Figure S1. Additional images for dengue hemorrhage in rhesus monkeys. The images of the other three monkeys (RM31, RM#5, and RM#6) with dengue hemorrhage are presented. The top and bottom panels indicate the images of the skin hemorrhage which were captured from different parts of the body within the same animal.
Figure S2. Viral load in plasma determined by focus forming units (FFU) assay. The viral titer and the infectivity of the virus in the collected specimens were determined using an immunocytochemical focus-forming unit (FFU) assay as previously described. Titers were expressed as FFU per ml. The pattern of the average viral titer was similar to that of qRT-PCR assays, peaking on day 3 after infection.
**Figure S3. Gating strategy for lymphocyte subset analysis.** Whole blood was stained with a panel of cell surface markers conjugated with various fluorochromes, lysis of red blood cells, and subjected to multi-color FACS analysis according to a protocol standardized in our lab. The following rhesus macaque cross-reactive anti-human monoclonal antibodies (mAbs) were utilized: anti-CD16 FITC (clone 3G8); anti-CD61 FITC (clone VI-PL2); anti-CD62P PE (clone A1.2); anti-CD45 PerCP (clone D058-1283); anti-CD4 PE-Cy7 (clone SK3); anti-CD56 APC (clone B159); anti-CD3 AlexaFluor700 (clone SP34-2); anti-CD20 APC-Cy7 (clone L27) and anti-CD8 Pacific Blue (clone RPA-T8) which were obtained from Becton Dickinson Biosciences (BDB: San Jose, CA). Anti-NKG2A PE (clone Z199) and anti-CD14 ECD (clone RM052) were obtained from Beckman Coulter (Miami, FL). Anti-CD41 AlexaFluor647 (clone PM6/243) was obtained from AbD Serotec (Raleigh, NC). The lymphocyte population was determined by gating on cells that expressed CD45 accompanied with low side scatter (SSC). This population was further gated by using forward (FSC) and side scatter (SSC) to include only viable lymphocytes. This gated lymphocyte population was then divided based on CD3 expression. The CD3 positive population was further gated and the frequency of CD4+, CD8+ and CD4+CD8+ T cells was determined based on the expression of CD4 and CD8 molecules. NHPs do appear to have significant numbers of double positive (CD4+/CD8+) T cells. The CD3 negative population was used to identify B cells as those that expressed CD20 and NK cells as those that expressed NKG2a.
A series of blood smear slides were used for staining with various antibodies to cell surface markers and dengue antigen. Blood smears were fixed with cold methanol, rinsed with PBS, and incubated with 10% human AB serum in blocking buffer (PBS containing 1%BSA) for 30 min to minimize non-specific binding. After washing twice, slides were incubated with monoclonal antibody against the dengue E protein (Clone 3H5, Chemicon), diluted in blocking buffer (1:100), for 1 hour at room temperature. Slides were rinsed twice with blocking buffer and incubated with HRP, FITC, or PE-conjugated goat anti-mouse IgG antibody (Vector lab) for 1 hour. Following two rinses with PBS, slides were incubated with HRP substrates and/or Sytox green (Molecular Probes) or Hematoxylin (Vector Lab) for nuclear staining for 30 min. After another rinse, the slides were mounted with mounting materials containing DAPI (Invitrogen). An appropriate Isotype antibody was used as a negative control (4A-1) in all experiments. Initially, dengue antigen was observed on the membrane edge of a cell lineage with an unknown phenotype (4A-2). Further investigation using a large panel of antibodies against several cell surface markers led to the observation that the cells that stained positively for the dengue antigen (red) expressed CD61+ marker (green) (4A-3). Due to the high background with immunofluorescence (IF) staining in blood smears,
immunohistochemical (IHC) staining was performed instead. Pockets of dengue antigen (brown) were observed either in the cytoplasm or on the edge of the cell membrane (4A-4), which was concordant with the results seen by IF staining. Furthermore, for purposes of control, a blood smear from samples of the same monkey obtained prior to infection was stained in parallel with Wright’s Giemsa stain in addition to IHC or IF staining. No platelets or dengue antigen positive cells were seen in these controls (4B-D0, prior to infection) as compared with the detection of dengue antigen (red) within cells that expressed the platelet associated marker (blue) (4B-D1, 1 day pi) or on the membrane edge (brown) of a cell (4B-D3, 3 days pi).