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Interaction of a dengue virus NS1-derived peptide with the inhibitory receptor KIR3DL1 on natural killer cells

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Abstract

Killer immunoglobulin-like receptors (KIRs) interact with HLA class I ligands and play a key role in the regulation and activation of NK cells. The functional importance of KIR-HLA interactions has been demonstrated for a number of chronic viral infections, but to date only a few studies have been performed in the context of acute self-limited viral infections. During our investigation of $CD8^+$ T cell responses to a conserved HLA-B57-restricted epitope derived from dengue virus (DENV) non-structural protein-1 (NS1), we observed substantial binding of the tetrameric complex to non-T/non-B lymphocytes in PBMC from a long-standing clinical cohort in Thailand. We confirmed binding of the NS1 tetramer to $CD56^{dim}$ NK cells, which are known to express KIRs. Using depletion studies and KIR-transfected cell lines, we further demonstrated that the NS1 tetramer bound the inhibitory receptor KIR3DL1. Phenotypic analysis of PBMC from HLA-$B57^+$ subjects with acute DENV infection revealed marked activation of NS1 tetramer-binding NK cells around the time of defervescence in subjects with severe dengue disease. Collectively, our findings indicate that subsets of NK cells are activated relatively late in the course of acute DENV illness and reveal a possible role for specific KIR-HLA interactions in the modulation of disease outcomes.

Introduction

Killer immunoglobulin-like receptors (KIRs) are expressed predominantly on natural killer (NK) cells and interact with specific human leukocyte antigen (HLA) class I ligands to transduce inhibitory or activating signals [1]. One of the best characterized and highly polymorphic members of the KIR family is the inhibitory receptor KIR3DL1, which is present in >90% of the human population and has at least 62 allotypes [2]. Interactions between KIR3DL1 and the HLA-Bw4 motif act to maintain NK cell inhibition. However, the downregulation of major histocompatibility complex (MHC) class I molecules that often follows viral infection or cellular transformation alleviates NK cell inhibition via KIR3DL1, leading to proinflammatory cytokine release and cytolytic activity. A role for KIR3DL1 in the control of chronic viral infections has been proposed on the basis of associations with disease outcome in HIV-infected individuals [3-8]. These studies suggest that both MHC class I and KIR genotypes may contribute to protection in the context of HLA-B57. Moreover, KIRs that interact with HLA-C have been linked epidemiologically to the development of liver disease in hepatitis C virus (HCV)-infected patients and protection from HCV infection in a cohort of intravenous drug users [9]. In contrast, the role of KIR-HLA interactions in acute self-limited viral infections remains largely unexplored.

Dengue virus (DENV) is a member of the flavivirus family comprising at least four distinct serotypes. Transmitted by the mosquito *Aedes aegypti*, DENV is endemic in the tropics/subtropics and causes an acute febrile illness known as dengue fever (DF). However, a small percentage of individuals experience a more severe syndrome known as dengue hemorrhagic fever (DHF). The key features of DHF are plasma leakage and a bleeding tendency, which develop as the fever subsides with clearance of viremia[10, 11]. Although both viral and host-specific factors likely influence clinical outcome, prospective cohort studies have identified secondary infection with a heterologous DENV serotype as a major risk factor for DHF [12]. At the mechanistic level, pre-existing antibodies [13], memory T cell responses [12, 14], and certain HLA genotypes [15-18] have all been linked with more severe dengue illness.

A number of reports describe associations between HLA class I genotypes and dengue disease severity [15-18]. In one earlier study, extended HLA region haplotypes including TNF, lymphotoxin alpha (LTA) and lymphotoxin beta (LTB), together with specific combinations of class I and class II alleles, were strongly associated with DHF during secondary DENV infection. Various aspects of disease outcome after DENV exposure have also been linked to functionally-defined HLA class I supertypes [19], as well as the MHC class I-related chains A/B (MICA/B) [20-22]. These latter proteins are upregulated in stressed cells and interact with NKG2D, an activating receptor on NK cells. More recently, two small genetic studies evaluated associations between KIR-ligand pairs and susceptibility to dengue in Gabon and Southern Brazil [23, 24]. Petitdemange *et al.* found no evidence of a role for KIR genotypes in patients infected with DENV-2. In contrast, Beltrame *et al.* detected an association between certain KIR genes and their cognate HLA ligands in the context of infection with DENV-3. Differences in population origin and the infecting DENV serotype may explain these disparate results. Other studies have noted NK cell activation during acute DENV infection. In particular, Azeredo *et al.* linked early activation of NK cells with mild DENV disease [25], whereas Green *et al.* found increased frequencies of NK cells expressing CD69 in children who developed DHF compared to those with attenuated disease [26]. The mechanisms by which NK cells contribute to immune protection and immunopathogenesis in DENV infection therefore require further elucidation [27, 28].

We recently characterized antigen-specific $CD8^+$ T cells directed against a highly conserved HLA-B57-restricted epitope derived from DENV non-structural protein-1 (NS1) [29]. In the present study, we examined binding of the corresponding $B57\text{-}NS1_{26\text{-}34}$ tetramer (NS1 TET) to enriched NK cell populations from samples obtained prior to, during and up to 1 year after the critical phase of illness (around the time of defervescence) in $HLA\text{-}B57^+$ subjects from a clinical cohort in Thailand. Using $KIR3DL1^+$ healthy donor peripheral blood mononuclear cells (PBMC), we confirmed that the NS1 TET bound mostly to $CD56^{dim}$ NK cells, which are known to express KIRs [30]. We then demonstrated that the

NS1 TET bound KIR3DL1. To determine whether there was an association between NK cell activation and dengue disease severity, we analyzed PBMC from our HLA-B57$^+$ cohort and found marked activation of NS1 TET$^+$ NK-enriched cells at the critical phase of illness in patients who developed DHF. Our results define a specific interaction between the inhibitory receptor KIR3DL1 and a DENV-derived CD8$^+$ T cell epitope with potential relevance to the immunopathogenesis of dengue disease.

Results

Binding of the NS1 TET to $CD8^-$ cells in PBMC from dengue patients

In a study of $CD8^+$ T cell responses to the HLA-B57-restricted epitope $NS1_{26\text{-}34}$ (HTWTEQYKF) [29], we observed binding of the corresponding tetrameric antigen complex (NS1 TET) to $CD8^-$ cells. As monocytes and B cells were eliminated by our gating strategy, we speculated that the NS1 TET bound a subset of NK cells. Furthermore, we hypothesized that the NS1 TET bound KIR3DL1 on NK cells given the extensive literature describing HLA-B57-restricted HIV-derived peptide ligands for this inhibitory receptor [5-7, 31, 32]. Initially, we used the NS1 TET to stain PBMC obtained at a convalescent time point from two HLA-$B57^+$ donors in our clinical cohort. The flow cytometric gating strategy is shown in Supplemental Figure S1A. In parallel, we used a variant B57-$Gag_{240\text{-}249}$ tetramer (TW10n TET) based on a $CD8^+$ T cell escape sequence (TSNLQEQIGW) of the wildtype HIV-derived epitope that abrogates HLA-B57 binding to KIR3DL1*001 [6]. We observed substantial binding of $CD8^-$ cells to the NS1 TET with minimal binding to the TW10n TET (Figure 1A and 1B).

Next, we tested PBMC obtained at multiple time points during and after acute DENV infection from 11 HLA-$B57^+$ children, two with primary and nine with secondary DENV infection (Table 1). As our staining panel for clinical samples was developed to phenotype $CD8^+$ T cells and did not include NK cell-specific markers, we first confirmed that live lymphocytes excluding monocytes, T and B cells were predominantly NK cells. We used convalescent samples for this purpose and found that >70% of the $CD3^-CD8^-CD14^-CD19^-$ population comprised $CD56^+$ NK cells in the majority of donors (Supplemental Figure S1B); these cells are hereafter referred to as the “NK-enriched” population. Although a significant proportion of NK cells can express CD8, these were excluded from our study to ensure the elimination of all T cells. This was considered important because CD3 downregulation during acute illness complicated the identification of T cells based solely on this marker. Evaluating the frequency of NS1 TET^+ $CD8^-$ cells in PBMC from the HLA-$B57^+$ Thai cohort, we were able to detect NS1 TET^+ NK-enriched cells at all time points tested in all donors (n=10; n=5 DF, n=5 DHF) (Figure 1C). The frequencies of these NS1 TET^+ NK-enriched cells varied over time (Figure 1C).

To confirm binding of the NS1 TET to NK cells, we used a staining panel with NK lineage-specific markers (Figure 2A, D) to analyze $KIR3DL1^+$ PBMC from healthy donors and convalescent PBMC from Thai cohort subjects (Figure 2B, C). A fluorescence minus one control excluding the NS1 TET, parallel staining with the TW10n TET, and KIR3DL1 antibody labeling were used to aid gate placement for the accurate identification of NS1 TET^+ NK cells. We observed NS1 TET^+ NK cell populations in all donors at variable frequencies and degrees of separation. Moreover, the NS1 TET bound mostly to $CD56^{dim}$ NK cells, which are known to express KIRs [30]. Given that NK cells are highly heterogeneous, we next determined whether NS1 TET^+ NK cells differed phenotypically from the total NK cell population. We found that NS1 TET^+ NK cells resembled typical NK cells in that they expressed CD161, NKp30, NKp46, and NKG2D (Figure 2D). Thus, the NS1 TET bound archetypal $CD56^{dim}$ NK cells.

Binding of the NS1 TET to KIR3DL1

We speculated that binding of the NS1 TET to NK cells was mediated via the inhibitory receptor KIR3DL1. To test this possibility, we used a magnetic separation protocol to deplete PBMC of $KIR3DL1^+$ cells and compared NS1 TET binding in parallel experiments with non-depleted PBMC (Figure 3A, B). We found that depletion of $KIR3DL1^+$ cells reduced NS1 TET binding by 66%, suggesting a specific interaction between these proteins on the NK cell surface. To confirm binding of the NS1 TET to KIR3DL1 directly, we used distinct KIR3DL1-transfected cell lines individually expressing the allotypes *001, *005, and *015, which represent the three major lineages of this inhibitory receptor [2]. We observed significant binding of the NS1 TET to all three KIR3DL1 allotypes in these experiments. As expected, HLA-B57 tetramers folded with the self-peptide LF9 (LSSPVTKSF) also bound all three allotypes of KIR3DL1 (Figure 3C, D, E, F) [33]. Moreover, pretreatment with a KIR3DL1-specific monoclonal antibody (DX9) blocked the binding of both tetramers to KIR3DL1 (Figure 3C, D, E, F). Collectively, these data indicate that the NS1 TET binds KIR3DL1 on the surface of NK cells.

Peak expression of CD38 on NS1 TET$^+$ NK-enriched cells occurs around fever day 0 and correlates with disease severity

To determine whether NS1 TET$^+$ and total NK cells were activated during acute infection in HLA-B57$^+$ subjects (n=2 DF 1^0, n=3 DF 2^0, n=5 DHF 2^0), we assessed the expression of CD38, CD69, and CD71 on NK-enriched populations in PBMC samples collected prior to, during and after the critical phase of DENV illness. The flow cytometric gating strategy used to identify NK-enriched populations in these experiments is shown in Figure 4A. Representative stainings for CD69 and CD71 expression on PBMC obtained at an acute and convalescent time point from a subject with DHF are shown in Figure 4B and 4C. We found that CD69 expression was mildly elevated early in disease, but remained relatively high at convalescent time points in patients with DF and DHF (Figure 4D). In addition, CD69 expression on NS1 TET$^+$ NK cells in individual donors was similar to the expression of CD69 on total NK-enriched cells. Peak CD71 expression occurred at fever day 0 on NS1 TET$^+$ and total NK cells in many donors, but the differences were not statistically significant between patients with DF and DHF. Mean CD71 expression at acute time points was significantly higher in the NS1 TET$^+$ NK cell population compared to total NK cells ($p<0.01$; Figure 4E).

Next, we examined CD38 expression on NK-enriched cell populations in this HLA-B57$^+$ cohort. We found that CD38 expression was highly elevated on NK cells in PBMC during acute illness, but decreased during early convalescence and remained present on up to 40% of NK-enriched cells 1 year after infection (Figure 5A). More careful examination revealed that CD38 expression clearly segregated into $CD38^{hi}$ and $CD38^{low}$ populations on NK-enriched cells at acute time points. Figure 5B shows CD38 expression on NK-enriched cells at fever day +1 and fever day +180 in a representative donor. Frequencies of $CD38^{low}$ cells followed the same pattern as CD69 expression on NK cells, with elevations early during infection that remained high even during convalescence (Figure 5C). However, a different pattern was observed for $CD38^{hi}$ cells in both the NS1 TET^{+} and total NK cell populations, with low frequencies early during acute infection becoming elevated between fever day 0 and fever day +1, then returning to baseline at 1 year post-infection (Figure 5D). The peak frequency of $CD38^{hi}$ cells was observed on fever days 0 and +1 for both the total NK-enriched and NS1 TET^{+} NK cell populations. Strikingly, very high frequencies of $CD38^{hi}$ NS1 TET^{+} and total NK cells were observed uniquely in patients with DHF (p=0.0571 compared to patients with DF).

As our original gating strategy excluded $CD3^{-}CD8^{+}$ cells in the NK-enriched population, we further evaluated the expression of CD38, CD69, and CD71 using an inclusive approach (Supplemental Figure S2). Activation levels of NK-enriched populations assessed using these markers were similar in the presence or absence of $CD3^{-}CD8^{+}$ cells. In addition, we used a quantitative PCR to measure viremia levels during early clinical illness in 9 of the 11 HLA-$B57^{+}$ subjects. As expected, plasma virus loads were high in all donors prior to defervesence and dropped significantly as the fever dissipated (Supplemental Figure S3). However, no statistically significant correlations were detected between viremia levels and $CD38^{hi}$ NK cell frequencies (data not shown).

Expression of KIR3DL1 on NK cells in PBMC from the HLA-$B57^{+}$ Thai cohort

To extend these findings, we examined KIR3DL1 expression on NK cells in PBMC from our Thai cohort using the KIR3DL1-specific antibody DX9. Expression levels of KIR3DL1 are known to vary between donors [4, 30, 34], and differential expression of inhibitory KIRs can significantly impact NK cell function [35]. We found substantial frequencies of $KIR3DL1^{+}CD56^{+}$ NK cells in 9 of 9 donors tested

(Figure 6A). The frequency of KIR3DL1 on NK cells varied from 3.5% to 15%, which is consistent with frequencies reported elsewhere [34]. PBMC were not available from two subjects, but genotypic studies indicated that both were KIR3DL1^{+}. The intensity of KIR3DL1 expression varied among donors, with mean fluorescence intensity (MFI) values ranging across an order of magnitude (881-7,094). However, the sample size was too small to draw any conclusions regarding associations between KIR3DL1 expression, KIR3DL1 subtyping and dengue disease severity (Figure 6A and Table 1).

Finally, we measured CD69 expression to assess NK cell activation in a limited number of PBMC samples obtained at fever day 0 (+/- 1 day) and fever day +180. Consistent with the results presented above, we found high frequencies of KIR3DL1^{+}CD69^{+} NK cells during acute infection (Figure 6B, C). At the same time, overall KIR3DL1^{+}CD56^{+} NK cell frequencies remained stable (data not shown). Collectively, these data indicate that NK cells are activated in HLA-B57^{+} individuals during the critical phase of illness.

Discussion

In this study, we demonstrate binding of the NK cell-expressed inhibitory receptor KIR3DL1 to an HLA-B57-restricted DENV NS1-derived peptide that also serves as a $CD8^+$ T cell epitope. Direct *ex vivo* staining of primary human NK cells was observed with the corresponding pMHC tetramer in peripheral blood samples isolated from Thai children during and after acute DENV infection. Moreover, NS1 TET^+ and total NK cells were activated to express CD38 during the critical phase of DENV illness only in HLA-$B57^+$ patients with DHF, suggesting that NK cell subsets may contribute to the immunopathogenesis of dengue disease. This phenotypic analysis provides the first indication of a role for KIR-HLA interactions in an acute self-limited viral infection and suggests that innate immune receptors may determine the outcome of DENV infection alongside traditional adaptive responses [12, 14].

Interactions between MHC class I molecules and NK cell-expressed KIRs have been associated with both beneficial and detrimental outcomes in various chronic viral infections [9] and with the development of autoimmune diseases [36]. Several studies have shown that certain KIR alleles and HLA-B loci strongly influence the rate of progression to AIDS in HIV-infected individuals and mechanistically implicate NK cells as key determinants of viremic control [3]. The interaction between HLA-B57 and KIR3DL1 has been extensively studied in this context. For example, Fadda *et al.* showed that naturally occurring single amino acid escape mutations in HLA-B57-restricted HIV-derived $CD8^+$ T cell epitopes could completely abolish KIR3DL1 binding [6, 33]. Similarly, the interaction between B57-$NS1_{26\text{-}34}$ and KIR3DL1 may represent a novel strategy by which DENV evades NK cell-mediated immunity. Functional studies are in progress to address this possibility. Polyfunctional assays with HLA-B57+ NK sensitive targets are critical to determine whether the DENV NS1 peptide can modulate NK cell function and are an active area of research in the laboratory.

In longitudinal phenotypic analyses, we found that CD69 expression on NK-enriched cells was elevated early during acute infection. In contrast, $CD71^{+}$ and $CD38^{hi}$ NK cells were rare at this time point and became more prevalent later, with peak frequencies around fever day 0 in several donors. The emergence of abundant $CD38^{hi}$ NK cells coincided with peak $CD8^{+}$ T cell activation in this cohort and the critical period for plasma leakage and thrombocytopenia in patients with DHF [29]. Moreover, $CD38^{hi}$ expression on NK-enriched cells differed substantially between patients with mild (DF) and severe (DHF) dengue disease. These distinct activation patterns may prelude the identification of clinically relevant biomarkers in acute DENV infection.

The late activation of NK cells could be a consequence of the cytokine storm associated with DHF. In this scenario, NS1 TET^{+} (and therefore $KIR3DL1^{+}$) NK cells might be driven to expand preferentially in HLA-$B57^{+}$ hosts due to more efficient licensing. Alternatively, NS1 TET^{+} cells may represent a subset of NK cells that are restrained early in infection due to interactions between B57-$NS1_{26-34}$ and KIR3DL1. As flaviviruses are known to upregulate MHC class I [37], we propose that the increased expression of HLA-B57 on target cells early in infection augments NS1 peptide presentation during the acute viremic phase, thus enhancing KIR3DL1 interactions and maintaining NK cell inhibition. As viral titers fall and MHC class I expression returns to normal during defervescence, B57-$NS1_{26-34}$ levels will also wane and allow “retuned” NK cells to respond vigorously.

Despite collection over a 15 year time period, we were only able to enroll a total of 15 HLA-$B57^{+}$ donors due to the low frequency of this allele in Thailand. This limitation impacted the power of our study and the differences in $CD38^{hi}$ expression did not quite achieve statistical significance ($p=0.0571$). In addition, the relative rarity of HLA-B*57 may confine the clinical relevance of DENV $NS1_{26-34}$ in the Thai population. The fact that not all HLA-$B57^{+}$ $KIR3DL1^{+}$ individuals develop DHF suggests the involvement of additional regulatory loops [38]. Given the stochastic expression of KIRs, different individuals will co-express different combinations of inhibitory and activating receptors within the KIR3DL1$^+$ NK cell subset. This constellation of receptor/ligand interactions will likely contribute to differential effects on NK cell function. In addition, elevated levels of cytokines known to be upregulated in patients with dengue will almost certainly influence the quality of NK cell and T cell responses. It is notable in this respect that the DENV envelope (E) protein interacts directly with the NK cell activating receptor NKp44 [39].

As with most clinical studies of dengue, the delay between initial viral infection and presentation to the clinic or hospital prevented a very early assessment of NK cell activation in this cohort. A rapid NK cell response that leads to pathogen elimination may reduce the levels of antigen available for presentation, thereby potentially impairing the development of memory T cell populations. Indeed, NK cells have been implicated in the regulation of T cell immunity during viral infections, purportedly acting to prevent pathological responses by attenuating T cell activation in the presence of high viral loads [40-42]. In this study, we found delayed activation of NK cells in HLA-B57$^+$ KIR3DL1$^+$ donors, which could hamper the development of protective memory T cell responses to DENV. This regulatory activity of NK cells could explain the modest CD8$^+$ T cell responses directed against this highly conserved NS1 epitope in secondary DENV infections [29].

In conclusion, our findings suggest that NK cell subsets play a role in the development of adverse immune responses associated with DHF in the context of HLA-B57. Further studies are warranted to identify determinative KIR-HLA interactions in other acute self-limited viral infections.

Materials and Methods

Study subjects and blood samples

The study design for patient recruitment and collection of blood samples has been reported in detail elsewhere [11, 43-45]. Briefly, the enrolled subjects were Thai children aged 6 months to 15 years with acute febrile illnesses (<72hrs) diagnosed as DF or DHF according to WHO guidelines [46]. Serology and virus isolation were used to confirm acute DENV infection, and primary and secondary infections were distinguished on the basis of serologic responses [11]. For donors undergoing a secondary infection, it was not possible to determine the previous infecting serotype(s). Blood samples were obtained daily during acute illness, once during early convalescence, and at various intervals during late convalescence. PBMC were isolated by density gradient centrifugation, cryopreserved, and stored at −70°C. Samples were numbered relative to the day of defervescence (designated fever day 0). Serologic HLA class I typing was performed as described previously using peripheral blood from immune Thai donors at the Department of Transfusion Medicine, Siriraj Hospital [15, 44]. Written informed consent was obtained from each subject and/or his/her parent/guardian prior to study participation. The study was approved by the Institutional Review Boards of the Thai Ministry of Public Health, the Office of the US Army Surgeon General and the University of Massachusetts Medical School (UMMS). For control purposes, PBMC were obtained with informed consent from healthy HLA-$B57^+$ dengue-naïve volunteers aged >18 years under approval granted by the UMMS Institutional Review Board.

Peptide-MHC tetramers

Peptide-MHC tetramers (pMHC TETs) were either obtained from the NIAID Tetramer Core Facility or generated in-house as described previously [47]. The following conjugates were used in this study: A2-$E_{213\text{-}221}$ TET-APC, B57-LF9 TET-PE, B57-$NS1_{26\text{-}34}$ TET-PE, B57-$NS1_{26\text{-}34}$ TET-APC, B57-TW10n TET-PE, and B57-TW10n TET-APC.

Flow cytometry

As described previously [29], cryopreserved PBMC from Thai subjects were thawed and washed in RPMI before resting in RPMI/10% FBS for 2 hours at 37°C. Cells were then washed in PBS and stained with 1µL of pre-diluted (1:80) LIVE/DEAD® Green (Molecular Probes, Invitrogen). After washing in FACS buffer (PBS/2% FBS/0.1% sodium azide), cells were incubated with 0.5-2µL pMHC TET (1µg/µL with respect to the monomeric component) for 20 minutes at 4°C. Pre-titrated monoclonal antibodies specific for CD3, CD8, CD14, CD19, CD28 or CD56, CD38, CD45RA, CD57, CD69, CD71, and CCR7 were then added for a further 30 minutes at 4°C. Monoclonal antibodies specific for CD3, CD14, CD16, CD19, CD56, CD69, and KIR3DL1 were used in a separate panel to identify NK cells. For NS1 TET staining of PBMC from healthy individuals, $1x10^7$ cells from $KIR3DL1^+$ subjects were washed in PBS and stained with LIVE/DEAD® Green. After washing in FACS buffer, cells were incubated with 2µL pMHC TET or a KIR3DL1-specific monoclonal antibody for 20 minutes at 4°C. Pre-titrated monoclonal antibodies specific for CD3, CD14, CD16, CD19, CD56, CD161, NKp30, NKp46, and NKG2D were then added for a further 30 minutes at 4°C. In all experiments, cells were washed and fixed with BD Stabilizing Fixative™ (BD Biosciences). Data were collected using a FACSAria™ flow cytometer (BD Biosciences) and analyzed with FlowJo version 10 (TreeStar Inc.). Details of all monoclonal antibodies used in this study are presented in Supplemental Table 1.

$KIR3DL1^+$ NK cell depletion and NS1 tetramer staining

PBMC were isolated from $KIR3DL1^+$ healthy subjects using standard density gradient centrifugation and depleted of $KIR3DL1^+$ cells via magnetic bead separation (Miltenyi Biotec). KIR3DL1-depleted PBMC were washed in FACS buffer and incubated with NS1 TET for 50 minutes at 4°C. After a further wash in FACS buffer, cells were fixed with 100µL of pre-diluted (1:4) BD Cytofix

(BD Biosciences) and kept at 4°C until acquisition. Flow cytometric data were collected and analyzed as described above.

Binding of pMHC tetramers to KIR3DL1-transfected cell lines

Detailed analyses of KIR3DL1-transfected lines were performed as reported elsewhere [33]. Briefly, HEK 293 cells were transfected with FLAG-tagged constructs of KIR3DL1*001, *005, or *015. An anti-FLAG monoclonal antibody was used to verify KIR3DL1 expression. Transfected cells were pre-incubated with 10µg/µL of the blocking monoclonal antibody DX9 or control IgG, then stained with 0.25µL of the NS1 TET or the well described LF9 TET, representing a self-derived peptide complexed with HLA-B57 that binds KIR3DL1 [48].

Statistical analysis

Comparisons between groups were conducted using the Mann-Whitney rank sum test for non-normally distributed variables. All statistical analyses were performed using GraphPad Prism (GraphPad Software).

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Author Contributions

ET, AM and ALR conceived and designed the experiments and wrote the manuscript text. ET, GOC and MW performed the experiments. ET and AM analyzed the data. ET, AM and DAP prepared the figures. MC, SJT, SK, IKY, AN, AS and SG enrolled patients and collected samples. CC, EG, DAP, MC, GA and DWM contributed reagents, protocols. HAFS provided HLA typing data. All authors reviewed the manuscript and agree with the results and conclusions.

Conflict of Interest Disclosure: The authors declare no commercial or financial conflict of interest.

Abbreviations

DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
KIR	Killer immunoglobulin-like receptor
LTA	Lymphotoxin alpha
LTB	Lymphotoxin beta
MICA/B	MHC class I-related chains A/B
NS1	Non-structural protein-1
NS1 TET	B57-NS1$_{26\text{-}34}$ tetramer
pMHC TETs	Peptide-MHC tetramers
TW10n TET	B57-Gag$_{240\text{-}249}$ tetramer

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Figure Legends

Figure 1: Binding of the NS1 TET to non-CD8 cells in PBMC from Thai children with dengue. (A, B) Using flow cytometry, frequencies of NS1 TET$^+$ (A) and TW10n TET$^+$ (B) CD3$^-$CD8$^-$CD14$^-$CD19$^-$ (NK-enriched) cells in PBMC from donors CHD01-018 and KPP94-041 at the 1 year time point. (C) Kinetics of NS1 TET$^+$ frequencies among NK-enriched cells during acute dengue illness and convalescence. Fever day 0 indicates the day of defervescence. Symbols distinguish subjects with primary (*n=2*, grey symbols) versus secondary (*n=8*, black symbols) DENV infections and lines distinguish those with DF (*n=5*, black line) versus DHF (*n=5*, dashed line).

Figure 2: Frequencies and phenotype of NS1 TET$^+$ NK cells. (A) Gating strategy to identify CD56$^+$ and/or CD16$^+$ NK cells. (B) Frequencies of NS1 TET$^+$ NK cells in PBMC from healthy KIR3DL1$^+$ donors. Representative flow cytometry plots from 4 of 13 donors are shown on the top row. Fluorescence minus one (FMO), NS1 TET$^+$ and TW10n TET$^+$ NK cell frequencies in PBMC from healthy donor LD093 are shown on the bottom row. (C) Frequencies of NS1 TET$^+$ NK cells in PBMC obtained from Thai study subjects 2 to 3 years after DENV infection. (D) Overlay of NS1 TET$^+$ NK cells (red dots) on the total NK cell population (zebra plot) in PBMC from a healthy KIR3DL1$^+$ donor. The expression pattern of CD161, NKp30, NKp46, and NKG2D was compared between NS1 TET$^+$ NK cells and the total NK cell population.

Figure 3: Binding of the NS1 TET to KIR3DL1. Using flow cytometry, (A, B) Frequency of NS1 TET$^+$ NK cells in PBMC from a KIR3DL1$^+$ donor before (A) and after (B) magnetic depletion of KIR3DL1$^+$ cells. Data represent one of three independent experiments. (C-F) HEK 293 cells were transfected with KIR3DL1 and stained with the NS1 TET (black) or the LF9 TET (grey). Histograms

show NS1 TET and LF9 TET binding (solid lines) to untransfected cells (C) or cells stably transfected with KIR3DL1*001 (D), KIR3DL1*005 (E), or KIR3DL1*015 (F). Binding of the NS1 TET and the LF9 TET in the presence of a monoclonal KIR3DL1-specific blocking antibody (DX9) is shown (dashed lines).

Figure 4: Activation of NS1 TET$^+$ and total NK cells over the course of acute dengue illness. (A) Gating strategy to identify NK-enriched cells in PBMC from Thai subjects. (B) Representative flow cytometry plot depicting CD69 expression on NK-enriched cells at fever day -1 and fever day +6 from a subject with DHF. (C) Representative flow cytometry plot depicting CD71 expression on NK-enriched cells at fever day 0 and fever day +180 from a subject with DF. (D, E) Kinetics of CD69 (D) and CD71 (E) expression on NS1 TET$^+$ and total NK cells during acute dengue illness and convalescence. The average frequencies of CD69$^+$ and CD71$^+$ total NK-enriched cells are shown using a solid red line for subjects with DF and a dashed red line for subjects with DHF. Symbols distinguish subjects with primary (*n=2*, grey symbols) versus secondary (*n=8*, black symbols) DENV infections and lines distinguish those with DF (*n=5*, black line) versus DHF (*n=5*, dashed line).

Figure 5: CD38 expression on NS1 TET$^+$ and total NK cells over the course of acute dengue illness. (A) Kinetics of CD38 expression on NS1 TET$^+$ and total NK cells during acute dengue illness and convalescence. (B) Representative flow cytometry plots depicting CD38^{hi} versus CD38^{low} NK cell populations at fever day +1 and fever day +180 from a subject with DF. (C, D) Frequencies of CD38^{low} (C) and CD38^{hi} (D) NK cell populations during acute dengue illness and convalescence. The average frequencies of CD38^{hi} and CD38^{low} total NK-enriched cells are shown using a solid red line for subjects with DF and a dashed red line for subjects with DHF. Symbols distinguish subjects with primary (*n=2*,

grey symbols) versus secondary (*n=8*, black symbols) DENV infections and lines distinguish those with DF (*n=5*, black line) versus DHF (*n=5*, dashed line).

Figure 6: KIR3DL1 staining of NK cells in PBMC from Thai study cohort subjects. (A) Frequencies of $KIR3DL1^+$ NK cells in PBMC obtained from Thai study subjects 2 to 3 years after DENV infection. PBMC were gated on $CD56^+$ and/or $CD16^+$ NK cells. Dot plots show CD56 versus KIR3DL1 staining. (B) Representative flow cytometry plots depicting CD69 versus KIR3DL1 expression on NK cell populations at fever day 0 and fever day +180 from a subject with DHF. (C) Frequencies of $KIR3DL1^+CD69^+$ NK cell populations (*n=9*) during acute dengue illness and convalescence.

TABLE 1: Clinical, virological and immunogenetic profiles of HLA-B57+ Thai study subjects

Donor	Serology[a]	Serotype[b]	Diagnosis[c]	KIR3DL1[d]	KIR3DS1
CHD95-039	P	DENV-1	DF	01502	+
CHD06-029	P	DENV-3	DF	01502, 01502	-
CHD05-023	S	DENV-1	DF	01502	+
CHD01-018	S	DENV-2	DF	020	+
KPP94-037	S	DENV-2	DF	01502,01502	-
KPP94-041	S	DENV-1	DHF-3	00501	-
CHD02-073	S	DENV-1	DHF	00501	-
CHD01-058	S	DENV-2	DHF-1	01502	+
CHD01-050	S	DENV-2	DHF-3	01502	-
CHD00-054	S	unknown	DHF-2	00701	+
CHD06-092	S	DENV-4	DHF-2	00701,01502	+

[a] Primary (P) versus secondary (S) infection as determined by IgM/IgG ratios [11].
[b] Of current infection. Unknown = could not be determined.
[c] *According to WHO guidelines 1997; DF = dengue fever; DHF = dengue hemorrhagic fever (grades 1-3).*
[d] *KIR3DL1 subtyping.*

Figure 1

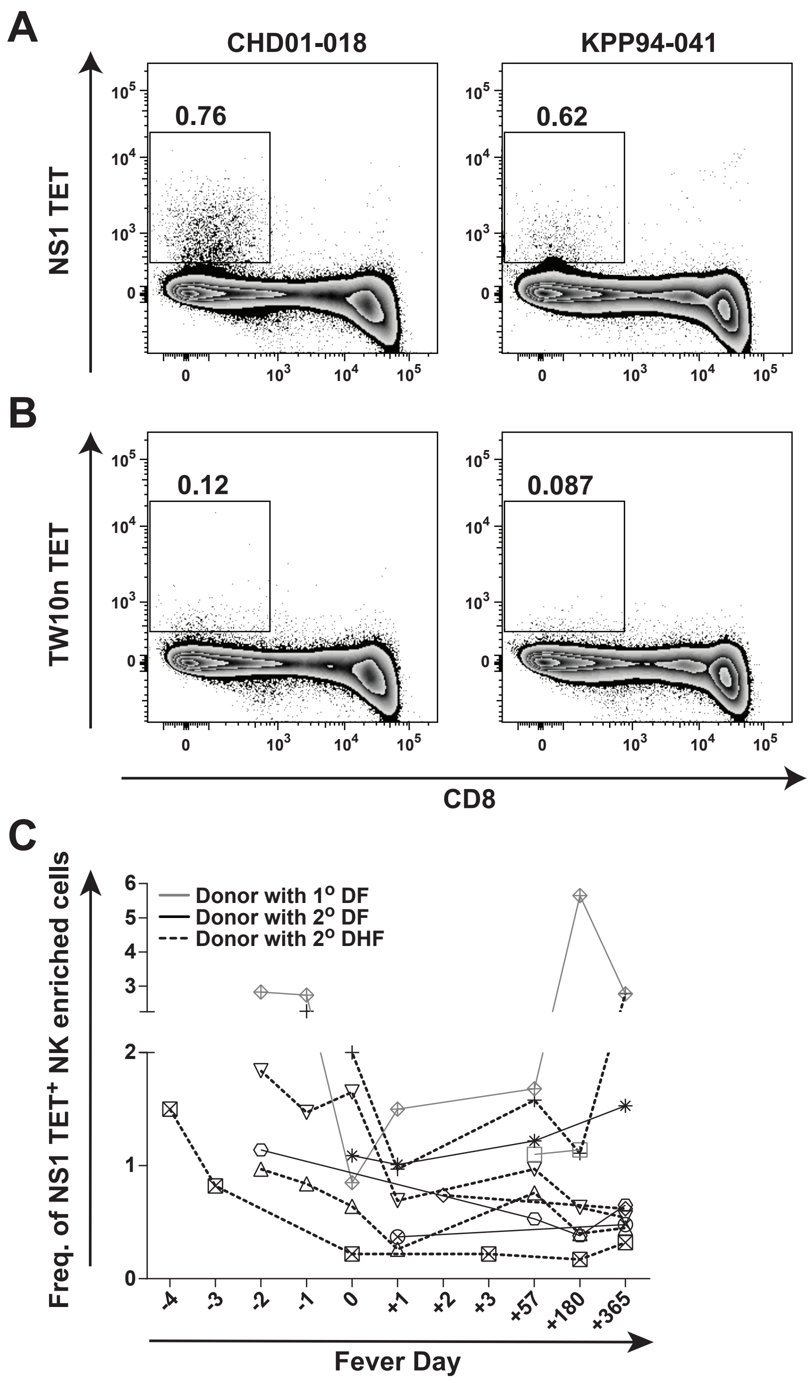

A
CHD01-018
KPP94-041
NS1 TET
0.76
0.62
B
TW10n TET
0.12
0.087
CD8
C
Freq. of NS1 TET+ NK enriched cells
Donor with 1° DF
Donor with 2° DF
Donor with 2° DHF
-4
-3
-2
-1
0
+1
+2
+3
+57
+180
+365
Fever Day

Figure 2

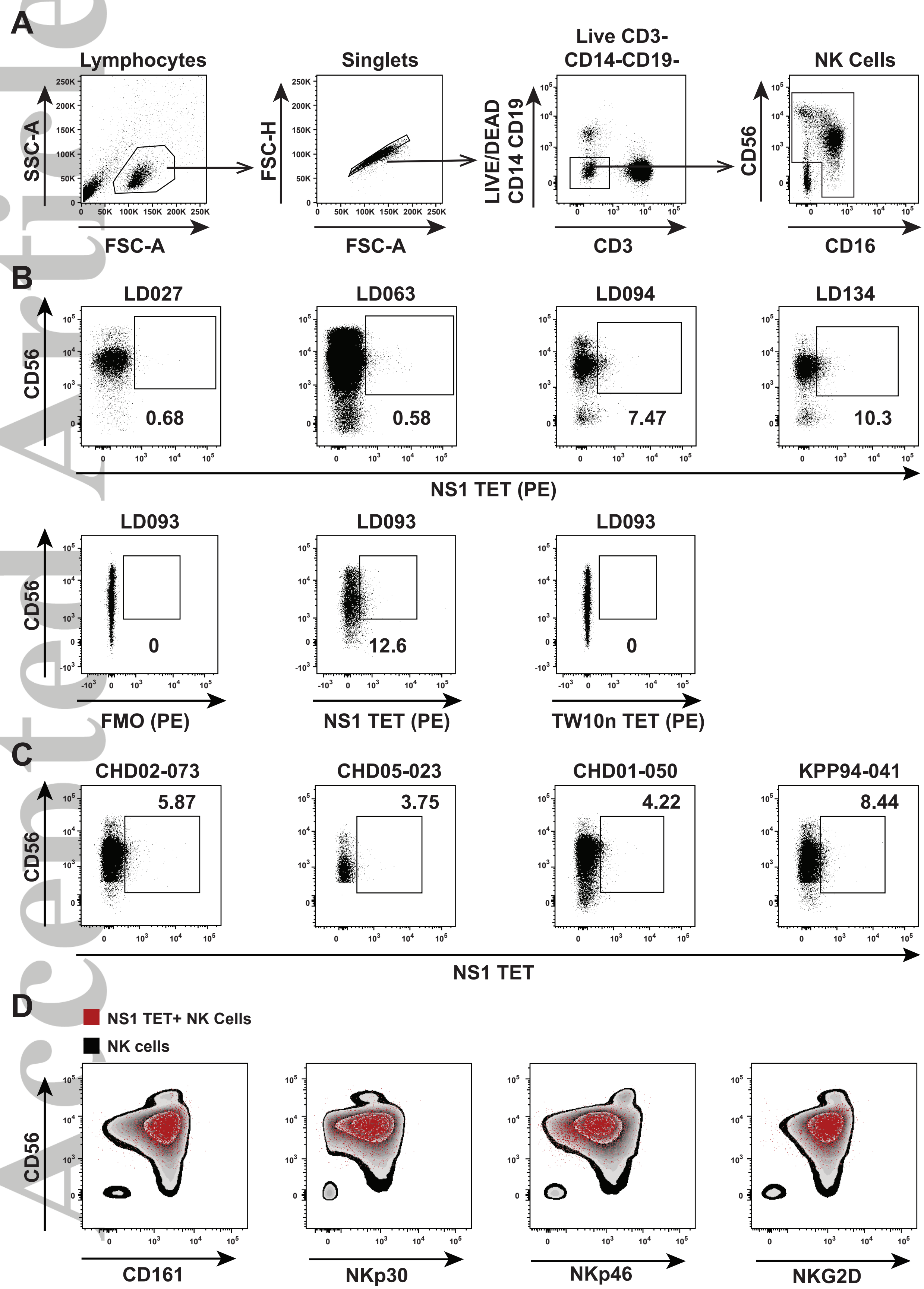

A
Lymphocytes
SSC-A
FSC-A
Singlets
FSC-H
FSC-A
Live CD3-
CD14-CD19-
LIVE/DEAD
CD14 CD19
CD3
NK Cells
CD56
CD16
B
LD027
0.68
LD063
0.58
LD094
7.47
LD134
10.3
CD56
NS1 TET (PE)
LD093
0
LD093
12.6
LD093
0
FMO (PE)
NS1 TET (PE)
TW10n TET (PE)
C
CHD02-073
5.87
CHD05-023
3.75
CHD01-050
4.22
KPP94-041
8.44
CD56
NS1 TET
D
NS1 TET+ NK Cells
NK cells
CD56
CD161
NKp30
NKp46
NKG2D

Figure 3

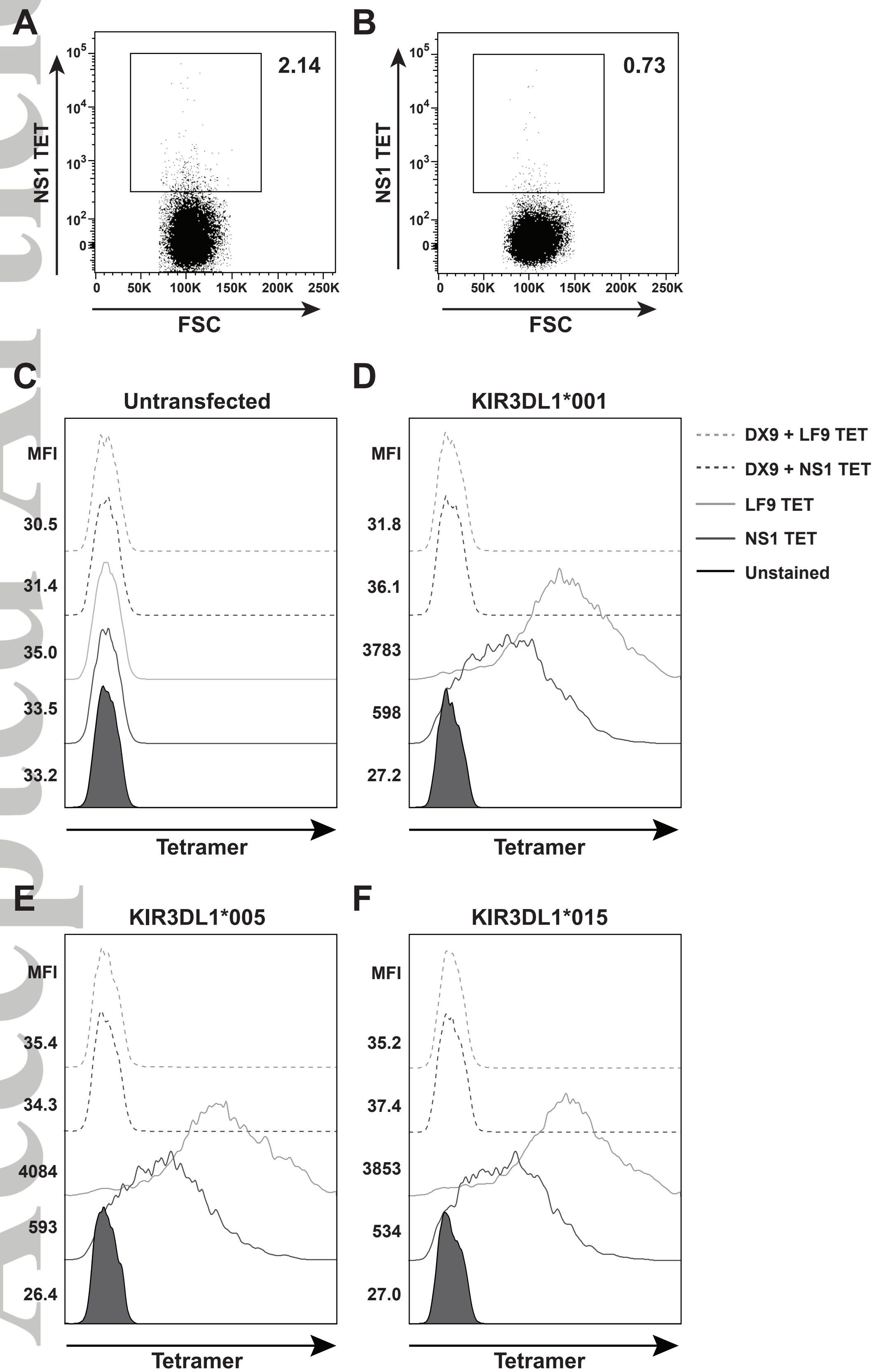

A
B
NS1 TET
FSC
2.14
0.73
0
50K
100K
150K
200K
250K
C
Untransfected
D
KIR3DL1*001
E
KIR3DL1*005
F
KIR3DL1*015
MFI
30.5
31.4
35.0
33.5
33.2
31.8
36.1
3783
598
27.2
35.4
34.3
4084
593
26.4
35.2
37.4
3853
534
27.0
Tetramer
DX9 + LF9 TET
DX9 + NS1 TET
LF9 TET
NS1 TET
Unstained

Figure 4

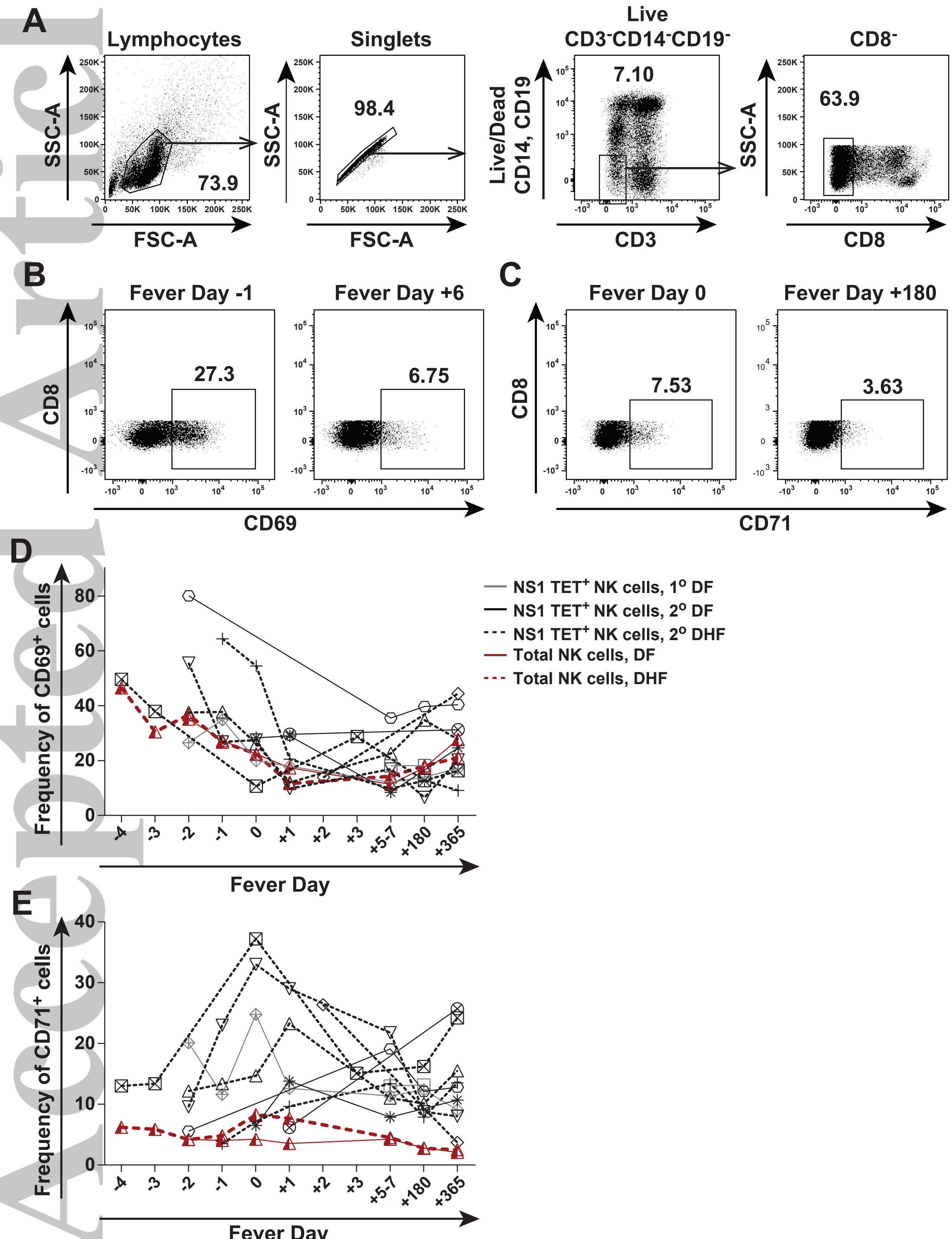

A
Lymphocytes
Singlets
Live
CD3-CD14-CD19-
CD8-
SSC-A
FSC-A
73.9
98.4
Live/Dead
CD14, CD19
CD3
7.10
CD8
63.9
B
Fever Day -1
Fever Day +6
27.3
6.75
CD69
C
Fever Day 0
Fever Day +180
7.53
3.63
CD71
D
Frequency of CD69+ cells
Fever Day
NS1 TET+ NK cells, 1° DF
NS1 TET+ NK cells, 2° DF
NS1 TET+ NK cells, 2° DHF
Total NK cells, DF
Total NK cells, DHF
E
Frequency of CD71+ cells
Fever Day

Figure 5

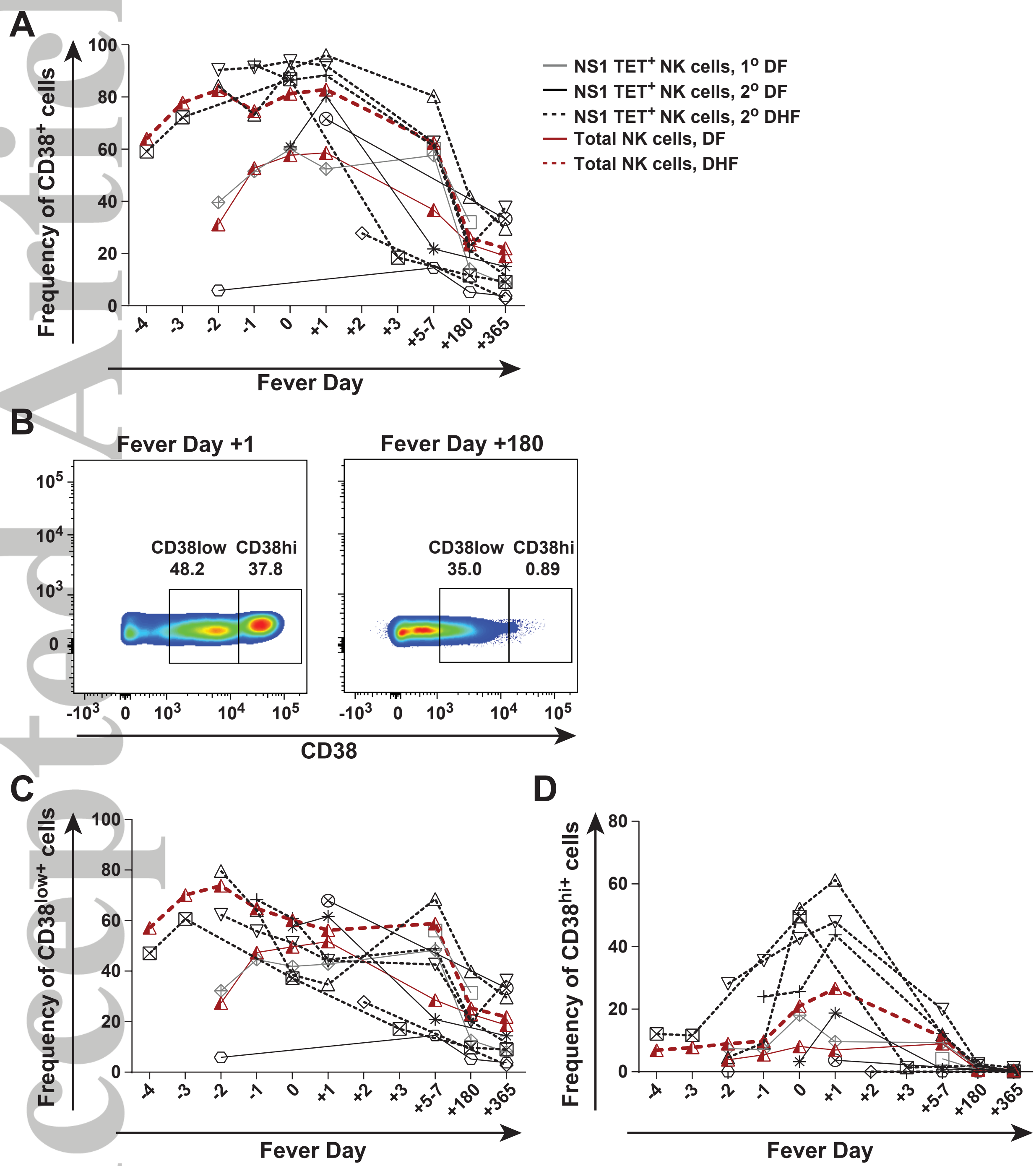
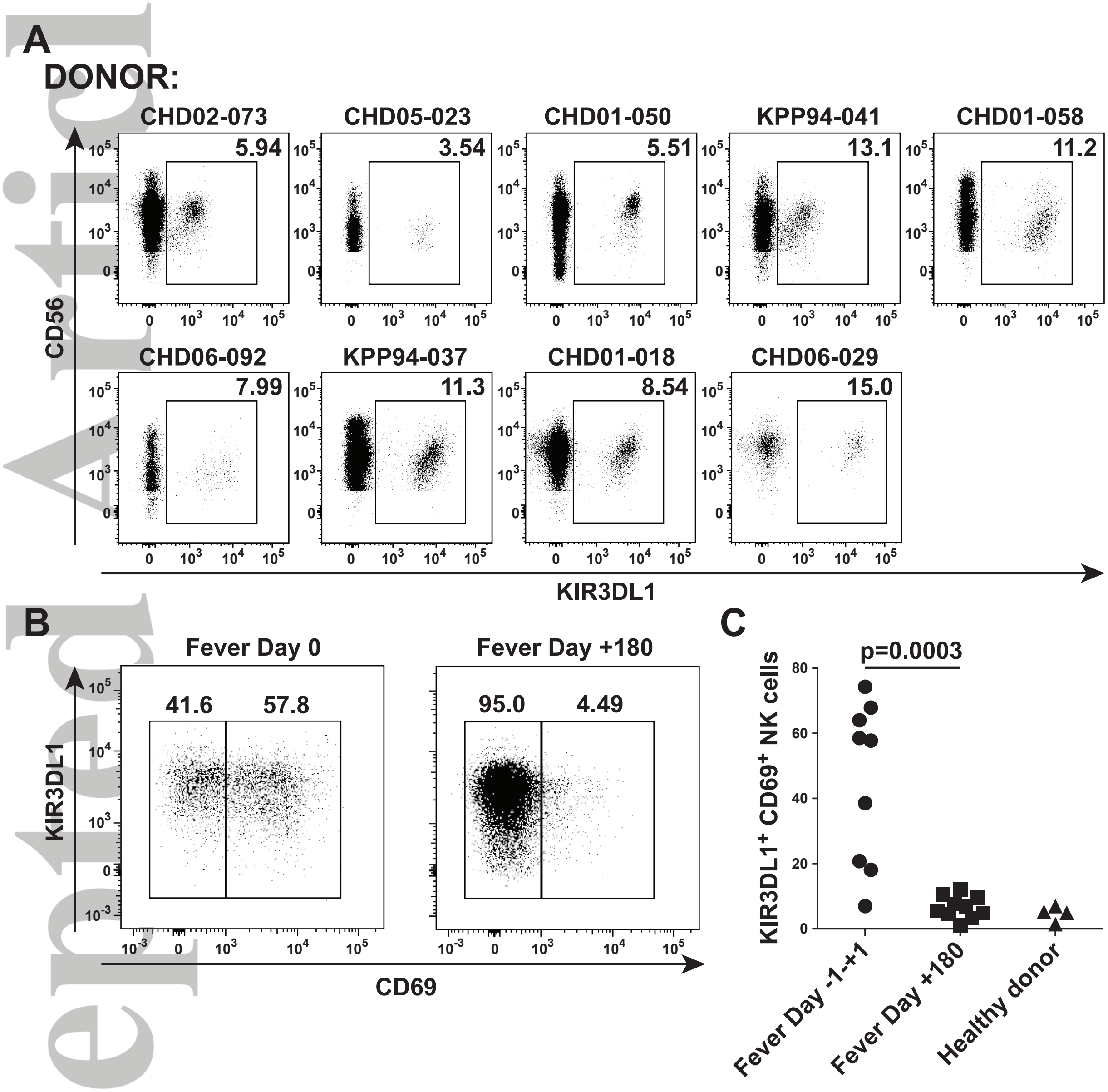
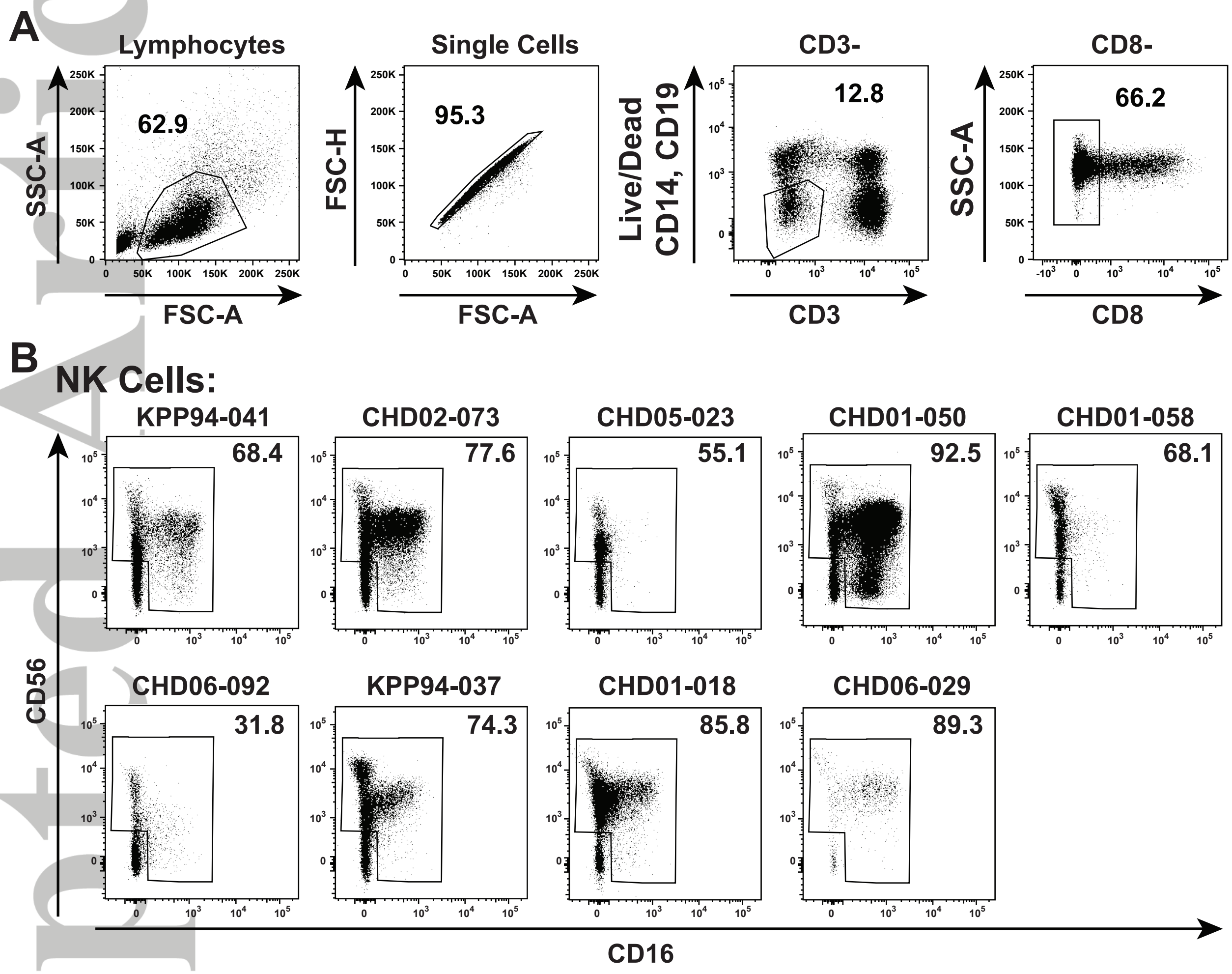

A
Frequency of CD38+ cells
Fever Day
NS1 TET+ NK cells, 1° DF
NS1 TET+ NK cells, 2° DF
NS1 TET+ NK cells, 2° DHF
Total NK cells, DF
Total NK cells, DHF
B
Fever Day +1
Fever Day +180
CD38low CD38hi
48.2 37.8
35.0 0.89
CD38
C
Frequency of CD38low+ cells
D
Frequency of CD38hi+ cells

Figure 6

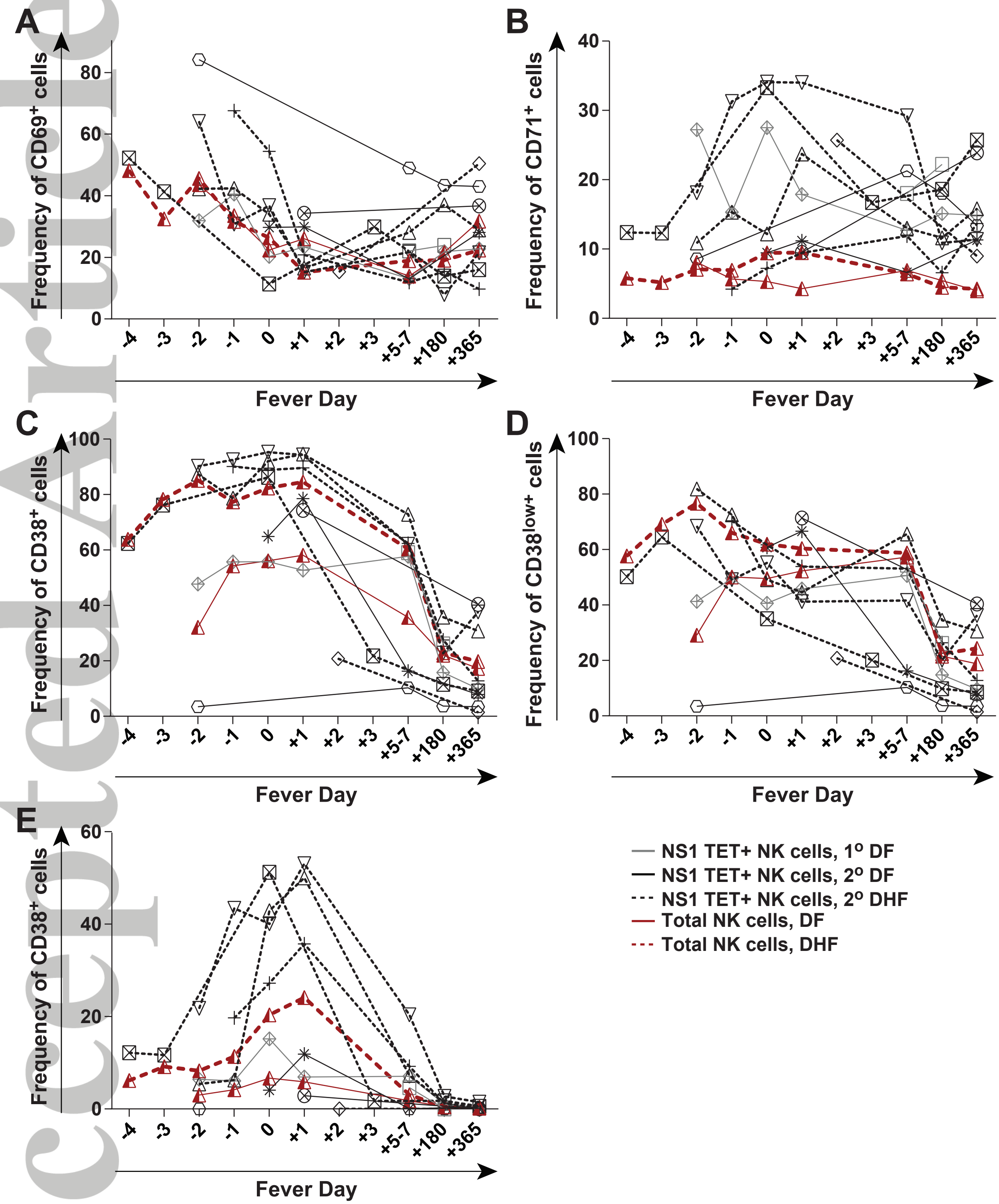

A
DONOR:
CHD02-073
5.94
CHD05-023
3.54
CHD01-050
5.51
KPP94-041
13.1
CHD01-058
11.2
CHD06-092
7.99
KPP94-037
11.3
CHD01-018
8.54
CHD06-029
15.0
CD56
KIR3DL1
B
Fever Day 0
41.6
57.8
Fever Day +180
95.0
4.49
KIR3DL1
CD69
C
p=0.0003
KIR3DL1+ CD69+ NK cells
Fever Day -1-+1
Fever Day +180
Healthy donor

Supplemental Figure 1



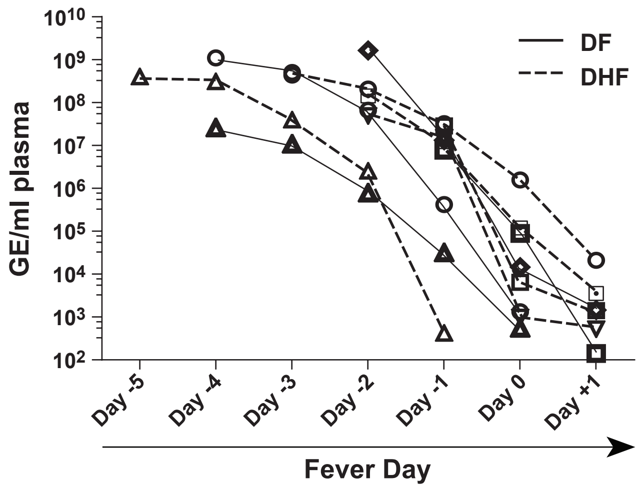
Supplemental Figure S1. Frequencies of NK cells in the CD3$^-$CD8$^-$CD14$^-$CD19$^-$ gate. (A) Gating strategy to identify CD3$^-$CD8$^-$CD14$^-$CD19$^-$ cells. Cells were first selected within the lymphocyte gate as defined by forward and side scatter profiles. Singlets were then identified and live CD3$^-$CD14$^-$CD19$^-$ cells were selected in a dump (LIVE/DEAD® Green with αCD14 and αCD19) versus CD3 bivariate plot. CD8$^-$ cells were gated within this population. (B) Frequencies of CD56$^+$ and/or CD16$^+$ NK cells in PBMCs collected from Thai cohort subjects 2 years after acute DENV infection. Plots are gated on live CD3$^-$CD8$^-$CD14$^-$CD19$^-$ cells.

Supplemental Figure 2



Supplemental Figure S2. Activation of NS1 TET$^+$ and total NK cells over the course of acute dengue illness. Kinetics of CD69 (A), CD71 (B), total CD38 (C), CD38^{low} (D), and CD38^{hi} (E) expression on NS1 TET$^+$ and total NK cells during acute dengue illness and convalescence. The average frequencies of CD69$^+$, CD71$^+$, total CD38$^+$, CD38^{low}, and CD38^{hi} total NK-enriched cells are shown using a solid red line for subjects with DF and a dashed red line for subjects with DHF. Symbols distinguish subjects with primary (*n=2*, grey symbols) versus secondary (*n=8*, black symbols) DENV infections and lines distinguish those with DF (*n=5*, black line) versus DHF (*n=5*, dashed line).

Supplemental Figure 3



Supplemental Figure S3. Magnitude of DENV viremia by day of illness. Levels of DENV genome equivalent (GE) cDNA (copies/mL) were determined in serial plasma samples from HLA-B57$^+$ patients. Symbols denote individual subjects and lines distinguish those with DF (*n=4*, black line) versus DHF (*n=5*, dashed line).

SUPPLEMENTAL TABLE 1: Antibodies used for flow cytometry studies

Marker	Clone	Manufacturer	Fluorochrome
CD3	UCHT1	BD Biosciences	V500
		BioLegend	BV510
	OKT3	BioLegend	BV510
CD8	SK1	Invitrogen	PE-AlexaFluor610
CD45RA	HI100	BD Pharmingen	APC-H7
CCR7	150503	BD Horizon	V450
CD69	CH/4	Invitrogen	PE-Cy5.5
	FN50	BioLegend	BV650
CD38	HB7	eBioscience	eFluor®650NC
CD57	HCD57	BioLegend	PerCP/Cy5.5 (Lightening Link)
CD71	OKT9	eBioscience	PE-Cy7 (Lightening Link)
	CY1G4	BioLegend	APC
CD28	CD28.2	BioLegend	AlexaFluor700
CD56	B159	BD Biosciences	AlexaFluor700
CD19	HIB19	BD Biosciences	FITC
CD14	HCD14	BioLegend	FITC
CD56	HCD56	BioLegend	BV711
CD16	3G8	BD Horizon	APC-H7
NKp30	P30-15	BioLegend	APC
NKp46	9E2	BioLegend	BV421
CD161	HP-3G10	BioLegend	BV605
NKG2D	1D11	BD Biosciences	PE-CF594
KIR3DL1	DX9	Beckman Coulter	PE
		BioLegend	PE
KIR3DL1/S1	Z27	Beckman Coulter	APC
HLA-A,B,C	W6/32	BD Biosciences	APC, PE, FITC
		BioLegend	PE
HLA-B57	BIH0243	One Lambda	PE-NeutrAvidin