Parallel Loss of Myeloid and Plasmacytoid Dendritic Cells from Blood and Lymphoid Tissue in Simian AIDS[1]

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The loss of myeloid (mDC) and plasmacytoid dendritic cells (pDC) from the blood of HIV-infected individuals is associated with progressive disease. It has been proposed that DC loss is due to increased recruitment to lymph nodes, although this has not been directly tested. Similarly as in HIV-infected humans, we found that lineage-negative (Lin^-) $HLA\text{-}DR^+CD11c^+CD123^-$ mDC and $Lin^-HLA\text{-}DR^+CD11c^-CD123^+$ pDC were lost from the blood of SIV-infected rhesus macaques with AIDS. In the peripheral lymph nodes of SIV-naive monkeys the majority of mDC were mature cells derived from skin that expressed high levels of HLA-DR, CD83, costimulatory molecules, and the Langerhans cell marker CD1a, whereas pDC expressed low levels of HLA-DR and CD40 and lacked costimulatory molecules, similar to pDC in blood. Surprisingly, both DC subsets were depleted from peripheral and mesenteric lymph nodes and spleens in monkeys with AIDS, although the activation status of the remaining DC subsets was similar to that of DC in health. In peripheral and mesenteric lymph nodes from animals with AIDS there was an accumulation of $Lin^-HLA\text{-}DR^{moderate}CD11c^-CD123^-$ cells that resembled monocytoid cells but failed to acquire a DC phenotype upon culture, suggesting they were not DC precursors. mDC and pDC from the lymphoid tissues of monkeys with AIDS were prone to spontaneous death in culture, indicating that apoptosis may be a mechanism for their loss in disease. These findings demonstrate that DC are lost from rather than recruited to lymphoid tissue in advanced SIV infection, suggesting that systemic DC depletion plays a direct role in the pathophysiology of AIDS. ***The Journal of Immunology,*** **2007, 178: 6958–6967.**

Dendritic cells (DC)[3] are a heterogeneous population of APC essential in bridging the innate and adaptive immune responses (1). The two major DC subsets in humans are $CD11c^+$ conventional or myeloid DC (mDC) and $CD123^+$ plasmacytoid DC (pDC) (2, 3). Although mDC and pDC circulate as immature cells in blood, a major stage of their life cycle takes place in lymphoid organs. mDC populate peripheral tissues as immature cells and migrate to lymph nodes through afferent lymphatics where they constitute the only mature DC population and stimulate Ag-specific T cells (1, 4, 5). In contrast, pDC migrate directly from blood to lymph nodes across high endothelial venules, residing in significant numbers and exhibiting increased recruitment to inflamed lymph nodes where they produce type I IFN and contribute to virus-specific T cell responses (6–9).

Numerous studies have demonstrated that both mDC and pDC are decreased in blood during HIV infection (10–15), with the decline being inversely correlated with viral load and associated with reduced $CD4^+$ T cell numbers (12, 15). Recently, pDC were shown to undergo phenotypic and functional activation following exposure to HIV-1 in vitro, inducing the bystander maturation of mDC (16). It has been hypothesized that HIV-induced maturation of both DC subsets may lead to increased DC migration to lymph nodes, partly explaining their disappearance from blood as infection progresses (13, 16). There is a lack of data regarding the changes in pDC number or phenotype within lymphoid tissue during HIV infection and few studies defining the alteration of mDC. In situ analysis of peripheral lymph node sections indicated that an accumulation of DC-$SIGN^+$ and $CD40^+$ DC occurred during acute HIV infection (17). However, in patients with chronic HIV infection the proportion of DC in the spleen was the same as that in uninfected controls, suggesting DC may not be recruited to lymphoid tissues in this later phase of infection (18). The current lack of a comparative analysis of mDC and pDC subsets in blood and lymphoid tissues leaves the question regarding the ultimate fate of DC during HIV infection unanswered.

Experimental infection of rhesus macaques with pathogenic SIV provides a valuable model to study the relationship between circulating and lymphoid-resident DC, with typical mDC and pDC identified in healthy monkeys (19–21). Although reports on the effects of SIV infection on circulating DC subsets have been conflicting (19, 20), there is evidence to suggest that SIV infection of macaques alters the status of DC in lymph nodes (22, 23). In the present study we defined mDC and pDC in lymphoid tissues and asked whether DC recruitment to lymphoid tissues during advanced SIV infection explains the loss of circulating DC in blood.

Materials and Methods

Animals and viral infection

Adult Indian rhesus macaques (*Macaca mulatta*) of both sexes were used. Seven animals were infected intrarectally with a characterized stock of the SIV/DeltaB670 primary isolate (24) and allowed to progress to AIDS as defined by $CD4^+$ T cell loss, increasing virus loads, lymphadenopathy, >20% weight loss, and/or opportunistic infections. Average time post-SIV

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[3] Abbreviations used in this paper: DC, dendritic cell; mDC, myeloid DC; pDC, plasmacytoid DC; Lin, lineage; mod, moderate; rh, recombinant human.

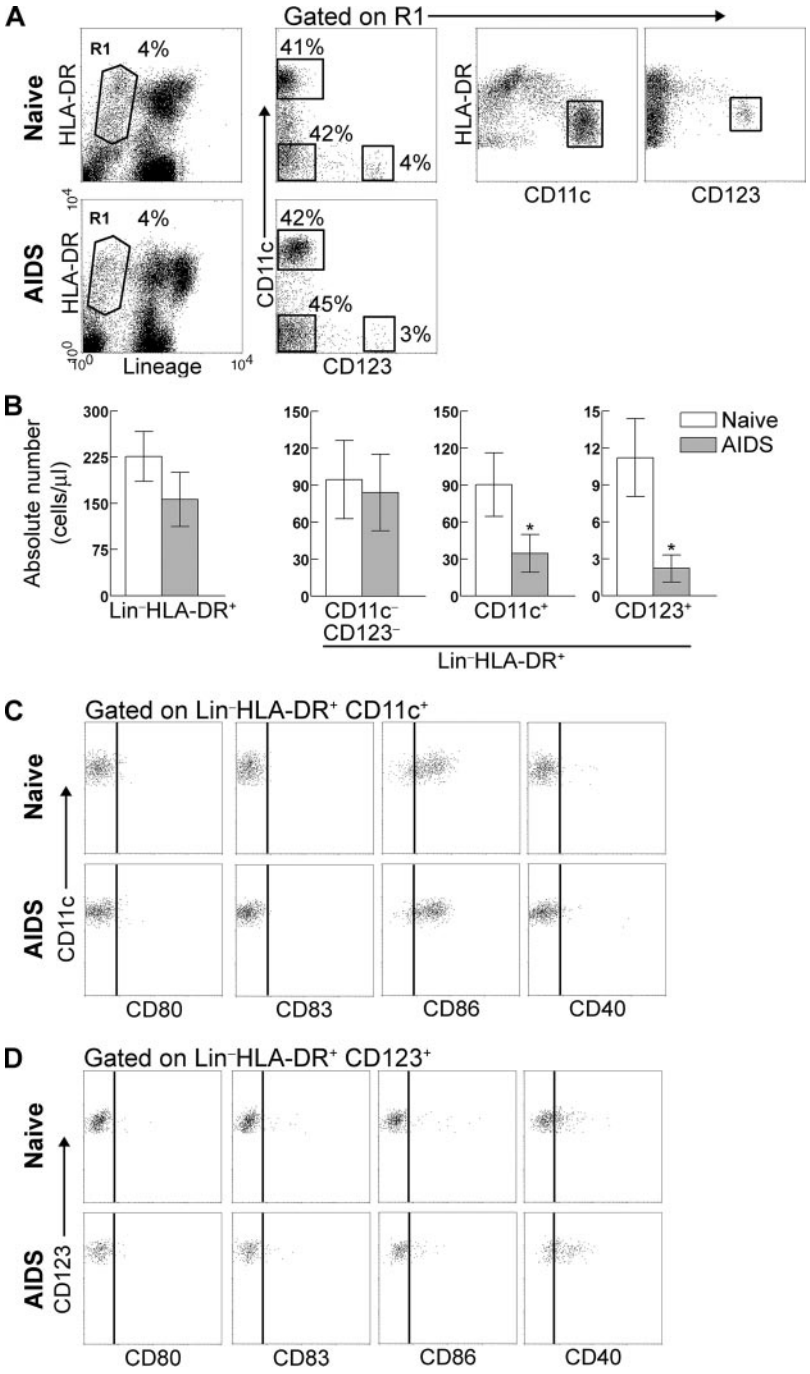

FIGURE 1. mDC and pDC are decreased in blood during simian AIDS. *A*, PBMC were gated based on forward/side scatter characteristics and DC were identified within the lineage (CD3, CD14, and CD20)-negative (Lin^-) $HLA\text{-}DR^+$ fraction (R1) from SIV-naive animals and monkeys with AIDS. One representative experiment of eight is shown to demonstrate the gating strategy used to identify DC subsets. Numbers represent the percentage of cells within R1. *B*, Absolute numbers of cells per microliter of blood for cell populations within the $Lin^-HLA\text{-}DR^+$ fraction (R1) are shown. Data represent the mean ± SEM for naive monkeys ($n = 6$) and animals with AIDS ($n = 4$). *, $p < 0.02$. *C* and *D*, Immunophenotype of $CD11c^+$ mDC (*C*) and $CD123^+$ pDC (*D*) in SIV-naive animals and macaques with AIDS. One representative experiment of four is shown for both groups. Vertical bars denote the level of isotype control Ab staining.

infection was 50 wk and the average time to AIDS following SIV/DeltaB670 infection was 11 mo (25). Eight animals were used as SIV-naive controls. All experiments were performed using protocols approved by institutional regulatory committees.

Cell isolation and flow cytometry

PBMC were isolated from heparinized blood by Ficoll gradient centrifugation. Single-cell suspensions of axillary and mesenteric lymph nodes and spleens were generated as described (26). For flow cytometric analysis, mAb were purchased from BD Pharmingen or BD Biosciences Immunocytometry Systems unless otherwise noted. Fresh or frozen PBMC, spleens, and lymph node cell suspensions were blocked with FcR blocking reagent (Miltenyi Biotec) for 15 min at 4°C before staining, except when analyzing CD16 expression. Simultaneous identification of mDC and pDC in PBMC and spleens was performed using a lineage (Lin) mixture of FITC-conjugated mAb against CD3 (clone SP-34), CD14 (clone M5E2) and CD20 (clone 2H7) with HLA-DR-CyChrome (clone G46-6), CD123-PE (clone 7G3), and CD11c-allophycocyanin (clone S-HCL-3). Expression of costimulatory molecules and other receptors on $CD11c^+$ mDC in PBMC and spleens was performed by staining with Lin-FITC, HLA-DR-CyChrome, CD11c-allophycocyanin, and PE-conjugated CD80 (clone L307.4), CD86 (clone FUN-1), CD83 (clone HB15a; Coulter-Immunotech), CD40 (clone EA-5; Ancell), and CD1a (clone SK9). The immunophenotype of $CD123^+$ pDC in PBMC and spleens was determined using Lin-allophycocyanin, HLA-DR-CyChrome, CD123-PE, and FITC-conjugated CD80, CD83, CD86, and CD40. Three-color analysis of axillary and mesenteric lymph node single-cell suspensions was performed using Lin-FITC and HLA-DR-CyChrome in addition to PE-conjugated CD11c, CD123, CD14 (clone MΦP9), CD20 (clone L27), CD80, CD83, CD86, CD40, or CD1a. Analysis of purified cells was performed using the previously mentioned mAbs in addition to CD11b (clone D12), p55 (clone 55K-2; DakoCytomation), CD16 (clone 3G8), and CD45 (clone D058-1283). Multicolor flow cytometric analysis of DC activation was performed using Lin-PE-Cy7, HLA-DR-PerCP, CD123-PE, CD11c-allophycocyanin, FITC-conjugated costimulatory markers, and CD20-Pacific Blue (clone 2H7; eBioscience) plus a violet fluorescent LIVE/DEAD fixable dead cell

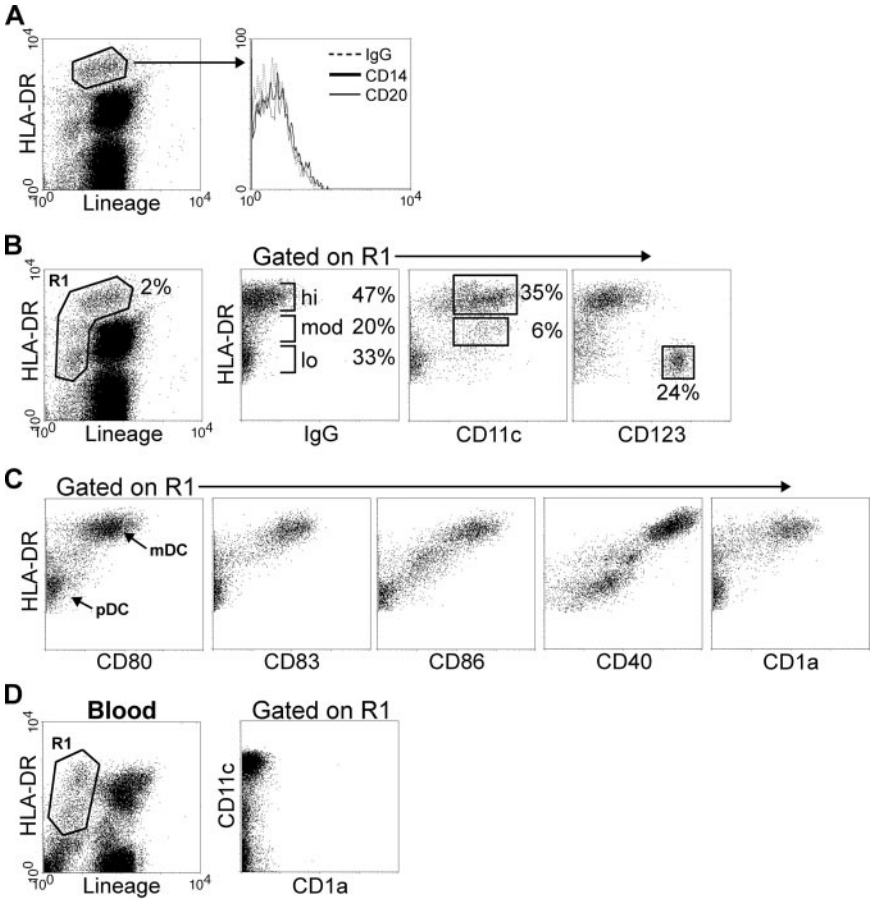

FIGURE 2. pDC and epidermal-derived mature mDC differentially express HLA-DR in superficial lymph node from normal monkeys. *A–C*, Axillary lymph node cell suspensions from SIV-naive monkeys were analyzed by flow cytometry. Total lymph node cells were gated based on forward/side scatter characteristics and DC were identified within the Lin$^-$HLA-DR$^+$ fraction (R1). *A*, Staining of cells expressing high levels of HLA-DR with isotype control Ab or Ab to CD14 and CD20. *B*, Representative dot plot demonstrating the gating strategy used to define the Lin$^-$HLA-DR$^+$ fraction (R1) of lymph node and the three populations of HLA-DR-expressing cells within R1. Numbers represent the percentage of cells within R1 indicated by gates or brackets. *C*, Expression of maturation and costimulatory molecules and CD1a by Lin$^-$HLA-DR$^+$ cells. *D*, Lack of CD1a expression by the Lin$^-$HLA-DR$^+$ fraction of PBMC. Data shown are representative of four (*A*, *C*, and *D*) and 16 (*B*) experiments.

stain (Invitrogen Life Technologies). A viability assay of cultured lymph node cell suspensions was performed in a similar manner using Lin-PE-Cy7, HLA-DR-PerCP, CD123-PE, CD11c-allophycocyanin, and the LIVE/DEAD stain. Isotype-matched control mAbs were included in all experiments. After staining, cells were fixed with 2% paraformaldehyde and 500,000 to one million events acquired using a FACSCalibur or BD LSRII flow cytometer and analyzed with CellQuest Pro (BD Biosciences Immunocytometry Systems) or BD FACSDiva software (BD Bioscience), respectively. Absolute DC numbers in blood were calculated by multiplying the percentage of Lin$^-$HLA-DR$^+$CD11c$^+$ or Lin$^-$HLA-DR$^+$CD123$^+$ cells in PBMC by the number of mononuclear cells per microliter of blood as determined by complete blood cell counts. Determination of absolute DC numbers in lymph node was made by multiplying the percentage of specific cell populations by the total lymph node cell count per gram of tissue.

Immunofluorescence and light microscopy

For immunofluorescence, lymph nodes were prepared as described (27) and sections were incubated with 5 μg/ml purified mouse anti-human CD83, CD123, CD1a, or isotype-matched control Abs for 2 h followed by goat anti-mouse IgG conjugated to Alexa 488 (Molecular Probes) for 2 h. Nuclei were labeled with Hoechst 33342 (Sigma-Aldrich) and slides were imaged using an Olympus Provis epifluorescence microscope. For light microscopy, purified Lin$^-$HLA-DR$^+$CD123$^-$ cells were spun onto glass slides using a Shandon cytocentrifuge and stained with the Hema 3 stain set (Fisher Scientific).

Cell purification and culture

Purification of Lin$^-$HLA-DR$^+$CD123$^-$ lymph node cells was performed by depletion of CD3$^+$, CD14$^+$, and CD20$^+$ cells using magnetic microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The depleted fraction was then labeled with anti-CD123-PE followed by anti-PE microbeads to remove CD123$^+$ pDC. The purity of the remaining cell population assessed by flow cytometry was >90%. Purified cells were cultured at 1×10^6/ml in medium alone or with 1000 U/ml recombinant human (rh) GM-CSF and 1000 U/ml rhIL-4 (both from Schering-Plough), 20 ng/ml rhIL-3 (R&D Systems), or 1.0 μg/ml soluble human trimeric CD40L (Immunex) for 24 h. For cell viability experiments, single-cell suspensions of peripheral lymph node or spleen were depleted of CD20$^+$ cells using magnetic microbeads (Miltenyi Biotec) according to the manufacturer's instructions and cultured at 1×10^6/ml for 24 h in medium alone or with 20 ng/ml rhIL-3.

Statistical analysis

Differences between SIV-naive monkeys and animals with AIDS were determined by comparison of means using the nonparametric Mann-Whitney *U* test. Two-tailed p values <0.05 were considered significant at an alpha level of 0.05. Differences between DC viability before and after culture were determined by comparison of means using the paired t test. One-tailed p values <0.05 were considered significant. All data are presented as the mean $\pm$ SEM.

Results

Blood mDC and pDC are lost in monkeys with AIDS

To clarify whether DC are reduced in the blood of monkeys with AIDS, the two major DC subsets were identified in PBMC as Lin$^-$HLA-DR$^+$CD11c$^+$CD123$^-$ mDC or Lin$^-$HLA-DR$^+$CD11c$^-$CD123$^+$ pDC (Fig. 1*A*). Previous studies of macaque DC included CD11b and CD16 as lineage markers to stain NK cells (20, 28). However NK cells are found within the Lin$^-$HLA-DR$^-$ fraction of PBMC from rhesus macaques (29), thus eliminating the need to include NK cell markers for exclusion from the Lin$^-$HLA-DR$^+$ fraction of PBMC. In addition, preliminary experiments demonstrated that mDC expressed CD16 as well as low levels of CD11b (data not shown), consistent with the findings of others (19). Hence, we did not include mAb to CD16 and CD11b in our analyses. In healthy monkeys and animals with AIDS, mDC predominated in the Lin$^-$HLA-DR$^+$ fraction whereas pDC comprised only a minor proportion of cells (Fig. 1*A*), consistent with previous findings (20, 21). In addition, mDC and pDC expressed

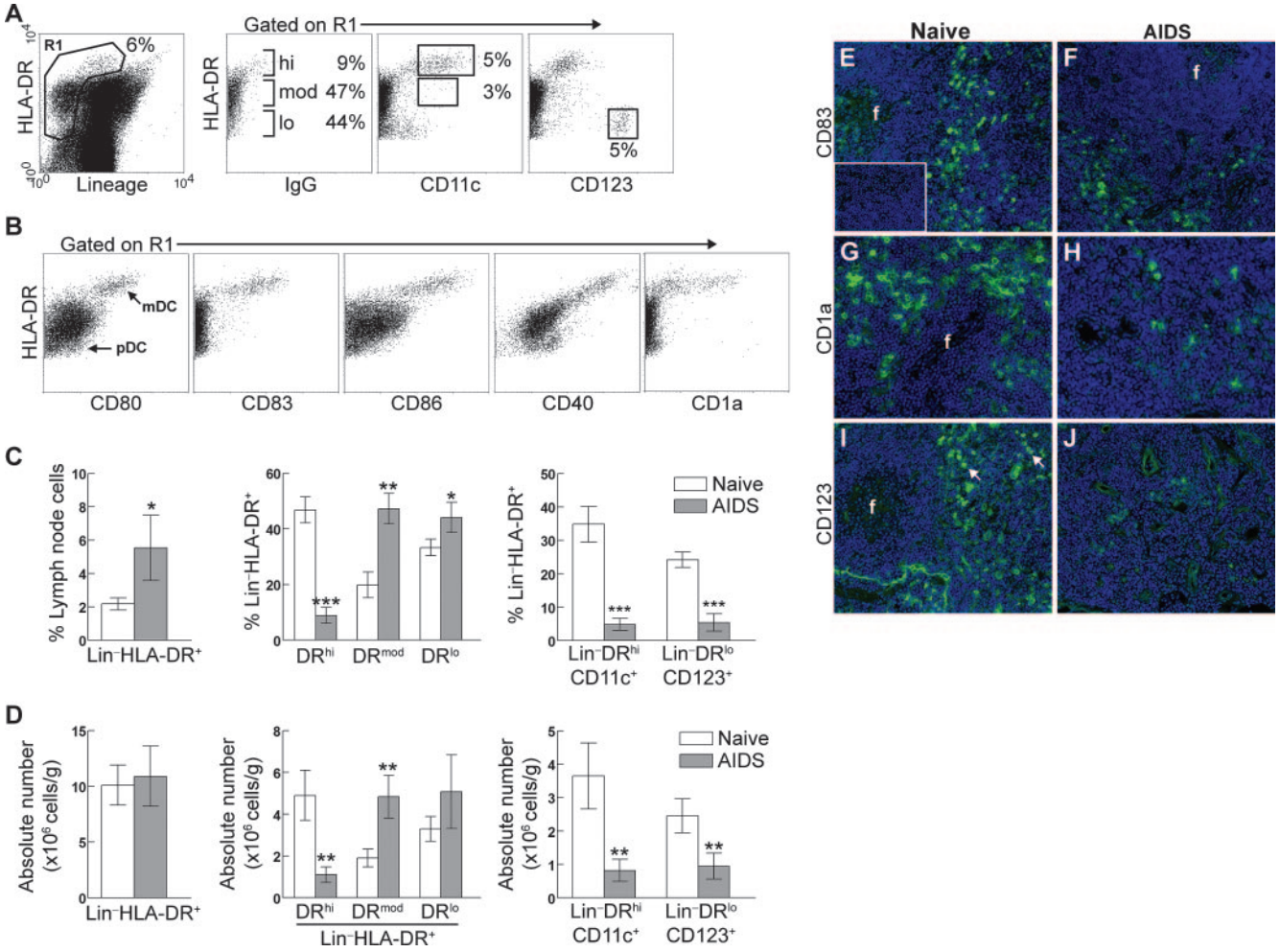

FIGURE 3. pDC and mature mDC are depleted in superficial lymph nodes from monkeys with AIDS. *A* and *B*, Axillary lymph node cells from monkeys with AIDS were analyzed by flow cytometry and DC were identified within the Lin$^-$HLA-DR$^+$ fraction (R1). *A*, Representative plot illustrating the gating strategy used to define R1 and the three HLA-DR-expressing populations within R1. Numbers represent the percentage of cells within R1 denoted by gates or brackets. *B*, Flow cytometric analysis of maturation and costimulatory molecule expression by cells within R1. *C* and *D*, Percentage (*C*) and absolute number (*D*) of cells/gram of tissue within the Lin$^-$HLA-DR$^+$ fraction of lymph node from SIV-naive monkeys ($n = 8$) and animals with AIDS ($n = 7$). Data represent the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *E–J*, Immunofluorescent analysis of axillary lymph node sections from both groups of monkeys for the expression of CD83 (*E* and *F*), CD1a (*G* and *H*), and CD123 (*I* and *J*). Isotype control Ab is shown in the *inset* in (*E*). Positive staining for the indicated markers is green and the cell nuclei stained with Hoechst dye are blue. f, Follicle. Arrows in *I* indicate individual cells expressing CD123. Images are representative of three animals per group. Original magnification was ×200.

similar and relatively low levels of HLA-DR in SIV-naive monkeys (Fig. 1*A*) and animals with AIDS (data not shown). When the absolute number of cells was calculated, mDC and pDC were significantly reduced in the blood of animals with AIDS while the number of Lin$^-$HLA-DR$^+$CD11c$^-$CD123$^-$ cells was relatively unchanged compared with that in SIV-naive monkeys (Fig. 1*B*). The phenotype of mDC from SIV-naive monkeys and animals with AIDS was similar, expressing low levels of CD86 and lacking CD80, CD83, and CD40 (Fig. 1*C*). pDC from both groups were also phenotypically similar, except for a slight increase of CD40 expression in monkeys with AIDS (Fig. 1*D*). These data indicate that circulating mDC and pDC in rhesus macaques are immature cells that are significantly decreased during simian AIDS, consistent with the findings in HIV-infected individuals (11–15).

High proportion of pDC and mature, epidermis-derived mDC in superficial lymph nodes of healthy macaques

To determine the effects of SIV infection on mDC and pDC in lymph nodes, we first characterized DC subsets from normal SIV-naive macaques in a manner comparative to that for blood. Initially, a population of cells not observed in blood and expressing high levels of HLA-DR was identified that appeared to express lineage markers, similar to recent studies using human lymph nodes (30). However, staining with different mAb clones than those used in the lineage mixture revealed a lack of CD14 and CD20 expression, indicating a high level of autofluorescence rather than lineage marker expression (Fig. 2*A*). Therefore, in all future analyses this unique population was included in the gate defining the Lin$^-$HLA-DR$^+$ fraction of the lymph node. In normal monkeys, the Lin$^-$HLA-DR$^+$ fraction represented only 2% of total lymph node cells and could be subdivided into three populations based on HLA-DR expression, HLA-DR^{high}, HLA-DR^{mod}, and HLA-DR^{low}, where mod is moderate (Fig. 2*B*). The majority of CD11c$^+$ mDC were HLA-DR^{high} with a minor proportion being HLA-DR^{mod}, whereas CD123$^+$ pDC were exclusively HLA-DR^{low} (Fig. 2*B*), demonstrating that mDC and pDC in lymph nodes could be differentiated based on HLA-DR expression, similar to recent findings in humans (30). Lin$^-$HLA-DR^{high} mDC uniformly expressed moderate to high levels of CD80, CD83, CD86, and CD40, a phenotype characteristic of mature DC (Fig. 2*C*). In

contrast, Lin$^-$HLA-DR^{low} pDC lacked CD80, CD83, and CD86 and expressed low levels of CD40, consistent with immature cells (Fig. 2*C*). Finally, the Lin$^-$HLA-DR^{mod} population displayed an intermediate phenotype, with the majority of cells expressing CD80, CD86, and CD40 but low to undetectable levels of CD83 (Fig. 2*C*).

In the steady state, the majority of lymphoid organ DC with a mature phenotype are those that migrate from epithelial surfaces (5). Mature DC in peripheral lymph nodes originate from either dermal DC or epidermal Langerhans cells, the latter identified by strong expression of CD1a (31). Notably, Lin$^-$HLA-DR^{high} mDC, but not other DC subsets in the superficial lymph node, expressed CD1a (Fig. 2*C*). In contrast, CD1a was not expressed by cells within the Lin$^-$HLA-DR$^+$ fraction of blood (Fig. 2*D*), consistent with findings in humans (32). Collectively, these findings demonstrate that in superficial lymph nodes from SIV-naive monkeys pDC are phenotypically immature and likely derive from blood, whereas the majority of mDC are mature, epidermis-derived cells, consistent with studies of lymph node DC in mice and humans (3, 8, 9, 33).

Major loss of pDC and epidermis-derived mature mDC from superficial lymph nodes in monkeys with AIDS

To determine the impact of SIV on DC populations in lymphoid tissue, we analyzed superficial lymph nodes from animals with AIDS. A major loss in the proportion of the Lin$^-$HLA-DR^{high} cells was noted, corresponding to a reduction of CD11c$^+$ mDC. In addition, there was a dramatic decrease in the proportion of CD123$^+$ pDC in monkeys with AIDS compared with SIV-naive animals (Fig. 3, *A* and *C*). Although reduced, the remaining mDC exhibited a mature phenotype expressing CD80, CD83, CD86, CD40, and CD1a, whereas pDC only expressed CD40 (Fig. 3*B*), similar to SIV-naive monkeys. Despite the reduction of pDC and mature mDC, the proportion of lymph node cells within the Lin$^-$HLA-DR$^+$ fraction increased in animals with AIDS, related to an increase of HLA-DR^{mod}-expressing cells (Fig. 3, *A* and *C*). This population was not accounted for by either mDC or pDC and displayed a less defined phenotype, expressing CD40, low CD80, heterogeneous CD86, but not CD83 or CD1a (Fig. 3, *A* and *B*). To determine whether decreases in the proportion of mDC and pDC translated to an absolute loss of cells, cell numbers were standardized against tissue mass. The total number/gram of Lin$^-$HLA-DR$^+$ cells did not change between naive animals and monkeys with AIDS, but the absolute number of Lin$^-$HLA-DR^{high} cells was significantly decreased while Lin$^-$HLA-DR^{mod} cell numbers increased (Fig. 3*D*). When the Lin$^-$HLA-DR$^+$ fraction was analyzed for the expression of CD11c and CD123, the absolute numbers of both pDC and mature mDC were significantly decreased compared with SIV-naive animals (Fig. 3*D*), consistent with the observed changes in cell proportions.

In general, lymph nodes from animals with AIDS contained ~50% fewer cells per gram of tissue than SIV-naive monkeys, likely due to tissue fibrosis and lymphadenopathy (data not shown). Hence, the assessment of DC subsets as an absolute number of cells per gram of tissue may be influenced by the stage of disease. Therefore, to confirm the reduction in both percentage and absolute number of DC in lymph nodes during AIDS, immunofluorescent analysis was performed on lymph node sections. CD83 and CD1a mAbs were used to identify mature mDC, as staining was superior to that of the CD11c mAb (data not shown). In naive animals, both CD83^{bright} and CD1a$^+$ cells resided in lymph node paracortical regions outside of follicles (Fig. 3, *E* and *G*). Consistent with our observations by flow cytometry, very few CD83^{bright} or CD1a$^+$ cells were found in lymph nodes from animals with AIDS (Fig. 3, *F* and *H*). Staining for CD123 demonstrated that pDC also resided in paracortical areas of lymph nodes from naive animals and confirmed their depletion in animals with AIDS (Fig. 3, *I* and *J*).

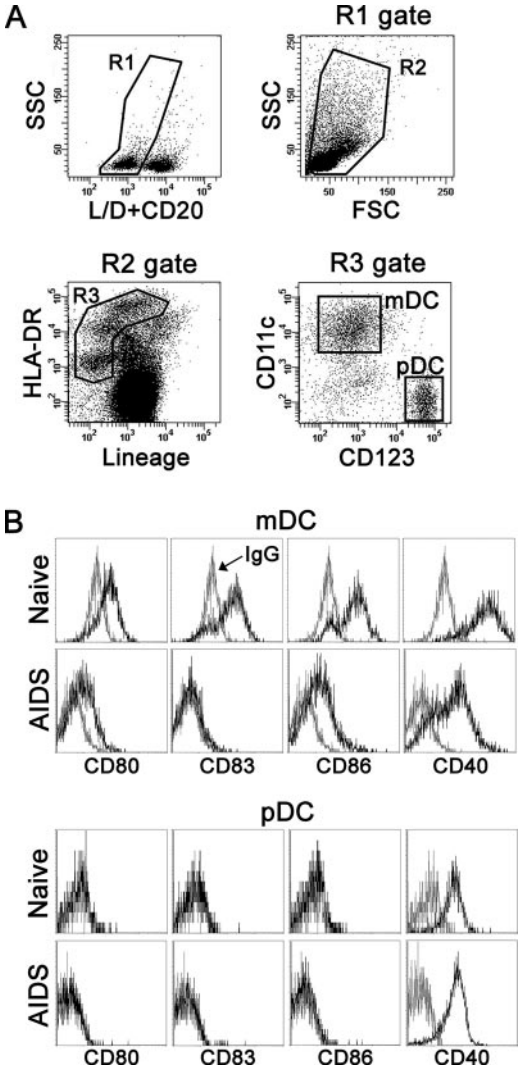

FIGURE 4. Multicolor analysis of DC subsets in peripheral lymph nodes. *A* and *B*, Lymph node single-cell suspensions were labeled with the indicated mAb followed by staining with the LIVE/DEAD cell viability dye (L/D). *A*, Representative plots from an SIV-naive animal demonstrating the gating strategy used to define DC subsets. Viable CD20$^-$ cells were gated against side scatter (SSC) (R1) followed by an additional forward scatter (FSC)/SSC gate (R2). DC subsets were simultaneously identified within the Lin$^-$HLA-DR$^+$ fraction (R3) and analyzed for maturation and costimulatory molecule expression in *B*. *B*, Costimulatory molecule expression (heavy line) vs isotype control staining (thin line) in mDC (*top panels*) and pDC (*bottom panels*) from SIV-naive monkeys and animals with AIDS. All data are representative of four SIV-naive animals and four monkeys with AIDS.

To further analyze the phenotype of DC subsets in peripheral lymph nodes, we used a six-color flow cytometry panel. We gated out nonviable and CD20$^+$ cells and analyzed pDC and mDC simultaneously (Fig. 4*A*). In SIV-naive animals, the majority of Lin$^-$HLA-DR$^+$CD11c$^+$ mDC expressed CD80, CD83, CD86, and CD40 (Fig. 4*B*), consistent with our previous results. However, in monkeys with AIDS the remaining mDC lacked CD83 but retained expression of CD40 and low levels of CD80 and CD86 (Fig. 4*B*). This likely reflects the preferential loss of mature skin-derived mDC from lymph nodes with the remaining mDC being

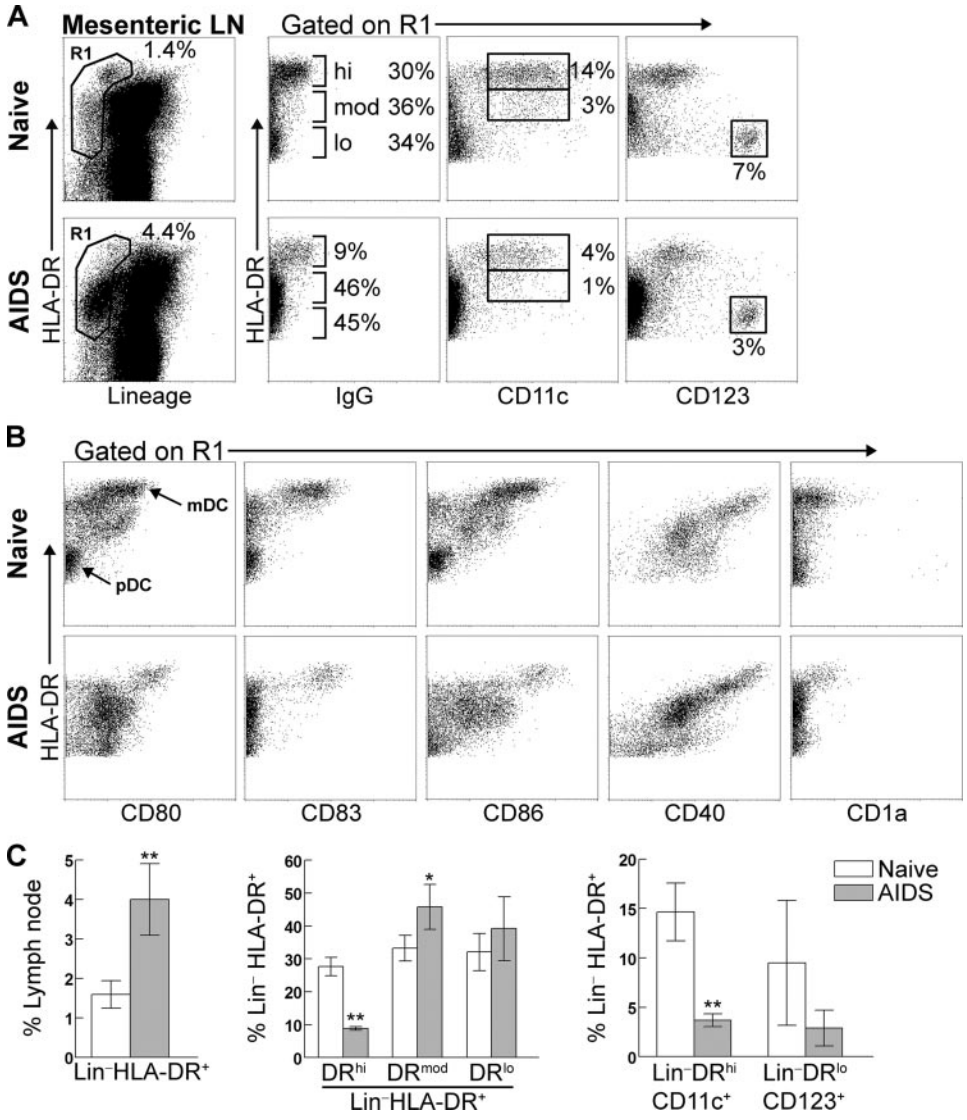

FIGURE 5. mDC and pDC are decreased in mesenteric lymph nodes from animals with AIDS. *A*, Mesenteric lymph node cells from SIV-naive monkeys and animals with AIDS were analyzed by flow cytometry and DC were identified within the $Lin^{-}HLA\text{-}DR^{+}$ fraction (R1). A representative dot plot demonstrating the gating strategy used to define R1 and the three HLA-DR-expressing populations is shown. Numbers represent the percentage of cells within R1 indicated by gates or brackets from one representative experiment of four (naive) and five (AIDS) animals. *B*, Dot plots demonstrating the expression pattern of maturation and costimulatory molecules and CD1a in an SIV-naive monkey and an animal with AIDS. mDC and pDC denote the location of these cells within the $Lin^{-}HLA\text{-}DR^{+}$ fraction. Results are representative of four animals per group. *C*, Percentage of cells within R1. Data represent the mean ± SEM for naive animals ($n = 4$) and monkeys with AIDS ($n = 4$). *, $p < 0.04$; **, $p < 0.02$.

primarily the relatively immature $HLA\text{-}DR^{mod}$ cells (Fig. 3*A*). pDC from SIV-naive animals and monkeys with AIDS were phenotypically similar and expressed similar levels of CD40 (Fig. 4*B*), suggesting that the remaining pDC did not have an altered state of activation in peripheral lymph nodes.

mDC and pDC are decreased in mesenteric lymph nodes and spleens during AIDS

To determine whether the decreases in pDC and mature mDC in superficial lymph nodes during AIDS reflected alterations in other lymphoid compartments, we extended our analysis to the mesenteric lymph nodes. As in superficial lymph nodes, three populations of HLA-DR-expressing cells could be distinguished within the $Lin^{-}HLA\text{-}DR^{+}$ fraction of mesenteric lymph node from SIV-naive animals, with the majority of mDC being $HLA\text{-}DR^{high}$ and that of pDC being $HLA\text{-}DR^{low}$ (Fig. 5*A*). Furthermore, the pattern of maturation and costimulatory molecule expression of DC closely resembled that of superficial lymph nodes from both groups, with $HLA\text{-}DR^{high}$ mDC having a mature phenotype and $HLA\text{-}DR^{low}$ pDC having an immature phenotype (Fig. 5*B*). Notably, $Lin^{-}HLA\text{-}DR^{high}$ $CD11c^{+}$ mature mDC from both groups lacked expression of CD1a (Fig. 5*B*), consistent with findings in human lymphoid tissues draining the mucosal epithelium (33, 34). Overall, the proportion of pDC and mature mDC decreased whereas the proportion of $Lin^{-}HLA\text{-}DR^{mod}$ cells increased in monkeys with AIDS (Fig. 5, *A* and *C*), similar to superficial lymph nodes.

Finally, we assessed whether DC may have accumulated in the spleen, which anatomically is accessible only via the blood. The spleen lacked the $HLA\text{-}DR^{high}$ population of cells corresponding to epithelium-derived mature mDC in lymph nodes (Fig. 6*A*), consistent with previous finding in mice (5, 35). Both mDC and pDC in spleen were significantly reduced in monkeys with AIDS compared with normal animals (Fig. 6*B*). The phenotype of splenic DC in both groups was similar, except for a marginal increase of CD40 expression in animals with AIDS (Fig. 6, *C* and *D*).

$Lin^{-}HLA\text{-}DR^{mod}$ cells that accumulate in lymph nodes from animals with AIDS do not acquire a DC phenotype following culture

In a previous study we showed that culture of $Lin^{-}HLA\text{-}DR^{+}$ $CD11c^{-}CD123^{-}$ cells from blood resulted in CD11c expression by a major fraction of cells indicative of DC precursors (21), and it is possible that the population of $Lin^{-}HLA\text{-}DR^{mod}$ $CD11c^{-}CD123^{-}$ cells in lymph nodes during AIDS similarly represent DC precursors. Light microscopic analysis of purified

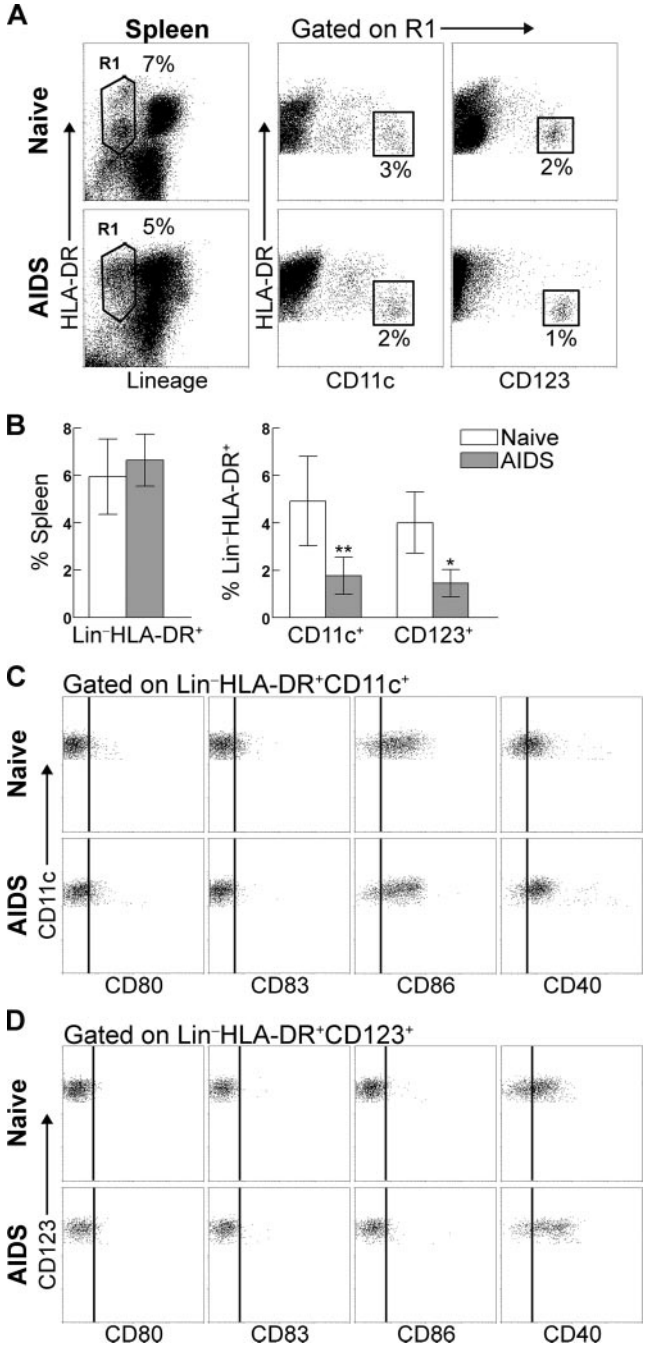

FIGURE 6. mDC and pDC are depleted in spleen from monkeys with AIDS. *A*, Single-cell suspensions of spleen from SIV-naive monkeys and animals with AIDS were labeled with the indicated mAb and DC were identified within the $Lin^-HLA\text{-}DR^+$ fraction (R1). *B*, Percentages of cells within R1 from SIV-naive animals ($n = 5$) and animals with AIDS ($n = 4$). Data represent the mean ± SEM. *, $p < 0.04$; **, $p < 0.02$. *C* and *D*, Dot plots showing the expression of maturation and costimulatory molecules by mDC ($Lin^-HLA\text{-}DR^+CD11c^+$) (*C*) and pDC ($Lin^-HLA\text{-}DR^+CD123^+$) (*D*) in an SIV-naive animal and an animal with AIDS. Vertical bars denote the level of isotype control Ab staining. All data are representative of five SIV-naive animals and four monkeys with AIDS.

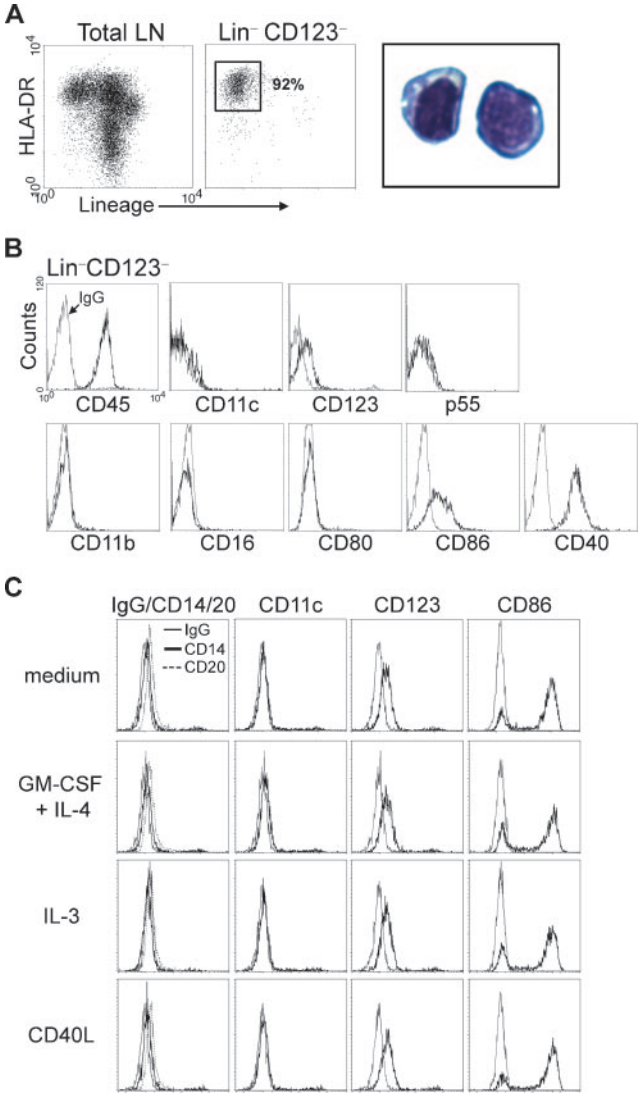

FIGURE 7. $Lin^-HLA\text{-}DR^{mod}$ cells from monkeys with AIDS are not DC precursors. *A*, Lymph node single-cell suspensions were depleted of Lin^+ and $CD123^+$ cells and the depleted fraction was analyzed by flow cytometry or stained with Hema 3 and examined by light microscopy. Original magnification was ×1000. *B*, Purified cells from *A* were analyzed with a panel of mAb by flow cytometry. *C*, Purified cells were analyzed by flow cytometry following 24 h of culture in the indicated conditions. Data are representative of three animals analyzed.

$Lin^-HLA\text{-}DR^{mod}CD123^-$ cells revealed a dull gray-blue cytoplasm and convoluted nuclei resembling those of monocytoid cells (Fig. 7*A*). Flow cytometric analysis of purified $Lin^-HLA\text{-}DR^{mod}CD123^-$ cells before culture demonstrated CD45 and CD40 expression by all cells, whereas ~50% expressed the costimulatory molecule CD86 (Fig. 7, *A* and *B*). The $Lin^-HLA\text{-}DR^{mod}$ population lacked expression of the DC markers CD11c and CD123, consistent with the analysis of total lymph node cells as well as the pan-DC marker p55 (Fig. 7*B*). In addition, freshly isolated cells did not express CD11b or CD16, suggesting they are not NK cells (Fig. 7*B*). To test the hypothesis that $Lin^-HLA\text{-}DR^{mod}CD11c^-CD123^-$ cells may represent DC precursors, purified cells were cultured in growth factors known to result in DC differentiation. Culture with GM-CSF and IL-4, IL-3, or CD40L did not result in CD11c or CD123 expression (Fig. 7*C*), indicating the $Lin^-HLA\text{-}DR^{mod}$ cells were unlikely to be DC precursors. In addition, ~50% of the cells were nonviable after 24 h as demonstrated by trypan blue exclusion (data not shown), indicating that growth factors had no impact on cell survival. Culture also did not result in CD14 or CD20 expression (Fig. 7*C*), thus ruling out the possibility that this unique population differentiated to a monocyte/macrophage or B cell. Nevertheless, under all conditions the majority of cells up-

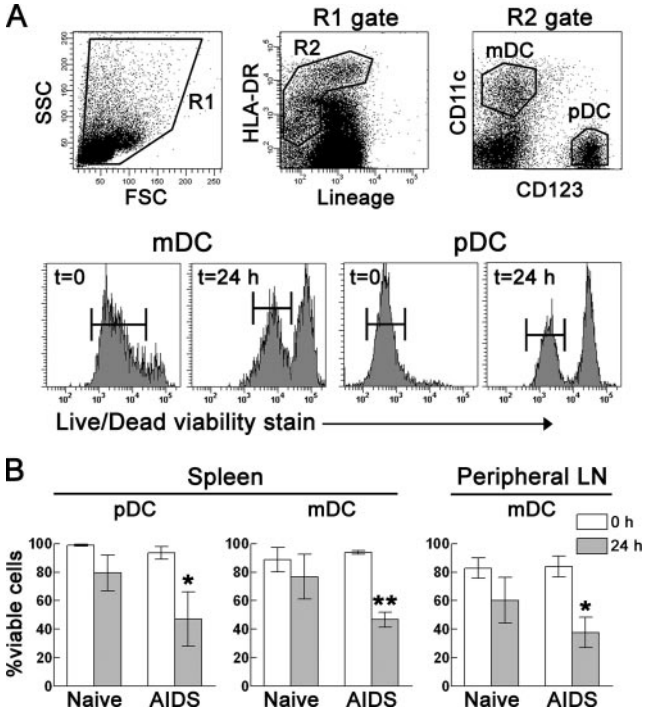

FIGURE 8. DC from animals with AIDS undergo increased cell death in culture. *A*, CD20-depleted single-cell suspensions of peripheral lymph nodes or spleen were analyzed before and 24 h after culture by flow cytometry. Representative dot plots from an SIV-naive peripheral lymph node sample illustrate the gating strategy used to identify mDC and pDC. The R2 gate used for spleen was similar to that in Fig. 6. Histograms represent the LIVE/DEAD staining pattern of peripheral lymph node mDC and splenic pDC from an animal with AIDS. Brackets denote the gate used to quantitate viable cells before and after culture. *B*, Percentage of viable cells for pDC and mDC from SIV-naive monkeys ($n = 4$) and animals with AIDS ($n = 4$) before and after culture. Data represent the mean ± SEM. *, $p < 0.02$; **, $p < 0.01$.

regulated CD86 expression, demonstrating an effect of culture (Fig. 7*C*).

Spontaneous death of mDC and pDC from lymphoid tissues of monkeys with AIDS

To begin to address the mechanism of DC depletion, we sought to determine whether DC in animals with AIDS may be susceptible to spontaneous cell death in culture. To increase the frequency of DC, we depleted lymph node or spleen cell suspensions of $CD20^+$ B cells and, to provide a survival factor for pDC, we included IL-3 (3). Before and 24 h after culture we used the LIVE/DEAD viability dye to identify post-apoptotic or necrotic cells within the mDC or pDC fraction (Fig. 8*A*). We first studied mDC and pDC from the spleen, which are phenotypically similar to their respective DC subsets in blood. At the onset of culture, mDC and pDC from both groups contained a similar high percentage of viable cells (Fig. 8*B*). However, there was a significant decrease in the viability of pDC from animals with AIDS, but not from SIV-naive monkeys (Fig. 8*B*). In addition, mDC viability significantly decreased in animals with AIDS following culture, whereas mDC from naive monkeys showed a minimal loss of viability (Fig. 8*B*). Similarly, mDC from the peripheral lymph nodes of animals with AIDS demonstrated a significant loss of viability following culture as compared with mDC from SIV-naive animals (Fig. 8*B*). Together, these data indicate that both mDC and pDC from animals with AIDS are more prone to spontaneous cell death.

Discussion

Our findings contribute considerable insight to the current understanding of DC dynamics during HIV infection and point to a more direct role of systemic DC loss in the pathophysiology of immunodeficiency. We demonstrate that the loss of circulating DC in progressive SIV infection is not due to recruitment to lymph nodes but rather is a component of the generalized loss of both DC subsets from blood and lymphoid tissues. The notion that DC recruitment to lymph nodes may in part explain their disappearance from blood arises from the observed phenotypic activation of DC subsets following in vitro or in vivo exposure to HIV (15, 16, 36). However, clear differences in the phenotypic activation of mDC and pDC was not observed by us or by others in SIV-infected macaques (20). Increasing evidence suggests that the interaction between mDC and pDC is important in the induction of virus-specific immune responses in lymph nodes (6, 7) and the stimulation of HIV-specific $CD4^+$ T cell responses (16). Therefore, the loss of both DC subsets from lymphoid tissues in advanced infection would clearly reduce the capacity to respond to HIV or opportunistic infections and could thus contribute to the onset of immunodeficiency. In contrast, early in HIV infection DC recruitment to lymph nodes could facilitate viral spread and the infection of susceptible T cells (37). Indeed, DC accumulate in the lymph node paracortex during acute infection with either SIV or HIV (17, 23), coincident with the massive depletion of memory T cells (38).

The mechanism of DC depletion from blood and lymphoid tissue during AIDS is likely to be complex. Our findings indicate that mDC and pDC undergo spontaneous cell death during culture, supporting the hypothesis that the loss of DC may be due to cell death (39, 40). Notably, the amine reactive viability dye used in this study identifies not only necrotic but also late or postapoptotic cells (41), and preliminary experiments have identified viable DC in the lymph nodes of monkeys with AIDS that are annexin V-positive (data not shown), suggesting that the DC may be undergoing apoptosis. pDC and mDC in blood do harbor virus, suggesting that direct infection may contribute to loss of cells from the circulation (42). However, exposure of pDC to HIV in vitro may promote pDC survival, not death, although the data are somewhat conflicting (16, 43). In addition, the frequency of infected pDC and mDC in tissues appears to be too low to support a major role of infection in depletion of tissue-resident DC (23, 44–46). It is possible that the apoptosis of DC in HIV and SIV infection is mediated by the binding of TNF family ligands to cognate death receptors involving FasL binding to Fas or TRAIL/DR5 interactions. Fas/FasL and TRAIL/DR5 have been shown to play a role in the apoptosis of T cells in HIV infection (47–50), and both TRAIL- and Fas-mediated pathways have been implicated in DC apoptosis during measles virus infection (51, 52).

We have previously shown that a significant factor in the loss of DC from lymph node may be the failed trafficking of DC from epithelial surfaces (23). Consistent with this finding, the predominant mDC population lost from lymph nodes of monkeys with AIDS was the mature skin-derived DC, while the relatively immature HLA-DR^{mod} mDC subset was decreased to a lesser degree. The mechanism of suppressed migration remains elusive, although it is unlikely to be due to the direct infection of DC. HIV and SIV can infect Langerhans cells (53, 54); however, evidence for the significant infection of Langerhans cells during progressive infection is lacking. Moreover, Langerhans cells migrate with normal efficiency from the skin in SIV-infected monkeys at the time of peak virus load, indicating that failed Langerhans cell migration is not simply a function of high virus burden (23).

Despite the reduction of pDC and mature mDC in lymph nodes, we observed an increase in a population of $Lin^-HLA\text{-}DR^{mod}CD11c^-CD123^-$ cells within the $Lin^-HLA\text{-}DR^+$ fraction of lymph nodes from animals with AIDS that resemble monocytoid cells. Recently, an increased frequency of circulating blood DC lacking CD11c and CD123 expression that elicited poor T cell proliferation and IFN-γ secretion was identified in cancer patients (55). In our study the $Lin^-HLA\text{-}DR^{mod}$ cells did not acquire CD11c or CD123 expression after 24 h of culture, although it is conceivable that a longer period of culture would promote their differentiation. It is also interesting to note that the lymph nodes of HIV-infected subjects were found to contain an abundance of semimature DC defined as being Lin^-CD83^+ $IL\text{-}12^-$, which induced allogeneic T cells to adopt a regulatory T cell phenotype (56). The low CD86 and lack of CD80 expression of the $Lin^-HLA\text{-}DR^{mod}CD11c^-CD123^-$ cells in the lymph nodes of monkeys with AIDS are consistent with these cells being regulatory rather than immunostimulatory DC.

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Disclosures

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