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Lineage Tree Analysis of High Throughput Immunoglobulin Sequencing Clarifies B Cell Maturation Pathways

Lena Hazanov, Ramit Mehr
The Mina and Everard Goodman Faculty of Life Science,
Bar-Ilan University,
Ramat-Gan, Israel

Yu-Chang Bryan Wu, Deborah K. Dunn-Walters
Department of Immunobiology, Faculty of Life Sciences & Medicine,
King's College London,
London, UK

***Abstract*—Transitional (TR) B cells are immature B cells that have migrated from the bone marrow to peripheral lymphoid organs, but can still undergo selection against autoreactivity. TR cells that survive selection eventually develop into mature naïve B cells (CD27-IgD+, NA). Upon exposure to antigen, NA cells may become IgM memory (CD27+IgD+, MM) or "classical", class-switched memory (CD27+IgD-, SM) B cells. Although MM immunoglobulin (Ig) genes do not undergo class switching, they do undergo somatic hypermutation, albeit with lower frequency than SM. It has been postulated that MM B cells originate from T-independent immune responses, while SM cells originate from T-dependent responses. Alternatively, MM cells may be early emigrants from T-dependent germinal centers. Double negative B cells (CD27-IgD-, DN) have been said to be exhausted memory cells, but their precise origin is unclear. Therefore, a definitive elucidation of lineage relationships between these different B cell subsets is needed.**

In this study, we used lineage tree analysis of Ig heavy chain gene sequences from five human B cell subsets (TR, NA, MM, SM and DN) from three individuals, to study the relationships between these B cell populations and garner insights regarding their roles in immune responses. Our analyses confirmed that both MM and SM branches can include DN Ig sequences, sometimes identical to SM Ig sequences. MM trees were significantly shorter than SM trees. Even when they belonged to the same clone, MM branches were shorter, consistent with either an early exit of MM cells from germinal centers in a T-dependent response, before accumulating many mutations, or their generation by T-independent responses. Our finding of combined trees that included both MM and SM sequences suggests that at least some MM cells originate from the same clones as SM, rather than develop separately.

Keywords—memory B cells; immunoglobulin; high-throughput sequencing; somatic hypermutation

I. INTRODUCTION

During B cell development, haematopoietic stem cells in the bone marrow (BM) undergo several stages of differentiation into immature B cells [1] that later enter the peripheral compartment [2]. In the developmentally intermediate stage between immature BM B lineage cells and fully mature naive B cells in the peripheral blood (PB) and secondary lymphoid tissues, the B cells are called 'transitional (TR) B cells' (Fig 1). These cells in humans are defined as $CD27^{-}IgD^{+}CD10^{+}$ and constitute less than 1% of B cells [3]. After the TR B cells leave the BM, they can undergo further selection against autoreactivity [4]–[6] and the remaining small fraction develops eventually into mature naïve B cells (NA, $CD27^{-}IgD^{+}$, Fig 1) [7]–[9]. This maturation is associated with the co-expression of IgM and IgD [10]; about 50% of all B cells in human peripheral blood are naïve [3]. Naïve B cells with mature phenotypic characteristics and a fully functional BCR recirculate between the secondary lymphoid tissues, dying after a few days.

Upon exposure to antigen the NA cells become IgM memory (MM, $CD27^{+}IgD^{+}$, Fig 1), which constitute about 14% of the B cells [3], or "classical", class-switched memory cells (SM, $CD27^{+}IgD^{-}$, Fig 1) which constitute about 7% [3]. Both MM and SM undergo SHM in their Ig variable region genes and show characteristics of antigen selection [11]–[13]. However, IgA and IgG SM, which are derived from T cell-dependent responses in the GC, have highly somatically hypermutated Ig genes [14]–[16]. The expression of CD27 correlates with greater cell size [14],[15], proliferative capacity [19]–[22], antigen presentation capacity [22], and differentiation into antibody secreting cells [20]. MM Ig genes also undergo SHM, though with a lower frequency then SM, but not class switch recombination, and have been shown to be important in polysaccharide responses [23],[24]. Therefore, it is assumed that MM cells mostly function in T-independent immune responses while SM cells function in T-dependent responses [25][26]. Alternatively, MM cells may be early immigrants from the T-dependent response in the GC [27] or part of the constitutive production of natural antibodies [28].
It has been said that double negative B cells (DN, $CD27^{-}IgD^{-}$, Fig 1). [29][30] are exhausted memory cells [31],[32] which proliferate less than MM or SM [33]. The DN cells, like SM, can be stimulated to secrete Igs against tetanus toxin and influenza virus [17], suggesting they were involved in prior vaccine responses. However, it is unclear which of the

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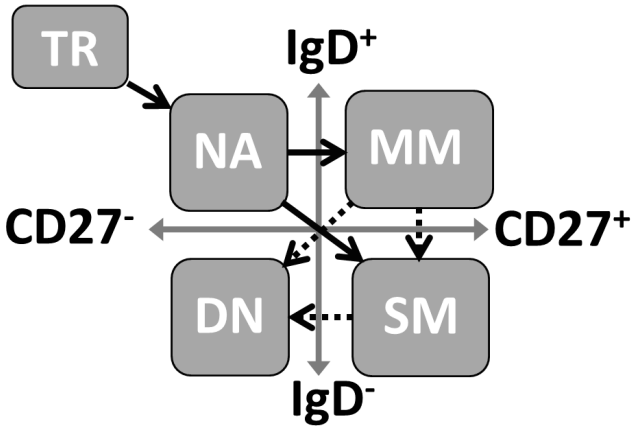

Fig. 1. The transitions between B cell subsets. Transitional (TR) B cells become mature naïve (NA, $CD27^{-}IgD^{+}$) cells that, upon activation, become either IgM memory (MM, $CD27^{+}IgD^{+}$) or "classical", class-switched memory cells (SM, $CD27^{+}IgD^{-}$). Solid – a known transition. Dashed – a possible transition.

memory populations, MM, SM or both, becomes DN (Fig. 1). Another option is that they originate via an altogether different maturation pathway [22].

The intraclonal variation achieved by SHM enables the construction and analysis of clonal lineage trees (also referred to as dendrograms or pedigrees). In a GC, there are usually several different B cell clones. Clonally related cells are identified by the use of the same V (D) and J segments and highly homologous sequences of the complementary determining region 3 (CDR3) of their Ig variable region (V) genes. Lineage trees depict the relationships between sequences in a clone, by representing each single mutation by a tree node [34].

We developed a method of mathematically analyzing lineage trees by measuring their graphical properties [35]. Previous studies demonstrated the usefulness of this method and resulted in new insights regarding differences in GC dynamics in aging or between different tissues [35]–[37],in autoimmune diseases [38], chronic inflammation [39], lymphomas [40], and chronic gastritis and gastric lymphomas [41]. This method was also used to determine clonal relationships of Ig genes from different tissues [39][42].

In this study, we used Ig gene sequences from five B cell subsets (Transitional – TR, Naïve – NA, IgM memory – MM, class-switched memory cells – SM and Double negative – DN) from three individuals [43] to study the relationships between these populations as they are revealed by lineage trees.

II. Methods

A. Sources of data for analysis

All sequences were taken from Wu et a l.[43] The dataset includes Ig sequences from peripheral blood of three young healthy volunteers (21 to 26 years) that were sequenced on a 454 GS FLX Titanium Sequencer.

B. Data processing

Sequence data were processed as described [41] [44]. Briefly, an in-house program deletes duplicate sequences (which might be a product of PCR amplification), and leaves only the unique sequences for the analysis. For each acceptable sequence, the V, D and J germline segments were identified using SoDA [45]. Groups of sequences having the same V(D)J segments were aligned by ClustalW [46] to identify groups of clonally related sequences (having the same junction regions). Characteristic deep sequencing errors, such as insertions/deletions near homopolymer tracts of three or more nucleotides, were also identified when possible by our in-house program Ig-Indel-Identifier [44] and, unless another sequence with the same mutation was found in the clone, the sequence with the suspected mutation was also discarded. Single -sequence clones with suspected insertions/deletions near homopolymer tracts were discarded as well, since the credibility of the mutation could not be confirmed. The numbers of sequences remaining after each cleaning step are provided in Table 1.

C. Lineage tree analysis

Clonal groups of Ig gene sequences were transformed into mutational lineage trees using our program IgTree© [34], implementing a distance method-based algorithm that finds the most likely tree with a high probability. All trees were measured using our program MTree©[36][35], quantifying shape properties of the trees, such as the number of leaves, number of nodes, etc. According to this method, defined distances between key tree nodes (root, split nodes or 'forks' and leaves) reflect dynamical features of clonal evolution

		Raw	Without duplicates	Clone of size 1 with indels	Sequences with illegitimate indels	Sequences without indels	Number of clones
	TR	125	124	30	25	69	39
	NA	668	666	171	174	321	186
	MM	404	402	68	105	229	172
	SM	930	925	202	372	351	233
	DN	2250	2242	450	912	879	607
A	All	---	4359	921	1588	1849	879
	TR	344	342	104	60	178	104
	NA	70	70	19	14	37	41
	MM	229	228	55	57	116	96
	SM	586	582	100	231	251	163
	DN	473	467	115	133	219	173
B	All	---	1689	393	495	801	555
	TR	86	86	16	33	37	14
	NA	57	56	8	8	40	52
	MM	1112	1100	76	214	252	183
	SM	313	311	98	85	128	92
	DN	245	244	97	46	101	98
C	All	---	1797	295	386	558	422

Table 1. The numbers of sequences in the dataset, in three individuals (A-C), at all stages of data cleaning from raw data to the clean, analyzed data [43].The number of clones of all subsets is smaller than the summary of the subsets of each patients because some of the clones are combined.

including the mutation rate, the affinity between the target antigen and the B cell receptor of the Ig clone and antigen-driven selection pressure [47]. The measurements were then compared between the subsets using the non-parametric Mann-Whitney U-test, as normal distributions (required by tests such as Student's T-test) could not be assumed.

D. Mutation targeting motif analysis

Mutation targeting motif analysis was based on previous work by Dunn-Walters et al. [48][49]. Briefly, the base composition at positions flanking a mutation (ten nucleotides on either side) was determined. For each nucleotide in each position and each type of mutation, a $\chi 2$ analysis was performed to check whether the frequency of the nucleotide is significantly different from its germline frequency. Then, the results for different B cell subsets were compared using the F-test.

III. RESULTS

A. Data preparation and lineage tree generation

Ig sequences of TR, NA, MM, MS and DN B cell subsets from PB of three young, healthy donors (Table 1)[43] were used to generate lineage trees. The generated trees were divided into two groups; combined lineage trees that include clonally related Ig sequences from between two and five subsets (mixed trees, Fig. 2) and lineage trees that contained sequences from only one subset (unmixed trees).

B. DN Lineage trees are almost as long as switched memory lineage trees

In order to compare the characteristics of the individual subsets we only used the unmixed lineage trees. The tree measurements of each subset separately (Fig. 3a-c), together with the average number of mutations per sequence (Fig. 3 d),

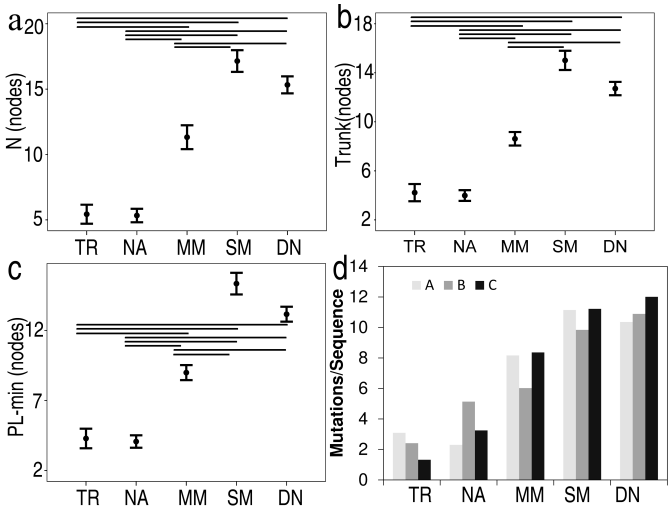

Fig. 3. CI plots of lineage tree measurements; number of nodes (a), trunk size (b) and PL-min (c). The plots present the clones from all donors; the results of each of the donors were similar. (d) The average number of mutations per sequence in each of the subsets in donors A-C. P values for all comparisons are given in supplemental Table 1, and lines in (a-c) represent significant differences. were found in trunk length (T) and minimal path length (PLmin). A line represents p-value <0.005.

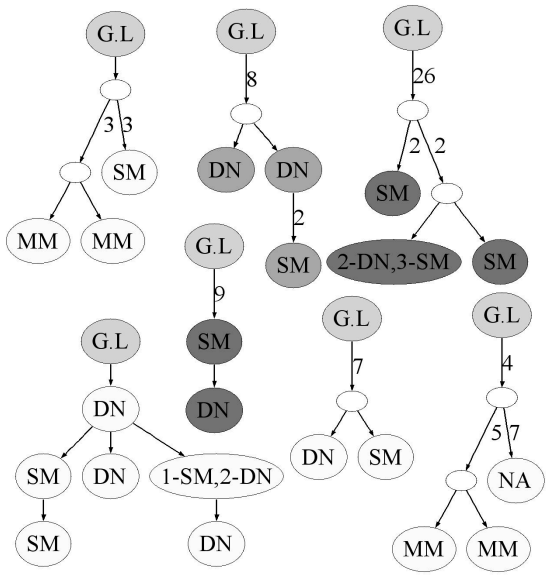

Fig. 2. Examples of combined lineage trees. The numbers in the nodes indicate the number of sequences; the numbers on the edges indicate the number of mutations (Single mutations are not indicated). IgM-white, IgG-light gray, IgA-dark gray

those two subsets can develop from the same lineage; however, such cases are rare (Fig. 4).

Second, some of the combined trees included SM and/or MM along with DN (Figures 2 and 4), confirming that the source of DN cells may be MM, SM or both. However, since the analyzed sequences represent a sample of the whole human repertoire, resulting in relatively small trees, sampling limitations precluded us from finding the exact temporal relationships between the different subsets. For example, MM cells can first become SM and then DN, but we would not

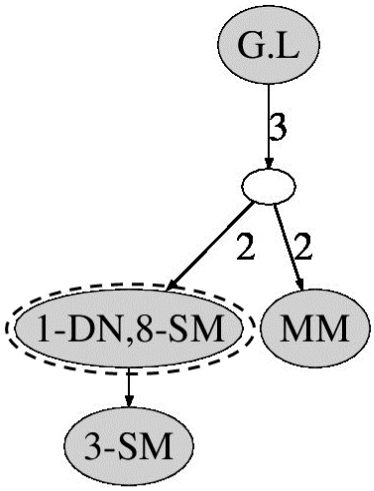

Fig. 4. A combined tree that includes SM, MM and DN cells with a DN sequence that is identical to SM sequences.

know this if the SM cells were not sampled. Out of 15 lineage trees that included DN along with SM or MM (or both), four trees included identical DN and SM Ig sequences (example in Figures 2 and 4), while none of the trees included identical DN and MM Ig sequences. This may indicate that SM cells can directly become DN, while such an indication for MM cells is lacking.

Third, in all the 15 "mixed" lineage trees, each DN sequence shares the isotype with the branch it belongs to (data not shown). This is consistent with the hypothesis that DN B cells are exhausted cells that do not change further. In summary, lineage trees that include both MM and SM are rare, and while DN cells appear in lineage trees with MM and SM cells, they only share identical sequences with SM.

D. Mutation characteristics are the same in all memory subsets

Finally, we looked for evidence for differences in the mutational mechanisms in sequences from different subsets. We assume that a change in the mutational mechanism will be reflected by a change in the mutational motifs. The average numbers of differences in the motifs (Fig. 5, Table 2) show that NA and TR cells have similar mutational motifs and SM, DN and MM cells also have similar motifs, however the motifs are different between these two groups of subpopulations. Since the "mutations" in NA and TR cells are not generated by SHM and are artifacts of the method, this difference was expected and confirms that our methods can distinguish between SHM and random errors, at least statistically. However, this analysis could not distinguish between MM, SM and DN cells, showing that the SHM mechanism does not change between them.

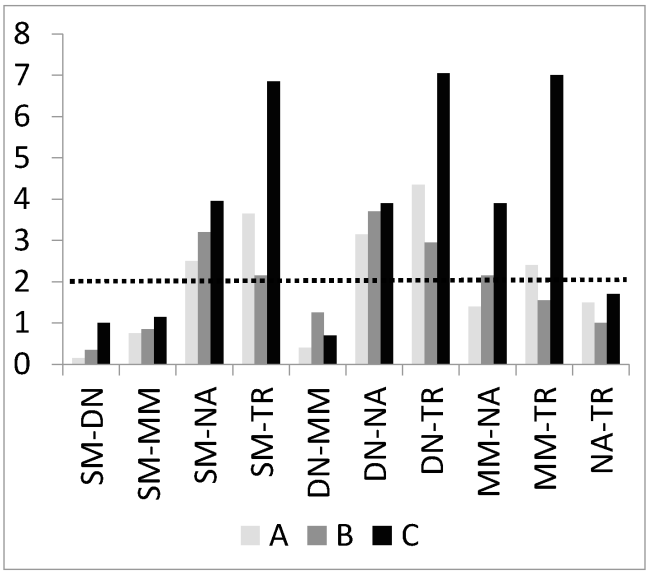

Figure 5: The average numbers of differences in sequence motifs around mutations. The distribution of nucleotides in each of the 10 positions up- and down- stream of each mutation was calculated per each mutation type and compared between the subsets by F-test. Then, the number of significant differences (P value <0.05) in each position (out of 12 options, as each nucleotide can be mutated to the other three) was calculated. The dotted line is the median, to split the high- from low- difference groups. The numbers of mutations included in this analysis are summarized in Table 2.

		A	C	G	T	Total
A	TR	66	52	58	37	213
	NA	158	192	228	160	738
	MM	478	479	656	256	1869
	SM	1036	1048	1311	517	3912
	DN	2414	2459	2962	1270	9105
B	TR	89	118	124	98	429
	NA	46	57	63	24	190
	MM	196	162	247	94	699
	SM	695	666	795	314	2470
	DN	671	623	783	308	2385
C	TR	5	14	20	10	49
	NA	28	34	52	16	130
	MM	569	553	635	350	2107
	SM	381	395	486	174	1436
	DN	331	336	370	176	1213

Table 2: Numbers of mutations in each patient, in each subset and from each nucleotide (A,C,G and T).

IV. Discussion

In this study, we used lineage tree analysis of Ig heavy chain gene sequences from five human B cell subsets (TR, NA, MM, SM and DN) from three individuals, to study the relationships between the populations and garner insights regarding their roles in immune responses. Our analyses confirmed that both MM and SM branches can include DN cell Ig sequences, and that SM had in some of the cases identical Ig heavy chains to those of DN cells the DN. In addition, rarely, MM and SM cells can share a lineage tree. The similarity of the motifs in MM, SM and DN suggests that the source of the mutations in DN is the same as in MM and SM, which is SHM, rejecting the assumption that they originate in a completely deferent maturation pathway. Taken together, these results support all the suggested DN origins. The small CI in the tree measurements of DN cells, however, suggests that that these trees are not a mixture of small trees that originated from MM cells and large trees that originated from SM cells, but a uniform group of trees. The differences in tree lengths (total number of mutations) imply that, had these trees originated from the smaller MM trees, the DN cells would have needed to accumulate more mutations afterwards. Alternatively, had they originated from SM cells, the DN cells would have had to leave the GC before some of the SM.

We have also seen that MM trees are significantly shorter than SM trees, consistent with either MM cells leaving the GC early, before accumulating many mutations, or their belonging to a separate pathway. Since combined lineage trees with both MM and SM cells were rare, it is more likely that MM cells belong to a separate pathway such as the TI response. However, at least some of the MM may originate from the same clones as SM, and therefore may be part of the classical TD response.

Although, it was shown that TI stimulus in mice sends cells to GC and mutations occurs [50], changes in the SHM

mechanism, and therefore in the motifs around the mutations, may be affected by the microenvironment and activating signals, which are likely different between TI versus TD response pathways. However, our motif analysis method has never been used to study the differences in motifs around SHM in B cells from TD and TI pathways, thus it remains to be seen whether these pathways are characterized by different mutational motifs. Even though we did not find differences in SHM motifs between MM, SM and DN, the fact that our method could distinguish between SHM and artifacts according to the mutational motifs confirms that this method can be applied to detect alterations from normal SHM.

Our study did not completely resolve the questions regarding the origin and function of the subsets, but we have shown that differentiation from MM and SM to DN is likely, while that between MM and SM is rare. The potential size of the human repertoire is estimated to be 10^{11} unique BCR sequences [51], therefore a larger sample size is required in order to achieve the full picture of the relationships between all B cell populations and determine the correct model. In the future, when larger data sets are available, a comparison of DN IgM repertoire to those of MM and DN IgG/A and of SM will help clarify the inter-subset relationships.

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