Octamer binding protein 2 (Oct2) regulates PD-L2 gene expression in B-1 cells through lineage-specific activity of a unique, intronic promoter

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Abstract

PD-L2 expression extends beyond macrophages/dendritic cells to B-1 B cells, a distinct B cell lineage that is responsible for natural immunoglobulin and which is repertoire skewed toward autoreactive specificities. PD-L2 expression is constitutive in B-1 cells, whereas it is inducible in other cell types, suggesting that PD-L2 is regulated differently in the former versus the latter, and this proved to be the case, both in transcription and promotion. B-1 cells express a PD-L2 transcript that lacks exon1, in contrast to macrophages/dendritic cells for which exon1 is included, reflecting a unique start site upstream of exon2. PD-L2 transcription in B-1 cells is regulated by a novel intronic promoter located between exon1 and exon2. This intronic promoter binds Oct1 and Oct2, and although these transcription factors are present in all B cells, Oct2 binding is found in vivo only in B-1 cells and not PD-L2 negative B-2 cells. Moreover, the proximal promoter upstream of exon1 that is active in macrophages is inactive in B-1 cells. Thus, PD-L2 expression is regulated by two different promoters that function in a lineage-specific fashion, with the B-1-specific promoter being constitutively active as a result of Oct1 and Oct2 binding.

Keywords

PD-L2; Oct1; Oct2; intronic promoter; B lymphocyte; B-1 cells

Introduction

Programmed death 1 (PD-1, CD279) is a 50–55 kDa type I transmembrane protein originally identified in apoptotic T cells that shares homologous sequence with CTLA4 and CD28 and is inducibly expressed following TCR and BCR activation.[2] Despite it's name, PD-1 appears to have little to do with cell death. Rather, like CTLA4, PD-1 negatively influences lymphocyte activation and in so doing acts to enhance tolerance.[3] This is most

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clearly evidenced by the phenotype of PD-1-deficient animals: these animals are afflicted with generalized lupus-like autoimmunity complicated by glomerulonephritis or by autoimmune dilated cardiomyopathy associated with troponin-specific autoantibodies, depending on background strain.[4–6] The general theme that PD-1 downmodulates lymphocyte activity is supported by numerous in vitro and in vivo experiments, including demonstrations of enhanced B and T cell stimulation, and more effective anti-viral responses, in PD-1-deficient, as compared to normal, animals.[4,7,8]

Two ligands for PD-1 have been identified, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273), both of which are type I transmembrane proteins.[9–12] PD-L1 is constitutively and broadly distributed on lymphoid and non-lymphoid cells, encompassing multiple organ systems. The combination of wide tissue expression of PD-L1 plus antigen receptor-induced upregulation of lymphocyte PD-1 has led to the proposal that the PD-L1/PD-1 system functions as a "fail-safe" mechanism to modulate aberrant autoreactivity. In contrast to PD-L1, PD-L2 expression is much more tightly restricted, and has traditionally been considered to be a characteristic only of activated macrophages and dendritic cells (with perhaps small amounts on thymic stroma and cultured mast cells and, in humans but not in mice, on vascular endothelial cells).[3,11–16]

Aside from differences in tissue distribution, there is evidence that PD-L2 and PD-L1 differentially affect immune responses and disease processes. For example, antibody blockade or knockout of PD-L1 exacerbated experimental autoimmune encephalitis (EAE), and accelerated heart, cornea and skin graft rejection, whereas interference with PD-L2 had no effect.[17–21] Conversely, PD-L2 expression on J558 tumor cells provoked a more vigorous immune response, and led to more rapid tumor rejection, than did PD-L1 expression, which was instead associated with tumor evasion of host immunity; along the same lines, infection with Leishmania mexicana was exacerbated by the loss of PD-L2 in comparison to WT hosts, whereas loss of PD-L1 was associated with host resistance.[22–24]

In addition, PD-L2, but not PD-L1, has been shown to mediate retrograde signaling, leading in dendritic cells to PI-3K-dependent activation of NF-κB with attendant FLIP-associated apoptosis resistance, TREM-2 mediated antigen uptake, activation of cytokine and chemokine genes, and enhanced T cell stimulation.[25–28] These results and others[29] add a unique dimension to PD-L2 expression, strongly suggesting that PD-L2 may act as a receptor, signaling for changes in PD-L2-expressing cells, as well as serving as a ligand to affect target cells. Of note, a specific PD-L2 polymorphism (47103 C/T) has been identified in association with SLE, whereas no association was found between SLE and any PD-L1 polymorphism,[30] suggesting a correlation between PD-L2 and human autoimmune disease.

Recently, PD-L2 expression was found to extend beyond macrophages and dendritic cells to the B cell lineage.[31] In particular, PD-L2 expression was shown to characterize a subset of B-1 B cells, whereas B-2 cells are devoid of PD-L2 and cannot be induced to express this surface antigen.[31,32] B-1 cells are a distinct B cell lineage whose members are responsible for the production of natural immunoglobulin which is a necessary effector for successful anti-microbial defense.[33–39] Moreover, B-1 cell natural immunoglobulin is often autoreactive and thus B-1 cells may play a role in autoimmune dyscrasias.

The subpopulation of B-1 cells marked by expression of PD-L2 differs from PD-L2$^-$ B-1 cells in several important ways. The repertoire skewing toward PtC-binding VH11 and VH12 that typifies B-1 cells is much more highly expressed in PD-L2$^+$ B-1 cells;[31] the immunoglobulin cross-reactivity with self-antigen that characterizes B-1 cells is much more evident in PD-L2$^+$ B-1 cells [50]; and the especially efficient antigen presentation that distinguishes B-1 cells is enhanced in PD-L2$^+$ B-1 cells [50]. These features specifically implicate the PD-L2$^+$ population of B-1 cells in the origin or perpetuation of autoimmune diseases that contain a strong autoantibody component.

PD-L2 expression on mature B-1 cells is strictly constitutive, and can be neither further upregulated on PD-L2$^+$ B-1 cells, nor induced on PD-L2$^-$ B-1 cells.[31] This contrasts with other cells, specifically macrophages and dendritic cells, where PD-L2 expression is only apparent after induction by an appropriate stimulus. The contrasting nature of constitutive PD-L2 expression on B-1 cells versus inducible PD-L2 expression on macrophages/ dendritic cells strongly suggests that the nature of PD-L2 regulation is different in the former as opposed to the latter, either in terms of the transcript produced or the nature of transactivation. The functional distinctions associated with PD-L2 expression by B-1 cells further raise the possibility that PD-L2 produced in B cells is not the same as that produced in other cell types. To address these issues, we undertook to elucidate the nature and regulation of PD-L2 expression in B-1 cells. We found that B-1 cells express a unique PD-L2 transcript that is regulated in a unique fashion by a novel, intronic promoter.

Results

PD-L2 transcripts differ between unstimulated B-1 cells and IL-4-stimulated macrophages in terms of exon composition

We suspected that the *Pd-l2* gene is expressed differently in B-1 B cells than in macrophages or dendritic cells, as it is constitutive in the former and inducible in the latter. The murine *Pd-l2* locus encompasses 6 exons (Figure 1) with an ATG start codon in exon2 and a stop codon in exon5 (Figure 1). To examine PD-L2 exon usage in B-1 cells, we analyzed mRNA expression by real-time PCR using inter- and intra-exon primer sets (Supplementary Table). Primary macrophages stimulated by IL-4 to upregulate PD-L2 expressed all 6 PD-L2 exons, in accordance with the NCBI database (Table 1). In direct contrast, we found that unstimulated primary B-1 cells, and B-1 cell lines, that are PD-L2 positive failed to express exon I. Because the ATG start codon for PD-L2 is located in exon2 (Figure 1), PD-L2+ B-1 cells would be expected to display the same PD-L2 protein as macrophages, and indeed B-1 cell and macrophage PD-L2 are recognized by the same specific monoclonal anti-PD-L2 antibody.[31,32] Interestingly, we found that primary splenic B-2 cells expressed exons 5 and 6 (at a level 20–40% less than that of B-1 cells), which, however, shouldn't yield any PD-L2 product because of the presence of a stop codon in exon5, and indeed, neither naive nor stimulated B-2 cells expressed immunoreactive PD-L2.[31,32] These data suggest that constitutive PD-L2 gene expression in unstimulated B-1 cells might be regulated in a fashion different from inducible PD-L2 in stimulated macrophages.

PD-L2 transcripts differ between unstimulated B-1 cells and IL-4-stimulated macrophages in terms of start site

The results above regarding PD-L2 exon usage suggested that the transcription start site in B-1 cells might differ from that in macrophages and could be located in the region around the junction of intron1 and exon2. To address this issue, we carried out further mRNA expression assays using intron1 and exon3 primer sets (Supplementary Table) in order to determine whether B-1 cells express the extra region upstream of exon2. We found that B-1 cells, but not IL-4-stimulated macrophages, expressed sequence −82 from exon2 but not sequence −198 nor −310 from exon2 (Table 2). These data suggest that the transcription start site in B-1 cells lies in the region −198 to −89 from exon2. To identify the exact start site in B-1 cells, we carried out 5′ RACE using total RNA from PD-L2$^+$ B-1 cells in comparison with total RNA from IL-4-stimulated macrophages. After amplifying 5′ PD-L2 cDNA by nested PCR, we evaluated the PCR products on 2% agarose gels and noted that each cell type yielded a single band (Figure 2a). Further, the band derived from B-1 cells appeared a little bit smaller than the band derived from macrophages, suggesting that a single transcription start site differs between B-1 cells and macrophages in a cell type-specific manner. These bands were then TA-cloned and their sequences analyzed, revealing that the transcription start site in PD-L2$^+$ B-1 cells was -146 bp from exon2 (all 3 of 3 bacteria-derived clones) while that in IL-4-stimulated macrophages was +34 bp from exon1 (all 3 of 3 bacteria-derived clones), in comparison with the known dendritic cell cDNA sequence according to the NCBI database (NM_021396) (Figure 2b). The difference in transcription start sites for PD-L2 expression between B-1 cells and IL-4-stimulated macrophages raises the possibility that the PD-L2 gene may be regulated differently in these cell types.

A proximal, upstream region promotes PD-L2 expression in macrophages but not in B-1 cells

To determine the genomic region responsible for PD-L2 gene expression in murine B-1 cells, we first analyzed the proximal promoter sequence recorded in the NCBI database (NM_021396), using PromoterInspector (http://www.genomatix.de/index.html), which predicts promoter or enhancer regions using database-derived patterns. This algorithm predicted that the PD-L2 promoter is located within the region −5,279 to −5040 in relation to exon1. With this in mind we cloned the region −5574 to +5 from exon1 into the pGL4.10 firefly-luciferase reporter vector, and examined luciferase expression following reporter gene transfection into B cell and macrophage cell lines, which were used as models for primary cells because of their superior transfection efficiency. We transfected 2 B-1 cell lines that constitutively express PD-L2 (BRD2 and BCL1), and one macrophage cell line that constitutively expresses PD-L2 (RAW264.7). We found that RAW264.7 cells transfected with the −5574/+5 reporter vector expressed approximately 9-fold more luciferase than did RAW264.7 cells transfected with empty reporter vector (Figure 3a). In marked contrast, the upstream promoter region was not active in B-1 cell lines inasmuch as the level of luciferase activity produced by transfection of the −5574/+5 reporter vector was the same as that produced by transfection of the empty reporter vector (Figure 3a). Because PD-L2 expression was induced in macrophages and dendritic cells by cytokines including IL-4,[32] we stimulated B-1 cell lines with IL-4 or peritoneal cavity fluid, which contains

IL-6 and IL-10, and which surrounds primary B-1 cells in situ. In spite of stimulation, B-1 cell lines failed to enhance −5574/+5 reporter activity (data not shown). These results indicate that the PD-L2 gene responds to a different promoter in B-1 cells as opposed to macrophages, where a proximal, upstream region regulates gene expression.

An intronic region promotes PD-L2 expression in B-1 cells

In view of the unique exon usage for PD-L2 transcripts in B-1 cells, and the novel start site for B-1 cell PD-L2 transcripts within the intronic region upstream of exon2, we speculated that PD-L2 expression may be controlled in B cells by an intronic promoter. To test this, we cloned the intron 1 genomic region (−3032 to +5 from exon2) into the pGL4.10 firefly-luciferase reporter vector (even though PromoterInspector predicted no promoter in this region), and examined luciferase expression following reporter gene transfection into B cell lines. In contrast to the negative results obtained with the proximal promoter region, we found that the intron 1 region promoted transcription of the luciferase reporter gene in both B-1 cell lines, in the absence of stimulation (Figure 3b). Thus, the promoter for PD-L2 expression in B-1 cells is likely located in the intron 1 region between −3032 to +5 in relation to exon2, and not in the region proximal to and upstream of exon1, as is the case for macrophages. Presumably this region contains sites for cell type-specific enhancing elements that promote PD-L2 expression.

A small sequence within the intronic region proximal to exon2 promotes PD-L2 expression in B-1 cells

In order to identify more precisely within the −3032 to +5 sequence (in relation to exon2) the active region for promotion of B-1 cell PD-L2 expression, we prepared and tested a series of mutant reporter constructs using the pGL4.10 vector. We transfected these deletion mutant reporter constructs (−1465 to +5, −911 to +5, −708 to +5 and −404 to +5 from exon2) into BRD2 B-1 cells and analyzed reporter activity. We found that all of the mutant intronic promoter constructs produced similar levels of luciferase activity (Figure 4a). These results indicate that the promoter region controlling PD-L2 transcription in B-1 cells is located within the intronic sequence −404 to +5 in relation to exon2.

In order to further narrow the location of the intronic sequence that regulates PD-L2 expression in B-1 cells, we prepared and tested additional mutant reporter constructs. We first focused on the 3′ edge of the −404/+5 region that is downstream of the transcription start site identified in primary B-1 cells (−146 in relation to exon2), by comparing the region −404 to −136 with the positive control region −404 to +5, in relation to exon2, after transfection into BRD2 cells. We found that luciferase reporter gene activity was as active, or more active, in the former as compared to the latter (Figure 4b). These results indicate that the sequence −136 to +5 from exon2 is not required for PD-L2 expression in B-1 cells and draw attention to the region −404 to −136. We then evaluated this sequence in more detail, focusing on the 5′ edge by comparing the region −313 to −36 and −240 to −136 with the positive control region −404 to −136, in relation to exon2, after transfection into BRD2 cells. We found that luciferase reporter gene activity was at least as active in the two test constructs as compared to the positive control (Figure 4c). In fact, the sequence −240 to −136 was more active than either −404 to −136 or −313 to −136, raising the possibility that

a negative regulatory site lies within the region from −313 to −241. These results indicate that positive elements responsible for PD-L2 expression in B-1 cells are contained within the relatively narrow (94 bp) range of intronic sequence downstream of −240 in relation to exon2, as confirmed by mutational studies summarized below.

Oct1 and Oct2 transcription factors bind to the PD-L2 intronic promoter

To identify factors responsible for promoting PD-L2 expression that bind within the 94 bp intronic segment, we carried out electrophoretic mobility shift assays (EMSA) on B cell nuclear extracts, using the region −240 to −147 as labeled probe. We tested extracts obtained from primary B-1 and B-2 cells as well as extracts obtained from B-1 (BCL2, BRD2) and B-2 (A20) cell lines. Four nucleoprotein complexes were detected, labeled A, B, C, and D, in Figure 5a. Two complexes (A and B) were shared by all the samples (Figure 5a). In contrast, complex C was most readily apparent in BRD2 extracts and complex D was most readily apparent in A20 extracts (Figure 5a). In contrast to the marked bands which were always observed as noted above, the faint bands located in between complexes C and D were not reproducible (data not shown). Thus, we detected no nucleoprotein complexes that consistently distinguished primary B-1 from primary B-2 cells, even though more than half of primary B-1 cells express PD-L2 whereas no B-2 cells do so (Figure 5a).

We focused further on the A and B nucleoprotein complexes because these were the only bands that were abundantly and consistently present in nuclear extracts from primary B cells. To more fully define the DNA binding sites participating in these complexes, we carried out "cold" competition analysis using subsections of the −240 to −147 sequence. Thus, to EMSA binding reactions using labeled −240 to −147 probe, we added unlabeled ("cold") DNA consisting of −240 to −213, −212 to −191, −190 to −169, −168 to −147, and, as a positive control, the (unlabeled) probe sequence of −240 to −147, all in relation to exon2. We found, as expected, that addition of unlabeled probe sequence (−240 to −147) completely competed the formation of both the A and B nucleoprotein complexes (Figure 5b). None of the other DNA competitors was as effective; however, two of the sequences produced partial inhibition of both the A and B nucleoprotein complexes: −240 to −213, and −190 to −169, from exon2 (Figure 5b). These results raised the possibility that important promoter binding sites might be located in these two regions. We then analyzed these sequences via the Transfac Database, and found that both regions contained Octamer binding sites, although the sites in these two regions are both non-canonical and are characterized by an affinity for Octamer protein that is less than that of the canonical (original) sequence. To determine whether these sites function as Octamer binding sites, we carried out "cold" competition analysis with an unlabeled consensus Octamer sequence derived from the Igκ locus (AATTCCAGTGAGGGT<u>ATGCAAAT</u>TATTAAGAAGCAG) added to the EMSA binding reaction. We found that the consensus Octamer sequence completely blocked the binding of both the A and B nucleoprotein complexes to the labeled −240 to −147 probe (Figure 5b). The A and B nucleoprotein complexes migrate similarly to nucleoprotein complexes containing Oct1 and Oct2. To elucidate the nature of A and B site-binding proteins, we carried out supershift analysis using anti-Oct1 and anti-Oct2 antibodies added to EMSA binding reactions. We found that anti-Oct1 supershifted the A nucleoprotein complex and that anti-Oct2 supershifted the B nucleoprotein complex, in extracts obtained

from primary B-1 and B-2 cells and B-1 and B-2 cell lines (Figure 5c). Whereas anti-Oct2 supershifted Band B completely, anti-Oct1 produced only partial depletion of Band A; although there are many possible technical reasons for partial supershift, it is known that Oct1 consists of multiple alternatively spliced variants,[40] and it is likely that the anti-Oct1 antiserum does not bind with equal efficiency to all isoforms. Regardless, these results indicate that the A nucleoprotein complex contains Oct1, and that the B nucleoprotein complex contains Oct2.

Octamer binding sites are responsible for PD-L2 expression in B-1 cells

To determine the role of the A and B Octamer binding sites in promoting B-1 cell expression of PD-L2, we prepared and tested luciferase reporter constructs encompassing the sequence −240 to −136, in relation to exon2, in native form or encompassing mutation of one or both of the A and B octamer binding motifs. These reporter constructs were transfected into BRD2 B-1 cells and luciferase activity evaluated. We found that mutation of a single site, either A or B, substantially blocked luciferase activity, and that mutation of both sites virtually eliminated expression of the luciferase reporter gene (Figure 6a). These results indicate that octamer binding sites within the intronic sequence −240 to −136 from exon2, that bind Oct1 and Oct2, are together responsible for PD-L2 gene expression in B-1 cells.

Oct2 binds to the PD-L2 intronic promoter in vivo

To more fully evaluate the physiological role of Octamer binding sites within the intronic promoter sequence in primary B cells, we carried out chromatin immunoprecipitation (ChIP) assays using anti-Oct2 antibody, as described in Materials and Methods. We found that Oct2 constitutively bound to the intronic promoter in isolated peritoneal PD-L2$^+$ primary B-1 cells but not in primary splenic B-2 cells (that do not express PD-L2 under any circumstances); further, we found that Oct2 constitutively bound intronic DNA in BRD2 B-1 cells that express PD-L2, but not in A20 B-2 cells that do not (Figure 6b). These results indicate that Oct2 is normally bound to the PD-L2 intronic promoter in B-1 cells, strongly supporting our work with B cell lines in concluding that octamer binding sites control B-1 cell-specific PD-L2 expression. Despite similar A and B nucleoprotein complexes generated from B-1 and B-2 cell nuclear extracts in vitro (using an artificial DNA probe sequence), these results indicate a distinct difference between B-1 and B-2 cells in terms of Oct2 binding in vivo.

Okadaic acid downregulates PD-L2 expression in primary B-1 cells

To further examine the role of Oct2 in directing primary B-1 cell PD-L2 surface expression, we evaluated the effect of okadaic acid (OA), a serine and threonine phosphatase inhibitor that promotes phosphorylation of Oct2.[41] This results in an observable shift in the molecular size of Oct2 and a decrease in binding affinity of Oct2 for non-canonical Octamer motifs.[41] We treated primary B-1 and primary B-2 cells with OA and carried out western blotting using anti-Oct2 antibody (Figure 7a). OA treatment produced a substantial increase in the apparent molecular size of Oct2 consistent with phosphorylation (Figure 7a). The loss of Oct2 at later time points after OA treatment that is apparent in Figure 7a has been noted elsewhere and is presumably due to targeting of phosphorylated Oct2 for degradation;[41]

importantly, there was no decrease in viability during 9 hours treatment with OA. We then analyzed PD-L2 surface expression in OA-treated cells by flow cytometry. OA treatment of primary B-1 cells produced downregulation of PD-L2 surface expression (Figure 7b). These results further suggest that Oct2 is responsible for PD-L2 expression in B-1 cell types.

Discussion

We previously reported that PD-L2 is constitutively expressed by a subpopulation of B-1 cells, whereas PD-L2 expression by other cell types such as macrophages and dendritic cells is not constitutive but rather cytokine-induced in nature. Along with the distinctive functional attributes of PD-L2[+] B-1 cells, this raised the possibility that PD-L2 is regulated differently in B cells as opposed to macrophages and dendritic cells. In pursuing this issue, we found that B-1 cell PD-L2 is generated via a novel transcript that is regulated by a novel promoter.

PD-L2 gene expression in B-1 cells differs from that in macrophages and dendritic cells in terms of exon usage, inasmuch as exon1 is omitted from the former but is a constituent of the latter. This difference is unlikely to alter the structure of the PD-L2 protein product, because the translation start site for PD-L2 lies in exon2, which is a component of PD-L2 transcripts even in the absence of exon1. Consistent with this, immunoglobulin reagents that recognize macrophage and dendritic cell PD-L2 similarly recognize B-1 cell PD-L2.[31,32] Still, this transcriptional difference suggests an underlying difference in promoter structure/function, particularly in view of the fact that the region −146 to −1 in relation to exon2 is expressed in B-1 cells, ruling out an alternative splice mechanism in the generation of exon1-less PD-L2 transcripts. Further study demonstrated that the proximal promoter that directs PD-L2 expression in macrophages is inactive in B-1 cell lines, wherein, instead, PD-L2 expression is controlled by a newly identified, novel intronic promoter upstream of exon2.

It has been reported that inducible expression of PD-L2 in macrophages and dendritic cells requires STAT family molecules which are activated by cytokines or NF-κB.[32] Conversely, B-1 cells that express PD-L2 do so constitutively, in the absence of stimulation, which suggests that transcription factors required for B-1 cell PD-L2 expression are continually expressed and active in B-1 cells. Deletional analysis of the PD-L2 intronic promoter identified a relatively small active region which was shown to contain two Octamer binding sites necessary for gene expression. This intronic promoter region specifically binds Oct1 and Oct2, two transcription factors known to be expressed in B cells.[42]

Although the proximal promoter that was active in macrophages was inactive in B-1 cells, the converse is not true, and the intronic promoter that was active in B-1 cells was also active in macrophages (data not shown), as assessed by reporter gene assay. Moreover, we found no difference in intronic promoter-binding, Oct1- and Oct2-containing, complexes produced by nuclear extracts of B-1 and B-2 cells, as assessed by EMSA and supershift. This apparent promiscuity of the intronic promoter raised the possibility that the intronic promoter might not account for lineage-specific, constitutive expression of PD-L2. For this reason we examined the intronic promoter in its native, chromatin configuration by ChIP

assay. Here we found a clear distinction between primary B-1 and B-2 cells, and between PD-L2$^+$ B-1 and PD-L2$^-$ B-2 cell lines, whereby Oct2 was bound to the PD-L2 intronic promoter sequence in B-1, but not in B-2, cells. Thus, although Oct2 is present in PD-L2 non-expressing B-2 cell nuclei, it fails to bind the PD-L2 intronic promoter. These results clearly show that the native intronic promoter functions quite differently in B-1 cells as opposed to B-2 cells, and indicate that the widespread activity of the intronic promoter apparent in reporter gene and EMSA assays is a technical artifact and a limitation of using naked DNA in plasmid or probe form to replicate the function of chromatinized DNA. These results exemplify the important distinction between the presence within nuclei of a given factor and the activity of an artificial promoter construct on the one hand, and the binding of a given factor to native, genomic DNA in vivo (detected by in vivo binding as determined by ChIP assay) on the other. These results suggest either that Oct2 binding to the PD-L2 intronic promoter is regulated by chromatin structure at that site, or that Oct2 binding to the PD-L2 intronic promoter is regulated by post-translational modification, as suggested by the results with okadaic acid, or both. Our results do not, however, rule out other potential contributing factors such as miRNA.

Beyond constitutive expression in a portion of B-1 cells, PD-L2 is not induced by stimulation of PD-L2 negative B cell populations (B-2 cells and PD-L2$^-$ B-1 cells) as it is in PD-L2 negative macrophages and dendritic cells.[31,32] The failure by B-1 cells to use the proximal promoter, as opposed to other cell types, may explain the absence of inducibility in mature B cells. Further evidence that the macrophage/dendritic cell proximal promoter is not used in B cells lies in the constitutive expression of nuclear NF-κB and activated STAT3 by PD-L2$^-$ B-1 cells (unpublished observations) that by definition fails to bring about PD-L2 expression.

A key issue concerns the use of distinct genetic mechanisms to produce the same protein. The functional consequences of lineage-specific promoter activity for PD-L2 are clear, in terms of constitutive versus inducible expression of this protein by different cell types resulting from reliance on intronic versus upstream sequences, respectively. As such, the present results provide a new dimension to understanding B-1 cell behavior and the lineage-specific generation of a key PD-1 ligand.

Materials and methods

Mice

Male BALB/cByJ mice at 8–14 wk of age were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed at least 1 week before experimentation. Mice were cared for and handled in accordance with National Institutes of Health and institutional guidelines.

Primary B-1 cells, B-2 cells, and macrophages

PD-L2$^+$ and PD-L2$^-$ B-1a cell populations were obtained by sort-purifying B220^{low}CD5$^+$PD-L2$^+$ and B220^{low}CD5$^+$PD-L2$^-$ cells from peritoneal washouts with a FACSAria instrument (Becton Dickinson, Franklin Lakes, NJ, USA), as previously

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described.[31,43] Splenic B2 cells were obtained by negative selection of splenocytes with anti-Thy1.2 antibody and rabbit complement, as previously described.[44] Isolated peritoneal B-1a cells and splenic B-2 cells were >95% pure on re-analysis. Macrophages were obtained from peritoneal washouts by adherence to plastic tissue culture dishes, and were then stimulated with IL-4 (10 ng/ml) (R&D Systems, Minneapolis, MN, USA). Primary cells were cultured in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-ME.

B lymphoma cell lines

A20 and BCL1 B cell lymphomas were obtained from the American Type Culture Collection (Manassas, VA, USA). BRD2 B-1 cell lymphoma was obtained from Dr. G. Denis (Boston University Medical Center, Boston, MA, USA). RAW264.7 macrophages were kindly provided by Dr. K. Takahashi (The Feinstein Institute for Medical Research, Manhasset, NY, USA). Cell lines were cultured in RPMI 1640 medium as above.

Antibodies and reagents

Antibody 2.4G2 (anti-mouse FcγR), FITC-conjugated antibody RA3-6B2 (anti-B220), PE-conjugated antibody TY25 (anti-PD-L2), and PE-Cy5-conjugated antibody 53–7.3 (anti-CD5) were obtained from BD Pharmingen (San Diego, CA, USA). Rabbit polyclonal antibodies directed against Oct1 and Oct2 were obtained from Chemicon (Temecula, CA) and Santa Cruz (Santa Cruz, CA), respectively. Okadaic acid was obtained from EMD Chemicals (Gibbstown, NJ) and dissolved in DMSO.

Rapid amplification of 5′ cDNA ends analysis (5′ RACE)

Total RNA from PD-L2$^+$ B-1 cells and from macrophages was isolated with Ultraspec RNA Reagent (Biotecx, Houston, TX, USA) for rapid amplification of 5− cDNA ends (5− RACE) using 5−-Full RACE Core Set (Takara Bio, Otsu, Japan) according to the manufacturer's instructions, as previously reported.[45] Briefly, the unknown region was amplified by nested PCR (2 step-PCR) using LA-Taq (Takara Bio). PCR products were separated by electrophoresis on 2% agarose gels and visualized with ethidium bromide. After checking the band pattern, PCR products were TA-cloned into pGEM-T vector (Promega, Madison, WI, USA). The insert was verified by sequencing (Genewiz, South Plainfield, NJ, USA) and the transcription start site was determined. Sequence information for the primers used in 5'RACE is shown in the Supplementary Table.

Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was carried out with reagents from Upstate Biotechnology (Lake Placid, NY, USA), according to the manufacturer's protocol, as previously described, with slight modification.[46] Briefly, primary B cells (2×10^6) were subjected to chromatin cross-linking in 1% formaldehyde for 2 min at 37°C, and quenched with glycine (125 mM). Cell lysates were sonicated to shear the DNA length to between 200 and 2,000 bp; DNA was extracted from a fraction of the lysate for input controls. Precleared lysates were incubated overnight at 4□ with rabbit anti-Oct2 antibody or with normal rabbit IgG (4 μg each). Cross-linking was reversed and input and immunoprecipitated DNA samples were assayed for the PD-L2

promoter sequence by real-time PCR. The sequences of the primer sets used in the ChIP assays are shown in the Supplementary Table.

Construction of reporter plasmid and transfection

The PD-L2 genomic region was amplified by PCR using LA-Taq. PCR products were cloned into the pGL4.10 luciferase vector (Promega). Mutant reporters were constructed using LA-Taq and QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The insert was verified by sequencing (Genewiz). Native and mutant firefly-luciferase vectors were co-transfected with pRL-TK (renilla-luciferase) vector using the nucleofector kit V (Amaxa, Walkersville, MD, USA) according to the manufacturer's instructions. At 24 hours after transfection, luciferase activity in cell lysates was analyzed by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions, using a 20/20n Single Tube Luminometer (Turner Biosystem, Sunnyvale, CA, USA). Relative luciferase activity was calculated as firefly-luciferase activity/renilla-luciferase activity and the data shown represent the mean fold induction of 2–3 experiments. The sequences of the primer sets used in cloning are shown in the Supplementary Table.

qPCR mRNA expression analysis

Total RNA was extracted from primary PD-L2$^+$ B-1a cells, PD-L2$^-$ B-1a cells, B-2 cells, and macrophages, and from B lymphoma cell lines, using Ultraspec according to the manufacturer's instructions. RNA was reverse transcribed (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA), amplified by real-time PCR, and normalized to expression of β2-microglobulin, as previously described.[47] The sequences of the primer sets are shown in the Supplementary Table.

Nuclear protein extraction and electrophretic shift assay (EMSA)

Nuclear extracts were obtained from B cells and analyzed by EMSA as previously described. [48] In brief, B cells (5×10^6) were washed with HBSS and disrupted for 10 min on ice by hypotonic lysis buffer (10 mM HEPES, pH7.9, 10 mM KCl, 1.5 mM MgCl$_2$). Nuclear fractions were collected and suspended for 20 min on ice in extraction buffer (20 mM HEPES, pH 7.9, 420 mM KCl, 1.5 mM MgCl$_2$, 20% glycerol). All buffers were supplemented with a protease inhibitor cocktail, PMSF (0.5 mM), sodium orthovanadate (1 mM) and DTT (1 mM). Nuclei were pelleted and supernatants were collected as nuclear protein extracts and were stored at −80° C. Protein concentrations were determined by the Bradford method (BioRad).

EMSA reaction mixtures (20 μl) contained 2–4 μg of nuclear protein, 2 μg of poly dI:dC (GE Healthcare, Piscataway, NJ, USA) and 4×10^4 cpm/2 μl of ^{32}P end-labeled probe. For EMSA competitor assays, $50 \times$ cold excess competitor was added before adding labeled probe. For EMSA supershift or nucleoprotein complex depletion assay, 2 μg of anti-Oct1, anti-Oct2 and normal rabbit IgG were added to reaction mixtures and incubated at 4□ for 1 hr before adding probe. The sequences of the probes and cold competitors used in EMSA are shown in the Supplementary Table. Reaction mixtures were incubated at room temperature for 30 min and the nucleoprotein complexes were resolved on a native 4%

polyacrylamide gel in $0.25 \times$ Tris-borate-EDTA buffer for electrophoretic analysis at 150 V. Gels were dried and subjected to X-ray films.

Flow cytometry

Cell surface phenotype was analyzed as previously described.49 In brief, for FACS analysis, cells pre-treated with 2.4G2 (5 μg/ml) were stained with FITC-, PE- or PE-Cy5 conjugated mAb in staining buffer (PBS containing 2% FCS and 0.05% NaN$_3$) on ice for 30 min and washed with staining buffer. Stained cells were analyzed on a FACSCalibur instrument (Becton Dickinson) equipped with CellQuest software. 7-amino-actinomycin D (2 μg/ml) (BD Pharmingen) was used to exclude dead cells from the analysis. For each sample, at least 1×10^4 cells were collected and analyzed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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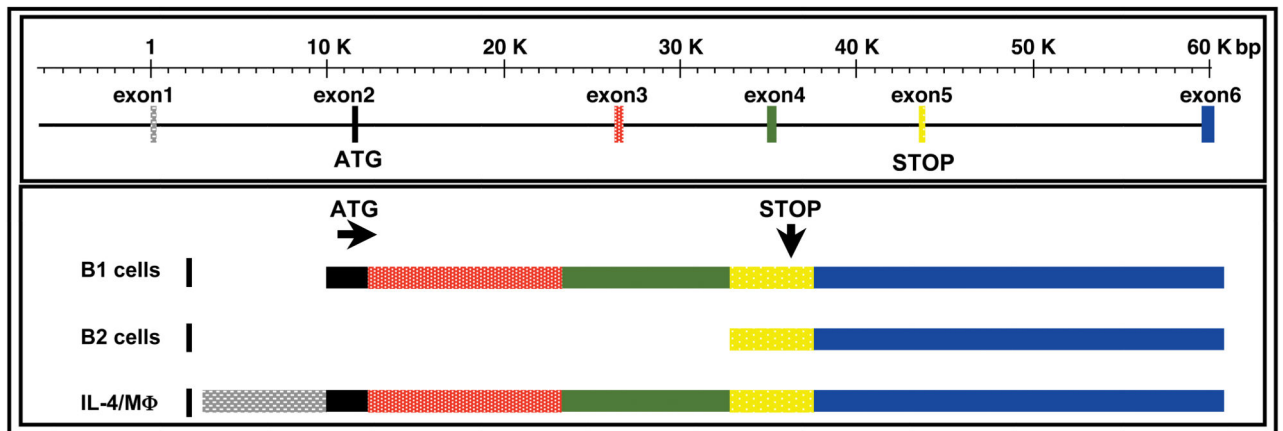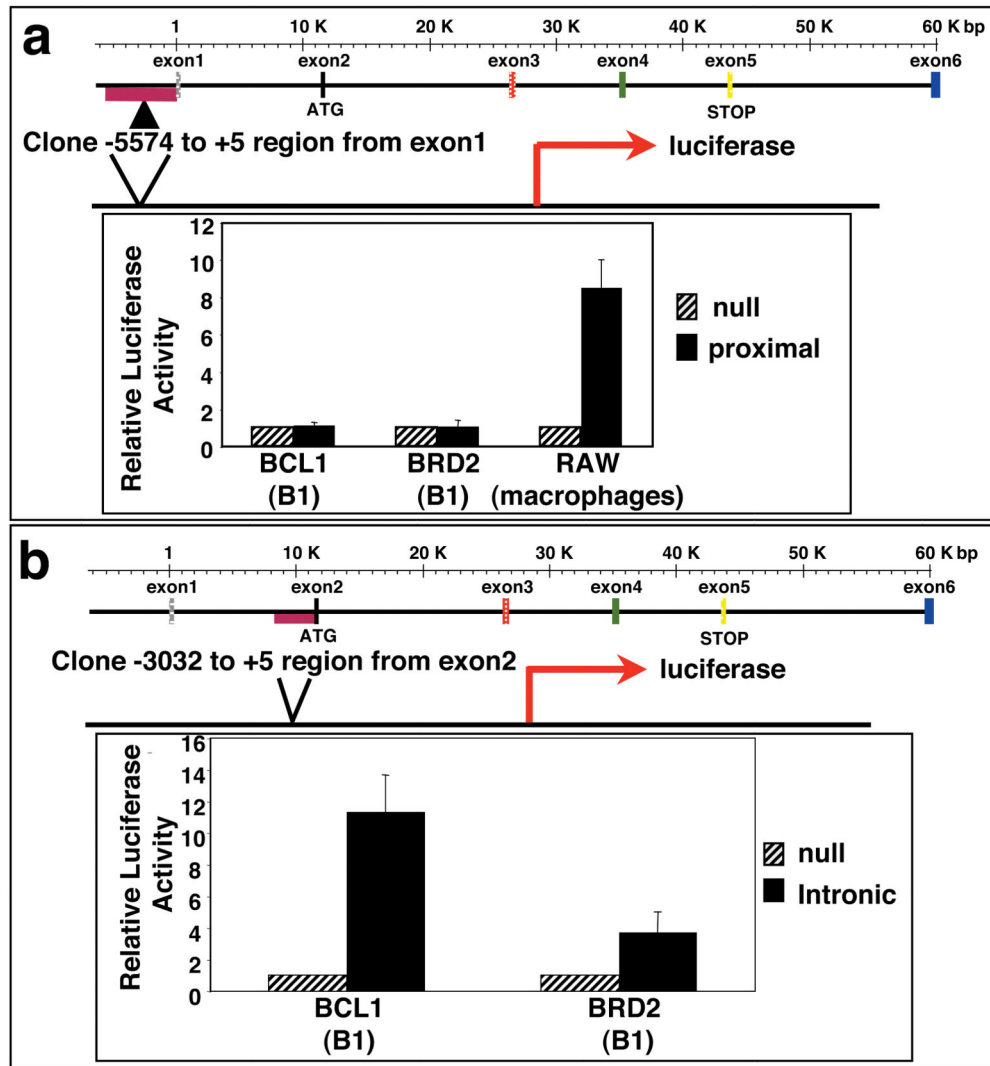
Figure 1.
PD-L2 transcripts differ between unstimulated B-1 cells and IL-4-stimulated macrophages in terms of exon composition. The mouse *Pd-l2* gene locus encompasses 6 exons with an ATG start codon in exon2 and a stop codon in exon5. IL-4-treated macrophages express all exons whereas B-1 cells fail to express exon1 and B-2 cells express only exon5 and 6, as determined by PCR-based expression assay results summarized in Table 1.
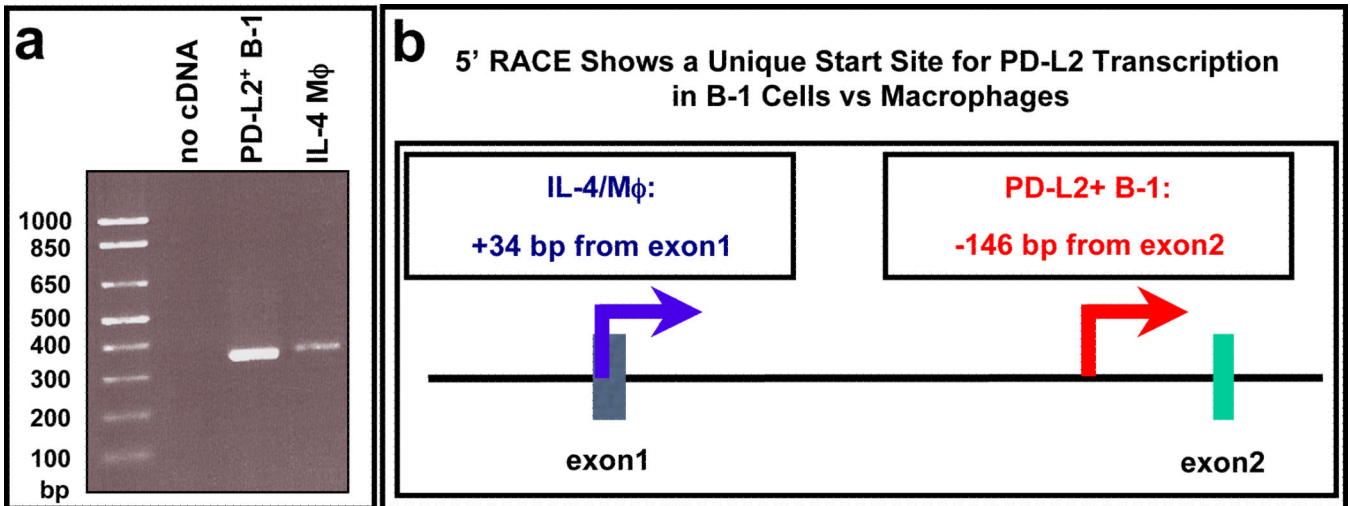
Figure 2.
PD-L2 transcripts differ between unstimulated B-1 cells and IL-4-stimulated macrophages in terms of start site. PD-L2 transcription start sites were identified by 5′ RACE as described in Materials and methods. RNA was obtained from sort-purified PD-L2+ B-1 cells and from IL-4-stimulated macrophages. After amplification of 5′ PD-L2 cDNA by nested PCR: (a) PCR products were separated by electrophoresis on 2% agarose gels and visualized with 2 μg/ml ethidium bromide; and, (b) PCR products were TA-cloned into the pGEM-T vector, after which the insert was verified by sequencing and the transcription start site was determined.

Figure 3.
A proximal, upstream region promotes PD-L2 expression in macrophages but not B-1 cells, whereas an intronic region promotes PD-L2 expression in B-1 cells. (a) BCL1, BRD2 and RAW264.7 cells were transiently transfected with an empty firefly luciferase construct, or with firefly luciferase reporter constructs containing genomic sequence from −5574 to +5 in relation to exon1, along with, in each case, a thymidine kinase promoter-dependent renilla luciferase expression vector. (b) BCL1 and BRD2 cells were transiently transfected with an empty firefly luciferase construct, or with firefly luciferase reporter constructs containing genomic sequence from −3032 to +5 in relation to exon2. For both (a) and (b), cell lysates were prepared and luciferase activity was measured in relation to the renilla luciferase control. Results are reported as multiples of the activity present with empty vector. For each of (a) and (b), mean values for 3 independent experiments are shown, along with lines corresponding to the standard errors of the means.
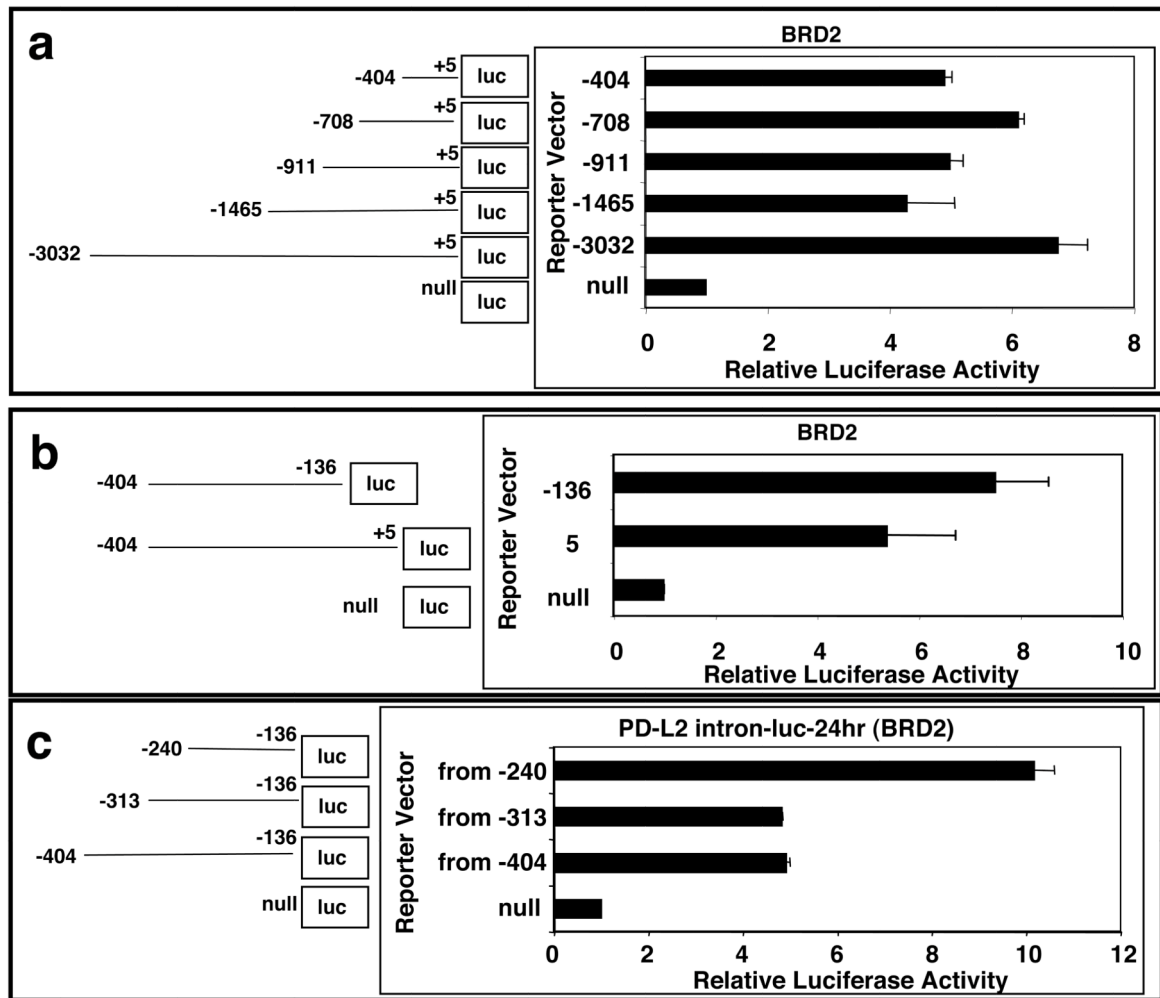
Figure 4.
A small sequence within the intronic region proximal to exon2 promotes PD-L2 expression in B-1 cells. (a) Serial 5′ deletion mutant reporter constructs were prepared from the −3023 to +5 intronic region as described in Materials and methods. BRD2 cells were transiently transfected with an empty firefly luciferase construct, or with firefly luciferase reporter constructs containing each of the 5− deletion mutants along with, in each case, a thymidine kinase promoter-dependent renilla luciferase expression vector. (b) A 3′ deletion mutant reporter construct was prepared from the −404 to +5 intronic region as described in Materials and methods. BRD2 cells were transiently transfected with an empty firefly luciferase construct, or with the firefly luciferase reporter construct containing the 3− deletion mutant, along with, in each case, a thymidine kinase promoter-dependent renilla luciferase expression vector. (c) Further serial 5− deletion mutant reporter constructs were prepared from the −404 to −136 intronic region as described in Materials and methods. BRD2 cells were transiently transfected with an empty firefly luciferase construct, or with firefly luciferase reporter constructs containing the 5− serial deletion mutants, along with, in each case, a thymidine kinase promoter-dependent renilla luciferase expression vector. For each of (a), (b) and (c), cell lysates were prepared and luciferase activity was measured in
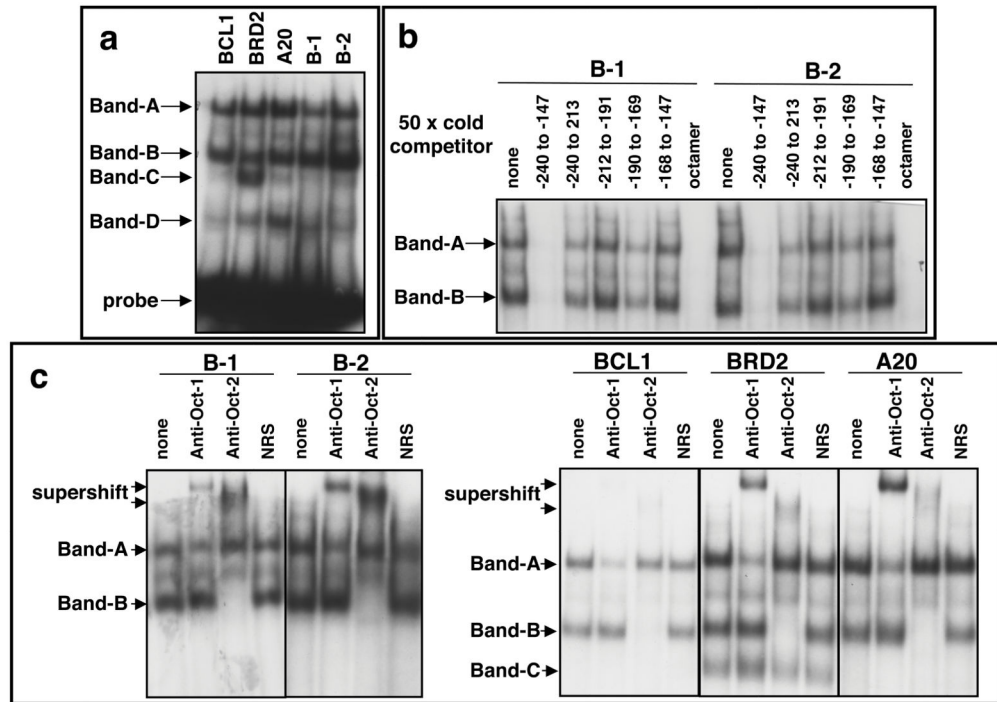
relation to the renilla luciferase control. Results are reported as multiples of the activity present with empty vector. For each of (a), (b) and (c), mean values for 2 independent experiments are shown, along with lines corresponding to the standard errors of the means.
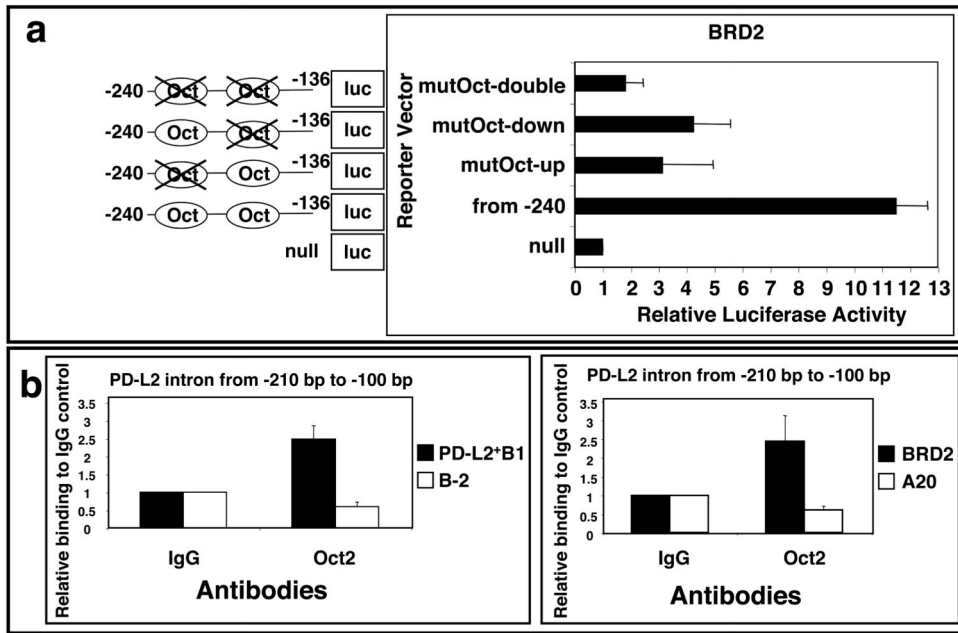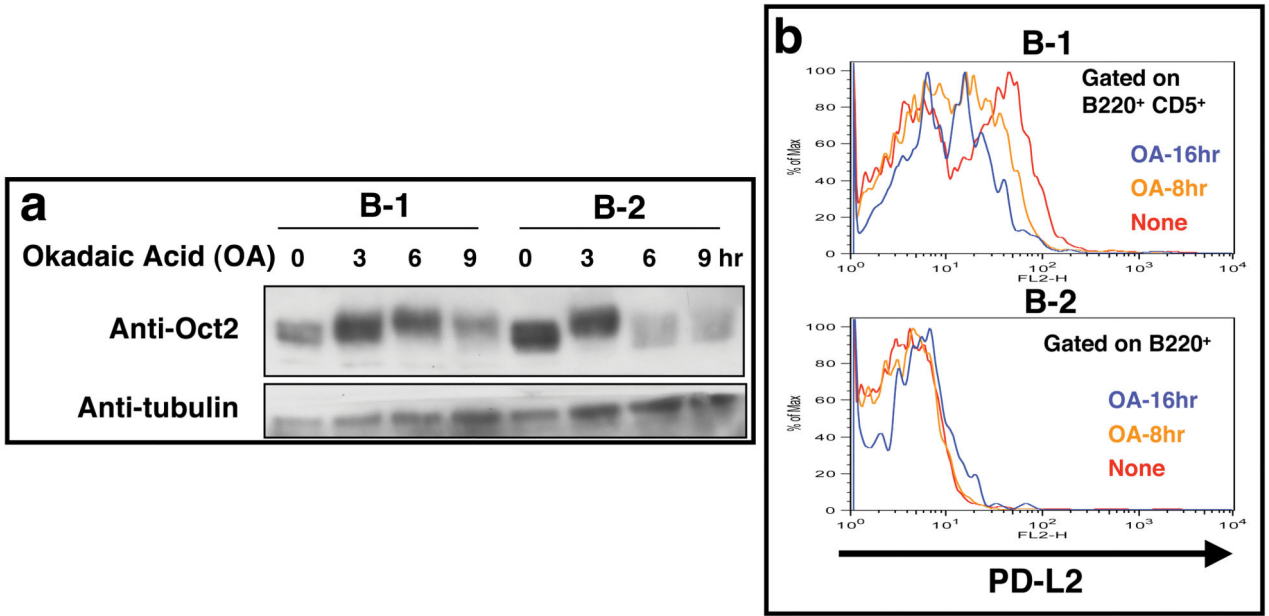
Figure 5.
Oct1 and Oct2 transcription factors bind to the PD-L2 intronic promoter in vitro. Nuclear extracts were obtained from B-1 (BCL1, BRD2) and B-2 (A20) cell lines and from sort-purified primary B-1 and B-2 cells, as indicated. (a) Nuclear extracts were tested for binding to the PD-L2 intronic promoter (−240 to −147 from exon2) by electrophoretic mobility shift assay (EMSA), as described in Materials and methods. Arrows indicate the major nucleoprotein complexes detected denoted A–D. One of 3 comparable experiments is shown. (b) Nuclear extracts were tested by EMSA for binding to the PD-L2 intronic promoter (−240 to −147 from exon2) in the presence of unlabeled ("cold") competitor consisting of 5− and 3− truncated versions of the PD-L2 intronic promoter, and a consensus octamer binding sequence, at 50-fold concentrations. The locations of the A and B nucleoprotein complexes are indicated. One of 3 comparable experiments is shown. (c) Nuclear extracts were tested for the presence of Oct1 and Oct2 by supershift EMSA in which the electrophoretic mobility of proteins binding to the PD-L2 intronic promoter (−240 to −147 from exon2) was altered by addition of anti-Oct1 or anti-Oct2 to the binding reaction. Normal rabbit serum (NRS) was used as a control for antibody addition. The locations of the A, B and C nucleoprotein complexes are indicated, along with the locations of complexes supershifted by anti-Oct antibodies. One of 3 comparable experiments is shown.

Figure 6.
Octamer binding sites are responsible for PD-L2 expression in B-1 cells and Oct2 binds to PD-L2 intronic promoter in vivo only in primary B-1 cells and B-1 cell lines. (a) Mutant reporter constructs were prepared from the −240 to −136 intronic promoter sequence by altering one or both Octamer binding sites, as described in Materials and methods. BRD2 cells were transiently transfected with an empty firefly luciferase construct, or with firefly luciferase reporter constructs with the indicated Octamer site mutations, along with, in each case, a thymidine kinase promoter-dependent renilla luciferase expression vector. Cell lysates were prepared and luciferase activity was measured in relation to the renilla luciferase control. Results are reported as multiples of the activity present with empty vector. Mean values for 3 independent experiments are shown, along with lines corresponding to the standard errors of the means. (b) Oct2 binding to the PD-L2 intronic promoter in vivo was assessd by Chromatin Immunoprecipitation assay (ChIP), as described in Material and methods. Sort-purified primary PD-L2$^+$ B-1 cells and B-2 cells (left hand panel) and BRD2 (B-1) and A20 (B-2) cells (right hand panel) were lysed and DNA was sheared by sonication, after which chromatin was immunoprecipitated with anti-Oct2 antibody or with control IgG, and the *Pd-l2* promoter sequence was amplified by real-time PCR using primers listed in the Supplementary Table. Results are expressed as the degree of amplification relative to input DNA. Mean values for 3 independent experiments are shown, along with lines corresponding to the standard errors of the means.

Figure 7.
Okadaic acid (OA) downregulates PD-L2 expression in primary B-1 cells. (a) Sort-purified primary B-1 and B-2 cells were treated with OA (100 ng/ml) for the indicated time periods in hours, and then total cell lysates were prepared and western blotted with anti-Oct2 antibody. Anti-tubulin antibody was used as a loading control. One of 3 comparable experiments is shown. (b) Primary B-1 and B-2 cells treated with OA for various periods of time, as indicated, were stained with anti-PD-L2-PE antibody and analyzed for PD-L2 surface expression by flow cytometry. One of 3 comparable experiments is shown.

Table 1

Primary B-1 Cells Differ from B-2 Cells and Macrophages in PD-L2 Exon Expression and B-1 Cell Lines (BCL1, BRD2) Express PD-L2 Similarly to Primary B-1 Cells

	B-1 cells	B-2 cells	MΦ	IL4-MΦ	genomic DMA	BCL1	BRD2
PDL2-ex1	–	–	–	+	+	–	–
PDL2-ex2	+	–	–	+	+	+	+
PDL2-ex3	+	–	–	+	+	+	+
PDL2-ex4	+	–	–	+	+	+	+
PDL2-ex5	+	+	–	+	+	+	+
PDL2-ex6	+	+	–	+	+	+	+
PDL2-ex1–2	–	–	–	+	–	–	–
PDL2-ex2–3	+	–	–	+	–	+	+
PDL2-ex3–4	+	–	–	+	–	+	+
PDL2-ex4–5	+	–	–	+	–	+	+
PDL2-ex5–6	+	+	–	+	–	+	+

Table 2

PD-L2 mRNA Expression in Intron1 Region

	−310	−198	−82
B-1 cells	−	−	+
IL-4/MΦ	−	−	−