Characterization of Plasmacytoid Dendritic Cells in Bone Marrow of Pig-Tailed Macaques\textsuperscript{\dag}

R. Keith Reeves and Patricia N. Fultz\textsuperscript{*}

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

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Plasmacytoid dendritic cells (pDCs), one of two types of bone marrow (BM)-derived blood DCs, play an important role in linking innate and adaptive immune responses. However, little is known about the nature of pDCs that reside in the BM. Because the simian immunodeficiency virus-macaque model closely mimics human immunodeficiency virus disease in humans, with both infections inducing a decrease in pDCs, we characterized and compared pDCs in the BM with in peripheral blood (PB) of healthy pig-tailed macaques. The results revealed that pDCs from both compartments had the same CD123\textsuperscript{+} HLA-DR\textsuperscript{−} Lin\textsuperscript{−} phenotype and were similar in size. Although BM-derived pDCs (BM-pDCs) were 3-fold greater in frequency and 10-fold greater in number, they had lower cell surface expression of both HLA-DR and the costimulatory molecule CD86 than did PB-pDCs. Both BM- and PB-pDCs responded ex vivo to synthetic CpG oligodeoxynucleotides and inactivated influenza virus by upregulating HLA-DR and CD86 and secreting cytokines; however, stimulated BM-pDCs secreted less alpha interferon and tumor necrosis factor alpha per cell than did PB-pDCs. These results suggest that while BM-pDCs appear to be phenotypically less mature than PB-pDCs, they do respond to pathogens. Thus, during acute infections, these cells could initiate immune responses either in the BM or after rapidly migrating from the BM into the periphery. A better characterization of pDCs in blood and tissues will be beneficial for future studies of macaques that focus on either pathogenesis or vaccine development.

Control of invading pathogens in vertebrates is dependent on the two major arms of the immune system: nonspecific innate immunity and antigen (Ag)-specific adaptive immunity. Components of the innate immune system mobilized during the acute phase of microbial infections include complement, granulocytes, natural killer (NK) cells, alpha/beta interferon (IFN-\(\alpha/\beta\)), and other proinflammatory cytokines. In contrast, mediators of adaptive responses, primarily T and B lymphocytes, specifically recognize an unlimited number of Ags by diversification of T-cell receptors and immunoglobulin genes. The principal links between these two protective components are cytokines and Ag-presenting cells (APCs), of which dendritic cells (DCs) are the most critical. In humans, blood DCs characteristically express high levels of major histocompatibility complex (MHC) class II molecules; lack common lineage markers (Lin\textsuperscript{−}) such as CD3 (T cells), CD14 (monocytes), CD56 and/or CD16 (NK cells), and CD20 (B cells); and are subdivided into CD11c\textsuperscript{+} myeloid DCs (mDCs) and CD123\textsuperscript{+} plasmacytoid DCs (pDCs). Although pDCs are generally less efficient as APCs than mDCs, pDC-mediated Ag presentation can be augmented in vitro by the addition to cultured cells of interleukin-3 (IL-3) and tumor necrosis factor alpha (TNF-\(\alpha\)) (27). Interestingly, activated pDCs secrete more IFN-\(\alpha\) per cell than any other cell type in the body and therefore have been designated natural IFN-producing cells (44).

Both mDCs and pDCs are activated and secrete cytokines after the recognition of diverse configurations that are characteristic of microbes; these motifs are known as pathogen-associated molecular patterns and include, among others, lipopolysaccharide, lipoproteins, peptidoglycan, and unmethylated CpG dinucleotides. Pathogen-associated molecular pattern recognition is a property of three families of receptors: Toll-like receptors (TLRs), NOD-like receptors, and RIG-I-like receptors, all of which overlap functionally, with TLRs having the broadest reactivity (12). TLRs are differentially expressed by human mDCs and pDCs; mDCs express primarily TLR-1 through TLR-6, while pDCs express TLR-7, TLR-9, and TLR-10 (26). TLR-7 and TLR-9 recognize and bind to single-stranded viral RNA and hypomethylated CpG motifs in viral or bacterial DNA, respectively, resulting in the activation of pathways that induce cellular maturation (2, 31, 32). Synthetic oligodeoxynucleotides containing one or more unmethylated CpG motifs (CpG ODNs), which are immunostimulatory in primates, are generally classified into one of three categories: CpG-A ODNs, which induce activation and maturation of pDCs; CpG-B ODNs, which potently activate B cells, which also express TLR-9; and CpG-C ODNs, which stimulate both pDCs and B cells (34). Following TLR ligation, pDCs not only produce and secrete high levels of IFN-\(\alpha\), TNF-\(\alpha\), and, to a lesser extent, IL-6 and -12 but also upregulate the expression of MHC class II proteins and the cell surface-costimulatory molecules CD80 and CD86 (2, 31, 32, 36, 49). IFN-\(\alpha\) and TNF-\(\alpha\) secreted by pDCs enhance NK cell-mediated killing of susceptible target cells and NK cell secretion of IFN-\(\gamma\) that synergizes with IL-12 to polarize CD4\textsuperscript{+} T cells for a Th1 response (23, 42); stimulated pDCs can also augment virus-specific memory T-cell activation (18, 22, 28, 49).

pDCs, which are commonly described as being sentinels in...
Peripheral blood (PB), have been identified in various tissues including tonsils, thymus, lungs, spleen, lymph nodes, Peyer’s patches, and colon (4, 6, 8, 35, 37, 45, 51). During microbial infections, pDCs can accumulate in inflamed tissues such as the nasal mucosa during acute respiratory viral infections and the central nervous system during bacterial meningitis (24, 25, 39, 53). In mice, pDCs can also infiltrate the vaginal mucosa, where they limit virus replication during acute infection such as herpes simplex virus infection (33). Furthermore, cells with a phenotype identical to that of pDCs have been identified in both murine and human bone marrow (BM) (5, 13, 15, 30, 40, 46). Although BM is considered to be a primary lymphoid organ because it is a major site of hematopoiesis, it can harbor bacterial, parasitic, and viral pathogens, including simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV), thus making it a site for immunosurveillance (14, 29, 43, 52). For example, memory and regulatory T cells migrate to and from the BM, where they proliferate extensively, suggesting that the BM serves as a central organ for long-term memory responses (3, 16, 54), and in mice, resident BM-DCs present Ag to naive T cells (21).

Because pDCs are an integral component of the innate immune system, the observation that the numbers of circulating pDCs are reduced in many viral infections, including primary and chronic HIV-1 infections, is of concern (17). Decreased numbers or impaired functions of pDCs could facilitate the establishment of opportunistic infections. We and others recently showed a similar loss of pDCs in blood and tissues of SIV-infected macaques, indicating that the SIV macaque model is appropriate for use in studying pDC-lentivirus interactions (7, 41). Since both cell-free virus and HIV- and SIV-infected cells, as well as pDCs, are found in the BM (29, 43), the purpose of our investigation was to characterize and compare the phenotypes and functions of BM- and PB-derived pDCs (PB-pDCs) in healthy macaques. SIV infection of macaques is the most reliable model to study immune responses to and the pathogenesis of HIV; therefore, a better understanding of the basic functional properties of macaque pDCs will provide a foundation for exploiting these cells in the design and development of novel vaccines with enhanced immunogenicity and for evaluating the role of pDCs in lentivirus infections.

Flow cytometry. For cell surface staining to determine percentages and to characterize macaque pDCs in EDTA-treated whole blood or in PBMC and BMMMC, three- and four-color flow cytometry was used. Mononuclear cells were first gated based on forward- and side-scatter characteristics, and CD123<sup>+</sup> HLA-DR<sup>+</sup> Lin<sup>-</sup> pDCs were then identified using phycoerythrin-labeled anti-CD123 (7G3), peridinin chlorophyll protein-cyanin 5.5-labeled anti-HLA-DR (G46–6), and a cocktail of fluorescein isothiocyanate-conjugated antibodies to the Lin markers CD3ε (SP34), CD14 (M6P9), CD16 (3G8), and CD20 (2H7). Expression of CD86 was evaluated using an allophycocyanin-conjugated antibody (231). To calculate percentages of positive cells, background fluorescence was determined with isotype-matched control antibodies conjugated to each fluorochrome. All antibodies and reagents for fluorescence-activated cell sorter analysis were purchased from BD Biosciences Pharmingen (San Diego, CA); acquisitions were performed using a BD LSRII flow cytometer (BD Biosciences).

Mononuclear cell cultures and stimulation assays. Approximately 1.0 × 10<sup>7</sup> PBMC or BMMC were cultured in 24-well plates in an enriched medium (RPMI 1640 supplemented with 10% fetal bovine serum, 10% normal macaque serum, and 20 ng of IL-3/ml) and stimulated with either 10 µg/ml Cpg-C274 ODN (Dynavax Technologies, Berkeley, CA) or 1 µg/ml formalin-inactivated influenza virus (A/Puerto Rico/8/34; Novavax, Rockville, MD); cells in medium only served as controls. After 18 h at 37°C, culture supernatants were removed; the cells were then washed and analyzed for CD86 expression on CD123<sup>+</sup> HLA-DR<sup>+</sup> Lin<sup>-</sup> cells by flow cytometry. Concentrations of IFN-α and TNF-α in culture supernatants were determined using cross-reactive human IFN-α and rhesus monkey TNF-α enzyme-linked immunosorbent assay kits (both from Biosource, Camarillo, CA) according to the manufacturer’s protocols; the lower limits of detection were 10 and 2 pg/ml for IFN-α and TNF-α, respectively.

Detection of intracellular cytokines. Using the same culture conditions as those described above, mononuclear cells were stimulated for 4 h with Cpg-C274 ODN or inactivated influenza virus; GolgiPlug (1 µ/ml) (BD Biosciences) was added, and the cells were cultured for an additional 2 h before being harvested, washed, and stained for the extracellular expression of CD123, HLA-DR, and Lin markers. To detect intracellular cytokines in pDCs, a Cytoxid CytoPerm kit (BD Biosciences) was used, according to the manufacturer’s protocol. For detection of IFN-α, polyclonal antibodies reactive to multiple isoforms were biotinylated with EZ-Link (Pierce, Rockford, IL) and then used with a streptavidin-allophycocyanin conjugate to stain mononuclear cells. Nonbiotinylated forms of the same polyclonal antibodies served as controls. Intracellular TNF-α was detected using a cross-reactive antibody (MAb11) directly conjugated to allophycocyanin (BD Biosciences). The percentages of cytokine-positive pDCs were determined as the fraction of CD123<sup>+</sup> HLA-DR<sup>+</sup> Lin<sup>-</sup> cells in cultured mononuclear cell populations.

Statistical analyses. To compare results and to determine statistical significance, the Welch alternate t test or the Mann-Whitney U test was used. Spearman’s correlation test was used to determine whether changes in two parameters were linked. All analyses were performed with InStat 2.0 software (GraphPad, San Diego, CA).

RESULTS AND DISCUSSION

Phenotype and frequency of macrophage pDCs in blood and BM. Using flow cytometry, PB-pDCs were found in an expanded lymphocyte/monocyte gate (Fig. 1A, R1) and were phenotypically CD123<sup>+</sup> HLA-DR<sup>+</sup> Lin<sup>-</sup> (Fig. 1A, R2 and R3), as are human PB-pDCs. Since there are DC precursors and resident DCs in the BM, we determined whether cells with a phenotype similar to that of PB-pDCs could be identified in macaque BM. BM aspirates were collected from six adult pig-tailed macaques; these aspirates contained a median of 2.9 × 10<sup>7</sup> mononuclear cells per ml (range, 4.7 × 10<sup>7</sup> to 8.0 × 10<sup>7</sup> mononuclear cells per ml). A homogeneous CD123<sup>+</sup> HLA-DR<sup>+</sup> Lin<sup>-</sup> cell population was identified among BMMCs using the same gating strategy as that used for PB-pDCs (Fig. 1B, R2 and R3). However, both the frequencies and absolute numbers of BM-pDCs were significantly greater than those of PB-pDCs (Table 1). This result was not unexpected, since Szabolcs et al. (46) identified pDCs in human BM and found them to be fivefold greater in number than those in blood. In addition, we...
found no correlations between the frequencies or the absolute numbers of macaque pDCs in the BM with those in PB (data not shown). Furthermore, while pDCs from both sites were similar in size, BM-pDCs consistently had lower levels HLA-DR cell surface expression, measured as mean fluorescence intensity (MFI), than PB-pDCs (Fig. 1 and Table 1), suggesting that BM-pDCs were less mature than PB-pDCs (19).

**Activation of pDCs.** The low frequencies of macaque pDCs in peripheral blood and tissues and the lack of sufficient cross-reactivity of human pDC-specific antibodies such as BDCA-2 and BDCA-4 (our unpublished data) that are used for isolation of these cells preclude the thorough characterization of purified macaque pDCs (1, 9, 11). Thus, we analyzed BM-pDCs and PB-pDCs in mononuclear cells (BMMC and PBMC) ex vivo either directly or after 18 h of culture with traditional pDC stimuli. As described above, in freshly isolated mononuclear cells, HLA-DR expression (MFI) was generally lower on BM-pDCs than on PB-pDCs and was upregulated minimally on both cell types after 18 h of culture in enriched medium (Fig. 2A and B). Furthermore, upon stimulation with CpG-C274 ODN or inactivated influenza virus, HLA-DR expression on BM-pDCs increased three- to fivefold above MFI levels detected ex vivo. While the mean MFI of HLA-DR on the surface of PB-pDCs increased to similar levels in response to CpG-C274 ODN or influenza virus, the increase was lower because of the inherently higher MFI before stimulation (Fig. 2A and B). The level of the activation/costimulatory molecule CD86 was also significantly (P < 0.01) lower on BM-pDCs ex vivo than on their peripheral blood counterparts (mean MFIs, 13.2 and 37.4, respectively) (Fig. 2A and C). After cultures were grown overnight in medium without one of the TLR agonists, CD86 was upregulated on pDCs from both sources, but the increase in MFIs on PB-pDCs was greater than that on BM-pDCs, as was reported previously for macaque pDCs cultured similarly (47). In response to CpG-C274 or influenza virus, mean MFIs associated with CD86 expression on BM-pDCs increased 6- and 14-fold, respectively, above those of uncultured cells, whereas mean increases in CD86 MFIs for PB-pDCs were six- and ninefold, respectively (Fig. 2A and C).

The upregulation of HLA-DR and CD86 in response to CpG-C274 and an RNA virus, such as influenza virus, is consistent

### TABLE 1. Quantification and characterization of macaque BM- and PB-pDCs

<table>
<thead>
<tr>
<th>pDC type</th>
<th>% Frequency among mononuclear cells (range)</th>
<th>Absolute no. of pDCs per ml (range)</th>
<th>HLA-DR MFI (range)</th>
<th>Relative size (range)</th>
<th>No. of animals</th>
</tr>
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<tbody>
<tr>
<td>PB</td>
<td>0.245 (0.147–0.34)</td>
<td>10,140 (4,358–14,161)</td>
<td>655 (104–1,230)</td>
<td>526 (445–676)</td>
<td>10</td>
</tr>
<tr>
<td>BM</td>
<td>0.764 (0.301–1.19)</td>
<td>101,495 (20,088–272,000)</td>
<td>229 (81.5–480)</td>
<td>514 (320–785)</td>
<td>6</td>
</tr>
</tbody>
</table>

* Statistical differences between BM- and PB-pDCs were calculated using the Mann-Whitney U test; P values of < 0.05 were assumed to be significant. The P value for frequency among MCs between BM- and PB-pDCs was < 0.001, that for the absolute number per milliliter was < 0.01, that for HLA-DR MFI was 0.09, and that for relative size was 0.64.

* Frequency of CD123+ HLA-DR+ Lin− pDCs among total BMMC or PBMC (based on forward- versus side-scatter characteristics) as determined by flow cytometry (Fig. 1).

* Absolute numbers (medians) of BM-pDCs were calculated by multiplying the percentages of CD123+ HLA-DR+ Lin− pDCs by the number of BMMC per ml of BM collected and of PB-pDCs by the total number of lymphocytes and monocytes per ml of blood calculated from complete blood counts and differentials.

* Median MFI of HLA-DR expression on BM- and PB-pDCs.

* All parameters were evaluated for PB-pDCs from 10 animals, except for the absolute numbers, which reflect data for 6 animals.
with data from previous reports for macaque and human PB-pDCs (2, 22, 48, 49); this result also suggests that BM-pDCs, like PB-pDCs, express TLR-7 and TLR-9, the cognate receptors for single-stranded viral RNA and bacterial DNA with hypomethylated CpG motifs. It is interesting, however, that CD86 levels were consistently lower on BM-pDCs than on PB-pDCs in identically treated cultures. Our experiments monitored pDCs after only 18 h of stimulation; therefore, it is possible that longer incubation times would overcome this difference. Also, the increases in MFI s of HLA-DR and CD86 (for all cultures) were directly correlated ($R = 0.979$ [$P < 0.001$] for BM-pDCs; $R = 0.916$ [$P < 0.001$] for PB-pDCs) (data not shown). This result was not unexpected since in murine cells, MHC class II and costimulatory molecules cluster together intracellularly, and the activation of APCs induces the coaggregation of MHC class II and costimulatory molecules on the cell surface, thereby enhancing Ag presentation to T cells (10). Although pDCs are generally poor stimulators of naïve T cells, the upregulation of both surface Ags might make pDCs more effective APCs. Feuerer et al. (21) previously showed that murine CD11c+ BM-DCs form clusters with CD3+ T cells in situ and process and present Ags by both MHC class I and class II pathways to prime local T cells; activated macaque BM-pDCs might behave in a similar manner.

One consequence of pDC activation is the production of IFN-α and TNF-α. After macaque BMMC and PBMC were cultured with CpG-C274 or influenza virus for 18 h, the concentrations of IFN-α and TNF-α in the medium were quantified. In control cultures, the amounts of these two cytokines were generally at or below the limits of detection (Fig. 2D and E). In contrast, when CpG-C274 or influenza virus was added at the time that the BMMC and PBMC cultures were established, the secretion of both cytokines was upregulated, and regardless of the stimulus, there was no statistically significant difference in the amount of either IFN-α or TNF-α that accumulated in the medium. Encabo et al. (20) recently reported that DCs from umbilical cord blood were phenotypically less mature and secreted less TNF-α than PB-DCs. This difference might be true for pDCs in the BM, consistent with the more immature phenotype of these cells. Since IFN-α and TNF-α augment Nk cell killing and IFN-γ secretion (23, 34), it appears that both BM- and PB-pDCs can support innate effector functions ex vivo and therefore might behave similarly in vivo.

**Frequency of cytokine-producing cells.** As shown in Table 1, the frequency of pDCs in BMMC is approximately threefold greater than that found in blood; however, comparable amounts of IFN-α and TNF-α accumulated in stimulated BMMC and PBMC cultures (Fig. 2D and E). These results suggest that either fewer BM-pDCs produce cytokines in response to stimuli or they produce lower amounts per cell. To define differences in cytokine secretion by BM- and PB-pDCs more accurately, BMMC and PBMC were stimulated with CpG-C274 or influenza virus for 6 h, and pDCs were then analyzed for intracellular IFN-α and TNF-α by multicolor flow cytometry. The frequencies of activated IFN-α-positive (IFN-α+) BM- and PB-pDCs were consistent with data from previous reports showing that not all human and macaque pDCs secrete IFN-α in response to CpG ODNs or RNA viruses (Fig. 3A) (1, 9). Low frequencies (up to 12%) of IFN-α+ pDCs were detected in unstimulated control cultures, indicating that either pDCs spontaneously produced IFN-α upon culture, pDCs may have been nonspecifically stimulated during mononuclear cell isolation, or pDCs were activated in vivo, perhaps by an infectious agent. Of interest, the frequencies of TNF-α+ pDCs appeared to be lower in BMMC than in PBMC, but this difference was not statistically significant (Fig. 3B). This observation is consistent with the lower concentrations of TNF-α.
that we found in activated BMMC culture supernatants (Fig. 2E). The frequencies of TNF-α− pDCs in both BMMC and PBMC were lower than those of IFN-α− pDCs, which is not unexpected, since pDCs are characterized by their ability to produce large amounts of IFN-α. In addition, our results agree with those that were obtained using macaque cells as described previously (9). However, the reason for comparable levels of IFN-α accumulating in culture supernatants, despite the fact that BMMC cultures contained far greater numbers of pDCs than PBMC cultures, is still unclear. Our intracellular cytokine analyses showed similar frequencies of IFN-α-secreting cells in both cultures, making it unlikely that differences in the relative numbers of IFN-α-secreting cells account for the disparity. Another possibility is that BM-pDCs secrete less IFN-α per cell than do PB-pDCs. Since the cellular and cytokine milieus of BMMC and PBMC are undoubtedly different, it is possible that other cells in the cultures are rapidly taking up IFN-α produced de novo, or some unknown regulatory pressures are being exerted on pDCs in BMMC that are less pronounced in PBMC.

If the BM-pDC population that we identified is a more immature form of pDC, it might be equivalent to the CD123−/H11001−/H9251−/H11002− “Pro-pDC” subset in primary lymphoid tissues described previously by Blom et al. (5), a population that also accumulates in culture supernatants, despite the fact that BM-pDCs in both cultures, making it unlikely that differences in the relative numbers of IFN-α-secreting cells account for the disparity. Another possibility is that BM-pDCs secrete less IFN-α per cell than do PB-pDCs. Since the cellular and cytokine milieus of BMMC and PBMC are undoubtedly different, it is possible that other cells in the cultures are rapidly taking up IFN-α produced de novo, or some unknown regulatory pressures are being exerted on pDCs in BMMC that are less pronounced in PBMC.

Regardless of whether macaque BM-pDCs are precursors of PB-pDCs or are resident DCs, it would be interesting to determine if a decrease in BM-pDCs occurs in HIV-infected persons, as was shown for human PB-pDCs (17) and, more recently, was documented for pDCs in macaques (7, 41). Since multilineage hematopoiesis is impaired during HIV infection (38, 50), pDC dendropoiesis might also be affected, which could contribute to the loss of these cells in blood and tissues.

The results presented herein provide a partial characterization of BM-pDCs in macaques and therefore should enhance our understanding of pDC dynamics in future studies of SIV infections of nonhuman primates.

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