of such cells and instead the generation of ATA BCR B cells with a marginal zone (MZ) B cell phenotype with the lck-Thy1-10 (the line expressing 1/10 the normal level of Thy1). Importantly, we could monitor the expression of BCR using an antibody specific for the ATA V_H/V_L combination (an anti-idiotype antibody), since strong selective pressures can act on relatively rare B cells with non-transgene encoded BCRs that are gen-

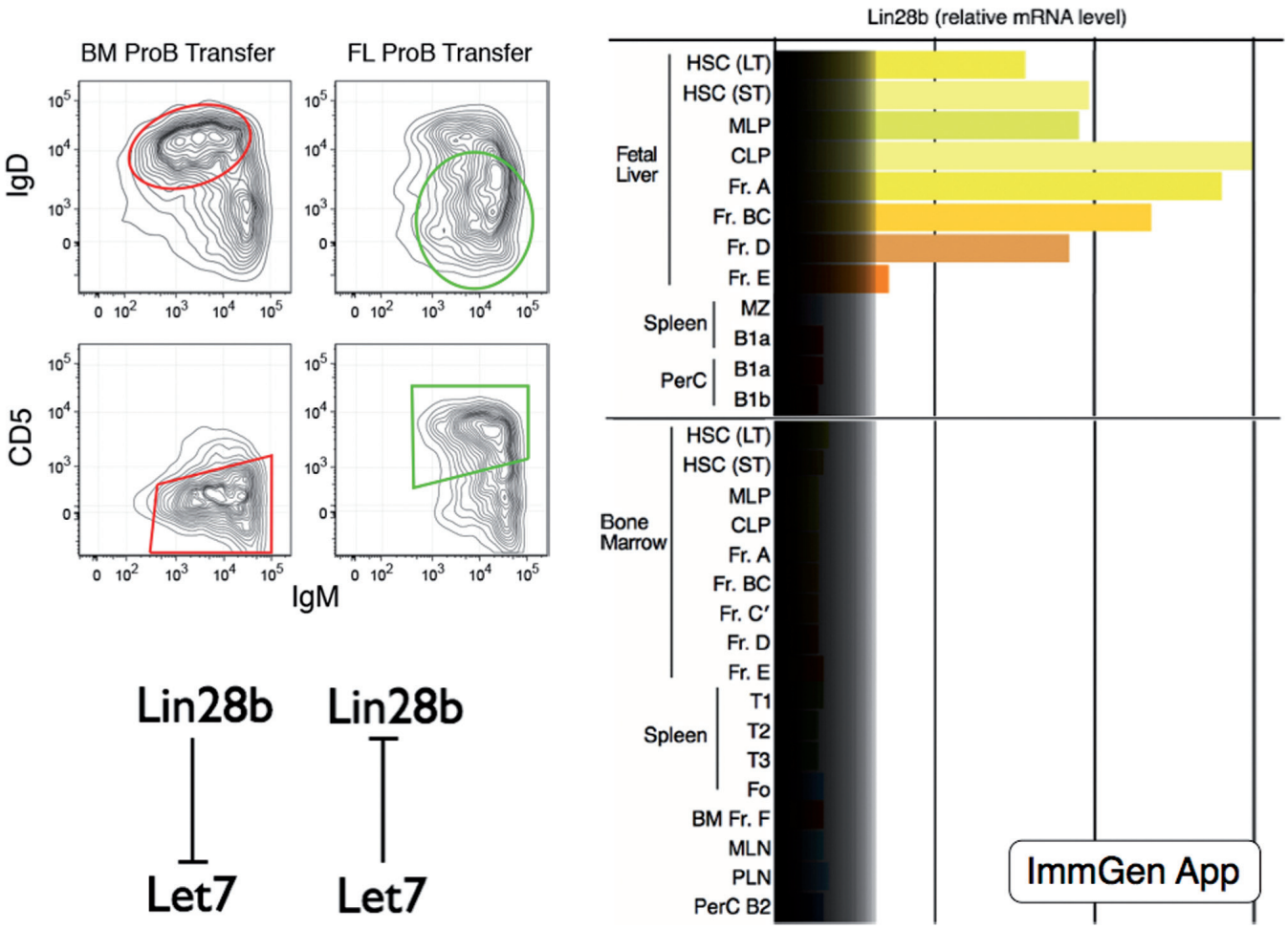

Fig. 5. Distinct developmental pathways, regulated by the Lin28b/let-7 axis, produce B cells with different phenotypes, referred to as B1 (foetal) and B2 (adult). Pro-B cell transfer of foetal and adult precursors illustrates the different phenotypes and global gene analysis reveals the distinctive foetal expression of Lin28b. Lin28b acts to sequester the let-7 microRNA and prevent its processing into functionally active miRNA; let-7 miRNA targets the Lin28b mRNA. Thus abundant Lin28b blocks let-7 function and abundant let-7 miRNA degrades Lin28b.

erated by infrequent rearrangement of the normal (endogenous) Ig heavy and light chain loci. Furthermore, when the ATA BCR was bred onto the Rag-1 null background, with even lower levels of serum Thy-1, we obtained the same result: most B cells in spleen showed a MZ B cell phenotype. This variation of ATA B cell fate depending on level of BCR crosslinking is diagrammed in Figure 4.

Importantly these MZ B phenotype cells localised in the spleen at the border of the white pulp and red pulp, the distinctive zone where marginal zone B cells are normally found. In addition, we characterised these cells for functions characteristic of such cells in normal mice and found that they exhibited such features. For example, these cells showed rapid calcium flux when exposed to Thy1 antigen, more rapid and more intense than ATA BCR B cells with a follicular B phenotype that were generated in the complete absence of antigen. Finally, it was even possible to generate MZ B cells in culture when we supplied immature ATA BCR B cells with an appropriate level of antigen and the proper non-BCR signals (activated Notch-2 and BAFF (14-16)). When immature B cells were instead supplied only with BAFF, ATA BCR B cells with a follicular cell type were produced. Thus we found that B cells with the same ATA BCR could enter functionally and phenotypically different B cell populations depending on the strength of BCR cross-linking that they experienced: the follicular fate with little or no crosslinking, the MZ B cell fate with some BCR crosslinking and appropriate non-BCR signals, or the B1 cell fate with a higher level of BCR crosslinking. An important caveat for B1 cell fate recruitment is that most bone marrow B cells did not progress to a long-lived mature B1 cell fate when their BCR was crosslinked at this high level, as described above. Since a significant portion of the B1 cell pool is generated during foetal/neonatal life, we consider this issue in the next section.

Developmental changes in natural autoantibody B cell selection

Our earliest cell transfer experiments designed to fully reconstitute $CD5^+$ B1 B cells in irradiated recipients were unsuccessful when we employed bone marrow from adult (>2 months old) mice as a source of B cell progenitors. Instead we could repopulate $CD5^+$ B1

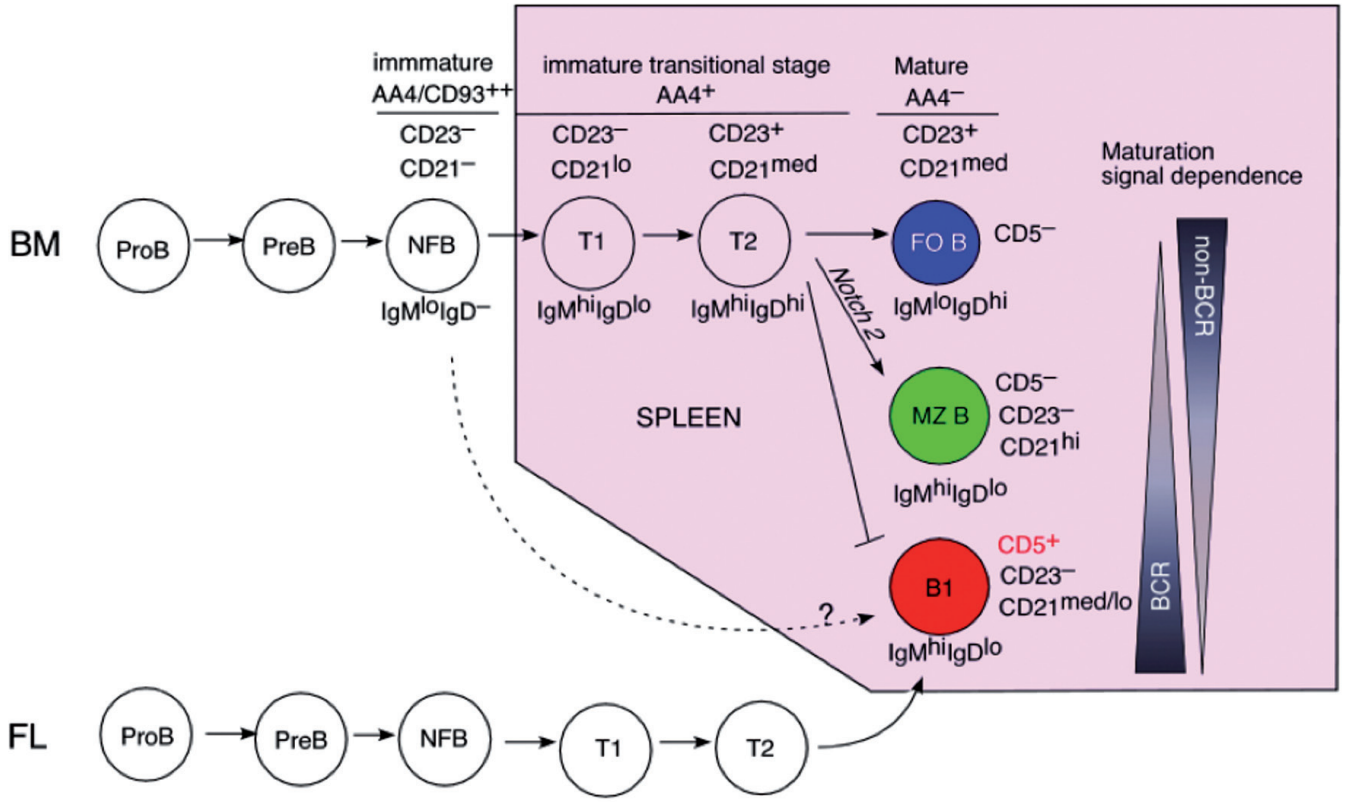

Fig. 6. Model for development of B cells into three mature subpopulations, follicular (Fo), marginal zone (MZ) and B1. In contrast to the largely negative selection experienced by bone marrow newly formed (NF) or transitional (T1, T2) B cells, a portion of B cells (predominating in foetal/neonatal life) respond to antigen encounter by adopting a B1 B cell phenotype and producing natural serum autoantibody. The figure indicates some of the cell surface markers that can be used to discriminate these different developmental stages and B cell subpopulations.

cells (and all other B cell populations) when we used progenitors in liver from foetal or newborn animals (17). This prompted us to suggest that CD5$^+$ B1 B cell generation was developmentally biased, predominantly from precursors present in foetal or neonatal animals. In later experiments we purified pro-B stage cells (B220$^+$CD43$^+$CD24$^+$) from foetal liver and bone marrow by cell sorting, then transferred the cells to SCID mice that lack lymphoid cells (18). These experiments demonstrated that most committed B cells in liver did not develop into classic follicular B cells in this transfer model, whereas those from bone marrow could (Fig. 5). Furthermore most bone marrow pro-B stage cells did not generate B1a (CD5$^+$) phenotype B cells, similar to earlier experiments with IgM$^-$ bone marrow. We now know that this developmental switch in B lymphopoiesis is regulated by the Lin28b/let-7 axis (19).

It seems reasonable to hypothesise that intrinsic differences at the pre-B and immature B cell stage in developing foetal/neonatal B lineage cells allow maturation of cells with moderately self-reactive BCRs; indeed in the absence of a developed immune system such self-reactivity may be a requirement, producing a repertoire of B cells biased toward the production of natural autoantibodies. In the context of BCR *versus* non-MCR survival signals, it is significant to note that the only B cell population that is largely intact in BAFF null mice is the B1 B cell subset, demonstrating that its development and maintenance is BAFF independent (and instead BCR-signalling dependent). In contrast, intrinsic differences at the pre-B and immature B cell stage in developing bone marrow B lineage cells appear to favour those with BCRs that do not show such levels of self-reactivity. Bone marrow B cells that do generated such BCRs are likely arrested at an immature stage, hence non-functional and short-lived, or else die by apoptosis, unless they revise their BCR by receptor editing, as demonstrated in several transgenic models of B cell tolerance. Probably some cells with moderately self-reactive BCRs can enter the marginal zone pool or possibly the CD5$^-$ B1b subset. Thus, as shown in Figure 6, we propose an overall model for B cell selection and production of natural antibodies that changes with development, and where the level of BCR crosslinking strongly influences entry into one of the three mature B cell pools, Fo, MZ, or B1.

Conclusions

CD5$^+$ B1 B cells are a major source of natural autoantibodies in healthy mice. In this review we have highlighted a key feature illustrated by our studies of ATA produced by B1 B cells: the critical role that antigen plays in establishing the B1 B cell pool and inducing production of natural autoantibody. Selection by self-antigen probably holds for all CD5$^+$ B1 B cell-derived natural autoantibodies, but remains to be assessed for natural autoantibodies that may be generated by other mechanisms. CD5$^+$ B1 B cells appear to "tol-

erate" higher levels of BCR signalling than B cells in other subsets, and entry into this population is dependent on a higher level of BCR signalling than for follicular B cells (20). In the future it will be important to determine the mechanism(s) mediating positive selection of natural autoreactive B cells, to understand how their population size is maintained throughout life, and to delineate the function(s) of the natural autoantibodies they produce in the immune system. Already is clear that certain specificities in B1 B cells play a critical role in host defense (21, 22). Finally, it is important to note that the breakdown of normal growth regulation in such a long-lived self-reactive cell population carries serious implications for development of autoimmune pathologies and even B cell leukemia (23, 24), highlighting the clinical relevance of such work.

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