Dendritic cell subsets in blood and lymphoid tissue of rhesus monkeys

and their mobilization with Flt3 ligand

P. Toby H. Coates, Simon M. Barratt-Boyes, Linyou Zhang, Vera S. Donnenberg,
Peta J. O'Connell#, Alison J. Logar, F Jason Duncan, Michael Murphey-Corb, Albert D. Donnenberg,
Adrian E. Morelli, Charles R. Maliszewski, and Angus W. Thomson

The Thomas E. Starzl Transplantation Institute and Departments of Surgery, Infectious Diseases and Microbiology, Medicine, Molecular Genetics and Biochemistry, and Immunology, University of Pittsburgh, Pittsburgh PA and Immunex Corporation, Seattle, WA

Corresponding author:
Dr Angus W. Thomson,
W1544 Biomedical Science Tower,
200 Lothrop St.
Pittsburgh PA 15217
Tel: 412 624 1116
Fax: 412 624 1172
Email: thomsonaw@msx.upmc.edu

Scientific section heading: Immunobiology
Short title: Characterization of rhesus monkey dendritic cell subsets

This work was supported by NIH grants UO1 A15169, RO1 AI 41101 and RO1 DK 49745, to AWT and R21 HL69725 and R21 AI55027 to AEM. PTC is a recipient of a CJ Martin Fellowship of the National Health and Medical Research Council of Australia. # Current address Robarts Research Institute, London, Ontario, Canada

CM is employed by a company (Immunex) whose potential product was studied in the present work.
Abstract

We provide phenotypic and functional evidence of pre-monocytoid DC and pre-plasmacytoid DC in blood and of corresponding DC subsets in secondary lymphoid tissue of rhesus monkeys. Subsets were identified and sorted by 4-color flow cytometry using anti-human monoclonal antibodies cross-reactive with rhesus monkey. To mobilize pre-DC subsets, fms-like tyrosine 3 kinase ligand (Flt3L; 100 µg/kg s.c.) was administered for 10 days. Presumptive pre-DC subsets were identified within the lineage-MHC class II+ fraction of blood mononuclear cells. Pre-monocytoid DC were CD11c+ CD123- (IL-3Rα-); pre-plasmacytoid DC were characterized as CD11c- CD123++. Flt3L increased the CD11c+ pre-DC (7-fold) and CD123++ pre-DC subsets (3-fold) in blood. The freshly-isolated CD11c+ pre-DC subset induced modest proliferation of naive allogeneic T cells. After overnight culture with GM-CSF and CD40L, both subsets upregulated surface costimulatory molecules and CD11c+ pre-DC became potent allostimulators. Freshly-isolated CD123++ pre-DC showed typical plasmacytoid morphology and when cultured with IL-3 and CD40L for 72 hr, developed mature DC morphology. Following stimulation with CD40L, CD11c+ pre-DC secreted increased levels of IL-12p40. Importantly, herpes simplex virus-stimulated CD123++ pre-DC but not CD11c+pre-DC secreted interferon-α (IFN-α). Corresponding DC subsets were identified by flow analysis and immunohistochemistry in lymph nodes wherein both populations were increased 2-3 fold by Flt3L administration. CD123+ pre-DC produced IFN-α in response to in vivo viral infection. Thus, rhesus monkeys exhibit two distinct DC precursor populations that closely resemble those of humans. Both are mobilized into blood and lymphoid tissue by Flt3L, offering potential for their further characterization and possible therapeutic application.
Introduction

Dendritic cells (DC) are rare, bone marrow (BM)-derived antigen-presenting cells present in blood and almost all other tissues. They are highly-specialized for antigen uptake and processing and for both the initiation and regulation of innate and adaptive immune responses. They play important roles in the outcome of BM and solid organ transplantation. In recent years, it has become increasingly clear that DC are a heterogeneous population comprised of several subsets that share common features, but that also exhibit distinct biological properties. In humans, blood-borne precursors (pre) of tissue DC may be classified into 2 subsets, CD11c+ pre-DC (monocytoid) and CD123++ pre-DC (plasmacytoid). Monocytoid DC are classical immunosurveillant cells that endocytose and subsequently present antigen to T cells in the context of cell surface MHC molecules. In the normal steady state, immature DC that engulf and present self-antigen to T cells in the absence of adequate co-stimulatory molecule expression may play a key role in the induction/maintenance of peripheral tolerance. Mature human monocytoid DC induce mostly Th1 responses. Plasmacytoid DC, the major type-1 interferon (IFN)-producing cells of human blood, are believed to be important in anti-viral responses. Following their in vitro maturation with IL-3 and CD40L, plasmacytoid DC promote polarization of Th cells towards Th2 responses, although the capacity of these cells to induce Th1 responses has also been described.

Hematopoietic growth factors (HGF), such as fms-like tyrosine kinase 3-ligand (Flt3L) and granulocyte colony-stimulating factor (G-CSF) mobilize CD11c+ pre-DC (Flt3L) and CD123++ pre-DC subsets (Flt3L, G-CSF) in humans. Flt3L also markedly increases CD34+ stem cells in rodents and rhesus monkeys. Mobilization of DC subsets into the peripheral circulation with HGFs renders these cells accessible for harvesting and potential use as immunologic therapy.
Non-human primates (NHP) are important in the assessment of new immunologic therapies of infectious diseases\textsuperscript{16} and allograft rejection\textsuperscript{17,18} as their immune systems closely resemble that of humans. Moreover, since there are differences between murine and human DC, both in phenotype and function, extrapolation from rodent data directly to humans can be difficult. Thus there is a clear need for the identification and functional characterization of NHP DC to define their relationship to human DC subsets.

In this report, we describe properties of DC subsets in the peripheral blood and lymph nodes of both normal and Flt3L-treated rhesus monkeys. As rhesus macaques are used for the study of a wide variety of human disorders, a detailed understanding of their DC subsets has important implications for interpretation of immune responses in these models. The major finding of this study is the identification and characterization of two pre-DC populations in rhesus monkeys with similar phenotypes and function to those in humans. We demonstrate that these rhesus pre-DC may be mobilized with Flt3L, making them amenable to collection by venesection or leukapheresis and subsequent investigation both in vivo and in vitro.

Materials and Methods

Animals

SIV-negative, herpes B virus-negative rhesus macaque monkeys (\textit{Macaca mulatta}), aged between 1-5 years, were housed within the Primate Infectious Disease Research Facility of the University of Pittsburgh. All monkeys were maintained in accordance with guidelines set forth by the Institutional Animal Care and Use Committee, which included a specific environmental enrichment program.

Reagents

FITC-, PE-, Cy-Chrome- or APC- conjugated mouse anti-human monoclonal antibodies (mAbs) used to detect cell surface MHC class II (clone G46-6), CD3 (clone SP34), CD11c (clone S-HCL-3), CD14 (clone M5E2), CD16 (clone 3G8), CD20 (clone 2H7), CD34 (clone 563), CD56 (clone MY31), CD80
(clone L307.4), CD86 (clone FUN-1) and CD123 (clone 7G3) expression by flow cytometry were purchased from BD PharMingen (San Diego, CA). The human DC-specific mAbs BDCA-1, BDCA-2, BDCA-3 and BDCA-4 were purchased from Miltenyi-Biotec (Bergisch Gladbach, Germany). Anti-human CD40 (clone MAB89) IgG mAb was purchased from Coulter Immunotech (Marseille, France). Anti-human Fascin (p55) and Ki-67 were both purchased from DAKO (Glostrup, Denmark).

Recombinant (r) chinese hamster ovary cell-derived human (h) Flt3L and trimeric rhCD40L were kindly provided by the Immunex Corporation (now Amgen; Seattle, WA). Human rIL-3, rhGM-CSF and rhIL-4 were purchased from Peprotech (NJ). RPMI-1640 (Life Technologies, Rockville, MD) was supplemented with 10% w/v FCS (Nalgene, Miami, FL), non-essential amino acids, L-glutamine, sodium pyruvate, penicillin-streptomycin and β-2-mercaptoethanol (all Life Technologies) and is referred to subsequently as complete medium.

**Flow cytometric analysis and cell sorting**

Leukocytes isolated from whole blood by Ficoll gradient centrifugation were first blocked with 10% v/v normal goat serum (Invitrogen, Carlsbad, CA) for 20 min at 4°C, then stained with mAb for 30 min at 4°C. Cells were also incubated with isotype-matched Igs (BD PharMingen) as negative controls. After staining, the cells were fixed with 1% w/v paraformaldehyde and analyzed on a Coulter EPICS Elite flow cytometer (Beckman Coulter Corporation, Hialeah, FL). For cell isolation, DC subsets were flow-sorted using a high speed MoFlow® cell sorter (Cytomation, Fort Collins, CO). In some flow sorting experiments, BDCA-1 was substituted for CD11c as an alternative myeloid DC marker. Absolute DC numbers were calculated by multiplication of the percentage of either CD11c⁺ or CD123⁺⁺ cells within the lineage⁻ MHC class II⁺ fraction by the percentage of lineage⁻ MHC class II⁺ cells of the total peripheral white cell count (as measured by Coulter counter).
Mixed leukocyte reaction

Allogeneic PBMC, isolated over Ficoll-Hypaque were used as responders. Two x 10^5 responder cells were placed in each well of a 96-well, round-bottom plate, and graded concentrations of γ-irradiated (2 Gy) flow-sorted CD11c^+ pre-DC or CD123^{++} pre-DC were added as stimulators. The cultures were incubated in complete medium for 5 days in a humidified atmosphere of 5% CO_2 in air. [^3\text{H}] TdR (1µCi in 10µl) was added to each well during the final 18 hr of culture. Cells were harvested using a multiple harvester (Tomtek, Turku, Finland) and [^3\text{H}] TdR incorporation determined in a liquid scintillation counter. Results are expressed as the mean c.p.m. ±1SD from triplicate cultures.

Immunohisto- and cytochemistry

Tissue blocks were fixed in 2% v/v paraformaldehyde for 1 hr at 4°C, then infused with 30% w/v sucrose overnight. Tissues were then frozen with an aerosol of 1,1,1,2 tetrafluoroethane (Fisher Scientific, Pittsburgh, PA) and then stored at -70°C. Frozen sections (8 µm) were collected onto superfrost slides (Fisher Scientific) and incubated with mAb against human CD11c, CD123, Fascin (p55) or isotype-matched control Ig. For single label experiments, a goat anti-mouse IgG conjugated with FITC was employed. For double label experiments, a goat anti-mouse FITC conjugate was used (Alexa 488, Molecular Probes, OR). In some experiments, CD123 biotin was employed with a streptavidin conjugate (both BD PharMingen). Cytospin preparations of flow-sorted Brefeldin A treated (10µg/ml;Sigma) pre-DC subsets were stained overnight at 4°C for IFN-α with biotinylated mouse anti-human IFN-α (clone MMHA-14, PBL Biomedical Laboratories, St Louis MO) followed by cytochrome 3 (Cy3) streptavidin (Jackson Immunoresearch Lab, West Grove PA) for 30 minutes at room temperature. Nuclei were stained with DAPI (4,6 diamidino-2phenylindole, Molecular Probes, Eugene OR).
Quantitation of cytokine secretion

The capacity of freshly-isolated rhesus monkey pre-DC subsets to secrete IFN-α was assessed by culture of sorted cells with HSV-1 (Kos strain) at 5,10 or 25 p.f.u. per cell. Culture supernatants were collected after 24 hr and analyzed using a multi-species IFN-α ELISA kit (PBL). The limit of detection was 156 pg/ml. Secretion of IL-12 by rhCD40L-stimulated DC was determined using a rhesus monkey IL-12 p40 ELISA kit purchased from Biosource (Camarillo, CA). The limit of detection was 11.7 pg/ml.

Morphologic analyses

Both flow-sorted CD11c+ pre-DC and CD123++ pre-DC were examined before and after culture by light and scanning electron microscopy (SEM). For cytospins, pre-DC were spun onto glass slides using a Shandon cytocentrifuge (Shandon, Cheshire, UK) (230 xg) then stained with May-Grunwald-Giemsa. For SEM, flow-sorted DC subsets were attached to glass coverslips pretreated with 0.1% w/v poly-L-lysine. Subsequently, the cells were fixed in 2.5% v/v glutaraldehyde, dehydrated, coated with 20 nm evaporated carbon, and analyzed in a JEOL 35 SEM.

Lymph node suspensions

Lymph nodes from either normal or Flt3L-treated animals were disrupted by incubation with DNAse I (Roche, Mannheim Germany)(20µg/ml) and collagenase (Sigma, St Louis MO) (1mg/ml), followed by a 30 min incubation at room temperature. Cell suspensions were then prepared by passage through a metal sieve and centrifugation prior to washing and either freezing for storage or immediate flow cytometric analysis.

Viral infection of monkeys with Influenza A

Influenza naïve monkeys were infected by intranasal instillation of 1 ml (0.5 ml per nostril) solution containing 10^6 TCID_{50} of A/Sydney/5/97-like virus (H3N2) as described^{20}. Infection was confirmed by nasal swabs performed on days 1-7 post infection for measurement of TCID_{50} in MDCK cells^{20}. In
some monkeys, Flt3L (100 µg/kg/day) was administered for 10 days prior to viral infection and blood and lymphoid tissues collected 3 and 5 days later.

**Statistical analyses**

Differences between control and treatment groups in hemoglobin, white cell count, platelet count and percentage of lymphocytes in peripheral blood were determined using the paired Student “t” test. A p value < 0.05 was considered significant. The Mann-Whitney U test was also used to determine differences between blood DC parameters pre and post Flt3L administration.
Results

Simultaneous identification of presumptive pre-DC subsets within rhesus monkey peripheral blood by rare event flow cytometric analysis

Presumptive pre-DC were identified within the heparinized PBMC fraction of both normal and Flt3L-treated rhesus macaques using anti-human mAbs known to cross-react with this species. Cells isolated over Ficoll-Hypaque were stained with a lineage cocktail of FITC-conjugated mAbs comprising anti-CD3 (pan T cell marker), CD14 (monocytes) and CD20 (B cells). Initially the NK cell marker CD16 was included in the lineage cocktail, however, during preliminary studies, a subset of human DC expressing CD16 was described. Therefore to avoid inclusion of these DC within the lineage gate, anti-CD16 was excluded from the lineage cocktail. The expression of CD56 on the lineage- MHC class II+ PBMC fraction was assessed using anti-CD56PE (see below). Cells were stained simultaneously with anti-MHC class II CyChrome, anti-CD11c APC and anti-CD123 PE mAbs. In humans, DC subsets are found within the lineage- MHC class II+ (HLA-DR) fraction of PBMC (approximately 1-1.5% of total PBMC). In normal rhesus macaques, this population comprises approximately 1% of PBMC. Rhesus monkey cells expressing similar markers to human pre-DC subsets (presumptive rhesus CD11c+ pre-DC and CD123++ pre-DC) were detected within this population, using a gating strategy that identified cells expressing CD11c (myeloid marker typically expressed on human CD11c+ pre-DC) and CD123 (IL-3Rα, a marker typically expressed on human plasmacytoid CD123++ pre-DC) (Figure 1).
Figure 1 Legend: Four-color gating strategy for identification of presumptive pre-DC subsets in blood of rhesus monkeys. Rhesus DC subsets were identified on the basis of forward (FSC) and side scatter (SSC) (A), within the lineage’ MHC class II$^+$ fraction of total PBMC after Ficoll gradient separation (indicated by gated region in B). Isotype-matched control Igs were included as appropriate negative controls (C). Subsequently, monocytoid DC precursors (CD11c$^+$ pre-DC) were identified as CD11c$^+$ CD123$^-$ and plasmacytoid DC precursors (CD123$^{++}$ pre-DC) as CD11c$^-$ CD123$^{++}$ and comprised between 10-50% and 1-4% of the lineage’ MHC class II$^+$ fraction respectively (D).
Plasmacytoid DC precursors (CD123++ pre-DC) were identified as CD11c- CD123++ cells within the lineage MHC class II+ population (D) and comprised typically between 1-4% of these cells. A third population of lineage- MHC class II+ cells expressing neither CD11c nor CD123 was also identified (D, left lower quadrant). Results are representative of 20 individual monkeys. Thus in normal rhesus blood, presumptive CD11c+ pre-DC showing the phenotype lineage- MHC class II+ CD11c+ CD123- (IL-3Rα-) and presumptive CD123++ pre-DC that were lineage-, MHC class II+ CD11c- CD123++ could both be identified. The gating strategy and results of a typical analysis conducted in a normal rhesus monkey are shown in Figure 1. This approach offered the advantage of simultaneous identification of both subsets on the same flow cytometric dot plot, thereby facilitating direct comparison of the relative percentage of each subset within the lineage- MHC class II+ fraction. An estimation of the percentage of putative rhesus CD11c+ pre-DC and CD123++ pre-DC within this fraction was made in 11 monkeys of either sex that ranged in age from 1-5 years (see Table 1).

**Table 1. Incidence of putative pre-DC subsets in normal rhesus monkey blood determined by rare event four color flow cytometric analysis**

<table>
<thead>
<tr>
<th>Number of monkeys</th>
<th>Lineage- MHC II+ (% PBMC)</th>
<th>CD11c+ pre-DC (CD11c+ CD123-)</th>
<th>CD123++ pre-DC (CD11c- CD123++)</th>
<th>CD11c- CD123-</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>1.1±0.1</td>
<td>39.3±16.8</td>
<td>3.3±1.1</td>
<td>39.2±21.8</td>
</tr>
</tbody>
</table>

**Table 1 Legend:** PBMC were isolated from heparinized blood and stained with mAbs directed against lineage markers, MHC class II, CD11c and CD123 as described in the Materials and Methods. Gated lineage- cells were analyzed for differential expression of CD11c and CD123 as depicted in Figure 1.
The mean percentage of CD11c+ pre-DC within the lineage- MHC class II+ fraction of PBMC was 39.3 (±16.8)%], a value lower than that which we have observed in normal humans using a similar approach (69.7 ±14.8%, n=13 subjects)22. Rhesus plasmacytoid DC (CD123++ pre-DC) however, were consistently present as a smaller percentage of the lineage- MHC class II+ PBMC fraction (mean 3.3 ± 1.1%) compared with human CD123++ pre-DC (9.4±3.5 %, n=13 subjects22. Recently, a number of DC-specific mAbs have been developed for the identification of human DC subsets19. We investigated the cross reactivity between human DC-specific mAbs BDCA 1-4 and rhesus PBMC in 30 monkeys. Only BDCA-1 (a myeloid marker that identifies CD1c in humans) showed significant cross-reactivity with rhesus monkey PBMC. No significant binding of BDCA-2,3 or 4 was detected and therefore further studies with these markers were not persued.

**Flt3L administration increases presumptive CD11c+ pre-DC and CD123++ pre-DC in peripheral blood**

Rhesus monkey PBMC expressing similar markers to human DC subsets could be identified in peripheral blood, however, in order to obtain sufficient cells for functional studies by flow cytometric cell sorting, the hematopoietic growth factor (HGF) Flt3L was used to increase the number of circulating DC in the peripheral circulation. Flt3L is known to markedly increase DC in rodents and humans 12,23-25. To determine the extent to which Flt3L could mobilize DC subsets in rhesus monkeys *in vivo*, 3 animals were given Flt3L (100µg/kg/day s.c.) for 10 days. The dosage regimen was chosen based on studies performed previously in humans and NHP. Venous blood was drawn every second day to determine changes in hematologic parameters and in DC subsets. Administration of Flt3L was uncomplicated, with no adverse reactions noted. **Figure 2** shows the peripheral blood white cell count (WCC) and % lymphocytes at various times up to 14 days after the start of Flt3L administration.
Figure 2: Changes in hematologic parameters in response to Flt3L administration.

Flt3L was administered to 3 rhesus monkeys (100 µg/kg/day) for 10 days. There was no significant change in white cell count (WCC) over the 10-day course of treatment, but the percentage of lymphocytes rose significantly by day 10, returning to within the normal range 4 days after discontinuation of growth factor administration.
Whilst there were no significant changes in total WCC and platelet count between days 0 and 10, the percentage of lymphocytes in peripheral blood increased significantly with Flt3L treatment (p=0.02; paired ‘t’ test), then returned to baseline within 4 days of Flt3L withdrawal. The percentage of blood monocytes in the peripheral circulation increased from 5% to 22% (absolute monocyte counts rose by 10-fold from 0.3 to 2.8 x10^4 cells/ml) in response to Flt3L treatment. The lineage^− MHC class II^+ fraction of PBMC increased substantially from 0.8±0.1 (day 0) to 10.4±5.3% (day10) (p=0.005; Mann-Whitney U test, - see Table 2). The percentage of lineage^− MHC class II^+ cells rose by 10-fold and absolute numbers of CD11c^+ pre-DC and CD123^++ pre-DC in the peripheral circulation were markedly increased. Circulating CD11c^+ pre-DC increased from a mean value of 18.0 ± 2.7x10^3 /ml on day 0 to 127.3 ±61.0x10^3 /ml (average 7-fold increase) on day 10 of treatment with Flt3L. Circulating CD123^++ pre-DC increased from a mean value of 1.3 ± 0.2x10^3 cells per ml on day 0 to 6.4 ± 0.8 x10^3 cells per ml (average 4.7-fold increase) by day 10. The immunophenotype of Flt3L-mobilized CD11c^+ pre-DC was further investigated by flow cytometric characterization of CD40, CD80 and CD86 on flow-sorted cells. As shown in Figure 3, moderate expression of CD40 and low-moderate surface expression of CD80 and CD86 was observed, indicating phenotypic immaturity. Freshly-isolated, flow-sorted rhesus CD123^++ pre-DC also displayed an immature phenotype, as indicated by moderate levels of CD40 and low levels of CD86. Both subsets upregulated surface costimulatory molecule expression upon overnight culture with soluble CD40L (Fig. 3).
Table 2. Mobilization of rhesus peripheral blood pre-DC subsets in response to Flt3L

<table>
<thead>
<tr>
<th>Time of sample</th>
<th>Lineage^- MHC II^+ (% PBMC)</th>
<th>CD11c^+ pre-DC</th>
<th>CD123^+ pre-DC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% lineage^- MHC II^+ PBMC</td>
<td>Absolute number (x10^3/ml)</td>
<td>% lineage^- MHC II^+ PBMC</td>
</tr>
<tr>
<td>Pre Flt3L (n=3)</td>
<td>0.82±0.14</td>
<td>38.0±1.1</td>
<td>18.0±2.6</td>
</tr>
<tr>
<td>Post Flt3L (n=3)</td>
<td>10.4±5.3*</td>
<td>15.2±13.0</td>
<td>127.3±61.0</td>
</tr>
</tbody>
</table>

*p=0.005 compared to pre Flt3L (Mann Whitney U test).

Table 2 Legend: Mobilization of rhesus CD11c^+ pre-DC and CD123^+ pre-DC subsets into the peripheral circulation following 10 days of rhFlt3L administration. Data show the % lineage^- MHC II^+ PBMC, pre- and post- Flt3L. Absolute cell numbers were calculated by multiplying the % of each DC subset within the lineage^- MHC II^+ fraction by the % lineage^- MHC PBMC, multiplied by the peripheral mononuclear cell count.
Figure 3: Costimulatory molecule expression on freshly-isolated Flt3L mobilized circulating rhesus monkey pre-DC subsets. Rhesus monkey pre-DC subsets were flow-sorted prior to secondary staining for costimulatory molecule expression. Flow-sorted CD11c+ pre-DC show moderate levels of CD40, minimal CD80 and moderate CD86 expression. Flow-sorted CD123++ pre-DC also showed moderate expression of CD40 and minimal CD86, indicating phenotypic immaturity. On overnight culture with CD40L, CD11c+ pre-DC and CD123++ pre-DC upregulated cell surface expression of CD40, CD80 and CD86. Data are representative of 3 separate experiments for each subset.
Flt3L also mobilizes lineage− MHC class II+ cells that express neither CD11c nor CD123

In both normal and Flt3L-treated monkeys, cells were identified within the lineage− MHC class II+ fraction of PBMC that expressed neither CD11c nor CD123 (Figure 1D). Backgating of these double-negative cells revealed similar forward and side scatter to either pre-DC subset (data not shown). Cytospin preparations of these flow-sorted cells stained with May-Grunwald Giemsa revealed this population to be heterogeneous in morphology (data not shown). Further analysis by mAb staining and flow cytometry revealed the phenotype of these cells to be lineage−, CD40lo CD80+ and CD86+. On further immunophenotyping, 50% of these cells exhibited the NK cell marker CD56 and 10% expressed CD34+ (stem cells) (data not shown). Flt3L is known to promote growth of both of these cell types in vivo. Recently, Comeau et al described several subclasses of plasmacytoid DC in Flt3L-mobilized normal human volunteers based on the expression of CD56. The precise relationship of these cells to those described above is unclear. Upon culture with GM-CSF/IL-4 (cytokines that promote the generation of human myeloid DC) up to 40% of these lineage− MHC class II+ cells acquired surface CD11c, indicating that they could differentiate into CD11c+ pre-DC following exposure to the appropriate cytokines. Whether it may be possible to generate rhesus plasmacytoid DC from the same initial population using the cytokines described for the growth of human CD123++ pre-DC in vitro is currently under investigation.

Morphological and functional characterization of rhesus peripheral blood pre-DC subsets

Circulating CD11c+ pre-DC and CD123++ pre-DC were isolated from Flt3L-mobilized rhesus monkeys by high-speed flow sorting to >90% purity. Freshly-isolated CD11c+ pre-DC showed reniform or multilobulated nuclei, with few dendrites and veil-like cytoplasmic projections (Figure 4A and E). Freshly-isolated, flow-sorted CD11c+ pre-DC cultured in rhGM-CSF (1000 IU/ml), rhIL-4 (400 IU/ml) and rhCD40L (3µg/ml) exhibited typical DC morphology with numerous elongated cytoplasmic projections within 24 hr (Figure 4C and G). Freshly-sorted CD11c+ pre-DC modestly to
P. Toby Coates et al

stimulated naïve allogeneic T cells in MLR, but following overnight culture (18 hr) with rhCD40L, CD11c⁺ pre-DC exhibited upregulated cell surface costimulatory molecules and enhanced T cell stimulatory ability (consistent with their functional maturation) (Figure 3 and 5). To assess the capacity of CD11c⁺ pre-DC to endocytose soluble antigenic material, freshly-sorted CD11c⁺ pre-DC were incubated with FITC-albumin for 60 min at 37°C. The CD11c⁺ pre-DC showed significant fluid phase uptake (data not shown), consistent with immaturity. Taken together, these data indicated that freshly-sorted Flt3L-mobilized rhesus CD11c⁺ pre-DC were functionally immature.
Figure 4: Morphology of flow-sorted rhesus DC subsets. Rhesus pre-DC subsets were stained with Giemsa (A-D) or examined by scanning electron microscopy (SEM) (E-H) pre-and post-culture with DC maturation-inducing factors GM-CSF and CD40L. (A), freshly-sorted CD11c+ pre-DC, showing a high nucleus-cytoplasm ratio, reniform or multilobulated nuclei, and few prominent dendrites. (B) Freshly-sorted CD123++ pre-DC with typical prominent Golgi region and lateralized reniform nucleus. (C) After culture with rhGM-CSF and rhCD40L for 24 hr, CD11c+ pre-DC show abundant and well-developed dendrites. (D), after culture with rhIL-3 and CD40L for 3 days, CD123++ pre-DC also acquire striking dendritic morphology. (E-H), SEM images of cells corresponding to populations A-D (magnification x 400); E x 7500, F x 9000, G x 5000 H x3500 .
Freshly-sorted CD123++ pre-DC showed a more uniform appearance compared to CD11c+ pre-DC, with eccentric reniform nuclei, prominent Golgi region and numerous short, veil-like projections (Figure 4B and F). Following culture (72 hr) with factors known to support the differentiation of CD123++ pre-DC in humans (rhIL-3 and rhCD40L) the CD123++ pre-DC developed more striking, typical mature DC morphology, with prominent extended cytoplasmic projections visible on Giemsa staining and SEM (Figure 4D and H). Freshly-isolated CD123++ pre-DC, like their CD11c+ pre-DC counterparts, failed to stimulate naive allogeneic T cell proliferation (Figure 5). Insufficient flow-sorted CD123++ pre-DC were available to allow overnight culture and subsequent testing for acquisition of allostimulatory capacity.
Figure 5. Flow-sorted rhesus CD11c⁺ pre-DC stimulate allogeneic T cells. Five-day MLR cultures were performed with freshly-sorted rhesus CD11c⁺ pre-DC, or CD123++ pre-DC subsets or with CD11c⁺ pre-DC matured following rhCD40L stimulation. Flow-sorted DC subsets were γ-irradiated prior to culture with bulk allogeneic rhesus PBMC at various stimulator: responder ratios (x-axis). T cell proliferation was measured by thymidine incorporation (y-axis). Data are means ± 1SD and are representative of 3 separate experiments. Freshly-isolated CD11c⁺ pre-DC were modestly immunostimulatory in comparison with CD123++ pre-DC that failed to induce proliferation.

CD40 ligation induces IL-12 secretion by Flt3L-mobilized CD11c⁺ pre-DC

A characteristic feature of human DC1 is their ability to secrete the Th1-promoting cytokine IL-12. We therefore examined the capacity of freshly-isolated flow-sorted rhesus monkey CD11c⁺ pre-DC to secrete IL-12 in response to stimulation with trimeric CD40L. As shown in Figure 6A, the level
of IL-12 p40 secreted by CD11c⁺ pre-DC was increased significantly (p<0.05) compared to non-stimulated controls.
Figure 6. Rhesus DC subsets display functional characteristics of CD11c⁺ pre-DC and CD123^{+++} pre-DC. (A) Freshly-sorted rhesus CD11c⁺ pre-DC show significantly augmented secretion of IL-12 p40 after 24 hr stimulation with rhCD40L (3µg/ml) in comparison with non-stimulated CD11c⁺ pre-DC cells. Data are means ±1SD and are representative of 2 independent experiments.
Flt3L-mobilized CD123++ pre-DC secrete IFNα in response to HSV stimulation

In humans, CD123++ pre-DC are the principal IFNα-producing cells of the immune system, therefore we sought to determine whether putative rhesus CD123++ pre-DC could secrete IFNα after in vitro challenge with HSV (a virus known to stimulate IFNα production in human CD123++ pre-DC9). Freshly-isolated, flow-sorted CD11c+ pre-DC and CD123++ pre-DC were cultured with human HSV-1 (Kos strain) at 3 multiplicities of infection (5, 10 and 25 pfu/cell). After 24 hr, supernatants were collected and analyzed for IFNα by ELISA. At all three viral concentrations tested, CD123++ pre-DC showed significant secretion of IFNα (Figure 6B), whereas no IFNα was detected in unstimulated cultures or in cultures of CD11c+ pre-DC stimulated with HSV (Figure 6B). These findings are similar to published data concerning the differential effect of HSV on IFNα production by human DC subsets9 and confirm the identity of the in vivo-mobilized rhesus DC subsets.
Figure 6 (B). Production of IFNα by flow-sorted rhesus CD123++ pre-DC but not CD11c+ pre-DC after stimulation with herpes simplex virus (HSV) (Kos strain). Freshly isolated pre-DC were stimulated with 10 pfu/cell for 24 hr in complete medium. In contrast to freshly-sorted, non-infected CD123++ pre-DC, or HSV-stimulated CD11c+ pre-DC exposed to an equivalent viral concentration, only the HSV-activated CD123++ pre-DC secreted detectable IFN-α (see also Figure 8B insert, which demonstrates INF-α production by intracellular cytokine staining under the same conditions). Data are representative of 3 separate experiments.
Identification of CD11c+ pre-DC and CD123++ pre-DC in lymphoid tissue of normal and Flt3L-mobilized rhesus monkeys

As DC comprise a significant cellular population within lymphoid tissues, inguinal lymph nodes (LN) were obtained from 3 rhesus monkeys before and after a 10-day course of Flt3L. Flow cytometric analysis of freshly-isolated lymph node cell suspensions performed using the same 4-color protocol employed to identify peripheral blood pDC subsets, revealed a mean CD11c+ pre-DC prevalence of 2.6% (±0.4%) of lineage− MHC class II+ cells. CD123++ pre-DC comprised 4.1% (±0.6%) of lineage− MHC class II+ cells, indicating a relative abundance of CD123++ pre-DC compared to CD11c+ pre-DC in normal LN. This contrasted with the relative proportions of these cells in peripheral blood. Direct immunofluorescence staining of lymph node sections confirmed both CD11c+ and CD123++ cells within normal tissue. To confirm that lymph node cells expressing CD123 were DC, dual labeling with anti-human Fascin (that identifies actin within dendrites) was performed. In normal lymph nodes (n=3), CD11c+ cells with dendritic morphology were identified within both follicular (F) and parafollicular (P) areas (Figure 7A). Within the parafollicular region of normal lymph nodes (n=3), isolated double positive cells (p55+CD123+) were observed within the T cell areas. Figure 7B demonstrates p55+ DC co-staining with CD123 (orange; white arrow) in normal rhesus lymph nodes.
Figure 7. Localization of rhesus DC subsets within normal and Flt3L-treated rhesus monkey lymph nodes. (A) Normal lymph node: germinal center CD11c+ myeloid DC (green) are visible as CD11c+ cells with dendritic morphology within follicular (F) and parafollicular regions (P) (original magnification x200). (B)p55 (fascin) -stained DC (green) co-localizing with CD123-biotin (red) to indicate pre-plasmacytoid DC within normal lymph node (orange/yellow; -insert magnification x600). (C) Cells expressing CD123 in Flt3L-mobilized rhesus monkeys tended to occur in aggregates (red), whereas CD11c+ cells (green) were visible as isolated cells (original magnification x100). (D) Ki 67 staining of lymph node from an Flt3L-treated rhesus monkey, showing CD123+ cells (red) in clumps distinct from Ki-67 positive cells (green), predominantly within the follicle (F) (x200).
Flt3L administration increased both CD11c⁺ pre-DC and CD123⁺⁺ pre-DC subsets in lymphoid tissue. Lymph node suspensions from Flt3L-treated animals (n=3) were analyzed by flow cytometry and revealed an increase in CD11c⁺ pre-DC from 2.6 to 7.5% of lineage⁻ MHC class II⁺ cells. The CD123⁺⁺ pre-DC subset increased from 4.1 to 11.3%. Freshly-sorted, Flt3L-mobilized CD123⁺⁺ LN cells showed moderate CD40 expression and low CD86 expression (data not shown). In tissue sections of Flt3L-treated animals, CD11c⁺ DC were usually identified as distinct cells, whereas CD123⁺ cells were typically found in clusters (Figure 7C), raising the question of whether these cells had migrated to the lymph node (presumably from the blood) or proliferated from precursors in situ in response to Flt3L. To address this question, LN from Flt3L-treated monkeys were co-stained with the cell cycle proliferation antigen Ki-67 and for CD123. Clusters of CD123⁺ cells in Flt3L-treated lymph nodes did not stain with Ki-67, indicating that these cells were not in active replication, and consistent with the hypothesis that they had migrated to the lymph node rather than arisen from precursors in situ (see Figure 7D).

**Response of rhesus monkey pre-DC subsets to in vivo viral infection**

Circulating levels of pre-DC subsets were determined during the acute viremic phase of infection of 3 normal monkeys with an influenza-A virus. Analysis of CD11c⁺ pre-DC and CD123⁺⁺ pre-DC was performed before and 72 hr after challenge. Circulating CD11c⁺ pre-DC levels rose from 11.3% of lineage⁻ MHC class II⁺ cells before challenge to 38% (p=0.02; paired ‘t’ test) at 72 hr. The incidence of blood CD123⁺⁺ pre-DC remained similar to pre-infection values *(Table 3).* The influence of influenza viral infection on lymph node DC populations was also examined at the same time points. No significant change in the incidence of either subset was observed *(Table 3).*
Table 3  Analysis of pre-DC subsets in response to in vivo viral infection

<table>
<thead>
<tr>
<th>Subset</th>
<th>Blood</th>
<th>Lymph Node</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre infection</td>
<td>Post infection</td>
</tr>
<tr>
<td>CD11c + pre DC*</td>
<td>11.3±4.1</td>
<td>38.0± 4.5**</td>
</tr>
<tr>
<td>CD123 ++ pre DC</td>
<td>1.4±0.5</td>
<td>0.9±0.1</td>
</tr>
</tbody>
</table>

Table 3 Legend: CD11c + and CD123 ++ pre-DC subsets were analyzed by 4-color flow in blood and lymphoid tissue 72 hours after intra-nasal instillation of influenza-A virus in 3 naïve rhesus monkeys (not Flt3L-mobilized). * % lineage - MHC class II + PBMC or lymph node cells ** p=0.02 compared to pre-infection value (paired ‘t’ test).

To further assess the influence of acute viral infection on pre-DC subsets, two rhesus monkeys were treated with Flt3L for 10 days prior to infection with influenza virus. Peripheral blood DC and lymph node cells were flow-sorted isolated 3-5 days later and immunostained for intracellular IFN-α. As shown in Figure 8 (D), IFN-α production was clearly evident within CD123 + pre-DC but not CD11c + pre-DC isolated from lymph nodes of virally-infected animals. IFN-α + cells were not detected within circulating pre-DC subsets.
Figure 8: Identification of IFN-α production by intracellular staining of flow-sorted lymph node CD123+ pre-DC following in vivo infection with influenza-A virus. Staining of cytospin preparations of Flt3L-mobilized blood CD11c+ pre-DC (panel A) and CD123++ pre-DC (panel B) subsets flow-sorted from peripheral blood of rhesus monkeys 5 days after in vivo infection with influenza-A virus shows no evidence of IFN-α production. (Panel B insert shows IFN-α production (cytoplasmic red dots) in CD123++ pre-DC mobilized by Flt3L and infected in vitro with HSV type-1(Kos strain;10 pfu/cell) as positive control for IFN-α production by DC). By contrast to peripheral blood, intracellular IFN-α could be readily detected (arrows) in lymph node CD123+ pre-DC (Panel D) but not CD11c+ pre-DC (panel C) on day 3 post influenza infection. IFN-α was detected by immunofluorescence staining with a biotinylated mouse anti-human IFN-α followed by streptavidin Cy3 conjugate (all figures x1000).
Discussion

Comparative/cross-species studies of DC subsets are important in our understanding of DC immunobiology and their potential for clinical application. NHP exhibit substantial immunological similarity to humans. To date, much of human DC biology has been uncovered by studying in vitro propagated, blood monocyte-derived DC, generated using GM-CSF and IL-4. Using similar protocols, it is possible to generate monocyte-derived DC from NHP, specifically chimpanzees, cynomologous monkeys (Macaca fascicularis) and rhesus macaques (Macaca mulatta). Whether in nature, such GM-CSF/IL-4-dependent pathways of DC generation exist has not been established. Thus, characterization of NHP DC subsets and the ability to manipulate their production in vivo are important steps toward the therapeutic application of DC in clinically relevant models.

In this report, we describe for the first time, rhesus monkey phenotypic and functional equivalents of human CD11c+ pre-DC (monocytoid) and CD123++ pre-DC (plasmacytoid) subsets. These were identified within the lineage- MHC class II+ fraction of PBMC and typically represented <1% of PBMC. Subsets were identified using cross-reactive anti-human mAbs, that recognized cell surface antigens known to be expressed by human DC. In humans, DC subsets are found within the lineage- (CD3-CD14-CD19-CD56-) HLA-DR+ fraction of PBMC (typically <2% PBMC), that appears to be slightly larger proportion than that in normal rhesus monkeys. Using mAbs against the myeloid marker CD11c and the IL-3R α chain (CD123), the CD11c+ pre-DC and CD123++ pre-DC fractions were identified by differential expression of these markers on cells within the lineage- MHC
class II\(^+\) fraction. Rhesus monkey CD11c\(^+\) pre-DC (lineage\(^-\) MHC class II\(^+\) CD11c\(^+\) CD123\(^-\)) represented 11-50\% of the lineage\(^-\) MHC class II\(^+\) PBMC, lower than in their human counterparts (69.7±14.8\%, A.F. Zahorchak, personal communication). Circulating rhesus CD11c\(^+\) DC have also been described, but not CD123\(^++\) DC\(^\text{32}\). In humans, approximately 36,000 CD11c\(^+\) pre-DC/ml have been reported\(^\text{14}\), whereas in rhesus monkeys, we identified about 18,000 CD11c\(^+\) pre-DC/ml. The difference in numbers between the species is more marked with respect to CD123\(^++\) pre-DC (lineage\(^-\), MHC class II\(^+\), CD11c\(^-\), CD123\(^++\)). In humans, there are approximately 28,000 CD123\(^++\) pre-DC/ml\(^\text{14}\), whereas in the rhesus monkey, we identified approximately 1,300 per ml. The reason(s) for this apparent difference in absolute numbers of both pDC subsets between humans and rhesus monkeys is not clear. A factor that may account for the numeric differences between the two species is the environment in which they are raised. Humans encounter a vast array of bacterial and viral pathogens throughout their lives whereas captive rhesus monkeys, are (as in this study) significantly younger (1-5 years old) and are raised under relatively pathogen-free conditions. It is possible that such environmental factors may account for these differences between human and NHP.

In order to study these rare APC further, Flt3L was administered (100\(\mu\)g/kg/day s.c.) to rhesus monkeys for 10 days. The animals showed a similar response to Flt3L administration to that reported in humans. Thus, whereas in normal human volunteers, Flt3L (75\(\mu\)g/kg/day) given for 14 days increased the lineage\(^-\) MHC class II\(^+\) fraction of PBMC from 1.8 to 14\%, the same fraction of PBMC increased from <1\% to 11\% in the rhesus. Pre-CD123\(^++\) DC in human blood were identified by Maraskovsky et al\(^\text{12}\) as lineage\(^-\) CD11c\(^-\) HLA-DR\(^+\) and expressed high levels of CD123. During HIV infection, a persistent decline in CD123\(^++\) pre-DC has been shown to correlate with viral load\(^\text{33}\), a response resistant to effective anti-retroviral therapy\(^\text{34}\). Circulating CD123\(^++\) pre-DC are increased in humans in response to Flt3L (3-fold increase)\(^\text{12}\), an effect similar to that observed in rhesus monkeys in the present study (4.7-fold over baseline values).
Freshly-isolated, flow-sorted, Flt3L-mobilized rhesus CD11c+ pre-DC showed typical immature DC morphology and stimulate allogeneic T cells modestly in primary MLR. As in human studies, these freshly-isolated rhesus CD11c+ pre-DC readily captured soluble antigen (FITC-albumin) by macropinocytosis and endocytosis. After exposure to CD40L however, they developed mature DC morphology, secreted significant amounts of the Th1 cell-driving cytokine IL-12p40, upregulated CD40, CD80 and CD86, and became potent stimulators in primary MLR. Taken together, these features suggest homology to human circulating CD11c+ pre-DC.

Freshly-isolated, Flt3L-mobilized lineage- MHC class II+ CD11c- CD123++ rhesus PBMC (presumptive CD123++ pre-DC) failed to stimulate allogeneic mononuclear cells. The defining functional characteristic of CD123++ pre-DC (pre-plasmacytoid DC) in both humans and mice, is their response to viral stimulation as the principal IFNα-producing cells. This response of CD123++ pre-DC to viral stimulation is determined by surface expression of Toll-like receptors (TLR) that are expressed differentially by DC subsets allowing plasticity of the cellular immune response to a variety of environmental pathogens. Human CD123++ pre-DC express TLR 7 and 9, in contrast to monocytoid DC that express TLR 1,2 and 3. When human CD123++ pre-DC are stimulated with HSV in vitro they secrete type 1-IFNs, including IFNα. In the present study, freshly-isolated rhesus CD123++ pre-DC, but not CD11c+ pre-DC stimulated with HSV-1, secreted high levels of IFNα, consistent with the known effects of the virus on human CD123++ pre-DC. These data suggest that like humans, rhesus monkeys possess a similar population of IFNα-secreting cells that may be identified by the expression of cell surface CD123. Here we show for the first time in primates, that the CD123+ population produces IFN-α in response to in vivo viral infection. Failure to detect IFN-α production by the corresponding circulating CD123++ pre-DC population may reflect the migration of these cells to inflamed lymph nodes where they produce IFN-α.
A third population of lineage- MHC class II+ PBMC was mobilized in Flt3L-treated rhesus monkeys. A significant proportion were NK cells (up to 50%) expressing CD56, whereas approximately 10% were stem cells (CD34), consistent with the known mobilizing effects of Flt3L on mobilization of both of these cell populations, in mice and humans. Previous studies of normal human blood have identified two DC subsets within the lineage- MHC class II+ fraction based on CD33 (myeloid marker) and CD123 expression, but not this third population. The most likely explanations for the absence of these cells in previous human studies is the inclusion, in these studies, of CD56 in the lineage cocktail and also (in the present study) the use of Flt3L as a DC-mobilizing agent. When rhesus lineage- MHC class II+ cells were cultured in GM-CSF and IL-4 for 48 hr, approximately 40% acquired the myeloid marker CD11c (data not shown). This suggests that this heterogeneous cell population contains functional progenitors of CD11c+ pre-DC. Conceivably, this population of lineage- MHC class II+ PBMC in Flt3L-mobilized animals represents cells in the process of acquiring their defining CD123/CD11c phenotype. The presence of a high number of CD56-expressing cells within this third cell population in the rhesus is of interest in light of recent work by Comeau et al, who have identified a precursor of human plasmacytoid DC, that expresses CD56 and CD7. The precise relationship between this third population of cells and CD11c+ pre-DC and CD123++ pre-DC in rhesus monkeys is under investigation in our laboratory. Significantly, the presence of putative precursors of either CD11c+ pre-DC or CD123++ pre-DC in the blood of Flt3L-mobilized animals may allow their collection and in vitro differentiation into the DC subset of choice, by exposure to the appropriate cytokine(s). This strategy may increase the yield of cells available for potential therapeutic application.

Previous studies of DC in rhesus lymph nodes have identified these cells on the basis of staining with anti-human fascin (p55), a DC-specific antibody that recognizes a 55 kDa actin-bundling protein within dendrites. Mature lymph node DC may also be identified by their co-expression of CD83. These cells decrease in rhesus monkeys infected with SIV. To date there have been no other descriptions of DC subsets in rhesus lymphoid tissue. Using mAbs directed against CD123, p55 and
CD11c, we identified 3 types of DC in normal monkey lymph nodes: germinal center DC, p55\(^+\)CD11c\(^+\)CD123\(^-\), and two types of interdigitating DC: p55\(^+\), CD11c\(^+\), CD123\(^-\) and p55\(^+\), CD11c\(^-\), CD123\(^+\).

In humans, up to five different DC subsets can be identified in secondary lymphoid tissue. In rhesus monkeys, germinal center DC were identified as rare, CD11c\(^+\) cells with dendritic morphology within the lymphoid follicle. By contrast, the interdigitating CD11c\(^+\) DC were more abundant and dispersed throughout the node, exhibiting characteristic dendritic morphology. In normal animals, another interdigitating DC population (CD11c\(^-\) and CD123\(^+\)) was found that was increased by Flt3L administration. Flt3L increased CD11c\(^+\) DC as discrete cells, in contrast to its effect on CD123\(^+\) DC, that aggregated in groups within lymphoid tissue. These CD123\(^+\) cells did not express Ki-67 (a nuclear antigen expressed by dividing cells but not resting cells) suggesting that they had migrated to the LN from blood, rather than proliferated in situ from a resident precursor population.

In conclusion, we have identified rhesus monkey phenotypic and functional equivalents of human CD11c\(^+\) pre-DC and CD123\(^++\) pre-DC in vivo. Both rhesus DC subsets can be mobilized in blood and lymphoid tissue by rhFlt3L. Identification of these important APC and their mobilization by HGF will allow their evaluation in rhesus monkey models of human diseases. The kinetic differences between human and rhesus DC subsets has implications for the interpretation of immunological responses where the outcome may depend on the role of DC in promoting tolerance or immunity.
Acknowledgments  We thank Mr. Alan F Zahorchak, Dr Laurent Galibert and Dr Pia Björck for helpful discussion, Dr Robert Hendricks for herpes-simplex virus, and the Immunex Corporation (now Amgen) for Flt3L.
References


P. Toby Coates et al


38. Brasel K, McKenna, HJ, Charrier, K, Morrissey, PJ, Williams, DE, Lyman, SD. Flt3 ligand synergizes with granulocyte-macrophage colony-stimulating factor or granulocyte colony-stimulating factor to mobilize hematopoietic progenitor cells into the peripheral blood of mice. Blood. 1997;90:3781-3788


Dendritic cell subsets in blood and lymphoid tissue of rhesus monkeys and their mobilization with Flt3 ligand

Patrick T Coates, Simon M Barratt-Boyes, Linyou Zhang, Vera S Donnenberg, Peta J O’Connell, Alison J Logar, F J Duncan, Michael Murphey-Corb, Albert D Donnenberg, Adrian E Morelli, Charles Maliszewski and Angus W Thomson

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include digital object identifier (DOIs) and date of initial publication.