iPS cell serves as a source of dendritic cells for *in vitro* dengue virus infection model

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Abstract

The lack of appropriate model has been a serious concern in dengue research pertinent to immune response and vaccine development. It remains a matter of impediment in dengue virus (DENV) studies when it comes to an *in vitro* model, which requires adequate quantity of dendritic cells (DC) with uniform characters. Other sources of DC, mostly monocyte derived DC (moDC), have been used despite their limitations such as quantity, proliferation, and donor dependent characters. Recent development of human iPS cells with consistent proliferation for long, stable functional characteristics and desired HLA background has certainly offered added advantages. Therefore, we hypothesized that iPS derived cells would be a reliable alternative to the traditional DCs to be used in *in vitro* DENV system. To develop DENV infection and T cell activation model, we utilized iPS cell (HLA-A*24) as the source of DC. iPS-ML-DC was prepared and DENV infectivity was assessed apart from the major surface markers expression and cytokine production potential. Our iPS-ML-DC had major DC markers expression, DENV infection efficiency and cytokine production properties similar to that of moDC. Moreover, DENV infected iPS-ML-DC demonstrated the ability to activate HLA-matched T cell (but not mismatched) *in vitro* as evidenced by significantly higher proportion of IFN-γ^+ $CD69^+$ T cells compared to non-infected iPS-ML-DC. This affirmed the antigen-specific T cell activation by iPS-ML-DC as a function of antigen presenting cell. To conclude, maturation potential, DENV infection efficiency and T cell activation ability collectively suggest that iPS-ML-DC serves as an attractive option of DC for use in DENV studies *in vitro.*

Keywords: iPS cell; dendritic cell; dengue virus; cellular immunity; antigen presentation; *in vitro* model.

Introduction

Dengue disease, caused by dengue virus (DENV), is one of the most catastrophic diseases of the current world annually affecting two-third of the global population with 96 million overt infections (including 500,000 severe ones), and responsible for huge socio- economic burden in more than 120 countries of the tropical and sub-tropical regions [1-3]. DENV has four genetically and antigenically related serotypes (DENV-1 to DENV-4), and infection with each of them triggers mild to severe manifestations [1, 3, 4]. Unfortunately, no specific drug for this disease has been approved yet.

DENV provokes peculiar immune response. Infection with any DENV serotype confers a long-term homotypic immunity against that serotype, however secondary infection with a different serotype often results into enhanced severity [5]. Therefore, balancing this tetravalent immunity and cross-protection is the biggest hurdle in dengue vaccine development [4, 6], despite the fact that several vaccine candidates are in pipeline [7-9] in addition to the one recently licensed (Dengvaxia™ by SANOFI PASTEUR) [10]. Dengvaxia™ induced sufficiently high level of neutralizing antibody against DENV serotypes, however it had a lower efficacy for DENV-2 [10]. Paucity of T cell immunity has been considered as a reason since the vaccine lacked protective T cell epitopes, particularly from non-structural (NS) proteins [11]. The effective dengue vaccine should induce both cellular and humoral immune responses (not mutually exclusive) [4, 6, 12].

Recent studies also demonstrated the protective roles of T cells in DENV infection in both human and mouse models [11, 13]. The exact mechanism on how T cells act in the pathogenesis or protection during DENV infection is still unclear and remained a matter of debate for long partly due to the lack of a perfect animal model [12, 14]. A reliable model has been a serious need either in the study of mechanisms or dengue immune responses/vaccines for decades now. Uninterrupted supply of functional dendritic cells (DCs) with constantly uniform characters is a prerequisite for a good *in vitro* system. For instance, to identify excellent protective epitopes presented by DC, a massive quantity of functional DCs with stable and uniform characters is needed [15]. Conventionally, monocyte derived DCs (moDCs) (induced by cytokines) have been used as antigen presenting cells (APCs) *in vitro* [16-18], however the number, quality and antigen presenting ability is donor dependent [19-21] which justifies the need of a better alternative source of DC to establish an *in vitro* system for DENV infection or vaccine studies.

At this juncture, DC is known to be a host of DENV and also an APC to activate T cell in antigen specific manner [16, 17, 22, 23]. iPS cells have recently made revolution in disease modelling and therapy [24, 25]. The iPS cell derived myeloid cell line (iPS-ML) had ability to proliferate for long and differentiate into iPS cell derived DC-like cell (iPS-ML-DC) in cytokine environment [19, 26]. Moreover, flexibility in generation of these iPS cells with different HLA background and quantity as required is its strength. Therefore, we hypothesized that iPS cell based *in vitro* system would also be appropriate for DENV infection to overcome limitations of moDC. In this study, we examined the characters of iPS-ML-DC and its ability to induce T cell upon DENV infection *in vitro*.

Results

General profile of iPS derived cells

iPS-ML originated from the human fibroblast showed a constant proliferation *in vitro* and expressed myeloid markers (CD14, CD33 and CD11b) (Fig. S1) On microscopic observations, the original iPS-ML cells were found small and round, which enlarged upon differentiation (i.e. iPS-ML-DC), and further stimulation with OK-432 induced the development of distinct dendrites (Fig. 1b). These morphological changes were concordant with the increase in forward scatter (FSC) and side scatter (SSC) observed in flow cytometric analysis (Fig. 1a).

Next, we analyzed the expression profile of major surface markers/co-stimulatory molecules (MHC-I, MHC-II, CD80, CD86 and DC-SIGN) of iPS-ML and iPS-ML-DC (with and without OK-432 treatment) before and after infection with DENV. Although both iPS-ML and iPS-ML-DC expressed MHC class I and II, expression levels varied with differentiation phases (Fig. 1a, S2a and S2b, Table S1). Expression of MHC-II, CD80 and CD86 increased in iPS-ML-DC after infection with DENV indicating a relationship with differentiation and activation status (Fig. S2a and S2b). These expression profiles of iPS-ML-DC were found comparable with that of moDC in our parallel experiments (Fig. S3). Also, our iPS-ML-DC expressed three types of Fc gamma receptors (FcγRI, FcγRII and FcγRIII) like other DCs do (Fig. S4) [27].

DENV efficiently infected iPS-ML-DC *in vitro*

DENV-2 (strain 16681) efficiently infected iPS-ML-DC *in vitro* as evidenced by immunofluorescence staining of cells and corresponding virus titers of the culture supernatant

(Fig. 2a). Virus titers in the culture supernatant peaked at day 2 post-infection for iPS-ML-DCs (both OK-432 treated and non-treated cells) while iPS-ML showed almost negligible infection with DENV (Fig. 2b). Additionally, similar infectivity was also confirmed with moDC in our experiments (Fig. S5a and S5b). This observation indicates that iPS-ML-DC can be efficiently infected by DENV similar to moDC.

DENV infected iPS-ML-DC produced high titers of IL-12p70 and TNF-α but not IFN-α

To observe the immune response of iPS-ML-DC after DENV infection, major cytokines known to produce by DC (IFN-α2, TNF-α and IL-12p70) were measured using a multiplex assay system (Fig. 3). iPS-ML-DC produced significantly higher titers of IL-12p70 after DENV infection compared to iPS-ML ($p = 0.0004$, day 3). OK-432 stimulated iPS-ML-DC also had higher TNF-α production compared to its non-stimulated counterpart ($p = 0.006$, day 3). In contrast, iPS-ML-DC produced relatively low levels of IFN-α2 even after stimulation (range: 133.8 - 210.4 pg ml^{-1}) (Fig. 3). When we performed the cytokine assay for DENV-infected moDC under the similar conditions, the cytokine profiles were comparable with that of the iPS-ML-DC (Fig. S5c).

IFN-α inhibited the infection of iPS-ML-DC by DENV in a dose dependent manner

IFN-α is considered as an essential cytokine to induce protection against viral infection in general [28, 29]. In our results, DENV infection did not induce significant IFN-α production even with stimulated iPS-ML-DC (Fig. 3). So, we further examined to find out whether some relationship exists between IFN-α production and protection against DENV. Exogenous IFN-α was supplied to the stimulated iPS-ML-DC during and after infection, and it reduced the DENV infectivity in a dose dependent manner as depicted by the immunofluorescence staining (Fig. 4a) and focus forming assay results (Fig. 4b). On day 1 and 2 post-infection, 10,000 pg ml^{-1} and > 10 pg ml^{-1} of IFN-α resulted into significant reduction in virus titers respectively ($p = 0.0176$; bootstrap CI: -6,650,000 to -425,000) indicating potential the role of IFN-α2 in infection/inhibition although the observed evidence may not prove the relation between level of IFN-α2 induction and efficiency of virus infection in OK-432 stimulated iPS-ML-DC (Fig. 4).

DENV infected iPS-ML-DCs activated T cells *in vitro*

One important function of DC is to stimulate T cells in an antigen specific manner. To examine whether the infected iPS-ML-DC could stimulate T cells, iPS-ML-DC was co-cultured with PBMC derived naïve HLA matched and mismatched T cell *in vitro* (Table S2). In a separate experiment, moDC was co-cultured with HLA matched T cell also. In the HLA matched experiment, we observed significantly higher proportion of IFN-γ^+ $CD69^+$ T cells (both $CD4^+$ and $CD8^+$ cells) with infected iPS-ML-DC compared to not-infected one ($p = 0.0129$ and $p = 0.0002$, respectively), and this activated proportion was also significantly higher than what was observed with the HLA mismatched combination ($p = 0.0089$ and $p = 0.0016$, respectively) (Fig. 5). Similarly, infected moDC co-cultured with naïve HLA matched T cell yielded significantly lower population of activated T cells (almost nil as also seen in the case of HLA mismatched iPS-ML-DC/ T cell combination) compared to the corresponding HLA matched combination of iPS-ML-DC/ T cell ($p = 0.0138$ and $p = 0.0022$, respectively) (Fig. 5). Despite the relatively smaller population of activated T cell we observed, these findings indicate that the DENV infected iPS-ML-DC had ability to activate the naïve T cells *in vitro* in an antigen specific manner.

From these results, we conclude that the iPS-ML-DC had comparable expression of key surface markers and cytokine production profiles as the DC does, and DENV infected iPS-ML-DC induced T cell *in vitro* indicating its ability as a professional APC.

Discussion

We have characterized the iPS cell derived iPS-ML-DC in the capacity of host cell for DENV infection and evaluated its T cell stimulation properties. The key surface markers and cytokine profiles were found not only comparable with the moDC but also infected iPS-ML-DC activated T cell suggesting their potential use as proxy DC in the DENV *in vitro* system to conquer the existing limitations of conventional moDC. Since DC is a crucial component in cellular immune response and acts as APC to induce T cells [30, 31], sole reliance on one cell source (monocytes) has become a barrier in several cell-based assays [20].

Apart from the morphological resemblance (e.g. presence of visible dendrites), our iPS-ML-DC exhibited MHC-I, MHC-II, CD80 and CD86 surface markers similar to that of DC. DC presents pathogen antigen to CD4 and CD8 T cell respectively via MHC-II and MHC-I, and co-stimulatory molecules CD80 and CD86 [31]. DC-SIGN which mediates DENV infection [32] was also

expressed on iPS-ML-DC *in vitro*. Increased expression of CD80, CD86 and MHC-II after DENV infection of our iPS-ML-DC brought it further closer to DC phenomenon since the increased expression of these markers are known to be associated with DC maturation during DENV infection [17]. This implies that the iPS-ML-DC was actually activated by DENV infection (Fig. 2, S2 and Table S1). Moreover, our iPS-ML-DC also expressed three type of Fc gamma receptors indicating its potential use as an *in vitro* model to study about mechanism of antibody dependent enhancement (ADE) in DENV infection.

Having seen the comparable phenomenology of iPS-ML-DC with DC, we carried out series of experiments to understand whether these cells had similarities in major cytokine secretion behaviors. We found that iPS-ML-DC produced IL-12 and TNF-α cytokines at high levels. IL-12 is one of the important cytokines produced by DC to propel Th1 response required for CD8 activation [33-35]. High TNF-α secretion by DC during DENV infection was also reported earlier [17]. Relatively low levels of IFN-α production by iPS-ML-DC is probably associated with the profound infectivity of DENV resulting into the inhibition of IFN-α production following the host cell (including human DC) infection as reported previously [36, 37]. Moreover, it is in agreement with the report that IFN-α promoted protection against DENV and vice versa [38]. It was also reflected in our experiment with exogenous IFN-α supply which reduced the DENV infectivity of stimulated iPS-ML-DC in a dose dependent manner (Fig. 4). Nevertheless, the post-infection cytokine profiles of our iPS-ML-DC were similar to that of moDC, which further suggests the functional similarities with the DC (Figs. 3 and S5c).

Next, we examined the most crucial function of iPS-ML-DC to know if it could activate T cell in a capacity of professional APC (Fig. 5). DENV infected iPS-ML-DC was able to induce T cell *in vitro* as revealed by the IFN-γ^+ $CD69^+$ T cell population when co-cultured with HLA matched T cell. In contrast, combination with the HLA mismatched T cell failed to induce T cell activation (IFN-γ^+ $CD69^+$ population close to nil) which indicates that the observed T cell activation with HLA matched combination was truly antigen specific regardless of the small positive population. Therefore, it can be stipulated that iPS-ML-DC works well as an alternative to DCs. As we were limited to iPS cell with only one HLA background (HLA-A*24), and single donor of the HLA matched T cells, further validation with different HLA background iPS cells and different T cell donors would certainly make iPS-ML-DC an attractive option of DC for *in vitro* experimental

systems. Potential implications of iPS-ML-DC may include, but not limited to, the use of iPS-ML-DC in DENV T cell epitope identification [15], or vaccine assessment to know antigen specific T cells induction [39]. Since there is growing interest in the T cell response in dengue, for instance, the identified role of CD8 T cell in dengue protection has great implication in vaccine strategies too [11, 13]. In that sense, iPS-ML-DC will be certainly useful as it is extremely flexible, and any cell background can be prepared in nearly unlimited quantity without losing the functional characters [19].

In conclusion, iPS-ML-DC showed the cell surface markers, maturation potential, DENV infection efficiency and T cell activation properties quite close to DC function. Therefore, iPS-ML-DC could potentially be used as an alternative source of moDC for *in vitro* system to study vaccine candidates, cellular immune response and mechanism of pathogenesis and protection in DENV infection.

Methods

Virus stocks, cells and antibodies

DENV-2 (strain 16681) propagated in C6/36 cells were stored below -80 °C until use. Vero cells were maintained in Minimum Essential Medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (HyClone, Utah, USA). Human peripheral blood mononuclear cells (PBMC) were stored at -80 °C until use, and RPMI (supplemented with 10% FBS, non-essential amino acids, sodium pyruvate) was used to culture human primary cells. All cell cultures were carried out at 37°C in a 5% CO_2 atmosphere for specified incubation time. Antibodies used were purchased from BioLegend Inc, CA, USA (anti-human antibodies: IFN-γ-PE, MHC class I-PE/Cy7, MHC class II-FITC, CD3-APC/Cy7, CD4-FITC/CD4-AlexaFluor488, CD8-PerCP/Cy5.5, CD14-PE, CD16-APC, CD25-Biotin, CD32-PE, CD33-PE/Cy7, CD64-PE/Cy7, CD69-Biotin, CD71-Biotin, CD80-PE, CD86-PerCP/Cy5.5 and their isotype matched controls), BD Biosciences, CA, USA (CD11b-APC, CD69-APC, CD209 (DC-SIGN)-APC and their isotype matched controls), HRP-conjugated anti-mouse antibody (American Qualex, San Clemente, CA, USA), and mouse anti-NS-1 antibody and mouse anti-DENV E-protein were prepared in house. All the experiments were performed independently at least three times unless stated otherwise.

243

244 **Generation of iPS cell derived DC like cells (iPS-ML-DC)**

245 iPS-ML-DC was generated as described previously [19], with some modifications in
246 differentiation and maturation steps. Briefly, iPS cells were derived from human fibroblasts
247 (HLA-A*24:02/11:01 and DRB1*01:01/04:06) after transduction with OCT3/4, SOX2, KLF4
248 and cMYC. Next, the differentiation into iPS derived myeloid cells (iPS-MC) was accomplished
249 by using M-CSF (50 ng ml^{-1}) and GM-CSF (50 ng ml^{-1}) containing α-MEM media supplemented
250 with 20% FBS. Differentiated iPS-MC were further transfected with cMYC, BMI1 and EZH2 (or
251 MDM2) to establish an iPS derived myeloid cell line (iPS-ML). Details on plasmid constructs
252 and transfections have been described elsewhere [19]. Briefly, human cMYC cDNA
253 fragment was cloned into the pENTR-TOPO vector (Invitrogen, Carlsbad, CA, USA). LR clonase
254 system (Invitrogen) was used to transfer cDNAs of BMI1, EZH2 and MDM2 to a lentiviral
255 expression vector, pCSII-EF. Additionally, two plasmids namely, pCMV-VSV-G-RSV-Rev and
256 pCAG-HIVgp were used for lentiviral vector packaging. Finally, using this iPS-ML (clone # WL-
257 59), iPS-ML-DC was generated by three-days culture in complete α-MEM supplemented with
258 recombinant human (rh) M-CSF (12.5 ng ml^{-1}) (Shenandoah Biotechnology, Warwick, PA, USA),
259 rhGM-CSF (100 ng ml^{-1}) (Gentaur, San Jose, CA, USA) and rhIL-4 (10 ng ml^{-1}) (Humanzyme,
260 Chicago, USA) at 37°C, 5% CO_2. Penicillin-killed *Streptococcus pyogenes* (OK-432) based
261 maturation was used in some experiments because previous reports showed that iPS-ML-DC [19]
262 or moDC [40] both achieved maturation when stimulated by OK-432. We used iPS-ML-DC
263 treated with OK432 particularly to compare with the DC maturation caused by DENV infection.
264 Thus, obtained iPS-ML-DC attained maturation upon additional three days of culture in the same
265 media (with same cytokines) in presence of OK-432 (1.25 μg ml^{-1}) (Picibanil, CHUGAI, Tokyo,
266 Japan).

267

268 **Generation of peripheral blood monocyte derived DC (moDC)**

269 Freshly obtained heparinized blood was subjected to PBMC separation by Lymphoprep™
270 (STEMCELL Technologies, Vancouver, Canada) gradient centrifugation method according to the
271 instruction manual. Briefly, after dilution with equal volume of PBS containing 2% FBS, blood
272 was layered on Lymphoprep™ and centrifuged (800 g, 25 min, 15-20°C). PBMC was collected,

washed twice (low speed) and stored frozen at -80°C using freezing medium (CELLBANKER™1 plus, ZENOAQ, Fukushima, Japan) when not used immediately.

$CD14^+$ cell was then positively selected from PBMC by MojoSort™ Human CD14 Selection Kit (BioLegend), and used for DC differentiation.

For DC differentiation, monocyte ($CD14^+$ cell) was seeded onto culture plates using complete RPMI medium containing 100 ng ml^{-1} rhGM-CSF and 75 ng ml^{-1} rhIL-4. Half of the culture medium was replaced every alternate day by fresh medium. Finally, moDC was harvested on day 7 and re-suspended in RPMI complete medium.

Flow cytometric analysis for cell surface markers

To block non-specific binding of antibodies, iPS cell derived cells or moDC were first treated with Human TruStain FcX (BioLegend) for 10 min prior to specific staining. After washing, cells were stained with antibodies and corresponding isotype matched controls for 30 min on ice. Cells were acquired by FACSVerse™ (BD Biosciences), and then data analysis was performed with FlowJo (FlowJo, LCC, OR, USA). MHC-I, MHC-II, CD80 and CD86 were selected since they are common DC surface markers/co-stimulatory molecules that participate in antigen presentation to T cell [31] while DC-SIGN was selected as it mediates DENV infection and expressed preferentially on immature DC [32]. Because the DC expresses Fc receptors (often used for ADE) [27], expression profiles of common Fc receptors (e.g. FcγRI, FcγRII and FcγRIII) were also assessed.

DENV infection

iPS cell derived cells or moDC were infected with DENV-2 for two hours at 37°C, 5% CO_2 using multiplicities of infection (MOI) 1. Mock infection was used as control. After washing (to eliminate unbound virus), cell concentration was adjusted to 2 x 10^5 cells ml^{-1} and cultured in 12-well or 24-well cell culture plates for up to three days. Cells and culture supernatants were collected at the different time points (non-infection (NI), day 1, 2 and 3 post-infection). Cells were processed immediately while the supernatant was stored at -80°C until assayed.

Immunofluorescence staining with DENV specific antibodies

DENV infected cells harvested at different time points were re-suspended with phosphate buffer saline (PBS) (pH 7.4), cell suspension (~20 µL) transferred onto a glass slide and air-dried. Fixation was carried out with cold acetone for 20 min. Immunofluorescence was performed immediately. Samples (cell spots) were incubated with primary antibody (anti-NS-1 antibody, 1 µg ml^{-1}) at 37°C for 1 h. Goat anti-mouse IgG conjugated FITC (Abcam, Cambridge, UK) was applied after washing. Finally, properly rinsed and dried samples were covered with cover-slip and visualized under a fluorescence microscope (BZ-9000) (Keyence, Osaka, Japan). Immunofluorescence was performed immediately after washing and fixation of cells at the different time points (NI, day 1, 2 and 3 post-infection).

Virus titer estimation by focus forming assay

Focus forming assay for virus titration in cell supernatant was carried out as described previously [41] with some modifications. In brief, Vero cells were prepared in 96-well cell culture plates. Then, 100 µL of 10-fold serially diluted culture supernatant of DENV infected cells were added and incubated for two hours, followed by addition of 100 µL of 1.25% methylcellulose (Wako Pure Chemical Industries, Osaka, Japan) in MEM supplemented with 2% FBS. After culturing for three days, the media was washed out and cells were fixed with 4% paraformaldehyde. Mouse anti-DENV E-protein antibody was added to each well after blocking and incubated for 1 h at 37°C, 5% CO_2. After washing out excess antibody, cells were stained with HRP-conjugated anti-mouse antibody. Following rinsing and drying, positive spots were counted by microscopy. Focus forming unit (FFU) was calculated.

Measurement of cytokine production by multiplex assay

DC related cytokines produced by iPS derived cells after DENV infection were measured by multiplex cytokine analysis of the cell supernatant using MILLIPLEX MAP Kit (Millipore, Billerica, MA) according to the manufacturer's manual. Acquisition and data analysis were executed with LABScan 100 (Luminex, Austin, TX, USA).

IFN-α mediated DENV infection inhibition assay

Different concentrations of recombinant human (rh) IFN-α (PBL Assay Science, Piscataway, NJ, USA) were continuously supplied to the medium during and following the DENV infection of

OK-432 treated iPS-ML-DC. Cell pellets and supernatants were recovered at indicated time points post-infection (day 1, 2, 3). DENV infectivity was determined by immunofluorescence staining and focus forming assay as described above.

Induction of T cell stimulation by DENV infected iPS DC-like cells

The HLA profiles of the T cells donors and iPS cells were determined by HLA typing (HLA Laboratory, Kyoto, Japan). Frozen HLA matched and mismatched PBMCs were thawed and rested overnight in complete RPMI medium at 37°C, 5% CO_2. $CD3^+$ T cells were negatively selected by MojoSort™ Human CD3 T cell Isolation Kit (BioLegend) according to the manual. Purified T cells (TC) were co-cultured with iPS-ML-DC (TC : DC = 5 : 1) for 48 h. Allo-reactive T cells (CD69 expressing cells) were removed by magnetic separation using biotinylated antibodies mix (CD25, CD71 and CD69) and CD3 T cell Isolation Kit (BioLegend) followed by incubation with Streptavidin Nanobeads (BioLegend). The resulting negative fraction of T cell was rested overnight. Next day, purified T cells were co-cultured with DENV infected iPS-ML-DC (MOI = 1) in 96-well cell culture plate (5 x 10^5 T cells/well in 250 µL complete medium; TC: DC = 5 : 1) and incubated for 96 h (i.e. 4 days). Non-infected iPS-ML-DC + TC (NI-DC) and TC-only (without DC) were used as controls. In a separate experimental set, moDC co-cultured with HLA-matched T cells was also used under similar conditions. On day 4, cells were supplied with Brefeldin A (10 µg ml^{-1}) for 5 h before harvesting. Cells were washed with PBS and stained with a panel of fluorescein-labelled antibodies against selected human cell surface markers (CD3, CD4, CD8, and CD69). Next, the cells were fixed and permeabilized using BD Cytofix/Cytoperm™ reagents (BD Bioscience) followed by fluorescein-labelled anti-human IFN-γ antibody staining (intracellular) in Perm/Wash Buffer (BD Bioscience). Stained cells were washed, resuspended in FACS buffer and acquired by flow cytometer (FACSVerse™). Results were analyzed by FlowJo software.

Statistical analyses

Data were analyzed by R version 3.4.4. Cell population proportion was expressed as percentages. Continuous variables were expressed as mean with standard deviation (SD) as indicated by error bars. Student t test or bootstrap confidence intervals test (with 1,000

sampling) was used to compare difference between two groups as appropriate. Statistically significant differences were determined when *p*-value was less than 0.05.

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Conflicts of interest

The authors declare that they have no conflict of interests.

Ethical statement

This study was approved by the ethics committee of Institute of Tropical Medicine (NEKKEN), Nagasaki University (App. No. 16 06 30 153). Informed consent was obtained from the voluntary blood donors. All experiments were performed in accordance with relevant guidelines and regulations.

Abbreviations

APC: antigen presenting cell

DC: dendritic cell

DENV: dengue virus

FFU: focus forming unit

iPS: induced pluripotent stem

iPS-MC: iPS cell derived myeloid cell

iPS-ML: iPS cell derived myeloid cell line

iPS-ML-DC: iPS cell derived DC-like cell

MFI: median fluorescence intensity

NI: non-infection

NS: non-structural

TC: T cell

Author contributions statement

D.H.M. performed the experiments and wrote the manuscript. S.M designed the study, performed experiments and prepared the manuscript and figures. S.P.D. performed the experiments and wrote the manuscript. M.R. performed the experiments. S.S., Y.N., J.K.L., N.T.H. and K.M. supervised the study. K.H. designed and supervised the study.

Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Figure legends

Figure 1. General profile of iPS-ML-DC. a: General characters of iPS-ML-DC and their precursor (iPS-ML, WL-59) as examined by flow cytometry. Expression of each maker on cell surface was indicated with black line, and control staining (isotype control) is indicated with grey shadow in the histogram, or dot blot as appropriate. **b:** Morphology of each cell type was observed with microscopy. Each assay was performed in triplicate, and representative result is shown. (+) indicates presence and (-) indicates absence of OK-432. iPS-ML: iPS cell derived myeloid cell line, iPS-ML-DC: iPS-derived DC like cell.

Figure 2. DENV efficiently infected iPS-ML-DC *in vitro*. Efficiency of DENV infection to iPS-ML and iPS-ML-DC was examined by immunofluorescence staining of cells (**a**) and virus titer of culture supernatant was measured by focus forming assay and expressed as focus forming units (ffu) /mL, results shown as mean±standard deviation (SD) of three independent experiments (**b**). DENV infection time line is indicated as non-infection (NI), and post-infection (day 1, 2 and 3). Immuno-staining results are shown as fluorescence staining and phase contrast panels (to show cells in the same field used in fluorescence panels). Green color indicates positive results with anti-NS1 staining. Each assay was performed in triplicate. (+) indicates presence and (-) indicates absence of OK-432. iPS-ML: iPS cell derived myeloid cell line, iPS-ML-DC: iPS-derived DC like cell.

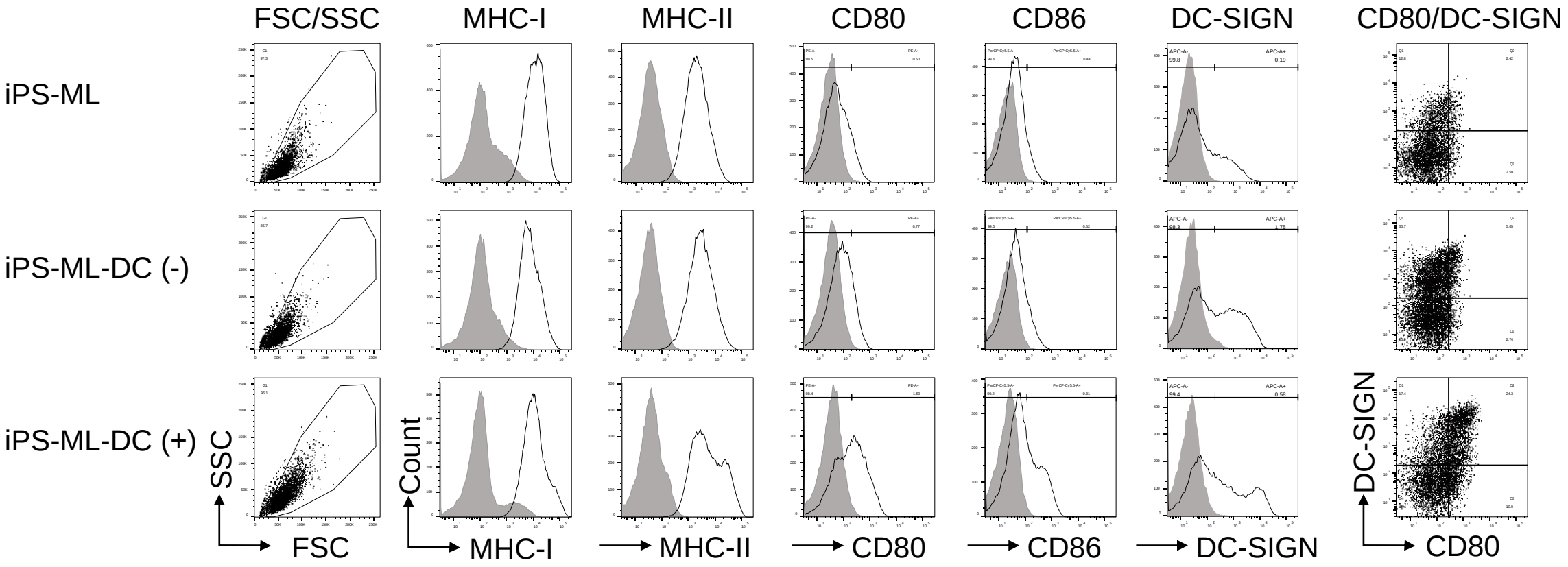
Figure 3. DENV infected iPS-ML-DC produced high titers of IL-12p70 and TNF-α but not IFN-α2. iPS-ML and iPS-ML-DC were infected by DENV and cytokine levels of culture supernatant were measured by multiplex assay. Cytokine production was monitored at non-infection (NI) and post infection (day 1, 2 and 3). Each assay was performed in triplicate, and mean±SD is shown. (+) indicates presence and (-) indicates absence of OK-432. iPS-ML: iPS cell derived myeloid cell line, iPS-ML-DC: iPS-derived DC like cell.

Figure 4. IFN-α inhibited the DENV infection of iPS-ML-DC in a dose dependent manner. (**a**) Immunofluorencence staining results, and (**b**) focus forming assay results showing the reduction in infection efficiency with IFN-α addition. Different concentrations of IFN-α were supplied, and cell pellets and supernatants were recovered. DENV infection in IFN-α treated iPS-ML-DC was monitored at day 1, 2 and 3 post-infection. Each assay was performed

561 in triplicate and expressed as mean ± SD of virus titers (ffu /mL). A representative figure of
562 immunofluorescence is shown together with the phase contrast panels. iPS-ML-DC: iPS-derived
563 DC like cell.
564
565 **Figure 5. DENV infected iPS-ML-DC activated T cells *in vitro*.** iPS-ML-DC function as
566 antigen presenting cell (APC) was examined and measured by flow cytometry. Purified HLA
567 matched or mismatched T cell (TC) and infected iPS-ML-DC (I-DC) were co-cultured for 96 h
568 followed by intracellular staining for IFN-γ. Non-infected iPS-ML-DC + TC (NI-DC) and TC-
569 only (without DC) were used as controls, in a separate experimental set, moDC co-cultured with
570 HLA-matched T cells was also used under similar conditions. IFN-γ and CD69 and expression
571 of samples after gated on $CD3^+$ and $CD4^+$ (or $CD8^+$) are shown by dot plots (**a**). Percentage of
572 IFN-γ^+ $CD69^+$ T cells are indicated by bar graphs with mean ± SD (**b**). In the bar graphs,
573 samples are indicated as I-DC (Infected-DC + TC), NI-DC (non-infected DC + TC) and without
574 DC (TC-only without DC). Each assay was performed in triplicate and a representative result is
575 shown for dot plot. Student t test or bootstrap test was used to compare difference between two
576 groups. Statistically significant differences were determined when *p*-value was less than 0.05
577 and showed as asterisk. iPS-ML-DC: iPS-derived DC like cell, moDC: monocyte DC without
578 OK-432 treatment. (-) indicates absence of OK-432

Figure 1

a


FSC/SSC
MHC-I
MHC-II
CD80
CD86
DC-SIGN
CD80/DC-SIGN
iPS-ML
iPS-ML-DC (-)
iPS-ML-DC (+)
SSC
FSC
Count
MHC-I
MHC-II
CD80
CD86
DC-SIGN
DC-SIGN
CD80

b


iPS-ML
iPS-ML-DC (-)
iPS-ML-DC (+)

Figure 2

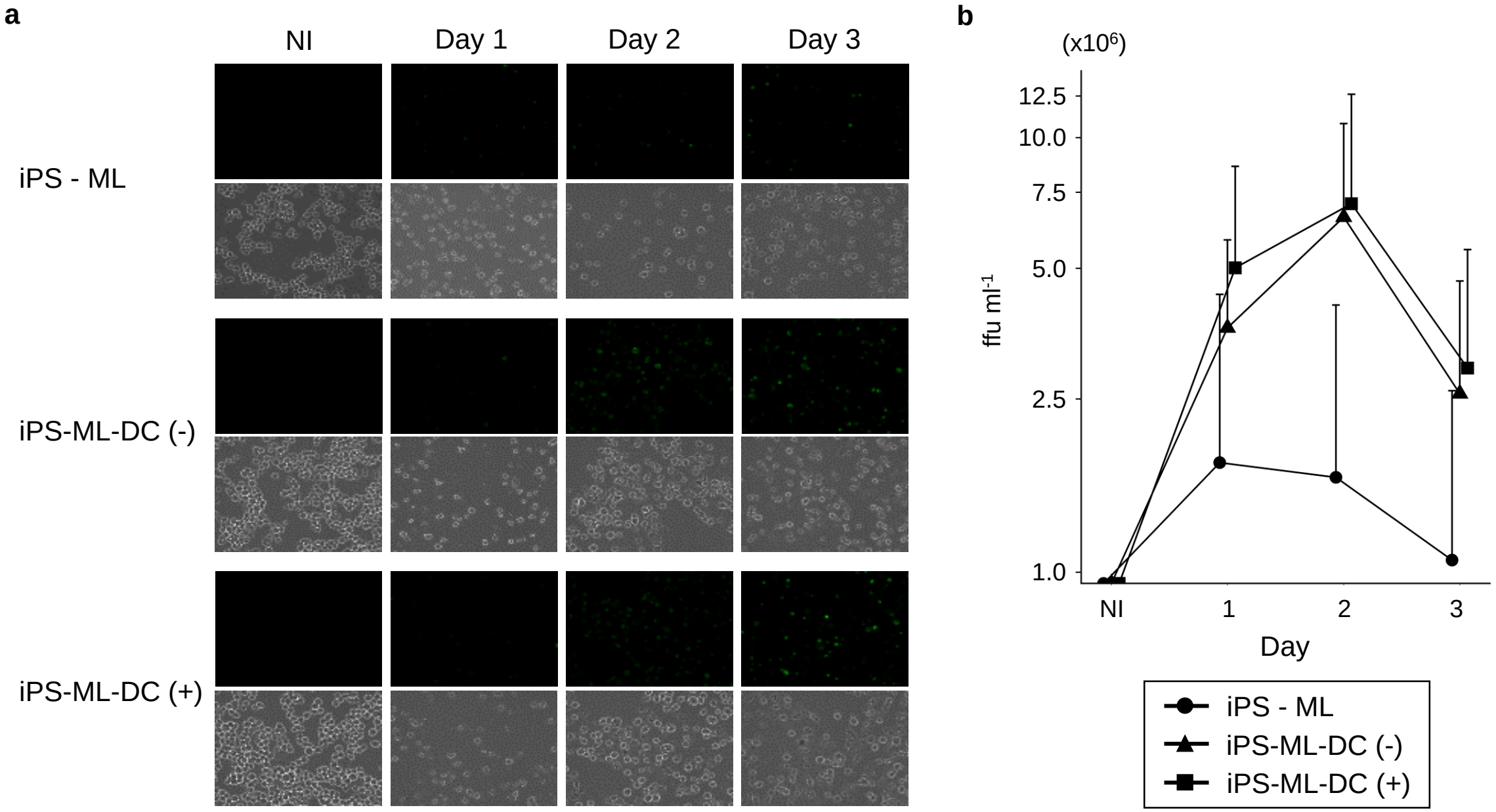

a
NI
Day 1
Day 2
Day 3
iPS - ML
iPS-ML-DC (-)
iPS-ML-DC (+)
b
(x10⁶)
ffu ml⁻¹
12.5
10.0
7.5
5.0
2.5
1.0
NI
1
2
3
Day
iPS - ML
iPS-ML-DC (-)
iPS-ML-DC (+)

Figure 3

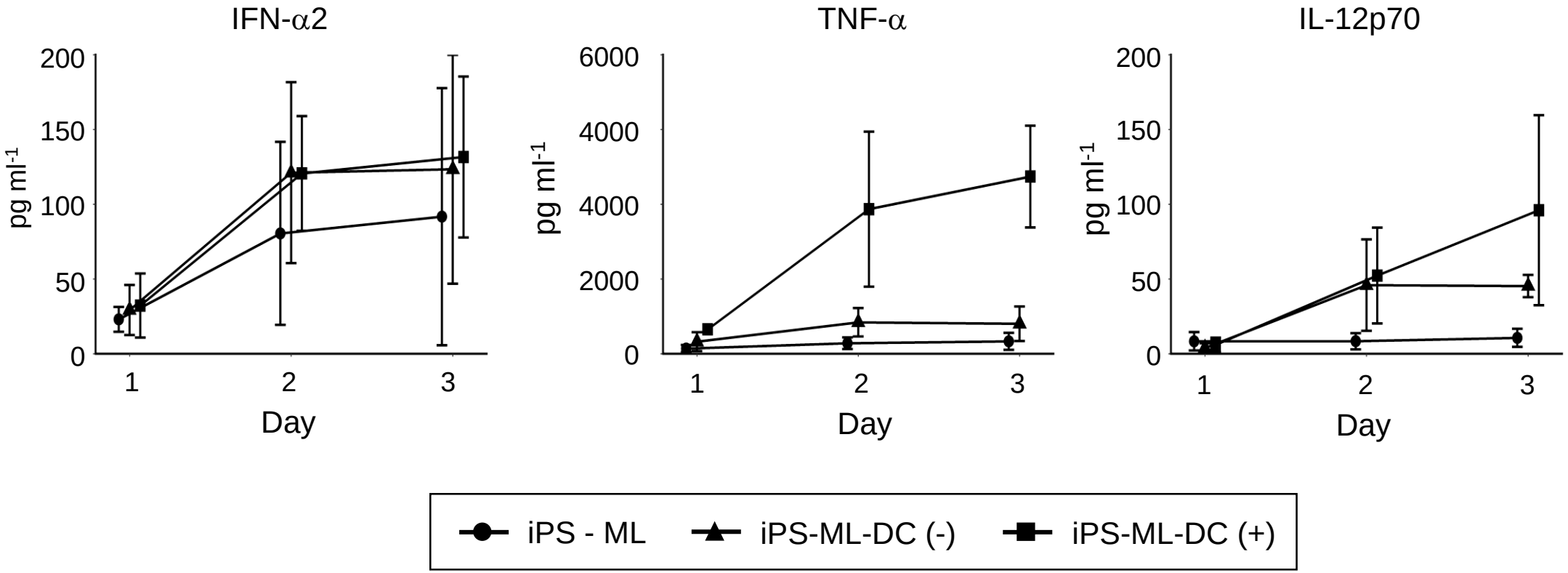

IFN-α2
TNF-α
IL-12p70
pg ml-1
200
150
100
50
0
6000
4000
4000
2000
0
1
2
3
Day
iPS - ML
iPS-ML-DC (-)
iPS-ML-DC (+)

Figure 4

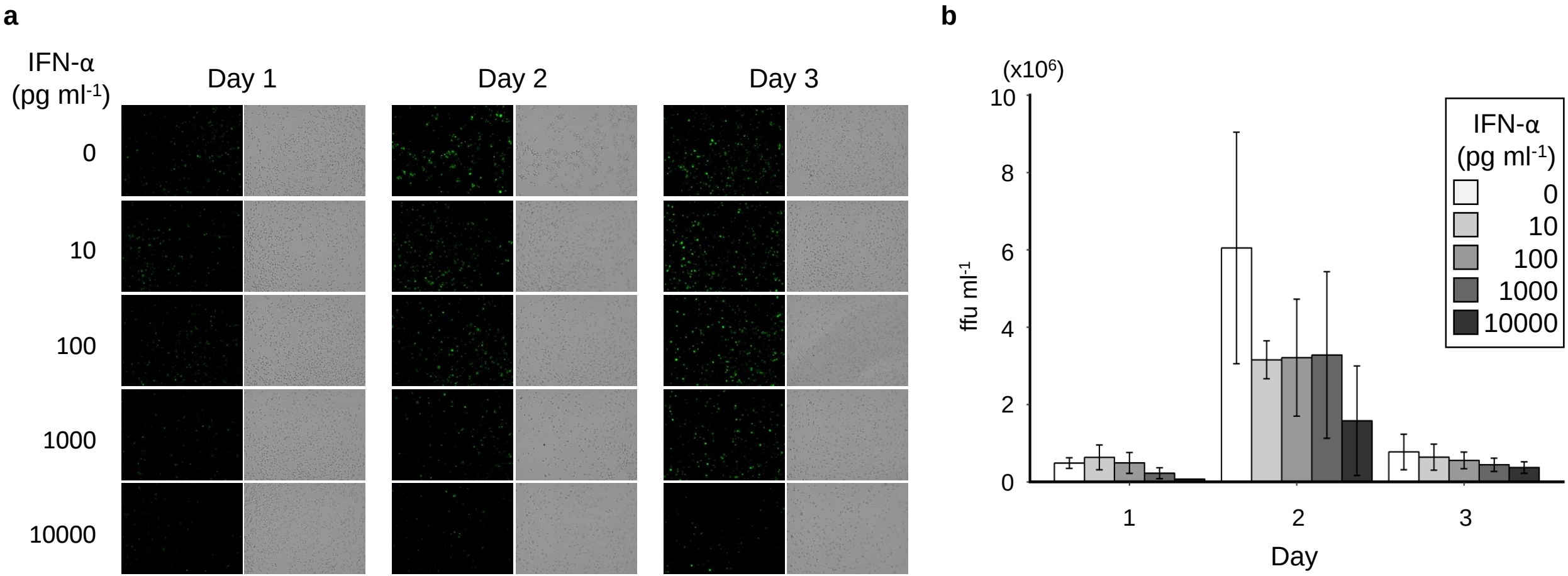
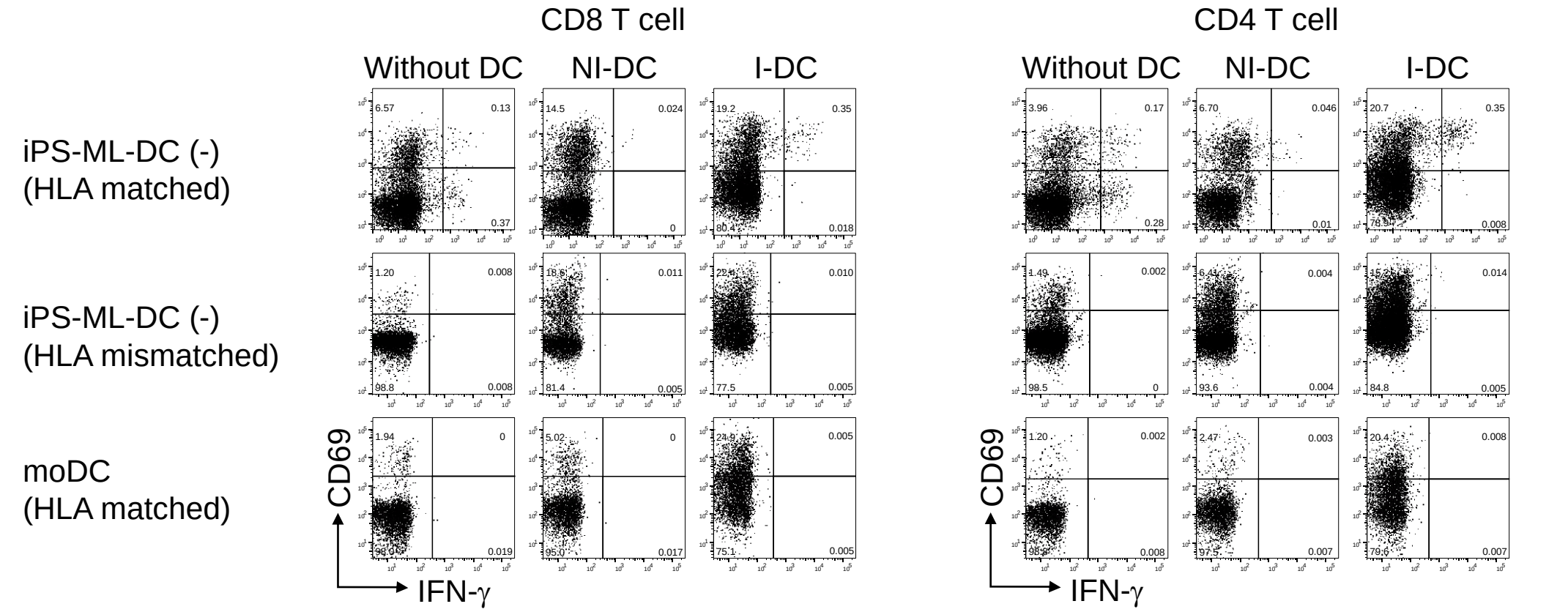
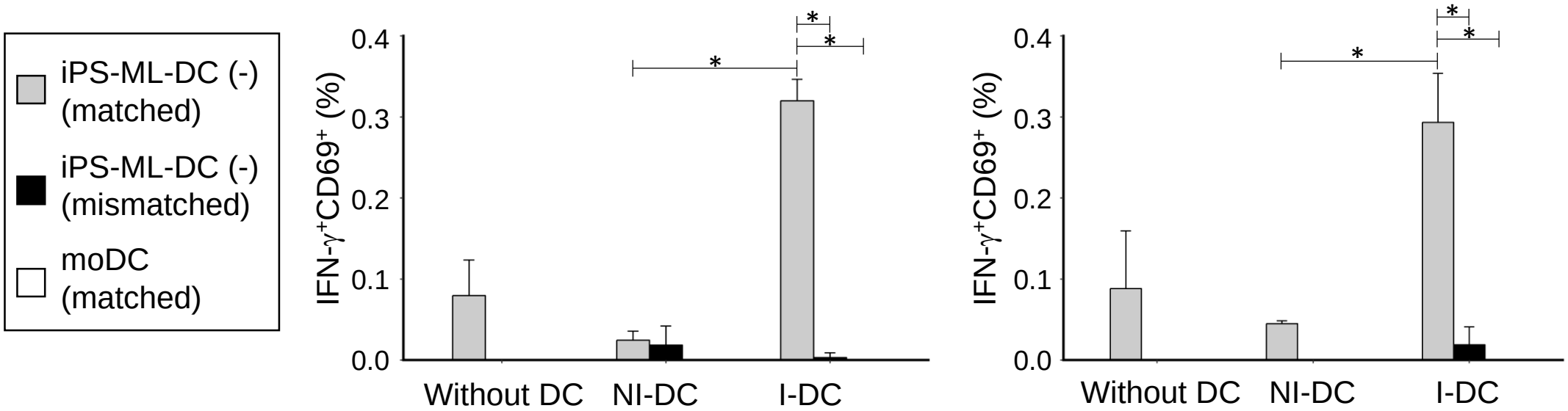

a
IFN-α
(pg ml-1)
Day 1
Day 2
Day 3
0
10
100
1000
10000
b
(x10^6)
10
8
6
4
2
0
ffu ml-1
IFN-α
(pg ml-1)
0
10
100
1000
10000
1
2
3
Day

Figure 5

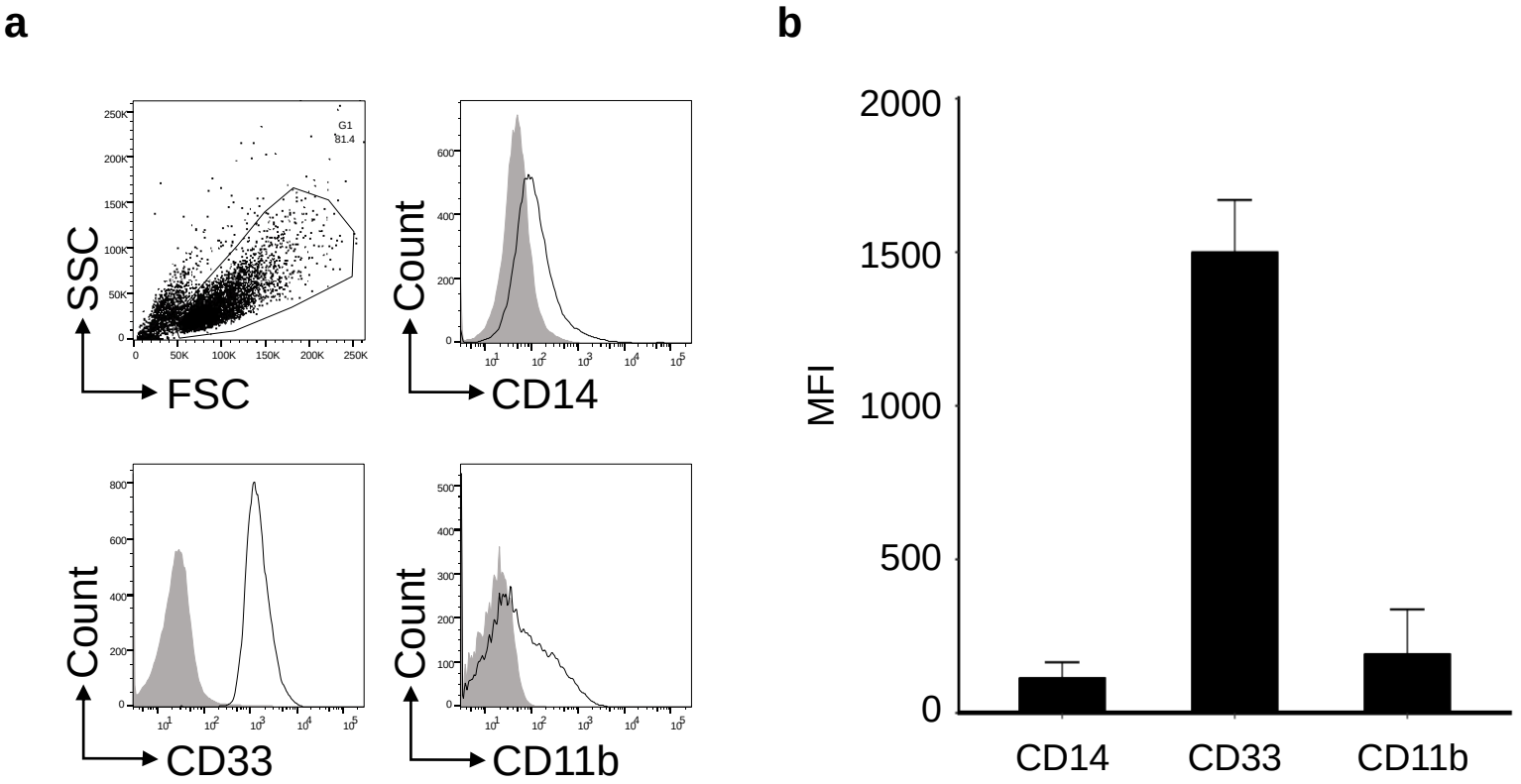
a


CD8 T cell
CD4 T cell
Without DC
NI-DC
I-DC
iPS-ML-DC (-)
(HLA matched)
iPS-ML-DC (-)
(HLA mismatched)
moDC
(HLA matched)
CD69
IFN-γ
6.57
0.13
0.37
14.5
0.024
0
19.2
0.35
80.4
0.018
1.20
0.008
98.8
0.008
18.6
0.011
81.4
0.005
22.4
0.010
77.5
0.005
1.94
0
98.0
0.019
5.02
0
95.0
0.017
24.9
0.005
75.1
0.005
3.96
0.17
0.28
6.70
0.046
0.01
20.7
0.35
78.9
0.008
1.49
0.002
98.5
0
6.44
0.004
93.6
0.004
15
0.014
84.8
0.005
1.20
0.002
98.8
0.008
2.47
0.003
97.5
0.007
20.4
0.008
79.6
0.007

b

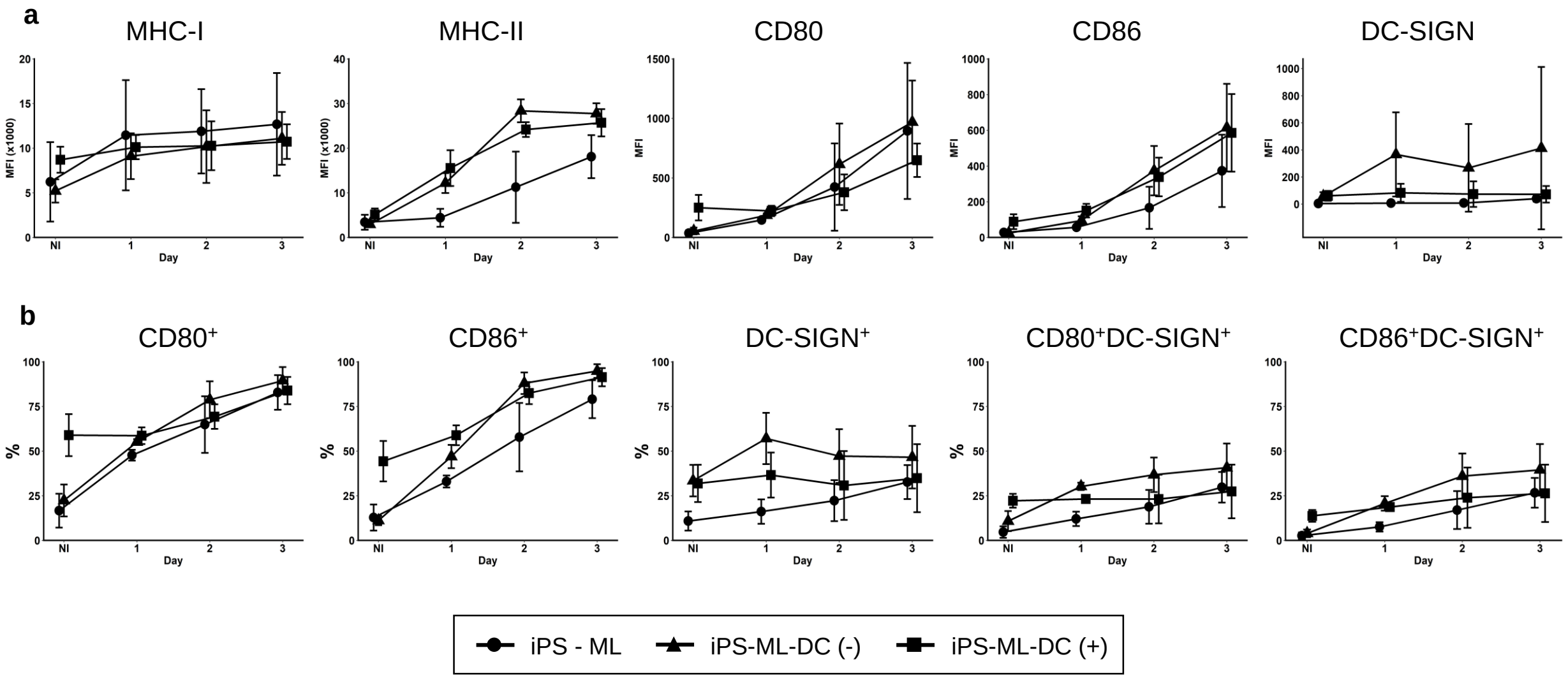

iPS-ML-DC (-)
(matched)
iPS-ML-DC (-)
(mismatched)
moDC
(matched)
IFN-γ+CD69+ (%)
0.4
0.3
0.2
0.1
0.0
*
*
*
Without DC
NI-DC
I-DC

Supplementary Figure 1



Supplementary Figure 1. iPS-ML cell expressed myeloid markers. a: Myeloid markers (CD14, CD33 and CD11b) of iPS-ML were examined by flow cytometry. Expression of each maker on cell surface was indicated with black line, and control staining (isotype control) is indicated with grey shadow in the histogram. Each assay was performed in triplicate, and a representative result is shown. **b:** Median of intensity (MFI) of each marker is expressed as mean±SD (error bar) derived from three independent experiments. iPS-ML: iPS cell derived myeloid cell line.

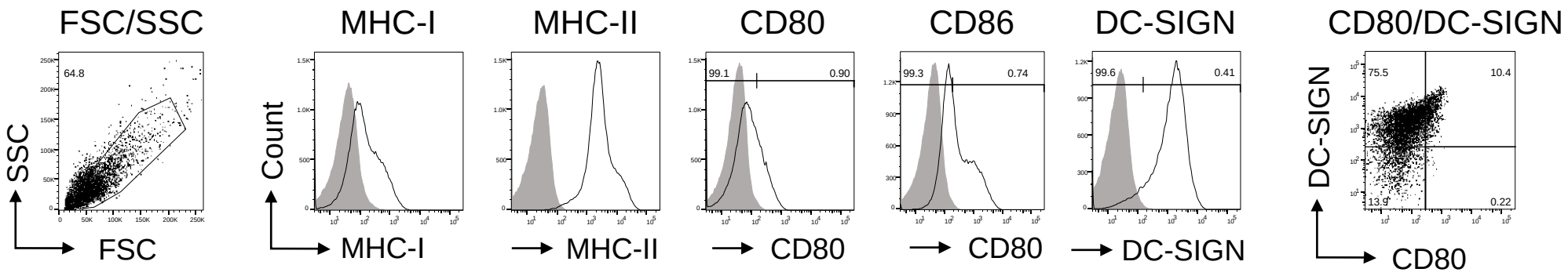
Supplementary Figure 2



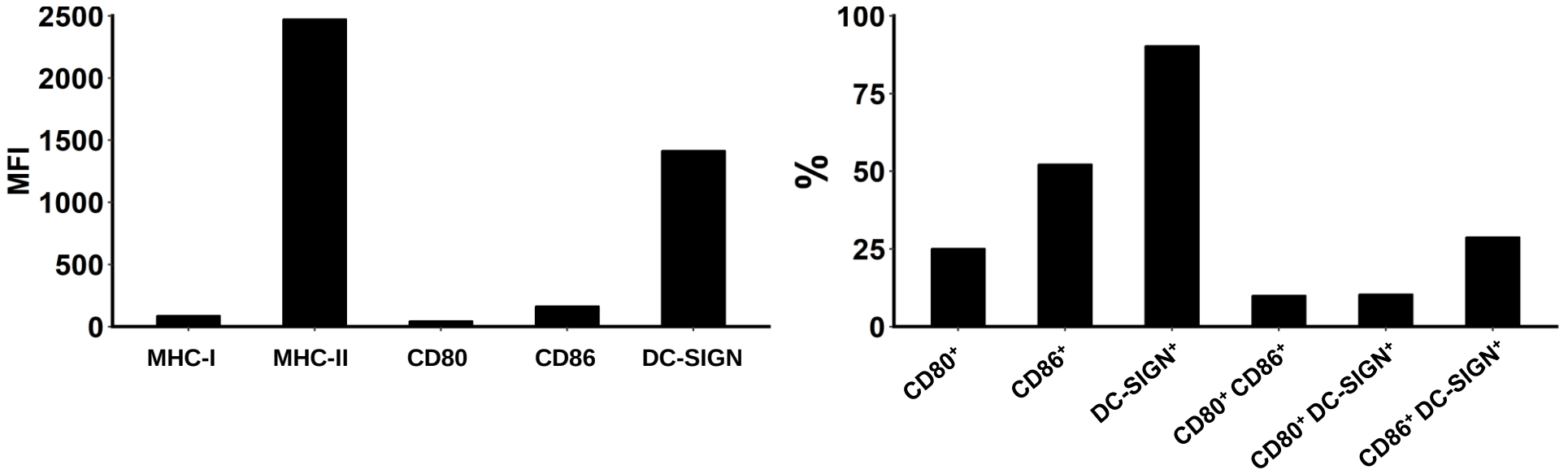
Supplementary Figure 2. Surface marker expression profiles of iPS-ML and iPS-ML-DCs before and after DENV infection. iPS-ML and iPS-ML-DCs were infected by DENV and expression levels of cell surface markers were examined non-infection (NI) and post-infection (day 1, 2 and 3) by flow cytometry. Results are presented as, **a:** Median of intensity (MFI) and **b:** percentage. Each assay was performed in triplicate, and expression level is presented as mean±SD (error bar). (+) indicates presence and (-) indicates absence of OK-432. iPS-ML: iPS cell derived myeloid cell line, iPS-ML-DC: iPS-derived DC like cell.

Supplementary Figure 3

a

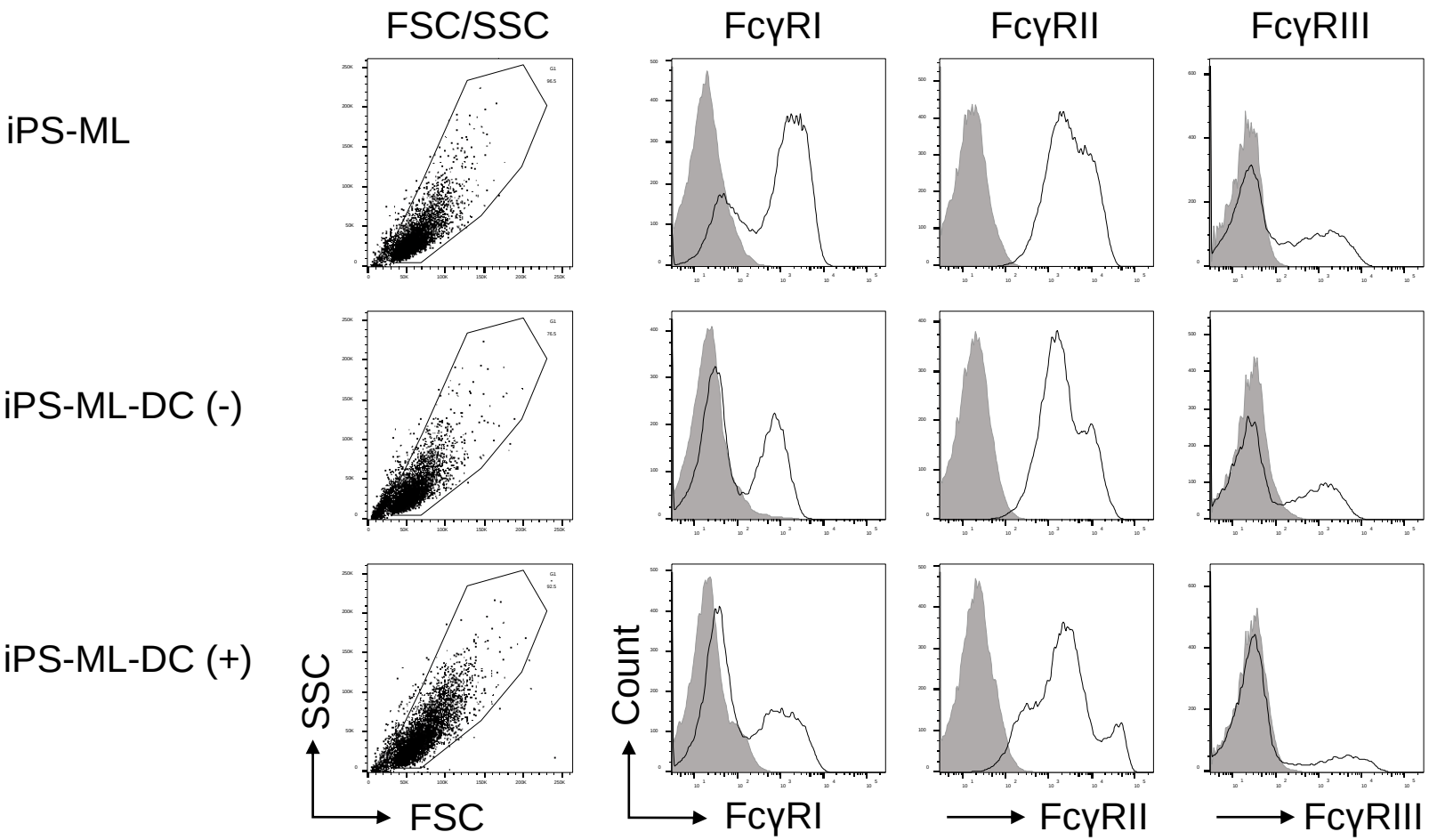


b



Supplementary Figure 3. General profile of moDC based on cell surface markers expression. a: General characters of moDC were examined by flow cytometry. Expression of each maker on cell surface was indicated with black line, and control staining (isotype control) is indicated with grey shadow in the histogram or dot blot as appropriate. **b:** Median of intensity (MFI) of surface markers of moDC and population of $CD80^+$, $CD86^+$, DC-$SIGN^+$, $CD80^+CD86^+$, $CD80^+$DC-$SIGN^+$ and $CD86^+$DC-$SIGN^+$ cells are shown as bar graphs. Each assay was performed in duplicate, and a representative result is shown. moDC: monocyte DC without OK-432 treatment.

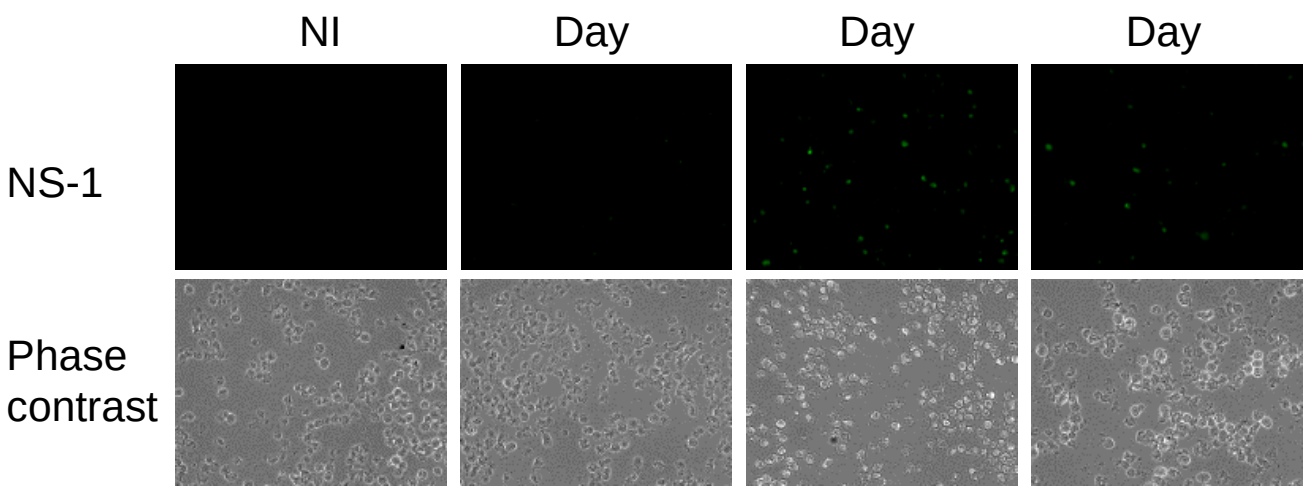
Supplementary Figure 4



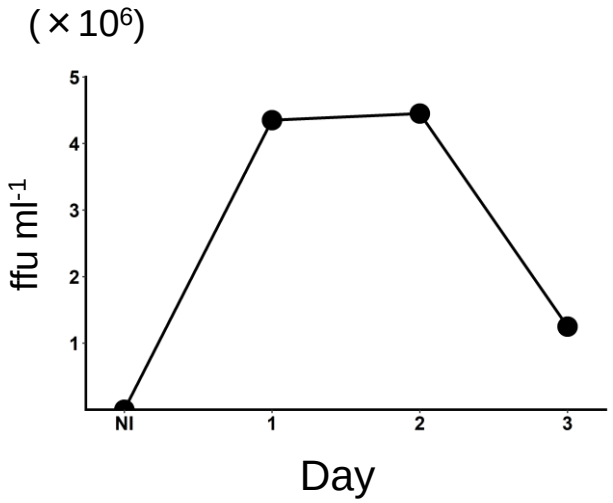
Supplementary Figure 4. iPS-ML-DC expressed Fc gamma receptors. Fc gamma receptors (FcγRI, FcγRII and FcγRIII) of iPS-ML and iPS-ML-DCs were examined by flow cytometry. Expression level of each maker is indicated with black line, and control staining (isotype control) is indicated with grey shadow in the histogram. Each assay was performed in duplicate, and a representative result is shown. (+) indicates presence and (-) indicates absence of OK-432. iPS-ML: iPS cell derived myeloid cell line, iPS-ML-DC: iPS-derived DC like cell.

Supplementary Figure 5

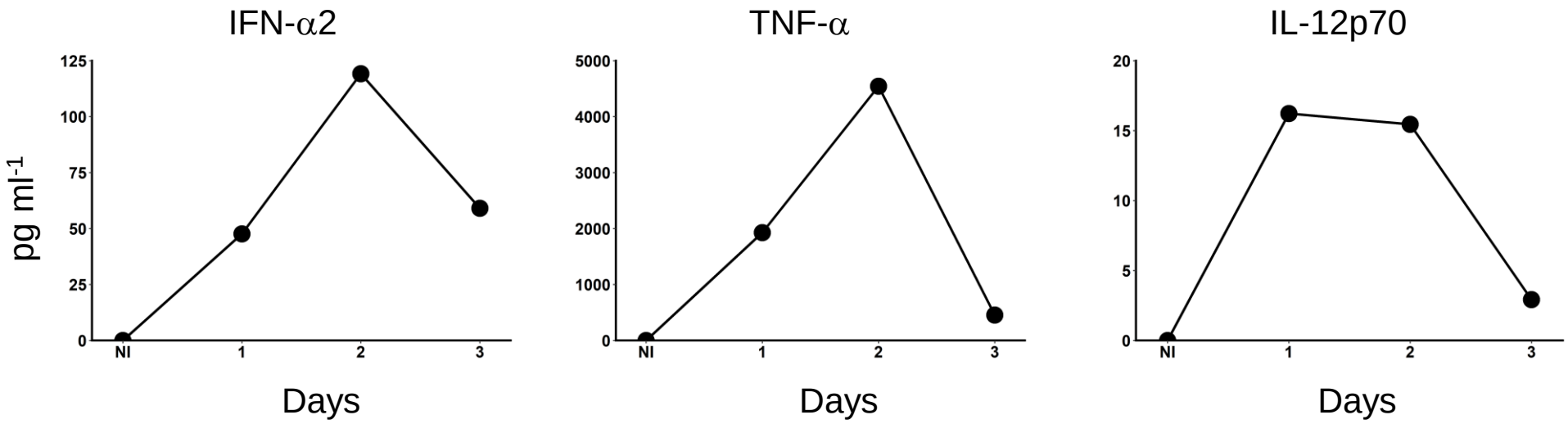
a



b



c



Supplementary Figure 5. Cytokine profiles of moDC before and after infection with DENV. moDC were infected by DENV and cytokine levels of culture supernatants were measured by multiplex assay. **a:** Efficiency of DENV infection to moDC was examined by immunofluorescence staining of cells. Immuno-staining results are shown as fluorescence staining and phase contrast panels (to show cells in the same field used in fluorescence panels). Green color indicates positive results with anti-NS1 staining. **b:** virus titer of culture supernatant was measured on each day before and after DENV infection (as mentioned above) by focus forming assay and expressed as focus forming units (ffu) ml^{-1}. **c:** Cytokine production was monitored as non-infection (NI) and post-infected (day 1, 2 and 3). Each assay was performed in duplicate and a representative figure is shown. moDC: monocyte DC without OK-432 treatment.

Supplementary Table 1. Surface marker expression profiles of iPS-ML and iPS-ML-DCs before and after DENV infection.

		CD80$^+$	CD86$^+$	DC-SIGN$^+$	CD80$^+$CD86$^+$	CD80$^+$DC-SIGN$^+$	CD86$^+$DC-SIGN$^+$
iPS-ML (Mean±SD)	Non infection	16.63±9.47	12.79±7.33	10.82±5.37	6.17±5.40	4.64±3.20	2.61±1.81
	Day 1	47.70±3.03	32.97±3.36	16.14±6.83	21.17±1.50	12.05±4.01	7.55±2.62
	Day 2	64.87±15.84	57.80±19.17	22.25±11.48	43.23±20.17	18.78±9.46	16.97±10.60
	Day 3	82.83±9.70	79.07±10.63	32.63±9.51	66.93±15.18	29.77±8.56	26.67±8.34
iPS-ML-DC (-) (Mean±SD)	Non infection	22.37±8.95	11.14±2.62	33.43±8.83	5.26±2.43	10.79±5.62	3.96±2.13
	Day 1	55.10±1.61	46.97±6.49	57.07±14.35	28.37±3.38	30.23±2.06	20.70±4.04
	Day 2	78.77±10.31	88.00±6.06	47.23±15.06	64.93±12.52	36.73±9.68	35.97±12.66
	Day 3	89.47±7.60	94.83±3.85	46.60±17.53	80.00±9.27	40.73±13.51	39.50±14.47
iPS-ML-DC (+) (Mean±SD)	Non infection	58.93±11.80	44.33±11.31	31.90±10.45	34.57±10.53	22.17±3.87	13.72±3.29
	Day 1	58.63±4.67	58.87±5.56	36.57±12.60	38.53±5.80	23.20±2.34	18.70±2.31
	Day 2	69.30±6.86	82.50±6.20	30.73±19.27	56.50±8.45	23.13±13.61	23.93±16.90
	Day 3	83.90±7.65	91.40±5.09	34.83±19.04	74.30±8.96	27.40±15.02	26.35±16.05

Supplementary Table 2. HLA typing results

	iPS-ML	HLA matched and moDC	HLA mismatched
HLA-A	11:01:01	24:02:01	11:02:01
	24:02:01	31:01:02	33:03:01
HLA-B	07:02:01	51:01:01	27:04:01
	15:01:01	59:01:01	58:01:01
HLA-C	04:01:01	01:02:01	03:02:02
	07:02:01	15:02:01	12:02:02
HLA-DRB1	01:01:01	04:05:01	12:02:01
	04:06:01	09:01:02	15:02:01
HLA-DRB3/4/5	4*01:03:01	4*01:03:01	3*03:01:03
		4*01:03:02	5*01:01:01
HLA-DQA1	01:01:01	03:03:01	01:02:01
	03:01:01	03:02	06:01:01
HLA-DQB1	03:02:01	03:03:02	03:01:01
	05:01:01	04:01:01	06:02:01
HLP-DPA1	01:03:01	01:03:01	01:03:01
HLA-DPB1	02:01:02	02:01:02	03:01//+
	04:02:01		04:01//+