Published in final edited form as:

Mol Immunol. 2011 June ; 48(11): 1292–1300. doi:10.1016/j.molimm.2010.12.006.

L2pB1: a new player in autoimmunity

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Abstract

L2pB1 cells (PD-L2 positive B1 cells) are a newly discovered subpopulation of B1 B cells. L2pB1 cells are noted for the expression of PD-L2 (CD273, B7-DC), a ligand for the inhibitory receptor PD-1, which distinguishes this subpopulation from other B1 B cells that lack PD-L2, namely, L2nB1 cells (PD-L2 negative B1 cells). PD-L2 gene expression is regulated differently in B1 B cells as compared to macrophages and dendritic cells. L2pB1 cells share many commonly known B1 cell features with L2nB1 cells. These include spontaneous IgM secretion, constitutive ERK activation, elevated co-stimulatory molecule expression, skewing of T cell differentiation, and unique proliferative responsiveness (to LPS, PMA, but not anti-IgM). However, L2pB1 cells express a biased Ig repertoire that is enriched for self-reactivity as compared with L2nB1 cells. Further, L2pB1 cells present antigen more potently than L2nB1 cells. In addition, L2pB1 cells switch Ig isotype more readily from IgM to IgG1 and IgG2b upon cytokine stimulation. Moreover, increased numbers of L2pB1 cells are present in murine models of lupus and this correlates with increased serum anti-dsDNA titers. These characteristics suggest that L2pB1 cells may play a pathophysiological role in autoimmune dyscrasias. In this report we review the special features of L2pB1 cells and how they may contribute to autoimmunity.

Keywords

PD-L2; B1 B cells; autoimmunity

1. Characteristics of L2pB1 cells

1.1 What is the L2pB1 cell?

In mice, B cells can be divided into at least two separate lineages, characterized by distinct progenitors: conventional B cells (also termed **B2** B cells), consisting of follicular and marginal zone (MZ) B cells, and **B1** B cells (Montecino-Rodriguez and Dorshkind, 2006). B1 B cells are readily distinguished phenotypically from B2 cells, most notably by expression of the macrophage marker CD11b and the T cell marker CD5 (reviewed in

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(Hardy, 2006)). B1, MZ and B2 B cells are said to form a layered immune system with MZ and B1 cells bridging the innate and adaptive branches of the immune system (Viau and Zouali, 2005a). Murine B1 B cells preferentially localize to the peritoneal cavity. However B1 B cells are also present in the spleen, in lymph nodes, and in various parts of the intestine, albeit in much smaller proportions in comparison to B2 B cells (Kantor and Herzenberg, 1993; Kroese et al., 1992; Marcos et al., 1989; Yeo et al., 2006). B1 B cells are subdivided into B1a and B1b B cells, depending on expression of CD5. CD5-expressing **B1a** cells are the dominant B1 cell population. Functionally B1a, but not B1b, B cells are the source of natural antibody (Forster et al., 1991; Mond et al., 1995a; Mond et al., 1995b) that is present in the “resting” state in the absence of infection or intentional immunization. This natural antibody is protective against viral and bacterial infections, in concert with B2 cell-generated adaptive immune responses (Baumgarth et al., 2000; Boes et al., 1998; Haas et al., 2005; Ochsenein et al., 1999).

L2pB1 cells are a major subpopulation of B1a cells and express PD-L2 (PD-L2 positive B1 cells). Depending on the strain, 50% to 70% of murine peritoneal B1a cells are L2pB1 cells (Figure 1). In addition, a small number of L2pB1 cells can be found in murine spleen and lymph nodes (Zhong et al., 2007b).

1.2 PD-L2 is uniquely expressed on L2pB1 cells

PD-L2, also termed B7DC, is a member of the B7 family and one of the two ligands (PD-L1 and PD-L2) for the inhibitory receptor Programmed Death 1 (PD-1) (Latchman et al., 2001). PD-L1 and PD-L2 located closely on the same chromosome in mice and have been reported having distinct expression and function in various diseases (reviewed by Singh et al. 2010). Prior to the identification of L2pB1 cells, macrophages and dendritic cells (DCs) were the only cell types reported to express PD-L2, and then only after stimulation with IL-4, IFN γ and GM-CSF (Liang et al., 2003; Loke and Allison, 2003; Yamazaki et al., 2002). In contrast, PD-L2 is constitutively expressed by L2pB1 cells and cannot be induced on B cells that do not initially express it (specifically, PD-L2 negative B1, L2nB1, cells and B2 cells) by these or other cytokines in vitro (Zhong et al., 2007b). Furthermore, after intravenous injection into RAG2 null mice, most L2pB1 and L2nB1 cells home to the peritoneum cavity whereas B2 B cells mostly home to the spleen (Figure 2). During such adoptive transfer, L2nB1 cells do not gain PD-L2 expression when “parked” in RAG2 null mice for 7 days, and L2pB1 cells do not lose PD-L2 after the same manipulation (Figure 2). Moreover, the inducible expression of PD-L2 on macrophages has been reported to be STAT6-dependent (Loke and Allison, 2003), whereas loss of STAT6 does not affect PD-L2 expression on L2pB1 cells (Zhong et al., 2007b), suggesting that PD-L2 expression might be regulated differently in B1 cells as compared to other cell types.

To further understand PD-L2 expression in L2pB1 cells, PD-L2 gene products were examined in several cell types. We found that PD-L2 transcripts differ in L2pB1 cells as opposed to macrophages and DCs, with the former beginning downstream of, and excluding, exon1 but the latter beginning upstream of exon1 (Kaku and Rothstein, 2010). This raised the possibility of a B1 cell specific promoter, which was evaluated by determining the location of sites that direct PD-L2 gene expression. We found that a unique intronic promoter located between exon1 and exon2 is responsible for PD-L2 expression in L2pB1 cells (Kaku and Rothstein, 2010). Two transcription factors, Octamer binding protein1 (Oct1) and Oct2 bind this intronic promoter in vivo in B1 cells but not in B2 B cells, despite their presence. Thus other factors such as chromatin structure and/or post-translational modification of transcription factors may be responsible for distinct PD-L2 promoter usage and activity in L2pB1 cells (Kaku and Rothstein, 2010). Recently, PD-L2 expression by germinal center (GC) B cells and by memory B cells has been reported (Good-Jacobson et

al., 2010). It is unknown at present whether GC and memory B cells share the intronic regulation of PD-L2 expression that characterizes L2pB1 cells.

The physiological function of PD-L2 on L2pB1 cells has not been fully elucidated. As the PD-1 receptor is expressed on activated B cells and T cells, it is reasonable to assume that PD-L2 is involved in an interaction between L2pB1 cells and PD-1 expressing cells, which is generally thought to produce suppression or inhibition of receptor-bearing cells. However, PD-L2 on germinal center B cells promotes the generation of long-lived plasma cells, apparently by enhancing follicular T helper cell (T_{FH}) function via interaction with T_{FH} PD-1 (Good-Jacobson et al., 2010). Notably, PD-L2 on B cells, but not on DCs and macrophages, is effective in this context. These results and others (Shin et al., 2005; Tseng et al., 2001) suggest that PD-L2 can produce positive, as well as negative, effects through PD-1 (or, perhaps, through a putative unidentified positive-signaling receptor). It will be important to determine whether GC B cell PD-L2, like L2pB1 cell PD-L2, differs from PD-L2 expressed by macrophages and dendritic cells, in primary structure or as a result of post-translational modification, or whether in this case PD-1 is wired differently in T_{FH} cells as compared to other cell types. Alternatively, it is possible that other co-stimulatory molecules work in conjunction with PD-L2 on GC B cells to influence T_{FH} cells.

1.3 L2pB1 cells express many typical B1 cell characteristics

First and foremost, L2pB1 cells are B1 cells, and thus manifest typical features previously identified for murine B1 cells in general. These include spontaneous secretion of IgM (Klinman and Holmes, 1990; Tumang et al., 2005); constitutive ERK activation (Holodick et al., 2009; Wong et al., 2002); defective BCR signaling (Morris and Rothstein, 1993); proliferative responsiveness to PMA (Rothstein and Kolber, 1988); and, elevated expression of CD44, CD80 and CD86 (Murphy et al., 1990; Tumang et al., 2004). In these characteristics, L2pB1 cells and L2nB1 cells are similar and both are very different from B2 cells. In other general B1 cell characteristics, including autoreactive repertoire skewing (Hardy et al., 2004), potent antigen presentation (Zhong et al., 2007a), and elevated replication history (unpublished observations), L2pB1 cells exceed L2nB1 cells, which in turn far outweigh B2 cells.

2. L2pB1 cells and autoimmunity

2.1 L2pB1 cells display a repertoire skewed toward autoreactivity

B1 cells have historically been linked to autoimmunity, for reasons that include production of autoreactive antibody and increased number in some autoimmune mice and in some autoimmune patients (Dauphinee et al., 1988; Wofsy and Chiang, 1987; Youinou et al., 1990). The recent finding that a polymorphism of PD-L2, but not PD-L1, is linked to SLE susceptibility (Wang et al., 2007) raises the possibility that PD-L2-expressing L2pB1 cells play a pathophysiological role in autoimmunity. Indeed, several characteristics of L2pB1 cells, most notably repertoire skewing, suggest that this may be the case.

2.1.1 Most PtC-binding B1 cells are L2pB1 cells—It has been known for a long time that the B1 cell repertoire is skewed toward recognition of phosphatidylcholine, (PtC), the major component of the phospholipid portion of the cell membrane (Mercolino et al., 1988). Up to 10% of all peritoneal B1 cells express this signature autoantibody which binds to PtC exposed on senescent or bromelain-treated red blood cells, presumably playing a role in clearing damaged or expired erythrocytes (Cunningham, 1974; Hardy et al., 1989; Pages and Bussard, 1975; Reininger et al., 1988). Ironically, this specificity may also be responsible for some forms of autoimmune hemolytic anemia (Cabral et al., 1990). To evaluate potential differences in repertoire composition between L2pB1 and L2nB1 cells, B1 cell populations

were tested for binding to PtC-bearing liposomes. We found that anti-PtC antibody-bearing B1 cells are primarily L2pB1 cells; anti-PtC binding is almost an order of magnitude more common in the L2pB1 cell population as compared to the L2nB1 cell population (Zhong et al., 2007b). We further showed that L2pB1 cells express substantially more transcripts of VH11 and VH12, heavy chain variable region genes that specify PtC-binding, as compared to L2nB1 cells (Zhong et al., 2007b). Thus, the BCR repertoire of L2pB1 cells differs from that of L2nB1 cells and manifests skewing toward autoreactivity. Another example of L2pB1 repertoire segregation lies in the recognition of phosphorylcholine (PC), a determinant found on pathogenic pneumococci and on oxidized LDL. Anti-PC antibodies defend against infection and may fulfill a housekeeping function in relation to pathogenic oxidized lipids associated with atherosclerosis (Binder and Silverman, 2005). Our preliminary data suggest that majority of PC-binding B1 cells might also be L2pB1 cells (unpublished observations). Thus, with anti-PC and anti-PtC as signature antibodies, L2pB1 cells represent a subset of B1 cells with an expressed repertoire skewed toward recognition of self-components.

2.1.2 L2pB1 cells react to dsDNA and are poly-reactive to other autoantigens

—In view of the repertoire skewing evident in the specificity of L2pB1 cells for PtC and PC, further work focused on more classical assays for autoreactivity. To elucidate the nature of B1 cell antibodies, culture fluids obtained from LPS-stimulated B1 cells were tested for HEp-2 cell staining. We found that supernatants from sort-purified L2pB1 cells contain much higher titer anti-HEp-2 antibody than L2nB1 cell supernatants. Looking specifically at anti-dsDNA antibody by ELISA, we found that L2pB1 cell supernatants contain much higher titer DNA-binding immunoglobulin than L2nB1 cell supernatants, which in turn contained much more DNA-binding Ig than B2 cell supernatants (Zhong et al., 2009). To address the degree of polyreactivity manifest in L2pB1 autoantibodies, hybridomas were generated from sort-purified L2pB1 cells; hybridomas that produced anti-dsDNA antibody were selected and tested by autoantigen protein array for additional autoreactive specificities. We found that many anti-dsDNA hybridoma supernatant samples reacted with multiple autoantigens (including glomerular basement membrane), recapitulating the polyreactivity of LPS-stimulated L2pB1 cell supernatants and of BXS^B-Yaa⁺ positive control sera (Zhong et al., 2009). Thus, L2pB1 cells not only display a repertoire skewed toward autoreactive specificities, but individual L2pB1 autoantibodies display polyreactivity for multiple autoantigens. This poly-autoreactivity may explain the heightened expansion of L2pB1 cells in comparison to L2nB1 cells, as reflected in replication history measured by KREC assay (van Zelm et al., 2007), which is greater for L2pB1 cells than for L2nB1 cells, and which in turn is much greater for any B1 cell than for B2 cells (unpublished observations). A further difference between L2pB1 and L2nB1 cells lies in the degree of immunoglobulin N-region addition; within the context of B1 cells that as a group contain far fewer immunoglobulin N-region additions than B2 cells, we found that L2pB1 cell immunoglobulin contained fewer N-additions as compared to L2nB1 cell immunoglobulin (unpublished observations).

Overall, these results show that L2pB1 cells manifest a very skewed self-reactive immunoglobulin repertoire. Moreover, L2pB1-derived autoantibodies are highly poly-reactive. However, it is not known at this point if these poly-autoreactive antibodies directly contribute to the pathogenesis of autoimmune diseases. The notion that B1 cell-derived autoantibodies produce autoimmunity is still controversial. On one hand, B1 cell autoantibody is considered to be broadly reactive (as noted above), low affinity, and IgM, whereas disease-associated autoantibody is typically relatively specific, high affinity, and IgG (Baudino et al., 2008; Giorgini et al., 2008; Yang et al., 2005). Thus, Reap and colleagues reported that in chimeric mice reconstituted with B1 and B2 B cells from congenic B6/lpr mice of different allotypes, it is B2 B cells that contribute to IgG anti-DNA

antibody (Reap et al., 1992), supporting the view that pathogenic autoantibodies do not come from B1 cells. On the other hand, Kanno and colleagues reported that B1 cells are the precursors of IgG anti-DNA producing B cells in autoimmune-prone NZB/W F1 mice (Kanno et al., 1992), suggesting that B1 cells can isotype switch to produce disease. In keeping with the latter, we found that murine B1 cells can switch isotype expression to IgG1 and IgG2b upon cytokine stimulation in vitro, and that L2pB1 cells switch more readily than L2nB1 cells (Zhong et al., 2009), again promoting the idea that L2pB1 cells enriched for autoreactive specificities may switch isotype in vivo to produce pathogenic autoantibodies.

Alternatively, it may be that specificity trumps isotype in contributing to pathogenicity. Thus, in finding that both IgM and IgG NZM2410 monoclonal antibodies administered to NZW mice are pathogenic, Liang and colleagues showed that mice receiving monoclonal antibodies that are polyspecific and recognize both dsDNA and glomerular tissue exhibit the most severe renal dysfunction, regardless of isotype (Liang et al., 2004). Moreover autoreactive B1 cell-derived natural IgM antibody has been reported to induce ischemia reperfusion injury, through binding to self-antigen which produces a site for mannan-binding lectin that subsequently leads to activation of complement and ultimate injury (Zhang et al., 2006). Inasmuch as L2pB1 cell-derived immunoglobulin is skewed to autoreactivity and poly-reactivity, the L2pB1 population is likely to directly contribute to the pool of pathogenic autoantibodies. Such a contribution could be produced by a mere increase in L2pB1 cell numbers resulting in autoantibody overload regardless of isotype switching and affinity maturation.

2.2 L2pB1 cells are associated with autoimmunity

As noted above, an increase in the size of the B1 B cell pool has been associated with autoimmunity. In human disease, an increase of CD5⁺ B cells, albeit an imperfect marker for human B1 cells, is correlated with autoantibody production and C-reactive protein in patients with rheumatoid arthritis (Becker et al., 1990; Youinou et al., 1990). Similar increases in CD5⁺ B cells have been reported in various other autoimmune diseases, including Sjogren's Syndrome and Systemic Lupus Erythematosus (Dauphinee et al., 1988; Markeljevic et al., 1994).

In mice, an increase in the B1 cell population is observed in NZB/W F1 lupus-prone mice. Further, among several lupus susceptibility loci identified in NZM2410 mice, increased numbers of B1 cells are specifically associated with Sle2, in particular, Sle2c (Mohan et al., 1998; Wofsy and Chiang, 1987). Although previous data suggested that Sle2c does not, by itself, result in disease manifestations beyond autoantibody production (Xu et al., 2005), more recent work indicates that a smaller region of Sle2c both increases B1 cell numbers and produces autoimmune disease, suggesting a causal relationship (Laurence Morel, manuscript submitted). Beyond that, the fact that combining Sle2 with either Sle1 or Sle3 increases or exacerbates disease manifestations (Morel et al., 2000; Xu et al., 2005) further suggests a role for B1 cells. A causal relationship between B1 cell numbers and autoimmunity is emphasized by the report that superantigen-mediated depression of B1 cell numbers reduces signs of lupus in NZB/W F1 mice (Viau and Zouali, 2005b).

These findings, though strongly suggesting a role for B1 cells in autoimmunity, do not speak to the role of L2pB1 cells in particular. To address this point, autoimmune BXSB-Yaa mice were examined for L2pB1 cell numbers. We found increased numbers of L2pB1 cells in the blood and lymphoid organs of these mice as compared to control animals (Zhong et al., 2009). Although B1 cells are increased in general in BXSB mice, L2pB1 cells are increased more than L2nB1 cells, indicating preferential recruitment or expansion of L2pB1 cells. Emphasizing this point, we found in BXSB mice a direct correlation between the proportion of peripheral blood L2pB1 cells and serum anti-dsDNA titer (Zhong et al., 2009). In related

work we found an increased number of L2pB1 cells in a murine model of experimental autoimmune myocarditis (unpublished observations). These results suggest a pathophysiological role for L2pB1 cells in autoimmunity, although it remains to be determined if the increase in L2pB1 cells is due to a fundamental alteration in B1 cell development and/or repertoire, an increase in circulating autoantigens and immune complexes, or a change in chemokine and chemokine receptor expression. It is important to keep in mind that an increase in L2pB1 cells can result from an increase in the proportion of L2pB1 cells among all B1 cells, as noted above for BXSB-Yaa mice, or from an increase in the total number of B1 cells wherein the proportion of L2pB1 cells is unchanged. The latter may occur in other autoimmune situations in which large increases in B1 cells have been described.

2.3 L2pB1 cells activate and differentiate T cells

The skewed, poly-autoreactive repertoire of L2pB1 cells, combined with the spontaneous immunoglobulin secretion that characterizes all B1a cells, (possibly aided and abetted by isotype switching), strongly suggests that the L2pB1 cell association with, and presumed pathogenic role in, autoimmune diseases results in a direct fashion from the production of autoantibody. However, an alternate or additional mechanism is suggested by the underappreciated capacity of B1 cells to present antigen to T cells and to direct T cell activation and differentiation.

2.3.1 L2pB1 cells stimulate T cells—It is well known that B1 cells far surpass B2 cells in allogeneically stimulating, and presenting specific antigen to, T cells. To elucidate differences in L2pB1 and L2nB1 cells, allogeneic stimulation of T cells by sort-purified B1 cell populations was evaluated. We found that L2pB1 cells are more effective than L2nB1 cells in stimulating T cells, when assessed by T cell thymidine incorporation or T cell cytokine secretion (Zhong et al., 2009). The mechanism for this is not obvious, because L2pB1 and L2nB1 cells do not differ in the expression of B7 co-stimulatory molecules CD80 and CD86, nor in the expression of MHC I and MHC II (unpublished observations). Based on the observation that PD-1 null mice develop autoimmune disease (Nishimura et al., 1999; Nishimura et al., 2001), it is generally considered that PD-1 ligands such as PD-L2 deliver inhibitory signals through the PD-1 receptor; however, in some experimental systems, PD-L2 has been reported to stimulate T cells (Ofiazoglu et al., 2004; Tseng et al., 2001). Thus, it is possible that PD-L2 itself is responsible for the enhanced T cell stimulation produced by PD-L2-expressing B1 cells. Alternatively, an inhibitory effect of PD-L2 on PD-1-bearing T cells might be masked by the anti-PD-L2 sorting antibody, allowing L2pB1 cells to express a higher level of T cell stimulation. However, addition of anti-PD-L2 antibody to total B1 cells does not alter their capacity to allogeneically stimulate T cells (unpublished observations), as might be expected if the antibody affected more than half of the B1 cells (that bear PD-L2). Thus it remains puzzling why L2pB1 cells, in the absence of PD-L2 interaction, manifest an intrinsically higher capacity for T cell stimulation than L2nB1 cells. Still another possibility is that PD-L2 engagement produces retrograde signaling in L2pB1 cells that somehow alters the L2pB1 cell/T cell interaction. Retrograde PD-L2 signaling eventuating in NF- κ B activation has, in fact, been reported for dendritic cells, although more recently this work has been questioned (Nguyen et al. 2007; Nguyen et al. 2010 retracted; Radhakrishnan et al., 2003; Radhakrishnan et al., 2007). To verify this, B1-like BRD2 cells that express PD-L2 (Greenwald et al., 2004) were examined for induction of NF- κ B after treatment with anti-PD-L2 antibody, as measured by a κ B-dependent luciferase construct. We found no increase in NF- κ B, even when we used biotinylated anti-PD-L2 crosslinked with streptavidin (unpublished observations). In sum, all B1 cells efficiently present antigen to, and stimulate, T cells; the means by which L2pB1 cells outweigh L2nB1 cells in this regard remains unknown, and raises the question of

whether PD-L2 is the motive force behind this function or merely a sign of B1 cells that are especially adept at T cell interaction.

2.3.2 L2pB1 cells induce Th17 cell differentiation—In concert with the potent B1 cell antigen presentation function described above, B1 cells induce differentiation of naïve CD4 T cells to proinflammatory Th17 cells and Th1 cells, and away from Treg cells, in contrast to B2 cells, that skew T cell differentiation in the opposite direction (Zhong et al., 2007a). This unique function of B1 cells is at least partly due to increased CD86 expression (Zhong et al., 2007a). To address potential segregation of T cell differentiating capacity, sort-purified B1 cell fractions were examined for induction of Th17 cell differentiation. We found that L2pB1 cells and L2nB1 cells behaved similarly in promoting Th17 differentiation (Zhong et al., 2009), which may be due to similar expression levels of co-stimulatory molecules, as noted above.

Th17 (and Th1) cells have been reported to contribute to autoimmunity in many diseases, including lupus, rheumatoid arthritis, multiple sclerosis, psoriasis, asthma, and inflammatory bowel disease. (Crispin and Tsokos, 2010; El-behi et al. 2010; Leung et al., 2010; Lubberts, 2010; Mai et al., 2010; Tesmer et al., 2008; Turner et al., 2010). Whether Th17 cells induced by B1 cells contribute to autoimmune disease in vivo is not known. Further, whether L2pB1 cells in particular preferentially contribute to putative Th17 cell-mediated pathology is also not known. While it may seem that L2pB1 cells would be no different than L2nB1 cells in promoting Th17 cell-mediated outcomes, since they are no different in promoting Th17 cell differentiation, it is important to remember that the L2pB1 repertoire is skewed toward autoreactivity to a much greater extent than the L2nB1 repertoire (and will thus more likely capture and present self-antigens during Th17 cell differentiation), and that L2pB1 cells stimulate T cell expansion more strongly than L2nB1 cells. Through these mechanisms it may be speculated that among B1 cells, L2pB1 cells are particularly effective at stimulating and expanding autoreactive Th17 cells.

2.4 L2pB1 cells normally home to the peritoneal cavity

B1 cells in general, and L2pB1 cells in particular, preferentially reside in the peritoneal cavity and other coelomic cavities of normal mice. This is emphasized by reconstitution/migration experiments, wherein L2pB1 cells administered intravenously in RAG2 null mice preferentially home to the peritoneal cavity, in contrast to B2 cells that preferentially home to the spleen (Figure 2). In these experiments, few L2pB1 cells end up in the spleen and few B2 cells end up in the peritoneal cavity. These relationships appear to be disrupted in autoimmunity as a result of aberrant homing (Ishikawa and Matsushima, 2007; Ito et al., 2004), which includes migration of B1 cells to the thymus gland (Sato et al., 2004).

As noted above, L2pB1 cells accumulate in the peripheral blood of lupus-prone NZB/W F1 mice in direct relation to anti-dsDNA titer, blood being a tissue that is normally devoid of B1 cells. Beyond blood, we found that L2pB1 cells especially are present in increased numbers in BXS-B-Yaa thymic tissue, as well as in spleen and lymph node (Zhong et al., 2009 and unpublished observation). To explore the mechanism responsible for abnormal homing, B1 cells were evaluated for expression of CXCR5. Whereas B1 cells are known to express elevated levels of CXCR5 and respond to CXCL13 (Ansel et al., 2002; Ishikawa and Matsushima, 2007), we found that both L2pB1 cells and L2nB1 cells express similar high levels of CXCR5 (unpublished observations), so differential expression of CXCR5 cannot explain the enhanced aberrant homing of L2pB1 cells in autoimmunity. Further investigation of other chemokines and receptors is needed to solve this mystery. However, regardless of the mechanism of such abnormal migration, release of autoreactive L2pB1 cells from the sequestering peritoneal environment into peripheral tissues is likely to have a

profound effect on activity and function. Outside the peritoneal cavity, L2pB1 cells are exposed to different cytokine, immune cell, and stromal cell interactions. This different milieu is likely responsible for the well known observation that the peritoneal environment is special, in some cases sequestering B1 cells from self-antigen, in other cases altering viability in response to apoptotic agents (Hamaguchi et al., 2005; Murakami et al., 1997). Thus, during infection and/or inflammation, in which chemokine expression is altered, L2pB1 cells may be released into the periphery to ill effect.

2.5 Regulatory potential of L2pB1 cells

The role of B1 cells in autoimmune dyscrasias has generally been considered a straightforward one, in which B1 cell production and secretion of autoreactive antibodies, or B cell stimulation and/or differentiation of pro-inflammatory T cells, is pathogenic and leads directly to tissue damage. However, the role of B1 cells could relate to a relative deficiency of B1 cell-derived immunomodulation in the form of regulatory B cells (Breg).

The existence of Breg was first suspected in an early study of experimental autoimmune encephalomyelitis (EAE). In this model for multiple sclerosis, mice develop EAE following immunization with myelin basic protein (MBP). Most of the mice recover spontaneously. In contrast, mice without B cells are unable to recover from EAE and develop chronic disease (Wolf et al., 1996). Further study revealed that recovery from EAE depends on the presence of auto-reactive B cells that secrete IL-10 (Fillatreau et al., 2002), sometimes termed B10 cells {Matsushita, 2010}. Adoptive transfer of B10 cells was also reported to ameliorate established arthritis in a murine model (Mauri et al., 2003). Though it has been reported that B10 cells express CD5 (Yanaba et al., 2008), the classification of IL-10-producing B cells remains elusive (Evans et al., 2007; Jamin et al., 2008), because IL-10 producing B cells display different phenotypes in different models, and because IL-10 producing B cells have been reported to encompass to varying degrees subsets of B1, marginal zone, and transitional 2-MZ precursor B cells (Bouaziz et al., 2008; Evans et al., 2007; Lenert et al., 2005; Sun et al., 2005).

Despite these uncertainties, B1 B cells have long been appreciated as the principal B cell source of IL-10, and IL-10 has long been viewed as a key mediator of regulatory function, for which reasons B1 B cells stand out as the likely dominant contributors to Breg cells (Blenman et al., 2006; O'Garra et al., 1992). To further evaluate the origin of B cell-derived IL-10, purified B1 cell sub-populations were evaluated for IL-10 expression. We found that resting L2pB1 and L2nB1 cells both expressed IL-10, and that there was little difference between them (unpublished observations). As in the case of Th17 cell induction, it is important to note the preferential autoreactive repertoire skewing that characterizes L2pB1 cells. This preponderance of self-antigen specificities within the L2pB1 cell population suggests that Breg that are L2pB1 will be activated by self-antigen to down-modulate potential autoimmunity. In other words, it may be speculated that L2pB1 cell deficiency, or deficient function, may contribute to autoimmunity through deficient production of IL-10 in key situations. However, the role of IL-10 in SLE is controversial. On one hand, elevated IL-10 levels correlate with disease in many SLE patients and in some murine SLE models (Amel Kashipaz et al., 2003; Blenman et al., 2006). Further, polymorphisms in the IL-10 promoter that lead to high IL-10 production are associated with SLE susceptibility (Chong et al., 2004). On the other hand, IL-10 has been reported to be protective in some murine SLE models (Yin et al., 2002). This raises the possibility that IL-10 possesses both immunostimulatory and immunosuppressive properties such that high levels of IL-10 promote, whereas low IL-10 levels suppress, autoimmunity (Blenman et al., 2006). Thus, it remains to be determined whether L2pB1 cell-derived IL-10 contributes to (high IL-10) or ameliorates (low IL-10) autoimmune disease.

One interesting point relates to aberrant thymic infiltration of B1 in lupus mice, noted above (Ishikawa and Matsushima, 2007; Sato et al., 2004). It has been suggested that upon locating there, B1 cells might act to stimulate autoreactive T cells. While this may indeed occur, we suggest that the contrary may also be possible and that thymic B1 cells may function in a protective manner to counteract autoimmunity and re-establish homeostasis by presenting autoantigen to thymic T cells and reinforcing thymic clonal deletion of autoreactive T cells through apoptosis. Indeed, in a schistosomal infection model, B1a cells but not other B cells express high levels of FasL and downmodulate T cell-mediated chronic granulomatous inflammation through FasL-induced T cell apoptosis (Lundy and Boros, 2002). Of course, in addition to FasL, L2pB1 cells express PD-L2, which negatively regulates TCR-activated T cells that express PD-1. And the autoreactive repertoire of L2pB1 cells suggests that this population would be a prime mover for any mechanism based on presentation of self-antigen. In sum, as much as L2pB1 cells may directly contribute to the pathogenesis of autoimmune disease through production of autoantibody and/or stimulation of pro-inflammatory T cells, L2pB1 cells may also, at the same time or at other times, act to modulate autoimmunity through production of IL-10 and/or re-enforcement of thymic deletion mechanisms via expression of FasL and PD-L2.

3. Take-home message

L2pB1 cells are B1 cells, and as such express numerous B1 cell characteristics, importantly including spontaneous secretion of immunoglobulin, skewing of T cell differentiation, and elevated expression of IL-10 and co-stimulatory molecules. In addition, L2pB1 cells are special—beyond the unique regulation of PD-L2 expression, L2pB1 cells exceed L2nB1 cells in expressing a biased germline-like autoreactive immunoglobulin repertoire, in potentially presenting antigen to and stimulating T cells, and in switching isotype upon cytokine stimulation. All together these characteristics support the notion that L2pB1 cells may be involved in autoimmune disease, although the mechanism(s) remains uncertain but potentially multifactorial, encompassing autoantibody production, T cell differentiation, activation, and cytokine production. Despite unknown functions, the association of increased L2pB1 cells with autoimmunity in murine models, and the identification of a PD-L2 polymorphism that is linked to human SLE, infers that a connection between L2pB1 cells and autoimmune disease exists. As illustrated in figure 4, the presence of chronic infection and inflammation might be a critical switch between protective and pathogenic functions of L2pB1 cells. Finally, regardless of physiological functions, an increase of L2pB1 cells may serve as an alarming indicator of autoimmune diseases. Further investigation of L2pB1 cells in various disease models is needed to narrow down their functions and regulation.

Acknowledgments

The authors thank Dr. Robert Lafyatis for assistance in initiating the search for human L2pB1 cells. The work described in this review was supported by grants from the American Heart Association (0730298N), the Evans Medical Foundation, and Boston University to X. Zhong, and from the National Institutes of Health (USPHS AI029690 and AI60896) to T.L. Rothstein.

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Figure 1. L2pB1 cells are a major B1 B cell population in the peritoneal cavity

(A) Peritoneal washout cells from BALB/c mice were stained with anti-B220 (clone RA3-6B2, eBioscience), anti-CD5 (clone 53-7.3, eBioscience) and anti-PD-L2 (clone TY25, eBioscience) and analyzed by flow cytometry. PD-L2 expression on each cell population is shown. L2pB1 cells and L2nB1 cells are highlighted by arrows. Isotype matched control antibody staining is shown as blue in the histograms. (B) A smear of peritoneal washout cells from BXSB mice was stained with anti-CD20 (clone L26, BioCare), anti-PD-L2 (clone TY25, eBioscience) and DAPI, as indicated, and examined by fluorescence microscopy.

Figure 2. Homing of L2pB1 cells after adoptive transfer into RAG2^{-/-}γc^{-/-} mice

Donor B cells (B2 B cells, L2pB1 cells, L2nB1 cells) were sort-purified from BALB/c mice. C57BL/6J-gCKO-Rag2KO (RAG2^{-/-}) mice were used as recipients. Each recipient mouse received 8×10^5 donor B cells through *i.v.* injection. On day 13 post-transfer, cells were isolated from recipient mice and analyzed by flow cytometry. Donor B cells were identified as IgMa⁺ B cells and recipient B cells were IgMa⁻. RAG2^{-/-} mice received no donor B cells were used as negative controls. Wild type C57BL/6J mice (WT) were used as positive controls for proper gating of each cell population. (A) Peritoneal washout cells from recipient mice were stained for B220 and CD5. B1 and B2 cells were gated as B220^{low}CD5⁺ and B220^{hi}CD5⁻ populations respectively (middle panel). After *i.v.* injection, donor-derived L2pB1 cells (PD-L2⁺IgMa⁺) and L2nB1 cells (PD-L2⁻IgMa⁺) were found mostly within the peritoneal B1 cell gate (red arrows). No donor B2 B cells were found in the peritoneum after transfer. (B) Total splenocytes from recipient mice were stained for B220 and CD5. B1 and B2 cells were gated as described above (middle panel). B2 B cells were found mostly in the spleen (red arrow) after transfer. Few L2pB1 and L2nB1 were found in the spleen. (C) Total serum IgM from recipients transferred with various donor B cells (L2+: L2pB1 cells, L2-: L2nB1 cells, B2s: splenic B2 cells) and controls (None: C57BL/6J-gCKO-Rag2KO mice received no donor cells, WT: C57BL/6J wild type mice) were analyzed by ELISA. Mean values of three mice in each group are shown, along with lines indicating standard errors of the means.

Figure 3. Potential roles of L2pB1 cells in autoimmunity

Immunity and autoimmunity may represent two sides of the same L2pB1 coin. On one hand, L2pB1 cells can be immune-protective and regulatory. Natural, autoreactive IgM secreted by L2pB1 cells can mask or dispose of self-antigens and thereby prevent immune sensitization and production of pathogenic IgG antibodies. Further, L2pB1 cells can modulate incipient autoimmune responses through engagement of PD-1, expression of FasL, and secretion of IL-10. Conversely, L2pB1 cells can be pathogenic. Broadly reactive IgM secreted by L2pB1 cells provides a first line of defense in acute viral and bacterial infection. However, chronic infection may over-activate L2pB1 cells and provide an opportunity for class-switching and affinity maturation which consequently leads to autoimmunity. L2pB1 cells may stimulate effector T cells and influence naïve T cells to differentiate into pro-inflammatory Th17 and Th1 cells. Thus, whether L2pB1 cells are protective or pathogenic may depend on environmental factors that arise during infection and inflammation.