CD93+ Early Stage B Cells Interact With Dll-1 Expressing Cells in Mouse Spleen

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Abstract

The region at the interface of the red pulp and the white pulp of the spleen and which separates them is called the marginal zone. The main type of cells in the marginal zone are marginal zone B (MZ B) cells as well as two unique macrophage populations. In mice, MZ B cells are a unique population of B cells, distinct from follicular(FO) B cells. MZ B cells have a $IgM^{hi}IgD^{lo}CD23^{-}CD21^{hi}CD1d^{hi}$ surface phenotype. Because of the high expression of CD21, MZ B cells can bind to immune complexes containing circulating antigens and blood-borne pathogens. Two key factors appear to influence immature B cells to develop into MZ B cells- the specificity and strength of the BCR and Notch signaling through Notch2. Notch signaling may be required for the differentiation of B cells into MZ B cells or for the maintenance of MZ B cells. The Notch family genes play a key role in cellular differentiation in many organisms. Previous studies showed the importance of the conserved cell-to-cell signaling cascade of Notch signaling in lymphocyte development. Notch2 is indispensable for MZ B cell development and must be ligated by the Dll-1Notch ligand. Expression of Notch ligands on different cells in the spleen has been reported. Interactions between Dll-1 expressing cells and B cells have not been previously demonstrated or described. Our results in this thesis demonstrate cell-cell contact between Dll-1 expressing cells and $CD93^+$early stage B cells, and it is suggested the $CD93^+$ cells are T2 cells by the expression level of Notch2 from IMMGen database. These data suggest that Dll-1 must ligate Notch-2 at an early transitional stage of peripheral B cell development for MZ B cell development to occur.

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Chapter 1: Background

1.1 Introduction

The region at the interface between the non-lymphoid red pulp and the lymphoid white pulp of the spleen which separates them from each other is called the marginal zone. The blood exits the circulation and flows into the spleen via splenic artery. Then the splenic artery branches into central or penicillar arterioles, which terminate in the red pulp. The central arterioles, surrounded by many lymphoid areas (including B cell zone and T cell zone), which are referred as the white pulp, branch into many follicular arterioles that direct the blood flow to the marginal sinus, which is best defined in rodent spleen. Before their termination into the red pulp, most of the naïve lymphocytes in the marginal sinus are driven by chemokine gradients into the white pulp. The blood flow then goes to the marginal zone before going back to the circulation [1,2,3]. Therefore, cells in the marginal zone are usually exposed to large amounts of blood and to pathogens or antigens that exist in the body circulation [4] (Fig 1).

The main types of cells retained in the marginal zone are marginal zone B (MZ B) cells, macrophages and some reticular cells. In mice, MZ B cells are considered to be a unique population of B cells, which are separate from follicular(FO) B cells. In contrast, in humans, MZ B cells are regarded as a subpopulation of memory B cells. MZ B cells have an $IgM^{hi}IgD^{lo}CD23^{-}$ $CD21^{hi}CD1d^{hi}$ surface phenotype. MZ B cells are thought to play an important role in host defense against blood-borne pathogens because of the high expression of CD21, which helps capture complement coated antigens. These immune responses are thought to be largely T-independent, activated by multivalent antigens like LPS or polysaccharide. However, MZ B cells also express higher levels of MHC II and B7 proteins than FO B cells, which are thought to be

ideal for antigen presentations to the T cells. Thus, MZ B cells are also thought to be involved in T-dependent immune responses [1].

There is a well-known mechanism for the entry of lymphocytes to the lymph nodes- lymphocytes are induced to roll by the interaction between selectin ligands on high endothelial venules (HEVs) and selectins on naive lymphocytes. But in the spleen, this system does not apply. Instead, integrins LFA-1 and α4β1 express on lymphocytes interact with their ligands, ICAM-1 and VCAM-1, respectively, which are expressed on splenic stromal cells, may mediate an adhesion event that is involved in lymphocyte entry into the spleen [6]

B-lineage cells differentiate from common lymphoid precursors in the bone marrow. After becoming immature B cells(IgM^+), they leave the bone marrow and migrate to the spleen where they are identified as Type 1 transitional B cells (T1) [1,10]. T1 cells have not yet expressed follicular markers like IgD and CD23, and BAFF (B cell–activating factor of the TNF family) is not required for their survival. But they express high level of CD93(AA4.1) and intermediate levels of CD21. When they reach the marginal sinus, they are driven into the follicle by a CXCL13 gradient, and become Type 2 transitional B cells (T2), which are $IgM^{hi}IgD^{hi}CD21^{int}$ but remain $CD93^+$. T2 cells differentiate into T3 cells that CD93+IgDhiIgMlo T3 cells, that have sometimes been considered to be anergic cells. And finally, T1, T2 or T3 cells differentiate into at least two types of mature B cells, FO B cells and MZ B cells. There are two main key factors affecting the immature B cells developing into MZ B cells. One is the specificity of the BCR. If the BCR has fair affinity with an antigen, this B cells will differentiate into an FO B cells. If the BCR has a poor affinity with an antigen, it will survive because of BAFF and constitutive BCR signaling, but without strong BCR stimulation signal, the cells will be driven to an MZ B cell fate. The second factor is the Notch signaling. When the B cells are induced to differentiate into

a MZ B cell lineage, Notch signaling is requirement for the differentiation(Fig2). NF-κB signal is also required to generate MZ B cells, since activation of NF-κB downstream of the BCR is thought to be associated with BCR weak signal [7,8,9,10].

The Notch family genes, which encode Notch1, 2, 3 and 4, play a key role in cellular differentiation in many organisms. The Notch ligands, including Delta-like(Dll)-1, Dll-3 and Dll-4 and Jagged1 and Jagged2, are found to expressed on many types of cells. The binding of Notch ligands to the Notch receptors leads to two proteolytic cleavages of the transmembrane domain of the receptor. The first cleavage is mediated by the protease called tumor-necrosis factor α-converting enzyme (TACE) near the extracellular area of the transmembrane domain. The cleaved subunit will be endocytosed by the Notch ligand expressing cell. After this first cleavage, an enzyme called Gamma secretase (γ-secretase), which is a multi-subunit protease complex cleaving single-pass transmembrane proteins at residues within the transmembrane domain, cleaves the remaining part of the notch protein just inside the inner leaflet of the cell membrane of the cell, which results in the release of the Notch intracellular domain (NICD). Then the NICD trans-locates to the nucleus as a transcriptional activator and associates with RBP-J/CBF1. RBP-J is uniquely expressed and associates with the intracellular domains of all four types of the Notch. This association upregulates the transcription of genes such as hes1 and hes5, which contribute to cell differentiation regulation [11,12,14,22,23,24]. From previous studies, the conserved cell-to-cell signaling cascade of Notch signaling has been shown to be involved in lymphocyte development and adaptive immunity [16]. The essential function of Notch1 in T cell fate specification is considered the most well characterized function of a Notch receptor in hematopoiesis. In the absence of Notch1, bone marrow progenitors enter the thymus but then develop into B cells, which indicates that Notch1 plays

a key role in T cell lineage commitment. [24] In addition to its role in the T cell lineage, Notch signaling is important in later stages of B cell development. Expressions of multiple Notch genes has been found in most stages of B cell development, but the significance of this expression has been unclear. Studies demonstrated that knock out of Notch2 did not affect T cell development, while a significant decrease of MZ B cells and a specific population of T2 cells which are CD1dhi in the spleen were observed in Notch-2 knock out mice. Among different Notch target genes, only Dll-1 expression is dominant in MZ B cells on a Notch2-expression dependent manner. All of them suggests that Notch2 is prominently expressed in B cells and indispensable for MZ B cell development through interacting with Dll-1[12].

Expression of Notch ligands on splenocytes has been studied. Dll-1 was expressed on a substantial part of macrophages, which is $CD11b^{hi}CD68^{+}F4/80^{+}Gr\text{-}1^{-}$, and a lower expression of Dll1 was also observed on $CD11c^{+}$ dendritic cells. Moreover, IHC staining showed that Dll1 was expressed on $CD11b^{+}$ and $F4/80^{+}$ macrophages in the red pulp but not on MOMA-1^{+} MMM or $MARCO^{+}$ MZM. Also, Dll-1 was found expressed on $TER119^{+}CD71^{+}$ erythroblasts in the spleen of phlebotomized mice. But not all the Dll-1^{+} cells in the red pulp expressed CD68, suggesting that other cells such as stromal cells may also express Dll1. Interestingly, the Dll1-expressing cells at the red pulp were found frequently in contact with $B220^{+}$ B cells. Some ER-$TR7^{+}$ reticular fibroblasts which also localize mainly in the red pulp of the spleen expressed Dll1, Dll4, Jagged1 or Jagged2 [13]. In addition, $CD31^{+}$ vessels in the spleen expressed Dll1 or Jagged1. Based on the above studies, it was shown that Dll1 can be expressed on different types of cells in the spleen, but which kind of cells are the indispensable niche cells for MZ B cells development still remain unknown [14].

To determine whether hematopoietic cells or non-hematopoietic cells provides the Dll1-mediated signals to generate MZ B cells, the Dll1 conditional knock out mice were lethally irradiated, the lineage^{-}c-kit^{+}Sca-1^{+} (KSL) bone marrow cells from WT GFP-transgenic mice were transplanted into the mice. After transplantation, following the deletion of expression of Dll1 on the splenic stromal cells of the recipient mice, the donor-derived hematopoietic cells were unable to differentiate into MZ B cells. The frequency of other subsets of lineages, like lymphoid, erythroid and myeloid remained normal. These results suggesting that the expression of Dll1 in splenic non-hematopoietic stromal cells is essential for the development of MZ B cells [15].

Further studies were also performed, trying to confirm the subtypes of hematopoietic cells. Blood endothelial cell (BECs) could be the niche cells of Notch signal for MZ B cell development, as Dll1 was found expressed in BECs in the red pulp of the spleen. The transgenic DL1$^{lox/lox}$ mice were crossed with PDGF-Cre mice to conditionally knock out the Dll1 expression on BECs. But neither the relative percentage nor absolute numbers of MZ B cells were dramatically altered in DL1$^{\Delta/\Delta}$PDGFβ mice compared to the control. Similar results were found in the DL1$^{\Delta/\Delta}$CD11c mice. MZ B cell development was unaffected significantly compared with control. These results suggested that neither BECs nor DCs are the niche cells for MZ B cell development.

Based on the previous literature studies and experiments, mesenchymal stromal cells of the spleen could be part of the Dll1-expressing niche for MZB development. Ccl19 promoter is known to be active in fibroblastic reticular cells (FRC) of the T zone [17]. In the Dll1$^{\Delta/\Delta}$Ccl19 mice, a complete loss of MZ B cells was observed, whereas inactivation of only one Dll1 allele

resulted in a 50% reduction in relative and absolute MZ B cell numbers, which suggested that Dll1-expressing ccl19-Cre^{+} splenic stromal cells are the essential niche cells for MZ B development.

Later the splenic stromal niche cells were further characterized. They intercrossed Ccl19-Cre with R26-EYFP and R26R^{mTmG} reporter mice. R26-EYFP x Ccl19-Cre mice were investigated and EYFP expression was predominantly found in a reticular pattern throughout the white pulp of the spleen, including Pdpn^{+} T zone FRCs. Besides that, EYEFP expression was also found on three types of follicular fibroblast populations: follicular DCs (FDCs), MAdCAM-1^{+} marginal reticular cells (MRCs) and B Zone Reticular cells(BRCs). To characterize the various splenic stromal cell populations, an isolation and gating strategy was established that allows the identification of three stromal cell subsets by flow cytometry: Pdpn^{+}CD31^{-} FRC, Pdpn^{-}CD31^{+} BECs, and Pdpn^{-}CD31- cells (double negative; DN). Also, Ccl-19 Cre-triggered GFP expression in another comparable R26R^{mTmG} reporter mice were found in a small subset of splenic stromal cells, with the vast majority of GFP^{+} cells belonging to T zone FRC and DN cells. DN cells are likely to include all three follicular fibroblast subsets (FDCs, MRCs, and BRCs). Histologically, DL1 expression was found in several desmin^{high} cell types, including MRCs, BRCs surrounding the desmin^{low} CD35^{+} FDC cluster, and Pdpn^{+} FRC [18]. Another study from other research group, also identified that *Ccl19-Cre*$^{+}$ lineage–traced stromal cells are the critical cellular source of DLL1/4 Notch ligands by conditionally inactivating Dll1 and Dll4 with *Ccl19-Cre* (*Tg*$^{Ccl19\text{-}Cre+}$ *Dll1*$^{\Delta/\Delta}$ *Dll4*$^{\Delta/\Delta}$). Also, Rosa26^{EYFP} reporter mice were used for further characterization of *Ccl19-Cre* activity within cellular subsets of PDPN^{+}CD31^{-} stromal

cells showed eYFP in more than 95% of cells from the $CD157^{hi}CD21^{-}$ subset (fibroblastic reticular cells [FRCs]) and from the $CD157^{hi}CD21^{+}$ subset (follicular dendritic cells [FDCs]), which is generally consistent with the results above [19].

However, the interaction or contact between Dll-1 expressing cells and B cells is seldom demonstrated. We hypothesized that when the BCR of the immature B cells has a low affinity for self-antigens, those immature cells will acquire a MZ B cell fate. But this would not happen spontaneously. The immature B cells must receive the Notch signal by interacting with Dll-1 expressing cells, as Notch2 is essential for MZ B cells development. Here, in our study, we used multiple color immunostaining on the spleen samples of B6 mice to show the localization of Dll-1 expression and early stage B lymphocytes. Also, we found some interactions between Dll-1 expressing cells and early stage B cells. The result indicated DLL-1 signals need to be acquired at early stage of B cells for MZ B cells development.

1.2 Schematic figures

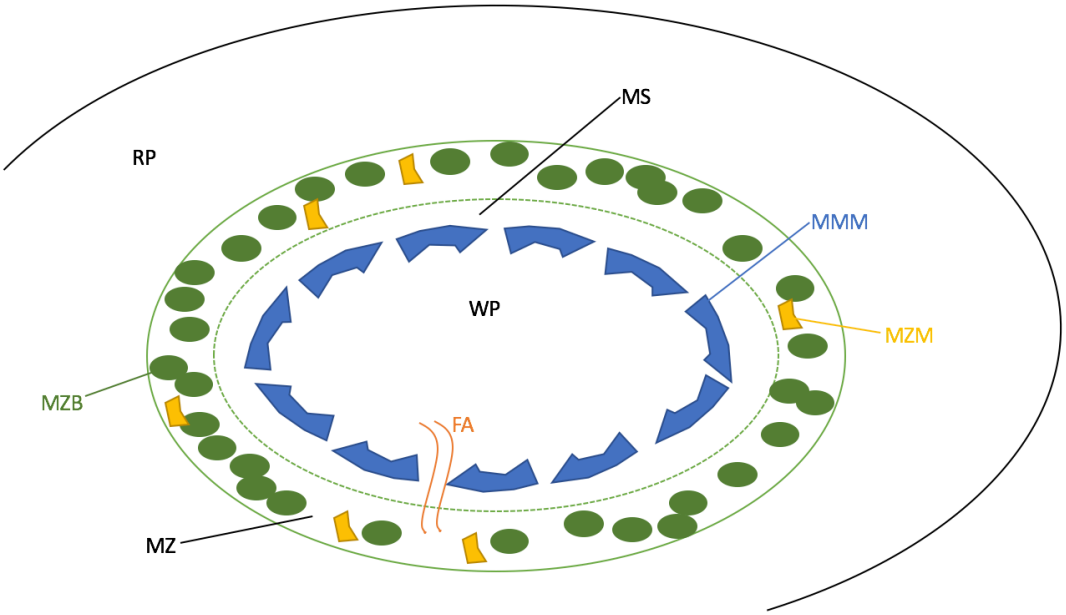

Figure 1 | Schematic view of the mouse marginal zone. WP: white pulp; RP: red pulp; MS: marginal zone sinus; MZ: marginal zone; MZB: marginal zone B cells; MZM: marginal zone macrophages; MMM: metallophilic marginal macrophages; FA: follicular arterioles.

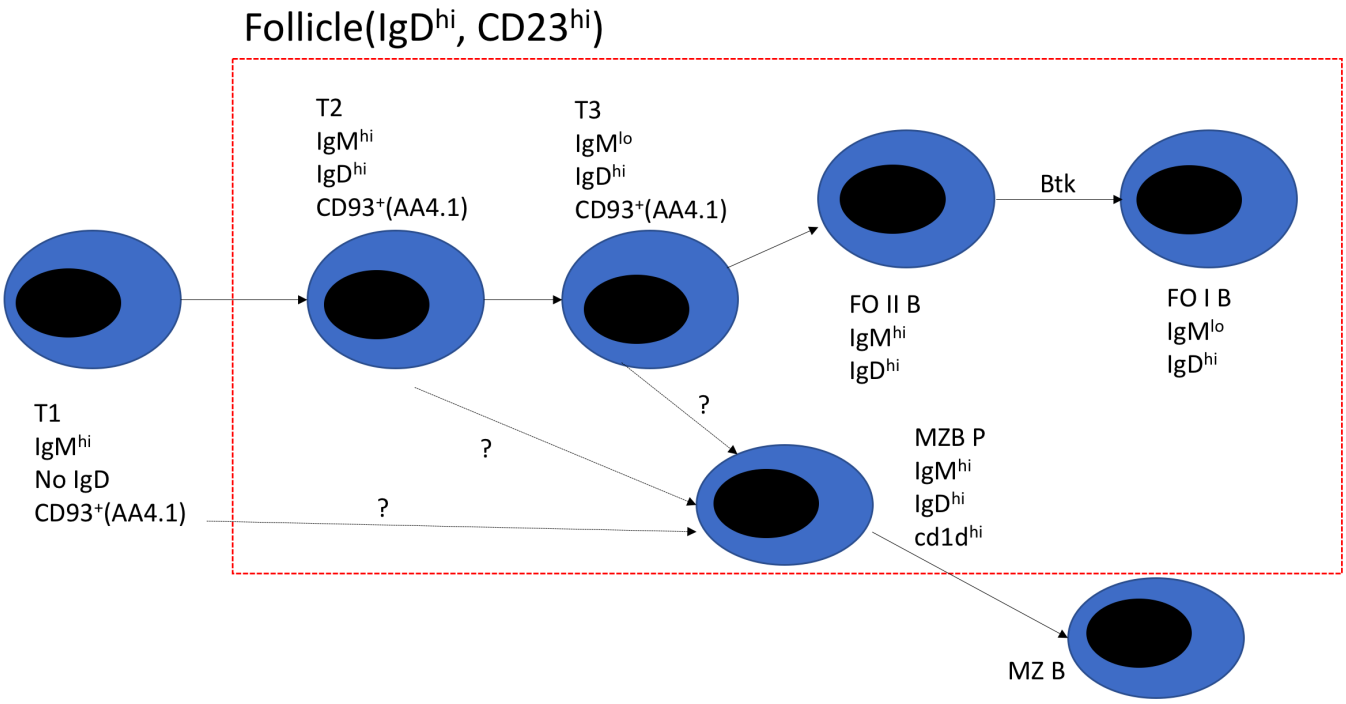

Figure 2 | Schematic view of Transitional B cell development. T1: Type 1 transitional B cell; T2: Type 2 transitional B cell; T3: Type 3 transitional B cell; FO I B: Type I follicular B cell; FO II B: Type II follicular B cell; MZP: marginal zone B cell precursor; MZB: marginal zone B cell.

Chapter 2: Methods and Data

2.1 Materials and Methods

Mice

All mice used in the study were B6 mice, 10-12 weeks old and female. The B6 mice were purchased from Jackson Laboratory. Mice were put in the mouse house in the animal facility at Ragon Institute until needed.

Antibodies

All antibodies used were conjugated anti-mouse antibodies and purchased from Biolegend. See the table below for detailed information.

Table1 | Detailed information of antibodies

Name	Clone	Conjugated Fluorophore	Reference number
IgM	MHM-88	APC	314509
CD169	MOMA-1	AF488	142419
CD93	AA4.1	FITC	136507
Dll-1	HMD1-3	APC	128314
IgD	11-26c.2a	AF594	405740

Preparation of the frozen spleen samples

After the spleen samples were harvested from the B6 mice, they were put in PBS with 4% paraformaldehyde(PFA) for fixation for 2 hours at 4 °C. Then they were washed for 5 times to remove the PFA. Samples were then put in 1X PBS with 10% sucrose overnight at 4 °C for cryo-protection before being embedded in OCT compound and frozen. After that 5 micrometers thick frozen sections were acquired at the Cryo-stat(Leica).

Immunostaining

The frozen sections were washed with 1X PBS for 5 times at room temperature(RT) to remove the OCT. Then the permeabilizing solution (5% BSA, 0.1% TritonX, 1X PBS) was used for blocking and permeabilization of the samples for 1h at RT. Then antibodies were diluted with the permeabilizing solution: IgM(1:25), CD169(1:100), CD93(1:25), Dll-1(1:10), IgD(1:50). After applying the antibodies to the section samples, they were incubated at RT for 1.5h. Next, washed the samples for 5 times with permeabilizing solution before mounting. After drying a little bit, samples were mounted with the anti-fade mounting medium with DAPI (Vector Laboratory). Stained samples were then scanned under TissueFax Scanner and analyzed with the TissueFax software.

2.2 Data and Results

Demonstration and Localization of Marginal Zone and Marginal Zone B cells

MZ B cells express high levels of IgM and low levels of IgD and CD23, and FO B cells express low level of IgM. It is easy to separate them by staining the cells with IgM. Transitional T2 cells are both IgM^{hi} IgD^{hi} and $CD93^{+}$. In order to identify MZ B cells, we needed to first find a way to distinguish MZ B and T2. As IgD is a follicular marker, T2 cells should be in the follicle in the white pulp and MZ B should be outside the follicle. In mice, marginal metallophilic macrophages(MMM) are located at the inner face of the marginal sinus. MMM express a sialic acid–binding lectin 1(Siglec-1), which can be targeted by the anti-moue MOMA-1 antibody [1]. This layer of MMM is always considered to be the innermost marker of the marginal zone, though the layer itself is part of the white pulp in the spleen. Considering that this layer can be used to separate the marginal zone the white pulp, we stained it with the CD169(MOMA-1) antibody. We observed the characteristic rim- the layer of MMM (green). The area within the rim is white pulp and the outer area around the rim is the marginal zone. Both the MZ B and T2 are demonstrated as IgM+ cells(red)(Fig3). The positive cells outside the rim are MZ B cells and the ones within the rim are T2 cells. From the result, we showed that MZ B cells inhabit the marginal zone around the white pulp. MZ B cells only consist a small proportion of the IgM + cells and the majority of the IgM+ cells are located in the B zone of white pulp, which are the T2 cells, or FO II B cells. This is consistent with previous studies and literature reviews.

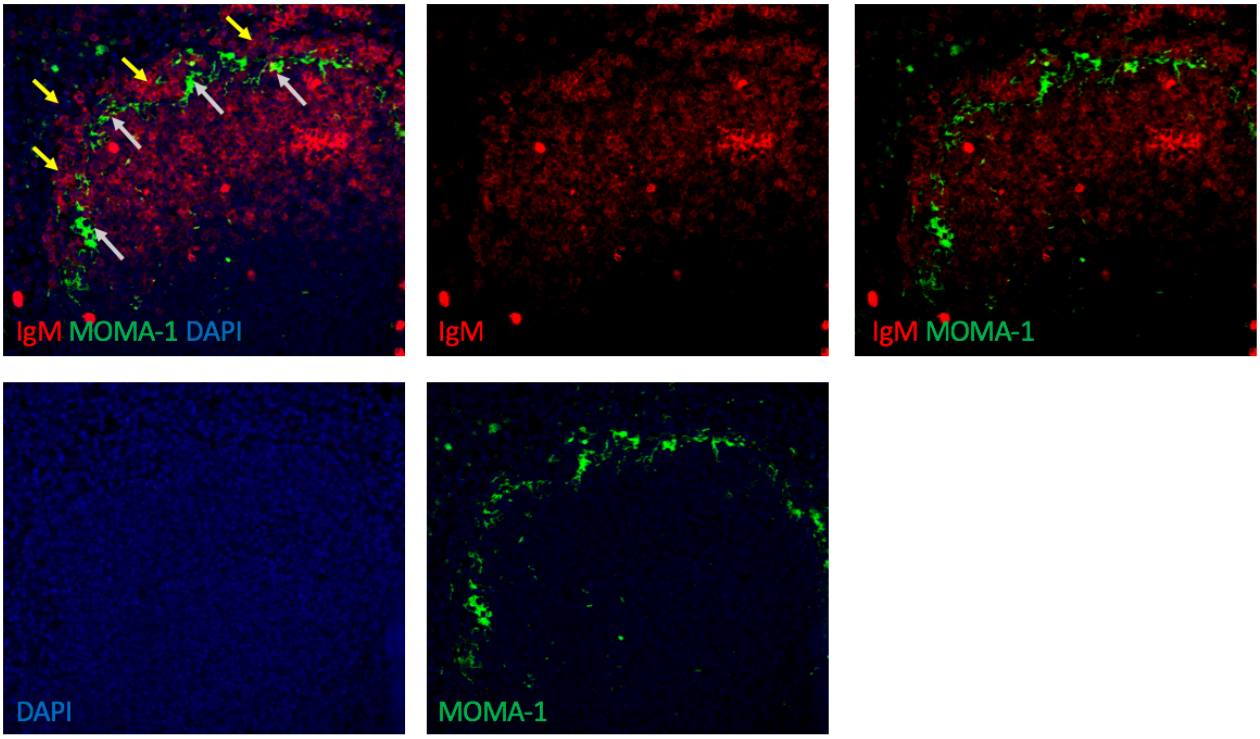

Figure 3 | Demonstration and Localization of Marginal Zone and Marginal Zone B cells: Immunofluorescent staining of B6 mice spleen sections shows the presence of IgM^+ MZ B cells and MOMA-1^+ MMM. IgM: red; MOMA-1: green; DAPI: blue. Yellow arrows: MZ B cells; Grey arrows: MMM.

Demonstrations of distribution of $CD93^+$ cells and Dll-1 expression in mouse spleen

As the conserved cell-to-cell signaling cascade of Notch2 signaling is essential for MZ B cells development, we hypothesized that the immature B cells must receive the Notch2 signal by interacting with Dll-1 expressing cells. Before we could look for the interactions between these two types of cells, we needed to demonstrate their locations in the mouse spleen. T1, T2 and T3 cells are all considered early stage B cells. They express CD93(AA4.1). As T1 cells are

$IgM^{hi}IgD^{lo}$, and T2 cells are $IgM^{lo}IgD^{hi}$ [1], and T3 cells is $IgM^{lo}IgD^{hi}$, we stained them with both anti-mouse CD93 and IgD antibodies. If the cells are double positive, they are T2 or T3 cells, and if they are only CD93 positive, that will be T1 cells. Interestingly, we found that the majority of $CD93^{+}$ cells(green) are mainly located in the red pulp of the spleen. IgD^{+} cells(blue), as expected, are located mainly in the B cell zone of white pulp. CD93 and IgD double positive cells are hardly to be found. Dll-1(red) cells are expressed in both of the red pulp and white pulp of the spleen, with a larger proportion in the red pulp (Fig 4). T2 and T3 cells are expected to be CD93 positive, but in the white pulp, a limited number of CD93 positive cells could be found, which suggested that the CD93 expression in T2 and T3 cells may be low and hard to detect, or there are very small amounts of T2 and T3 cells in the white pulp. Many $CD93^{+}$ cells and Dll-1 expressing cells are located closely, indicating that there are interactions between them. Our results demonstrated the locations of early stage B cells and Dll-1 expressing cells, providing the possibility to look for the interactions between these two types of cells.

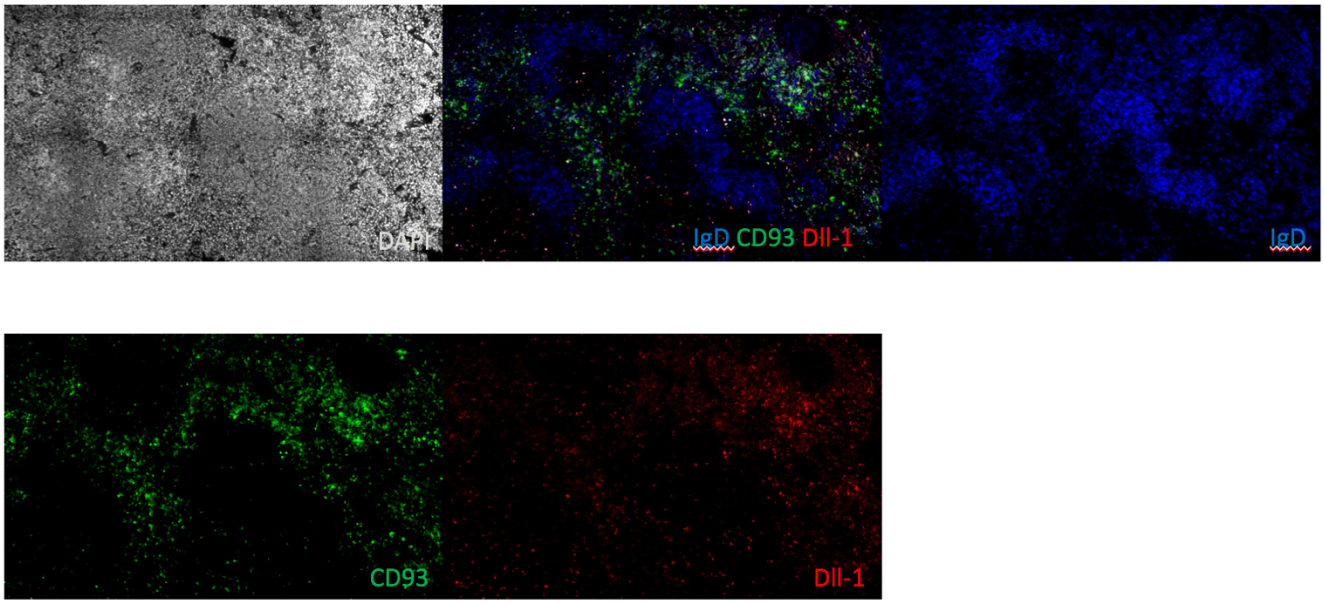

Figure 4 | Demonstrations of CD93+ cells and Dll-1 expressing cells in mouse spleen: Immunofluorescent staining of a frozen mouse spleen section sample to demonstrate the localization of $CD93^+$ cells, IgD^+ cells and Dll-1 expressing cells. Dll-1: red; CD93: green; IgD: blue.

Cell-cell contacts between $CD93^+$ cells and Dll-1 expressing cells were observed

Next, we looked for the cell-cell contacts between early stage B cells and Dll-1 expressing cells. The criteria of determining cell-cell contact is that the distance between the nucleus of two cells is less 0.54 micrometers. As discussed above, the $CD93^+$ and Dll-1 expressing cells are located close enough to each other. But cell-cell contacts were hard to be seen. While we can still find some interactions between these cells. The location of the contact observed is at the interface between the white pulp and red pulp, closer to white pulp. The distance between the nucleus of the two cells are 0.2 micrometers (Fig 5). This result indicate that cell-cell contact does occur between early stage B cells and Dll-1 expressing cells.

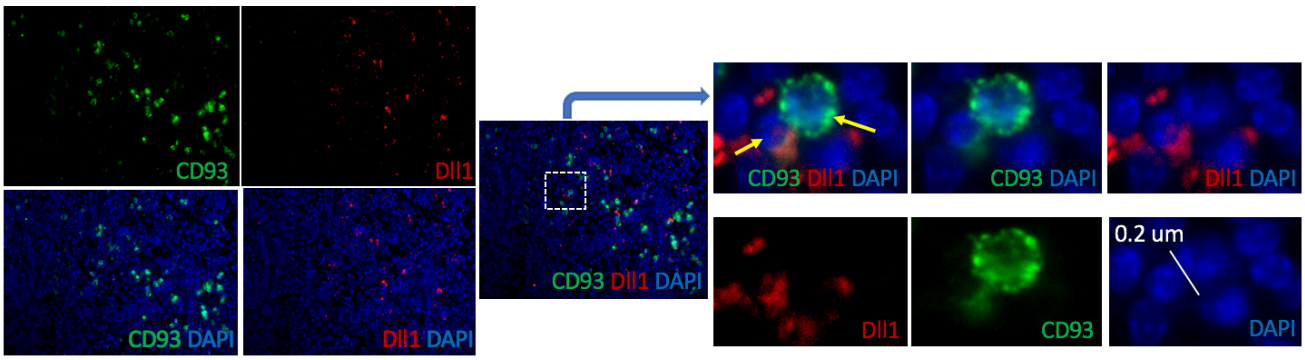

Figure 5 | Demonstration of cell-cell contacts between CD93$^+$ cells and Dll-1 expressing cells: Immunofluorescent staining of a frozen mouse spleen section sample to show the contact of CD93$^+$ cells and Dll-1 expressing cells (Yellow arrows pointed). Dll-1: red; CD93: green; DAPI: blue.

T2 cells are the potential CD93$^+$ cells interacting with Dll-1 expressing cells

As the cell-cell interaction of CD93+ cells and Dll-1 expressing cells was observed at the interface between the white pulp and red pulp of the spleen. The CD93$^+$ cells could be T1, T2 or T3. In order to further understand which subtype of transitional B cells are interacting with Dll-1, we reviewed the IMMGEN Database for the microarray data of Notch2 expression level on T1, T2 and T3 cells extracted from mouse spleen (Fig 6). We found that T2 cells have the highest expression level of Nothch2. It is strongly suggested that T2 cells are acquiring Dll-1 signals from Dll-1 expressing cells to become marginal zone precursor B cells (Fig 7).

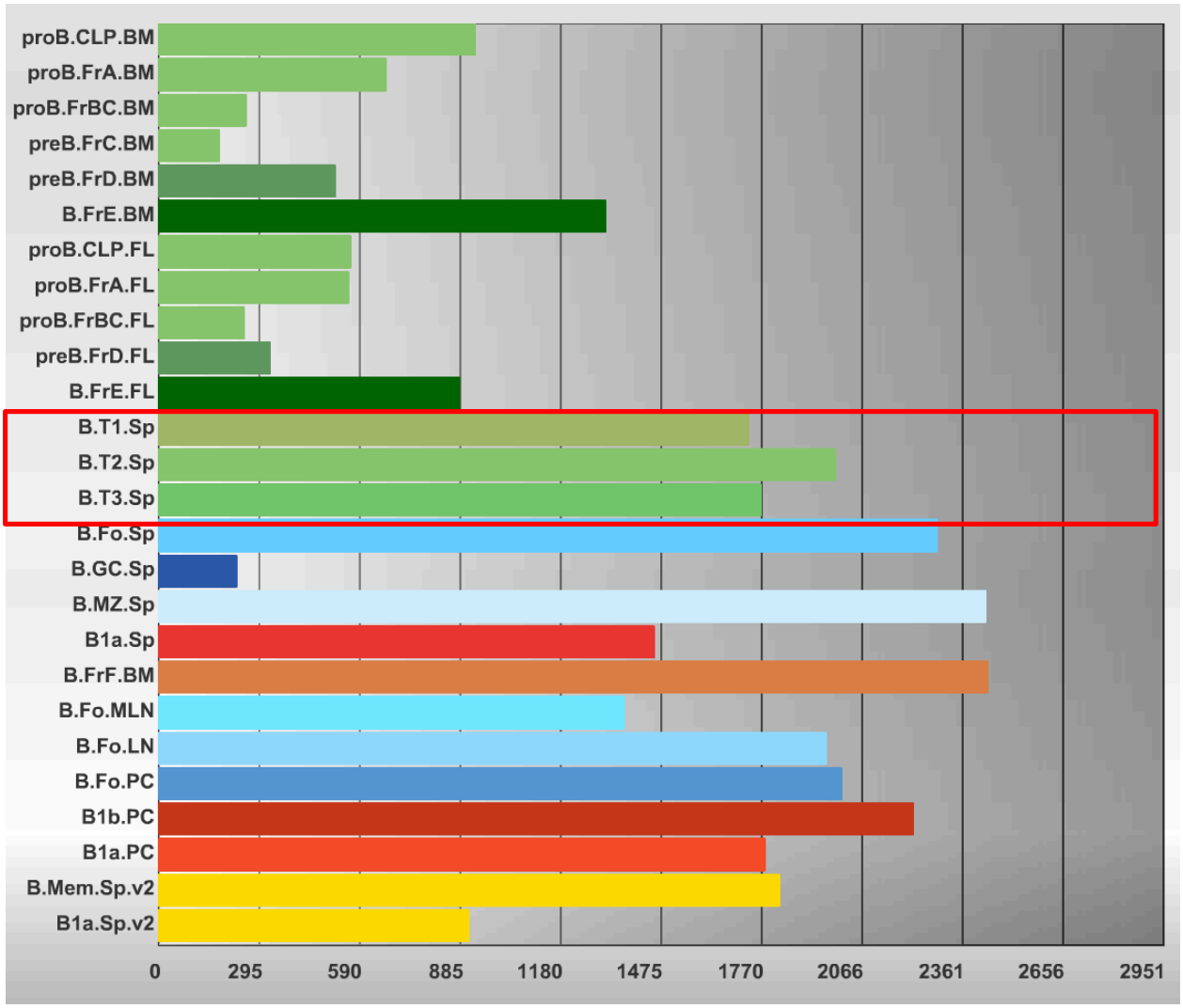

Figure 6 | Microarray data from IMMGen: Demonstration of Notch2 expression level of T1, T2 and T3 cells from mouse spleen (In the red frame). T2 cells have the highest level of expression of Notch2 among them.

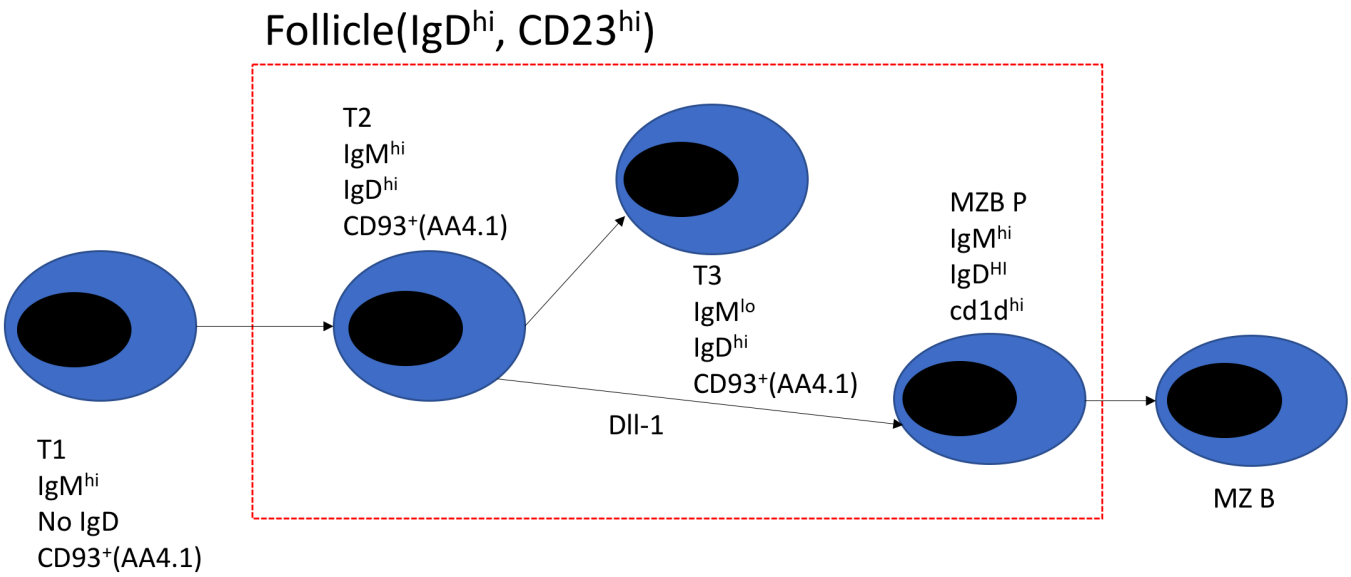

Figure 7 | Proposed schematic view of Marginal Zone B cell development: T2 cells receive Dll-1 signals (by interacting with Dll-1 expressing cells) to develop into Marginal zone precursor B cells.

Chapter 3: Discussion and perspectives

Two main key factors affect the immature B cells developing into MZ B cells. One is the specificity of the BCR. If the BCR has fair affinity with an antigen, then B cells will differentiate into FO B cells. If the BCR has a poor affinity with an antigen, without strong BCR stimulation signal, the cells will be driven to an MZ B cell fate. The second factor is the Notch signaling. When the B cells are induced to differentiate into a MZ B cell lineage, Notch signaling is requirement for the differentiation. In Notch2 conditional knock out mice, defects in MZ B cell development was observed, which indicated that Notch2 is indispensable for M Z B cell development through interacting with Dll-1. However, Notch2 is found prominently expressed in B cells, and which stage of B cells receive Notch signal to become MZ B cells is not clear. Also, the characteristics of the MZ B precursors are not well defined. It is suggested that T2 cells receive Notch signal and upregulate expression of CD1d to become MZ B precursor cells. In contest, other studies indicated that certain proportion of splenic FO B cells might be the MZ B precursors, since the knock-out of Notch2 expression also leads to the defect of a particular fraction of FO B cells besides the defect of MZ B cell population [1,20]. We hypothesized that the Notch signal should be acquired at early stage B cells before the cells are driven to MZ B cell fate, then the cells will develop into MZ B precursor cells. Our results showed the cell-cell contact of $CD93^{+}$ cells and Dll-1 expressing cells, which indicate that early stage B cells receive Notch2 signal by interacting with Dll-1 expressing cells, as CD93(AA4.1) is considered to be an early stage B lineage marker. However, further questions need to be addressed based on this: Since some studies demonstrated that the fibroblastic stromal cells may be the Notch signal niche cells for MZ B cells, the location of the cell interaction is expected to be in the white pulp. While in our study, the location of the cell-cell contact was observed in the area between red pulp and

white pulp, closer to white pulp. Some papers claimed that T2 cells or possibly some FO B cells (probably FO-II B cells) may migrate to the red pulp of the spleen, interact with Dll-1 expressing cells (Notch ligands) and are induced to differentiate into MZ B precursor cells and then MZ B cells. Therefore, it's really interesting and important to identify the real location for the cell-cell contact. For the $CD93^{+}$ cells, most of them were found located in the red pulp, which were also IgD^{-}. This indicated they are largely T1 cells. While T2 cells are thought to be the cells receiving Notch2 signal to differentiate into MZ B precursor based on previous studies. It's of essential to confirm whether T1 cells, rather than T2 cells contact with Dll-1 expressing cells to receive signals, as well as the mechanism of it. By reviewing the microarray data of Nothch2 expression level on T1, T2 and T3 from IMMGen database, we found the T2 cells have the highest expression of Notch2. It is indicated that T2 cells receive the signal from Dll-1 expressing cells and develop into marginal zone precursor B cells. Also, more work needs to be done continually: showing the percentage of the cell-cell contact between $CD93^{+}$ and Dll-1 expressing cells can prove the significance of the interaction. To further explore whether and how this physical contact contributes to MZ B cells development, as well as identifying the Dll-1 expressing cell would be interesting. Much more further work could be followed. More fun is coming.

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