Immunological Role of Megakaryocytes and Platelets during Influenza A Virus Infection

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***Abstract*—Influenza viruses pose a serious threat to public health, with severe cases often characterized by lung damage and inflammation. However, the underlying mechanisms of these processes remain poorly understood. This study aimed to investigate the essential role of megakaryocytes (MKs) and platelets (PLTs) in influenza A virus (IAV) infections. Conducted at the Department of Rare Respiratory Diseases, Cystic Fibrosis, and Pulmonology, Nord University Hospital, Marseille, France, the study collected seventy blood samples between October 2018 and March 2019. Samples were obtained from healthy individuals and patients diagnosed with IAV. Messenger RNA was extracted from isolated PLTs and subjected to quantitative real-time-polymerase chain reaction using sets of primers targeting immune marker genes. Western blot analysis was also performed for confirmation, focusing on Fas Ligand (FasL). Results showed that PLTs from IAV-infected individuals expressed the FasL, tumor necrosis factor-related apoptosis-inducing ligand, and Granulysin (GNLY) receptors when activated. Furthermore, an *in vitro* assay revealed the presence of FasL receptors on infected CMK cell lines. *In vivo* investigations demonstrated that activated MKs and PLTs in mice also expressed FasL. Interestingly, none of the immune receptors under investigation were found in both MKs and PLTs in mouse model studies. In conclusion, MKs and PLTs play a significant role in influencing immune responses that may help prevent viral spread during infection. However, further examination of their mechanisms of action is warranted. Understanding the involvement of these cells in influenza pathogenesis could offer valuable insights for developing potential therapeutic strategies.**

***Index Terms*—Influenza A virus, Megakaryocytes, Platelets, Killer activation receptors, FasL.**

I. Introduction

The Annual report of flu outbreaks indicated that this disease result in 250 000 to 500 000 mortalities worldwide (Organization, 2022). Recently, the GLaMOR Project has reported an uncertainty range of 294 000 - 518 000, which is close to the previous global estimate by the World Health Organization (Paget, et al., 2019). Moreover, the H5N1 and H7N9 subtypes of human pathogenic avian viruses pose a serious threat to public health, highlighting the need for better understanding of influenza A viruses (IAV) and development of new therapies (Iuliano et al., 2018; Li et al., 2018; Paget, et al., 2019).

The multifactorial process of severe influenza is primarily influenced by the interaction between the virus' ability to replicate, the downregulation of host response to infection, and vascular hemorrhages that cause alveolar injury (Perrone, et al., 2008). The most frequent components in the inflamed lungs following endothelial cell injury are platelets (PLTs), which undertake immunological activities differently from leukocytes; but their actual function is still unknown (Tate, et al., 2009). In addition to their function in hemostasis, PLTs are crucial components of immunity. The production of cytokines by PLTs, which contribute to the acute inflammatory process, is well documented (Morrell, et al., 2014).

Megakaryocyte (MKs) is a key element of early thrombosis, hemostasis, and wound healing. More evidences are indicated that MKs may perform a different intrinsic biological function in addition to manufacturing PLTs (Arabanian, et al., 2012). Furthermore, they are known to convey to PLTs a number of genes with well-known immunomodulatory properties (such as CD154, tumor necrosis factor-related apoptosis-inducing ligand [TRAIL], TNF-a, and FasL) (Crist, et al., 2004; Crist, et al., 2008).

PLTs are therefore crucial participants in IAV disease; however, their specific mechanisms of action are still unknown. The previous studies were believed that the MK precursor cells produced all of the PLTs at the bone marrow (Deutsch and Tomer, 2006; Machlus and Italiano, 2013; Patel, Hartwig and Italiano, 2005). However, recently, a milestone report provided evidence that a reservoir of MKs is also present in the lungs, which is believed the site for 50% of PLT biogenesis from total PLT (Lefrancais, et al., 2017). Furthermore, several studies emphasized that PLTs are cytotoxic to microorganisms and abnormal cells (Yeaman, 2010; Hamzeh-Cognasse, et al., 2015; Assinger, et al., 2019). *In vitro* studies highlighted similar to natural killer (NK) cells; PLTs destroy a range of tumor cell lines as

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well as parasites (Ibele, et al., 1985; McMorran, et al., 2009). According the previous report, cell death is caused by an antibody-dependent cell-mediated cytotoxicity (Joseph, et al., 1983). However, the mechanisms driving PLT-mediated cell death remain unexplained, with the exception of new reports that show FasL may be involved (Schleicher, et al., 2015).

From this context, this study was designed to understand the immunological effects of MKs and PLTs during the pathology of influenza and help in the development of novel therapy. In addition, this highlights the critical requirement for innovative antiviral approaches, as the study's strategy focuses on cellular host characteristics rather than the virus.

II. Materials and Methods

A. Study Design

The study was designed as a retrospective cohort study, aiming to investigate the significant immunological functions of MKs and PLTs in the progression of IAV pathogenesis. The total study population consisted of forty patients and thirty healthy individuals. The study was conducted between October 2018 and March 2019, at the Department of Rare Respiratory Diseases, Cystic Fibrosis, and Pulmonology at Nord University Hospital in Marseille-France.

B. Reagents

The study used the reagents listed below: Anti-viral N1 protein, monoclonal anti-hemagglutinin (HA) and anti-viral M2 protein (Santa Cruz Biotechnology, Heidelberg, Germany, catalogue numbers [Cat. No.]: sc-56968, sc-393579, and MABF2165, respectively); Purified Mouse Anti-Human FasL CD178 (BD Bioscience, Cat No: 556372); monoclonal anti-tubulin antibody (Sigma Aldrich, Lyon, France, Cat No: T6199); anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) antibody (Millipore, Cat No: AB2302); Anti-Mouse CD178 (Fas Ligand) Monoclonal Antibody (MFL3), allophycocyanin (APC) (eBioscience, Cat No: 50-152-48); polyclonal antiplatelet CD41 (Bioss, Woburn, MA, Cat No: bsm-30032M-APC); monoclonal CD62P (P-Selectin) antibody (BioLegend, France, Cat No: 304903); ketamine/xylazine anesthesia (Bayer HealthCare, France, Cat No: 424946-01-6). The Coomassie (Bradford) Protein Assay Kit was used to measure total protein (Thermo Fisher Scientific, Cat No: 23236).

C. Viruses

IAV (H1N1) strain A/PR/8/34 and IAV (H3N2) strain A/Udorn/72 were used in this study. Virus stocks were produced on MDCK cells and stored at -80°C with titer of 10^7 virus particles/mL.

D. Cell Culture

Human acute megakaryocytic leukemia cells (CMK), DSMZ (Brauschweig, Germany, Cat No: ACC 392) was cultivated in RPMI 1640 (Gibco, Cergy, France, Cat No: 52400017), 10% fetal bovine serum (Gibco, Cat No: A3160501), 100 IU/mL penicillin/streptomycin, 2 mM L-glutamine, and 1% sodium pyruvate (Gibco, Cat No: 15070063, 25030149, and 11360039 respectively) were added as supplements. The cell line growth maintained at a density between $5*10^5$ cells/mL and $1*10^6$ cells/mL. A NucleoSpin RNA kit from (Macherey-Nagel, Cat No: 740955.50) was used to extract RNA from CMK cells. Quantitative real-time-polymerase chain reaction (qRT-PCR) was used to evaluate changes in gene expression, which will be detailed later. Primer sequences are available on request.

The American Type Culture Collection (ATCC, Manassas, VA, USA) provided the MDCK (Maddin-Darby canine kidney) cell line (ATCC CCL-34). Thermo Fisher Scientific provided 1% penicillin-streptomycin, 10% fetal bovine serum, 2 mM L-glutamine, and Eagle's Minimum Essential Medium (EMEM, Cat No: 12491) for the maintenance of MDCK cells. The DMEM Dulbecco's modified Eagle's media (Thermo Fisher Scientific, Cat No: A1443001) with or without phenol red, supplemented with 2 mM L-glutamine, 1% pen-strep, and 10% FBS was used to grow the human alveolar A549 cells (ATCC CCL-185) and mouse fibroblast NIH/3T3 cells (ATCC CRL-1658). All cell lines were kept at 37°C in 5% CO_2 in a humid incubator. The RPMI 1640 culture media with the addition of 100 IU/ml interleukin-2 (IL-2) (Proleukin) (Chiron Corporation, Cat No: C210879), 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 0.1 g/ml streptomycin was used for the cultivation of the human Natural Killer cell line, NK-92 (ATCC CRL-2407).

E. IAV In Vitro Replication Experiment

RPMI 1640 was used to cultivate CMK cells in 96-well plates, accompanied by 2 mM L-glutamine, 1% sodium pyruvate, 10% fetal bovine serum, and 1% penicillin/streptomycin. At 70–80% of confluence cells had been infected by IAV A/PR/8/34 (H1N1) and IAV A/Udorn/72 (H3N2) at a multiplicity of infection (MOI, 3), suspended in complete low-serum (2%) DMEM medium without phenol red. Virus infection was performed for 2 h at 37°C using gentle agitation to increase the rate of virus adsorption, then viral inoculate was removed. Unless otherwise stated, viral titers in all conditions were measured by plaque assay in the supernatant 16 h after inoculation. Then, CMK cells were harvested at (0, 3, and 6) days after infection and RNA was extracted according to the instruction protocols. All analysis was performed in duplicate for three times.

F. Titration Experiment

In DMEM 10% fetal calf serum, $1*10^6$ MDCK cells were cultivated in each well of a 6 well culture plate. The diluted samples were prepared the following day by repeated dilution to a tenth of the original samples. For infection, diluted samples were added after the cells had been cleaned twice with phosphate buffer saline (PBS). The supernatant was removed following an hour of adsorption at 37°C, and MEM medium supplemented with 2% agarose and 1 μg/mL trypsin was then added. To identify plaque lysis, live cells were stained with purple crystal for five minutes when agarose had been removed after 72 h. Then, each sample's

infectious viruses were assessed, with each plaque standing for one infectious virus, after the cells had undergone a thorough cleaning procedure.

G. Human Sampling Experiment

A group of 22 women and 18 men, aged between 35 and 55, were referred to the Department of Rare Respiratory Diseases, Cystic Fibrosis, and Pulmonology at Nord University Hospital in Marseille, France, providing a demonstration for IAV infection; whereas the blood collecting center at la Timone Teaching Hospital in Marseille, France, received thirty volunteers who were then asked to provide control samples. None of the subjects were taking any medications or had any illnesses that are known to affect PLT function.

Regarding this study, which adhered to the principles of the Helsinki Declaration, all patients and healthy participants supplied written informed permission. In addition, the approval of this study was given by the research ethics committee of the University of Aix-Marseille.

H. Evaluation of PLT and Leukocyte Numbers

The previously published methods for preparing human PLT-rich plasma (PRP), PLT-poor plasma (PPP), and washed PLTs were used (Miller, et al., 1988). Tyrode's buffer was used to resuspended washed PLTs (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 Mm NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, 3.5 mg/ml HSA, and 5.5 mM glucose, pH 7.3) supplemented with 0.02 U/ml apyrase. Studies were carried out six hours after blood collection. RPMI 1640 was used to modify the PLT counts in PRP and washed PLTs to 3–4 10^8 cells/mL. On a BD-Accuri™ C6 flow cytometer (BD Biosciences), analysis was done using PE-CD62P (clone AK-4; eBioscience) and fluorescein isothiocyanate (FITC)-CD42b (clone HIP1; eBioscience) antibodies to assess granule secretion.

The mouse smart card 7030 was used to count PLTs on the Vet ABC Hematology Analyzer from (SCIL Animal Care Company Sarl, Altorf, France). Mouse PLTs in various sample fluids are distinguished by the automated cell counting according to their size.

I. Flow Cytometry of Blood PLTs

Mouse blood was drawn by retrobulbar venous plexus puncture (The least amount of PLT activation is likely to occur when blood is collected through a retrobulbar venous plexus puncture) (Jirouskova, Shet and Johnson, 2007), while venipuncture is used to collect human blood into a plastic tube containing buffered 3.2% sodium citrate, which is then gently mixed right away after filling. In whole blood, PLT activation and CD41-positive cells were assessed utilizing FITC-conjugated P-selectin and APC and phycoerythrin (PE)-conjugated CD41/CD61 antibodies.

J. In Vivo Experiments

Six-week-old C57BL/6 mice from Janvier Laboratories in (Le Genest Saint-Isle, France) were kept in a pathogen-free environment and provided with unlimited access to food and water. Mice were kept in cages inside stainless steel isolation cabinets for infection tests, and the air was filtered with high-efficiency particle air ventilation. At day 0, mice were given a ketamine and xylazine mixture (43 mg/kg and 5 mg/kg, respectively) to make them unconscious before being given 20 µl of a PBS solution containing the A/PR/8/34 virus intranasally, as previously described (Rahman, et al., 2021). Infection was performed with either 500 or 750 Plaque-Forming Units (PFU). Then as a control (placebo), the same volume of PBS was used to treat mice intranasally. Daily survival rates and weight loss were measured following virus inoculation. Days three and six following infection, mice were anesthetized and peripheral blood were taken for PLTs isolation, while blood was collected on day 0 for isolation of PBMC or/and natural killer (NK) cells as a positive control for immune markers. A broncho-alveolar lavage (BAL) sample from euthanized mice was collected and processed with PBS to assess virus replication. Infectious virus titers were then calculated using a plaque assay. Statistical difference between control and treated groups was estimated by unpaired t-test or one-way ANOVA, $p \leq 0.01$ was considered as statistically significant.

Ethical approval for the *in vivo* mouse studies was indeed obtained and adhered to the highest standards of animal welfare and regulatory guidelines. The experimental protocols were designed following the INRAE guidelines, which comply with the European animal welfare regulations. In addition, the study received explicit approval (number: G130555) from the Aix-Marseille University/ Faculty of Medicine la Timone's committee on animal research. This approval was further endorsed by the Directorate of Veterinary Services under the authority of the "Ministère de l'éducation nationale, de l'enseignement supérieur et de la recherche" with the authorization number 693881479 (APAFIS#14660). The animal welfare was take seriously and ensured that all experimental procedures were conducted in a biosafety level 2 facility, adhering to strict safety measures.

K. qRT-PCR Analysis

For every experiment, total RNA was isolated from MKs and PLTs of infected and non-infected mice, healthy individual and flu patient's, peripheral blood mononuclear cells (PBMC), [CMK, NK-92, A549, and NiH3T3] cell lines using QIAzol reagent (Qiagen) in accordance with the manufacturer's guidelines. Following that, the M-MLV Reverse Transcriptase kit (Invitrogen, Cat No: 10338842) was used to reverse-transcribe 5 µg of the obtained RNA. As previously mentioned, Uni12 primer was utilized for vRNA (viral RNA) reverse transcription (Hoffmann et al., 2001). As a housekeeping gene, GAPDH was reverse transcribed using a particular primer (Baier, et al., 1993) (All primer sequences are provided on request). The 5X HOT Pol EvaGreen qPCR Mix Plus (Euromedex, France, Cat No: 08-24-00001) was then used to conduct qRT-PCR. A Light-Cycler 480 type II apparatus (Roche, France) was carried out for the reactions,

amplifications, and measurements. The LC480 software (Roche) was employed to create amplification plots, and GAPDH was used to normalize the data. Fold induction was determined using the threshold cycle method $2^{-\Delta\Delta Ct}$. Changes were scaled to the control values' mean, which was set to 1.

L. Gene Expression Assays in Mouse Bone Marrow MKs

Following euthanasia, mice will have their tibias and femurs from both legs removed to collect their bone marrow. PBS/ethylenediaminetetraacetic acid (EDTA) will be used to flush bone marrow cells prior to filtering (using 70 μm cell strainers), and flow cytometry will be used to sort MKs after red blood cells lysis. In addition, Ficoll gradient (Eurobio, Courtaboeuf, France, Cat No: CMSMSL01-01) was also used to enrich MKs from the bone marrow of infected and uninfected mice before they were chosen using fluorescence-activated cell sorting (Aria III SORP, Becton Dickinson) based on ≥8 N polyploidy (Staining with 10 μg/mL Hoechst 33342, Sigma-Aldrich, France) and CD41+ cells (anti-mouse-CD41-PE, Thermo Fischer Scientific, Cat No: CD4-112AP). Total RNA was extracted and gene expression assay was performed as mentioned before.

M. Western Blotting

Isolated PLTs from healthy individuals and flu patient's, MKs isolated from uninfected and infected mouse, PBMC, non-infected and infected CMK cell lines, NK-92 cell lines, NiH3T3 cells, and A549 cells were gathered as previously mentioned, lysed in buffer after being rinsed with PBS (Tris 50 mM, NaCl 250 mM, 0.1% NP-40, 0.1 mM PMSF, pH 7.4 added EDTA as a supplement – complementary combination of protease inhibitors) for 20 min on ice. The cell lysate was mixed with an equivalent volume of 2X Laemmli buffer, boiled for 5 minutes and was subjected to an SDS/PAGE. Proteins were then electro-transferred onto nitrocellulose membranes and stained with antibodies to FASLG (G247-4) (1:500) or mouse monoclonal anti-γ-Tubulin antibody (1:1000) for one hour at ambient temperature (Immunoblots were performed along with antibodies directed against human and mouse). After washing in PBS-Tween 0.05% buffer (3 min, 3 times) an anti-mouse secondary HRP-conjugated antibody was applied to the membranes using this concentration (1:2000) or goat anti-mouse (1:10000) antibody for one hour at ambient temperature. Moreover, the membranes were then cleaned in PBS -Tween 0.05% buffer (5 min, 3 times) and revealed with Pierce ECL Plus Western Blotting Substrate. Chemiluminescent immunoassay (CLIA) CCD imager ImageQuant LAS 4000 was used for image acquisition (GE Healthcare, Velizy-Villacoublay, France).

N. Statistical Analysis

The software GraphPad Prism (version 9.0, GraphPad, La Jolla, CA, USA) was carried out for all statistical analyses. Some statistical analysis was performed using the Mann-Whitney test, and the findings were deemed statistically significant at $p < 0.01$ (*). To contrast the mean ranks between groups, statistical analysis of other data was carried out using one-way ANOVA and multiple comparison tests, $p < 0.01$ (*) was used to determine if the results were statistically significant. Standard error of the mean (± SEM) is the measure used to displayed data.

III. Results

A. Contribution of MKs and PLTs to the Pathogenesis of IAV

To eliminate any risk of contamination in blood cells components, peripheral blood was taken from patients and healthy individuals, then after each of PLTs, MKs, and leucocytes were isolated as described in material and methods, then stained with specific antibodies corresponding to each blood cells and plotted by flow cytometry (Fig. 1a). It is noteworthy that in my experiments, PLTs and MKs (CD41-positive) were highly purified and were not contaminated (or at least undetectable with my technic) with leukocytes (PBMC) (CD45-positive cells) (Fig. 1a and e) and NK cells (CD56-positive) (Fig. 1a). In addition, the negative controls (A549 and NIH/3T3) cells were also stained to reduce any possible contamination with blood cell components (Fig. 1b and f). Importantly, in addition to PLTs and MKs purifications, the P-selectin surface expression was also observed on IAV infections in both PLTs and MKs compared to non-infected (Fig. 1c and d).

B. Definition of the IAV Infection Window

To determine the most powerful activity of IAV infection in MKs and PLTs. CMK cell lines were infected with IAV (MOI 3) for different indicated time points (Fig. 2d). Then mRNA was harvested and qRT-PCR was conducted with specific IAV primers to non-structural protein 1 (NS1). The result shows that pathogenicity of IAV was most active starting from day 3 to day 9 post-infection (Fig. 2d). Interestingly, the significant expression of NS1 gene was observed in infected mouse in day 3 and day 6 post-infection in both isolated MKs (Fig. 2b) and PLTs (Fig. 2c). The same results were also obtained for infected CMK cell lines at day 3 and day 6 post infections in comparison to uninfected CMK cells (Fig. 2a).

C. Analysis of the Expression of Cytotoxic Molecules in Human PLTs

Except for FASL, the analysis of killer molecules in PLTs was never investigated. To test this point, PLTs isolated from non-infected/IAV-infected patients at several stages of the disease were thus analyzed by flow cytometry (data not shown). Moreover, to examine the activities of immune markers in PLTs during IAV infection in healthy individuals and infected patients by IAV at (day 3) and (day 6) of sign and symptoms onset, peripheral blood was taken from both groups. First, after PLTs isolation, the PLT activations were checked by two specific PLT markers p-selectin (CD62P) (Fig. 1d) to differentiate between PLTs of uninfected (healthy individuals) and IAV-infected patients (Flu patients), and also PLTs population were determined by using CD41 polyclonal

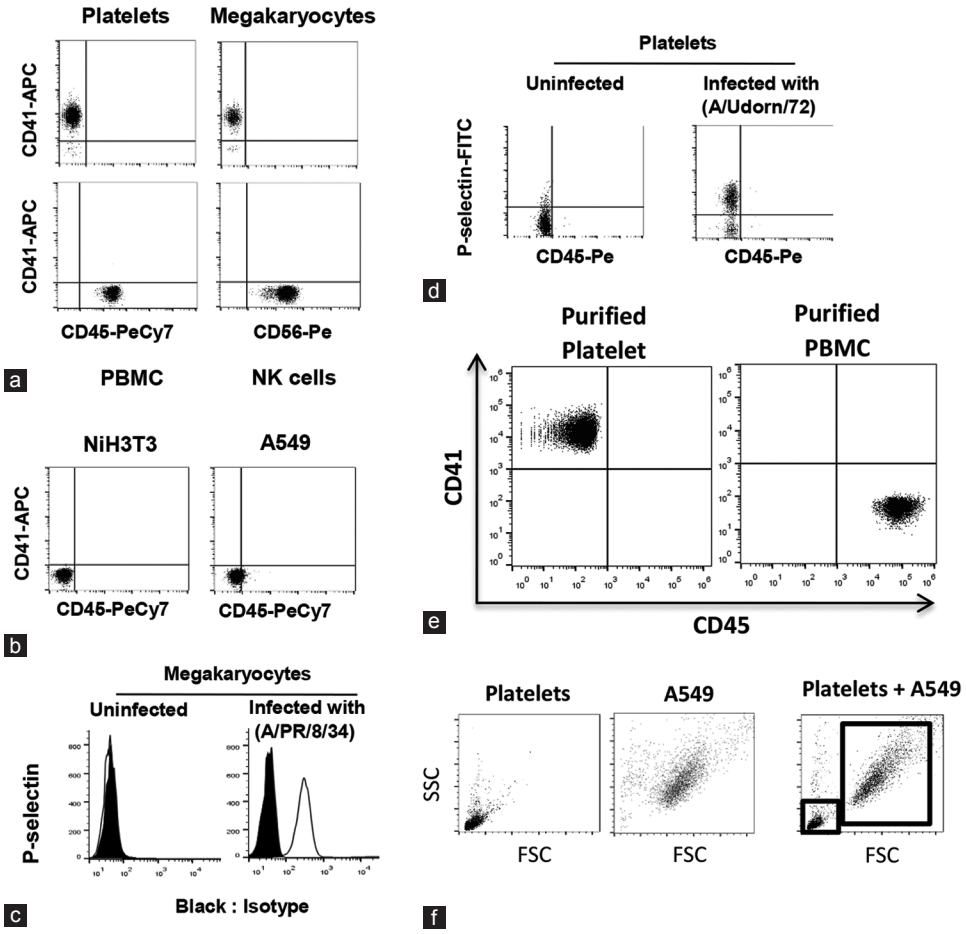

Fig. 1. During influenza A virus (IAV) infection, megakaryocytes (MKs) and platelets (PLTs) are stimulated and contribute to the pathogenesis of influenza. (a) Blood samples from uninfected or infected mice were double-stained with anti-CD45 and anti-CD41 antibodies as leukocytes and platelets identifiers, respectively from side then after double stained with anti-CD56 and anti-CD41 antibodies as NK cells and megakaryocytes identifiers from other side. (b) A549 and NIH/3T3 cell lines were double stained with anti-CD41 and anti-CD45 antibodies as platelets and leukocytes identifiers to confirm their purity and contamination rates by leukocytes and platelets/megakaryocytes throughout the study. (c) Isolated MKs from non-infected or infected mice with (A/PR/8/34) were evaluated for p-selectin secretion as indicator for MKs activation upon IAV infection, black color is correspond to Isotype. (d) Isolated PLTs from infected mouse by (A/Udorn/72) and non-infected were double stained with anti-CD62P (P-Selectin) as platelets activation marker and anti-CD45 antibody as leukocytes identifier. (e) Purification of isolated PLTs and PBMC were determined by double staining with anti-CD45 and anti-CD41 antibodies as PBMC and platelets identifiers, respectively. (f) PLTs and A549 were identified and gated alone or together in regards to size and shape by flow cytometry.

antiplatelet (Fig. 1a and e). Total RNAs were extracted and qRT-PCR was applied using specific probes and primers for the expression of different immune markers and receptors as a large panel of killer activation receptors (KARs) including (*CD16, CD56, Granulysin (GNLY), FasL, Granzyme A, Granzyme B, NKG2C, NKG2D, TRAIL 1, TRAIL 2, and TRAIL 3*) (Fig. 3a) to indicate the important roles of PLTs through the expression of these markers during IAV infection. Inhibitory receptors (KIR) were also investigated (data not shown) such as (*ILT2, ILT4, KIR2DL-1-4, CD94/NKG2A*). NK92 and A549 cell lines have been used as positive and negative controls for receptors expression. Similar studies were also performed using infected versus non-infected mice (Figs. 4 and 5).

Result clearly shows that both PLTs of infected IAV patients and healthy individuals (control) were not significantly affected the expression of these markers except for *FasL, TRAIL*, and *GNLY* which are markedly and significantly expressed in infected patients (activated PLTs) compared to healthy individuals (resting) PLTs (Fig. 3a). Interestingly, all mRNA coding for killer activation and inhibitory receptors were not upregulated for most cytotoxic molecules, suggesting that *FasL, TRAIL*, and *GNLY*, were highly upregulated upon IAV infection is MKs and PLTs specific and not due to contaminants of NK cells. Altogether, these data suggest that activated PLTs have important roles during IAV pathogenicity.

D. PLTs Activation by IAV Exhibits Membrane-bound FasL

To investigate the potential influence of PLT activation on *FasL* gene expression, a known pro-apoptotic molecule, this study is aimed to analyze the relationship between these two factors. To find this relation, peripheral blood was taken from non-infected and infected patients and PLTs were isolated

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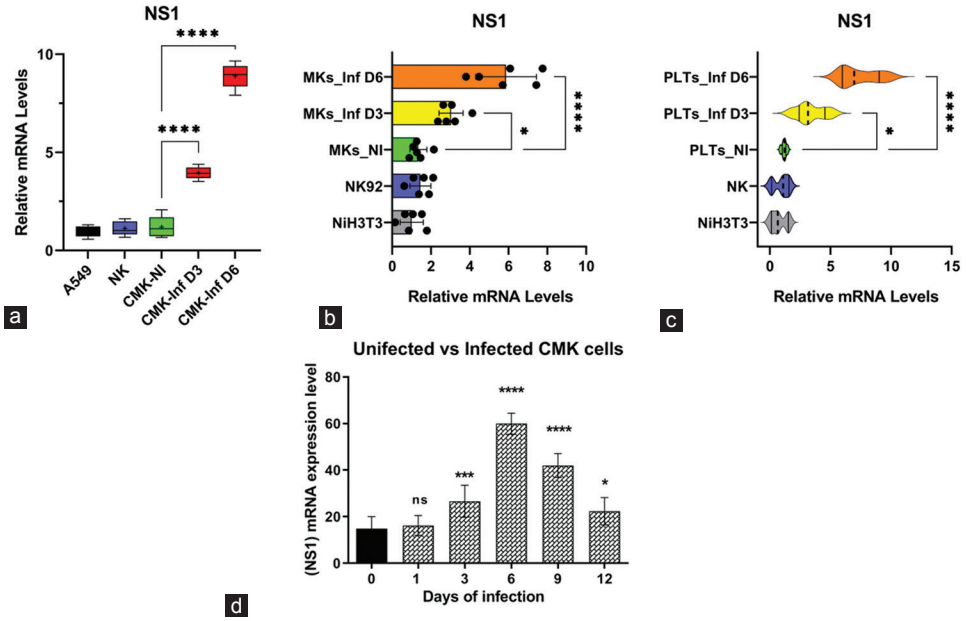

Fig. 2. Viral NS1 protein gene expression upon influenza A virus (IAV) infection. (a) CMK cells were non-infected or infected with IAV A/PR/8/34 virus (MOI 3), and then at day 3 and day 6 post-infection, RNA was extracted and real-time qPCRs were performed with specific primers to quantify gene expression of the non-structural protein 1 (NS1) of influenza A virus (mRNA or vRNA) at the indicated time point post-infection. A549 and NK92 cells were used as negative and positive controls for immune receptors, respectively. Data are represented as means ± SEM, n = 3–6 replicates. (b and c) Mice were non-infected or intranasally infected with (A/PR/8/34) virus (PFU 500), and then at day 3 and day 6 post-infection, megakaryocytes and platelets were isolated and RNA was extracted and real-time qPCRs were performed with specific primers to quantify (NS1) gene expression of influenza A virus (mRNA or vRNA) at the day 3 and day 6 post-infection. NiH3T3 and NK92 cells were used as negative and positive controls for immune receptors, respectively. Data are represented as means ± SEM, n = 6 mice. (d) CMK cell lines were grown on the 12 wells plates. At confluence 70-80% cells were non-infected (NI) or infected with A/PR/8/34 viruses (MOI 3) for the indicated time point (1, 3, 6, 9, and 12 days). CMK cell lines were then harvested and then after mRNA was extracted and converted to cDNA the quantitative real-time-polymerase chain reaction (qRT-PCR) were performed by using specific primers and probes for viral NS1 gene. Presence of the expressed gene was analyzed by qRT-PCR. Relative mRNA levels were calculated and plotted by GraphPad software as described in materials and methods. Analysis was performed three (3) times in duplicate for all experiments.

from both groups (as mentioned before); then after, RNAs were extracted and using specific primers for *FasL* gene, then qRT-PCR was performed for determining gene expression in activated (infected patients) and resting PLTs (healthy individuals). The present work shows that activated PLTs was significantly increased the expression of *FasL* gene when compared to inactivated (resting) PLTs in control group, A549 is used as negative and PBMC as positive control for the expression of *FasL* gene (Fig. 3a).

E. FasL mRNA Expression is Dependent on MKs Activation

To examine if MKs as PLTs precursor has the same response to *FasL* gene expression when it's activated much like PLT, CMK cell lines were cultivated and infected or not by IAV (see material and methods) then after were harvested at day 6 post infection, qRT-PCR was carried out after mRNA extraction using the same primers specific to *FasL* gene expression. The results show that like activated PLTs, *FasL* gene expression was greatly increased in activated CMK by IAV at day 6 post infection when compared to non-infected or resting CMK (Fig. 6a). An NK92 cell line was used as positive control, and A549 as negative control (data not shown). Thus, this finding confirmed that *FasL* gene expression required activation of MKs by IAV as PLTs.

F. Infected Human MKs and PLTs Induces Expression of FasL Protein

To assess protein expression by immunoblot, proteins were extracted from non-infected and infected CMK cells in indicated time points (Fig. 6b), and from infected and uninfected PLTs (Fig. 3b), also from PBMC of infected patients, (NK-92 cell line as positive) and (A549 cells as negative) controls. Furthermore, the extracted proteins were examined using a western blot. The findings showed that FasL protein for size of (37–40) kDa were detected in PBMC and NK-92 cell lines as positive controls and not detected in A549 as negative control (Fig. 3b and 6b). FasL protein expression was only detectable in infected PLTs and CMK cell lines at day 1, day 3, and day 6 post infection in a dose dependent manner, while no FasL protein detection was observed in uninfected CMK cell line and resting PLTs (Fig. 3b and 6b). Thus, IAV infection is crucial for promoting FasL protein secretion in activated PLTs and CMK cell lines. This discovery supported some studies which suggested that killing was FASL-dependent.

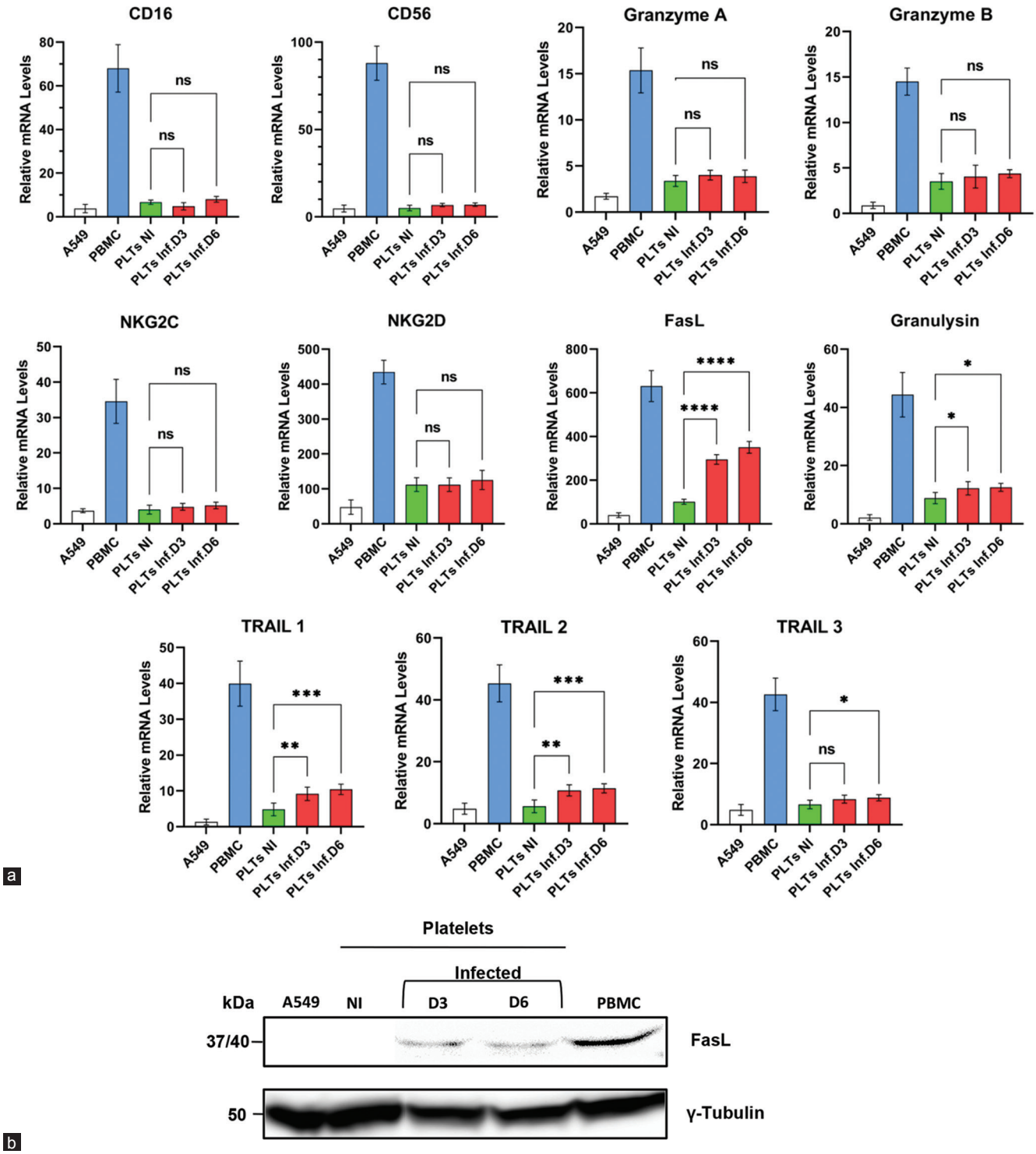

Fig. 3. Effect of influenza A virus (IAV) infection on killer activation receptors expression in human platelets (PLTs). (a) PLTs were isolated from patients infected by IAV (n = 10) and healthy individuals (n = 10), with isolated PBMC and A549 cells as positive and negative controls, respectively. PLTs and PBMC were isolated from healthy and flu patients and A549 was harvested as negative control. mRNA was extracted and converted to cDNA the quantitative real-time-polymerase chain reaction (qRT-PCR) were conducted using specific primers and probes for each known (KARs). Presence of the expressed genes was analyzed by qRT-PCR. Relative mRNA expression levels were calculated and plotted by GraphPad software as described in materials and methods. Analysis was performed 3 times in duplicate. (b) Platelets from healthy individuals (NI) and infected patients with IAV at day 3 (D3) and day 6 (D6) post influenza infections were isolated then lysed and presence of the FasL protein was analyzed by western blot. A549 cells and PBMC were lysed as negative and positive controls respectively, and presence of the FasL protein was analyzed by western blot. Presence of γ-Tubulin protein considered as internal loading control.

It is noteworthy that throughout this study, PLTs (CD41-positive) were highly purified and were not contaminated (or at least undetectable with our technic) with leukocytes (CD45 positive cells) (Fig. 1a and e).

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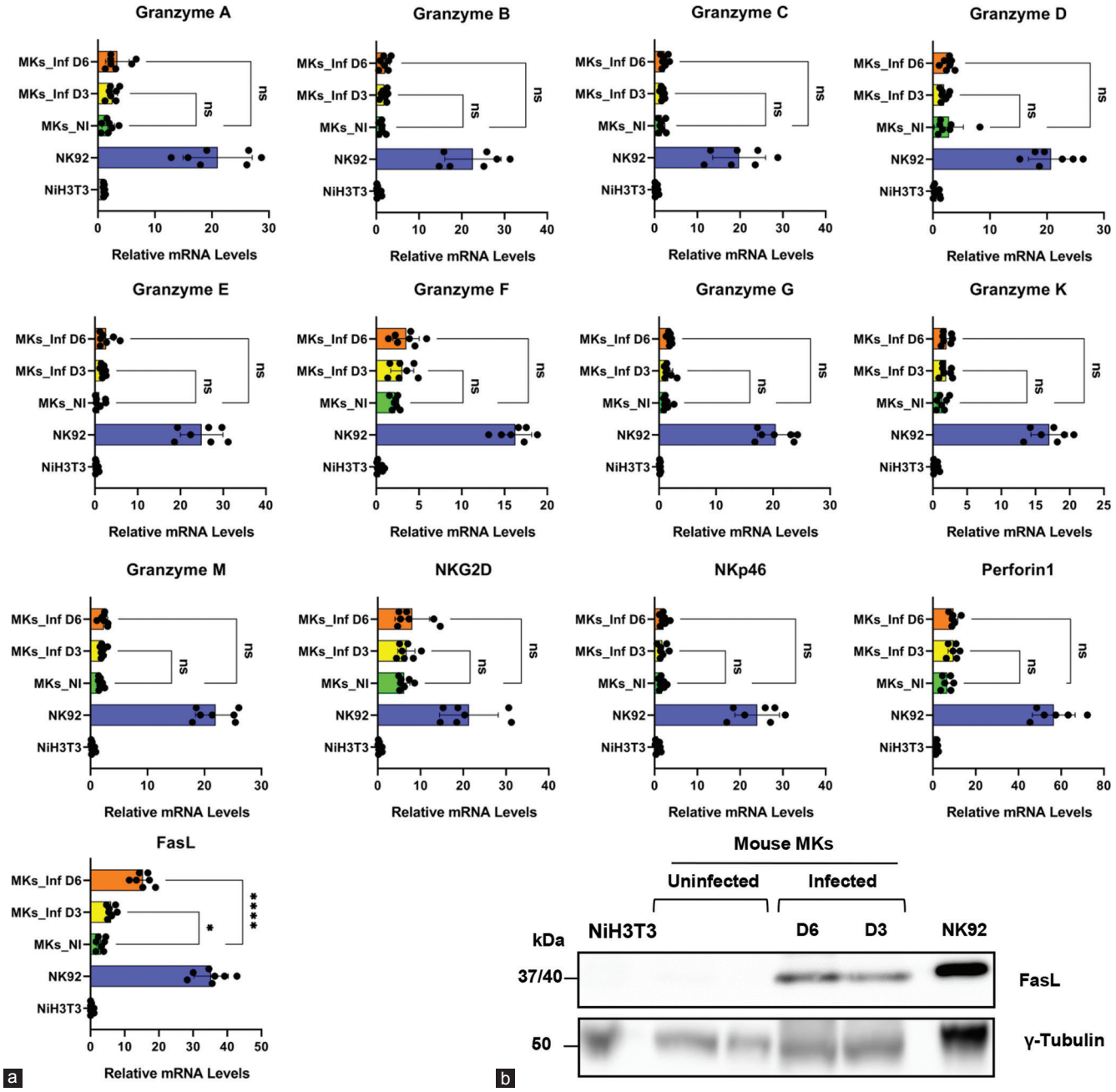

Fig. 4. Effect of influenza A virus infection on killer activation receptors expression in mouse megakaryocytes (MKs). (a) Three groups of mice (n = 7 mice/group) were uninfected or infected and inoculated with A/PR/8/34 virus, at a 50% lethal dose (LD_{50}) (500 pfu per mouse; Day 3 and 6 post-infection), and NK92 cells and NiH3T3 cell lines were used as positive and negative controls, respectively. MKs were isolated from bone marrow of infected mice or not and then NiH3T3 cells were harvested as negative control and NK92 cell lines were also harvested as positive control. mRNA was extracted and quantitative real-time-polymerase chain reaction (qRT-PCR) were performed by using specific primers and probes for each known genes of (KAR). Presence of the expressed genes was analyzed by qRT-PCR. Relative mRNA levels were calculated and plotted by GraphPad software as described in materials and methods. Analysis was performed 3 times in duplicate. (b) Mouse was uninfected (NI) or infected (inoculated) with A/PR/8/34 viruses (750 PFU) for 3 (D3) or 6 (D6) days post-infection, MKs were isolated from bone marrow (as described in materials and methods) then lysed and presence of the FasL protein was analyzed by western blot. NIH/3T3 and NK92 cell lines were lysed as negative and positive controls respectively, and presence of the FasL protein was analyzed by western blot. Presence of γ-Tubulin protein considered as internal loading control.

G. GNLY is Present in Human MKs and PLTs and is Released on Their Activation

To analyze whether the lysis granules secreted from the activated or/and resting PLTs and MKs. Specially to evaluate the secretion of *GNLY*, CMK cell lines, NK92, and A549 cells were cultivated from side, then after, not infected or infected with IAV (Fig. 6a); In addition, *GNLY* gene expression was estimated from other side by infected PLTs versus uninfected (Fig. 3a). Moreover, total RNAs were extracted and converted to cDNA then qRT-

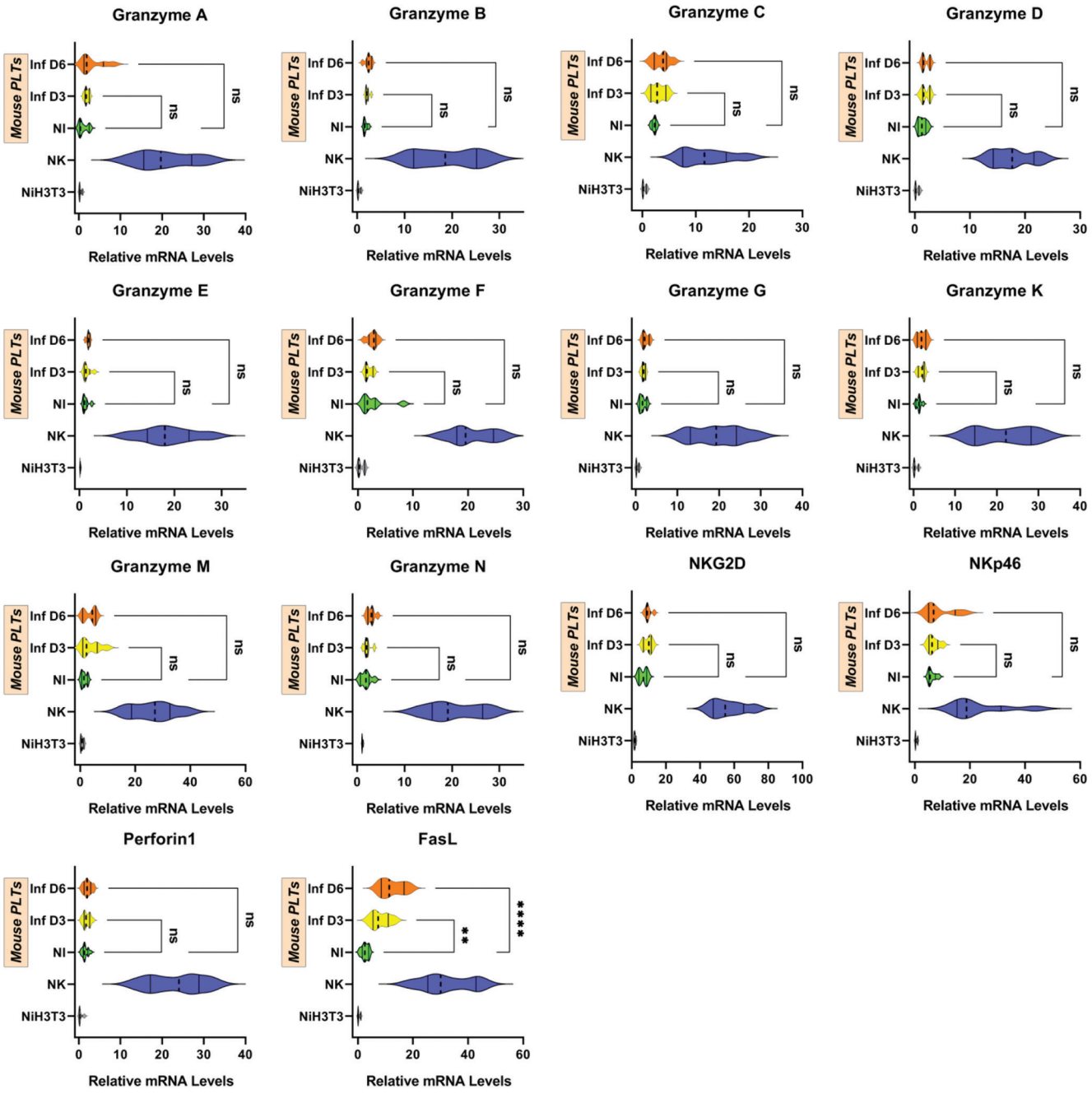

Fig. 5. Effect of influenza A virus (IAV) infection on killer activation receptors expression in mouse platelets (PLTs). Mouse PLTs (n = 7 mice/group) were infected or not by IAV at 3 days and 6 days post-infection, and NK92 cells and NiH3T3 cell lines were used as positive and negative controls, respectively. PLTs were isolated from non-infected or infected mice and then NiH3T3 cells and NK92 cell lines were harvested as negative and positive controls, respectively. mRNA was extracted and quantitative real-time-polymerase chain reaction (qRT-PCR) were conducted using specific primers and probes for each known genes of (KAR). Presence of the expressed genes was analyzed by qRT-PCR. Statistical analyses were performed using one-way ANOVA as mentioned in materials and methods. Analysis was performed 3 times in duplicate.

PCR was achieved to estimate the expression rates of *GNLY* gene among the infected and non-infected PLTs and CMK cell lines in parallel with NK cells and A549 cell lines as positive and negative controls, respectively. The present results demonstrated that expression of *GNLY* gene was moderately and significantly increased in infected CMK cells and PLTs in comparison to non-infected (Fig. 3a and 6a).

H. Most of Lytic Granules with Cytotoxic Actions were not Secreted by Activated or Resting Human MKs

Approximately most of KARs and KIRs were not expressed by CMK cells in resting situation or on their activation. To evaluate the secretion capabilities of lytic granules by MKs (CMK cells) in non-infected or IAV-infected status, CMK cell lines were harvested and total RNAs were extracted, upon its conversion to cDNA, qRT-PCR was conducted for

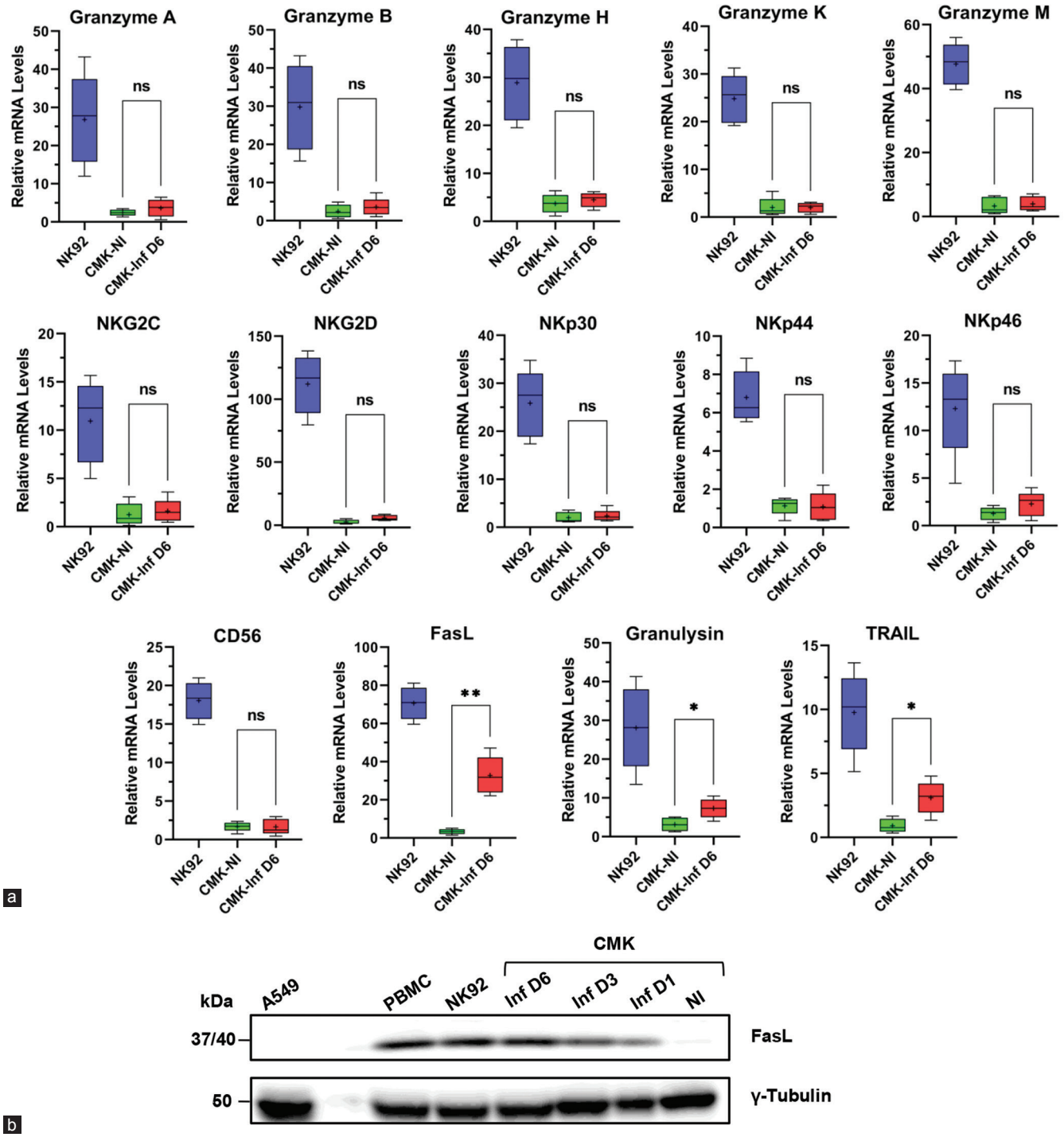

Fig. 6. Killer activation receptors (KARs) gene expression in CMK cell lines upon influenza A virus infection. (a) CMK cells were grown on the 6 wells plates. At confluence 70-80% cells were infected with A/PR/8/34 or A/Udorn/72 (MOI 3) or uninfected for 6 days post-infection, and then NK92 and A549 cells were used as positive and negative controls, respectively. Uninfected or infected CMK cells, NK92 cell lines and A549 cells (data not shown) were harvested, and then after mRNA was extracted and converted to cDNA the quantitative real-time-polymerase chain reaction (qRT-PCR) were conducted by using specific primers and probes for each known (KAR) genes. Presence of the expressed genes was analyzed by qRT-PCR. Relative mRNA levels were calculated and plotted by GraphPad software as described in materials and methods. Analysis was performed 3 times in duplicate. (b) CMK cell lines were non-infected (NI) or infected with A/PR/8/34 viruses (MOI 3) for the indicated time point (days), PBMC and NK92 cells were used as positive controls and A549 cells were included as negative control. All cell lines were then lysed and presence of the FasL protein was analyzed by western blot. Presence of γ-Tubulin protein considered as internal loading control.

approximately all of lytic granule's genes. Data indicated that there are no any significant differences between infected and uninfected CMK cells when compared to NK92 cell lines as positive control (Fig. 4a, and Fig. 7). Furthermore, these outcomes emphasize that both non-infected and infected CMK cell lines were unable to secret perforin and all

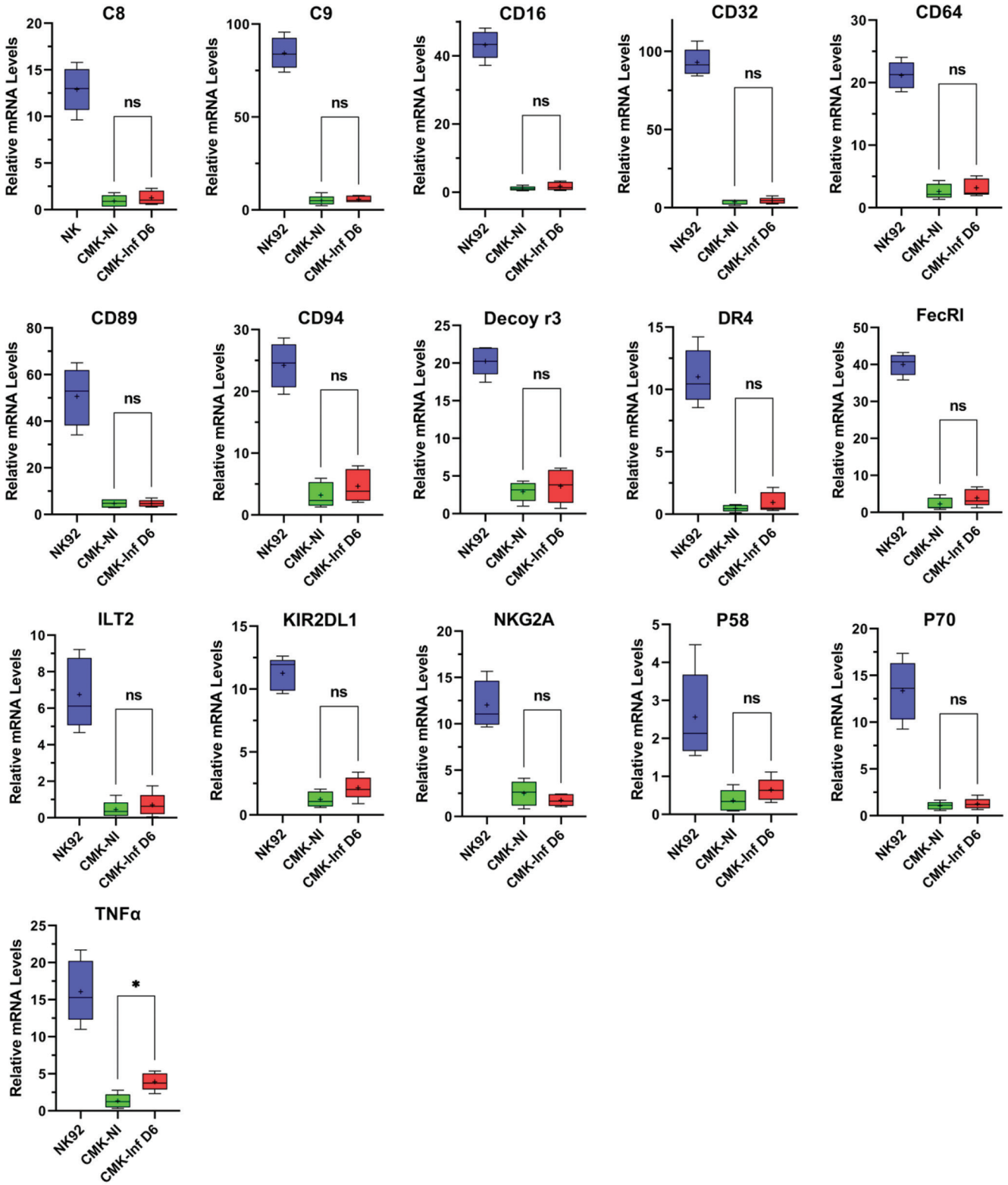

Fig. 7. Killer inhibitory receptors (KIRs) gene expression in CMK cell lines upon influenza A virus infection. CMK cells were grown on the 6 wells plates. At confluence 70-80% cells were infected with A/PR/8/34 or A/Udorn/72 (MOI 3) or uninfected for 6 days post-infection, and then NK92 and A549 cells were used as positive and negative controls, respectively. Uninfected or infected CMK cells, NK92 cell lines and A549 cells (data not shown) were harvested, and then after mRNA was extracted and converted to cDNA the quantitative real-time-polymerase chain reaction (qRT-PCR) were performed by using specific primers and probes for each known (KIRs) gene. Presence of the expressed genes was analyzed by qRT-PCR. Relative mRNA levels were calculated and plotted by GraphPad software as described in materials and methods. Analysis was performed 3 times in duplicate.

granzymes lytic granules. These results show that PLTs and MKs in human were not exhibits the cytotoxic activities and defense mechanisms as like immune cells. However, infected MKs was expressed membrane bound *FasL, TRAIL*, and *GNLY* lytic granule as KARs (Fig. 6a) and *TNFα* as KIRs (Fig. 7) on activation in contrast to resting status. Altogether, these findings suggest that activated MKs express some killer and inhibitory activation receptors, which point its pivotal roles during IAV pathogenicity.

I. Analysis of the Expression of Cytotoxic Receptors among Mouse MKs and PLTs (In Vivo).

As mentioned before, this work also tested whether the expression of killing receptors in murine PLTs on infection results from a modification of the transcriptome/proteome in the MKs (precursors of PLTs). To this end, mice had the infection intranasally by (A/PR/8/34) virus (500 PFU) and after 3 and 6 days, bone marrow MKs were isolated. The present result showed that *NKG2D, NKp46, perforin1* and all of granzyme types were not upregulated at the transcriptional level in bone marrow MKs upon mice infection; while *FasL* gene was significantly upregulated in infected mouse MK's when compared to resting MKs (uninfected mouse) in different time points (Fig. 4a). In addition, the expression of FasL protein was more certain through western blot. To do this study, MKs was isolated from bone marrow of infected and uninfected mouse, then after protein was extracted and lysed at (3 and 6) days post-infection (Fig. 4b), protein from negative control NIH/3T3 cells and NK92 cells as positive control were also extracted and lysed. Furthermore, the protein expression was analyzed by Immunoblot. The outcome amply demonstrates that FasL protein was observed in mice MKs at both day 3 and day 6 post-infection, while this protein was not detected in non-infected MKs (Fig. 4b). Altogether, this data is certainly indicated that MKs activation is required to express killing FasL.

As previously described, obtained outcome reveals that *FasL* gene is upregulated at the transcriptional level in bone marrow MKs upon mice infection. Moreover, the expressions of the same markers were screened for isolated PLTs from non-infected and infected mice at day 3 and day 6 as much like MKs (Fig. 5). Results demonstrated that except of *FasL* gene expression which was highly upregulated in infected PLTs in comparison to uninfected, while PLTs of both infected and non-infected mouse were unable to express genes related to all of *granzymes, perforin1, NKG2D*, and *NKp46* (Fig. 5). Thus, these results in the animals are important to investigate *in vivo* involvement of cytotoxic PLTs in IAV pathogenesis.

Here, this study extended to checking a large panel of other killing receptors. In all conditions, mRNA was extracted and whole-transcriptome analysis was performed on MKs and PLTs, the results of qRT-PCR revealed the differential gene expression in the different conditions. Indeed, no any receptors were significantly upregulated upon infected mice compared to uninfected (results not shown). Approximately all types of granzyme and perforin were not secreted by MKs and PLTs in resting situation or on their activation by IAV.

IV. Discussion

The goal of the current research was to better understand the unexpected roles that PLTs and MKs play in immunity and as cytotoxic agents. Furthermore, to investigate the role of PLT activation and *FasL* gene expression in a physio-pathological model of influenza virus infections, the current study aimed to comprehensively analyze their relationship and potential implications. A recent study shows that PLTs engulf influenza viruses *in vitro*, therefore, PLTs migration to the lungs is probably moving to do the same *in vivo* (Danon, Jerushalmy and De Vries, 1959). This could appear as the passive absorption of particles in a manner similar to bacterial ingestion (White, 2005). It is also possible to compare the uptake of IAVs by neutrophils and macrophages during the phagocytosis of human immunodeficiency viruses (Youssefian, et al., 2002). PLTs support the body's immunity against pathogenic bacterial infections by accelerating damage healing and reducing vascular lesions (Petaja, 2011; Engelmann and Massberg, 2013). PLT-neutrophil cross-talk mediates the early intrinsic resistance against influenza, it tightly controls host immune and complement responses but can potentially cause thrombotic vascular blockage (Koupenova, et al., 2019).

Supporting previous observations, obtained results demonstrated that plasma from infected mice contained signs of PLT activation. In response to a fatal IAV infection, PLTs and MKs were subsequently activated in the peripheral circulation and bone marrow, respectively. It's interesting to understand that the influenza virus activates PLTs by producing thrombin (Boilard, et al., 2014); thrombin primarily activates PAR4 and PAR1 to facilitate signal transduction (Kahn, et al., 1999; Kataoka, et al., 2003). This increases the possibility that thrombin may have a negative impact on IAV infection.

PAR4 and GPIIIa are two molecules that are crucial for PLT function. Due to the absence of PAR1 in mouse PLTs, thrombin-mediated PLT activation most likely happens through activating PAR4. As a result, fibrinolysis is a method by which PAR1 and plasminogen control pathogenesis. In addition, the results of the current study showed that IAV infection activated MKs and PLTs. This method was used to demonstrate several activation markers *in vivo* and in vitro (Figs. 3-5). These results specified that MKs and PLTs were highly activated upon infection with different strains of IAV in murine and in humans.

Additional result support current study which demonstrates that through thrombin production or FcgRIIA signaling, the influenza virus stimulates PLTs (Boilard, et al., 2014). Moreover, PLTs' TXA2 and serotonin are released upon the thrombin activation, which later activates the GPIIb/IIIa complex, and enhances the movement of P-selectin to the PLT plasma membrane (Polley, et al., 1981).

The arsenal is potent proinflammatory chemicals generated by active PLTs that improve adhesion, recruitment, and neutrophil rolling (Mayadas, et al., 1993; Diacovo, et al., 1996; Von Hundelshausen, et al., 2001; Zarbock, Polanowska-Grabowska and Ley, 2007). According to recent study, during

the intense inflammation brought on by influenza, PLT interaction with neutrophils is anticipated to be considerable. The physical interaction from neutrophils to PLTs is another element in neutrophil retention and activation (Zarbock, Polanowska-Grabowska and Ley, 2007).

The particular *in vivo* and *in vitro* expression of killer receptors in MKs and PLTs has not yet been described. This study also showed that the *FasL* gene was highly expressed and upregulated among activated PLTs in both mice and humans, which is consistent with some reports suggesting that killing was FASL-dependent (Garcia-Garcia and Ramos, 2006). However, it was suggested that murine PLTs induce apoptosis through FasL on activation (Volpe, et al., 2016).

These findings were also confirmed among human PLTs, when apoptosis was induced in Fas-positive tumor cells after human PLTs activation (Ahmad, et al., 2001). These observations open a door to the discovery of a novel immunological role of PLTs and their regulation function during pathological conditions.

To the best of our knowledge, this investigation is the initial to use *in vivo* models to investigate the expression of immunological receptors in MKs and PLTs as viral infections progress. The member of the tumor necrosis factor (TNF) family includes Fas and Fas Ligand (FasL). Apoptosis, which is governed by the protein Fas-FasL, is a crucial process for maintaining immunological homeostasis (Volpe, et al., 2016). A caspases cascade that starts apoptosis is activated when Fas ligates with FasL (Ashkenazi and Dixit, 1998).

The present results show that MKs and PLTs exhibit *FasL* expression (see figures) which effectively shielded mice from the IAV pathogenesis brought on by a variety of influenza strains (data not shown). These outcomes are in agreement with earlier research demonstrating that through membrane-bound FasL, PLTs trigger apoptosis (Schleicher, et al., 2015). It is interesting to note that membrane-bound FasL was shown to be expressed by activated MKs; however, its exact purpose is yet unknown (Arabanian, et al., 2012).

The present study's findings showed that MKs and PLTs express *FasL* (see figures), which is successfully protecting mice from multiple influenza strains that cause IAV pathology in mice (data not shown). These findings align with those of prior research papers indicating that through membrane-bound FasL, PLTs trigger apoptosis (Schleicher, et al., 2015). It is interesting that activated MKs have been proven to express membrane-bound FasL, but its specific function is yet unknown (Arabanian, et al., 2012).

There are two main hypotheses that can be formulated to explain this increased PLT content in immune properties molecules. First, as PLTs arise from the membrane budding of MKs, they largely inherit their transcriptome and proteome from them. Differences in the expression of intracellular PLT cytotoxic molecules might be the result of their upregulation in MKs on direct virus infection or an exposition of an inflammatory environment (cytokines). Second, PLTs have mRNA and the entire translational machinery for protein synthesis, even though they lack a nucleus (Han and Baker, 1964; Rowley, et al., 2011). Accordingly, PLTs that are directly exposed to viruses might also differ in their protein profile compared to uninfected PLTs. Both hypotheses are not exclusive and could act synergistically.

To test the first hypothesis, we isolated MKs from bone marrow of uninfected (NI) or infected mice (days 3 or 6 post-infection – ID3, ID6) by flow cytometry (based on the polyploidy of MKs) and found that on IAV infection, the content of murine MKs in mRNA coding for most (KARs) *Granzymes, perforin1*, and *NKG2D* was significantly not upregulated [except *FasL*] when compared to MKs of uninfected mice (Fig. 4a), whereas western blot analysis revealed that infected mice had higher levels of FasL protein expression than uninfected (Fig. 4b). NIH/3T3 cells and peripheral blood mononuclear cells (PBMC) were used as negative and positive controls, respectively. Interestingly, all mRNA coding for cytotoxic molecules were not upregulated such as *NKp46*, suggesting that this regulation is MK specific and not due to contaminants of NK cells.

It has previously been proven that T cells require the membrane-bound form of FasL (LA, et al., 2009). Following PLT activation, the cell membrane has FasL exposed is triggered the apoptosis of the target cell in 6 h (Schleicher, et al., 2015). Moreover, as a result of viral infection in the eye, it has been discovered that FasL-dependent apoptosis can stop damaging inflammatory reactions (Griffith, et al., 1995). As a result, the protein profile of PLTs that are directly exposed to IAV or an inflammatory environment (cytokines) may be different from uninfected PLTs. In addition, the current study found that, despite the highly upregulated *FasL* gene in activated MKs and PLTs in both humans and mice, the *TRAIL, GNLY*, and *TNF* genes, which function as killer activation receptors and inhibitory receptors, respectively, were all significantly upregulated in infected human PLTs and CMK cell lines at the indicated time points compared to non-infected. These findings were consistent with some recent studies, which indicated that *TRAIL* (Morrell, et al., 2014), *CD154* (Arabanianm, et al., 2012; Henn, et al., 2001), and *TNFα* (Liu, et al., 2001; Morrell, et al., 2014) were markedly expressed on MKs and PLTs activation. In addition, soluble and membrane-bound forms of functional *FasL* are expressed by activated PLTs and MKs (Josefsson, et al., 2014; Schleicher, et al., 2015).

This work show, for the first time, that neither in humans nor in mice were nearly all of the NK cytotoxic receptors expressed by MKs and PLTs in the resting or active states. Hence, the current investigation showed that MKs and PLTs have no cytotoxic role during the pathogenesis of *in vivo* and *in vitro* influenza virus infections. However, it's important to mention, these results show that PLTs and MKs were efficiently expressed (*FasL, TRAIL* and *TNFα*) after activation by IAV in human and mouse, while cytotoxic granules production were not demonstrated either among human or in murine in resting PLTs and MKs and also on their activations by IAV, except of *GNLY* granule gene expression in Flu patient *in vivo* (Fig. 3a) and among infected CMK cell lines *in vitro* (Fig. 6a).

The current data suggest that a novel treatment strategy for treating severe influenza would require looking into *FasL* expression in activated MKs and PLTs. FasL was found in

this work as an apoptotic receptor that is produced from MKs and PLTs. This finding requires additional work to investigate the part that possible pathways play in PLT-induced apoptosis. In addition, it also highlights the importance of FasL as an apoptotic immune element in activated MKs and PLTs during IAV pathogenicity.

It was suggested that PLTs express several pattern recognition receptors (PRRs) families as C-type lectin receptors (CLRs), toll-like receptors (TLRs), and NOD-like receptors (NLRs), which are highly conserved receptors, identify virus as a stranger (Chao, et al., 2019). Due to the activation of these receptors, the initial inflammation following IAV infection is linked to the release of cytokines and chemokine's (Koupenova, et al., 2019). It's interesting to note that PLT activation's increased cytokine production was only discovered later following infection (Dib, et al., 2020; Koupenova, et al., 2019).

Moreover, several immune cells are modulated by PLTs, which help to coordinate the immunological response (e.g., monocytes, lymphocytes, neutrophils, and dendritic cells). As well, during this process, immune cells, and PLTs work together to generate a physical barrier that stops pathogens from escaping and triggers immune system reactions that are innate and adaptive (Ebermeyer, et al., 2021).

Due to the fact that severe influenza virus infections are defined by the dysregulation of inflammation, it's probable that PLTs have a role in promoting inflammation and play a significant role in the development of IAV. In view of IAV outbreaks that consistently result in severe human infections, serious concerns have been raised about the therapeutic options available for these pathogens. This shows how urgently new antiviral techniques are required. New approaches that use drugs that target cellular proteins are thought to be less likely to promote resistance. Such cellular targets would be valuable for the treatment of most if not all influenza strains infections. In addition, the anti-influenza medications that are now on the market are only (modestly) effective when taken within 48 hours of infection. This treatment interval is usually exceeded in patients who are admitted to the hospital with severe influenza. It is well known that infectious respiratory viruses such as influenza A/B viruses, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and respiratory syncytial virus (RSV) infection, they attack from prominently the respiratory system and PLTs play a central role during this pathogenesis. Thus, antiplatelet drugs can circumvent the severity of the pathogenicity of these viruses.

In fact, the distinct role of PLTs is poorly understood, and the few findings that are available have been published in top publications. Considering that (KIRs) and (KARs) are little understood, this study investigated (KIRs) and (KARs) in PLTs and MKs in both physiological and pathological contexts using *in vitro, in vivo*, and clinical tools. Indicating that this study will definitely have an impact on the medical field and ultimately on the definition of PLT as a completely new type of immune cell. It is also noteworthy that many antiplatelet medications are already approved for humans use and are commercially available. Antiplatelet drugs are well-known and documented; their pharmacovigilance (PV) and adverse effects have also been extensively established. Interestingly, future research is most required to offer a long-term perspective and provides an alternative management option for severe influenza, especially if the disease is caused by viral strains resistant to any of the currently available anti-influenza drugs.

V. Conclusions

Altogether, these results suggest that MKs and PLTs have no cytotoxic function that can be induced in response to IAV infection. However, their role is limited to membrane-bound *FasL, TRAIL, GNLY*, and *TNFα* expressions and might open a new window to explore their immunological role during viral pathogenicity.

VI. Acknowledgment

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