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ORIGINAL ARTICLE

Reprogramming transgenes reactivation in AML-M5-iPSCs causes doxorubicin resistance in differentiated monocytic-like cells

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Abstract

Introduction: Acute monocytic leukaemia (AML-M5) disease models are scarce. To address this, we reprogrammed THP-1 cells from a patient into AML-M5-specific induced pluripotent stem cells (AML-M5-iPSCs) and differentiated them into monocytic-like cells. Unexpectedly, reprogramming transgenes *Oct3/4*, *Sox2* and *c-Myc* were reactivated in the AML-M5-iPSC genome. This study examined how transgene reactivation influences responses to doxorubicin in differentiated monocytic-like cells; *Materials and Methods*: AML-M5-iPSCs were differentiated into monocytic-like cells using hM-CSF and IL-3. Cell morphology, phagocytotic activity and surface markers expression of monocytic-like and THP-1 cells were assessed and compared. Cytotoxicity and apoptotic effects of doxorubicin were investigated with CCK-SK assay and flow cytometry; *Results:* Monocytic-like cells showed morphology, size, and phagocytosis comparable to THP-1 cells. However, they expressed lower surface markers CD4, CD117, CD33, CD64 and HLA-DR than THP-1 cells. Following 24h doxorubicin exposure, THP-1 cells exhibited an IC_{50} of 0.59 μM, while the IC_{50} for monocytic-like cells could not be determined. Upon similar treatment conditions, 92.47±3.90% of THP-1 cells underwent late apoptosis. In contrast, only 0.26±0.21% of monocytic-like cells entered late apoptosis, 37.23±1.52% underwent necrosis and 62.47±1.63% remained viable; *Conclusion:* Reactivation of *Oct3/4*, *Sox2* and *c-Myc* in AML-M5-iPSCs induced lower surface marker expression and doxorubicin resistance in monocytic-like cells. Moreover, the apoptotic effect of doxorubicin had been switched to necrotic effect. Surprisingly, morphology and phagocytic function were unaffected. We postulate that transgene reactivation disrupts epigenetic stability and downstream apoptotic pathways. Further investigations are warranted to clarify mechanisms underlying transgene-mediated drug resistance in iPSC-derived disease models.

*Keywords***:** Doxorubicin, iPSC, transgenes reactivation, disease model, AML-M5

INTRODUCTION

Acute monocytic leukaemia (AML-M5) is a disease that affects mostly children below two. It is classified as M5 subtype of acute myeloid leukaemia according to the French-American-British Classification. A patient is diagnosed of having AML-M5 if 80% or more of the non-erythroid cells in his bone marrow are identified as monoblasts, promonocytes or monocytes.[1] Clinically AML-M5 is distinguished by its high leukocyte counts, propensity for extramedullary infiltrates and association with disseminated intravascular coagulation.[2] Genetically, AML-M5 is often linked with key chromosomal translocations including t(8;16)(p11;p13) and MLL locus translocations at 11q23 such as t(10;11)(p13;q23), t(9;11)(p22;q23) as well as t(11;19)(q23;p13).[3]

Over the years, the debate of whether AML-M5 has a worse prognosis than other subtypes of acute myeloid leukaemia is still ongoing because the mechanism leading to AML-M5 treatment failure still remains unclear.[4] Advancement in the study of haematological malignancies such as AML-M5 has been profoundly hindered by the limitation in obtaining sufficient tissue sample

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and inefficiency in maintaining haematopoietic stem or progenitor cells in their multipotent stage. The discovery of induced pluripotent stem cells (iPSCs) has tremendously overcome these limitations and changed the landscape of disease modelling for drug screening.

A pivotal breakthrough was achieved in 2006 when Takahashi and Yamanaka[5] demonstrated that somatic cells could be reprogrammed into iPSCs using defined transcription factors *Oct3/4*, *Sox2*, *Klf4* and *c-Myc*. iPSCs hold several advantages for disease modelling: (i) they can self-renew indefinitely while maintaining pluripotency, providing an inexhaustible source of cells for experimentation;[6] (ii) their derivation evades ethical issues associated with embryonic stem cells (ESC);[7] and (iii) they capture the patient's genetic background, thereby enabling disease modelling under physiologically relevant conditions.[8] These attributes make iPSCs an invaluable platform for studying pathogenesis and drug responses.

Our group previously established an AML-M5 iPSC model by reprogramming the THP-1 cell line using retroviral vectors.[9] These AML-M5-derived iPSCs (AML-M5-iPSC) demonstrated similar morphology, gene expression and antigen expression with that of human ESC. Remarkably, AML-M5 associated *MLL-AF9* fusion gene was expressed in all iPSC clones together with parental cells, providing a functional AML-M5 model. However, we observed that the reprogramming transgenes *Oct3/4*, *Sox2* and *c-Myc* were unintentionally reactivated in the genome of AML-M5-iPSCs post-reprogramming. Since it has never been reported, the effects of reprogramming transgene reactivation in AML-M5-iPSC on drug responses in the subsequent differentiated cells were studied.

Here, we investigate whether reprogramming transgenes reactivation influences the behaviour of AML-M5-iPSC-derived monocytic-like cells and their responses to chemotherapy. We characterised these cells in terms of morphology, phagocytic function and surface marker expression, and subsequently evaluated their cytotoxic and apoptotic responses to doxorubicin, a frontline drug for AML-M5 treatment, with THP-1 parental cells serving as controls. Findings from this study would provide a clearer indication on whether reprogramming transgenes reactivation in the iPSCs would affect drug responses in the downstream differentiated cells.

MATERIALS AND METHODS

Cell culture

THP-1 cells (ATCC, USA) were maintained in RPMI-1640 medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and 0.5% penicillin-streptomycin (Gibco, USA) at 37 °C, 5% CO_2. Experiments were conducted on cells between passages 10–25. AML-M5-iPSCs were reprogrammed and generated as previously described.[9] They were cultured on inactivated mouse embryonic fibroblast using DMEM/F12 (Gibco, USA) medium supplemented with 20% KOSR (Gibco, USA), 1% GlutaMAX™ (Gibco, USA), 1 % NEAA (Gibco, USA), 0.143 molar β-mercaptoethanol (Merck, USA) and 10 ng/mL FGF2 (Miltenyi Biotec, Germany). Cells were maintained at 37 °C in a humidified atmosphere (Esco Lifesciences, Malaysia) containing 5% CO_2.

Detection of reprogramming transgene

Genomic DNA extraction of AML-M5 iPSCs and THP-1 cells was performed using genomic extraction kit (Promega, USA) according to manufacturer's instruction. The genomic DNA products were stored at -20°C prior to analysis. The PCR mixture (total volume of 15 µl) to amplify each transgene (*Klf4*, *Oct3/4*, *Sox2* and *c-Myc*) was prepared with nuclease-free water (Merck, Germany), 200 nM forward and reverse primers (Integrated DNA Technologies, Singapore), 2× PCR master mix (Promega, USA) and 1 µl of DNA template. Negative controls were likewise prepared except that the volume of DNA template was substituted with nuclease-free water. The primers used in transgene amplification were listed in TABLE 1. The amplification reaction was carried out in a PCR machine (Applied Biosystems, USA) using the thermal profile of 96°C/3 min for initial denaturation, (96°C/30 s, 60°C/30 s, 72°C/30 s) for 25 cycles and a final extension of 72°C/5 min.

PCR products were analysed on 2% agarose gels that pre-stained with 2 µl gel red (Biotium, USA) and visualised using a gel imager (Azure Biosystems, USA). A 100 bp DNA ladder (New England Biolabs, USA) was used as reference.

Haematopoietic differentiation of AML-M5-iPSCs

AML-M5-iPSC colonies were dissociated mechanically using scalpel blade (Swann-Morton, England) and transferred into an ultra-low adherence plate (Thermo Fisher Scientific,

TABLE 1: Forward and reverse primer sequences for *Oct3/4*, *Klf4*, *Sox2* and *c-Myc* genes

PCR	Primers	Nucleotide sequence (5'to 3')	Gene	Amplicon (bp)
Oct3/4	Forward	CTGCACTGTACTCCTCGGTC	*Oct3/4*	201
	Reverse	TCCTTACGCGAAATACGGGC		
Klf4	Forward	TTCCCATCTCAAGGCACACC	*Klf4*	353
	Reverse	TCCTTACGCGAAATACGGGC		
Sox2	Forward	ATGGGTTCGGTGGTCAAGTC	*Sox2*	379
	Reverse	TCCTTACGCGAAATACGGGC		
c-Myc	Forward	CAAGAGGCGAACACACAACG	*c-Myc*	401
	Reverse	TCCTTACGCGAAATACGGGC		

Denmark) containing ESC medium with 10 µM rock inhibitor (Targetmol, USA). After 5 days of incubation, embryoid bodies (EBs) were formed. Large EBs were selected using 40 µM cell strainer (Biologix, USA) and cultured in six-well plates (Trueline, USA) with DMEM (Gibco, USA) supplemented with 10% FBS, 1% penicillin-streptomycin, 25 ng/ml of human IL-3 (STEMCELL Technologies, Canada), 100 ng/ml of hM-CSF (STEMCELL Technologies, Canada), 1 % GlutaMAX™ and 0.055 mM of β-mercaptoethanol. Within 7 days, myeloid-cell-forming complexes (MCFCs) were formed. Differentiated monocytic-like cells continuously produced from MCFCs were used for subsequent experiments.

Morphological observation

Monocytic-like cells that differentiated from AML-M5-iPSCs were subjected to morphological observation using an inverted microscope (Thermo Fisher Scientific, USA). The differentiated cell images were captured at day 0, 5, 10, 15, 20 and 25 under 400× magnification and the cell diameters were measured using imaging analysis software version 3.21.00 (NIS-Elements, Japan).

Phagocytosis assay

THP-1 and monocytic-like cells were seeded in 96-well U-bottom microplates (Cellstar, Austria) at a density of 5000 cells/well in RPMI1640 medium supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. Carboxylate-modified red fluorescent latex beads with a mean diameter of 1 µm (Merck, Germany) were added at a concentration of 1:400 into the culture medium. Cells were incubated for 2 h at 37 °C and 5% CO_2. Following that, fluorescent quantification was measured using a microtiter plate reader (Tecan, Switzerland) at 575 nm.

Immunotyping assay

THP-1 and monocytic-like cells were harvested and washed twice with cold 0.2% BSA (Nacalai Tesque, Japan). The cells were pellet down by

centrifugation (Beckman Coulter, USA) at 300g, 4 °C for 5 min. The supernatant was discarded. A 100 µl aliquot of each THP-1 and differentiated monocytic-like cells suspension (1× 10^6 cells) was distributed into 2 flow tubes (STEMCELL Technologies, Canada). Antibodies CD4 (BD Biosciences, USA), CD33 (BD Biosciences, USA), CD64 (BD Biosciences, USA), CD117 (BD Biosciences, USA) and HLA-DR (BD Biosciences, USA) were added according to the suggested volume from the manual to the cells followed by short vortex (BioCote, UK) and incubated for 15 min at room temperature in dark. Cells were then washed 2 times with 1 ml 0.2% BSA and centrifuged at 300×g, 4 °C for 5 min. Supernatant was discarded and the cell pellets were resuspended in 0.5 ml of 0.2% BSA. Viability dye 7AAD (BD Biosciences, USA) was added into sample tube before sample acquisition and incubated for 5 min at room temperature in dark. The cells were then analysed by flow cytometry (BD Biosciences, USA).

Cytotoxicity assay

Cytotoxicity of doxorubicin (Merck, Germany) on THP-1 and monocytic-like cells were tested with a rapid colorimetric assay CCK-SK (Dojindo, Germany) and compared with untreated controls. An aliquot of 100 µl of 5000 cells/well suspension was seeded into 96-well U-bottom microplates. Doxorubicin prepared at 0.5, 1, 2 and 4 µM were added into the wells and incubated for 24 h at 37°C, 5% CO_2. The absorbance was then measured at 450 nm with a microtiter plate reader. The cytotoxic effect of doxorubicin was accounted for as the relative viability (% control) according to the calculation shown below. Percentage of cell viability in the untreated control was assumed as 100%.

Relative viability = (experimental absorbance - background absorbance) / (absorbance of untreated controls - background absorbance) × 100 %

Apoptosis assay

An aliquot of 1× 10^5 cells of THP-1 and monocytic-like cells were treated with doxorubicin at a final concentration of 4 µM. After 24 h of incubation, the cells were harvested and centrifuged at 400×g for 5 min and the supernatant was decanted. Then, the cells were washed with 1× ice cold PBS (Oxoid, USA) and centrifuged at 400×g for 5 min. Following that, the cells were resuspended in 190 µL 1× binding buffer. A volume of 10 µL Annexin-FITC (Life Technologies, USA) was added to the cell suspension and mixed well. The cell suspension was incubated for 10 min at room temperature. Subsequently, the cells were washed with 1× binding buffer and centrifuged at 400×g. After the supernatant was decanted, the cells were resuspended in 190 µL 1× binding buffer. After that 10 µL of 20 µg/ml propidium iodide (Life Technologies, USA) were added into the cell suspension and analysed by flow cytometry and compared with that of their untreated controls.

Statistical analysis

All assays were performed in triplicate and repeated at least three times. Data were analysed with GraphPad Prism 8.3.0 using one-way ANOVA with Tukey's post hoc test or Student's t-test where appropriate. $p < 0.05$ and $p < 0.01$ were considered statistically significant.

RESULTS

Reactivation of reprogramming transgenes Oct3/4, Sox2 and c-Myc detected in AML-M5-iPSCs

The reactivation of reprogramming transgenes for AML-M5-iPSCs was screened by using the genomic DNA template. Based on the gel image shown in FIG 1, distinct bands were observed from *Oct3/4* iPSC (lane 5), *Sox2* iPSC (lane 8) and *c-Myc* iPSC (lane 11) with expected band size of 201 bp, 379 bp and 401 bp, respectively. These results indicate that *Oct3/4*, *Sox2* and *c-Myc* genes were reactivated in the genome of AML-M5-iPSCs.

Monocytic-like cells exhibited similar morphology and size as THP-1 cells

AML-M5-iPSCs generated EBs comprising three germ layers; those of 100–300 µm diameter were selected for haematopoietic differentiation (FIG 2A). In the presence of IL-3 and M-CSF, MCFCs continuously produced monocytic-like cells for up to 25 days (FIG 2D). Morphological observation of monocytic-liked cells showed large, round cells with irregular nuclei and primary granules, closely resembling THP-1 cells (FIG 2B and 2C). Cell diameters increased from 13–14 µm (day 5) to 16-20 µm (day 25), comparable to the reported monocyte range (12–18 µm).[11]

Monocytic-like cells showed comparable phagocytotic activity to THP-1 cells

Phagocytosis assays using fluorescent latex

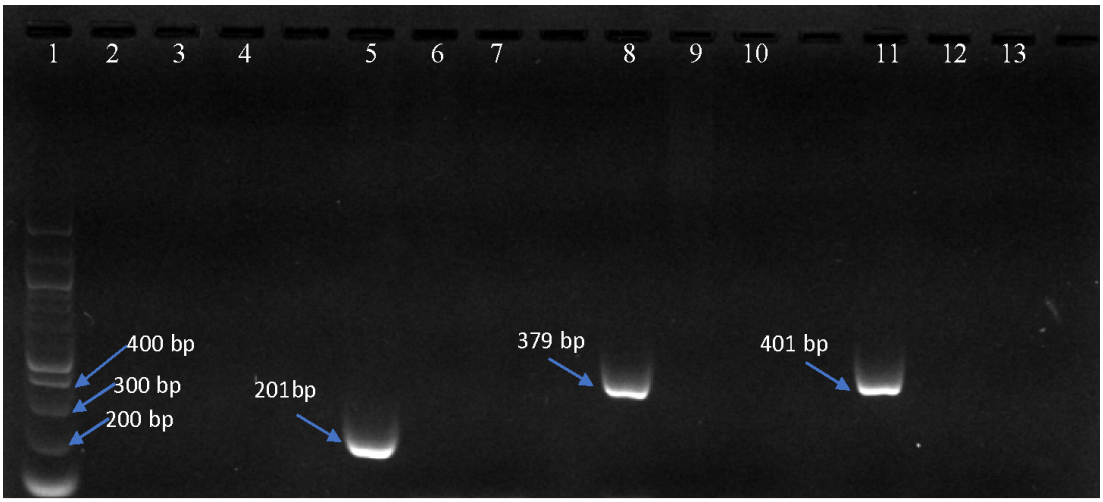


FIG 1: Gel image of reprogramming transgenes reactivation screening result. Lane 1: 100 bp DNA ladder; Lane 2: *Klf4* iPSC; Lane 3: *Klf4* THP-1; Lane 4: non-template control; Lane 5: *Oct3/4* iPSC; Lane 6: *Oct3/4* THP-1; Lane 7: non-template control; Lane 8: *Sox2* iPSC; Lane 9: *Sox2* THP-1; Lane 10: non-template control; Lane 11: *c-Myc* iPSC; Lane 12: *c-Myc* THP-1 and Lane 13: non-template control. Lanes 2 to 13 were loaded with 3 µL PCR product, 2 µL nuclease-free water and 1 µL 6× loading dye.

beads showed no significant differences ($p > 0.05$) between THP-1 and monocytic-like cells across days 5–25 (FIG 3). This indicates that the differentiated cells retained monocyte-like phagocytic function.

Monocytic-like cells expressed surface markers CD4 and CD117, but not CD33, CD64 and HLA-DR

THP-1 cells expressed CD33 (99.95 ± 0.07%), CD64 (93.9 ± 3.68%), CD4 (90.6 ± 2.26%), HLA-DR (81.3 ± 8.06%), and CD117 (53.85 ± 10.68%). In contrast, monocytic-like cells showed lower expression: on day 5, CD4 (60.0 ± 7.4%) and CD117 (11.57 ± 6.42%) were detectable, while CD33 (1.5 ± 2.34%), CD64 (0.47 ± 0.47%), and HLA-DR (0.2 ± 0.17%) were minimal. Expression further declined by day 15 to CD4 (43.63 ± 1.29%), CD117 (2.37 ± 0.38%), CD33 (0.2 ± 0.01%), CD64 (0.37 ± 0.21%), and HLA-DR (0.33 ± 0.21%) (FIG 4A to 4D). These results suggest partial maturation and incomplete immunophenotypic alignment with THP-1 cells.

Monocytic-like cells showed high resistance to cytotoxic doxorubicin

Doxorubicin caused a dose-dependent reduction in cell viability (FIG 5A). THP-1 cells showed significant cytotoxicity at all tested concentrations ($p < 0.05$), with an IC_{50} of 0.59 µM. In contrast, monocytic-like cells exhibited minimal reduction in viability and no calculable IC_{50} within the tested range. Viability differences between THP-1 and monocytic-like cells were significant ($p < 0.01$), indicating resistance of the latter to doxorubicin.

Apoptotic effect of doxorubicin had been switched to necrotic in monocytic-like cells

After 24 h treatment with doxorubicin at 4 µM, the viable population of THP-1 cells greatly decreased from 91.53±2.54% to 0.03±0.03%. Upon similar treatment conditions 92.47±3.90% of THP-1 cells entered late apoptosis stage (FIG 5C). Meanwhile, for monocytic-like cells, only 0.26±0.21% entered late apoptosis stage, 37.23±1.52% entered necrosis stage and 62.47±1.63% remained in viable stage (FIG 5C). These data suggest that firstly, the monocytic-like cells were more resistant to the apoptotic effect of doxorubicin at 4 µM as compared to the parental THP-1 cells; and secondly, doxorubicin was inducing necrosis instead of apoptosis in these monocytic-like cells.

DISCUSSION

The development of iPSC technology has enabled the generation of disease-relevant, genome-specific and previously unattainable cells covering a vast range of genetic,[12] neurodegenerative[13] and cardiovascular diseases.[14] This special cellular material can be produced in scalable quantities attributed to its ability to self-renew

and maintain in undifferentiated pluripotent state indefinitely. Due to such advantages, iPSCs have proven instrumental for drug screening, disease pathogenesis investigation as well as novel therapeutics identification.

With increasing usage of iPSCs as disease models for drug screening, we notice that most studies skipped quality check after reprogramming. This oversight could tremendously interfere with drug screening results because reprogramming using traditional retrovirus integrative method that relies on the virus's ability to integrate the transgenes into the host cell's genome could result in potential disruption of normal gene function and interruption of endogenous transcription factor network, leading to the failure of iPSC differentiation.[15,16] Normally, reprogramming transgenes are epigenetically silenced after reprogramming through DNA methylation and histone modifications, ensuring stable pluripotency.[17] However, these epigenetic modifications can be unstable, leading to the potential for transgene reactivation.[18]

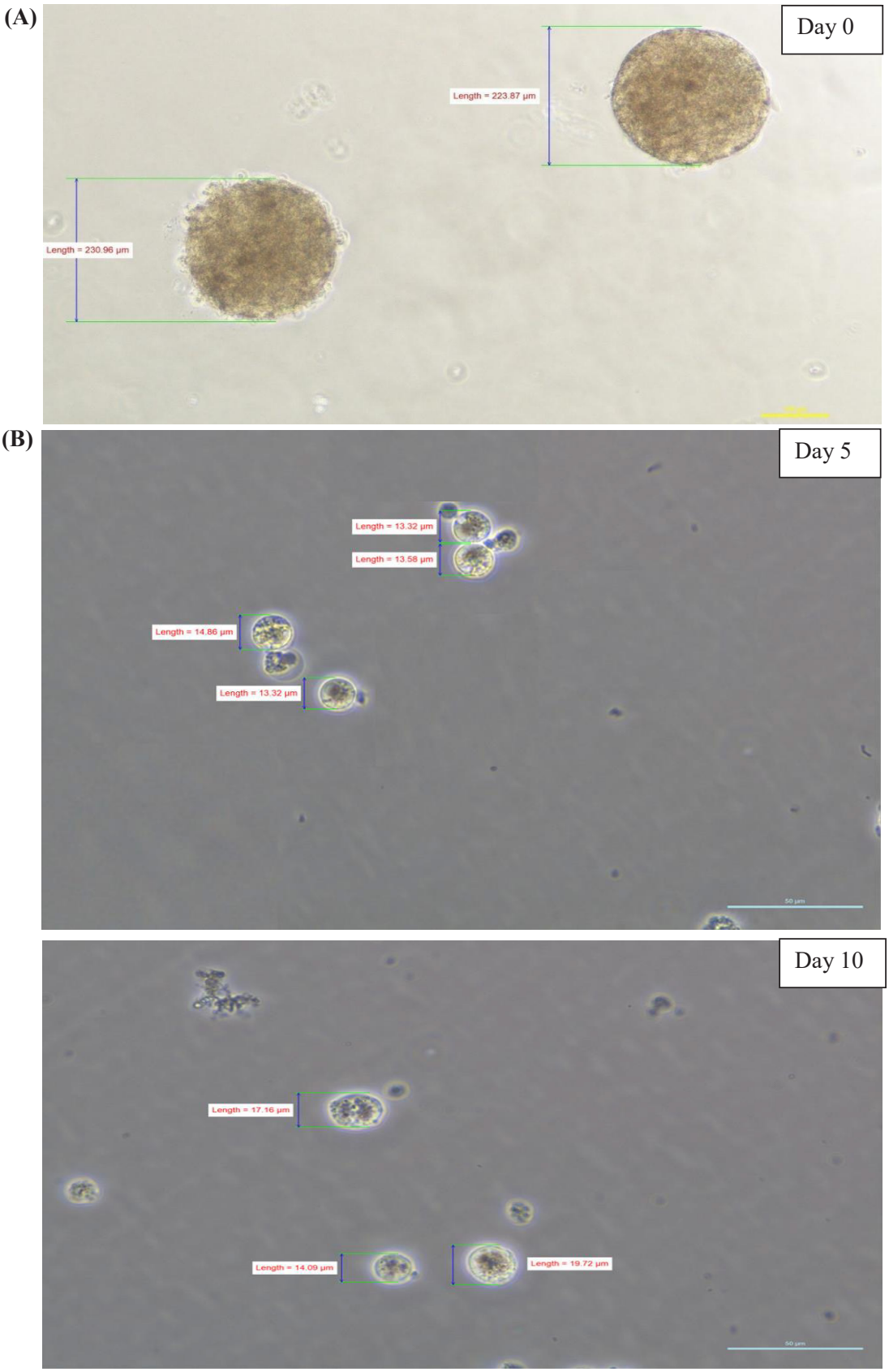
To address this challenge, safer approaches have been developed. Transgene integration into genomic "safe harbour" sites such as the AAVS1 locus minimises insertional mutagenesis and supports predictable expression.[19] In parallel, non-integrative reprogramming methods including microRNAs,[20] synthetic mRNAs,[21] and small molecules[22] circumvent permanent genomic modifications. Notably, Peng *et al*.[23] reported the successful generation of chemically induced pluripotent stem (hCiPS) cells from small volumes of blood using small molecules, highlighting the translational potential of integration-free approaches. Nonetheless, such methods often have lower efficiencies than retroviral systems due to transient and unstable factor expression.[15]

As mentioned earlier, we observed that reprogramming transgenes *Oct3/4*, *Sox2* and *c-Myc* were reactivated in the genome of AML-M5-iPSC. To fill the current research gap and to highlight the importance of performing proper quality checks after reprogramming, we decided to proceed with investigating the effects of transgene reactivation on doxorubicin drug responses in monocytic-like cells derived from AML-M5-iPSCs. This study is interesting because contradictory observations have been reported thus far. Some groups reported that residual transgene expression impaired differentiation potential for instance, reduced hepatocyte marker expression[24] or poor haematopoietic markers expression.[25] In contrast, other studies argued that *c-Myc*, did not alter proliferation or differentiation outcomes.[26] These discrepancies highlight the need to evaluate each system empirically, especially for disease models intended for drug testing.

We were confident to proceed with AML-M5-iPSCs haematopoietic differentiation into monocytic-like cells because the retroviral reprogrammed AML-M5-iPSCs did express *MLL-AF9* fusion gene, a critical oncogenic driver in AML-M5.[9] In clinical settings, *MLL-AF9* fusion gene causes poor prognosis of AML-M5 because the cellular mechanisms involved still remain poorly understood.[27]

Differentiation was initiated through EB formation (FIG 2A), an established approach for promoting multilineage commitment.[28] In order to establish haematopoietic *in vitro* differentiation, EBs were exposed to M-CSF and IL-3 to produce intermediate MCFC (FIG 2D), based on a well-established protocol described by Lachmann *et al*.[29] M-CSF promotes the differentiation of myeloid progenitors into monocytes and macrophages,[30] it is also an important cytokine used in terminal lineage differentiation from haematopoietic stem cell sources.[31] Meanwhile IL-3 regulates proliferation and maturation of haematopoietic progenitors.[32] Thus far, many protocols for haematopoietic differentiation of iPSCs used a multitude of cytokines or small molecules to imitate signalling pathways at different stages of embryonic development,[33,34] yet most of the factors involved in the differentiation process remain unclear. It is worth noting that adopting a stable protocol will ensure proper differentiation process and functionality of the target cells.[29]

Morphological and functional analyses confirmed the successful generation of monocytic-like cells. The cells exhibited round, lacking visible cytoplasmic extensions and non-adherent morphology, with diameters of 12–18 μm, similar to THP-1 parental cells (FIG 2) and consistent with prior descriptions of monocytes.[35,36] Functional validation through phagocytosis assays showed comparable activity to THP-1 cells across differentiation time points (FIG 3). This functional equivalence likely reflected similar activation of Dectin-1 and Mac-1 receptors on functional monocytes, which mediate recognition and engulfment of foreign particles.[37,38] Together, these findings indicate that monocytic-like cells were functionally


(A)
Day 0
Length = 223.87 µm
Length = 230.96 µm
(B)
Day 5
Length = 13.32 µm
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Length = 13.32 µm
Day 10
Length = 17.16 µm
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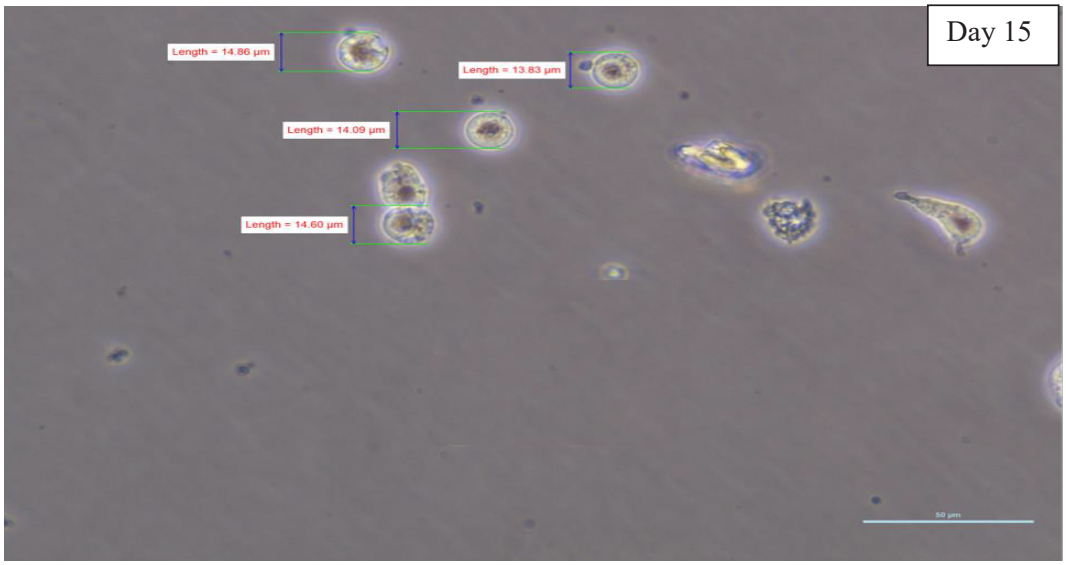
Day 15
Length = 14.86 µm
Length = 13.83 µm
Length = 14.09 µm
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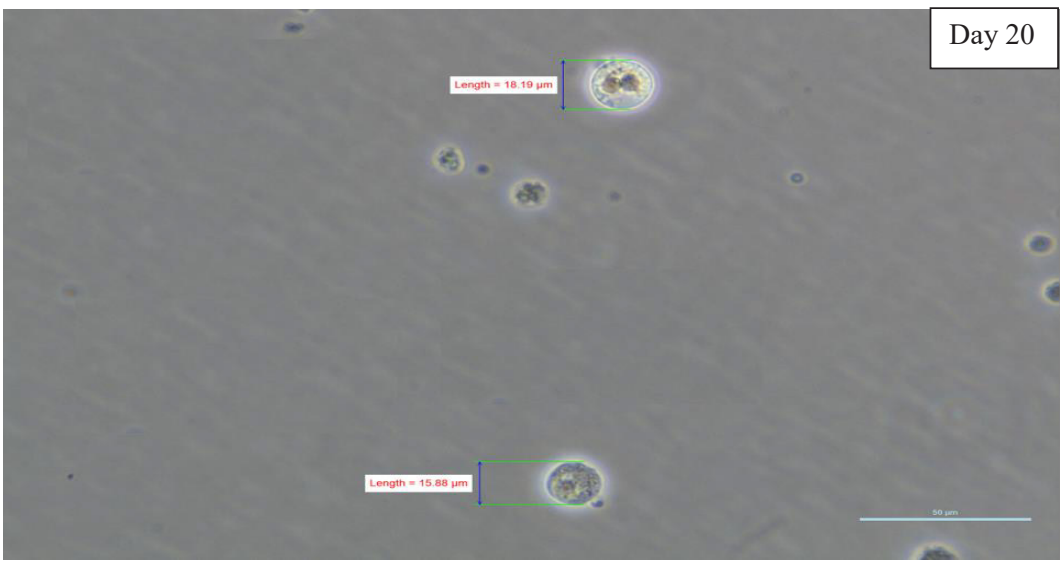
Day 20
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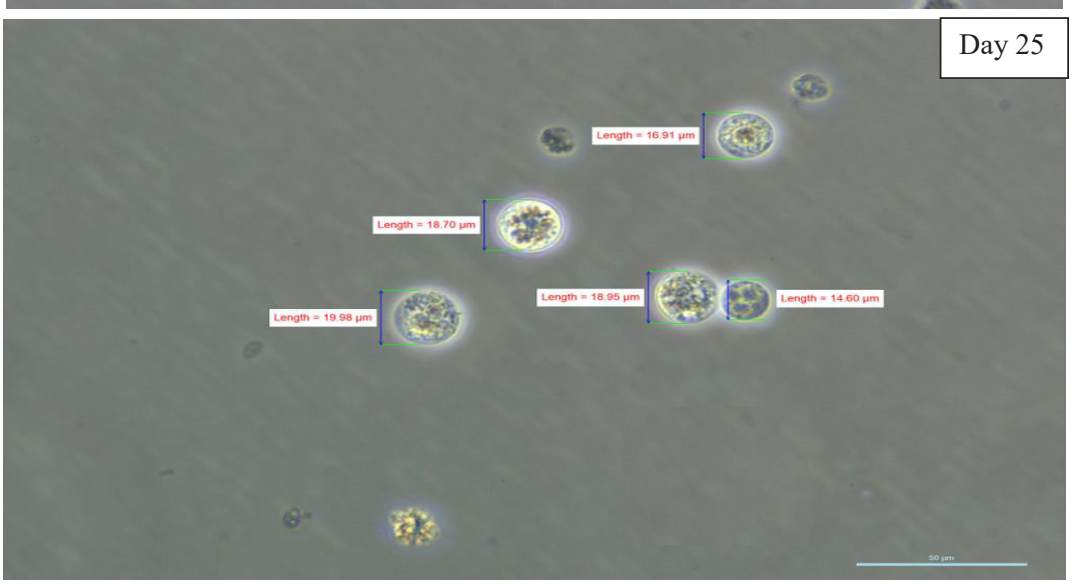
Length = 15.88 µm
50 µm


Day 25
Length = 16.91 µm
Length = 18.70 µm
Length = 18.95 µm
Length = 14.60 µm
Length = 19.98 µm
50 µm

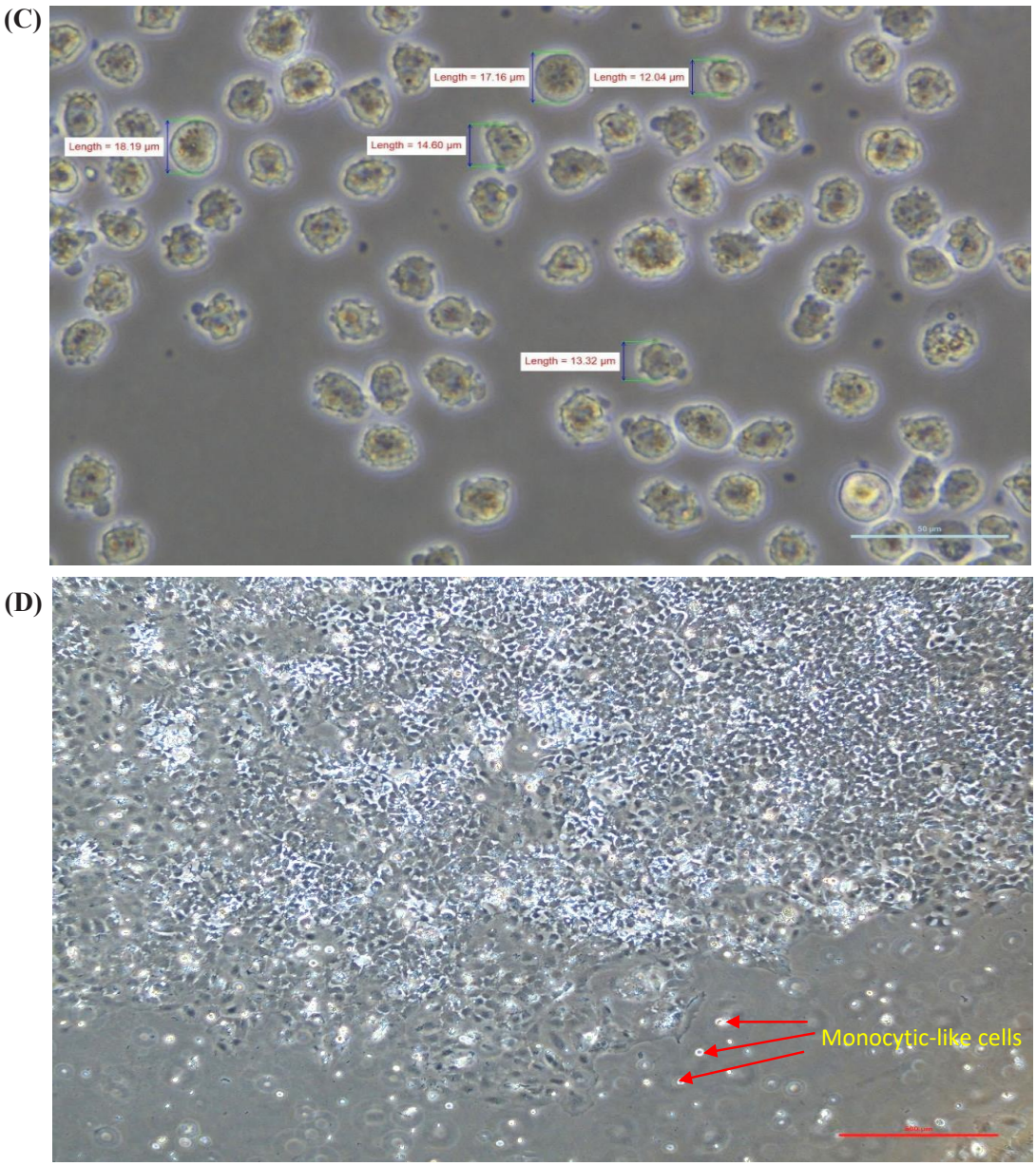


FIG 2. (A) Haematopoietic differentiation at day 0 under 40× magnification. The embryoid bodies were yet to attach to the culture plate. (B) Haematopoietic differentiation of AML-M5-iPSC from day 5 to 25 under 400× magnification. Monocytic-like cells with a diameter between 13-20 µm were observed from day 5-25. (C) THP-1 cells at passage 24 under 400× magnification. The diameter of the cells was in the range of 12-18 µm (D) Intermediate myeloid-cell-forming complex (MCFC) at day 25 under 40× magnification. Small spherical shape monocytic-like cells were constantly generated from MCFC.

competent despite their derivation from transgene-reactivated iPSCs.

Surface marker profiling revealed important distinctions between monocytic-like and parental THP-1 cells. While THP-1 cells expressed high levels of AML-M5-associated markers (CD4, CD33, CD64, CD117, and HLA-DR) (FIG 4),[39] monocytic-like cells displayed significantly reduced expression across all markers (FIG 4B-D). Although both CD4 and CD117 expressions somehow indicate that AML-M5-iPSC had been differentiated into monocytic lineage, but from the decreasing trend from day 5 to day 15, we somehow suspect that the monocytic-like cells were slowly maturing into macrophages. This postulation was based on similar observations

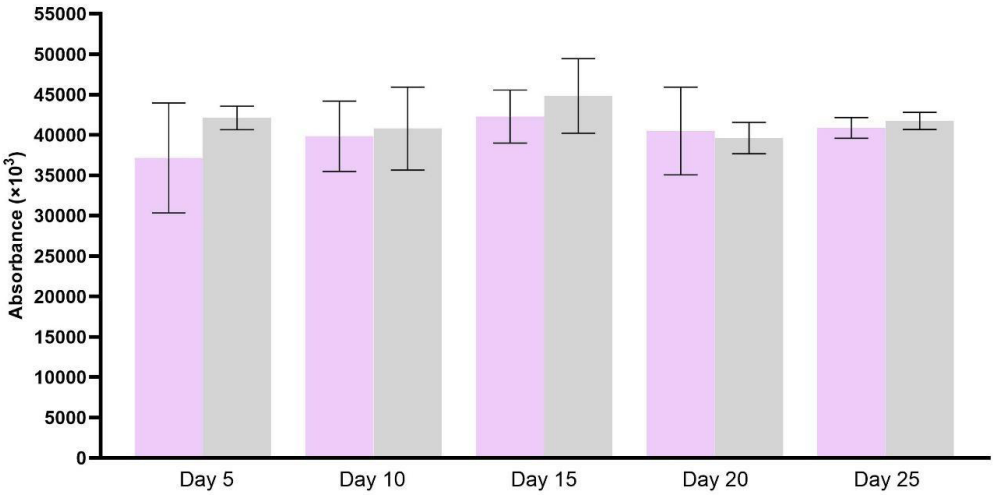


FIG 3. Phagocytosis of carboxylate-modified red fluorescent latex beads by THP-1 and monocytic-like cells on day 5-, 10-, 15-, 20- and 25-post differentiation. Data presented are means ± SEM (n=3).

reported by two different studies. Graziani-Bowering *et al*.[40] reported that CD4 was lost during the differentiation of monocytes into tissue culture-derived macrophages. Frumento *et al*.[41] reported that the expression of CD117 was also lost upon cellular differentiation of germ cells, haematopoietic stem cells as well as haematopoietic progenitors. In addition, the low expression of CD33, CD64 and HLA-DR could reflect an incomplete monocytic differentiation process possibly due to insufficient exposure duration or differentiation factors.[29] However, we tend to believe that the overall low expression of AML-M5 surface markers in monocytic-like cells was a direct outcome of transgenes reactivation in the genome of AML-M5-iPSCs.

Drug response assays revealed striking differences between monocytic-like cells and parental THP-1 cells. Doxorubicin treatment induced a clear dose-dependent cytotoxic response in THP-1 cells (FIG 5A), with an IC_{50} of 0.59 µM, consistent with prior reports of AML sensitivity to this drug.[42] By contrast, monocytic-like cells exhibited marked resistance, with no definable IC_{50} within the concentration range tested (FIG 5A). This resistance could not be attributed to technical limitations, as extended exposure was precluded by excessive THP-1 death at later time points (data not shown). Instead, the findings strongly implicate transgene reactivation as a driver of chemoresistance. Several mechanisms may explain this phenomenon. For instance, transgenes reactivation could give rise to insertional mutagenesis, especially when the transgene insertional site was located at the genes that were involved in drug metabolism or drug response pathways.[43] Also, various reprogramming transgenes have been associated with chemotherapeutic drug resistance in human cells. *c-Myc* has been shown to enhance drug efflux in myeloid and colon cancer stem cells via ABC transporter upregulation.[44,45] *Sox2* similarly induces resistance in colorectal cancer through ABCC2 transporter expression.[46] *Oct3/4* contributes to glioblastoma drug resistance by regulating efflux pumps such as ABCG2.[47] Knowing all of these, although our work did not directly investigate the cytotoxicity of doxorubicin in AML-M5-iPSCs, but the reactivation of *c-Myc*, *Sox2* and *Oct3/4* transgenes in AML-M5-iPSCs could be directly translated to doxorubicin resistance observed in the monocytic-like cells after differentiation.

Apoptosis assays provided further insight into the altered drug response. In THP-1 cells, doxorubicin induced classical late apoptosis in over 90% of cells, consistent with its known mechanisms of DNA intercalation and topoisomerase-II inhibition.[48] By contrast, monocytic-like cells exhibited negligible apoptosis but a substantial increase in necrosis (37.23±1.52%) (FIG 5B-C), suggesting a shift in cell death modality. This aberrant necrotic response may reflect epigenetic instability introduced by transgene reactivation, which could dysregulate p53 signalling or mitochondrial homeostasis.[49,50] While necrosis also contributes to cell clearance, but it is generally less

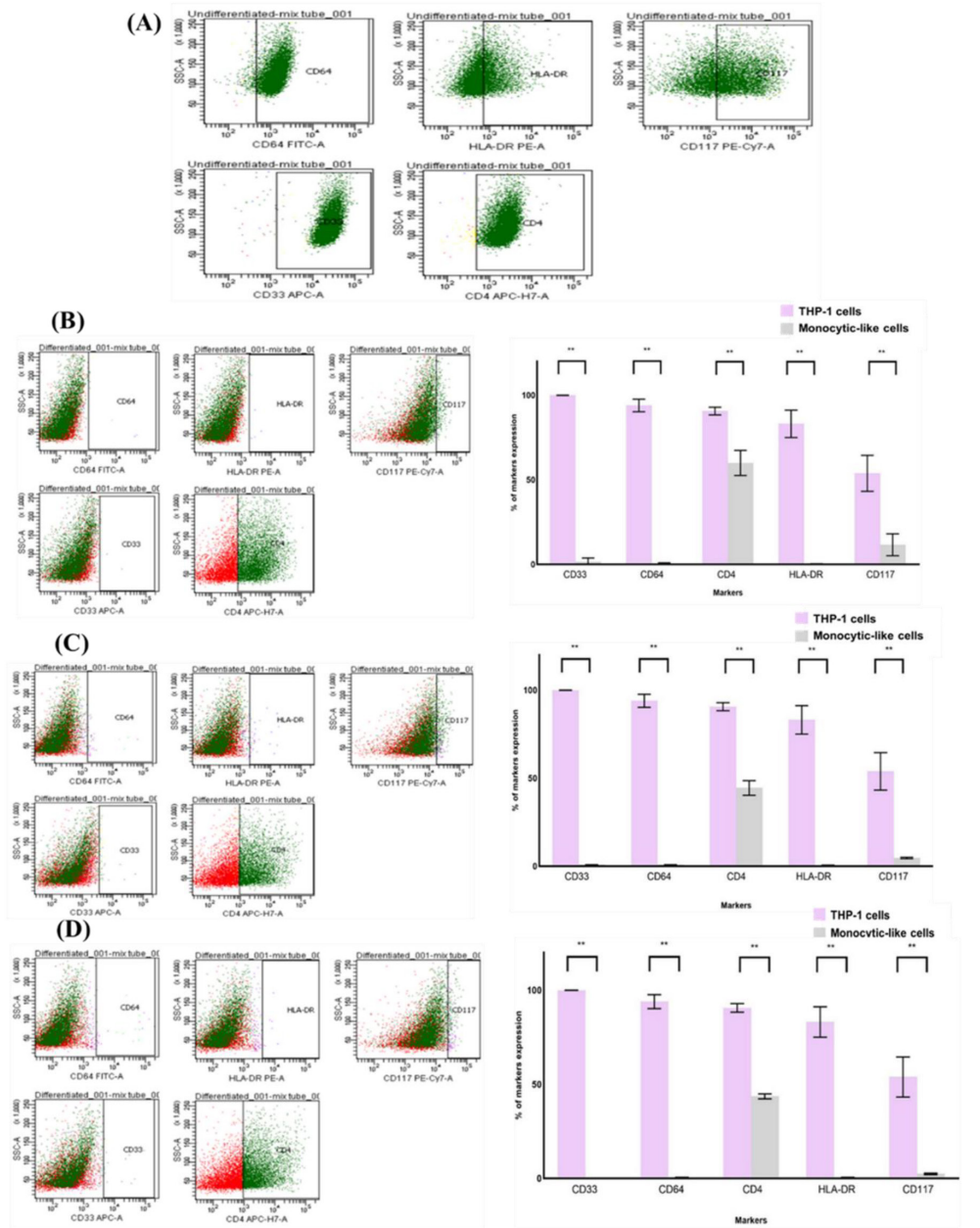


FIG 4. (A) Flow cytometry plots of surface markers CD64, HLA-DR, CD117, CD33 and CD4 expression of THP-1 cells. (B-D) Flow cytometry plots of surface markers CD64, HLA-DR, CD117, CD33 and CD4 expression of monocytic-like cells on day 5, 10 and 15 post-differentiation respectively. Bar graphs highlight the differences (%) in surface markers expression between THP-1 and monocytic-like cells at day 5, 10 and 15. Data presented are means ± SEM (n=3). Significant differences of surface markers expression in comparison with THP-1 cells are marked with asterisks (**$p < 0.01$).

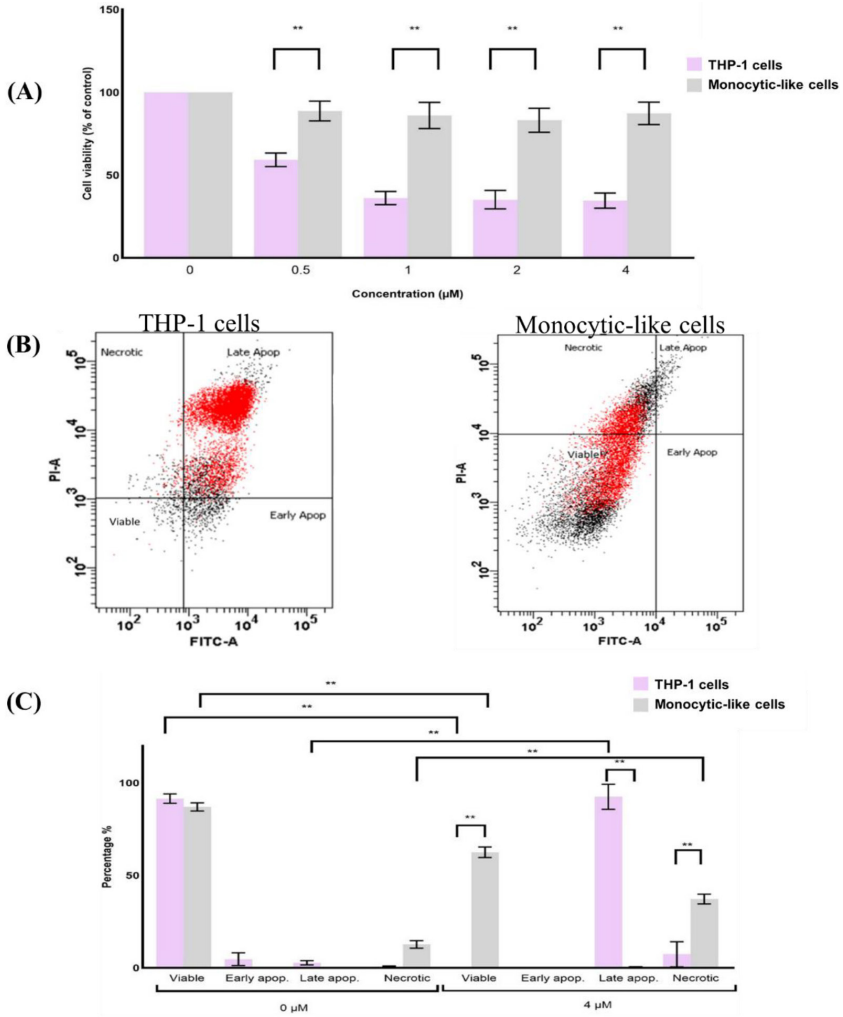


FIG 5. (A) Cytotoxicity of doxorubicin towards THP-1 and monocytic-like cells after 24-hour treatment. IC_{50} value determined for THP-1 cells was 0.59 μM, while the IC_{50} value remained undetermined for monocytic-like cells. Data presented are means ± SEM (n=3). Significant differences of cell viability in comparison with THP-1 cells are marked with asterisks (**p<0.01). (B) Flow cytometry plot of the effect of doxorubicin towards THP-1 cells and monocytic-like cells after 24-hour treatment. Data presented are representative of triplicates. (C) Apoptotic effects of doxorubicin towards THP-1 and monocytic-like cells after 24-hour treatment. Data presented are means ± SEM (n=3). Significant differences between THP-1 cells and differentiated cells within each population (viable, early apoptosis, late apoptosis and necrosis) under similar treatment condition, and significant differences between each cell type in similar population across different treatment conditions, are marked with asterisks (**p <0.01).

desirable in chemotherapy because of associated inflammatory responses which would lead to uncontrolled cell rupture that impairs efficient macrophage-mediated clearance.[51,52]

CONCLUSION

In conclusion, we have successfully differentiated AML-M5-iPSCs into monocytic-like cells that exhibited similar morphological and phagocytotic properties as the parental THP-1 cells albeit lower cell surface marker expression. The reactivation of reprogramming transgenes *Oct3/4*, *Sox2* and *c-Myc* seemed to cause doxorubicin resistance in monocytic-like cells, as well as switching the apoptotic effect to necrotic. In future studies, these reprogramming transgene reactivated AML-M5-iPSCs could serve as a distinct model to explore the mechanisms

underlying doxorubicin resistance especially in blood disorders. Otherwise, by adopting an alternative reprogramming method or silencing the reprogramming transgenes, AML-M5 iPSCs could still be useful for studying drug response and pathogenesis of AML-M5.

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