Article Title: Plasmacytoid dendritic cells regulate B cell growth and differentiation via CD70

Short Title: pDC stimulate human peripheral B cells via CD70

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ABSTRACT

The ability of plasmacytoid dendritic cells (pDCs) to promote plasma cell differentiation and Ig secretion through the production of type I interferon (IFN) and IL-6 has been well documented, although the role of additional factors including TNF receptor-ligand interactions has not been addressed. Upon stimulation with the TLR ligand CpG (B type, 2006) we found that pDCs exhibit strong and stable expression of CD70, a TNF family ligand that binds to its receptor CD27 expressed on memory B cells, and promotes plasma cell differentiation and Ig secretion. Using a pDC / B cell co-culture system, we found that, CpG-stimulated pDCs can induce the proliferation of CD40L-activated human peripheral B cells and Ig secretion. This occurs independently of IFN and residual CpG, and requires physical contact between pDCs and B cells. CpG-stimulated pDCs can induce the proliferation of both naive and memory B cells although Ig secretion is restricted to the memory subset. Blocking the interaction of CD70 with CD27 using an antagonist anti-CD70 antibody reduces the induction of B cell proliferation and IgG secretion by CpG-stimulated pDCs. We have therefore identified CD70 as an important factor in the regulation of B cell growth and differentiation by pDCs.
INTRODUCTION

Dendritic cells are a heterogeneous population of cells that play an important role in the initiation and regulation of both innate and adaptive immune responses. Plasmacytoid dendritic cells (pDCs), also known as type I interferon (IFN) producing cells, are one of the two main populations of dendritic cells in human peripheral blood. They selectively express TLR7 which allows them to respond to RNA viruses and TLR9 which allows them to respond to DNA viruses and CpG oligonucleotides. Upon exposure to virus pDCs produce vast amounts of IFN directly inhibiting viral replication and contributing to the activation of B cells, T cells, NK cells and myeloid dendritic cells.

Several recent studies have indicated an important role for pDCs in the regulation of B cell differentiation. Influenza virus-stimulated pDCs are capable of inducing the differentiation of human B cells into plasma cells secreting virus-specific antibodies. IFN secretion by pDCs mediates the differentiation of B cells into plasmablasts and pDC-derived IL-6 promotes the subsequent development of plasmablasts into Ig-secreting plasma cells. Blocking the function of IFN and IL-6 significantly reduces the production of IgG by B cells stimulated with virus-activated pDCs, however, CD40L-activated B cells cultured with IL-2, IFN-α and IL-6 produce levels of IgG that are lower than those observed in pDC/B cell co-culture experiments potentially indicating a requirement for additional factors. Also B cells cultured with pDCs preferentially secrete IgG indicating that pDCs might specifically target memory B cells. Indeed, in a recent study comparing the ability of pDCs and myeloid dendritic cells (MDCs) to promote B cell proliferation...
and differentiation, it was observed that pDCs, but not MDCs, could enhance the plasma cell differentiation of memory, but not naïve B cells stimulated with TLR7/8 ligands, via an IFN-dependent mechanism. It has also been observed that pDCs enhance the activation, plasma cell differentiation and Ig secretion of B cells activated by B cell receptor cross-linking and CpG-C. Separating the pDCs and B cells in a transwell system or blocking the function of IFN reduced but did not eliminate the activation of B cells observed in the presence of pDCs again indicating a role for additional factors including direct cell-to-cell contact.

CD27 is a member of the TNF receptor family and is a well established memory B cell marker. Engagement of CD27 with its ligand CD70 promotes the production of IgG, IgM and IgA by human peripheral B cells cultured with IL-2 and IL-10. It also enhances the differentiation of CD40L-activated B cells into plasma cells. CD40L stimulation strongly enhances B cell proliferation induced by Staphylococcus aureus Cowan I strain (SAC) however engagement of CD27 has little effect. Instead engagement of CD27 promotes plasma cell differentiation and IgG production from B cells cultured with SAC and IL-2. B cells cultured with agonist anti-CD40 antibody and IL-4 produce a significant amount of IgE but do not undergo plasma cell differentiation. Engagement of CD27 significantly enhances the production of IgE from B cells cultured with agonist anti-CD40 antibody and IL-4 and also promotes plasma cell differentiation and expression of the plasma cell-specific gene BLIMP1. Thus CD27 plays an important role in the regulation of plasma cell differentiation and Ig secretion from human memory B cells. Here we examine the expression of CD70 in pDCs and
investigate the role of CD70 in the regulation of B cell growth and differentiation by pDCs in order to better understand the mechanism of interaction between pDCs and B cells.
MATERIALS AND METHODS

pDC and B cell isolation

pDCs and B cells were isolated from buffy coat of healthy adult donors obtained from the Gulf Coast Regional Blood Center. This study was approved by the Institutional Review Board of the University of Texas M. D. Anderson Cancer Center and informed consent was provided in accordance with the Declaration of Helsinki. A pDC enriched population was isolated from PBMC by positive selection using CD304 (BDCA-4 / Neuropilin-1) microbead kit (Miltenyi Biotec) and pDCs were isolated by sorting CD3− (HIT3a) CD14− (MOP9) CD16− (3G8) CD56− (B159) CD19− (HIB19) CD11c− (Bly6) CD4+ (RPA-T4) CD123+ (6H6, Biolegend) cells. A B cell enriched population was isolated from PBMC by negative selection using human B cell isolation kit II (Miltenyi Biotec) and sorting CD3− CD14− CD16− CD56− BDCA-2− (AC144, Miltenyi Biotec) CD19+ cells. For some experiments naïve and memory B cells were isolated by sorting total B cells on the basis of CD27 (M-T271) expression. Unless indicated, all antibodies were purchased from BD Bioscience. Purity of sorted cells is greater than 98%.

pDC / B cell co-culture

pDCs and B cells are cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (Atlanta Biologicals), 2mM L-glutamine, 100 U/ml Penicillin, 100 U/ml Streptomycin, 1mM Sodium Pyruvate and 10mM HEPES. pDC were cultured for 24 hours at a density of 10^6/ml in medium alone or with 1µM CpG (B type, 2006, Alpha DNA) and washed three times with culture medium to remove residual CpG before use in subsequent culture. B cells were cultured over CD40L-transfected L cells irradiated 6500
– 7500 Rad at an L cell to B cell ratio of 1:5. In some experiments cells were cultured with recombinant CD40L (Alexis) at a concentration of 50 – 200 ng/ml. Similar results were obtained in both culture systems (data not shown). For analysis of B cell proliferation, 2x10⁴ B cells were cultured in 96 well round bottom plates with 1x10⁴ activated pDCs for a total of four days. One µCi of [³H]-Thymidine was added to each well for the last 18 hours and cellular incorporation was measured. Alternatively, to analyze B cell proliferation by CFSE dilution, 5x10⁴ B cells were labeled with CFSE and cultured with 2.5x10⁴ activated pDCs. Cells were harvested after 5 days of culture, stained with APC-labeled anti-CD19 to allow residual pDCs to be excluded, and CFSE dilution analyzed by flow cytometry. For analysis of immunoglobulin secretion, 2x10⁴ B cells were cultured with 1x10⁴ activated pDCs, IL-2 (50U/ml) and IL-10 (10ng/ml) for 14 days at which time supernatant was collected and IgG and IgM levels determined by ELISA (Bethyl Laboratories, Montgomery, TX). In some experiments, neutralizing anti-IFN-α (2000 neutralizing U/ml; PBL Biomedical Laboratories), rabbit polyclonal anti-IFN-β Abs (1000 neutralizing U/ml; PBL Biomedical Laboratories), and mouse anti-IFN-αβ receptor mAb (10 µg/ml, MMHAR-2; PBL Biomedical Laboratories)

**Flow Cytometry**

To analyze CD70 expression, freshly isolated pDCs or pDCs cultured with CpG-A (1µM, Alpha DNA), CpG-B (1 µM, Alpha DNA), irradiated HSV-1 (10 pfu/cell), influenza A virus (10 pfu/cell) or IL-3 (10 ng/ml, R&D Systems) were stained with purified anti-CD70 (clone KI-24, BD Bioscience) or purified isotype control (mouse IgG1 BD Bioscience) followed by PE-labeled goat anti-mouse Ig (BD Bioscience). To analyze
CD27 expression freshly isolated B cells or B cells cultured with CD40L with or without IL-2 (50 U/ml) and IL-10 (10 ng/ml) were stained with PE labeled anti CD27 (BD Bioscience).

**Generation of CD70 transfectants**

Full-length CD70 cDNA was amplified from cDNA derived from CpG-B-stimulated pDCs and cloned into the lentiviral vector FG9. The plasmid was cotransfected into HEK293T cells with the packaging constructs pMDLg/pRRE, RSV-Rev and CMV-VSVg. After 2 days, the culture supernatant containing the virus was collected and used to infect parental L cells. Expression of CD70 was confirmed by flow cytometry and cells with a high-level of CD70 expression were sorted and used in the generation of the antagonist anti-CD70 antibody.

**Generation of antagonist anti-CD70 antibody**

A BALB/c mouse less than six months of age was immunized with CD70-transfected L cells. Hybridomas secreting monoclonal antibodies recognizing CD70 were identified by ELISA and flow cytometry, and antagonist function was identified by screening clones for the ability to block the induction of proliferation of B cells cultured with IL-2 and IL-10 by CD70. Clone 113-16, which is IgG1 isotype, was used in all experiments.

**Microarray Analysis**

Microarray analysis was carried out as described previously.

15, 24.
RESULTS

Expression of CD70 and CD27 in human immune cells

Microarray analysis (Figure 1a) indicates that CD70 was not expressed in resting pDCs although expression of CD70 by pDCs was observed following stimulation with certain TLR7 and TLR9 ligands, including Influenza virus (Flu) and CpG oligonucleotides but not Herpes Simplex virus (HSV) or IL-3. CD70 expression in pDCs was confirmed using flow cytometry. Strong and stable upregulation of CD70 expression in pDCs was observed within 12 hours of CpG-B stimulation reaching peak levels after 48 hours of stimulation (Figure 1b). Expression of CD70 in pDCs was observed after stimulation for 48 hours with influenza virus and CpG-A, but not HSV or IL-3 (Figure 1c). CD27, the receptor for CD70, is specifically expressed on memory B cells 16-18. Microarray analysis indicates that CD27 was expressed by B cells, CD8+ T cells and CD4+ T cells subsets (Figure 1a). Total B cells were used for microarray analysis which can account for the relatively low level of CD27 expression that was observed. Expression of CD27 on human peripheral B cells, both resting and upon stimulation with CD40L or CD40L plus IL-2 and IL-10, was confirmed using flow cytometry (Figure 1d). CD27 expression was observed on a subset of resting B cells and decreases upon activation. Since CD70 is expressed on activated pDCs, it could potentially play an important role in the interaction between pDC and CD27+ memory B cells.
pDCs stimulated with CpG promote the proliferation and Ig secretion of human peripheral B cells

To examine the role of pDCs in the induction of B cell proliferation and Ig secretion, an \textit{in vitro} pDC / B cell co-culture system was established. CpG-B was selected to stimulate pDCs since it has a strong capacity to activate pDCs and induce the expression of CD70 but does not lead to significant IFN production\textsuperscript{25}. B cells were labeled with CFSE and cultured over irradiated CD40L L cells alone or with IL-2, IL-10 or IL-2 plus IL-10. Resting pDCs or pDCs which had been stimulated for 24 hours with CpG-B (CpG-B-pDCs) were added to B cells and proliferation was examined after five days of co-culture (Figure 2a.) The data indicates that CpG-B-pDCs had a strong capacity to promote the proliferation of CD40 L–activated B cells. Addition of IL-2 had little effect on the induction of B cell proliferation by CpG-B-pDCs. Addition of IL-10 or IL-2 plus IL-10 enhanced B cell proliferation in response to CD40L stimulation, but did not enhance the ability of CpG-B-pDCs to promote B cell proliferation. Similar results were observed when proliferation was measured using $[^3]$H-Thymidine incorporation (data not shown) and when B cells were activated with recombinant CD40L instead of CD40L-transfected cells (data not shown). A maximum two-fold increase in actual B cell numbers was observed in pDC / B cell co-culture experiments (data not shown). CpG-B alone was able to enhance the proliferation of B cells cultured over irradiated CD40L L cells alone or with IL-2, IL-10 or IL-2 plus IL-10 (Figure 2a). Using a similar pDC / B cell co-culture system, the ability of CpG-B-pDCs to promote the secretion of IgM and IgG from B cells was examined (Figure 2b). The addition of CpG-B-pDCs to B cells cultured with CD40L alone could not promote secretion of IgM or IgG. A moderate increase in IgG
secretion was observed when CpG-B-pDCs were added to B cells cultured with CD40L and IL-2 as compared to resting pDCs, although the increase in IgM secretion observed was minimal. Addition of CpG-B-pDCs to B cells cultured with CD40L and IL-10 resulted in a significant increase in IgG but not IgM secretion. However, when CpG-B-pDCs were added to B cells cultured with CD40L and IL-2 plus IL-10, a significant increase in the production of both IgG and IgM was observed. These data show that CpG-B-pDCs have the ability to promote the proliferation of CD40L-activated B cells and Ig secretion from B cells cultured with CD40L, IL-2 and IL-10.

**Induction of B cell proliferation and Ig secretion by CpG – stimulated pDC is contact dependent and independent of type I interferon and residual CpG.**

Previous studies have indicated that the interaction between pDCs and B cells is largely mediated by soluble factors including IFN and IL-6. We have shown that CD70 is expressed by CpG-B-pDCs (Figure 1a, b, c) and that CpG-B-pDCs are capable of inducing B cell proliferation and Ig secretion. If the induction of B cell proliferation and Ig secretion by CpG-B-pDCs is mediated by CD70, then it should occur in a contact dependent manner. To address whether the induction of B cell proliferation and Ig secretion by CpG-B-pDCs is mediated by cell-cell interactions or soluble molecules, we conducted a transwell experiment by culturing pDCs in the upper chamber and B cells in the lower chamber. Both wells were coated with CD40L L cells and proliferation was measured by CFSE dilution. Blocking the interaction between pDCs and B cells completely abrogated the induction of proliferation and Ig secretion by CpG-B-pDCs (Figure 3a). Using a flow cytometry based assay, conjugate formation between pDCs
and B cells was observed further supporting the physical interactions between pDCs and B cells (data not shown). Published data indicates that the induction of B cell Ig secretion by pDCs is largely mediated through IFN-α-10. To exclude a role for IFN in the induction of B cell proliferation and Ig secretion by CpG-B-pDCs, pDC / B cell co-culture was carried out in the presence of a cocktail of antagonistic antibodies against IFN-α, IFN-β and IFN-α/β receptor. In the presence of the antagonistic antibody cocktail, the induction of B cell proliferation by pDCs was significantly enhanced although the induction of IgM and IgG secretion was not significantly affected (Figure 3b). Since CpG-B alone was able to induce B cell proliferation (Figure 2a) and Ig secretion (Figure 2b), experiments were conducted to ensure that the induction of proliferation and Ig secretion observed upon the addition of pDCs was not due to carry-over of residual CpG-B. To rule out the possibility that the B cell proliferation and Ig secretion observed after co-culture with CpG-B-pDCs was due to residual CpG-B carried over on the pDCs after stimulation, chloroquine was added to culture wells. At a chloroquine concentration of 1 μg/ml, the induction of proliferation of CD40L-activated B cells by CpG-B was completely blocked although the induction of B cell proliferation by CpG-B-pDCs was only slightly reduced (Figure 3c). The ability of CpG-B to induce IgG and IgM secretion from B cells activated by CD40L, IL-2 and IL-10 was blocked by 0.5 μg/ml of chloroquine (Figure 3c). At this concentration, the induction of IgM secretion by CpG-B-pDCs was reduced to a level similar to that observed with resting pDCs although the induction of IgG secretion by CpG-B-pDCs was unaffected (Figure 3c). These data show that pDCs have the ability to promote B cell proliferation and Ig
secretion in a contact dependent, interferon independent manner, and that the induction of B cell proliferation and the secretion of IgG occurs independent of residual CpG-B.

**pDCs stimulated with CpG-B preferentially promote the proliferation and Ig secretion of CD27⁺ memory B cells.**

If CpG-B-pDCs promote B cell proliferation and Ig secretion via engagement of CD27 on B cells with CD70, then it would be anticipated that CpG-B-pDCs would preferentially promote the induction of proliferation and Ig secretion from CD27⁺ memory B cells. Naïve and memory B cells were sorted from total CD19⁺ B cells based on CD27 expression and used in pDC / B cell co-culture with resting pDCs or CpG-B-pDCs. Proliferation was measured by CFSE dilution on day 5. CpG-B-pDCs induced proliferation of both naïve and memory B cells stimulated by CD40L although a higher level of proliferation was observed for memory B cells as compared to naïve B cells (Figure 4a). Supernatant was collected on day 14 and IgG and IgM levels were determined by ELISA. CpG-B-pDCs were able to promote a slight but significant increase in IgM secretion from naïve B cells, but did not have a significant effect on IgG secretion (Figure 4b). CpG-B-pDCs were able to promote a significant increase in the induction of both IgG and IgM from CD27⁺ memory B cells (Figure 4b). The data indicate that while CpG-B-pDCs are able to induce the proliferation of both naïve and memory B cells, the induction of Ig secretion is restricted to the memory subset.
Antagonist anti-CD70 antibody limits the induction of B cell proliferation and Ig secretion by pDC

In order to specifically investigate the role of CD70 in the induction of B cell proliferation and Ig secretion by CpG-B-pDCs an antagonist anti-CD70 antibody was generated that could completely block the function of CD70 at a concentration of 10 μg/ml (data not shown). Resting or CpG-B-pDCs were pre-incubated with antagonist anti-CD70 antibody or purified mouse IgG1 isotype prior to the addition of B cells to the culture. In the presence of antagonist anti-CD70 antibody, the ability of CpG-B-pDCs to induce the proliferation of total B cells was significantly reduced (Figure 5a). In the presence of antagonist anti-CD70 antibody, IgG secretion was significantly reduced to a level comparable to that induced by resting pDC (Figure 5b). The induction of IgM secretion was reduced in some but not all donors but the result is not statistically significant. This is consistent with earlier data indicating that the induction of IgM secretion by CpG-B-pDCs was largely due to residual CpG-B (Figure 3c). These data indicate an important role for CD70 in the regulation of B cell proliferation and IgG secretion by CpG-B-pDCs.
DISCUSSION

The ability of pDCs to promote plasma cell differentiation and Ig secretion through the production of soluble factors including IFN and IL-6 has been well documented\textsuperscript{8-10}, although the role of additional factors including TNF receptor-ligand interactions has not been addressed. We have shown that CD70 is highly expressed on pDCs in response to stimulation with the TLR9 ligand CpG-B. Using a pDC / B cell co-culture system, the interaction between pDCs and B cells was further characterized and CD70 was identified as an important factor in the regulation of B cell proliferation and Ig secretion by pDCs.

CpG-B-pDCs can promote the proliferation of CD40L-activated peripheral B cells in the absence of additional cytokines. However the addition of IL-2 and IL-10 is required for CpG-B-pDCs to enhance the secretion of IgG and IgM from CD40L-activated B cells. The induction of B cell proliferation and Ig secretion by CpG-B-pDCs was shown to be contact-dependent, and independent of IFN. This data conflicts with published studies that indicate an important role for IFN in the induction of IgG secretion from CD40L-activated B cells by virus stimulated pDCs, and a marginal role if any for cell-cell contact\textsuperscript{8-10}. Upon stimulation with CpG-B pDCs produce very little IFN\textsuperscript{25} but exhibit robust expression of CD70. In contrast, pDCs stimulated with influenza virus produce a large amount of IFN, but express a relatively low level of CD70 on only a subset of cells. The level of CD70 expressed by pDCs in response to flu stimulation may be too low to influence B cell proliferation or differentiation. Also, IFN is produced very rapidly upon stimulation with flu, usually within the first 12 hours\textsuperscript{15} whereas peak levels of CD70 expression are observed after 48 hours of stimulation. It is possible that the B cell would
be exposed to IFN prior to CD27 ligation which might alter the response to CD70, or that there are two distinct phases of interaction between pDCs and B cells, a rapid IFN-dependent phase, followed by a later IFN-independent CD70-dependent phase. It is also possible that IFN stimulation might affect the expression level of CD27 on B cells or the level of CD70 expressed by pDCs. Initial studies indicated that pDCs promoted mostly IgG secretion from B cells activated by CD40L stimulation or B cell receptor cross-linking in the presence of CpG-C and led to speculation that pDCs may target the memory B cell subset. It was shown recently that pDCs could specifically promote the proliferation of memory B cells in response to CpG-B stimulation. CpG-B-pDCs were shown to promote the proliferation of both naïve and memory B cells, however Ig secretion was restricted to the CD27+ memory subset. Also CD70 was shown to have an important role in the induction of B cell proliferation and Ig secretion by pDCs. This provides a mechanism to explain the ability of pDCs to specifically influence the growth and differentiation of CD27+ memory B cells.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by tissue damage resulting from immune complex deposition, and high titers of autoantibodies specific for DNA and nucleosomes. Low numbers of pDCs are found in the blood of SLE patients as they have migrated to sites of inflammation. However, increased levels of IFN-α, most likely produced by pDCs, are observed in the serum of approximately 20-30% of SLE patients. IFN-α enhances the differentiation of monocytes into dendritic cells that can take up and express antigens from apoptotic cells leading to autoimmunity. IFN-α also promotes the development of autoreactive plasmablasts. Abnormalities in the
composition of the peripheral B cell compartment have been observed in SLE patients. Although decreased numbers of naïve and CD27+ memory B cells are found in SLE patients, increased numbers of CD27$^{\text{high}}$ plasma cells $^{29}$ and CD27$^{\text{high}}$ plasma cell precursors $^{30}$ have been observed and levels of these cells have been found to correlate with disease severity $^{31}$. SLE patients treated with rituximab, an antibody that depletes CD20$^+$ B cells, who exhibit a delayed reconstitution of CD27$^+$ memory B cells after treatment, have a better response to the therapy than other patients with better relief from symptoms and reduced levels of autoantibodies in the serum $^{32}$. There is considerable evidence that pDCs are activated in SLE and that CD27$^+$ B cells have an important role in the disease. It is possible that activated pDCs expressing CD70 may drive the expansion of these cells and promote their differentiation into antibody secreting plasma cells thereby promoting autoantibody production and exacerbation of symptoms.
ACKNOWLEDGEMENTS
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AUTHORSHIP
JS designed and performed research, analyzed data and wrote the manuscript. YHW performed research and analyzed data. TI performed research and analyzed data. KA designed and performed research. YJL designed research and reviewed and approved the manuscript.

CONFLICT OF INTEREST
The authors declare no competing financial interests.
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FIGURE LEGENDS

Figure 1. Expression of CD70 and CD27 in immune cells. (A) Microarray analysis showing expression of CD70 and CD27 in immune cells. (B) The expression of CD70 on resting pDCs or pDCs stimulated with CpG-B for 12, 24, 48 or 72 hours was analyzed by flow cytometry. (C) The expression of CD70 on pDCs stimulated with IL-3, CpG-A, CpG-B, Flu or HSV for 48 hours was analyzed by flow cytometry. (D) The expression of CD27 on resting B cells and B cells cultured for 12, 24, 48 or 72 hours with CD40L with or without IL2 and IL-10 was analyzed by flow cytometry. Shaded histogram indicates isotype control and open histogram indicates specific staining.

Figure 2. Induction of B cell proliferation and Ig secretion by pDC. Freshly isolated pDCs were stimulated for 24 hours with 1 μM CpG-B and then added to B cells cultured over irradiated CD40L transfected L cells alone or in the presence of IL-2 (50 U/ml), IL-10 (10 ng/ml) or both, as indicated. (A) B cells were labeled with CFSE prior to culture. After 5 days of co-culture, cells were harvested and stained with APC-labeled anti-CD19 to allow residual pDCs to be excluded, and CFSE dilution was analyzed by flow cytometry. In pDC / B cell co-culture experiments, results of which are shown in the left panel, the shaded histogram indicates B cells cultured with CD40L, the dashed histogram indicates B cells cultured with resting pDCs, and the solid histogram indicates B cells cultured with CpG-B-pDCs. In control experiments, results of which are shown in the right panel, the shaded histogram indicates B cells cultured with CD40L and the dotted histogram indicates B cells cultured with CD40L plus 1 μM CpG-B. The results shown, which are representative of at least five individual donors, have been gated on the CD19+...
population. (B) Similar cultures were established with unlabeled B cells; supernatant was collected on day 14 and IgG and IgM concentrations were measured by ELISA. Results shown are representative of three independent experiments. The average of duplicate results from two individual donors +/- SEM is shown. Results were analyzed using unpaired t test, * indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001.

**Figure 3. The induction of B cell proliferation and Ig secretion by pDC is contact-dependent and independent of IFN and residual CpG-B.** (A) pDC / B cell co-culture was established with pDCs sequestered in the upper transwell chamber and B cells in the lower chamber. B cells were labeled with CFSE prior to culture and proliferation was assessed by measuring CFSE dilution on day 5. The shaded histogram indicates B cells cultured with CD40L, the dashed histogram indicates B cells cultured with resting pDCs, and the solid histogram indicates B cells cultured with CpG-B-pDCs. Data shown indicate one donor representative of three. Supernatant was collected on day 14 and IgG and IgM concentrations were determined by ELISA. The mean +/- SEM of duplicate results from three individual donors is shown. (B) pDC /B cell co-culture was carried out as described previously except that a cocktail of antagonist antibodies against IFN-α, IFN-β and IFN-α/β/R were added. Proliferation was assessed by measuring [³H]-Thymidine incorporation on day 3 and IgG and IgM levels in supernatant were measured by ELISA on day 14. Mean results +/- SEM from one experiment representative of three are shown. (C) B cells were labeled with CFSE and cultured with CD40L and either 1 μM CpG-B, resting pDCs or CpG-B-pDCs. Chloroquine was added to the B cells at the indicated concentrations thirty minutes prior to the addition of CpG-B or pDCs.
Proliferation was assessed by measuring CFSE dilution on day 5. In control experiments, results of which are shown in the upper panel, the shaded histogram indicates B cells cultured with CD40L and the dotted histogram indicates B cells cultured with CD40L plus 1 μM CpG-B. In pDC / B cell co-culture experiments, results of which are shown in the lower panel, the shaded histogram indicates B cells cultured with CD40L, the dashed histogram indicates B cells cultured with resting pDCs, and the solid histogram indicates B cells cultured with CpG-B-pDCs. Supernatant was collected on day 14 and IgG and IgM concentration determined by ELISA. Chloroquine was titrated to determine the optimal concentration. Fixed numbers of B cells and pDCs were added. Results of one experiment representative of three are shown. Data were analyzed by unpaired t test, * indicates p < 0.05 and ** indicates p < 0.01.

**Figure 4.** pDCs promote the proliferation of both naïve and memory B cells although Ig secretion is restricted to the memory subset. Total B cells or B cells sorted into naïve and memory subsets based on CD27 expression were cultured with resting or CpG-B-pDCs. (A) Proliferation was assessed by analyzing CFSE dilution on day 5. The shaded histogram indicates B cells cultured with CD40L, the dashed histogram indicates B cells cultured with resting pDCs, and the solid histogram indicates B cells cultured with CpG-B-pDCs. Results of one donor representative of three are shown. (B) Supernatant was collected on day 14 and IgG and IgM levels were determined by ELISA. Mean results +/- SEM from one experiment representative of three are presented. Data was analyzed by unpaired t test, * indicates p< 0.05 and *** indicates p< 0.001.
Figure 5. Antagonist anti-CD70 antibody blocks the induction of B cell proliferation and Ig secretion by pDC.  pDC / B cell co-culture was established and antagonist anti-CD70 antibody or purified mouse IgG1 isotype control at a concentration of 10 μg/ml was added to the wells as indicated. Antibody and pDCs were added and incubated at 37°C for thirty minutes prior to the addition of B cells.  (A) Proliferation was assessed by measuring [3H]-Thymidine incorporation on day 3.  Results shown indicate the average of triplicate results from one donor representative of five.  (B) Supernatant was collected on day 14 and IgG and IgM levels were determined by ELISA.  Each point represents the average of duplicate results for one individual donor.  Data was analyzed by unpaired t test, * indicates p< 0.05.
Figure 1

A. CD70

B. PDC

CpG-B (1mm)

Time (Hours)

0 12 24 48 72

CD70

C. IL-3 CpG-A CpG-B Flu HSV

CD70

D. B Cell

Time (Hours)

0 12 24 48 72

CD40L

CD40L

IL-2/10

CD27
Figure 2

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<td>+ pDC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ CpG-B-pDC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ CpG-B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4

A

Naïve

Memory

Total

CFSE

B

Naïve

Memory

Total

IgG (ng/ml)

CD40L IL-2/10  pDC  CpG-B-pDC

***

IgM (ng/ml)

CD40L IL-2/10  pDC  CpG-B-pDC

***
Figure 5

A. 3H Thymidine Incorporation (cpm) x 10^3

- Medium
- CD40L IL-2/10
- + pDC
- + CpG-B-pDC
- + CD70
- + CpG-B-pDC αCD70
- + CpG-B-pDC Isotype

B. IgG1 (ng/ml)

- Medium
- CD40L IL-2/10
- + pDC
- + CpG-B-pDC
- + CD70
- + CpG-B-pDC αCD70
- + CpG-B-pDC Isotype

C. IgM (ng/ml)

- Medium
- CD40L IL-2/10
- + pDC
- + CpG-B-pDC
- + CD70
- + CpG-B-pDC αCD70
- + CpG-B-pDC Isotype
Plasmacytoid dendritic cells regulate B cell growth and differentiation via CD70

Joanne Shaw, Yui-Hsi Wang, Tomoki Ito, Kazuhiko Arima and Yong-Jun Liu