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Differences in the Constitutive and SIV Infection Induced Expression of Siglecs by Hematopoietic Cells from Non-Human Primates¹

Jiraporn Jaroenpool^{2,4}, Kenneth A. Rogers², Kovit Pattanapanyasat^{3,4}, Francois Villinger², Nattawat Onlamoon², Paul R. Crocker⁵, and Aftab A. Ansari^{2,6}

²Department of Pathology & Lab Medicine, Emory University School of Medicine, Atlanta, GA 30322

³Division of Instruments for Research, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand ⁴Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, Thailand Research Fund Senior Scholar ⁵Wellcome Trust Biocentre, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom

Abstract

The expression of the Siglec family of molecules by hematopoietic cells from uninfected and SIV infected disease susceptible rhesus macaques (RM) and SIV infected disease resistant sooty mangabeys (SM) and for comparison humans was carried out. The predominant cell lineage in all 3 species expressing Siglec's was monocytes. The major finding by both a cross sectional and a prospective SIV infection study showed that whereas monocytes from RM show marked increase in each Siglec constitutively expressed, monocytes from SM showed marked decreases in Siglec-1 expression. While monocytes from all 3 species constitutively expressed Siglec-3, human monocytes in addition expressed Siglec-5 and 9 and to a lower density 7, monocytes from RM expressed Siglec-7 and those from SM expressed Siglec-1. Monocytes from all 3 species however expressed mRNA for Siglec's-1, 5, 7 and 9. The reasons for the failure to detect these molecules at the protein level and the mechanisms for such distinct effects of SIV infection on Siglec expression are discussed.

Keywords

Siglec; non-human primate; SIV infection; monocytes; hematopoietic cell lineages

INTRODUCTION

A majority of the sialic acid-binding immunoglobulin-like lectins (Siglecs) are cell surface molecules expressed by mammalian hematopoietic cell lineages [1;2;3]. The Siglecs are type I membrane proteins that display a N-terminal V-set immunoglobulin-like domain and variable numbers of immunoglobulin-like constant region type 2 (C2) domains. The amino terminal domain of Siglecs has the binding specificity for sialic acid [1;2;3;4]. Siglecs are broadly categorized into 2 subsets based on sequence similarity and evolutionary conservation [2]. The

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⁶ Corresponding author: Room, 2309 WMB, Emory University School of Medicine, 101, Woodruff Circle, Atlanta, GA 30322, USA (pathaaa@emory.edu).

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first subset includes the sialoadhesin/CD22/myelin associated glycoprotein (MAG) and the recently described Siglec-15 molecule [5], whereas the second subset comprises the CD33-related Siglecs (reviewed in 1). Each of these molecules is also named by an individual Siglec number and some of them have been assigned a CD nomenclature. Since each Siglec has unique specificity and preference for distinct sets of sialylated ligands, it is reasoned that each of these may have distinct but overlapping functions making it complex to sort out their individual roles.

Our interest in the study of Siglecs was initiated by 3 sets of findings. Firstly, there was a report that the expression of Siglec-5 by lymphoid cells including CD4⁺ T cells from chimpanzees but not humans was associated with a relative decreased potential for proliferation following activation via the TCR [6] and this decreased proliferative capacity was speculated to contribute to the relative disease resistance of chimpanzees infected with HIV-1 as compared to humans. These observations provided an evolutionary basis for species differences in susceptibility to HIV-1 pathogenesis and since our lab has been studying the simian immunodeficiency virus (SIV) infections of non-human primates, this finding piqued our interest. Secondly, the CD33-related (Siglecs-5 to 14) were shown to contain a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) with the potential to recruit SHP-1/2. Suppressor of cytokine signaling (SOCS3) that is normally up-regulated during inflammation has been shown to bind to the ITIM of a variety of cytokine receptors and inhibit signaling via these receptors [7]. SOCS 3 has also been shown to compete for SHP 1/2, bind to phosphorylated ITIM of Siglec-7 and target it for proteasomal degradation [8] contributing to the dysregulation of a number of intracellular signaling pathways characteristic of HIV-1 infected cells. Thirdly, many Siglecs are known to be expressed by cells of the innate immune system including natural killer cells (NK cells) where they can function as inhibitory receptors by suppressing signaling mediated by receptors that have ITAMs within their intracellular domains [9]. The recent findings of a prominent role for NK cells in dictating the course of HIV-1 disease progression [10;11] provide an additional rationale for the studies reported herein.

The fact that these Siglecs have been shown to bind to sialylated glycans of a variety of pathogenic micro-organisms [8] coupled with the three sets of findings listed above and our interest in defining the role of the innate immune system in lentiviral infection, prompted us to carry out a detailed initial study of the precise lineages of hematopoietic cells of non-human primates that express Siglecs. Our lab has been involved with studies of 2 nonhuman primate species that show distinct clinical outcomes following infection with SIV [12;13;14;15]. Thus, experimental infection of Asian rhesus macaques (*Macaca mulatta*) with SIV leads to an acute phase which is highlighted by a massive depletion of CD4⁺ T cells in the gastrointestinal tract followed by a chronic phase during which there is persistent plasma viremia, gradual immune dysfunction and a terminal disease stage marked by increased susceptibility to opportunistic infections followed invariably by death [16]. In contrast, West African sooty mangabeys (*Cercocebus atys*) one of the natural hosts for SIV (bred in captivity at the Yerkes National Primate Research Center of Emory University) remain disease resistant despite high plasma viral loads. In some cases, these mangabeys remain asymptomatic despite having higher plasma SIV viral loads than those noted for rhesus macaques that have died following SIV infection [12;14;17;18]. Experimental infection of sooty mangabeys with SIV isolates (that causes disease and AIDS in rhesus macaques) leads to rapid viremia and is associated with a small but readily detectable loss of CD4⁺ T cells but no recognized disease. Identification of the mechanism(s) that distinguish disease resistance as compared with disease susceptibility in these 2 SIV infected non-human primates has been and continues to be one of the goals of our laboratory with the rationale that such findings would have an important impact on our understanding of lentiviral pathogenesis.

During the course of such studies, our laboratory has determined a prominent role for innate immune effector mechanisms in setting the course and relative rate of disease progression of these two non-human primate species following SIV infection (submitted for publication). Since Siglecs are predominantly expressed by cell lineages that comprise the innate immune system, it was reasoned that the identification of differences (if any) of the expression of these molecules may shed light on their role in the pathogenesis of SIV infection of these 2 non-human primate species. The results from these studies form the basis of this report.

MATERIAL & METHODS

Non-Human Primates

The adult SIV negative sooty mangabeys (SM), adult rhesus macaques (RM) and the naturally SIV infected SM that served as donors of peripheral blood samples were from the breeding colonies of the Yerkes National Primate Research Center (YNPRC) of Emory University. SIV infected RM blood donors were adult monkeys that were infected IV with 200 TCID₅₀ of SIVmac239 and blood samples from these SIV infected RM were obtained at various times post infection (pi). SM blood samples were obtained from naturally SIV infected animals as well as from SM that were experimentally infected with an SIV isolate termed FUo (a kind gift from Dr. S. Staprans, Emory University) that replicates efficiently in CD4⁺ T cells from sooty mangabeys. All animals were housed and cared for at the YNPRC in conformance to the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council and the Health and Human Services guidelines "Guide for the Care and Use of Laboratory Animals" and all protocols were reviewed and approved by the Emory IACUC.

Human PBMC

Normal healthy adult laboratory workers served as blood donor volunteers for the studies reported herein.

Viral load determination

Plasma SIV viral load was routinely monitored in each of the SIV infected monkey species by the NIAID, NIH CFAR sponsored Virology Core Laboratory of Emory University School of Medicine.

Monoclonal antibodies

Eleven clones of mouse anti-Siglec monoclonal antibodies (mAb) were used in this study including Siglec-1 (clone 7D2), Siglec-3 (clone 6C5), Siglec-5 (clone 1A5 and clone 8H2 denoted as Siglec 5.1 and 5.2, respectively), Siglec-6 (clone E20-1232, purchased from BD Pharmingen, San Diego, CA), Siglec-7 (clone 7.5A and clone 7.7A denoted as Siglec-7.1 and Siglec-7.2, respectively), Siglec-8 (clone 7C9), Siglec-9 (clone Kalli), Siglec-10 (clone 5G6), and Siglec-11 (clone 4C4). Sheep anti-human-Siglec-5,-7,-8,-9,-10 are polyclonal antisera (all anti-Siglec antibodies kindly provided by Dr. Paul R. Crocker, University of Dundee, Scotland). Secondary antibodies, R-PE-conjugated donkey polyclonal anti-sheep IgG (H+L), (Abcam, Cambridge, MA), R-PE-conjugated goat anti-mouse IgG (H+L) and APC-conjugated goat (Fab')₂ anti-mouse IgG (H+L) (Southern Biotech, Birmingham, AL) were obtained commercially. FITC-conjugated- anti-CD3 (clone SP34), -CD8 (clone RPA-T8), -CD14 (clone M5E2), -CD16 (clone 3G8), -CD20 (clone 2H7) and a cocktail of FITC-conjugated lineage specific antibodies also termed lin antibodies, PE-conjugated-anti-CD123 (clone 7G3), -CD11c (clone S-HCL3), PerCP Cy5.5-conjugated- anti-HLA-DR (clone G46-6) were purchased from BD Pharmingen, FITC-conjugated-CD11b (clone Bear-1) was purchased from Beckman Coulter, (Somerset, NJ, USA).

Flow cytometry

Media referred throughout these studies consisted of RPMI 1640 containing 5 g/ml of gentamicin, 2 mM L-glutamine and 10% heat inactivated fetal calf serum. PBS pH 7.4 was utilized for washing the cells in between the staining steps. PBMCs isolated by standard Ficoll hypaque gradient technique were washed and re-suspended in RPMI 1640 medium at 10×10^6 cells/ml. The cells were dispensed in a volume of 0.1 ml to individual test tubes. The expression of Siglec molecules by lymphocytes and monocytes were measured by incubating one million PBMCs in a volume of 100 μ l of media containing a previously optimized concentration of anti-Siglec antibodies (1:100 of Siglec-5 and Siglec-6 and 1:5 of the other anti-Siglec antibodies). The cell suspension was incubated at 4°C for 30 min followed by washing once with wash buffer. Ten μ l of PE-conjugated goat anti-mouse IgG (dilution 1:25) was added and the cells incubated at 4°C for 30 min. After labeling, cells were washed and fixed with 2% PFA. One hundred thousands events were analyzed using the FACSCalibur assisted by the use of Flow Jo software (BD). Lymphocytes and monocytes were gated according to FSC and SSC characteristics. An aliquot of the cells was incubated with isotype control antibodies followed by the addition of secondary antibody for purposes of control. The frequency and intensity of fluorescence by the isotype control was utilized to calculate the net specific mean fluorescent intensity (MFI) and percentage of cells expressing the appropriate Siglec molecules.

Siglec expressions by monocytes, granulocytes, eosinophils and neutrophils were analyzed using EDTA blood samples. Peripheral whole blood samples (150 μ l) were incubated with anti-Siglec monoclonal antibodies (Siglec-5.1,-5.2,-7.1,-7.2,-8,-9,-10) or polyclonal sheep antisera (Siglec-5,-7,-8,-9,-10) at 4°C for 30 min. The cell suspension was washed in media and then incubated at 4°C for 30 min with RPE-conjugated goat anti-mouse IgG or RPE-conjugated donkey polyclonal anti-sheep IgG (dilution 1:100). The cell suspension was washed in media and then incubated at 4°C for 30 min with FITC conj. anti-CD16 mAb (5 μ l) followed by the addition of 2 ml of red blood cell lysing solution (BD). After 10 min incubation, cells were washed and fixed with 2% PFA. Monocytes and granulocytes were distinguished according to FSC and SSC characteristics as described utilizing a strategy outlined in Fig. 4A. To discriminate between neutrophils and eosinophils, an identification strategy was utilized based on eosinophils' high auto-fluorescence and SSC and lack of CD16 expression, in contrast to neutrophils which have lower SSC and are CD16 positive. Briefly, cells were gated on dot plots representing cell size based on forward scatter cytogram (FSC) and granularity based on side scatter cytogram (SSC). All data were corrected for auto-fluorescence as well as for non-specific binding using isotype-matched and secondary Ab negative controls. These gated populations of cells were then analyzed for CD16 expression and the CD16⁻ cells (eosinophils) and CD16⁺ cells (neutrophils) were analyzed for Siglec expression as outlined in Fig. 5A.

The expression of Siglecs by dendritic cell subsets was performed using ficollhypaque gradient purified PBMCs. Aliquots of 150 μ l of the cell suspension at 10^7 cells/ml were stained with 100 μ l of a previously optimized concentration of the anti-Siglec antibodies at 4°C for 30 min. The cells were washed in media followed by the addition of 10 μ l of APC-goat (Fab')₂ anti-mouse IgG (dilution 1:25) and incubation at 4°C for 30 min. Cells were washed once more with media and one aliquot incubated with FITC-Lin, PE-CD11c, PerCPCy5.5-HLA-DR and the other aliquot incubated with FITC-Lin, PE-CD123, PerCP Cy5.5-HLA-DR to define myeloid dendritic cells (mDC's) and plasmacytoid dendritic cells (pDC's), respectively. The FITC-conjugated lin (lineage specific) antibodies were utilized to exclude other cell lineages (T cells, B cells, monocytes and NK cells) from DC subsets. The lineage (lin) cocktail included the use of FITC-conjugated (CD3, CD14, CD16, CD19, CD20, CD56) for human cells and the combination of FITC-labeled antibodies (CD3, CD8, CD11b, CD14, CD20) for cells from the 2 monkey species. Fig. 6 shows the gating strategy used to define DC sub-populations. As seen

the mononuclear cells were first gated according to FSC and SSC properties followed by the exclusion of the Lin⁺ cell lineages. This gated population was then analyzed for HLA-DR⁺ cells that express CD11c⁺ mDCs or the HLA-DR⁺ cells that express CD123⁺ pDCs, respectively. Data on at least 3.5×10^5 events per sample were acquired by flow cytometry and the net percentage/MFI of Siglec expressions were determined for each of the 2 dendritic cell subsets by deducting the values from those observed with the isotype control.

Intracellular expression of Siglecs

To determine the intracellular expression of Siglecs, PBMCs were washed once with media followed by permeabilization with 200 μ l of CytoFix/CytoPerm solution (BD) for 20 min at 4°C. The permeabilized cells were re-suspended in media at 10^7 /ml and 100 μ l aliquots dispensed into individual tubes. To individual tubes were added pre-determined optimal concentrations of the anti-Siglec-1,-5.1,-5.2,-7.1,-7.2 or -9 mAbs followed by incubation for 30 min. The cell suspensions were washed in media and then incubated with PE-conjugated goat anti-mouse IgG. The cells were washed and one hundred thousands events/sample were analyzed by flow cytometry. Controls consisted of cells incubated with isotype control antibody followed by the same secondary antibody. For each analysis, an aliquot of the same cell sample was also utilized in a non-permeabilized form and stained using the same reagents in efforts to compare the display of cell surface expression (if any) with the intracellular staining of the Siglec molecules.

RT-PCR analysis

To determine the expression of Siglec at the mRNA level, one step RT-PCR were performed using PBMCs as a source of RNA. Total RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA) and treated with RQ1 RNase free DNase (Promega, Madison, WI) to eliminate contaminating DNA. RNA was then amplified using a one step access RT-PCR System (Promega) with primer pairs for Siglec-1 (Forward: 5'-CACTAGGGCTGATACTGGCTT-3' Reverse: 5'-TCCAGGAAGGCTGTCTCAGGACTG-3') Siglec-5 (Forward: 5'-CTTGAGGTGACAGCCCTGA-3', Reverse: 5'-TCCAGGAGAATGTGAGAGGT-3'), Siglec-7 (Forward: 5'-CCTGCCAGGCTGAGCTGGAC-3', Reverse: 5'-CCTCATTTTGCCGTGTACTC-3') and Siglec-9 (Forward: 5'-GATGCTCCAGTGGCCACAAACAAC-3', Reverse: 5'-CCAGGGCACAGAGCAGGTCAGATT-3'). First strand cDNA was transcribed at 45°C for 45 min followed by RT inactivation at 94°C for 2 min. PCR amplification was performed at 94°C for 30 sec, annealing at 55–60°C for 30 sec and extension at 68°C for 30 sec. A total of 35 amplification cycles were utilized with a final 7 min extension at 68°C. The PCR products were analyzed by agarose gel electrophoresis.

Cloning of a full length cDNA encoding Siglec-5

Total RNA was prepared from PBMC from human, rhesus macaque and sooty mangabey donors as described above. First strand cDNA was synthesized by reverse transcription of 1 μ g of RNA using SIGLEC-5 RT primer (5'-CAGTGAATTGCTTGATGATGACAGTG-3'). The reaction product was subjected to nested PCR using high fidelity polymerase (Roche Molecular Biochemicals) using the forward primer (5'-ACATTGGRGCATTTTCCTTC-3') and reverse primer (5'-CAGTGCCAGGCCTAGGTTTGAGC-3') initially. The product was further amplified by nested PCR using forward primer (5'-GGGCCCTGGCGGATGGA-3') and reverse primer (5'-AGGGCTCTTYGTGCTTCAG-3'). The PCR products were ligated to the pGEMT vector and individual clones were isolated. Plasmids were purified and sequenced.

In vitro infection of monocyte derived macrophages with SIV

Monocytes were isolated by using Easysep® magnetic nanoparticles for negative selection using a customized antibody cocktail for macaque cells (Stem Cell Technologies Inc., Vancouver, BC, Canada). The enriched preparation of monocytes was re-suspended at 0.5×10^6 /ml in macrophage culture medium (RPMI 1640, 20% FBS, 1mM L-glutamine, gentamicin 5 µg/ml, 100 ng/ml of M-CSF (PeproTech Inc. Rocky Hill, NJ)) and dispensed in a volume of 2 ml into individual wells of a 24 well-plate and incubated at 37°C, 7% CO₂ for 7–10 days. At day 3, the contaminating red blood cells were removed and the media replenished with fresh medium. At the end of the 7-day culture period, an aliquot of the cells was subjected to flow analysis for the purity of the cell population using PE-CD3, FITC-CD14, and PerCP cy5.5-HLA-DR. A high frequency (65%) of CD14⁺ cells were noted in the monocytes/macrophages cultures. Duplicate wells of the remaining in vitro matured macrophages were incubated with either media (control), SIVmac239 at a MOI of 1.0 (stock at 10^6 TCID₅₀/ml) or a MOI of 0.1 of SHIV33 (stock at $10^{4.8}$ TCID₅₀/ml) overnight and the cultures incubated for an additional 3 days, washed 3 times with 37°C pre-warmed serum free RPMI 1640 medium. The cells were then harvested by adding warmed PBS containing 5 mM EDTA and incubated at 37°C for 15 min to collect all adherent cells. Controls consisted of non-infected media treated cells treated identically. The control and SIV infected macrophages were then harvested and aliquots of 5×10^4 were stained with Siglec-1 mAb and the frequency and MFI of Siglec-1 expression determined as outlined above.

Statistical Analysis

Statistical Analysis was performed using the SPSS software (SPSS Inc., Chicago, IL). Comparison of values for each separate variable in two independent groups was analyzed using the two-tailed Mann-Whitney U tests for nonparametric independent comparisons. A probability value of less than 0.05 was considered statistically significant.

RESULTS

Differential expression of Siglecs by cell lineages from humans, rhesus macaques and sooty mangabeys

A series of monoclonal and polyclonal antibodies that have been previously characterized for their specificity against the family of human Siglecs [19;20;21;22;23;24;25;26] were first screened for their ability to cross react with cells from rhesus macaques (RM) and sooty mangabeys (SM) using human cells as a reference control. Data obtained using mAb clones 1A5 and 8H2 (specific for Siglec-5) and mAb clones 7.5A and 7.7A (specific for Siglec-7) gave essentially similar data, however, some exceptions were noted and thus select results using both the clones are illustrated. In addition, the profiles of only the positively reacting anti-Siglec antibodies are shown and those not shown should be considered non-reactive for the lineage being studied. Results of this initial screen showed that both the anti-Siglec monoclonal and the polyclonal antibodies utilized reacted predominantly with peripheral blood monocytes from otherwise healthy RM and SM. Thus, except for the expression of Siglec-7 by > 95% of the NKG2a expressing NK cells (data not shown), none of the other Siglec specific antibodies showed detectable reactivity on other lymphoid cell lineages. Flow cytometric gating techniques were subsequently utilized to focus the analysis first on peripheral blood monocytes from RM and SM and for comparison healthy human adults.

As seen in Fig. 1 and Table 1, whereas a high frequency (> 85%) of human monocytes expressed Siglec-3, 5 and 9, a much lower signal was detected for Siglec-7, suggesting low density staining for Siglec-7 by these cells under our experimental conditions. In contrast, about a third of the monocytes from healthy RM expressed Siglec-3 and Siglec-7 and a small readily detectable frequency (5–8%) of RM monocytes expressed Siglec-1. On the other hand, a high

frequency (> 85%) of peripheral blood monocytes from healthy SM expressed Siglec-3 and interestingly while gating techniques show about 25% of the monocytes express Siglec-1, the entire population shifts to the right, denoting that most of them express low levels of this Siglec molecule. Thus, whereas Siglec-3 was expressed by a significant frequency of monocytes from humans and both non-human primate species, Siglec-5 and 9 were expressed primarily by human monocytes, Siglec-7 primarily by monocytes from RM and Siglec-1 primarily by SM.

The failure of reactivity of some of these anti-Siglec antibodies with cells from the non-human primates were reasoned to be due to the lack of cross reactivity of some of these reagents secondary to species specific differences. In efforts to address this issue, RT-PCR techniques were first utilized for the detection of mRNA that are discordant in their expression between human and the 2 non-human primate species. Thus, mRNA analysis was performed specifically for Siglec-5 and 9 which reacted with human monocytes but not monkey monocytes, Siglec-1 that reacted with monocytes from the monkeys but not human, and Siglec-7 that reacted with monocytes from human and RM but not monocytes from SM. As seen in Fig 2, readily detectable mRNA coding for Siglec-1, 5, 7 and 9 was noted in samples from not only humans but also from RM and SM. Thus, the failure to detect Siglec-1 in monocytes from humans, Siglec-5 and Siglec-9 in monocytes from RM and SM and Siglec-7 in SM could be due to differences in the sequences that code for these molecules. These findings prompted us to define the degree of potential differences between humans and these non-human primates and we thus chose to clone and sequence Siglec-5 as a prototype to address this issue. Primer pairs were thus designed which were utilized to amplify, clone and sequence Siglec-5 from enriched population of monocytes from the PBMC's of RM and SM. As seen in Fig. 3, the protein sequence of Siglec-5 differed significantly (87% homology only) between the 2 monkey species and the published sequence of human Siglec-5, which could contribute to the failure of the Siglec-5 mAb to react with Siglec-5 if these were indeed expressed by monocytes from the 2 monkey species. It is of interest to note that upon close scrutiny, the protein sequence showed 8 amino acid residues that were identical between human and SM but not RM. The biological significance of this closer homology of Siglec-5 between human and mangabeys as compared to macaques remains to be determined.

Finally, it was also reasoned that the failure to detect cell surface expression of Siglec's could be due to a failure of the Siglec's to traffic from the intracellular compartment to the cell surface. This issue was addressed by taking aliquots of highly enriched population of monocytes from humans, RM and SM which were fixed, permeabilized and analyzed for intracellular reactivity with mAbs to the discordant Siglec-1, 5.1, 5.2, 7.1, 7.2 and 9 using standardized techniques for intracellular localization of cell surface molecules [27]. These extensive staining studies, unfortunately did not reveal any difference from the results of the cell surface staining studies except for Siglec-7. Thus, 30–50% of the monocytes from SM showed readily detectable intracellular staining by the Siglec-7 mAbs. The failure to detect the other Siglec molecules was not secondary to technical issues since Siglec-1 was readily detected intracellularly in monocytes from SM, Siglec-5 and 9 in monocytes from humans and Siglec-7 from RM (data not shown).

We next examined the frequencies of myeloid and plasmacytoid dendritic cells (mDC and pDC) that expressed Siglecs in the PBMC samples from humans, RM and SM. Appropriate mAb reagents previously shown to cross react with cells from RM and SM were utilized to gate and identify highly enriched populations of mDCs and pDCs from humans RM and SM (Fig. 4A). Results of these studies showed that whereas a high frequency of mDCs from humans expressed Siglec 3, 5 and 9, only approximately 17% expressed Siglec-6 (Fig. 4B). In contrast, a significant frequency of human pDCs expressed Siglec-3, 5 and 6 but a low frequency expressed Siglec-9. In the case of RM, whereas a high frequency (> 60%) of the mDCs only expressed Siglec-7, a lower frequency (30%) of the pDCs only expressed Siglec-3 (Fig. 4C).

In the case of the SM, it was interesting to note that this species appeared to have 2 sub-populations of mDCs (Fig. 4D) based on the relative density of HLA-DR and each of these were thus analyzed separately. Whereas > 75% of the high density HLA-DR⁺, CD11c⁺ mDCs expressed Siglec-3, about 40% of the lower density of HLA-DR⁺, CD11c⁺ mDCs expressed Siglec-3 which was also at a lower MFI. The mDCs did not express detectable levels of Siglec-1. In the case of the pDCs from the SM whereas a high frequency (> 60%) expressed Siglec-1, a very low frequency expressed Siglec-3.

Effect of SIV infection on the expression of Siglecs by hematopoietic cells from non-human primates

As outlined above, one of our major interests in studying the expression of Siglec's was to determine if the differences in the clinical outcome of SIV infection in RM (disease susceptible) and SM (disease resistant) correlated with differential constitutive expression of Siglec's by cell lineages from these 2 species. In addition, it was reasoned that the effect chronic SIV infection would have on the expression of Siglec's would be of interest. Overall, except for Siglec-7 and 8 expression by a low (< 10%) frequencies of pDCs from mangabeys (data not shown), there was no neo-expression of any of the Siglec's following SIV infection in the RM and SM. The same Siglec's were thus expressed by the monocytes from uninfected and SIV infected cells from the 2 species of non-human primates. The only major difference noted was the marked increases in the frequency of monocytes that expressed Siglec-1 in RM post SIV infection (Fig. 5A, Table 1) and of interest a reproducible and significant decrease in the frequency of monocytes that expressed Siglec-1 in SM (Fig. 5B, Table 1). There was also a marked increase in the frequency of pDCs from SIV infected RM that expressed Siglec-1 in > 70% of the cells (data not shown). Minor differences included slight increases in the frequencies of monocytes that expressed Siglec-3 and 7 following SIV infection of RM. As stated above, the only neo-Siglecs expressed following SIV infection were Siglec-7 and 8 by a low frequency of pDCs from the SM. SIV infection had no other effect on Siglec expression by cells from SM post chronic SIV infection.

Since there was a marked increase in the frequency of monocytes and pDC's from RM that expressed Siglec-1 post SIV infection, additional studies were carried out such as the influence of plasma viral load and the disease stage on Siglec-1 expression in this species. As seen in Fig. 5C, there appeared to be a clear relationship between plasma viral load and Siglec-1 expression by monocytes from RM with > 80% showing Siglec-1 expression in animals with high viral load and about 50% expressing Siglec-1 in monkeys with relatively lower viral loads. In addition, a prospective study was performed on blood samples from a minimum of 3 RM and 3 SM prior to and following experimental SIV infection to determine the effects of disease stage on Siglec-1. As seen in Fig. 6A, whereas there is a steady increase in the frequency of monocytes that express Siglec-1 from baseline (pre-infection) following experimental infection of RM with SIV, there is a transient increase in the frequency of monocytes that express Siglec-1 from SIV infected SM. This increase sharply drops to below baseline levels in the SIV infected SM. While there is a correlation between increased Siglec-1 expression by monocytes with increased plasma viral loads in SIV infected RM, there did not appear to be clear relationship between viral loads/CD4 counts and changes in Siglec-1 expression in the 2 species (see Fig. 6B). Thus both species showed a decline in absolute CD4 counts and a rapid increase in plasma viral loads during acute infection. As noted previously, the CD4 count thereafter appears to be maintained in SM but steadily declines in RM. The reasons for such differences in the response to SIV infection in the 2 species thus remain to be defined.

In efforts to determine whether the reason for the increased/decreased expression of Siglec-1 by monocytes from RM and SM following SIV infection was a direct result of SIV infection or due to chronic immune activation characteristic of SIV infection of RM but not SM, highly

enriched populations of monocytes from uninfected RM and SM were infected in vitro with SIV as described in the methods section. Results from these studies showed that in vitro culture of monocytes from either uninfected RM or SM in the culture media containing M-CSF led to marked increases in the frequency and density of expression of Siglec-1 (80% positive with MFI > 400) prior to in vitro infection with SIV (data not shown). Following SIV infection, the in vitro cultured monocytes from both species continued to show the same level of increased Siglec-1 expression. Thus, while it was difficult to assess any further increase in Siglec-1 expression by SIV infection in cells from RM, it was intriguing to note that such increased expression was also maintained in cells from SM which showed marked decreases in Siglec-1 expression following SIV infection in vivo. These data suggest that in vitro activation of monocytes alone is sufficient for the synthesis and cell surface expression of Siglec-1 in cells from both species. In addition, these data support the view that differences exist in the form and results of chronic immune activation in SIV infected disease susceptible RM but not disease resistant SM.

DISCUSSION

HIV-1 infection has been documented to affect the function of a variety of cell lineages that comprise the innate immune system including natural killer cells [28;29;30], macrophages [31;32], and dendritic cells [31]. Impairment of the phagocytic capability of macrophages [33] and impairment of intracellular transport of cholesterol within macrophages [34] are among some of the functions altered by HIV-1 infection. Germane to the studies presented herein, HIV-1 infection has been shown to impair mannose-receptor-mediated phagocytosis of pathogens considered major contributors to opportunistic infections in patients with HIV-1 such as *P. carinii*, *Mycobacterium TB*, *Mycobacterium Avian Complex (MAC)* and *C. neoformans* [35]. Mannose receptors are a prototype of host defense proteins expressed by cells comprising the innate immune system which mediate recognition of specific carbohydrate moieties expressed by pathogens such as listed above. A similar role for Siglecs can be envisaged. Thus, while a primary role for a select class of Siglecs as mediators of cell adhesion has been documented [2], the role of Siglecs as molecules that can serve as receptors for pathogens and as molecules that can deliver inhibitory signals much like killer cells immunoglobulin-like receptors (KIR's) [36;37;38] is rapidly emerging. Since Siglec's are expressed predominantly by cells that comprise the innate immune system, it seemed reasonable to examine the expression of these molecules on these cell lineages specially in SIV infected animals. In addition, it also seemed logical to study the comparative expression of the Siglec's in the 2 non-human primate species studied herein since they demonstrate such distinct clinical outcome following SIV infection [12;14;17;18]. Thus, it was reasoned that differences in Siglec expression identified either due to species specific differences or following SIV infection of these 2 species may provide some clues as to their potential differential role in the pathogenesis of SIV infection with a potential for a follow up studies in HIV-1 infected humans.

Results of the studies reported herein show that a high frequency (80–90%) of peripheral blood monocytes from humans constitutively express several members of the Siglec family of molecules such as Siglec-3, 5, and 9 (and to a significantly lower density Siglec-7). Only about 30% of monocytes from RM express Siglec-7 and a low frequency express Siglec-1. In contrast, a high frequency of monocytes from the SM only express Siglec-3 and a lower frequency Siglec-1. Thus, expression of Siglec-3 by a significant frequency of monocytes appears to be conserved in humans and non-human primates but differences exist in the type of Siglec's and the level of constitutive expression of other Siglec's by monocytes from the 3 species examined.

Since Siglecs have been shown to be expressed by a number of other hematopoietic cell lineages such as neutrophils and granulocytes, these other lineages from the 2 non-human primate species were also studied. Data showed that whereas human neutrophils in particular expressed

Siglec-5, and -9 in humans, low to moderate frequencies of granulocytes from RM expressed Siglec-7 and none of the Siglec's examined were expressed by granulocytes from SM (data not shown). As far as dendritic cells, whereas human mDCs constitutively expressed not only the same Siglec's as their monocytes, a significant frequency also expressed Siglec-6. On the other hand, whereas mDCs from RM constitutively expressed only Siglec-7, mDCs from mangabeys only expressed Siglec-3. Of interest was the finding that pDCs from human constitutively expressed the same Siglec's as mDCs except for Siglec-9, which was predominantly expressed by the mDCs. On the other hand while the pDCs from RM only expressed Siglec-1, pDCs from SM constitutively expressed not only Siglec-1 but also low levels of Siglec-3. The significance of the differences in the type of Siglec's and the frequency of cell lineages that constitutively express these Siglec's is far from clear and needs to be addressed individually within the context of the function ascribed to each of these Siglec's.

The failure to detect Siglec-5 and 9 in the 2 non-human primate species and Siglec-1 and 7 in humans suggests that not only are their losses in the expression of Siglecs in humans but that there also evolutionary gains. Thus, as described above, humans appeared to have lost the expression of Siglec-5 on T cells [6] as compared with chimpanzees that readily express Siglec-5 on lymphoid cells which was shown to account for their decreased levels of proliferation following TCR ligation. However, as noted herein, Siglec-5 is readily expressed by human monocytes but not by lymphoid or monocytoid cells from RM and SM. Siglec-5 has been classified as an inhibitory receptor with a cytoplasmic domain that can bind SHP-1 and SHP-2 signaling molecules and mediate inhibition of cytokine based signaling [7;39] which maybe a function needed for regulating monocyte activation in humans but not non-human primates. In addition, Siglec-5 has also been shown to bind to NeuAcalpha 2,3 Gal, which is a terminal capping structure that is part of the lipopolysaccharide molecule of *N. meningitidis* [40] and thus facilitates its phagocytosis by monocytes in humans. This function may have been replaced by other receptors in non-human primates. A similar role for Siglec-9 has been proposed by its ability to function as an endocytic receptor [41], which is again expressed by human but not non-human primate monocytes. There is a growing awareness of the potential of Siglecs serving a function similar to KIRs. Thus, since KIRs appear to have evolved in response to and in concert with specific MHC molecules (their ligands), it is tempting to speculate that a similar association for Siglecs may become apparent both within a species and between evolving non-human primate species. Again, it is important to remember that the lack of detection could be due to a myriad of technical and biochemical reasons and thus interpretation of these data has to be guarded.

As outlined above, one of the major goals of the present study was to identify changes if any that SIV induces on the qualitative and quantitative expression of Siglec's by the hematopoietic lineage of cells from the disease susceptible and disease resistant RM and SM, respectively. Data obtained indicate that SIV infection primarily induces quantitative changes in the expression of Siglec's except for the induction of Siglec 7 and 8 on a low frequency of pDCs from SM, which is discussed below. Thus, of the quantitative changes noted, SIV infection in vivo clearly increases the frequency of monocytes that express Siglec-1 in RM and interestingly down regulates Siglec-1 expression by monocytes in vivo from SIV infected SM (see Fig. 6A and B). Siglec-1 also termed sialoadhesin was one of the first Siglec's identified [2]. It is the largest transmembrane member of the immunoglobulin super family (IgSF) with an extracellular domain made up of 17 immunoglobulin domains. Since it lacks tyrosine binding signaling motifs within its cytoplasmic domain and this domain is poorly conserved, it is likely that its primary function is more to serve as a binding partner for appropriate ligands and involved in cell adhesion and functions such as serving as a phagocyte receptor [42]. It has also been implicated in influencing T cell function and activation [43]. Increased expression of Siglec-1 following SIV infection in RM but not SM suggests that the above noted functions are likely to be perturbed following SIV infection of RM. In this regard it is of interest to note

that the frequency of monocytes that expressed Siglec-1 was correlated not only with plasma viral load but also with progressive stages of SIV infection in RM. Clearly since the frequency of monocytes in the blood that are infected with SIV are quite low, the high frequency of these monocytes that express Siglec-1 denotes that it is not directly due to infection of the cells but more likely a reflection of the activated state of the immune system during chronic and progressive SIV infection of RM similar to human HIV-1 infection [44; 45]. The fact that plasma viral loads do not influence the frequency of Siglec-1 expressing monocytes from SIV infected SM coupled with the finding that there is in general a low immune activated state in such SIV infected SM [18; 46], is consistent with the previous statement. The chronic activated state of SIV infected RM is also likely the basis for increased frequency of monocytes and granulocytes that express Siglec-7 and since this is likely an inhibitory receptor [47], it is reasonable to assume that its ligation on monocytes and granulocytes must deliver inhibitory signals and decrease macrophage and granulocyte function in SIV infected RM.

In summary, the data presented herein show that differences exist in not only the types of Siglec's but also the lineage of cells that express the Siglec's in hematopoietic cells from humans as compared with the 2 species of non-human primates. The reasons for these differences clearly require additional studies but in keeping with our overall rationale to conduct these studies, the data clearly demonstrate that there is significant increase in the frequency of monocytes that express Siglec-1 and to some degree Siglec-7 following SIV infection selectively in RM but not SIV infected SM. These findings are consistent with our general observation of a lack of immune activation state in SM despite viral loads that far exceed those that result in progression to disease and ultimately death in RM.

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Fig. 1. Aliquots of Ficoll hypaque purified PBMCs from healthy adult humans ($n = 10$), normal uninfected adult rhesus macaques ($n = 13$) and uninfected adult sooty mangabeys ($n = 12$) were stained with antibodies with specificity for human Siglec-1, 3, 5 (2 clones), 6, 7 (2 clones), 8, 9, 10 and 11 and analyzed by flow cytometry by gating on monocytes based on scatter profiles. Only those showing significant staining with one of 3 species is depicted. Data are representative profiles from each of the 3 species.

Fig. 2.

RT-PCR assisted detection of mRNA coding for Siglec-1 (128 bp), Siglec-5 (134 bp), Siglec-7 (192 bp) and Siglec-9 (293 bp) in RNA isolated from enriched populations of monocytes from humans, rhesus macaques and sooty mangabeys is shown. (M = molecular weight standards; S1, 5, 7, and 9 refer to Siglecs). Data are representative of 3 similar assays.

Leader peptide | Ig-like Domain1
 Human MLPLLLPLLWGGSLQEKPVYELQVQKSVTVQEGLCVLVPCSFSPWRSWYSSPPLYVYVFRDGEIPYYAEVVAT
 Rhesus1A.....E.G.....N.....PS.....SQ.....A...
 Rhesus2A.....E.G.....N.....PS.....SQ.....A...
 Mangabey1A.....E.G.....GN.....PS.....N.....FG.P...
 Mangabey2A.....ESG.....GN.....PS.....N.....FG.P...

Human NNPDRRVKPEQTQGRFRLLDGVQKKNCSLSIGDARMEDTGSYFFRVERGRDVKYSYQONKLNLEVTALEKPDHIF
 Rhesus1RN.....G...N.Y.....N...N.V.....T.R..VR.
 Rhesus2RN.....G...N.Y.....N...N.V.....T...VR.
 Mangabey1N.E...S.....W...G.....G.....S...A..K...T.L.....T...V...
 Mangabey2N.E...S.....W...G.....G.....S...A..K...T.L.....T...V...

Ig-like Domain2
 Human LEPLESGRPTRLSCSLPGSCEAGPPLTFSTGNALSPDPETTRSELTLTPRPEDHGTNLTCQMKRQGAQVTE
 Rhesus1L.....V.R.....DV.....G.....HV.H.....
 Rhesus2L.....V.R.....DV.....G.....HV.H.....
 Mangabey1L.....V.R.....D..N.....HV.H.....
 Mangabey2L.....V.R.....D..N.....HV.H.....

| Ig-like Domain3
 Human RTVQLNVSYPQTITIFRNGIALEILQNTSYLPVLEGQALRLLCDAPSNPFAHLSWFGSPALNATPISNTGILE
 Rhesus1N.....T.....T.L.....E.....E.....A.S.P.T...A...
 Rhesus2N.....T.....T.L.....E.....E.....A.S.P.T...A...
 Mangabey1N.....T.....H...T.L.....E.....A.S.P...A...A
 Mangabey2N.....T.....H...T.L.....E.....A.S.P...A...A

| Ig-like Domain4
 Human LRRVRSAAEEGGFTCRAQHPLGFLQIFLNLSVYSLPQLLGPSCSWEAEGHCRCSFRARPAPSLCWRLEEKPLEGN
 Rhesus1 .P...EFTK...V.....S.H.....S...S...W.....W.G.....
 Rhesus2 .P...EFTK...V.....S.H.....S...S...W.....W.G.....
 Mangabey1 .P...EFTK...V.....S.H.....G...S...S...W.....W.G.....
 Mangabey2 .P...EFTK...V.....S.H.....S...S...W.....W.G.....

Human SSQGSFKVNSSSAGPWANSSLILHGGLSSDLKVSCKAWNIYGSQSGSVLLQLQGRSNLGTGVVPAALGGAGVMALL
 Rhesus1T.V.....G..T.....V....L..R.....
 Rhesus2T.V.....G..T.....V....L..R.....
 Mangabey1F.....T.G...Y..G...N.....V....L..R.....
 Mangabey2F.....T.G...G...N.....V....L..R.....

Transmembrane domain | cytoplasmic tail
 Human CICLCILIFFLIVKARRKQAAGRPEKMDDEDPIMGTTITSGSRKKPWPDSPGDQASPPGDAPPLEEQKELHYASLSF
 Rhesus1V.....VSWD.....A...T...G...Q.....
 Rhesus2V.....VSWD.....A...T...G...Q.....
 Mangabey1V...T.....G.....VSWD.....A...T...G...Q.....
 Mangabey2V...T.....VSWD.....A...T...G...Q.....

Human SEMKSREPKDQEAPSTTEYSEIKTSK.
 Rhesus1V...N...
 Rhesus2V...N...
 Mangabey1V...N...
 Mangabey2V...N...

Fig. 3.

The protein sequence of Siglec-5 from rhesus macaques and sooty mangabeys and their alignment with the published protein sequence of human Siglec-5 is shown. Note there is 87% homology between the human and non-human primate sequences and there are 8 amino acid residues that are similar between human and mangabeys but not rhesus macaques.

A.

B.

Human

Fig. 4.

Flow cytometric analysis of Siglec expression by gated populations of myeloid (mDC) and plasmacytoid dendritic (pDC) cells from rhesus macaques and sooty mangabeys. The gating strategy utilized for human samples is shown in Fig. 6 A and for rhesus macaques and sooty

mangabeys on left portions of Fig. 6C and 6D, respectively. PBMC samples were first gated on forward and side scatter profiles and then on cells that were lineage negative, HLA-DR⁺ and were either CD11c⁺ (mDC) or CD123⁺ (pDC). Representative data from one of the 3 similar experiments is depicted. Fig. 6B, C and D show the profiles of human, rhesus macaques and sooty mangabeys, respectively. Note the 2 distinct populations of mDC in samples from sooty mangabeys based on differences in the expression of HLA-DR.

Fig. 5.

Effect of chronic SIV infection and viral load on the expression of Siglec's by monocytes from rhesus macaques (n = 13) and sooty mangabeys (n = 13) as determined by flow cytometry. Aliquots of gated population of monocytes in the PBMCs from 13 uninfected and 13 SIVmac239 infected rhesus macaques and 12 uninfected and 13 naturally SIV infected sooty mangabeys were analyzed for the expression of Siglec-1, 3, 5, 6, 7, 8, 9, 10 and 11. Data presented illustrate the Mean $\pm$ S.D. of the frequency of monocytes from Rhesus macaques (Fig. 5A) and sooty mangabeys (Fig. 5B) that express the appropriate Siglecs. Fig. 5C shows the effect viral load has on the expression of Siglec-1 by monocytes from 12 chronically infected rhesus macaques with low (< 10,000 viral copies/ml of plasma), 11 samples with high (> 100,000 viral copies/ml of plasma) viral loads and for comparison uninfected monkeys of each of the 2 species.

Fig. 6.

Prospective analysis of the effect of SIV infection on Siglec-1 expression by monocytes from a minimum of 3 rhesus macaques and 3 sooty mangabeys as determined by flow cytometry.

A) Aliquots of PBMC samples from each of the 6 monkeys prior to and at 4, 8, 16 and 36 weeks post experimental SIV infection were stained with anti-Siglec-1 antibody and the frequency of the gated population of monocytes evaluated. B) The absolute number of CD4⁺ T cells and plasma SIV viral load were determined in aliquots of the same blood sample as

used for Siglec studies. Data shown are Mean $\pm$ S.D. of the 3 samples from each of the 2 species.

Table 1

Comparative Frequencies of Monocytes from SIV Infected and SIV Chronically Infected Rhesus Macaques and Sooty Mangabeys that Express SIGLECs						
SIGLECS	HUMAN (n = 10)	SIV – RM (n = 13)	SIV + RM (n = 13)	SIV – SM (n = 12)	SIV + SM (n = 13)	
SIGLEC-1	<1	7.9 (4.4)	55.6 (22.9)	25 (13.6)	8.3 (3.0)	
SIGLEC-3	94.11(2.4)	32.3 (15.4)	50.3 (23.2)	87.3 (3.9)	80.1 (6.0)	
SIGLEC-5.1	87.2 (3.8)	<1	<1	<1	<1	
SIGLEC-5.2	86.9 (4.4)	<1	<1	<1	<1	
SIGLEC-6	1.9 (0.6)	<1	<1	<1	<1	
SIGLEC-7.1	3.6 (1.3)	33.5 (10.5)	58.7 (17.0)	<1	<1	
SIGLEC-7.2	3.3 (1.9)	33.2 (11.2)	60.3 (18.8)	<1	<1	
SIGLEC-8	<1	<1	<1	<1	<1	
SIGLEC-9	86.4 (3.6)	<1	<1	<1	<1	
SIGLEC-10	<1	<1	<1	<1	<1	
SIGLEC-11	<1	<1	<1	<1	<1	

Data are mean (=/- S.D). Frequency of normal human monocytes that express SIGLECs are shown as a reference.