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Probing the Interaction between Feline Immunodeficiency Virus and CD134 by Using the Novel Monoclonal Antibody 7D6 and the CD134 (O×40) Ligand▽

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The feline immunodeficiency virus (FIV) targets activated CD4-positive helper T cells preferentially, inducing an AIDS-like immunodeficiency in its natural host species, the domestic cat. The primary receptor for FIV is CD134, a member of the tumor necrosis factor receptor superfamily, and all primary viral strains tested to date use CD134 for infection. We examined the expression of CD134 in the cat using a novel anti-feline CD134 monoclonal antibody (MAb), 7D6, and showed that as in rats and humans, CD134 expression is restricted tightly to $CD4^+$, and not $CD8^+$, T cells, consistent with the selective targeting of these cells by FIV. However, FIV is also macrophage tropic, and in chronic infection the viral tropism broadens to include B cells and $CD8^+$ T cells. Using 7D6, we revealed CD134 expression on a B220-positive (B-cell) population and on cultured macrophages but not peripheral blood monocytes. Moreover, macrophage CD134 expression and FIV infection were enhanced by activation in response to bacterial lipopolysaccharide. Consistent with CD134 expression on human and murine T cells, feline CD134 was abundant on mitogen-stimulated $CD4^+$ T cells, with weaker expression on $CD8^+$ T cells, concordant with the expansion of FIV into $CD8^+$ T cells with progression of the infection. The interaction between FIV and CD134 was probed using MAb 7D6 and soluble CD134 ligand (CD134L), revealing strain-specific differences in sensitivity to both 7D6 and CD134L. Infection with isolates such as PPR and B2542 was inhibited well by both 7D6 and CD134L, suggesting a lower affinity of interaction. In contrast, GL8, CPG, and NCSU were relatively refractory to inhibition by both 7D6 and CD134L and, accordingly, may have a higher-affinity interaction with CD134, permitting infection of cells where CD134 levels are limiting.

The initial event in the process of viral entry into a target cell is the interaction between the virus and its cellular receptor, and the specificity of this interaction determines both the cell tropism and the pathogenicity of the virus. The primary receptor for the feline immunodeficiency virus (FIV) is CD134 (OX40), a member of the tumor necrosis factor receptor (TNFR) superfamily. Ectopic expression of feline CD134 in nonpermissive cells renders the cells permissive for binding of both virus (29) and soluble envelope glycoprotein (Env) (12). CD134 expression alone is insufficient to confer susceptibility to infection with FIV; infection requires the expression of a coreceptor which for FIV is the chemokine receptor CXCR4 (CD184) (37, 40). In the presence of the CXCR4 antagonist AMD3100, CD134-dependent infection is ablated. Further, the interaction between FIV and CD134 is species specific; human CD134 does not support FIV infection (29) and, thus, FIV is not xenotropic for human cells. The species specificity of the FIV-CD134 interaction has facilitated the mapping of the determinants on CD134 that mediate both the binding of soluble Env and viral entry. Using chimeric molecules generated by exchanging fragments of feline and human CD134, the binding site for soluble (dimeric) Env was mapped to the first cysteine-rich domain (CRD1) of CD134 (11); however, although expression of the feline CRD1 in the context of human CD134 rendered cells permissive for infection with the PPR strain of FIV, additional determinants in the second CRD (CRD2) are essential for infection with primary strains, such as GL8 (39). CRD2 contributes to the CD134 ligand (CD134L) binding site on feline CD134 (38), indicating that FIV infection may be sensitive to modulation by CD134L (OX40L, CD252).

Previous analyses of CD134 expression on feline cells have relied on a cross-species-reactive anti-human CD134 monoclonal antibody (MAb) and have yielded inconsistent data (11, 21, 29). Observations regarding CD134-dependent targeting of FIV have relied on inferred data from binding experiments with recombinant dimeric envelope glycoprotein immunoglobulin G (IgG)-Fc fusion protein (SU-Fc) and subsequent immunoblotting and could not examine surface expression of CD134 due to the absence of reagents with sufficient specificity and sensitivity (12). Similarly, the effect of CD134L on FIV infection has not been assessed rigorously, as a feline CD134 binding ligand was not available. The effect of CD134L on FIV infection was inferred from binding studies using human

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CD134L and chimeric CD134 molecules bearing CRD1 of feline CD134 in the context of human CD134 (11). Bearing in mind this caveat, the binding site for dimeric SU-Fc was mapped to CRD1 of CD134, while the binding site for human CD134L appeared to be formed by CRDs 2 and 3 (11). The failure of human CD134L to block binding of dimeric FIV SU-Fc to cells expressing a chimeric feline CRD1 in the context of human CD134 was interpreted as suggesting that FIV infection would not be modulated by native feline CD134L (11). Subsequent structural analyses of the human and murine CD134-CD134L interaction have revealed that the receptor and ligand make multiple contacts using regions from all three CRDs of CD134 (7), including CRD1, the region bearing the predicted FIV Env binding site. Moreover, recent studies have demonstrated that many primary strains of FIV require a complex determinant on CD134 that requires determinants in CRD2, indicating a possible overlap with the CD134L binding site (38, 39) and the potential for modulation of FIV infection by CD134L. In this study we evaluated the role of CD134 and CD134L in FIV infection. To facilitate our analyses we generated a novel monoclonal antibody recognizing feline CD134 with a high affinity and we cloned the feline CD134L homologue. Using these reagents, we analyzed the expression of CD134 in the domestic cat and we show that FIV infection is modulated by both antibody and ligand binding.

MATERIALS AND METHODS

Cells and viruses. MYA-1 (23), MCC (6), CLL (39), and NSO cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.11 mg/ml sodium pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin (complete RPMI). 293T and NP2 cells were maintained in Dulbecco's modification of Eagle's medium supplemented as above. The medium for MYA-1 cells was supplemented with conditioned medium from a murine cell line (L2.3) transfected with a human interleukin-2 (IL-2) expression construct (equivalent to 100 U/ml of recombinant human IL-2) and 50 μM 2-mercaptoethanol. All media and supplements were obtained from InVitrogen Life Technologies Ltd. (Paisley, United Kingdom). Stable cell lines expressing constructs based on the pDONAI (Takara) or PCR3 (InVitrogen) expression vectors were maintained in G418-supplemented medium (InVitrogen, Paisley, United Kingdom), while those based on pTorsten (31) were maintained in hygromycin-supplemented medium. Splenic macrophages were isolated from spleen tissue collected post mortem. Cells were separated from tissue by teasing with crossed scalpels, washed by centrifugation, and plated overnight at 37°C. Nonadherent cells were removed by pipetting, while adherent cells were cultured in complete RMI supplemented with supernatant from Chinese hamster ovary (CHO) cells stably transfected with feline granulocyte-macrophage colony-stimulating factor (GM-CSF; kindly provided by S. Dunham). Lipopolysaccharide (LPS) stimulation experiments used LPS from *Escherichia coli* 0127:B8 (Sigma, Poole, United Kingdom).

Antibodies and flow cytometry. Fluorescein isothiocyanate (FITC)-conjugated anti-feline CD4 (VPG34), R-phycoerythrin (PE)-conjugated anti-feline CD8αβ (VPG9), and anti-human CD14 (Tuk4)–FITC conjugate were obtained from AbD Serotec (Oxford, United Kingdom). Anti-human CD134 (BerACT35) was obtained from Alexis Biochemicals (Axxora Ltd., Nottingham, United Kingdom). Anti-mouse B220 (CD45R) was from BD Biosciences (Pharmingen, Oxford, United Kingdom). Anti-human CXCR4 (no. 44701) was obtained from R&D Systems, Abingdon, Oxford, United Kingdom. Cells to be processed for flow cytometry were resuspended in phosphate-buffered saline (PBS) supplemented with 1.0% (wt/vol) bovine serum albumin, 0.1% (wt/vol) sodium azide (PBA). Cells were incubated with 1 μg of primary antibody for 30 min at 4°C and then washed twice with PBA by centrifugation at 1,000 rpm for 5 min. Bound primary antibody was detected with the appropriate anti-mouse IgG secondary antibody (AbD Serotec) corresponding to the isotype of the primary antibody and conjugated to either FITC or PE. Cells were incubated with secondary antibody for 30 min at 4°C and then washed twice with PBA by centrifugation at 1,000 rpm for 5 min and resuspended in 1 ml of PBA for analysis. All samples were analyzed on a Beckman Coulter EPICS MCS-XL flow cytometer, 10,000 events being collected for each sample in LIST mode. Data were analyzed using EXPO 32 ADC Analysis (Advanced Cytometry Systems).

Production of anti-feline CD134 monoclonal antibody. Feline CD134 cDNA (29) was amplified from pDONAI.fCD134 using the oligonucleotides 5′-CGATCTAGAATGAGGGTGGTTGTGGGGGCT-3′ and 5′-ATGGGATCCCGCTGGGGACCCCTGGGGGGCTC-3′ and the Expand high-fidelity PCR system and cloned as an XbaI/BamHI fragment into the pTorsten expression vector (30). CHO cells were then transfected with the pTorsten.fCD134-Fc construct using Superfect (QIAGEN Ltd., Crawley, United Kingdom) and selected in hygromycin (Sigma, Poole, United Kingdom). CD134–IgG-Fc was then purified by affinity chromatography using protein A-Sepharose (HiTrap Protein A HP; GE Healthcare, Chalfont St. Giles, United Kingdom) and bound protein eluted with 0.1 M glycine pH 3.0, neutralizing the eluate immediately to pH 7.0 by collection into 1.5 M Tris pH 8.6. The integrity of the purified protein was assessed by immunoblotting, and the fusion protein was detected with goat anti-human IgG-Fc horseradish peroxidase conjugate (Sigma, Poole, Dorset, United Kingdom) and visualized with amino-9-ethylcarbazole (Vectastain AEC substrate kit; Vector Laboratories Ltd., Peterborough, United Kingdom). BALB/c mice received one immunization of 25 μg CD134–IgG-Fc protein in complete Freund's adjuvant followed by two further immunizations at monthly intervals with 25 μg protein in incomplete Freund's adjuvant. Prebleed samples were analyzed for anti-CD134 reactivity by enzyme-linked immunosorbent assay (ELISA) prior to a final single immunization 1 week in advance of splenectomy. Lymphocytes were isolated from spleens by centrifugation over Ficoll (GE Healthcare), washed, and fused with NSO myeloma cells. Cells were then plated in complete RPMI supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT medium supplement Hybri-Max; Sigma). Approximately 2 weeks postplating, supernatants from 1,008 wells were screened by ELISA for anti-CD134-Fc reactivity. A 0.25-μg aliquot from each well of CD134-Fc was bound overnight to 96-well high-binding ELISA assay plates (Greiner Bio-One) in coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) at room temperature. Plates were then washed and blocked with 1.0% skimmed milk (Marvel) in phosphate-buffered saline containing 0.1% Tween 20 (PBS-Tween). Wells were then incubated with hybridoma culture supernatants for 1 h, washed in PBS-Tween, and then incubated for a further hour with sheep anti-mouse IgG–horseradish peroxidase conjugate (Sigma) in 1.0% skimmed milk–PBS-Tween. Wells were washed once again with PBS-Tween, and then 3,3′,5,5′-tetramethylbenzidine substrate (Sigma) was added and incubated for 30 min, at which time the absorbance at 450 nm was recorded using a Microscan Ascent plate reader (Thermo Life Sciences). Supernatants from 129 hybridoma wells containing anti-CD134 activity were screened by flow cytometry against CLL-fCD134 cells (38), identifying seven supernatants with activity against native CD134 on mammalian cells. Of these seven hybridomas, a single hybridoma (7D6) proved stable and had a reactivity that was confirmed as specific for CD134; this hybridoma was cloned and expanded. Culture supernatants were pooled and filtered at 0.45 μm, and the monoclonal antibody was affinity purified on protein A-Sepharose, eluted with 0.1 M sodium citrate pH 3.0, and neutralized with 1.5 M Tris buffer pH 8.6.

Reactivity of the MAb was confirmed by flow cytometry and immunoblotting. In the latter case, bound MAb was detected using biotinylated horse anti-mouse IgG (Vector Laboratories Ltd.) followed by development with VECTASTAIN ABC-AmP reagent and 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium substrate.

Molecular cloning of feline CD134L. The 2X cat genome database (http://www.ncbi.nlm.nih.gov/genome/guide/cat/) was searched using the NCBI gene symbol TNFSF4. The genomic fragments identified were then aligned with published murine and human CD134L sequences (U12763 and X79929, respectively) and used to identify the 5′ and 3′ regions of the orthologous feline CD134L coding sequence from which oligonucleotide primers could be designed based on regions of consensus sequence. Total RNA was prepared from MCC and MYA-1 cells using RNAqueous (Ambion, Applied Biosystems, Warrington, United Kingdom), and first-strand cDNA was synthesized using oligo(dT) primer and RETROscript (Ambion) as per the manufacturer's instructions. Feline CD134L cDNA was then amplified by PCR from total cDNA, using the primers 5′-GAA GAT GGA AGG GGT CCA ACC CCT GGA TGA-3′ and 5′-CTC AAA GGA CAC AGA A(G/T)C CAC CAG GAT TTT-3′ and Roche Expand high-fidelity PCR reagent (Roche) on a Perkin-Elmer 9700 thermal cycler. Products were then cloned into the pCR-TOPO vector (TOPO TA cloning kit; InVitrogen) and their nucleic acid sequence determined by cycle sequencing on an Applied Biosystems 9700 thermal cycler using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) followed by analysis on an Applied Biosystems 3700 genetic analyzer. For ectopic expression of native feline CD134L (fCD134L) in

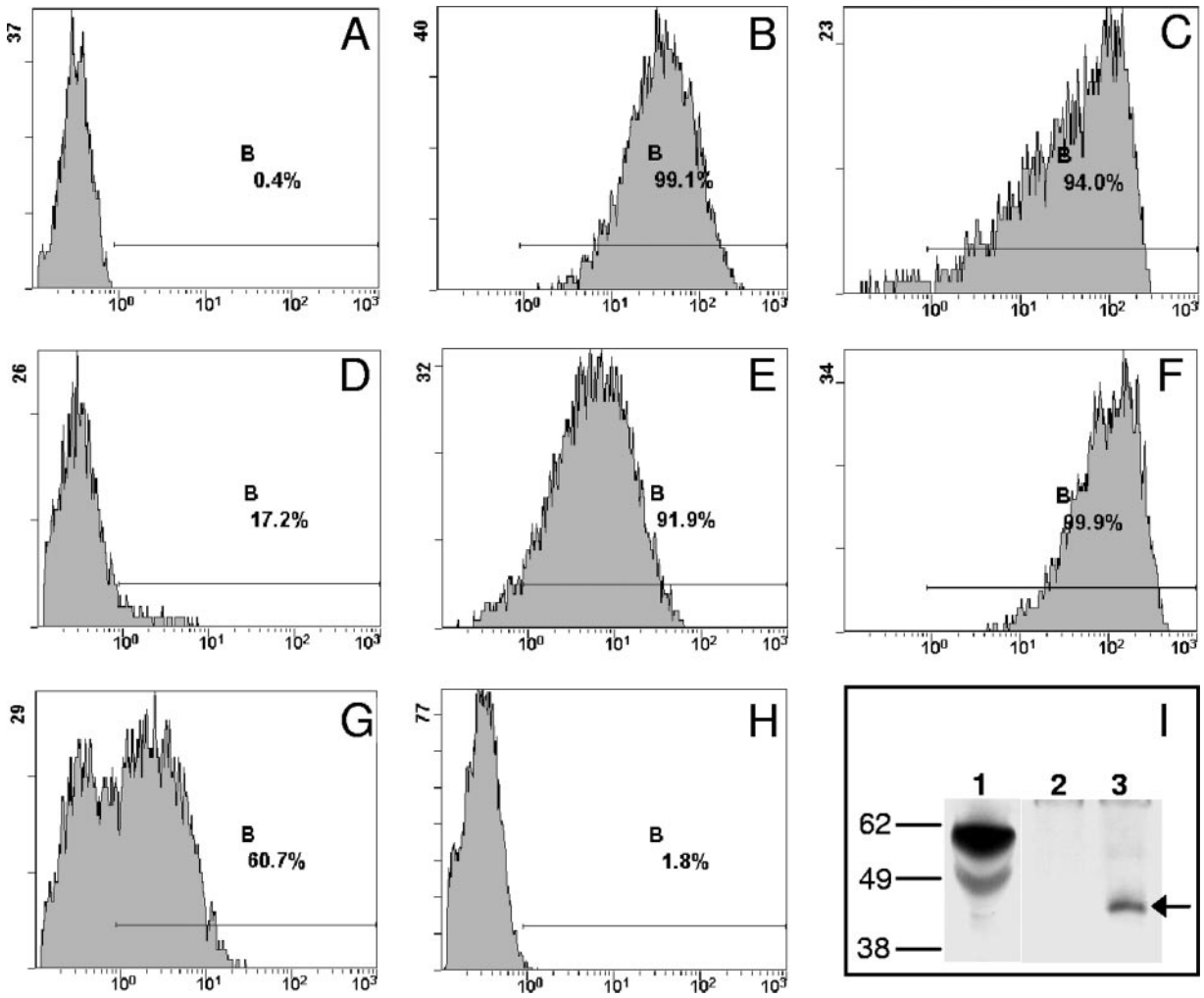


FIG. 1. Characterization of anti-feline CD134 MAb 7D6. MCC cells expressing feline CD134 (B and E), human CD134 (C and F), or vector only (A and D) were stained with 7D6 (A to C) or BerACT35 (D to F). MYA-1 cells (G and H) were stained with 7D6 (G) or BerACT35 (H). Primary antibodies were detected with phycoerythrin-conjugated anti-mouse IgG. Cells were processed for flow cytometry, and 10,000 events were collected in LIST mode. (I) Reactivity of 7D6 in an immunoblot assay. Affinity-purified soluble feline CD134-Fc (lane 1) or lysates from MCC vector-only control (lane 2) or CD134-transduced cells (lane 3) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with 7D6 MAb (endogenous; full-length CD134 is indicated by the arrow, lane 3).

feline cells, the fCD134L cDNA was subcloned into pDONAI retroviral vector (Takara) and packaged into murine leukemia virus particles by cotransfection into HEK 293T cells with pHIT60 and PCI-VSV-G as previously described (35). Viral pseudotypes were then used to transduce MCC cells before selection 72 h posttransduction in G418-supplemented medium.

Preparation of HIV (FIV) pseudotypes. FIV *env* gene expression constructs GL8, B2542, CPG41, and 0827 have been described previously (29, 38, 39). Five μg of each VR1012-*env* and 7.5 μg of pNL4-3-Luc-E$^-$R$^-$ were cotransfected into HEK 293T cells by using the calcium-phosphate coprecipitation technique essentially as described previously (19). Culture supernatants were collected at 48 h posttransfection, filtered at 0.45 μm, and frozen at −80°C until required. Adherent target cell lines were seeded at 1×10^4 cells per well of a CulturPlate-96 assay plate (Perkin-Elmer, Life and Analytical Sciences, Beaconsfield, United Kingdom) and cultured overnight, while suspension target cell lines were seeded at 5×10^4 cells per well and used immediately. The cells were then infected with 50 μl of human immunodeficiency virus (HIV) (FIV) luciferase pseudotypes and cultured for 72 h, and then luciferase activity was quantified by the addition of 50 μl of Steadylite HTS (Perkin-Elmer) luciferase substrate and measurement by single-photon counting on a MicroBeta TriLux luminometer (Perkin-Elmer).

Inhibition of viral entry. A total of 5×10^4 MCC-fCD134 or MYA-1 cells were incubated with increasing concentrations of purified 7D6 monoclonal antibody or CD134L in complete medium for 30 min at room temperature. Viral pseudotypes were added, incubated for a further hour, and then washed once by centrifugation. Cells were then plated in 96-well culture-treated luciferase assay plates (CulturPlate 96) in complete medium in the presence of maintenance antibody or ligand at a 1:3 dilution of the blocking concentration. Cultures were maintained for 72 h postinfection, at which point glow luciferase assay substrate was added and luminescence quantified.

Production of recombinant IgG-Fc fusion proteins. Fc-murine CD134L (mCD134L) was produced from Fc-mCD134L-expressing 293 cells in CELLine AD1000 bioreactor flasks (Integra Biosciences [Scientific Laboratory Supplies, Nottingham, United Kingdom]) in medium supplemented with low-IgG serum (Integra Biosciences). Culture supernatant was filtered at 0.22 μm and frozen at −80°C prior to use in order to preserve optimal bioactivity. Protein concentration was estimated by immunoblotting using protein A-purified Fc-mCD134L of known concentration as a standard. Fc-mCD134L and Fc-hCD134L were also expressed from vectors described previously (pPS1763 and PS1466) (5). The extracellular domain of feline CD134L was subcloned as a PstI/EcoRI fragment into the same vector (pPS1297) (20). For this purpose, fCD134L cDNA was amplified using primers 5′-GCTGCAGGTGCCACCTCAGTATCCTCCAATT-3′ and 5′-CGAATTCTCAAAGGACACAGCCACCAGG-3′. FIV Env SU-Fc fusion proteins were produced by amplifying the SU coding sequence with the oligonucleotide primers 5′-CGATCTAGAAACAATAATTATGGCAGAAG-3′

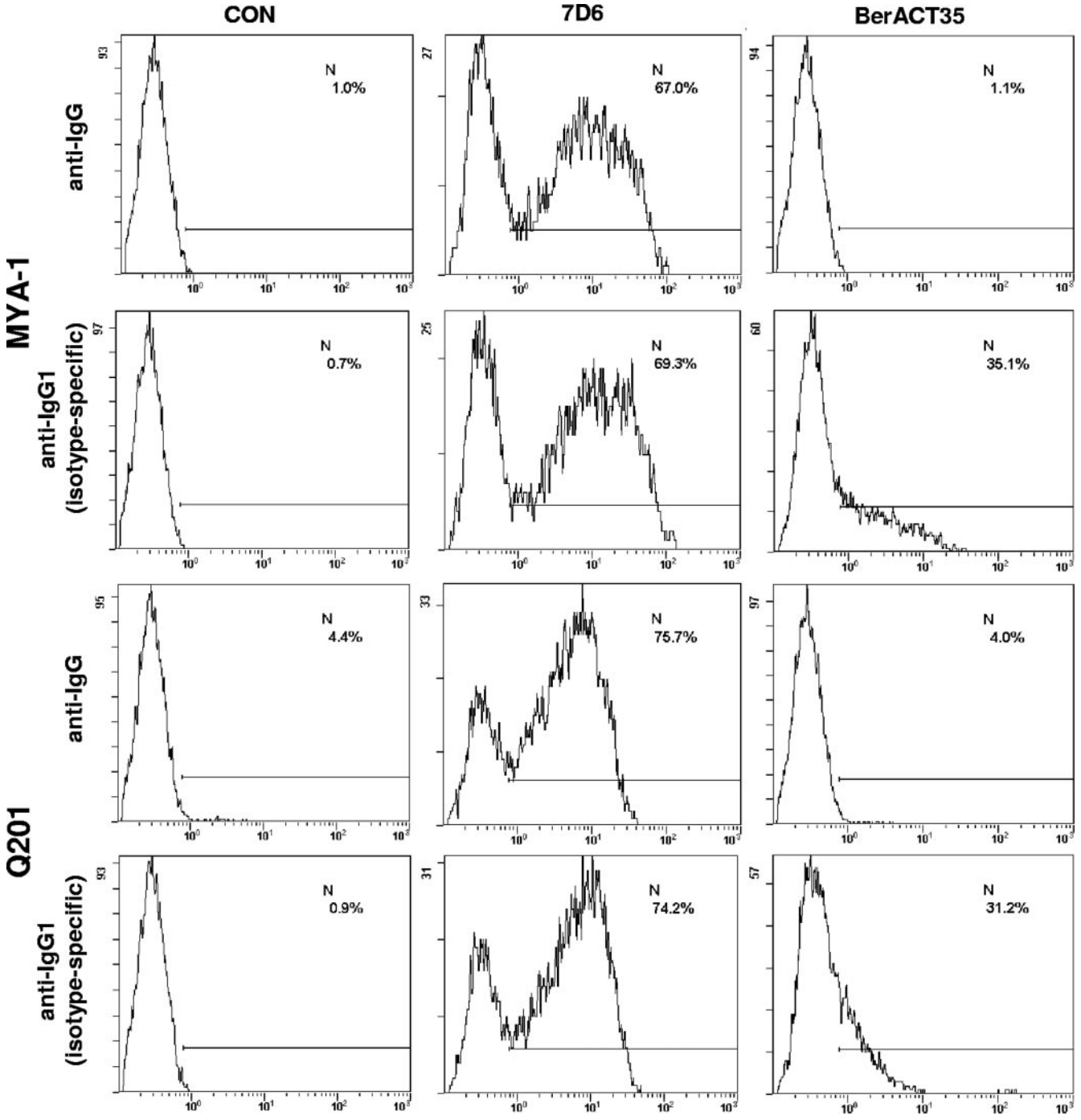


FIG. 2. Detection of CD134 on IL2-dependent T cells using BerACT35. MYA-1 or Q201 T cells were stained for CD134 using anti-feline CD134 MAb 7D6 or the cross-species-reactive anti-human CD134 MAb BerACT35. Bound antibody was detected using either PE-conjugated $F(ab')_2$ fragment of rabbit anti-mouse IgG (anti-IgG) or isotype-specific goat anti-mouse IgG1 (anti-IgG1 isotype specific). A total of 10,000 events were collected for each sample. Plots display fluorescence intensity (abscissa) versus the number of events (ordinate).

and 5′-GGCGGCCGCTGGTACCAC(C/T)AAGTAATC-3′ corresponding to the start codon for the Env leader sequence and the SU/TM junction, respectively. The amplified products were cloned as XbaI/NotI fragments into pTorsten (30), expressed in CHO cells, and purified as above.

Binding of FIV SU-Fc to CD134. MCC cells expressing feline CD134 were incubated with increasing concentrations of monoclonal antibodies 7D6, BerACT35 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-feline CD8 (VPG9; AbD Serotec) for 30 min at room temperature in complete RPMI supplemented with 10 mM *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid). A 0.5-μg aliquot of SU Fc fusion protein was added and incubated for a further 30 min at room temperature. Cells were then washed twice by centrifugation through PBA at 1,000 rpm and incubated with PE-conjugated anti-human IgG-Fc (eBioscience, San Diego, CA) for 30 min at 4°C. Cells were then washed twice with PBA by centrifugation at 1,000 rpm for 5 min and resuspended in 1 ml of PBA for analysis and analyzed by flow cytometry as above.

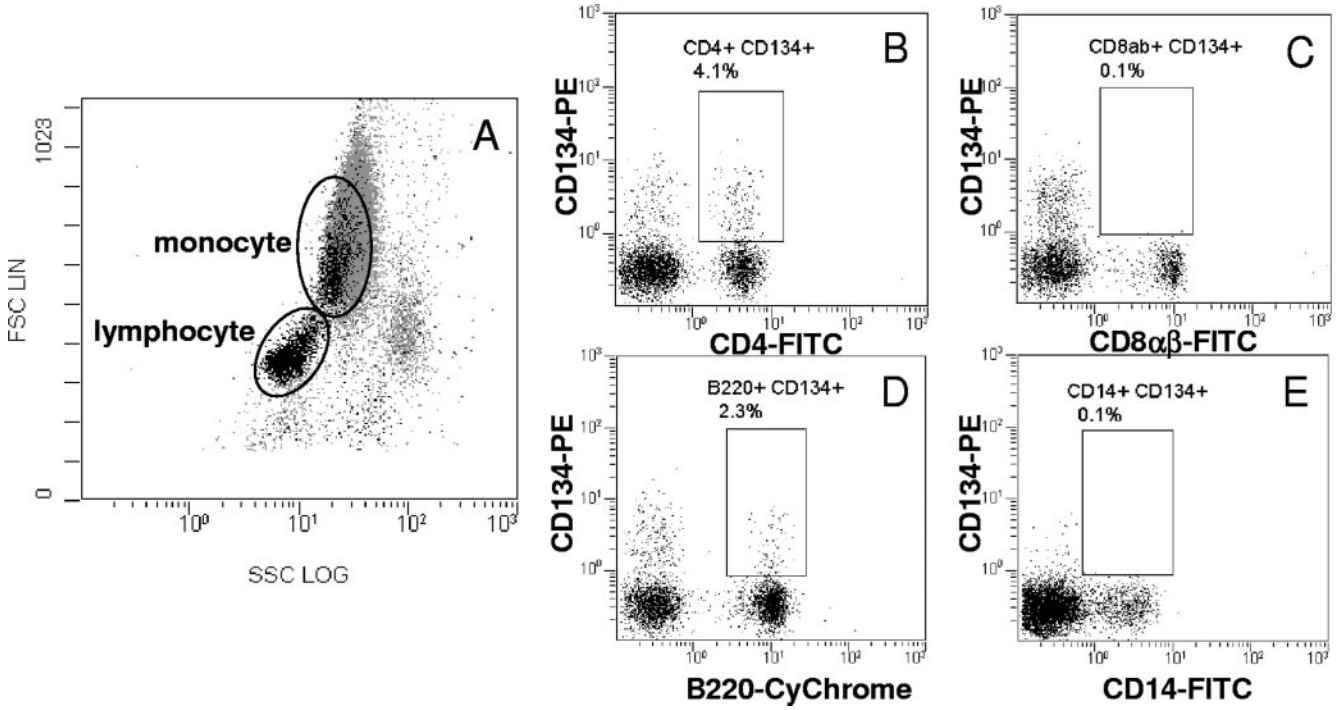


FIG. 3. CD134 expression on feline PBMC. Lymphoid and monocytoid analysis gates (A) were set based on forward scatter (FSC) versus 90° side scatter (SSC) and expression of CD14. CD134 expression was detected on the total population using MAb 7D6 followed by a PE–anti-mouse conjugate, and cells were then double-stained with FITC-conjugated anti-CD4 (B), CD8αβ (C), or CD14 (E) or CyChrome-conjugated B220 (D). Events were recorded in the lymphoid gate (B to D) and monocytoid gate (E). Cells were processed for flow cytometry, and 10,000 events were collected in LIST mode. Data are representative of multiple analyses ($n = 13$).

Nucleotide sequence accession number. The sequence of feline CD134L has been lodged with GenBank, accession number DQ269941.

RESULTS

An anti-feline CD134 monoclonal antibody. The immunoreactivity of the 7D6 monoclonal antibody was examined on feline MCC cells stably transduced with feline or human CD134 (Fig. 1). 7D6 labeled 99.1% of feline CD134-expressing cells (Fig. 1B; mean fluorescence intensity [MFI], 50.4) and 94.0% of human CD134-expressing cells (Fig. 1C; MFI, 64.2) with no background on vector-only control cells (Fig. 1A; 0.4%). Anti-human CD134 (BerACT35) recognized 99.9% of human CD134-expressing cells (Fig. 1F; MFI, 116.3) and 91.9% of feline CD134-expressing cells (Fig. 1E; MFI, 9.6), a reduction in mean fluorescence intensity of ~50-fold. It was notable that BerACT35 also gave significant background binding on the vector-only control cells (Fig. 1D; 17.2%). Thus, where antigen is abundant, as in transduced MCC cells, either antibody performs well. However, the anti-feline CD134 antibody may perform more efficiently at low antigen levels. To test this hypothesis, CD134 expression on IL-2-dependent T cells (MYA-1 cells) was analyzed using either 7D6 or BerACT35. Antibody binding was detected using the same PE–anti-mouse IgG (whole molecule) as in Fig. 1A to F. While 7D6 recognized 60.7% of MYA-1 cells (Fig. 1G), BerACT35 failed to detect CD134 expression on the MYA-1 cells (Fig. 1H; 1.8% compared with isotype-matched control of 0.3% [not shown]), indicating that the 7D6 antibody was more sensitive in the detection of low levels of feline CD134 expression. Staining of IL-2-dependent T cells, such as MYA-1 or Q201 with BerACT35 is weak and requires amplification with a high-affinity secondary antibody conjugate (29). Therefore, we compared the detection of CD134 on MYA-1 and Q201 cells using MAbs 7D6 and BerACT35 in conjunction with a broad-specificity conjugate (anti-IgG) or an isotype-specific conjugate (anti-IgG1). While 7D6 detected CD134 on both cell lines irrespective of the conjugate, BerACT35 only detected CD134 when used in conjunction with the isotype-specific anti-IgG1 conjugate (Fig. 2). The specificity of the 7D6 antibody was confirmed further by immunoblotting against the original immunogen against which it was raised, soluble feline CD134-IgG Fc fusion protein (Fig. 1I, lane 1) and against lysates of MCC cells transduced with vector-only control (Fig. 1I, lane 2) or with feline CD134 (Fig. 1I, lane 3). A single ~43-kDa species was recognized in the latter cells.

CD134 expression in the cat. Having confirmed the sensitivity and specificity of the 7D6 antibody, we analyzed CD134 expression on feline peripheral blood mononuclear cells to ascertain whether CD134 expression correlated with the cellular targets for FIV. CD134 expression was measured on $CD4^+$ and $CD8\alpha\beta^+$ T cells, $B220^+$ ($CD45R^+$) B cells and $CD14^+$ monocytes. Peripheral blood mononuclear cells were prepared from feline blood samples that had been submitted to the Companion Animal Diagnostics laboratory for routine virological testing and which were feline leukemia virus, FIV, and feline coronavirus negative, and then the cells were processed

TABLE 1. CD134 expression on lymphocytes and monocytes in peripheral blood

Cell population	% Positive[a]	Cell population	% Positive[a]
$CD4^+$	30.0 (3.2)	$B220^+$	39.9 (3.9)
$CD4^+$ $CD134^+$	3.8 (0.5)	$B220^+$ $CD134^+$	2.0 (0.5)
$CD8^+$	15.2 (1.7)	$B220^+$ $CD4^+$ $CD134^+$	0.4 (0.1)
$CD8^+$ $CD134^+$	0.1 (0.04)	$CD14^+$	30.4 (4.6)
		$CD14^+$ $CD134^+$	0.2 (0.04)

[a] Mean percent positive (standard error) ($n = 13$).

for flow cytometry. T-cell expression of CD134 was restricted to the $CD4^+$ (helper) subpopulation (3.8% ± 0.5% of total cells, 12% of $CD4^+$ cells), while $CD8^+$ T cells were consistently negative (0.1% ± 0.04% of total cells, <1% of $CD8^+$ cells) (Fig. 3 and Table 1). CD134 expression was also detected on a subpopulation of $B220^+$ lymphocytes (2.0% ± 0.5% of total cells, 5% of $B220^+$ cells) (Fig. 3). Although B220 is expressed predominantly on feline B cells, a subpopulation of both $CD4^+$ and $CD8^+$ T cells also express B220 (not shown). However, only 1% of $B220^+/CD134^+$ cells were also positive for CD4, suggesting that the remaining 4% of $B220^+/CD134^+$ cells were bona fide B cells (Table 1). $CD14^+$ monocytes in peripheral blood were consistently CD134 negative (0.2% ± 0.04% of total cells, <1% of $CD14^+$ cells) (Fig. 3). Thus, in peripheral blood, CD134 expression is restricted to $CD4^+$ T-cell and $B220^+$ B-cell subpopulations. The levels of CD134 expression on $CD4^+$ T cells (Table 1) are similar to those described previously for OX40 in rats, mice, and humans (2, 27).

Given that peripheral blood monocytes were ostensibly negative for CD134 and yet cells of the monocyte/macrophage lineage are known to support FIV infection, we analyzed CD134 expression on enriched splenic macrophages following culture in the presence of GM-CSF. A 75.2% proportion of the GM-CSF-cultured cells expressed CD14 (Fig. 4A), as expected for cells of the macrophage lineage. A 16.1% proportion of $CD14^+$ cells coexpressed CD134 (Fig. 4B), while 92.1% expressed CXCR4 (Fig. 4C). Following incubation for 48 h with LPS, 31.9% of $CD14^+$ cells expressed CD134 (Fig. 4E) while 88.5% expressed CXCR4 (Fig. 4F). These data are consistent with CD134 being up-regulated on macrophages following activation, although expression levels remain low but comparable to that of the IL-2-dependent T-cell line MYA-1 (Fig. 1G). We next asked whether the increase in CD134 expression correlated with enhanced infection with FIV. The splenic macrophages analyzed in Fig. 4A to F were infected with HIV (FIV) luciferase pseudotypes bearing GL8, CPG41, or B2542 Envs (Fig. 4G). LPS-stimulated macrophages showed an enhanced susceptibility to infection with all three viruses (1.9-, 1.5-, and 2.5-fold enhancement for GL8, CPG41, and B2542, respectively), consistent with the increased CD134 expression having functional consequences.

Analysis of CD134 expression on peripheral blood mononuclear cells (PBMC) had indicated that expression was restricted largely to $CD4^+$ T cells and $B220^+$ cells. We next examined the expression of CD134 on cells of peripheral lymph nodes, mesenteric lymph nodes, spleen, and thymus collected post mortem. While CD134 expression was detected on both $CD4^+$ T cells and $B220^+$ cells on PBMC (Fig. 5A to C), the majority of CD134 expression in peripheral lymph nodes (Fig. 5D to F), spleen (Fig. 5G to I), thymus (Fig. 5J to L), and mesenteric lymph nodes (Fig. 5M to O) was restricted to $CD4^+$ T cells. Even in the thymus, where CD4 and CD8 are coexpressed on T-cell precursors, CD134 expression was restricted to $CD4^+$ cells, indicating expression by mature (CD8-negative) $CD4^+$ T cells only. While the majority of FIV-infected cells in early infection is $CD4^+$ T cells (10, 17), in late infection, both B cells and $CD8^+$ T cells are infected. Given the absence of CD134 expression on $CD8^+$ T cells from either peripheral blood or tissues, we investigated whether, as in humans and mice, expression would be induced following mitogenic stimulation. Lymph node cells from two animals were incubated in the presence of the T-cell mitogens concanavalin

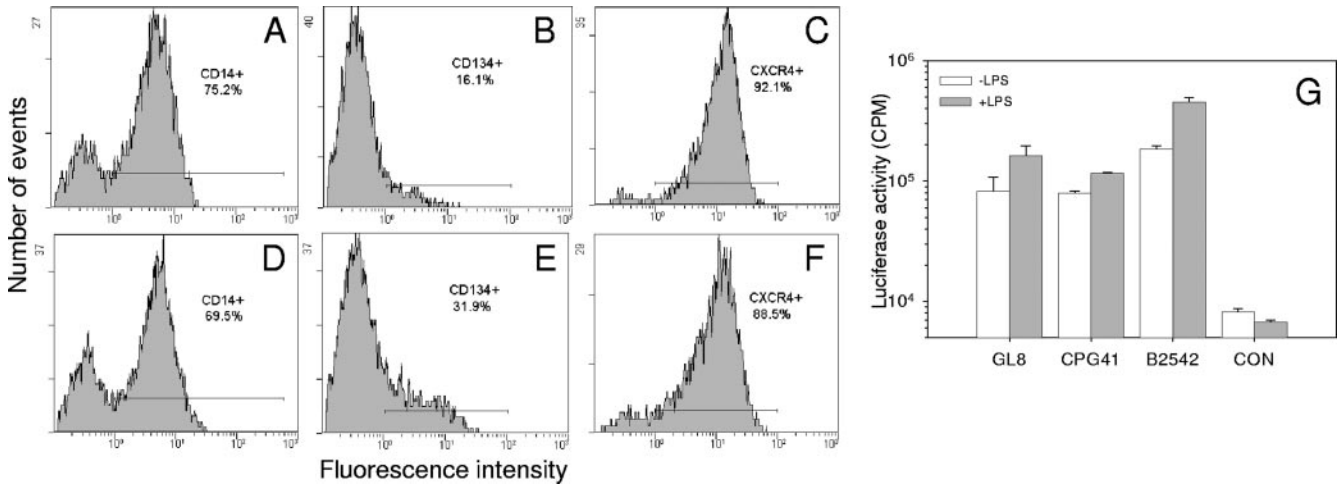


FIG. 4. Macrophage expression of CD134. Splenic macrophages were cultured in the presence (D to F) or absence (A to C) of bacterial LPS and analyzed for expression of CD14 (A and D). CD14-positive macrophages were gated and analyzed for expression of CD134 (B and E) and CXCR4 (C and F). (G) Parallel cultures were infected with HIV (FIV) luciferase pseudotypes bearing GL8, CPG41, or B2542 Envs, and luciferase activity was analyzed at 72 h postinfection. CON, control without infection. Data are means ± standard errors ($n = 3$).

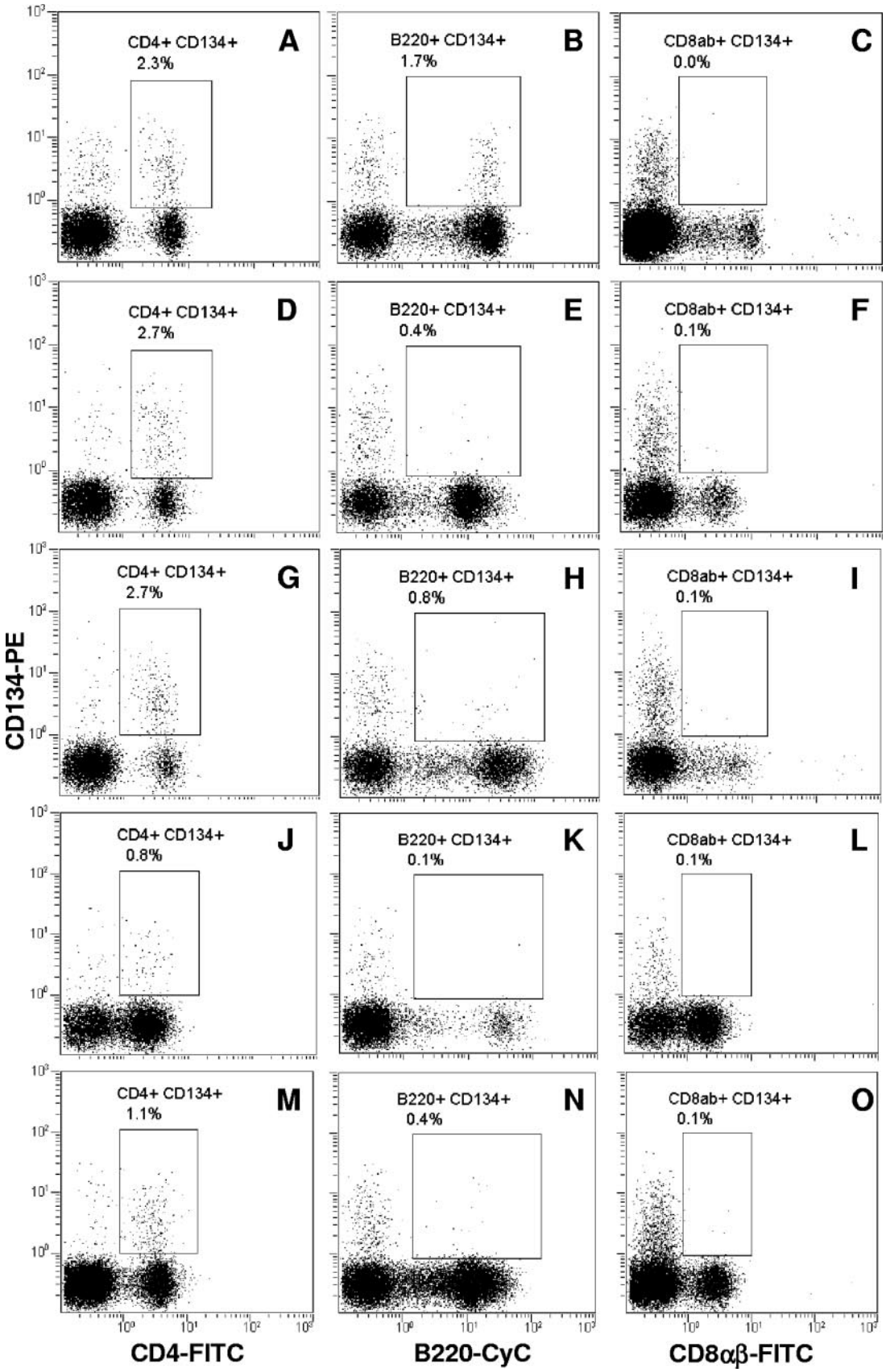
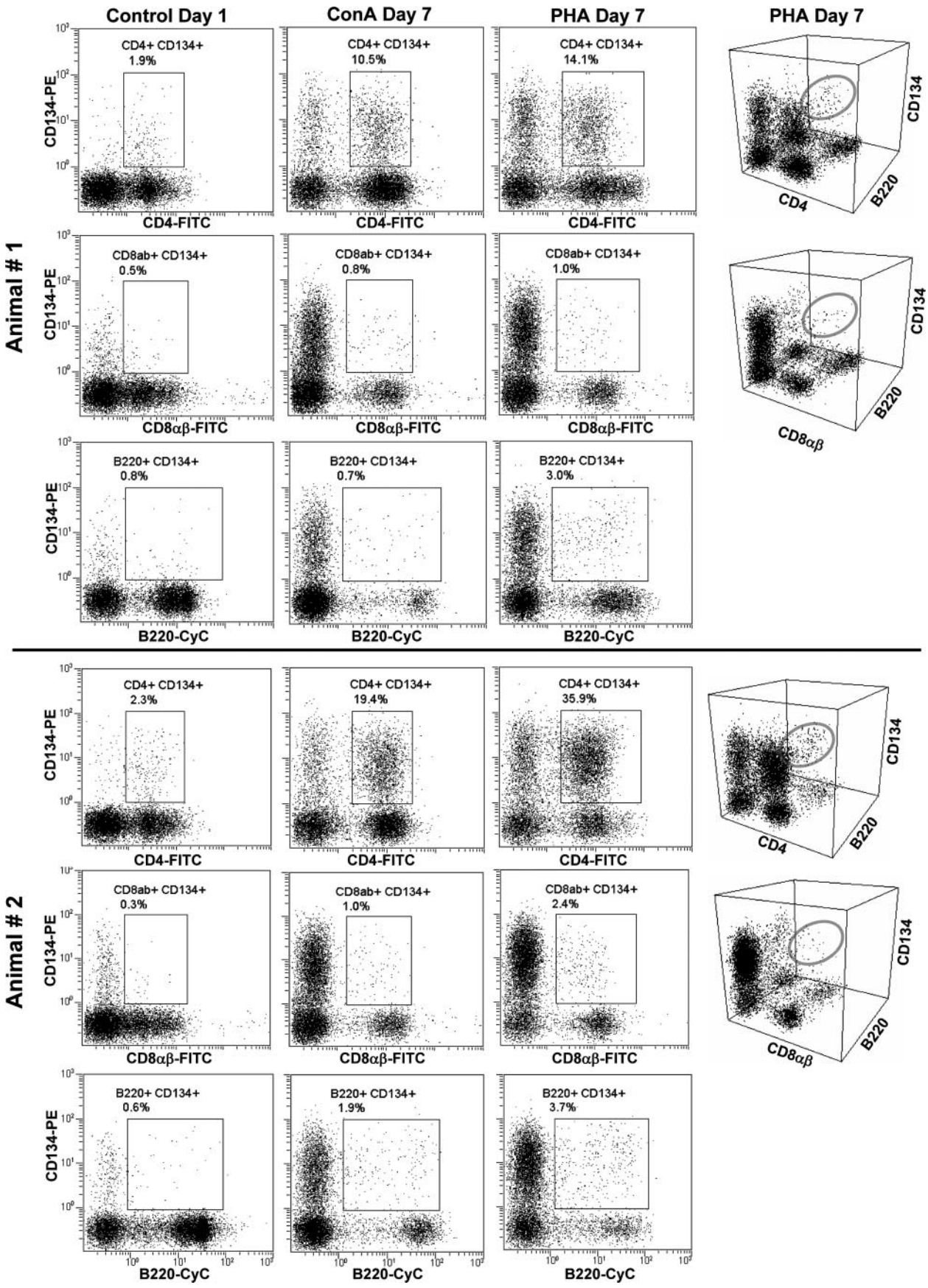


FIG. 5. CD134 expression on cells from peripheral blood (A to C), popliteal lymph node (D to F), spleen (G to I), thymus (J to L), and mesenteric lymph node (M to O). Cell suspensions were stained indirectly with anti-CD134 followed by PE–anti-mouse followed by double-staining with CD4-FITC and CD8αβ-FITC or CyChrome-B220. Cells were processed for flow cytometry, and 10,000 events were collected in LIST mode. Data are representative of multiple analyses ($n = 12$).



A (ConA) and phytohemagglutinin (PHA) for 1 week and analyzed by flow cytometry for CD134 expression (Fig. 6) on $CD4^+$ and $CD8\alpha\beta^+$ T cells and $B220^+$ cells. Following ConA stimulation, CD134 expression on $CD4^+$ T cells increased from 1.9% and 2.3% on day 1 to 10.5% and 19.4% on day 7. PHA stimulation had an even more striking effect, increasing CD134 expression on $CD4^+$ T cells to 14.1% and 35.9%. Thus, CD134 expression is increased by mitogenic stimulation of $CD4^+$ T cells. ConA stimulation increased CD134 expression marginally on $CD8^+$ T cells (from 0.5% and 0.3% on day 1 to 0.8% and 1.0% on day 7). PHA stimulation increased CD134 expression on $CD8^+$ T cells to 1.0% and 2.4% by day 7 postexposure, confirming a modest but detectable increase of CD134 expression in postmitotic $CD8^+$ T cells. A modest increase in CD134 expression was also observed on $B220^+$ T cells. However, the majority $B220^+$ $CD134^+$ cells in these cultures coexpressed CD4 or CD8 (Fig. 6, tomogram plots), consistent with ConA and PHA acting as T-cell (and not B-cell) mitogens.

Anti-CD134 inhibition of FIV infection. HIV (FIV) luciferase pseudotypes bearing Envs from GL8, B2542, CPG41, and 0827 were used to infect MCC cells stably expressing fCD134 (MCC-fCD134) (Fig. 1B) in the presence of increasing concentrations of the 7D6 anti-CD134 antibody (Fig. 7). The ability of 7D6 to block viral entry was reproducible but Env dependent. GL8 and CPG41 were the least sensitive to the inhibitory effects of 7D6; infection with both viruses was reduced by 65% at 4 μg/ml 7D6. In contrast, infections with B2542 and 0827 were reduced by 88% and 83%, respectively. It is noteworthy that with increasing antibody concentration the inhibitory effect was either reduced (B2542 and 0827) or was no longer significant (GL8 and CPG41). Infection mediated by the G protein of vesicular stomatitis virus (VSV-G) was not affected by 7D6 (not shown). If there were a direct competition between 7D6 and Env for CD134 binding, one might expect infection to be ablated completely in a dose-dependent manner. Thus, these data may indicate an indirect or allosteric effect.

The binding site for CD134L on CD134 utilizes residues from the first, second, and third cysteine-rich domains (CRD1, CRD2, and CRD3) (7), while residues in CRD2 are critical in determining the species specificity of the human CD134L-CD134 interaction (38). The binding site for soluble FIV SU-Fc on CD134 has been mapped to CRD1 (11), while additional determinants in CRD2 have been shown to contribute to infection mediated by viruses such as FIV GL8 (38, 39). Previous studies have demonstrated that the herpes simplex virus glycoprotein D (HSV-gD) and LIGHT (*l*ymphotoxin-like, exhibits *i*nducible expression and competes with HSV *g*lycoprotein D for *H*veA and is expressed on *T* lymphocytes) bind distinct sites on the herpes simplex virus entry mediator A (HveA) and yet cross-compete for binding (28) (indicating an allosteric effect). Given that 7D6 inhibited infection with FIV Env-bearing pseudotypes, we asked whether 7D6 would block binding of soluble FIV SU-Fc fusion protein to MCC-fCD134. Preincubation of MCC-fCD134 with 7D6 reduced binding of either FIV GL8 SU-Fc (Fig. 7C) or PPR SU-Fc (Fig. 7F) at 1 μg/ml and more substantially at 10 μg/ml, indicating that the antibody did indeed disrupt binding of FIV SU. Mean fluorescence intensity of GL8 SU-Fc binding was reduced from 69.3 to 29.8 (Fig. 7I), while that of PPR SU-Fc was reduced from 101.3 to 25.4 (Fig. 7J). Although SU-Fc binding was reduced reproducibly, it was never abolished completely, suggesting the 7D6 antibody may target a specific high-affinity binding and that there is a substantial 7D6-resistant binding component (e.g., CXCR4 or heparin sulfate-dependent binding [12, 15]). Binding of either FIV GL8 SU-Fc (Fig. 7E) or PPR SU-Fc (Fig. 7H) was unaffected by incubation with isotype-matched anti-CD8 (VPG9). Given that MAb BerACT35 had a weaker reactivity with feline CD134 than human CD134 (Fig. 1), we asked whether BerACT35 would also interfere with SU-Fc binding to MCC-fCD134. BerACT35 had no effect on GL8 SU-Fc (Fig. 7D), while with PPR SU-Fc (Fig. 7G) there was a modest enhancement of SU-Fc binding. The significance of this increase in binding was not ascertained but may reflect the BerACT35 antibody altering the configuration of CD134 such that the PPR-SU Fc binding site is more readily accessible.

Effect of CD134L on FIV infection. The binding site for soluble FIV PPR SU-Fc fusion maps to CRD1 of CD134 (11), and expression of CRD1 of feline CD134 alone in the context of human CD134 is sufficient to facilitate binding and infection with FIV PPR. In contrast, other strains of FIV require determinants in CRD2 for infection to proceed (39), determinants that map to the alternate face of CD134 and which contribute to the binding site for CD134L (38). Infection with viruses such as GL8 is critically dependent on these determinants in CRD2, raising the possibility that infection may be susceptible to modulation (inhibition or enhancement) by the engagement of CD134 by CD134L. To investigate the role of CD134L in FIV infection, we cloned feline CD134L from MYA-1 cells and confirmed its ability to bind fCD134. Soluble fCD134-Fc fusion protein bound specifically (Fig. 8A, 92.7%) to MCC cells stably transduced with fCD134L, compared with vector-only transduced control cells. fCD134L bound fCD134-Fc selectively (Fig. 8B), and binding was dependent on determinants in CRD2 in that soluble chimeric fCRD1 in the context of human CD134 (Fig. 8B, fCRD1/h) fusion protein did not bind to MCC-fCD134L cells. Binding of the fCRD1/h fusion protein could be restored partially by mutation of residues ^{78}SSK^{80} of the human CD134 CRD2 in the fCRD1/h fusion protein to ^{78}NYE^{80} (Fig. 8B, fCRD1/h+NYE). Thus, the feline CD134L was expressed in a biologically relevant configuration that supported feline CD134 binding specifically. We next asked whether a biologically active soluble fCD134L could be gener-

FIG. 6. Effect of T-cell mitogens on CD134 expression. Lymph node cells from two animals (upper and lower panels, respectively) were cultured with the T-cell mitogens ConA and PHA for 7 days. Cells were then stained indirectly with anti-CD134 followed by PE–anti-mouse followed by double staining with CD4-FITC and CD8αβ-FITC or CyChrome-B220. Cells were processed for flow cytometry, and 10,000 events were collected in LIST mode. Data were analyzed using Expo32 v1.2. Tomograms represent three-color staining of PHA day 7 cells, and an indicative $CD4^+$ $CD134^+$ $B220^+$ population is circled.

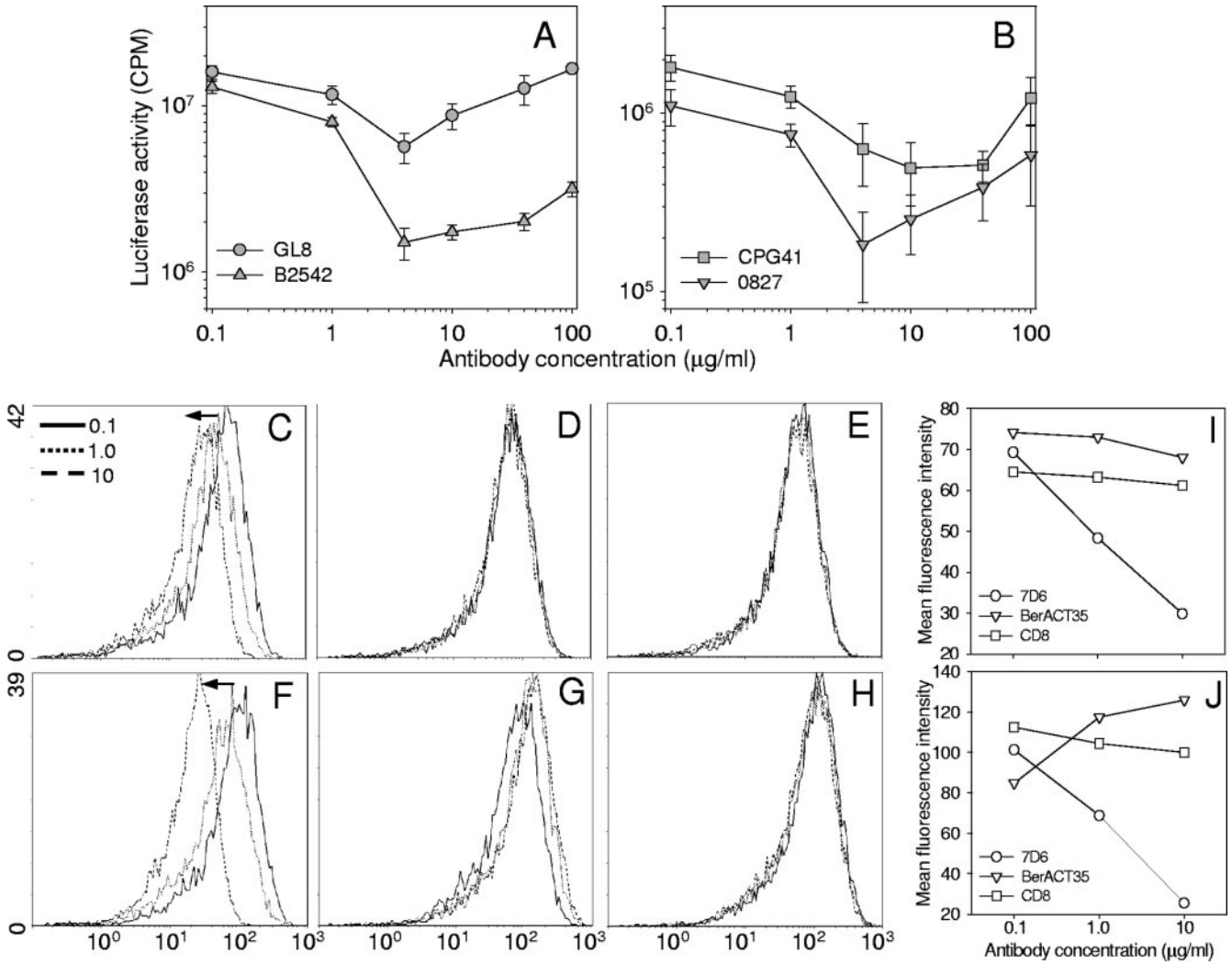


FIG. 7. Inhibition of FIV infection by anti-CD134 MAb 7D6. MCC cells expressing feline CD134 were preincubated with 7D6 and then exposed to HIV (FIV) pseudotypes bearing GL8 or B2542 (A) and CPG41 or 0827 (B) Envs. Cells were cultured for 72 h postinfection, and luciferase activity was quantified. Data are means ± standard errors ($n = 3$) and are representative of three experiments. MAb 7D6 reduces binding of SU-Fc fusion proteins. MCC cells expressing feline CD134 were preincubated with 0.1, 1.0, or 10 μg/ml MAb 7D6 (C and D), BerACT35 (D and G), or isotype-matched control anti-CD8, VPG9 (E and H), prior to addition of GL8 SU-Fc (C to I) or PPR SU-Fc (F to J). (I and J) Effects of MAbs on mean fluorescence intensity for GL8 SU-Fc (I) and PPR SU-Fc (J). Bound SU-Fcs were detected with PE-conjugated anti-human IgG-Fc. The arrow indicates the reduction in fluorescence intensity. Data are representative of three experiments.

ated from the cDNA clone. Soluble feline, murine, and human CD134Ls were expressed as Fc fusion proteins and adjusted to equivalent concentrations, and their binding properties were examined. While there was no specific binding of the proteins to control MCC cells (Fig. 8C), murine Fc-CD134L bound to MCC cells expressing either feline (Fig. 8D) or human (Fig. 8E) CD134, consistent with the promiscuous binding of this ligand (5). Human Fc-CD134L bound specifically to human CD134-expressing cells (Fig. 8E) and not feline CD134-expressing cells, in agreement with the species specificity of this ligand (5, 11, 38). In contrast, feline CD134L binding to either cell line could not be detected. Similar results were obtained when the soluble CD134Ls were prepared as FLAG-tagged fusion proteins (not shown). The data suggest that feline CD134L does not retain its specific binding properties when expressed in a soluble form, perhaps due to inappropriate aggregation (loss of ligand activity for related molecules [mFasL and mLIGHT] following expression as Fc-fusion proteins has been observed previously [5]). However, given that murine CD134L bound feline CD134 well (Fig. 8D), we were able to proceed with experiments to examine the effect of CD134L engagement on FIV infection using murine CD134L as a surrogate ligand.

We investigated the nature of the 7D6 binding site on fCD134. Firstly, we examined the effect of 7D6 on binding of soluble feline CD134-Fc to MCC cells stably transduced with a feline CD134L expression vector. sfCD134-Fc bound well to MCC-fCD134L cells (97%) (Fig. 8F); however, preincubation of sfCD134-Fc with 1 μg/ml 7D6 reduced binding to 7.0% (Fig. 8F), while binding was blocked completely by preincubation with 10 μg/ml 7D6 (0.2%) (Fig. 8F). Thus, 7D6 competes with fCD134-Fc for binding to fCD134L. In the converse experi-

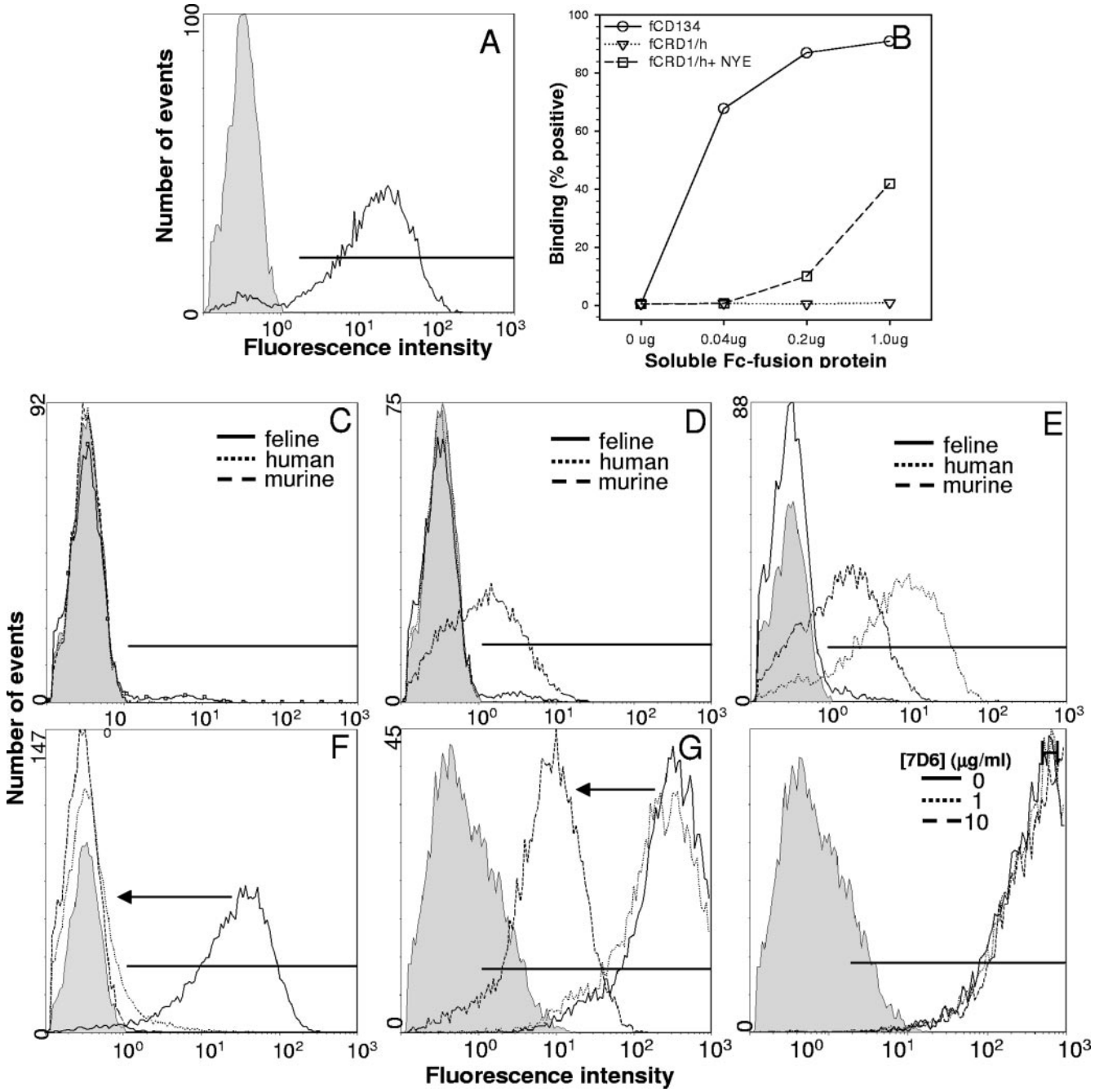


FIG. 8. Interaction between CD134 and CD134L. (A) MCC cells stably transduced with feline CD134L (open histogram) or vector-only control cells (filled histogram) were incubated with 1 μg soluble feline CD134-Fc fusion protein followed by PE–anti-human IgG-Fc. (B) CRD1-dependent binding of CD134 to feline CD134L-expressing MCC cells. Cells were incubated with feline CD134-Fc (fCD134), a human CD134 CRD1 × feline CD134 chimera (fCRD1/h), or fCRD1/h with ^{78}SSK^{90} mutated to ^{78}NYE^{90}. (C to E) Binding of soluble feline, human, or murine Fc-CD134L to vector-only control MCC (C) or MCC expressing feline CD134 (D) and human CD134 (E). Bound ligand was detected with PE–anti-human IgG-Fc. (F) Inhibition of soluble feline CD134-Fc to MCC-fCD134L cells by anti-CD134 MAb 7D6. Cells were preincubated with 0, 1, or 10 μg/ml 7D6 prior to incubation with 1 μg feline CD134-Fc. The arrow indicates a reduction in fluorescence intensity. (G and H) Effects of MAb 7D6 on murine Fc-CD134L binding to MCC cells expressing feline (G) or human (H) CD134. Cells were preincubated with 0.1, 1, or 10 μg/ml of MAb 7D6 followed by 50 μl (~1 μg) soluble murine Fc-CD134L. The arrow indicates a reduction in fluorescence intensity. All bound ligands were detected with PE–anti-human IgG-Fc, while filled histograms represent binding of PE–anti-human IgG-Fc in the absence of ligand. Data are representative of three experiments.

ment, we looked at the ability of 7D6 to block binding of soluble Fc-mCD134L to MCC-fCD134 cells. Human and murine CD134Ls were transiently expressed as Fc fusion proteins in 293T cells. We then examined the effect of 7D6 on the binding of soluble Fc-mCD134L to MCC-fCD134. 7D6 blocked binding of Fc-mCD134L to MCC-fCD134 at 10 μg/ml (Fig. 8G), indicating that the antibody interferes with the site of interaction between CD134 and CD134L either directly or

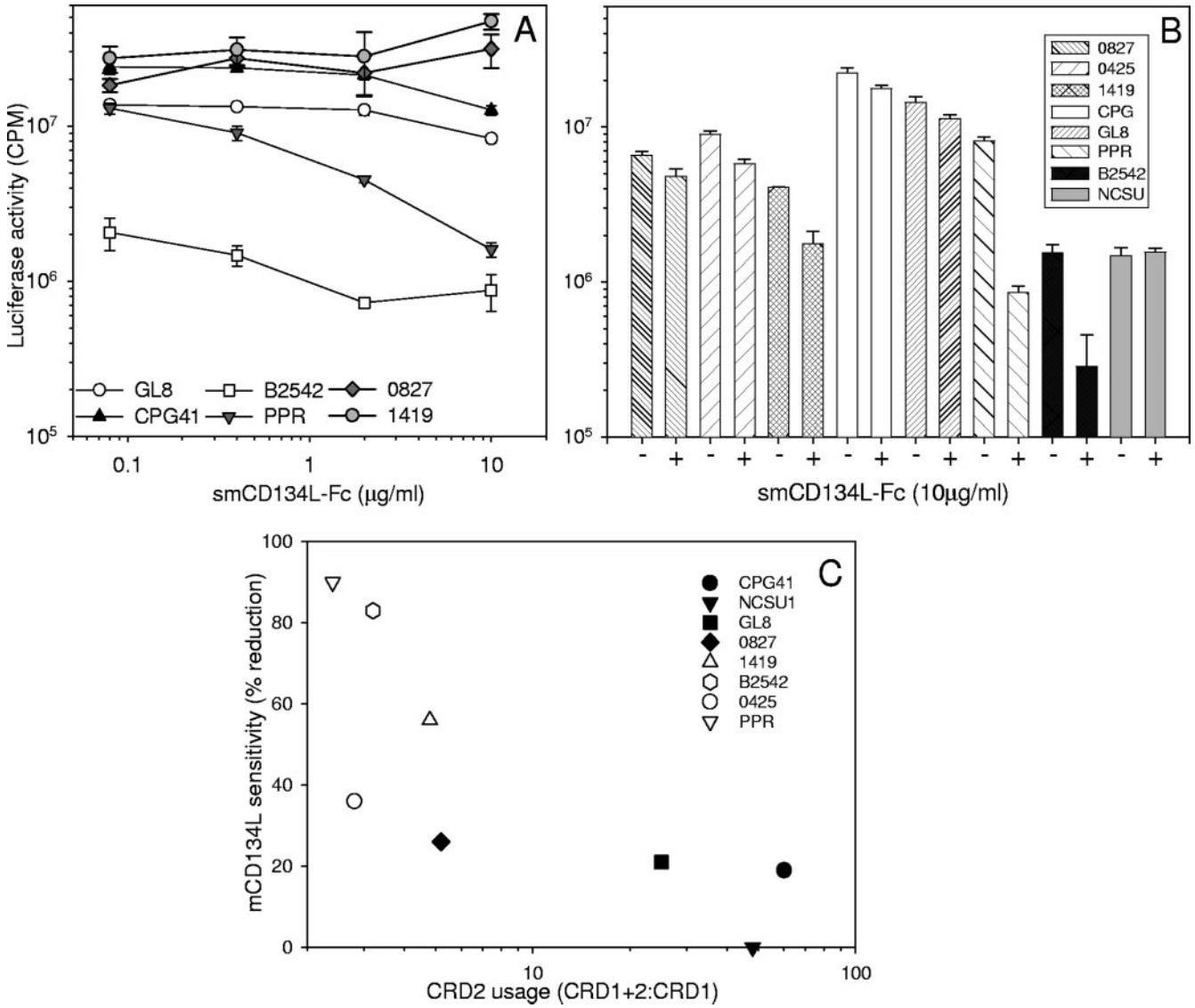


FIG. 9. Effect of soluble murine Fc-CD134L on FIV infection. (A) MYA-1 cells were preincubated with soluble Fc-mCD134L followed by HIV (FIV) luciferase pseudotypes bearing GL8, B2542, 0827, CPG41, PPR, or 1419 Envs. Cells were cultured for 72 h, and luciferase activity was quantified. (B) MYA-1 cells were preincubated with 10 μg/ml soluble Fc-mCD134L followed by HIV (FIV) luciferase pseudotypes bearing GL8, B2542, 0827, CPG41, PPR, NCSU, 0425, or 1419 Envs. At 2 h postinfection, cells were washed and plated. Cells were cultured for 72 h, and luciferase activity was quantified. All data are means ± standard errors ($n = 3$) and are representative of three experiments. (C) Graphical representation of the dependence of a viral strain on CRD2 of CD134 for infection (39) (CRD2 usage is the luciferase activity in fCD134-expressing MCC cells divided by luciferase activity in MCC cells expressing fCRD1 alone) and sensitivity to mCD134L (percent reduction in infection at 10 μg/ml Fc-mCD134L).

through an allosteric affect. In contrast, 7D6 did not inhibit binding of Fc-mCD134L to MCC-hCD134 at 10 μg/ml (Fig. 8H), consistent with the antibody recognizing human CD134 with a reduced affinity compared to feline CD134 (Fig. 1).

The blocking of HSV infection by LIGHT (22) and the cross-competition between HSV gD and LIGHT for binding to HveA (28) indicate that even if the Env and CD134L binding sites on CD134 are noncontiguous, viral entry may be blocked by ligand engagement through an allosteric mechanism. We therefore examined the effect of soluble CD134L on FIV infection. MYA-1 cells were incubated sequentially with mCD134L followed by HIV (FIV) pseudotypes. Preincubation of feline T cells with soluble mCD134L blocked infection with B2542 and PPR in a dose-dependent manner (Fig. 9A). In contrast, CPG41 and GL8 were relatively resistant to the blocking effect of mCD134L, while 0827 and 1419 displayed a marginal enhancement of infection at the highest concentration, indicating that there may be strain-specific differences in sensitivity to antagonism by CD134L and that, depending on the strain of virus, the ligand may either inhibit or even facilitate infection. We repeated the assay for inhibition of viral entry using a single fixed dose of mCD134L (10 μg/ml) with the modification that following the incubation with HIV (FIV) pseudotypes for 2 h, unbound pseudotypes were removed by centrifugation and

cells were reseeded in medium supplemented with mCD134L (Fig. 8B). Again, using the modified "washout" protocol, the most marked effect was observed with B2542 and PPR (82% and 90% reductions in viral entry, respectively). Under these conditions, mCD134L also reduced infection with 0827, 0425, 1419, and CPG41 Env-bearing pseudotypes to some extent (20 to 50%), but NCSU was entirely resistant to inhibition by the mCD134L at 10 μg/ml. We next examined a possible correlation between sensitivity to inhibition by CD134L and the interaction pattern of the different viral strains on CD134. Indeed, we have shown previously that some strains require only determinants in CRD1 for infection (e.g., PPR) whereas others also make use of CDR2 determinants, in particular the sequence ^{78}NYE^{90} (e.g., GL8) (38, 39). As shown in Fig. 9C, there was an inverse correlation between CD134L sensitivity and CRD2 usage, such that viruses with a greater dependence on CRD2 were least sensitive to inhibition by mCD134L and vice versa. This is in line with our previous hypothesis that an extended and complex interaction of the virus with CD134 is of higher affinity(38, 39) and, therefore, more difficult to displace with CD134L. The dependence on CRD2 for infection may thus represent a surrogate measure of receptor affinity and of the ability to displace CD134L.

DISCUSSION

FIV is the only nonprimate lentivirus that in its natural host species, the domestic cat, induces a disease similar to AIDS (26). Acute infection with FIV is accompanied by a selective targeting of $CD4^+$ T lymphocytes, resulting in a progressive decline in $CD4^+$ T helper cell number (1). In chronic infection, an immunodeficiency syndrome develops that is characterized by wasting, neurological manifestations, chronic stomatitis and gingivitis, and an increased incidence of lymphoma (25). In contrast, the ungulate lentiviruses induce diseases reminiscent of chronic inflammatory conditions. The progression from the acute to the chronic phase of infection is accompanied by an expansion of the viral cell tropism, perhaps indicative of a shift in the receptor usage analogous to the emergence of CXCR4-using variants in HIV infection with disease progression (8). The early targeting of FIV to $CD4^+$ helper T cells would be explained if CD134 expression in the domestic cat were restricted to this cell population, as has been observed in the rat (24). Human CD134 expression is also restricted predominantly to $CD4^+$ T cells, although expression has been noted on $CD8^+$ T cells following stimulation with allogeneic monocyte-derived dendritic cells (18) and more rarely on B-cell blasts (16). In comparison, murine CD134 is expressed not only on $CD4^+$ T cells but also in substantial amounts on mitogen-stimulated $CD8^+$ T cells (2). Using the 7D6 anti-feline CD134 monoclonal antibody, we have now shown that CD134 expression in the domestic cat is restricted largely to $CD4^+$ T cells, not only in peripheral blood but also in spleen, thymus, and lymph nodes. Indeed, the degree of restriction of CD134 expression to $CD4^+$ T cells is striking, suggesting tight regulation of gene expression. On thymocytes, CD134 expression remained restricted to $CD4^+$ T cells, suggesting that $CD4^+$ $CD8^+$ thymocytes are CD134 negative and that CD134 is only expressed on mature $CD4^+$ T cells. Thus, the restriction of feline CD134 expression to $CD4^+$ T cells mirrors the reported expression pattern in the rat.

CD134 expression was induced, albeit weakly, on $CD8^+$ T cells in vitro following exposure to the T-cell mitogens ConA and PHA. These findings are similar to observations with mouse and human T cells, where CD134 expression is abundant on activated $CD4^+$ T cells but is low on activated $CD8^+$ T cells (2, 18). In contrast, $CD8^+$ rat T cells are CD134 negative (2), although expression was detected on a $CD4^+$ $CD8\alpha^+$ population (2). In this study, we used double staining with CD134 and CD8αβ to confirm the cellular lineage. Whether CD134 expression is induced in vivo under conditions of immune activation remains to be established. However, these data would explain how FIV can also infect $CD8^+$ T cells in chronically infected animals. In early infection, the virus would target the cell population upon which expression of CD134 is most abundant, activated $CD4^+$ T cells. As the infection progresses, the virus may then expand into less-abundant CD134-positive populations, such as $CD8^+$ T cells.

CD134 expression was observed on a peripheral $B220^+$ population, the majority of which were CD4 and CD8 negative, consistent with the B-cell lineage (these cells were distinguishable from the $B220^+$ populations of mitogen-stimulated $CD4^+$ and $CD8^+$ T cells generated in vitro, which coexpressed CD134). B220/CD45R is a marker used widely for the B-cell lineage; it is expressed on B cells at all stages of development but is reduced on plasma cells and a memory B-cell subset. B cells are known to be a significant reservoir of FIV proviral DNA in peripheral blood in chronic infection (10, 17), and the expression of CD134 would be consistent with these cells being targeted. B220 is also expressed on $NK1.1^+$ $CD8\alpha^+\beta^-$ TCRαβ $B220^+$ lymphokine-activated killer cells (3); however, the $CD134^+$ $B220^+$ cells in feline peripheral blood in this study reacted with neither anti-CD8α nor anti-CD57 (NK1.1), further supporting the B-cell lineage of the cells. A third population of CD134-positive cells in the "lymphoid" gate in peripheral blood were CD4, CD8αβ, CD8αα, and B220 negative. The lineage of this population in the domestic cat remains to be determined, and identification remains problematic due to the paucity of reagents recognizing feline leukocytes. However, it is possible that this population may include either NK cells or a B220-negative (memory) subset of B cells.

The levels of CD134 detected on PBMC populations in this study are consistent with those reported for murine or human PBMC; however, they are significantly lower than those inferred by binding studies using soluble FIV Env SU-Fc proteins, where SU-Fc bound to approximately 60% of PBMC (12), the majority of which (77%) was ablated by the CXCR4 antagonist CXCR4 (12). Given that CXCR4 expression on freshly isolated feline PBMC is restricted to B cells and monocytes (36), the data suggest that SU-Fc binding is an unreliable measure of CD134 expression. It is likely that this reflects the monomeric/dimeric structure of the SU-Fc protein where the CXCR4 binding site may be more readily exposed. Indeed, although we found that MAb 7D6 reduced binding of GL8 SU-Fc significantly, there was a high degree of binding that was refractory to 7D6 and found subsequently to be CD134 independent (not shown). Given that the GL8 strain is highly CD134 dependent, the high degree of nonspecific binding associated with the GL8 SU-Fc suggested to us that this ap-

proach was not representative of the virus-receptor interaction and was not pursued further.

Diverse strains of FIV target cells of the monocyte/macrophage lineage (9, 32, 33), indicating that the cellular receptor(s) for FIV may be expressed in cells of this lineage. Flow cytometric analyses of CD134 expression on PBMC indicated that $CD14^+$ cells (monocytes) were ostensibly negative. We therefore postulated that CD134 may be expressed on macrophages and that expression may be enhanced by activation. CD134 expression was indeed detected on cultured macrophages, expression was up-regulated following exposure to bacterial LPS, and the enhanced expression correlated with an increase in susceptibility to infection. Thus, while HIV targets monocytes/macrophages via expression of CD4, FIV achieves a similar targeting by using CD134 (CD4 is not expressed on feline monocytes/macrophages). The restricted expression of CD134 to macrophages (and not monocytes) would indicate that targeting of this lineage FIV most likely occurs in immunological tissues, at sites where helper T cells and antigen-presenting cells are brought into close proximity. Previous studies have demonstrated that FIV interacts with DC-SIGN (14), providing a useful means of delivering the virus to areas of lymphoid tissues that are rich in the target cells for the virus. It would appear then that the use of CD4 and CD134 by HIV and FIV may be a means to an end, the viruses acquiring the use of a primary receptor to enhance their ability to replicate in helper T cells and macrophages.

The interaction between the TNF superfamily and TNFR superfamily molecules TRAILR(DR5)-TRAIL(Apo2L) and TNFR-LT/TNF is mediated by residues in CRD2 and CRD3 of the TNFR superfamily molecule and the ligand (reviewed in reference 4), leading to speculation that the interaction between FIV and CD134 is not modulated by engagement of CD134 by CD134L (11). Accordingly, human CD134L bound to a chimera expressing feline CD134 CRD1 in the context of human CD134, and infection via this chimeric receptor with FIV pseudotypes bearing the FIV PPR Env was not blocked by human CD134L. However, subsequent studies demonstrated that diverse strains of FIV require determinants in CRD2 of CD134 for infection, indicating that infection with these viruses may be susceptible modulation by CD134L. Moreover, the crystal structure of the complex between human CD134 and either human or murine CD134L has revealed that residues in not only CRD2 and CRD3 but also CRD1 contribute to the receptor-ligand interaction (7).

In order to probe the FIV-CD134 interaction, we cloned fCD134L. Surface-expressed fCD134L bound feline but not human CD134, and this interaction was dependent on CRD2. MAb 7D6 blocked this interaction efficiently. However, fCD134L did not retain biological activity when prepared in soluble forms, impeding studies of antagonism of the FIV-CD134 interaction. However, given that mCD134L binds not only to human but also murine CD134 (5), we postulated that this promiscuity could extend to feline CD134. Indeed, mCD134L bound feline CD134, and this interaction was inhibited by MAb 7D6. We were then able to utilize mCD134L to probe the FIV-CD134 interaction and showed that mCD134L blocked FIV infection in an apparently strain-specific and dose-dependent manner. Critically, the two viral strains that were most sensitive to the inhibitory effects of mCD134L (PPR and B2542) were also sensitive to inhibition by MAb 7D6 and able to utilize chimeras expressing CRD1 of feline CD134 alone efficiently (38, 39). Conversely, strains such as GL8 and CPG41 show a strict dependence on CRD2 of CD134 and are less sensitive to inhibition by either MAb 7D6 or mCD134L. While the concordance between dependence on CRD2 for infection and refractoriness to inhibition by MAb 7D6 or mCD134L is compelling, it is likely that additional factors will contribute to the efficiency of viral entry in vivo, for example, affinity for CXCR4. Indeed, NCSU shows high dependence on CRD2 on feline (MCC) cells but low dependence on human (HeLa) cells (39); HeLa cells express high levels of human CXCR4, which is, paradoxically, a more efficient coreceptor for FIV than feline CXCR4 (34). Accordingly, infection with NCSU has been reported to correlate primarily with level of CXCR4 expression (21). These data may implicate a model for infection where the viruses that are transmitted and which are present in early infection (asymptomatic phase in HIV infection), such as CPG and GL8, may interact with CD134 with a higher affinity and thus may target cells more efficiently where CD134 levels are limiting. As a result of their strict dependence on a complex (and high-affinity) interaction with CD134, these viruses may be less sensitive to neutralizing antibodies targeting the CXCR4 binding site. With disease progression (late infection, the symptomatic phase in HIV infection), viruses such as B2542 and PPR may be more prevalent. These viruses require only a minimal interaction with CD134 before interacting with CXCR4, the CXCR4 binding site being readily exposed, and may be more sensitive to both CD134L and neutralizing antibodies. Accordingly, infection with the PPR strain can be facilitated by simply overexpressing CXCR4 (13), while CXCR4 overexpression has a minimal effect on viruses such as GL8 (35). Thus, with disease progression, viruses may evolve towards CD134 independence, the cell tropism mirroring the broad cellular distribution of CXCR4, while in early infection the viral cell tropism may reflect the limited distribution of CD134.

The binding of FIV Env, MAb 7D6, and CD134L to CD134 would appear to be inextricably linked, analogous to the binding of HSV gD, LIGHT, and LT-α to HveA (28). Given that engagement of CD134 by CD134L promotes the survival of antigen-specific T cells, future studies should address whether the FIV Env, either oligomeric on the viral particle or in a soluble form, shed into the extracellular milieu, antagonizes this survival signal or mimics it, promoting the expansion of a population of $CD4^+$ helper T cells that provide the ideal home for an AIDS-causing lentivirus.

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